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Enhancing Chrysanthemum (*Dendranthema* × *grandiflorum*) tissue culture for improved ornamental flower production: Genotypic insights and growth regulator optimization

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Abstract

This study addresses the need for refining *in vitro* regeneration protocols for chrysanthemum (*D. grandiflorum*), the second most prominent ornamental cut flower globally, through the strategic implementation of tissue culture techniques. The objective of the study was to evaluate the response of four *Dendranthema* genotypes (CO-1, Pink Marble, Snapper and Super White) to various plant growth regulators (PGRs) during callus culture, shoot formation, rooting and acclimatization. For this, ray floret explants were utilized and PGRs including 2,4-D, BAP, IBA, GA_3, IAA and NAA were tested at different concentrations. Results revealed the superior performance of genotypes CO-1 and Super White across all stages of *in vitro* regeneration. Optimal conditions were identified, including a synergistic combination of 2.0 mg L^{-1} 2,4-D and 0.100 mg L^{-1} BAP for callus induction, 2.0 mg L^{-1} BAP with 0.100 or 0.250 mg L^{-1} NAA for shoot formation and 1.0 mg L^{-1} IBA with 0.100 mg L^{-1} NAA for rooting. Acclimatization was successful using vermicompost + red soil + coir pith (1:1:1) as a substrate. Furthermore, tissue culture-raised chrysanthemum plants exhibited a significant increase in flower numbers upon treatment with 100 mg L^{-1} GA3, indicating the potential for enhanced flower quality and yield compared to conventionally grown plants. These findings provide crucial insights for optimizing large-scale chrysanthemum production and underscore the importance of tissue culture techniques in ornamental cut flower industry advancements.

Keywords

Recalcitrant; cut flower; indirect organogenesis; *in vitro*

Introduction

Chrysanthemum, specifically *Dendranthema* x *grandiflorum* (Ramat.) Kitam. is a botanical entity of profound significance, with versatile applications in the realms of medicine, culinary arts, floral ornamentation, religious ceremonies, cut flower production and insecticide development, notably pyrethrins (1). *D. grandiflorum*, a prominent species within the Chrysanthemum genus, holds a pivotal economic role as it is predominantly cultivated for the yield of cut flowers, loose flowers and potted plants. This botanical gem is believed to have its origins rooted in East Asia and has earned the illustrious epithet of the "Autumn Queen or Queen of the East" (2).

Taxonomically, *Dendranthema* belongs to the Compositae family and exhibits variable growth patterns, achieving heights of up to 1.5 meters. This

cultivar's prevalence extends worldwide, with the largest production acreage found in China (8475 ha) and Japan (5230 ha). Thailand and India serve as influential leaders in the domestic market, cultivating *Dendranthema* across 1900 and 2199 ha respectively (3). India, in particular, contributes substantially to the global production, generating approximately 470.14 tonnes of *D. grandiflorum* annually, with key states such as Andhra Pradesh, Karnataka, Tamil Nadu, Telangana and Maharashtra playing pivotal roles (4).

The economic productivity of *Dendranthema* is closely linked to propagation methods. Traditional vegetative techniques like root suckers and stem cuttings face limitations, including propagule availability, labour intensity, high costs, disease susceptibility and scalability issues. Tissue culture, costing Rs. 1-2 per plant and capable of producing up to 10 million plants annually, offers a more economical solution. Compared to traditional methods, garden mums sold at Rs.187 per pot yield profit margins of 36.8 % for delivered plants and 44.2 % for non-delivered plants. In response to these constraints, tissue culture emerges as a promising avenue for rapid multiplication of *D. grandiflorum* through *in vitro* regeneration (iihr.res.in/ and https://www.uky.edu/Ag/Horticulture/mumbudgets.htm-assessed on 03.06.2024).

Previous studies have demonstrated successful regeneration through various explants, including shoot tips (5), nodal cuttings (6-8), leaf segments (9-10) and disc and ray florets (11-14). In this context, this study embarks on an experimental journey utilizing ray florets as explants in 4 distinct genotypes of *D. grandiflorum*, with the primary objective of optimizing *in vitro* system for accelerated plant multiplication and flowering. The overarching aim is to ensure a steady supply of superior quality seedlings and to foster the growth of the cut flower industry, thereby contributing to the sustainability and economic viability of *Dendranthema* cultivation.

Materials and Methods

Explant Materials

The explant materials used for this experiment were 'ray' florets. The mother plant consisted of four *Dendranthema* genotypes: CO-1, Pink marble, Snapper and Super White. These genotypes were obtained from the Indian Institute of Horticultural Research (IIHR), Bangalore and Anbil Dharmalingam Horticultural College and Research Institute (ADHC & RI), Trichy. The mother plants, aged 3 months, were cultivated in pots under a shade net for 30 days with controlled irrigation and management to induce flowering. Inflorescences, referred to as 'heads' or 'capitula', were harvested from each of the 4 genotypes individually during the early morning. Culture initiation was carried out using ray floret explants.

Disinfection and Preparation of Explants

The collected inflorescence heads underwent a series of meticulous preparations. Initially, they were washed in tap water, followed by rinsing with distilled water for 15 min. Subsequently, the inflorescence heads were subjected to sequential disinfection, which included treatment with 30 % ethanol for 30 seconds, exposure to a 1.5 % sodium hypochlorite solution (NaOCl) containing a droplet of Tween 20 for 3 min with constant agitation and multiple rinses with sterile deionized distilled water. The dried inflorescence heads were gently handled to extract the 'ray florets' within a laminar airflow chamber.

Establishment of the culture

Culture conditions and callus induction

Healthy ray floret explants were selected and subjected to a surface sterilization process. The disinfected explants were subsequently placed onto Murashige and Skoog (15) (MS) medium for callus induction. The MS medium was composed of MS basal media (including vitamins and salts), 3 % sucrose and 0.8 % agar. Various concentrations of plant growth regulators (PGRs), specifically 2,4-Dichlorophenoxyacetic acid (2,4-D) ranging from 1.0 - 2.0 mg L^{-1} (milligram per litre) and benzylaminopurine (BAP) ranging from 0.100 - 0.150 mg L^{-1}, were incorporated into the medium. The pH of the medium was adjusted to 5.8 before autoclaving at 121 °C for 15 min. The experiment was conducted with four replications and each treatment included 15 ray florets per genotype. Additionally, a control treatment was maintained, which utilized MS basal medium without PGRs. The cultures were incubated under controlled conditions, including a temperature of 25 ± 1 °C and a 16/8-h light/dark photoperiod with a photon flux density of 36 µ mol m^{-2} s^{-1}.

Callus Induction

Callus induction was monitored at approximately three weeks post-inoculation. The percentage of callus induction and the number of days required for induction were documented. The initial weight of the callus was determined at four weeks, after which the callus was subcultured onto fresh variants of MS callus induction media. After six weeks, the final weight of the callus was recorded and the relative growth rate (RGR) of the callus was calculated using the following formula (16):

$$\text{RGR/6weeks} = \frac{\text{Final weight (FW) - Initial weight (IW)}}{6}$$

Shoot induction, multiplication and elongation

Following six weeks of callus culture, the calli were transferred to MS regeneration media. This regeneration medium contained MS basal media and specific concentrations of PGRs, with benzylaminopurine (BAP) ranging from 1.5 - 2.5 mg L^{-1} and α-naphthaleneacetic acid (NAA) ranging from 0.100 - 150 mg L^{-1}. The experiment was conducted with four replications, each consisting of 10 calli per treatment. Shoot induction was observed in all genotypes approximately three weeks after transferring to the regeneration media and they were allowed to grow for an additional week. After four weeks, shoots were isolated and subcultured two times at two-week intervals onto fresh variants of MS shoot induction media for multiplication. The *in vitro* shoots aged eight weeks, were subjected to a subculture on shoot elongation media on fresh variants of

Murashige and Skoog (15) (MS) basal medium was enriched with cytokinin (6-Benzylaminopurine - BAP) at concentrations ranging from 1.5- 2.5 mg L^{-1} and auxin (α-Naphthaleneacetic acid - NAA) within the range of 0.150 - 0.200 mg L^{-1}. The purpose of this phase, spanning two weeks, was to promote shoot growth and elongation. Each treatment and genotype included four replicates, with 10 shoots per replicate. After eight and ten week period, shoot multiplication rate (%), shoot length and the number of shoots were recorded.

In vitro rooting

Subsequently, shoots that aged ten weeks and measuring 7-9 cm in length were transferred to a rooting medium formulated on the MS basal medium. This medium included either indole-3-butyric acid (IBA) or indole-3-acetic acid (IAA) at a concentration of 1.0 mg L^{-1}, in conjunction with NAA within the range of 0.150 - 0.250 mg L^{-1}. The primary objective of this phase was to stimulate root development. Similar to the shoot elongation step, each treatment was replicated four times, with ten shoots per replication. After three weeks on the rooting medium, the *in vitro* plantlets were assessed for rooting percentage and the number of roots they had formed.

Acclimatization and flowering

Following successful root development, well-rooted plantlets, at 13 weeks of age, were transferred to acclimatization media. These plantlets were then potted in 15 x 25 cm pots covered with transparent polythene bags to maintain high humidity and were placed inside a mist chamber set at 86 % relative humidity, with ambient room temperature and partial exposure to sunlight. Various soil substrates were employed for acclimatization, including sandy loam soil (control), vermicompost + red soil + sand (1:1:1, v:v), vermicompost + red soil (1:1, v:v), Coir pith + red soil (1:1, v:v), FYM + red soil + sand (1:1:1, v:v), vermicompost + FYM + red soil (1:1:1, v:v:v), and vermicompost + red soil + coir pith (1:1:1, v:v). Ten days post-transfer, the terminal shoots of tissue culture-raised plants underwent a crucial experimental phase involving the removal of buds and application of gibberellic acid (GA_3) at concentrations of 0, 50, 100, 150 and 200 mg L^{-1}. Field-grown Pink Marble variety plants, recognized for early flowering, served as control for assessing the flowering response of *in vitro*-raised plants. The acclimatization phase involved four replicates, each comprising ten well-rooted plantlets for every treatment and genotype. Plant survival was assessed ten days after transfer and upon successful acclimatization, the established plantlets were carefully transplanted into alluvial soil to facilitate subsequent flowering. In chrysanthemum, root cuttings transplanted during July-August at the 3 to 5-leaf stage, exhibit flowering in October-November. Observations were meticulously recorded, focusing on the days to flower initiation and the number of flowers.

Statistical Analysis

We adopted a Completely Randomized Design (CRD) for the experiment. Data was analyzed using ANOVA, followed by Duncan's Multiple Range Test ($p > 0.05$) for mean comparisons. All statistical analyses were conducted using SPSS software, ensuring the reliability of the results.

Results

Callus induction and callus relative growth

Callus induction was noticed as a slightly greenish unorganized mass of cells projecting out from ray florets when cultured on MS callus induction media consisting of 2,4-D and BAP at various concentrations. A significant difference was observed between each genotype for callus induction (%), days to callus induction, relative growth rate, shoot multiplication rate, number of shoots, shoot length, rooting, number of roots and acclimatization at various PGR combinations (Table 1), showing that the *in vitro* callus induction response varied with genotype.

Table 1. ANOVA for significance of factors and their interaction on the growth characteristics of *in vitro* plants.

Traits / Source of variation	Treatment	Genotype	T x G	Error
Degrees of freedom	6	3	18	84
Mean sum of square ($p \leq 0.05$ significance level)				
Callus induction (%)	4342.7	981.9*	157.4	5.1
Days to callus induction	1690.9	113.3*	12.1	4.9
Relative growth rate (g)	276.1	16.6*	6.04	1.02
Shoot multiplication rate	9886.4	500.5*	17.3	5.4
Number of shoot	1054.7	70.6*	3.4	4.0
Shoot length	330.3	27.7*	7.01	2.0
Rooting (%)	1222.9	236.7*	2.4	4.5
Number of root	129.4	58.0*	2.0	3.8
Acclimatization (%)	4327.0	818.3*	56.7	6.4

* $p \leq 0.05$

The callus induction rate of the four *Dendranthema* genotypes varied from 17.3 – 71.0 % depending on the concentration of PGRs supplemented in MS medium. The statistically significant highest rate of callus induction was recorded by genotypes CO-1 (71.0 and 59.3 %) and Super White (53.8 and 46.5 %) on media enriched with 2.0 mg L^{-1} 2,4-D + 0.100 – 0.200 mg L^{-1} BAP (Fig. 1, Table 2). The lowest callus induction (%) was recorded by genotype Pink marble (17.3 and 19.0 %) at 1.0 mg L^{-1} 2,4-D, implying that increasing the concentration of 2,4-D results in an increasing callus induction rate. None of the genotypes registered callus induction in the control treatment (MS basal media).

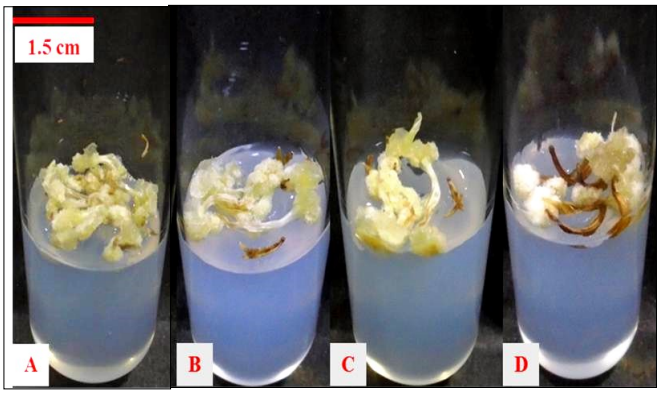

Fig. 1. *In vitro* callus induction responses of chrysanthemum genotypes over different PGR combinations: (**A**) CO-1; (**B**) Pink Marble; (**C**) Snapper; (**D**) Super White.

The response of genotypes for days to callus induction varied with different PGR treatments. The callus induction process and the nature of callus induction were influenced to a lesser extent by the amount of 2,4-D combined with the type of cytokinin supplemented. The days to callus induction ranged from 19.0 – 36.3 days (Table 2). A significant very early callus induction was observed for tered for Snapper and Pink marble on media supplemented with 1.0 mg L^{-1} 2,4-D + 0.100 – 0.150 mg L^{-1} BAP (Fig. 3 and 7, Table 2). The embryogenic calli were found to maintain higher relative fresh mass at a given PGR treatment. The results show that 2,4-D at 2.0 mg L^{-1} was optimum for recovering calli with high fresh mass.

Table 2. Response of genotypes for callus induction over different PGR combinations.

2,4-D mg L^{-1}	BAP mg L^{-1}	Callus induction (%)			
		CO-1	Pink marble	Snapper	Super White
Control	0.0	0.0 ± 0.0^{e}	0.0 ± 0.0^{e}	0.0 ± 0.0^{f}	0.0 ± 0.0^{e}
1	0.100	21.8 ± 1.3^{d}	17.3 ± 1.3^{d}	20.0 ± 1.5^{e}	27.8 ± 1.0^{d}
	0.150	21.5 ± 1.5^{d}	19.0 ± 1.1^{d}	21.8 ± 1.3^{de}	26.8 ± 1.6^{d}
1.5	0.100	33.0 ± 1.8^{c}	26.8 ± 1.7^{c}	26.0 ± 1.2^{c}	39.3 ± 1.3^{c}
	0.150	31.5 ± 1.4^{c}	24.0 ± 1.5^{c}	24.0 ± 1.7^{cd}	37.0 ± 1.1^{c}
2	0.100	71.0 ± 1.2a	39.5 ± 1.4a	38.5 ± 1.2a	53.8 ± 1.7a
	0.150	59.3 ± 1.3b	36.0 ± 1.5b	33.0 ± 1.2b	46.5 ± 1.5b
Days to callus induction					
Control	0.0	0.0 ± 0.0^{e}	0.0 ± 0.0^{d}	0.0 ± 0.0^{e}	0.0 ± 0.0^{d}
1	0.100	30.5 ± 1.2^{b}	24.8 ± 1.0^{a}	25.8 ± 1.0^{b}	30.0 ± 1.2^{b}
	0.150	34.8 ± 1.0^{a}	26.0 ± 1.1^{a}	29.0 ± 1.2^{a}	36.3 ± 1.3^{a}
1.5	0.100	25.3 ± 1.0^{c}	22.8 ± 1.0^{ab}	23.3 ± 1.3^{cd}	28.3 ± 1.0^{b}
	0.150	25.5 ± 0.7^{c}	24.5 ± 0.7^{ab}	24.5 ± 1.2^{bc}	29.5 ± 1.8^{b}
2	0.100	21.3 ± 1.0^{d}	19.0 ± 1.1^{c}	20.3 ± 1.0^{d}	21.3 ± 1.2^{c}
	0.150	24.0 ± 1.1^{cd}	21.3 ± 1.0^{bc}	21.5 ± 1.2^{cd}	23.0 ± 1.5^{c}
Relative growth rate of callus (g)					
Control	0.0	0.0 ± 00^{e}	0.0 ± 0.00^{f}	0.0 ± 0.0^{e}	0.0 ± 0.00^{e}
1	0.100	2.7 ± 0.07^{b}	2.3 ± 0.07^{cd}	2.1 ± 0.10^{d}	2.4 ± 0.07^{cd}
	0.150	2.4 ± 0.14^{d}	2.1 ± 0.10^{e}	2.0 ± 0.07^{d}	2.2 ± 0.13^{d}
1.5	0.100	2.8 ± 0.07^{c}	2.6 ± 0.05^{bc}	2.5 ± 0.12^{bc}	2.6 ± 0.06^{bc}
	0.150	2.6 ± 0.07^{cd}	2.4 ± 0.12^{c}	2.4 ± 0.07^{c}	2.5 ± 0.07^{c}
2	0.100	3.4 ± 0.10a	2.8 ± 0.12^{a}	2.8 ± 0.12^{a}	3.1± 0.13^{a}
	0.150	3.0 ± 0.07^{b}	2.7 ± 0.10^{b}	2.6 ± 0.12^{b}	2.8 ± 0.08^{b}

* Means ± standard errors within a column followed by the same letter are not significantly different according to Duncan's multiple range test at $p \leq 0.05$.

Pink marble (19.0 days) and Snapper (20.3 days) on medium enriched with 2.0 mg L^{-1} 2,4-D + 0.100 mg L^{-1} BAP.

However, a very high mean number of days to callus induction was recorded for Super White (36.3 days) and CO -1 (34.8 days) on media containing 1.0 mg L^{-1} 2,4-D + 0.150 mg L^{-1} BAP. The nature of calli induced either embryogenic (friable) or non embryogenic (compact) was to a great extent mediated by the concentration of 2,4-D in the media (Fig. 2). The friable callus observed was more likely at the concentration of 2.0 mg L^{-1} 2,4-D used in the media. With the increased concentration, the possibility of a compact callus was recovered.

The relative fresh mass of the callus significantly varied with genotype under different PGR treatments. The relative growth rate of the callus ranged from 2.0 - 3.4 g. The genotypes CO-1 and Super White recorded significantly high relative growth rates of 3.4 and 3.1 g respectively, on media enriched with 2.0 mg L^{-1} + 0.100 mg L^{-1} BAP. Likewise, the lowest growth rates of 2.0 and 2.1 g were regis-

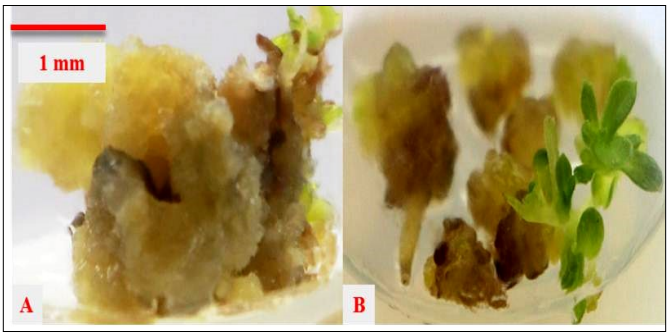

Fig. 2. Effect of 2,4-D on the nature of the callus: (**A**) Nonembryogenic; (**B**) Embryogenic.

Shoot induction, multiplication and elongation

All genotypes appeared to induce shoots when calli were cultured on a medium containing a balanced cytokinin:auxin ratio (Fig. 4, Table 3). The shoot induction rate ranged from 5.0 – 84.5 %. The highest mean rate of shoot induction was observed for Super White (84.5 %) and CO-1 (79.5 %) on MS shoots induction medium when

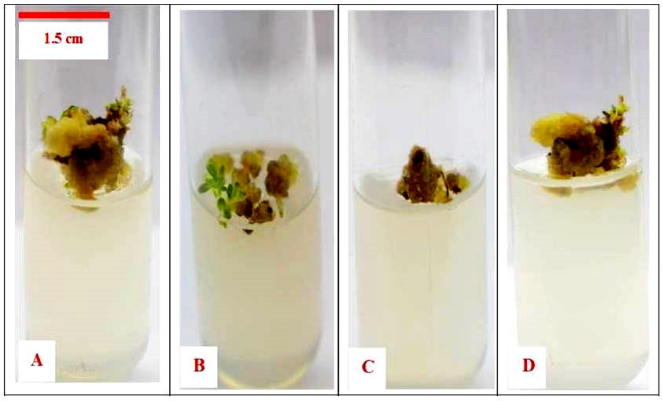

Fig.3. Relative growth of callus in response to different PGRs: (**A**) CO-1; (**B**) Pink Marble; (**C**) Snapper; (**D**) Super White .

supplemented with 2.0 mg L^{-1} BAP + 0.100 mg L^{-1} NAA. However, a significantly low rate of shoot induction was observed for Snapper (28.8 %) on medium enriched with 1.5 mg L^{-1} BAP + 0.100-0.150 mg L^{-1} NAA. In the control treatment (devoid of any PGRs), all genotypes recorded no shoot induction, showing that PGRs play an important role in shoot organogenesis.

The ability of a genotype to produce multiple shoots at a rapid rate was accessed through the numbers at which the shoots were produced. The highest mean number of shoots was recorded for Super White (30.3 shoots) and CO-1 (28.5 shoots) on media supplemented with 2.0 mg L^{-1} BAP + 0.100 mg L^{-1} NAA. The lowest mean number of shoots was observed for Snapper (15.0 shoots)

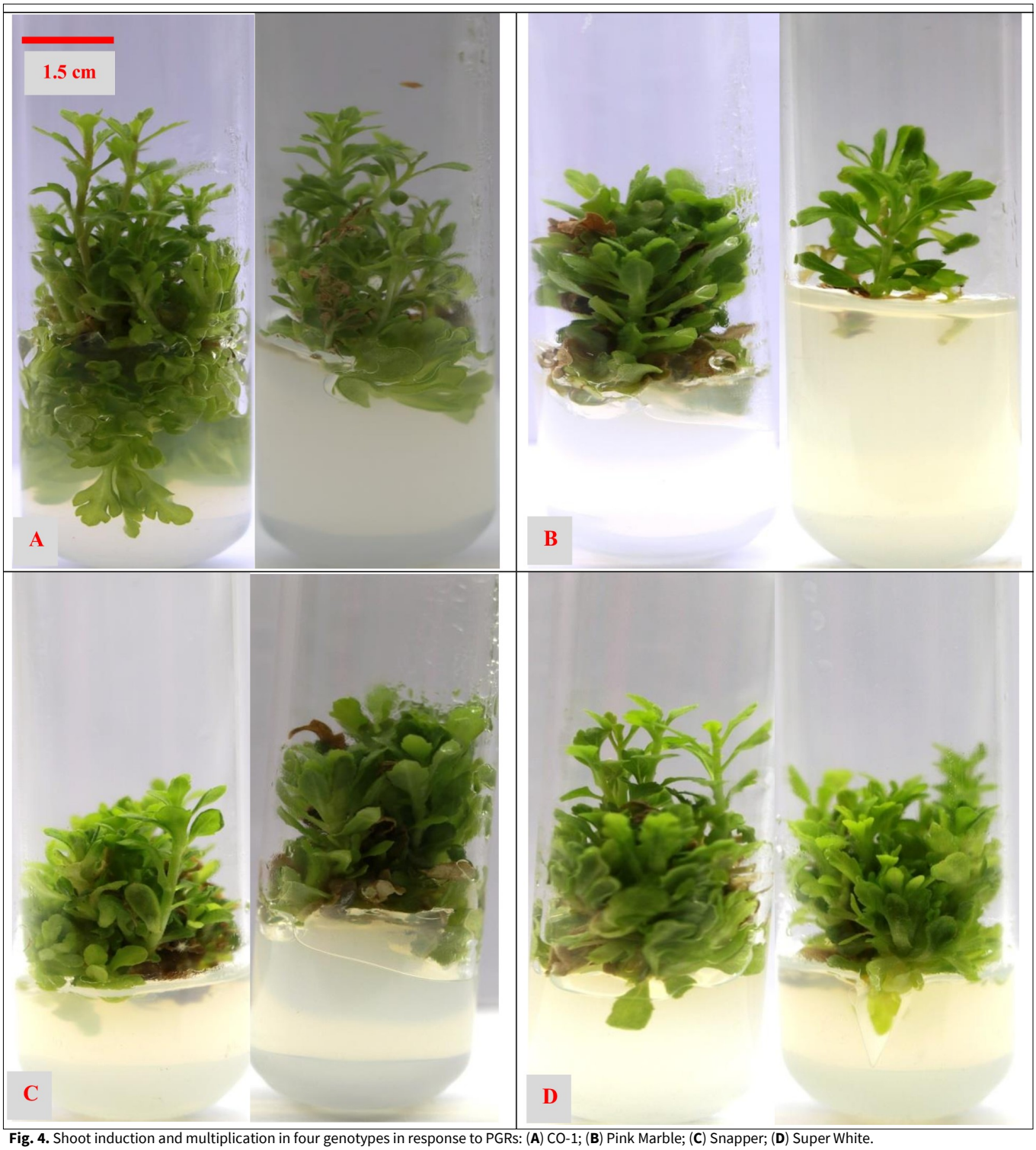

Fig. 4. Shoot induction and multiplication in four genotypes in response to PGRs: (**A**) CO-1; (**B**) Pink Marble; (**C**) Snapper; (**D**) Super White.

Table 3. Shooting response of *Dendranthema* genotypes over different PGR treatments.

BAP mg L^{-1}	NAA mg L^{-1}	Shoot induction (%)			
		CO-1	Pink marble	Snapper	Super White
Control	0.0	0.0 ± 0.0^{f}	0.0 ± 0.0^{e}	0.0 ± 0.0^{e}	0.0 ± 0.0^{f}
1.5	0.100	36.5 ± 1.5^{e}	33.5 ± 1.5^{d}	30.8 ± 1.3^{d}	38.8 ± 1.0^{e}
	0.150	$34.3 \pm 1.3e$	32.3 ± 1.0^{d}	28.8 ± 1.0^{d}	35.5 ± 0.7^{e}
2.0	0.100	79.5 ± 1.8^{a}	72.0 ± 1.1^{a}	69.0 ± 1.2^{a}	84.5 ± 1.1^{a}
	0.150	75.3 ± 1.8^{b}	65.5 ± 1.5^{b}	62.5 ± 1.2^{b}	77.3 ± 1.3^{b}
2.5	0.100	66.5 ± 1.4^{c}	62.3 ± 1.0^{b}	61.0 ± 1.1^{b}	68.5 ± 1.5^{c}
	0.150	62.3 ± 1.4^{d}	58.8 ± 2.0^{c}	56.8 ± 1.7^{c}	63.3 ± 1.8^{d}
Number of shoots					
Control	0.0	0.0 ± 0.0^{e}	0.0 ± 0.0^{e}	0.0 ± 0.0^{f}	0.0 ± 0.0^{e}
1.5	0.100	20.5 ± 1.4^{c}	18.8 ± 1.0^{d}	17.3 ± 1.0^{d}	22.5 ± 1.2^{c}
	0.150	18.3 ± 1.0^{d}	17.0 ± 1.1^{d}	15.5 ± 1.2^{e}	19.5 ± 1.1^{d}
2.0	0.100	28.5 ± 1.5^{a}	25.8 ± 1.4^{a}	25.0 ± 1.2^{a}	30.3 ± 1.0^{a}
	0.150	27.5 ± 0.7^{b}	24.5 ± 0.7^{a}	23.0 ± 1.2^{b}	28.5 ± 0.7^{b}
2.5	0.100	21.8 ± 1.6^{c}	22.5 ± 0.7^{b}	21.8 ± 1.0^{bc}	22.8 ± 1.4^{c}
	0.150	21.0 ± 1.1^{c}	20.3 ± 1.4^{c}	19.5 ± 1.2^{c}	21.8 ± 1.5^{cd}
Shoot elongation (cm)					
Control	0.0	0.0 ± 0.0^{d}	0.0 ± 0.0^{d}	0.0 ± 0.0^{e}	0.0 ± 0.23^{e}
1.5	0.150	8.2 ± 0.11^{b}	8.4 ± 0.09^{b}	8.3 ± 0.05^{bc}	7.4 ± 0.07^{b}
	0.200	8.8 ± 0.11^{c}	8.7 ± 0.10^{bc}	8.6 ± 0.09^{bc}	8.3 ± 0.14^{c}
2.0	0.150	$8.0 \pm 0.07c$	8.6 ± 0.12^{c}	8.5 ± 0.15^{d}	7.7 ± 0.12^{d}
	0.200	9.1 ± 0.13^{a}	9.4 ± 0.20^{a}	9.2 ± 0.15^{a}	8.9 ± 0.10^{a}
2.5	0.150	8.3 ± 0.11^{b}	8.5 ± 0.06^{ab}	8.4 ± 0.12^{b}	7.5 ± 0.15^{b}
	0.200	8.7 ± 0.12^{b}	8.9 ± 0.10^{b}	8.7 ± 0.07^{bc}	8.7 ± 0.13^{b}

* Means ± standard errors within a column followed by the same letter are not significantly different according to Duncan's multiple range test at $p \leq 0.05$.

and Pink marble (17.0 shoots) at 1.5 mg L^{-1} BAP + 0.150 mg L^{-1} NAA, implying that 2.0 mg L^{-1} BAP along with trace amounts of NAA was optimum for shoot multiplication (Fig. 4 and Fig. 8, Table 3).

After multiplication, the shoots were isolated and cultured on MS shoot elongation media consisting of NAA along with BAP, as it promotes internodal elongation. The shoot with the highest mean length was registered by pink marble (9.4 cm) and Snapper (9.2 cm) with a shoot length of 7.4 and 7.7 cm while, 7.5 cm was observed for Super White at 1.5 - 2.5 mg L^{-1} BAP + 0.150 mg L^{-1} NAA implies that the addition of NAA results in an increase in the shoot length with respect to their concentration (Table 3).

In vitro rooting of shoots

n vitro rooting of shoots was observed when the shoot was cultured on MS rooting media enriched with auxins, IBA/ IAA + NAA. When compared to IAA, more *in vitro* rooting was observed on a medium with IBA. Statistically significant very high rooting was recorded for Super white (92.3 %), CO-1 (91.5 %) and Pink marble (87.3 %) at 1.0 mg L^{-1} IBA + 0.150 mg L^{-1} NAA. However, the genotype Snapper recorded 71.3 % and 72.8 % at 1.0 mg L^{-1} IAA + 0.200 and 0.250 mg L^{-1} respectively, which was comparatively lower than that of IBA-enriched media. All the genotypes showed poor rooting in the control treatment, implying the importance of auxins for rooting (Fig. 5 and Fig. 8, Table 4). Better survival of tissue culture-raised plants requires the firm establishment of roots attributed to the number of roots. The Super white group recorded 19.3 and 18.3 roots at 1.0 mg L^{-1} + 0.150 and 0.200 mg L^{-1} NAA respectively,

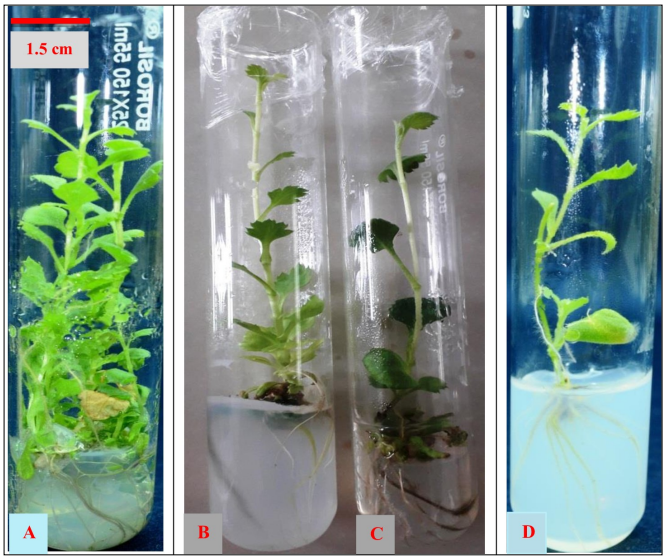

Fig. 5. *In vitro* rooting of shoots in response to PGR treatment: (**A**) CO-1; (**B**) Pink Marble; (**C**) Snapper; (**D**) Super White.

which was significantly higher than that of the control and other treatment combinations enriched with IAA. Likewise, the lowest mean number of roots was recorded by Pink marble (12.0 roots) at 1.0 mg L^{-1} IAA + 0.200 mg L^{-1} NAA (Table 4).

Acclimatization and flowering

In vitro-raised plants were transferred to pots containing various soil substrates and observed for acclimatization. The genotypes Super White (71.3 %) and CO-1 (68.3 %) registered the highest acclimatization on vermicompost +

Table 4. *In vitro* rooting of shoots in four *Dendranthema* genotypes over different rooting media.

IBA/IAA mg L^{-1}	NAA mg L^{-1}	Rooting (%)			
		CO-1	Pink marble	Snapper	Super White
Control	0.0	11.5 ± 0.7^{e}	10.3 ± 0.6^{d}	8.8 ± 1.0^{e}	12.5 ± 0.7^{d}
	0.150	91.5 ± 1.2^{a}	87.3 ± 1.0^{a}	84.0 ± 1.1^{a}	92.3 ± 1.3^{a}
IBA 1 mg L^{-1}	0.200	81.8 ± 1.3^{b}	79.8 ± 1.3^{b}	77.0 ± 1.5^{b}	82.8 ± 1.3^{b}
	0.250	80.8 ± 1.6^{c}	78.0 ± 2.1^{b}	76.0 ± 2.4^{b}	82.3 ± 1.3^{b}
	0.150	81.0 ± 1.2^{bc}	77.8 ± 1.2^{bc}	75.3 ± 1.4^{c}	82.5 ± 0.7^{b}
IAA 1 mg L^{-1}	0.200	77.8 ± 0.9^{d}	74.8 ± 1.0^{cd}	71.3 ± 1.0^{d}	79.5 ± 0.7^{c}
	0.250	77.5 ± 0.7^{d}	75.3 ± 1.0^{c}	72.8 ± 1.4^{cd}	79.0 ± 0.5^{c}
Number of root					
Control	0.0	10.5 ± 0.7^{e}	5.8 ± 1.0^{e}	7.5 ± 0.7^{f}	12.3 ± 1.0^{e}
	0.150	18.0 ± 1.2^{a}	16.0 ± 1.1^{a}	17.8 ± 0.9^{a}	19.3 ± 1.1^{a}
IBA 1 mg L^{-1}	0.200	17.0 ± 1.3^{b}	15.3 ± 1.2^{b}	16.3 ± 1.1^{b}	18.3 ± 1.3^{ab}
	0.250	16.0 ± 1.2^{c}	14.3 ± 1.4^{c}	15.0 ± 1.2^{c}	17.0 ± 1.2^{b}
	0.150	16.0 ± 1.^{1c}	13.8 ± 1.3^{cd}	14.3 ± 1.4^{cd}	16.0 ± 1.1^{c}
IAA 1 mg L^{-1}	0.200	15.3 ± 1.0^{cd}	13.0 ± 1.2^{cd}	13.8 ± 1.5^{d}	15.8 ± 1.0^{c}
	0.250	14.0 ± 1.2^{d}	12.0 ± 0.5^{d}	12.8 ± 0.6^{e}	14.3 ± 1.5^{d}

* Means ± standard errors within a column followed by the same letter are not significantly different according to Duncan's multiple range test at $p \leq 0.05$.

Red soil + coir pith media over the control and other substrates used (Fig. 6 and 8, Table 5). Similarly, the lowest acclimatization rate of 25.5 % was observed for Snapper on vermicompost + Red soil media. On control (sandy loam soil) media, the acclimatization rate was low for all genotypes.

During the period from the second week of October to December, field-grown chrysanthemum plants, encompassing both non-tissue culture and tissue culture-raised

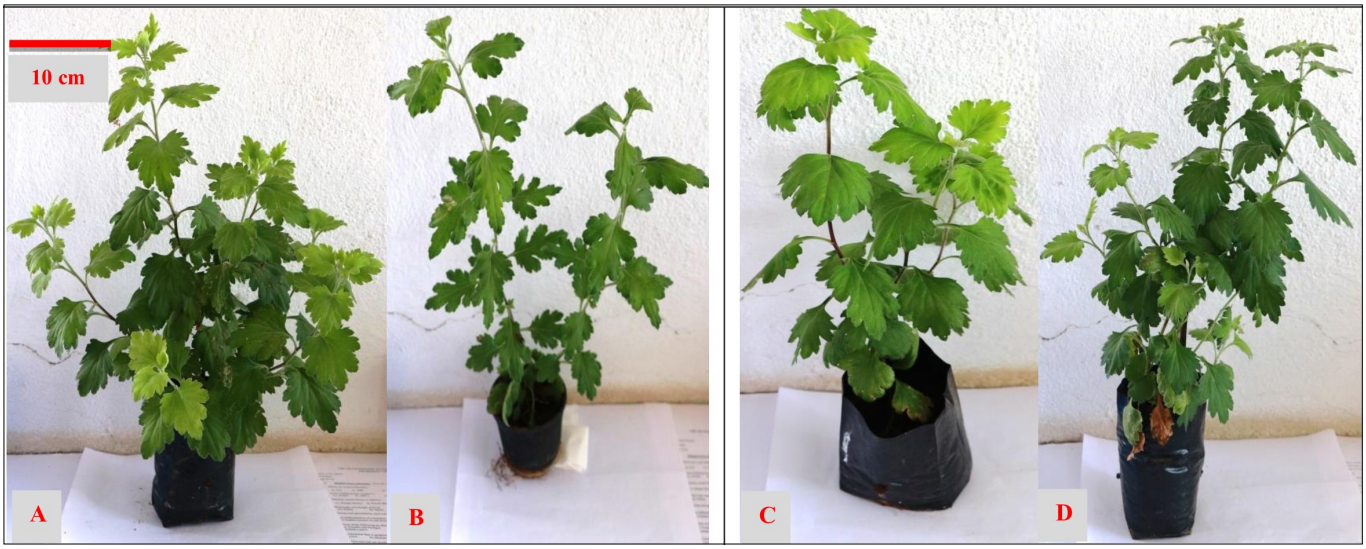

Fig. 6. *Ex vitro* acclimatization of tissue culture- raised plants: (**A**) CO-1; (**B**) Pink Marble; (**C**) Snapper; (**D**) Super White.

Table 5. *Ex vitro* acclimatization of *Dendranthema* genotypes over different soil substrates.

	Acclimatization (%)			
Soil substrate (*v*:*v*)	**CO-1**	**Pink marble**	**Snapper**	**Super White**
Sandy loam soil (Control)	14.5 ± 1.5^{e}	10.8 ± 1.5^{e}	9.8 ± 1.0^{e}	15.3 ± 1.8^{e}
Vermi Compost: Red soil: Sand	43.0 ± 1.7^{b}	32.0 ± 1.1^{c}	30.5 ± 1.4^{c}	45.5 ± 1.2^{c}
Vermi Compost: Red soil	35.3 ± 1.7^{c}	30.3 ± 0.9^{d}	25.5 ± 1.2^{cd}	35.5 ± 1.4^{d}
Coir pith: Red soil	30.3 ± 0.6^{d}	26.8 ± 1.0^{de}	27.0 ± 1.5^{d}	31.0 ± 1.7
FYM: Red soil: Sand	33.5 ± 1.2^{cd}	32.5 ± 0.7^{cd}	31.3 ± 0.9^{c}	34.0 ± 1.8^{de}
Vermi Compost: FYM: Red soil	60.3 ± 1.3^{ab}	49.0 ± 1.1^{b}	43.0 ± 1.2b	65.5 ± 1.1^{ab}
Vermi Compost: Red soil: Coir pith	68.3 ± 3.3^{a}	54.5 ± 1.5^{a}	52.5 ± 1.7^{a}	71.3 ± 1.4^{a}

* Means ± standard errors within a column followed by the same letter are not significantly different according to Duncan's multiple range test at $p \leq 0.05$.

varieties, exhibited flower bud initiation. The Pink Marble (Control) variety demonstrated the earliest mean flowering across all GA_3 spray treatments, including the control, in comparison to tissue culture-raised Pink Marble plants. Pink Marble in particularly at 100 and 150 mg L^{-1} GA_3 spray treatments, displayed a comparatively early mean flowering of 111.7 and 113.3 days respectively, as opposed to the control (125.5 days) (Table 6). Tissue culture-raised Pink Marble plants showed a consistent and significantly high mean number of flowers per plant at all GA_3 treatment levels. Notably, at 100 mg L^{-1} GA_3 concentration, tissue culture -raised Pink Marble plants exhibited a very high mean number of flowers per plant (53.3 flowers/plant^{-1}) compared to the control (36.3 flowers/plant^{-1}) (Table 6, Fig. 7). The application of exogenous GA_3 significantly influenced both the days to flowering and the number of flowers per plant, with specific concentrations producing distinct effects.

2,4-D in combination with BAP stimulates the callus induction process. A study reported that callus induction in *C. grandiflorum* using ray florets cultured on media containing 2,4-D + BAP was in line with our result (17). Conversely, another study reported callus induction in *C. morifolium* using ray florets on media enriched with BAP + NAA instead of 2,4-D, in contrast to our result (18). Similar to callus induction, the days to callus induction and the relative growth rate of calli also appeared to be significantly influenced by the exogenous supply of auxins. Furthermore, the embryogenic callus (capable of producing plantlets) undergoes caulogenesis, which yields shoot organogenesis. This result firmly implies that while increasing the 2,4-D level above 2.0 mg L^{-1} is likely to cause more callus proliferation, it results in compact calli (nonembryogenic). It was observed that organogenesis from the chrysanthemum species *D. grandiflora* using a wide range of PGRs was in agreement with the present result (19).

Table 6. Flowering of *in vitro*- raised plants in *Dendranthema* genotypes over different GA_3 concentrations.

GA_3 mg L^{-1}	Days to flowering		No. of flowers/plant^{-1}	
	Pink Marble Non tissue culture plants	Pink Marble tissue culture plants	Pink Marble Non tissue culture plants	Pink Marble tissue culture plants
Control	97.3 ± 1.3cd	125.5 ± 0.9^{e}	26.3 ± 1.6^{d}	36.3 ± 1.8^{e}
50	96.9 ± 1.7d	120.3 ± 1.7^{d}	31.2 ± 1.9^{bc}	45.7 ± 2.1^{d}
100	93.3 ± 0.6c	111.7 ± 0.8a	34.1 ± 1.1^{c}	53.3 ± 1.3^{b}
150	91.7 ± 1.4^{a}	113.3 ± 1.3^{b}	38.7 ± 1.3^{a}	51.6 ± 1.7^{c}
200	92.5 ± 0.7^{b}	117.8 ± 1.1^{c}	33.9 ± 1.7^{b}	50.7 ± 1.5^{a}

* Means ± standard errors within a column followed by the same letter are not significantly different according to Duncan's multiple range test at $p \leq 0.05$.

Fig. 7. Exogenous application of GA_3 and flowering response of chrysanthemum (**A**) Non-tissue culture Pink Marble (**B**) Tissue culture-raised Pink Marble.

Discussion

Dendranthema is the most accessible ornamental plant with reduced seed setting and is prone to severe disease infection through vegetative propagation. *In vitro* techniques offer a possible means of rapid multiplication of species; otherwise, propagation *via* seed is difficult. The present regeneration experiment in *Dendranthema* through indirect organogenesis *via* callus culture shows that each genotype varied with call induction in response to PGR treatment. In this species, callus induction through ray florets seemed to be simple and effective while initiating the culture on MS media supplemented with 2.0 mg L^{-1} 2,4-D + 0.100 mg L^{-1} BAP. This result suggests that the highest call induction was registered by CO-1, indicating that

Shoot organogenesis is the most crucial stage of *in vitro* regeneration. Caulogenesis is directed by a balanced ratio of cytokinin and auxin. The results showed that the shoot induction rate was at a maximum (Super White) when calli were cultured on media supplemented with 2.0 mg L^{-1} BAP along with 0.100 mg L^{-1} NAA. A concentration beyond this level results in the inhibition of shoot induction and multiplication occurs as a result of the interaction between PGRs and polyamines synthesized in *in vitro* culture (9, 18, 20), observed that caulogenesis and somatic embryogenesis on media containing BAP + NAA were similar to our results. However, a study reported a maximum rate of shoot induction when transferring calli to MS shoot induction media supplemented with 9.3 µM kinetin in combination with 4.9 µM IBA (17), which was counter to our present report. The shoot number is an important attribute of the genotype for rapid multiplication. The results revealed that the genotypes Super White and CO-1 registered a high number of shoots at 2.0 mg L^{-1} BAP in combination with 0.100 mg L^{-1} NAA, indicating that *in vitro* shoot induction was positively associated with genotype and concentration of BAP in the medium. A similar trend was noticed (19) who cultured calli from mutagenized ray florets of single standard Chrysanthemum on MS medium enriched with 0.5 mg L^{-1} BAP and 0.2 mg L^{-1} NAA. The mean shoot length result revealed that the genotype Pink Marble registered the highest shoot elongation on media enriched with 2.0 mg L^{-1} in combination with 0.200 mg L^{-1} NAA.

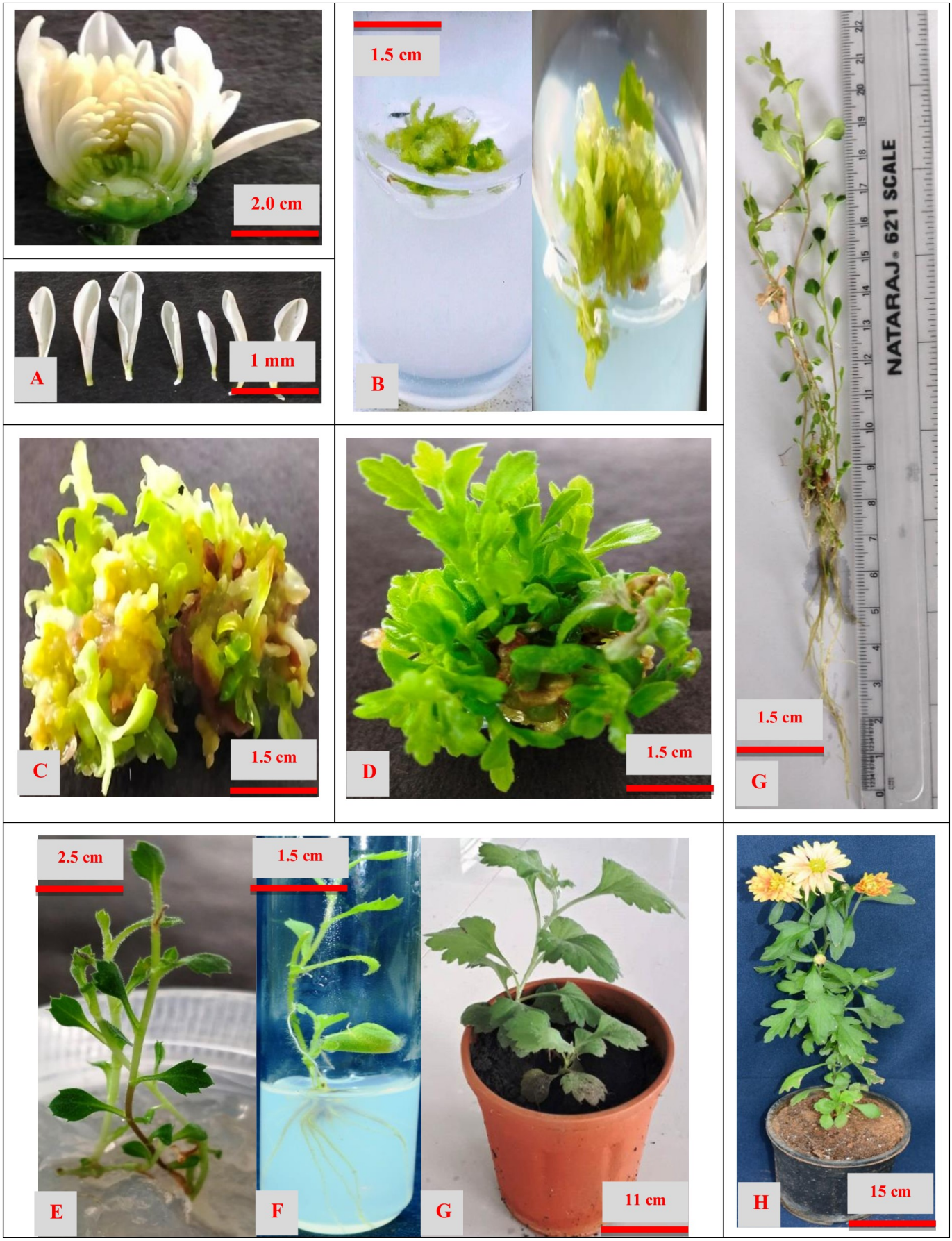

Fig. 8. *In vitro* regeneration of Chrysanthemum, (A) Inflorescence – ray florets explant; (B) Callus induction; (C) Axillary shoot induction; (D) shoot multiplication; (E) Axillary shoot elongation; (F) *In vitro* rooting of axillary shoots; (G) *In vitro*–raised plant; (H) *Ex-vivo* acclimatization; (I) Flowering of tissue culture-raised plant.

Supplementing auxin and NAA in MS shoot elongation media in the presence of cytokinin, BAP promotes shoot elongation by adjusting shoot nodal length. While culturing calli on BAP-enriched media with lower concentrations of NAA, slight or no shoot elongation was observed. The present report supports findings in other varieties of Chrysanthemum, where shoot elongation of shoots was achieved

when calli were cultured on media supplemented with BAP and NAA (21, 22).

In vitro rooting of shoots required the presence of either IBA/IAA or trace amounts of NAA in the media. A small number of roots was observed even on auxin-free medium (control), indicating the role of endogenous auxin in *in vitro* rhizogenesis. The results showed that genotypes Super White and CO-1 had the highest rooting (%) and number of roots at 1.0 mg L^{-1} IBA in combination with 0.150 mg L^{-1} NAA, indicating an added advantage of rooting in combination rather than alone. When compared to IAA, a greater genotypic response was observed on IBA-supplemented medium. The earliest report of (23, 24) on *in vitro* rooting of shoots from ray florets on IBA- and NAA-containing medium was in line with our result. Acclimatization is one of the crucial stages in the tissue culture of plants of commercial importance. In the present study, *in vitro* plantlets were transferred to various soil substrates for acclimatization and showed a significantly high acclimatization rate by Super White and CO-1 on vermicompost + red soil + coir pith when compared to others and the control treatment. The result represents an impression of the vermi compost + red soil + coir combination for better survival of *in vitro*-raised plants under *ex vivo* conditions. A study transferred *in vitro* plantlets to polybags with vermicompost (25); another study transferred plantlets to glass jars with peat:soilrite (1:1, v:v) (13); other 2 studies transferred regenerated plantlets to pots with vermiculite:soil (1:1, v:v) (26) and (27), which was the earliest report available on the acclimatization of tissue culture-raised plants in Chrysanthemum.

Flowers serve as the specialized reproductive organs in plants and their significance is particularly notable in ornamental species such as chrysanthemums (28). These flowers hold considerable importance and find diverse applications across various sectors. In this study, we investigated the impact of the growth regulator GA_3 on the flowering of *in vitro*-raised chrysanthemum plants. Our results revealed a significant influence of GA_3 on flower bud initiation, particularly in tissue culture-raised plants compared to conventionally grown ones. The exogenous application of GA_3 demonstrated a remarkable ability to expedite the flowering process, with notable effects observed within a short span of 7-8 days. The most favorable outcome was achieved at a concentration of 100 mg L^{-1} GA_3, where tissue culture plants exhibited an earlier initiation of flowering and produced a higher quantity of quality flowers compared to normal field-grown plants. The results convincingly demonstrate that there are minimal differences in duration between plants arising from tissue culture and conventional methods. Plants propagated from non-tissue culture sources, through rooted cuttings, take approximately one month from cutting to sprouting and rooting to field planting. Our result was in accordance with the report of (29-31) in *Chrysanthemum*. In contrast, (32, 33) found that spraying 150 mg L^{-1} of GA_3 resulted in the earliest and highest number of flowers in chrysanthemums. These findings underscore the potential of GA_3 to efficiently adjust flowering timing, presenting a promising avenue for the cut flower industry. The rapid multiplication and superior flower yield observed in tissue culture-raised plants emphasize the practical applications of this approach in enhancing ornamental plant production.

Conclusion

Optimizing plant growth regulator concentrations in tissue culture media is crucial for efficient *in vitro* regeneration of *Dendranthema*, impacting callus induction, shoot organogenesis and rooting. Genotypic variations in response to these treatments underscore the need for tailored protocols. These findings hold promise for enhancing ornamental plant production through tissue culture techniques.

Authors' contributions

GT led the conception and design of the study, performed key tissue culture experiments, statistical analyses, interpretation of results, drafting and revising the manuscript. NN supplied necessary materials, aided in data analysis and interpretation and provided substantial contributions to drafting and revising the manuscript. VP conducted the statistical analyses and contributed to the interpretation of results. He was involved in writing sections of the manuscript and made important revisions. TV contributed to the experimental design and carried out a significant portion of the tissue culture work.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None.

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