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Efficient *in-vitro* regeneration protocol in chrysanthemum (*Chrysanthemum morifolium*) from ray florets

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Abstract

The experiment was conducted to standardize regeneration protocol from ray florets in chrysanthemum cv. Pusa Arunodaya (TQP-06). For callus induction, the highest callusing percent (82.67%) with a minimum number of days for callus initiation (10.87 days) was observed in MS medium supplemented with Kinetin (10 mg/l) + NAA (1.0 mg/l) in treatment (T_3). Highest shooting percent (69.33 %) with minimum number of days for shoot initiation (6.27 days) along with maximum number of shoots per explant (5.67 shoots) in MS medium supplemented with Kinetin (5.0 mg/l) + NAA (1.0 mg/l). In shoot proliferation, the highest number of shoots after 30 days (51.67), 60 days (72.00), and 90 days (98.67) in MS medium supplemented with Kinetin (5.0 mg/l) + NAA (1.0 mg/l). The highest rooting percent (85.00%) with least days for root initiation (7.00 days) along with maximum roots per shoot (8.00) and optimum root length (5.00 cm) in MS medium supplemented with IBA (0.2 mg/l). The rooted plants were successfully acclimatized in 3-4 weeks and survived under field conditions.

Key words: Chrysanthemum, *in-vitro,* ray florets, callus induction, regeneration, proliferation.

Chrysanthemum (*Chrysanthemum morifolium* Ramat) is vital cut flower in international markets and is placed in second place in the global cut flower trade (Datta and Gupta, 2012). It is from the Asteraceae family and is an important traditional flower that can be used as both loose and cut flowers (Lone and Shah, 2013).

India has commercially released 46 mutant cultivars of chrysanthemum (Verma and Prasad 2019). Micropropagation, an *in-vitro* method, has the ability to quickly produce a significant amount of healthy, disease-free and true-to-type stock (Karn *et al.* 2022). Different explants were used for shoot organogenesis and plant regeneration like petal, leaf and stem (Xue *et al.,* 2004; Nahid *et al.*, 2007) to improve the micropropagation rate. However, there are very few examples of new mutants being successfully established in chrysanthemums using irradiation and *in-vitro* regeneration techniques (Prasad *et al.*, 2008). Therefore, Pusa Arunodaya (TQP-06) which is a mutant developed by gamma irradiation from cv. Thai Chen Queen was used.

Materials and Methods

The explants were collected from fully mature flowers of Pusa Arunodaya (TQP-06) which were maintained at Research Farm of ICAR Directorate of Floriculture Research (DFR), Pune. The explants were washed with detergent (Tween 20) solution for 4-5 minutes, and washed with double distilled water 2-3 times then treated with fungicide (carbendazim) for 30 minutes, followed by 3-4 washings of double distilled water. During inoculation explants were surface sterilized with 0.1% $HgCl_2$ solution for 2-3 minutes and immediately rinsed with double distilled water 3 times to remove all traces of $HgCl_2$. The pH of medium was adjusted to 5.8 before autoclave (121^{0}C for 20 minutes) and the medium was solidified by the addition of agar (0.8%).

The surface sterilized ray florets were inoculated on MS medium supplemented with different concentrations of cytokinins, i.e. kinetin (7.5 and 10.0 mg/l) or BAP (2.0, 3.0 and 6.0 mg/l) along with NAA (0.1, 0.2, 0.5 and 1.0 mg/l) and 2, 4-D (1.0 mg/l). MS medium supplemented with different concentrations of cytokinins, i.e. kinetin (5.0 mg/l) or BAP (2.0 and 5.0 mg/l) along with NAA (0. 5 and 1.0 mg/l) was used for micro-shoots regeneration from ray florets and MS medium supplemented with different concentrations of cytokinins, i.e. kinetin (5.0 mg/l) or BAP (2.0 and 5.0 mg/l) along with NAA (0.5, and 1.0 mg/l) was used for shoot proliferation.

Half-strength and full-strength MS medium supplemented with different concentrations of auxins, indole butyric acid (IBA) (0.1 and 0.2 mg/l) were used

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for *in vitro* rooting to the well-developed and elongated shoots. The experiments were laid out in a completely randomized design (CRD). Each treatment had 15 units with three replications and rooting had 20 units with four replications. All the percentage values were arcsine transformed and counted data below 10 was square root transformed before calculating ANOVA. The data having common superscripts are statistically non-significant or otherwise significant.

Results and Discussion

Callus induction : The callusing (82.67 %) was observed after 10.87 days when the surface sterilized and pinched (wounded) ray florets were cultured on MS medium supplemented with Kinetin (10 mg/l) and NAA (1.0 mg/l) (T-3) as compared to those cultured on other media (Fig-1). Present findings are in line with those of Kumar *et al.* (2012). Kumar *et al.* (2017) and Verma and Prasad (2019) reported that wounded parts of ray florets showed faster callusing as compared to those inoculated without wounding (Table 1).

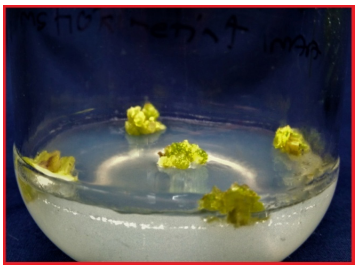

Fig. 1: Callus induction

According to Verma and Prasad (2019), kinetin and NAA in combination in the culture medium resulted in highest callus initiation with a good morphogenic response from nodal and ray floret explants in chrysanthemum. The present results on survival and callusing percentage confirm with earlier findings of Nahid *et al.* (2007) and Kumar *et al.* (2017).

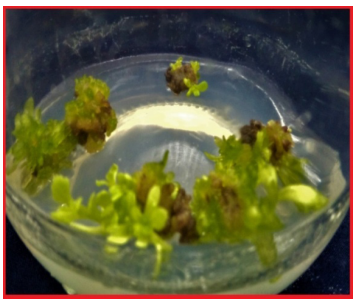

Fig. 2: Shoot regeneration

Micro-shoot regeneration and shoot proliferation: Data shows highest regeneration (69.33 %), maximum number of shoots/explant (5.67) in minimum days for shoot initiation (6.27 days) in ray florets cultured on MS medium supplemented with Kinetin (5.0 mg/l) and NAA (1.0 mg/l) as compared to those cultured on other media (Fig. 2 and Table 2). Similar results were obtained by other workers working on chrysanthemums (Kumar *et al.,* 2012, Kumar *et al.*, 2017 and Verma and Prasad 2019).

Table 1: Effect of BAP, Kinetin, 2, 4-D, and NAA on callus induction

Treatment	Medium	Callusing (%)	Days required for initiation
T_1	MS blank (control)	0.000 (0.00)	0.000
T_2	MS+ Kinetin (10 mg/l) + NAA (0.5 mg/l)	77.33^{cdc}(61.69)	12.53^{b}
T_3	MS + Kinetin (10 mg/l) + NAA (1.0 mg/l	82.67^{e} (65.40)	10.87^{a}
T_4	MS + Kinetin (7.5 mg/l) + NAA (0.5 mg/l)	72.00^{abc}(58.07)	13.27^{c}
T_5	MS + Kinetin (7.5 mg/l) + NAA (1.0 mg/l)	80.00^{de}(63.48)	12.47^{b}
T_6	MS + BAP (2.0 mg/l) + NAA (0.1 mg/l)	68.00^{a}(55.64)	13.73^{c}
T_7	MS + BAP (2.0 mg/l) + NAA (0.2 mg/l)	70.67^{ab}(57.26)	13.60^{c}
T_8	MS + BAP (3.0 mg/l) + 2, 4-D (1.0 mg/l)	74.67^{bcd}(59.98)	13.20^{c}
T_9	MS + BAP (6.0 mg/l) + 2, 4-D (1.0 mg/l)	81.33^{e}(64.48)	11.93^{b}
	C.D. @ 0.01	6.27	0.62
	S.E. (m) ±	2.10	0.21
	C.V. (%)	6.72	3.18

Table 2: Effect of BAP, Kinetin, and NAA on shoot regeneration

Treatment	Medium	Shoot Regeneration (%)	Days for initiation of shoots	Shoots/ explant
T_1	MS Blank (control)	0.00 (0.00)	0.000	0.000 (1.00)
T_2	MS + BAP (2.0 mg/l)	45.33^{a} (42.30)	10.07^{d}	2.47^{a} (1.90)
T_3	MS + BAP (2.0 mg/l) + Kinetin (1.0 mg/l)	48.00^{ab} (43.83)	9.80^{d}	2.73^{a} (1.93)
T_4	MS + BAP (2.0 mg/l) + Kinetin (0.5 mg/l)	49.33^{bc} (44.60)	9.73^{cd}	2.80^{a} (1.95)
T_5	MS + BAP (5.0 mg/l) + NAA (0.5 mg/l)	52.00^{cd} (46.13)	8.87^{c}	3.80^{b} (2.20)
T_6	MS + BAP (5.0 mg/l) + NAA (1.0 mg/l)	53.33^{d} (46.90)	7.33^{b}	4.20^{b} (2.28)
T_7	MS + Kinetin (5.0 mg/l) + NAA (0.5 mg/l)	57.33^{e} (49.20)	6.33^{a}	4.33^{b} (2.31)
T_8	MS + Kinetin (5.0 mg/l) + NAA (1.0 mg/l)	69.33^{f} (56.36)	6.27^{a}	5.67^{c} (2.60)
	C.D. @ 0.01	2.74	0.89	0.14
	S.E. (m) ±	0.91	0.29	0.05
	C.V. (%)	3.81	6.99	3.90

The direct adventitious micro-shoot regeneration begins with cells that are located either in the epidermis or just below the surface of explant which apparently originate from single cells. The data showed maximum number of shoots after 30 days (51.67), 60 days (72.00), and 90 days (98.67) in MS + Kinetin (5.0 mg/l) + NAA (1.0 mg/l) as compared to other treatment combinations (Table 3). These results lend support to the report of earlier workers (Liu and Gao 2007, Waseem *et al.* 2011, Kumar *et al.* 2012, Kumar *et al.* 2017 and Verma and Prasad 2019). Shoot proliferation might be due to the optimum doses of cytokinins and auxins, which enhances axillary branching.

Rooting and establishment of plantlets in greenhouse : The micro-shoots cultured on full-strength MS medium supplemented with 0.2 mg/l IBA took minimum days to root initiation (7.00 days) and produced maximum rooting (85 %), higher average number of roots/shoot (8.00) and optimum and manageable length of longest root (5.00 cm) (Table 4 and Fig. 3). These findings are in line with reports of Prasad *et al.* (2008), Waseem *et al.* (2011), Kumar *et al.* (2012), and Kumar *et al.* (2017).

Fig. 3: Rhizogenesis

Plantlets were successfully acclimatized by transferring them in pots each filled with sterilized coco peat devoid of any nutrients and frequent watering. After 3-4 weeks of acclimatization, the plants were transferred to open conditions. (Fig. 4). The mutant plants produced uniform bright pinkish colour flower as compared with the parent.

Fig. 4: Acclimatized plantlets in open conditions

Table 3: Effect of BAP, Kinetin, and NAA on shoot proliferation

Treatment	Medium	Number of shoots		
		after 30 days	after 60 days	after 90days
T_1	MS Blank (control)	0.00	0.00	0.00
T_2	MS + BAP (2.0 mg/l)	10.33[a]	19.33[a]	25.67[a]
T_3	MS + BAP (2.0 mg/l) + Kinetin (1.0 mg/l)	18.33[b]	24.00[b]	32.33[b]
T_4	MS + BAP (2.0 mg/l) + Kinetin (0.5 mg/l)	26.67[c]	30.67[c]	41.33[c]
T_5	MS + BAP (5.0 mg/l) + NAA (0.5 mg/l)	29.67[d]	38.33[d]	58.33[d]
T_6	MS + BAP (5.0 mg/l) + NAA (1.0 mg/l)	32.33[de]	49.33[e]	61.00[d]
T_7	MS + Kinetin (5.0 mg/l) + NAA (0.5 mg/l)	40.67[e]	58.00[f]	79.33[e]
T_8	MS + Kinetin (5.0 mg/l) + NAA (1.0 mg/l)	51.67[f]	72.00[g]	98.67[g]
	C.D. @ 0.01	2.92	2.98	3.32
	S.E. (m) ±	0.97	0.99	1.10
	C.V. (%)	6.38	4.68	3.84

Table 4: Effect of auxins on rooting of micro-shoots

Treatment	Medium	Rooting (%)	No. of days for root initiation	No. of roots/ shoot	Length of longest root (cm)
T_1	MS (1/2 strength) control	59.00[a] (50.17)	9.95[e]	1.90[a] (1.70)	6.20[e]
T_2	MS (1/2 strength) + IBA (0.1 mg/l)	79.00[b] (62.71)	8.25[d]	6.55[b] (2.75)	4.00[a]
T_3	MS (1/2 strength) + IBA (0.2 mg/l)	81.00[b] (64.21)	7.85[c]	6.78[c] (2.79)	4.40[b]
T_4	MS + IBA (0.1 mg/l)	82.00[c] (64.90)	7.50[b]	7.23[d] (2.87)	4.70[c]
T_5	MS + IBA (0.2 mg/l)	85.00[d] (67.22)	7.00[a]	8.00[e] (3.00)	5.00[d]
	C.D. @ 0.01	2.77	0.21	0.04	0.02
	S.E. (m) ±	0.91	0.07	0.01	0.01
	C.V. (%)	2.95	1.71	1.03	0.31

Conclusion

The best treatment for callus induction was MS medium with Kinetin (10 mg/L) with NAA (1.0 mg/L), for shoot regeneration and proliferation was Kinetin (5.0 mg/L) with NAA (1.0 mg/L) and for rooting was MS + IBA (0.2 mg/l). Therefore, these treatments can be recommended for large-scale multiplication of mutant plantlets.

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