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# REGENERATION DYNAMICS AND SECONDARY METABOLITES IN *DENDRANTHEMA GRANDIFLORUM* (RAMAT.) KITAM THROUGH TISSUE CULTURE

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**Abstract**

Shoot tips and ray florets were utilized as explants for rapid mass propagation of *Dendranthema grandiflorum. In vitro* shoot regeneration frequencies of 88 and 80 % were observed in shoot tips and ray florets, respectively, when cultured on MS medium supplemented with 1.0 mg/l BAP and 1.0 mg/l Kn, and BAP (1.0 mg/l) + NAA (0.5 mg/l). For rhizogenesis, microshoots transferred to half-strength MS medium supplemented with IBA; 0.2 mg/l) and IAA; 0.2 mg/l) achieved 80% root induction within 3-4 weeks. Comparative phytochemical profiling confirmed the presence of total phenols, tannins, and flavonoids in the one month old micropropagated clones. Quantifying the foundational phenolic and flavonoid pools prior to transplantation allows researchers to gauge whether the juvenile tissues possess a sufficient biochemical buffer to survive the acclimatization phase. *Ex vitro* hardening of well-developed plantlets yielded a 95% survival rate, followed by successful field acclimatization. Notably, the regenerated plants exhibited accelerated phenology, displaying earlier flowering compared to the donor mother plant.

## Introduction

*Dendrenthema grandiflorum* (Ramat.) Kitam commonly known as Chrysanthemum and Queen of the East, belongs to the family Asteraceae (Roopa *et al.* 2018). It is one of the most popular cut flowers in the world (Negi *et al.* 2015). Chrysanthemum is native to China and was first cultivated as a flowering herb back in the 15$^{th}$ century BC. It is the world's second economically important floricultural crop after roses. About 2000 varieties of chrysanthemum have been reported from the world, and about 1000 varieties from India, and about 160 species in the genus Chrysanthemum (Kalia 2015). Chrysanthemum holds a very important position in the medicinal world and its medicinal properties are due to the presence of natural antioxidants. The health benefits of chrysanthemum flowers have been reported to include anti-inflammatory, antipyretic, sedative, antiarthritic, and antihypertensive properties (Han *et al.* 2019). The economic and medicinal value of chrysanthemum increases its demand for large scale production.

Traditionally, this crop is propagated through root suckers and terminal cuttings which have slow growth, higher chances of diseases, pest attack and delayed flowering which results in low production. Hence, the *in vitro* propagation technique is a better approach to produce large scale disease free quality planting material throughout the year irrespective of the season (Chee *et al.* 2015, Kazmi *et al.* 2015). The importance and demand of chrysanthemum has never diminished. In view of its economic value and demand in the market, there is a need to enhance the productivity of high quality planting materials. Embracing micropropagation ensures that the supply chain can dynamically meet commercial volumes, thereby securing both agricultural productivity and market profitability for this globally prized ornamental. Keeping in view the

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importance and demand of Chrysanthemum. This study investigates the Regeneration Dynamics and Secondary Metabolites in *D. grandiflorum* establishing an efficient tissue-culture framework to meet growing commercial demand.

**Materials and Methods**

*Dendranthema grandiflorum* was maintained in the field of the Department of Biotechnology, College of Horticulture and Forestry, Neri, Hamirpur. The healthy shoot tips and ray florets were excised from mother plants, washed thoroughly and proceeded for explant sterilization (Roopa *et al*. 2018). The ray florets were placed horizontally on MS medium containing NAA and IAA in combination with BAP and kinetin. The concentrations of auxins were 0.1 - 1.0 mg/land cytokinins were at 0.1 to 2.0 mg/l. All the cultures were kept under a 16 hrs photo period and 8h dark period cycle in a growth room with intensified 1000 LUX at 24 ± 2°C. The percentage and the days of shoot regeneration was recorded. Same medium was used for direct shoot induction from shoot tips. Individual shoots of 2-3 cm length were excised and inoculated on shoot multiplication MS medium containing different concentrations of BAP (1.0 -2.0 mg/l), NAA (0.5-2.0 mg/l) and Kn (0.5 -1.0 mg/l). Average number of shoots per explant and average length of shoots were recorded. After 4 weeks, the elongated shoots were transferred to half-strength MS medium containing different concentrations and combinations of IBA and 1AA. The number of days taken for root induction and the percentage of root induction were recorded.

The plantlets were removed from the culture tubes and washed to remove traces of media on the root surfaces. The plants were first treated with fungicide and then transferred to a potting mixture containing perlite and cocopeat with a ratio of 2 : 1. The pots were placed in plastic covers with some holes at 26 ± 2°C under a 16 hrs photoperiod for one month. After the first week, the plastic cover was removed. The one month old plants were used for phytochemical analysis.

Leaves of *in vitro* raised as well as mother plants were collected, dried at room temperature and grounded into a fine powder. 1g of powdered tissues was extracted with 25 ml of methanol and kept for 48 h in an orbital shaker at 25°C. The extract was filtered through Whatmann filter paper, the filtrate was dried using a hot water bath and the powder was used for phytochemical analysis.

Total phenolic content and tannins of plant extracts was estimated by the Folin-Ciocolteu method (Yeasmin *et al*. 2016). Total phenolic compounds were expressed as mg gallic acid equivalent per 100g (mg GAE/100g) of the dry plant extract. Total flavanoid content was measured by using the aluminum chloride colorimetric method (Aryal *et al*. 2019). The study was conducted in controlled environment using a completely randomized design (CRD) (Gomez and Gomez 1984) and all the experiments were performed in replicates. Statistical analysis was conducted using MS-Excel and OPSTAT. All the data was expressed as mean ± SE .The data for shoot tips was calculated as three explants per flask and for ray florets as four explants per flask.

**Results and Discussion**

The morphogenic plasticity of *D. grandiflorum* was heavily modulated by the choice of explant type and the exogenous balance of plant growth regulators (PGRs). In all experimental controls involving MS basal medium was devoid of PGRs (T1), zero morphogenic response or shoot tip regeneration was documented, validating that exogenous hormonal triggers are essential to disrupt cell dormancy in *D. grandiflorum*.

For direct organogenesis, the highest *in vitro* shoot tip proliferation rate (80.0%) was achieved on the T13 medium within 29 days (Table 1). This rapid direct development circumvents a callus phase, making it highly valuable for clonal fidelity. Thirteen treatments were tested for *in vitro*

shoot regeneration from ray floret explants. MS medium without growth regulators did not induce any shoot formation whereas, media optimized with specific concentration of auxin-cytokinin initiated callogenesis within 12-15 days, followed by visible shoot organogenesis after 20-25 days of culture. Maximum indirect shoot initiation and regeneration from callus was observed on T2 (78.3%) within 40 days window (Table 2), whereas T11 exhibited the minimum regenerative capacity, achieving only 19.66% shoot formation over an extended period of 52 days. Kn and IAA along with NAA+ BAP, were also used for shoot regeneration in a number of studies (Khan *et al.* 2020).

**Table 1. *In vitro* shoot regeneration of *Dendranthema grandiflorum* using shoot tips and ray florets.**

| Treatments | Shoot regeneration (%) | Average days for shoot regeneration |
|---|---|---|
| T1 (0.0+0.0) | - | - |
| T2 (1.0 BAP+0.5 NAA) | 74.33 (59.56) | 29 |
| T3 (1.0 BAP+1.0 NAA) | 69.33 (56.35) | 30 |
| T4 (1.5 BAP+0.5 NAA) | 63.66 (52.51) | 35 |
| T5 (1.5 BAP+1.0 NAA) | 58.66 (49.97) | 36 |
| T6 (2.0 BAP+1.0 NAA) | 57.00 (49.01) | 37 |
| T7 (2.0 BAP+1.5 NAA) | 51.33 (45.74) | 29 |
| T8 (2.0 BAP+2.0 NAA) | 48.66 (44.21) | 32 |
| T9 (2.5 BAP+1.0 NAA) | 47.66 (43.64) | 36 |
| T10 (1.0 BAP+2.0 NAA+0.1 IAA) | 50.66 (45.36) | 35 |
| T11 (1.0 BAP+2.0 NAA+0.2 IAA) | 53.66 (47.08) | 29 |
| T12 (2.0 BAP+2.0 NAA+0.2 IAA) | 44.66 (41.91) | 36 |
| T13 (1.0 BAP+1.0 Kn) | 80.00 (63.44) | 29 |
| T14 (1.5 BAP+1.0 Kn) | 78.00 (62.03) | 30 |
| T15 (2.0 BAP+1.0 Kn) | 67.66 (55.32) | 36 |
| T16 (2.5 BAP+0.5 Kn) | 61.00 (51.33) | 36 |
| T17 (1.0 BAP+0.5 NAA+0.5 Kn) | 54.66 (47.25) | 35 |
| T18 (2.0 BAP+1.0 NAA+0.5 Kn) | 50.33 (41.17) | 32 |
| **$C.D_{0.05}$** | **2.250** | |

Values in paranthesis are arc sine transformed values.CD : Critical difference

The proliferated shoots were transferred on shoot multiplication media. The maximum average number of multiple shoots (6.0) was achieved on T5 with 7.5cm of shoot length while, minimum (2.0) shoots with a length of 2.1cm on T2. Similarly, MS medium with 1.0 mg/lBAP and 0.5 mg/l NAA proved effective for shoot multiplication (Pant *et al.* 2015). Among the 10 medium compositions, T6 was identified as the best medium for *in vitro* root induction which showed 80% rooting with 2.6 cm root length after 21 days. Many researchers have similarly verified that half-strength MS medium supplemented with IAA or Indole-3-butyric acid (IBA), either alone or combined, provides the ideal osmotic and hormonal environment for robust root induction in *Dendranthema* species (Faisal *et al.* 2018, Khan *et al*. 2020, Imtiaz *et al.*2019). The highest survival rate (95.0%) of raised plants was observed on Cocopeat : Perlite (1 : 2). This

loose, highly porous mixture provided excellent aeration for the fragile *in vitro* root systems, minimizing transplant shock.

**Table 2. *In vitro* shoot regeneration of *Dendranthema grandiflorum* using ray florets.**

| Treatments | Shoot regeneration (%) | Average days for shoot regeneration |
|---|---|---|
| T1 (0.0+0.0) | - | - |
| T2 (1.0 BAP+0.5 NAA) | 78.33 (62.23) | 40 |
| T3 (1.0 BAP+1.0 NAA) | 67.66 (55.32) | 42 |
| T4 (2.0 BAP+0.5 NAA) | 63.66 (52.91) | 42 |
| T5 (2.0 BAP+1.0 NAA) | 70.33 (56.97) | 52 |
| T6 (2.0 BAP+2.0 NAA) | 67.00 (54.92) | 49 |
| T7 (0.2 BAP+0.5 IAA+0.1 Kn) | 75.66 (60.42) | 45 |
| T8 (0.5 BAP+0.5 IAA+0.2 Kn) | 52.33 (46.32) | 44 |
| T9 (1.0 BAP+1.0 IAA+0.5 Kn) | 46.66 (43.07) | 42 |
| T10 (0.1 NAA+1.0 Kn) | 25.00 (29.92) | 45 |
| T11 (0.2 NAA+2.0 Kn) | 19.66 (26.31) | 52 |
| T12 (2.0 BAP+1.0 NAA+0.1 Kn) | 45.66 (42.49) | 51 |
| T13 (2.0 BAP+1.0 NAA+0.2 Kn) | 50.33 (45.17) | 44 |
| **$C.D_{0.05}$** | **2.427** | |

Spectrophotometric monitoring of the methanolic extracts demonstrated that the mother plant possessed a significantly higher concentration of total phenolics (8.84 ± 0.08 GAE mg/g leaf weight). In comparison, the one-month-old *in vitro* raised plantlets exhibited a reduced accumulation of total phenolics (5.49 ± 0.07 mg/g leaf weight). A parallel trend was observed in the flavonoid profiles. The leaf extracts of the mother plant displayed a significantly superior total flavonoid content value of 4.49 ± 0.19 mg QE/g, whereas *in vitro* raised plants had noticeably lower (1.6 ± 0.40 mg QE/g) baseline content. Methanolic extract of leaf from the mother plant showed higher tannin content i.e 7.62 ± 0.10 mg TAE/g leaf weight, as compared to *in vitro* plant extract (4.86 ± 0.06 mg TAE/g). The screening results showed that total phenolics, flavonoids and tannins are heavily accumulated in the mother plant. In contrast, in one month old micropropagated plants phenolic-to-flavonoid ratio confirms that downstream flavonoid synthesis is restricted in tissue culture. However, the prioritized foundational phenolic pool (5.49 ± 0.07 mg/g leaf weight) an essential antioxidant reservoir that the plantlet utilizes to counter transplantation shock and light-induced oxidative stress during *ex vitro* hardening (Mansinhos *et al*. 2022).

Following an initial seven to eight week hardening phase and quantification of phytochemicals, these robust plantlets were successfully shifted to standard earthen pots containing a soil and farmyard manure matrix. The micropropagated plants reached full maturity and displayed normal phenological development, ultimately producing healthy, true-to-type flowers. The complete micropropagation scheme from explant establishment to *ex vitro* hardening is sequentially detailed in Fig. 1.

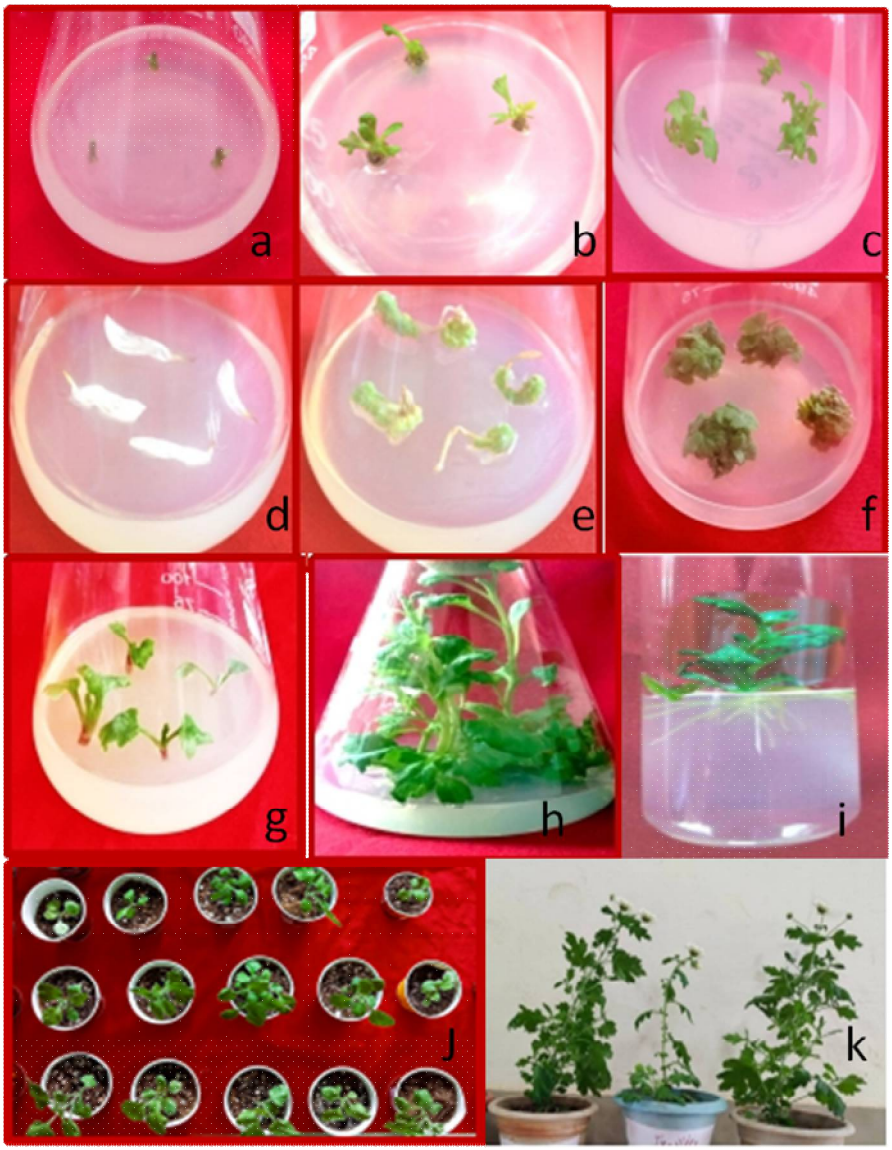


Fig. 1. *In vitro* regeneration of *Chrysanthemum morifolium.* a: shoot tip explant in T13, b: shoot proliferation in T13, c: shoot elongation in T15, d: ray florets explant in T2, e: callus induction in T2, f: shoot clump formation, g: subculturing in shoot, h: shoot multiplication, i: rooting in T6 , j: one month old plants , and k: flowering after 55 days.

This study establishes an efficient, high-frequency *in vitro* micropropagation protocol for *Dendranthema grandiflorum* using shoot tip and ray floret explants. Early accunulation of phenolic and flavonoid provided the biochemical resilience necessary for successful hardening of plants.The accelerated flowering of the regenerants confirms this protocol's commercial viability for the rapid mass production of this important ornamental species.

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