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Influence of BAP with TDZ growth regulators on *in vitro* regeneration in chrysanthemum (*Dendranthema grandiflora* T.) cv. Marigold

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Abstract
An efficient protocol for *in vitro* regeneration of Chrysanthemum (*Dendranthema grandiflora* T.) cv. Marigold was developed. In the present study shoot, tips and nodal segments were used as explants. Nodal explants responded better when compared to shoot tips. Explants were cultured on Murashige and Skoog (MS) medium with different concentrations of 6-benzylaminopurine (BAP) and Thidiazuron (TDZ) alone and with combinations. An intermediate level of BAP (1.0 mgL^{-1}) showed its superiority over other concentrations by giving early shoot initiation (12.10 days) with 3.54 5.33 and 6.25 number of shoots per explant. and maximum shoot length (3.25, 5.85 and 6.99 cm) obtained at 30, 60 and 90 days after culture. MS medium supplemented with BAP 1.5 mgL^{-1} + TDZ 0.4 mgL^{-1} gave early shoot initiation (12.40 days), maximum number of shoots per explant (5.25, 8.65 and 11.90) and maximum shoot length (4.45, 5.55 and 7.45 cm) at 30, 60 and 90 days after shoot initiation. Satisfactory rooting response was obtained in half strength MS medium supplemented with 0.2 mgL^{-1} indole butyric acid (IBA).

Keywords: Chrysanthemum, *In vitro* culture, shoot tips, nodal segments, cytokinins, auxins

Introduction
Chrysanthemum (*Dendranthema grandiflora* T.) is an herbaceous flowering plant extensively grown in all around the world. The chrysanthemum belongs to family Asteraceae. It is mainly grown in the southern part of the country and supplying loose flowers to the market for making garlands, decoration and for worship purpose. It is mainly grown in the states of Bihar, Gujarat, Karnataka (Bengaluru, Kolar, Hassan, Tumkur etc.) Madhya Pradesh, Maharashtra, Rajasthan and Tamil Nadu.

Chrysanthemum name is derived from the Greek words "Chryos" meaning golden and "anthemon" meaning flower. C*hrysanthemum coronarium* var. *coronarium*, commonly named as garland chrysanthemum. In chrysanthemum, the Marigold is an introduced cultivar, which is very popular in Southern India and is being cultivated by the local farmers. This cultivar has high demand due to its bright yellow colour, ray florets orientation and especially high shelf life alongside with high rate of production (Ghosh *et al*., 2018) [4]. The flowers does not fade away soon after plucking and remains fresh for more period provided shade and cool atmosphere. Availability of planting material through conventional cultivation of this crop is very difficult due to its commercial importance, therefore the production of quality planting material and highly demand for cut flowers it is difficult to mitigate the gradual increase in demand through vegetative means of propagation, which is time consuming with low multiplication rate.

Micro propagation is a boon in such cases to augment the multiplication rate faster. Many researchers developed micro propagation techniques for chrysanthemum by using explants such as shoot tip, leaf, petals, stem and nodal segment. It offers production of great number of disease free, true to type planting material within short period of time irrespective of growing season. So *in vitro* culture is a greatly acceptable and dependable method of propagation for chrysanthemum (*Dendranthemum grandiflora).*

Material and Methods
The study was carried out at the Plant Tissue Culture Laboratory, Department of Horticulture, University of Agricultural Sciences, Gandhi Krishi Vignana Kendra, Bengaluru-560065, during 2019 to 20.

Explants
Chrysanthemum plants were maintained in the greenhouse at Plant Tissue Culture Laboratory,

Department of Horticulture, GKVK, Bangalore. The explants were collected from 5 to 6 months old plants. The healthy and matured Nodes (1 to 1.5 cm) and Shoot tips (1cm) were selected as explants.

Sterilization

Explants were kept under running tap water for 20 minutes and washed with soap solution for 5 minutes. The separated nodes and shoot tips were washed with sterile water for 2 times with 3 minutes per wash, then the explants were kept in antiphenol solution (ascorbic acid + citric acid solution) for 45 minutes. After this, the explants were transferred to 0.2 per cent (w/v) Bavistin solution with 1 to 2 drops (v/v) of Tween 20 (10 minutes for nodes and 5 minutes for shoot tips). The explants were given 3 sterile water wash (5 minutes per wash). After this, the explants were surface sterilized with 0.1 per cent (v/v) Mercuric chloride 2 to 3 minutes. The solution was drained and the explants were washed 3 times with sterile water for 5 minutes in each wash. These explants were then transferred on to 0.2 per cent (w/v) streptomycin solution for a period of 5 minutes.

Shooting

The explants were placed on medium consisting of MS salts (Murashige and Skoog, 1962) [10] supplemented with 3 per cent (w/v) sucrose and the media were solidified by 0.6 per cent agar having different concentrations of BAP (0.5, 1.0, 1.5, and 2.0 mgL^{-1}) and TDZ (0.1, 0.2, 0.3 and 0.4 mgL^{-1}) combinations were tested for shoot proliferation. The culture bottles were incubated at temperature of 24 ± 2^0C and light intensity of 2000 lux with 8 hours of light and 16 hours dark period.

The data was recorded for different parameters *viz*., number of days for shoot initiation (number of days from the date of the culturing to the date of emergence of shoot), number of shoots per explants (avg. of number of shoot produced per explant was recorded) and length of shoots (average of length of the shoot was recorded) at 30, 60, 90 DAR

Rooting

After 90 days of shoot initiation, the regenerated shoots were placed in the half strength MS medium supplemented with IBA and IAA both at a concentration of 0.1, 0.2, 0.3, 0.4 and 0.5 mgL^{-1} to induce roots. The data was recorded for different parameters *viz.*, number of days for root initiation (number of days from the date of the culturing to the date of emergence of root), number of roots per shoot (average number of root produced per shoot was recorded) and length of roots (Length of the root was recorded) at 15, 30, 45 days interval after root initiation.

Statistical analysis

The experiments were laid out in completely randomized design (CRD). In all the experiments ten replications were taken to record the data. The complete data was analyzed using SPSS software.

Results and Discussion

The shoot tip explants did not responded positively when compared to nodal segments. The shoot tips turned brown and remained as same. They did not produce any further growth even after 7 weeks of culture.

Effect of different concentration of BAP and TDZ alone on shoot induction from nodal segment

The influence of BAP and TDZ differed significantly among the different concentration used on shoot induction (Table 1). Among the different concentrations of BAP used, the medium with. intermediate level of BAP (1.0 mgL^{-1}) found to be the best hormone concentration in respect of early shoot initiation (12.10 days), production of highest number of shoots per explant. (3.54, 5.33 and 6.25) and maximum shoot length of 3.25, 5.85 and 6.99 cm at 30, 60 and 90 DAS respectively compared to the basal medium (control). It was followed by BAP 0.5 mgL^{-1} for early shoot initiation (17.40 days). Whereas, for number of shoots per explant and shoot length, BAP 1.5 mgL^{-1} follows the BAP 1.0 mgL^{-1} as BAP 1.5 mgL^{-1} produced 3.00, 4.35 and 5.90 number of shoots per explant with shoot length of 2.65, 5.0 and 6.15 cm at 30, 60 and 90 DAS respectively. These results variation in shoot length might be because of the fact that higher doses of BAP failed to express superiority could be due to obnoxious effect at high concentration, whereas the ineffectiveness of lower dose shows the insufficient of hormone to produce more number of shoots (Waseem *et al*. 2011) [12]. The similar results were in conformity with Waseem *et al*. (2009) [11] and Zafarullah *et al*. (2013) [15]. Our results are in line with Ali *et al*. (2008) who reported that BAP 1.0 mgL^{-1} showed superior results in regeneration of calendula.

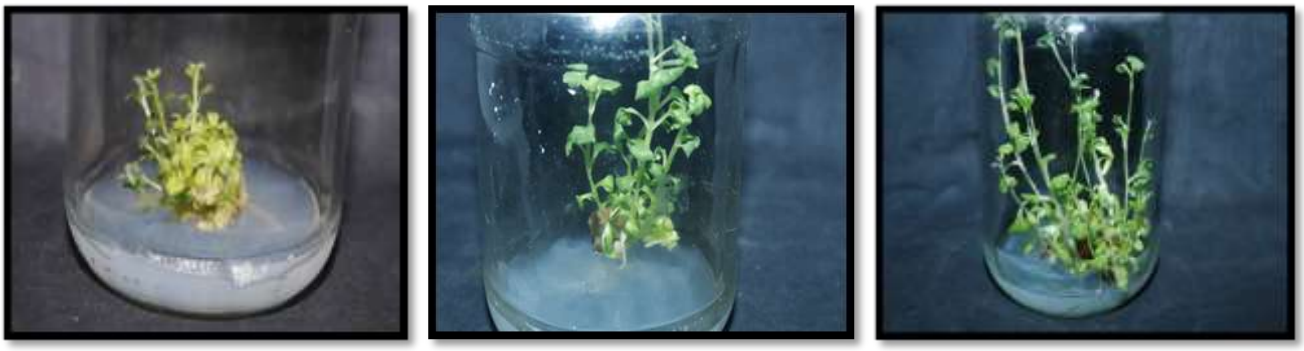
Table 1: Effect of different concentration of BAP and TDZ alone on shoot induction from nodal segment

Treatments	Days for shoot initiation	Number of shoots per explant			Shoot length (cm)		
		30 (Days)	60 (Days)	90 (Days)	30 (Days)	60 (Days)	90 (Days)
T_1	27.10	0.98	1.02	2.18	1.09	1.55	2.09
T_2	17.40	2.86	4.20	5.45	2.25	4.65	5.75
T_3	12.10	3.54	5.33	6.25	3.25	5.85	6.99
T_4	19.00	2.98	4.35	5.86	2.65	5.00	6.15
T_5	22.30	2.17	3.43	4.99	2.15	4.36	5.24
T_6	24.10	1.02	1.95	3.75	1.15	2.35	3.54
T_7	19.30	1.23	2.11	3.99	1.44	2.65	3.99
T_8	19.70	1.54	2.54	4.31	1.99	3.15	4.35
T_9	18.40	1.99	3.26	4.75	2.25	3.55	5.15
F-test	*	*	*	*	*	*	*
S.Em±	0.514	0.052	0.024	0.036	0.027	0.026	0.037
CD (1%)	1.917	0.193	0.089	0.133	0.100	0.098	0.140

*Significant at 1%
S.Em - Standard Error of Mean
DAS - Days after shoot initiation
CD - Critical difference
T_1- Basal medium (Control)

T_2 - BAP 0.5 mgL^{-1}
T_3 - BAP 1.0 mgL^{-1}
T_4 - BAP 1.5 mgL^{-1}
T_5 - BAP 2.0 mgL^{-1}
T_6 - TDZ 0.1 mgL^{-1}
T_7 - TDZ 0.2 mgL^{-1}
T_8 - TDZ 0.3 mgL^{-1}
T_9 - TDZ 0.4 mgL^{-1}

Among the medium which are supplemented with TDZ, the medium with TDZ 0.4 mgL^{-1} took 18.40 days for initiation of shoots, highest number of shoots per explant (1.99, 3.26 and 4.75) and maximum shoot length (2.25, 3.55 and 5.15 cm) at 30, 60 and 90 days after shoot initiation respectively. The higher concentration of TDZ may have more influence on shoot proliferation than lower concentration. Similar results were reported by Lindiro *et al*. (2013) [9]. Arinaitwe *et al*. (2000) [2] stated that, among BAP, TDZ, 2Ip, KN and ZN. BAP was good, as the cultivars of banana responded more, than other cytokinin.



30 DAYS **60 DAYS** **90 DAYS**

Plate 1: Shoot proliferation from nodes in MS medium supplemented with BAP 1.0 mgL^{-1} at a) 30 days b) 60 days and c) 90 days

Effect of different concentration of BAP with TDZ in combination on shoot induction from nodal segment

The influence of BAP with TDZ on shoot induction was differed significantly (Table 2). The data observed that, least number of days (12.40 days) for shoot initiation was noticed in MS medium contains with BAP 1.5 mgL^{-1} + TDZ 0.4 mgL^{-1}. Maximum number of shoots per explant 5.25, 8.65 and 11.90 and maximum shoot length of 4.45, 5.55 and 7.45 cm at 30, 60 and 90 DAS respectively was recorded in the same medium. BAP 1.5 mgL^{-1} + TDZ 0.4 mgL^{-1} was followed by BAP 1.5 mgL^{-1} + TDZ 0.3 mgL^{-1} as it showed shoot initiation in 14.20 days, with 4.98, 8.25 and 9.85 number of shoots and 4.15, 5.41 and 7.25 cm of shoot length at 30, 60 and 90 DAS. Other combination of BAP with TDZ also resulted in proliferation of multiple shoot but the number of days taken for shoot initiation, number of multiple shoots and shoot length was best in MS medium with BAP 1.5 mgL^{-1} + TDZ 0.4 mgL^{-1}. This might be due to the use of combination of BAP and TDZ, which was more effective than the use of growth hormone alone.

The similar results were reported by Ghauri *et al.* (2013) [3], who stated that, BAP 1.25 mgL^{-1} + TDZ 0.5 mgL^{-1} was best among the various concentration of BAP + TDZ in stevia micro propagation from root explants. Imtiaz *et al*. (2019) [5] also stimulated shoot proliferation of chrysanthemum through shoot bud explants by using TDZ in combination of BAP.

30 DAYS **60 DAYS** **90 DAYS**

Plate 2: Shoot proliferation from nodes in MS medium supplemented with BAP 1.5 mgL^{-1} with TDZ 0.4 mgL^{-1} at a) 30 days b) 60 days and c) 90 days

Table 2: Effect of different concentration of BAP with TDZ on shoot induction from nodal segment

Treatments	Days for shoot initiation	Number of shoots per explant			Shoot length (cm)		
		30 (Days)	60 (Days)	90 (Days)	30 (Days)	60 (Days)	90 (Days)
T_1	25.60	1.08	2.27	3.36	0.90	2.44	3.57
T_2	24.40	2.15	4.25	5.15	1.99	3.85	4.49
T_3	25.00	1.75	4.00	4.75	1.35	3.55	4.25
T_4	24.20	2.35	4.55	5.45	2.05	3.98	4.65
T_5	23.20	2.85	5.15	5.65	2.35	4.45	5.13
T_6	21.40	3.15	5.50	6.15	2.53	4.65	5.37
T_7	23.50	2.55	4.65	5.55	2.25	4.25	4.85
T_8	22.10	3.15	5.25	5.85	2.43	4.59	5.35
T_9	19.40	3.65	5.98	6.45	2.75	4.76	5.45
T_{10}	18.20	3.94	6.35	7.35	3.35	4.98	6.05
T_{11}	16.50	4.65	7.15	8.55	3.85	5.25	6.55
T_{12}	14.20	4.98	8.25	9.85	4.15	5.41	7.25
T_{13}	12.40	5.25	8.65	11.90	4.45	5.55	7.45
T_{14}	17.20	4.35	6.85	8.25	3.75	5.30	6.45
T_{15}	15.20	4.85	7.55	9.21	3.98	5.34	6.98
T_{16}	17.60	4.25	6.55	7.75	3.55	5.16	6.25
T_{17}	18.70	3.75	5.99	6.96	3.15	4.85	6.00
F-test	*	*	*	*	*	*	*
S.Em±	0.434	0.030	0.034	0.046	0.029	0.035	0.043
CD (1%)	1.600	0.110	0.126	0.168	0.106	0.126	0.159

*Significant at 1% ; S.Em - Standard Error of Mean; DAS - Days after shoot initiation ; CD - Critical difference ; T_1 - Basal medium (Control) ; T_2 - BAP 0.5 mgL^{-1} + TDZ 0.1 mgL^{-1}; T_3 - BAP 0.5 mgL^{-1} + TDZ 0.2 mgL^{-1}; T_4 - BAP 0.5 mgL^{-1} + TDZ 0.3 mgL^{-1}; T_5 - BAP 0.5 mgL^{-1} + TDZ 0.4 mgL^{-1}; T_6 - BAP 1.0 mgL^{-1} + TDZ 0.1 mgL^{-1}; T_7 - BAP 1.0 mgL^{-1} + TDZ 0.2 mgL^{-1}; T_8 - BAP 1.0 mgL^{-1} + TDZ 0.3 mgL^{-1}; T_9 - BAP 1.0 mgL^{-1} + TDZ 0.4 mgL^{-1}; T_{10} - BAP 1.5 mgL^{-1} + TDZ 0.1 mgL^{-1}; T_{11} - BAP 1.5 mgL^{-1} + TDZ 0.2 mgL^{-1}; T_{12} - BAP 1.5 mgL^{-1} + TDZ 0.3 mgL^{-1}; T_{13} - BAP 1.5 mgL^{-1} + TDZ 0.4 mgL^{-1}; T_{14} - BAP 2.0 mgL^{-1} + TDZ 0.1 mgL^{-1}; T_{15} - BAP 2.0 mgL^{-1} + TDZ 0.2 mgL^{-1}; T_{16} - BAP 2.0 mgL^{-1} + TDZ 0.3 mgL^{-1}; T_{17} - BAP 2.0 mgL^{-1} + TDZ 0.4 mgL^{-1}

Effect of different concentration of IBA and IAA on rooting from the micro shoots regenerated from nodal segments.

Effect of different concentration of IBA and IAA on root regeneration is depicted in the Table 3. Among the different concentrations of auxins (IBA and IAA) used, half strength MS medium fortified with IBA 0.2 mgL^{-1} showed minimum duration (5.20 days) for initiation of roots, production of maximum number of roots per. shoot (4.49, 6.10 and 8.21) and root length (3.85, 5.54 and 6.42 cm) at 15, 30 and 45 days after root initiation. IBA 0.1 mgL^{-1} was on par with IBA 0.2 mgL^{-1} for number of days for root initiation (5.80 days). IBA 0.2 mgL^{-1} was followed by IBA 0.1 mgL^{-1} for number of root per shoot (4.10, 5.81 and 7.16) and root length (3.45, 5.22 and 5.95cm) at 15, 30 and 45 days of root initiation respectively.

Table 3: Effect of different concentration of IBA and IAA on rooting from the micro shoots regenerated from nodal segments

Treatments	Days for shoot initiation	Number of shoots per explant			Shoot length (cm)		
		30 (Days)	60 (Days)	90 (Days)	30 (Days)	60 (Days)	90 (Days)
T_1	12.10	1.09	2.24	4.13	1.26	2.64	3.52
T_2	5.80	4.10	5.81	7.16	3.45	5.22	5.95
T_3	5.20	4.49	6.10	8.21	3.85	5.54	6.42
T_4	6.90	3.11	5.04	6.31	2.54	4.07	5.11
T_5	8.60	2.05	3.20	5.31	1.82	3.18	4.36
T_6	7.50	2.99	4.61	6.13	2.28	4.02	4.96
T_7	7.70	2.64	3.77	5.97	2.08	3.93	4.75
T_8	6.40	3.41	5.28	6.35	2.77	4.56	5.23
T_9	8.30	2.35	3.56	5.61	1.91	3.84	4.57
T_{10}	8.90	1.94	2.91	4.86	1.34	3.03	4.10
T_{11}	9.10	1.45	2.74	4.88	1.53	3.53	4.28
F-test	*	*	*	*	*	*	*
S.Em±	0.281	0.084	0.075	0.065	0.047	0.050	0.048
CD (1%)	1.045	0.313	0.285	0.240	0.175	0.184	0.179

*Significant at 1%
S.Em - Standard Error of Mean
DAR - Days after root initiation CD - Critical difference
T_1 - Basal medium (Control)
T_2 - IBA 0.1 mgL^{-1}
T_3- IBA 0.2 mgL^{-1}
T_4-IBA 0.3 mgL^{-1}
T_5- IBA 0.4 mgL^{-1}
T_6- IBA 0.5 mgL^{-1}
T_7- IAA 0.1 mgL^{-1}
T_8- IAA 0.2 mgL^{-1}
T_9- IAA 0.3 mgL^{-1}

T_{10}- IAA 0.4 mgL^{-1}
T_{11}- IAA 0.5 mgL^{-1}

The lower concentration of IAA showed more influence on root initiation than the higher concentration. The results revealed the supremacy of IBA over IAA in root induction. This might be due to the fact that IAA photo-oxidized rapidly than IBA in tissue culture. So IAA degraded soon after initial root induction in the rooting medium. The IBA even at lower concentration remained active for longer period, which positively affected the root length. These finding are more or less similar with earlier reports of Yesmin *et al*. (2014) [14] who reported 12.27 number of roots with 6.65 cm of root length in IBA 0.2 mgL^{-1}. Waseem *et al*. (2009) [11] stated that, 9.00 cm root length in IBA 0.2 mgL^{-1} in 5.00 days. Several earlier workers Waseem *et al*. (2011) [13]; Keresa *et al.* (2012) [8] and Khan *et al*. (1994) [7] reported maximum root length in chrysanthemum by using 0.2 mgL^{-1} IBA in half MS.

Plate 3: Root regeneration in MS medium supplemented with IBA 0.2 mgL^{-1}

This might be due to the fact that IAA photo-oxidized rapidly than IBA in tissue culture. So IAA degraded soon after initial root induction in the rooting medium. The IBA even at lower concentration remained active for longer period, which positively affected the root length. These finding are more or less similar with earlier reports of Yesmin *et al*. (2014) [14] who reported 6.65 cm of root length on the same medium. Waseem *et al*. (2011) [12] stated that, 9.00 cm root length in IBA 0.2 mgL^{-1}. Among the two different auxins IBA showed best response towards root formation. Several earlier workers Waseem *et al*. (2009) [11]; Karim *et al*. (2002) [6]; Keresa *et al.* (2012) [8] and Khan *et al*. (1994) [7] reported 0.2 mgL^{-1} IBA in half MS best hormone concentration for rooting in chrysanthemum.

Conclusion

Present study reveals that, intermediate concentration of BAP (1.0mgL^{-1}) alone and combination of BAP 1.5 mgL^{-1} + TDZ 0.4 mgL^{-1} showed better response for shoot regeneration and the IBA 0.2 mgL^{-1} was better for root regeneration in the production of chrysanthemum plants through *in vitro* culture. The established protocol may be utilized for production of virus free, true to type plants and for large scale multiplication of desired types to fulfill the demand of quality planting material of Chrysanthemum (*Dendranthema grandiflora* T.) cv. Marigold.

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