Efficient *In Vitro* Regeneration of Chrysanthemum (*Chrysanthemum morifolium Ramat*.) Through Nodal Explant Culture

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Abstract

An efficient *in vitro* plant regeneration protocol was developed for *Chrysanthemum morifolium*, an ornamental plant. Nodal segments were used as explants in the present experiment. The explants were cultured onto Murashige & Skoog's (MS) nutrient medium containing plant growth regulators (cytokinins or auxins) with various combinations and concentrations for the study. Maximum percentage (93.33%), best shoot induction per culture (21.73 ± 1.19) and highest shoot length (6.43±0.31) were achieved on MS medium supplemented with 1.0 mg/l BAP and 0.1 mg/l IAA. With repeated subculture on the same medium increase the number of shoots per culture. For rooting, *in vitro* raised well developed shoots were cultured onto half strength MS medium supplemented with different concentrations of IBA, IAA and NAA. The isolated shoots rooted well (98%) within 4 weeks on half-strength MS fortified with 0.2 mg/l IBA, where average number of roots per shoot was 12.27 ± 0.53. After transplantation, regenerated plantlets developed in natural condition variation.

Keywords: Regeneration, *in vitro*, *acclimatization*, *Chrysanthemum morifolium* Ramat, nodal explant

1. Introduction

Chrysanthemum morifolium Ramat. is a herbaceous, perennial, ornamental plant belongs to the family of Compositeae (Asteraceae). It is highly valued as a cut flower worldwide with its diverse floral types and colours and globally is an important flower and pot plant species usually cultivated by vegetative cuttings [1]. *Chrysanthemum* is native to Asia and northeastern Europe and most species originate from East Asia and the center of diversity is in China. There are lots of horticultural varieties and cultivars [2]. It has a great commercial value in flower industry all over the world as well as in Bangladesh. It is the world's second most economically important floriculture crop following the rose [3]. The commercial production of this ornamental plant is growing worldwide. Its monetary value has significantly increased over the last two decades and there is a great potential for further growth both in domestic and international markets. Flowers bloom in early winter with a wide shape & sizes and color range from white and cream through the shades of yellow, pink, bronze, red, deep purple and green [4]. They are appreciated for their high keeping quality and also their ability to produce desired grades and types at anytime during the year adds to their popularity [5].

The commercial cultivars are usually propagated vegetatively through terminal cuttings and root suckers which are very slow, time consuming and tiring. These conventional propagation methods not only take longer time to flower, but also not useful for large scale production. Regeneration through *in vitro* culture has become now a viable alternative to the conventional propagation methods [6]. Chrysanthemums are susceptible to infection by many viruses. Problems of virus infection can also be solved by micropropagation methods [7].

In recent years various reports on *in vitro* propagation of different plant species have shown that tissue culture technique is suitable for rapid propagation of selected plant species [8- 9]. A number of studies have been carried out on *in vitro* propagation of *C. morifolium.* Many researchers developed micropropagation techniques for Chrysanthemum using different parts such as shoot tip, leaf, petals, stem, apical meristem and few report using nodal explants in the Asian sub-continent, but very few reports on Chrysanthemum in Bangladesh. Present study is designed using nodal explants which may be fruitful to cultivate the plant in our soil and environment. Most of reports regarding *in vitro* regeneration of Chrysanthemums from different explants via organogenesis [1, 10-13] somatic embryogenesis [7, 14] and callus culture [6, 15]. Tissue culture is alternatively called cell, tissue and organ culture through *in vitro* condition. It can be employed for large-scale propagation of disease free clones and gene pool conservation.

The present study aims at developing a simple, rapid, economical and high frequency regeneration protocol from nodal explants of *Chrysanthemum morifolium* Ramat. so as to give rise to true-to-type clones for potential application in large scale propagation.

2. Materials and methods

The experiments were carried out in the plant biotechnology division of National Institute of Biotechnology of Bangladesh. Chrysanthemum plants collected from local nursery and conserved in the experimental garden of the National Institute of Biotechnology for obtaining explant. Nodal segments were used for this purpose. The nodal segments (1.0 – 1.5 cm) with one node were excised from five to six month old plant and thoroughly washed under running tap water for 30 minutes. Then they were treated with mild detergent followed by ringing several times with distilled water. The explants were dipped in 0.8% antifungal bavistin for 10-12 minutes and again washed 4-5 times with sterilized distilled water. Further sterilization was done in laminar air flow

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cabinet under aseptic conditions. The explants were dipped in 70% (v/v) ethanol for 1 minute and then washed three times with sterilized distilled water. For surface sterilization, the explants were dipped in 0.1% aqueous solution (w/v) of $HgCl_2$ for 2-3 minutes, then they were washed in sterilized distilled water for 3-4 times, till the sterilents were removed completely. The nodal segments were then cut at both ends prior to inoculation on culture media. The explants were cultured on MS medium with 3% sucrose and supplemented with different concentrations of BAP, Kn (0.5-5.0 mg/l) and IAA (0.1- 0.5 mg/l) alone or in combination with IAA (0.1- 0.3 mg/l). The pH of the medium was adjusted to 5.8 before the agar addition. The medium was solidified with 0.8% agar and autoclaved at 121^0 C for 20 minutes. After inoculation, the cultures were incubated at 26 ± 2 ºC under 16 hours photoperiod with a light intensity of 3000 lux. The cultures were regularly subcultured at 15 days intervals. For induction of roots, well developed shoots were excised and cultured onto half strength MS medium fortified with auxins (IBA, IAA and NAA) separately. After sufficient development of roots plantlets were taken out from the culture vessels and rinsed with sterilized distilled water to remove all trace of medium attached to the roots. After washing, plantlets were transplanted in small plastic pots containing autoclaved garden soil and compost (1:1). In order to maintain a high humidity the pots were covered with transparent polythene bags and kept in the growth room temperature for 10 days. After 10 days these pots were uncovered and then they were exposed to partial and then complete direct sun light. Finally, these hardened plantlets were transferred to the natural condition for their further growth and development.

3. Results and Discussion

Nodal explants of Chrysanthemum were cultured on MS medium supplemented with various concentrations of BAP, Kn, IAA alone and different concentrations & combinations of BAP and IAA for multiple shoot regeneration (Table. 1). Between the two cytokinins (BAP and Kn), BAP showed better response compared to Kn (Table 1). The maximum percentage of shoot induction (90%), maximum number of shoots per explants (5.73±0.21) and shoot length (6.10±0.35 cm) were observed on MS medium supplemented with 1.0 mg/l BAP whereas maximum percentage (60%) of shoot induction, maximum shoots per explant (3.00±0.24) and highest shoot length (3.60±0.18) were found in 1.0 mg/l Kn. A group of researchers reported that BAP is an important growth regulators for multiple shoots regeneration of *C. Morifolium* [1, 10, 12, 13]. Generally, percentage of shoot formation, average number of shoot per explant and average length of shoots were increased up to a certain concentration of plant growth regulators. In the present study it was observed that an intermediate level (0.5- 1.0 mg/l) of BAP showed the best results in all the parameters. Higher concentrations of BAP reduced shoot bud induction, shoot multiplication and decreased shoot length which result is similar with another group of scientists [1, 10]. Among the different concentrations (0.1, 0.2, 0.3, 0.4 and 0.5 mg/l) of IAA, MS medium supplemented with 0.1 mg/l IAA showed best response towards regeneration. The maximum percentage of shoot induction (86.66%), maximum number of shoots per explants (4.07±0.30) and highest shoot length (5.01±0.27) were achieved on above mentioned medium.

Table 1. Individual Effect of different concentrations of BAP, Kn and IAA on multiple shoot regeneration from nodal segment explants of Chrysanthemum after eight weeks of culture.

Growth regulators (mg/l)	Shoot induction (%)	Number of shoots/explants	Shoot length (cm)
	Mean	Mean ± SE	Mean ± SE
BAP			
0.5	73.33	4±0.22	5.11±0.29
1.0	90	5.73±0.21	6.10±0.35
2.0	76.66	3.67±0.19	4.35±0.26
3.0	66.66	3.33±0.23	4.00±0.33
4.0	60	3.07±0.18	3.24±0.12
5.0	46.66	2.60±0.27	2.31±0.20
Kn			
0.5	53.33	2.40±0.16	3.35±0.19
1.0	60	3.00±0.24	3.60±0.18
2.0	50	2.60±0.16	2.47±0.14
3.0	43.33	2.20±0.20	2.22±0.13
4.0	40	2.07±0.21	1.97±0.10
5.0	33.33	1.93±0.21	1.68±0.13
IAA			
0.1	86.66	4.07±0.30	5.01±0.27
0.2	76.66	3.60±0.29	4.18±0.20
0.3	66.66	3.33±0.23	3.93±0.19
0.4	56.66	2.87±0.19	3.54±0.14
0.5	50	2.67±0.21	3.19±0.12

Results are Mean ± SE of 15 replications.

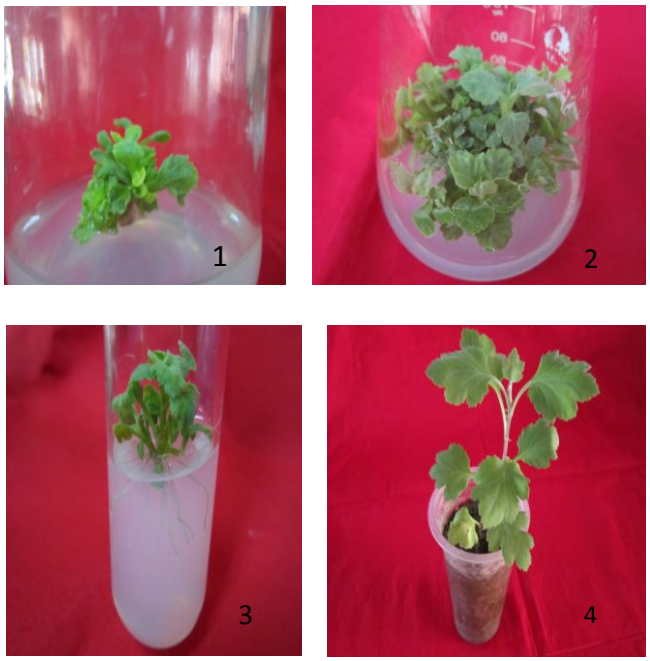
For further development of multiple shoot regeneration, various concentrations and combinations of BAP and IAA were used with MS medium (Table 2). The effect of BAP in combination with an auxin has been reported and most of the cases BAP, NAA and IAA were used for the induction of multiple shoots of various ornamental plants [16-18]. The percentage of responded explants, number of shoots per explants and length of shoots were differed with the medium constituents. Among the four different media used the explants in MS medium supplemented with 1.0 mg/l BAP and 0.1mg/l IAA showed better response. The explants were started producing shoots within three weeks in MS medium supplemented with BAP and IAA (Fig. 1). The highest percentage of responsive explants (93.33%) was recorded on MS + 1.0 mg/l BAP + 0.1mg/l IAA. The maximum number (21.73±1.19) of shoots per explants and highest shoot length (6.43±0.31) were found in this medium (Table 2). MS supplemented with 1.0.mg/l BAP and 0.1

mg/l IAA was most effective for shoot induction in *C. morifolium* reported by different researchers [1]. In the present study it was observed that explants with small shoots were subculture in the same medium the number of multiple shoots was increased. Repeated subculture in the same medium resulted profuse shoot multiplication (Fig. 2). Observed result is almost similar with the previous result [1, 12].

Table 2. Combined Effect of different concentrations and combinations of BAP and IAA on shoot regeneration from nodal segments explants of Chrysanthemum after six weeks of culture.

Growth regulators (mg/l)		Shoot induction (%)	No.of shoots/explants	shoot length (cm)
BAP	IAA	Mean	Mean±SE	Mean±SE
0.5	0.1	83.33	10.13±0.49	5.33±0.25
0.5	0.2	60	5.33±0.39	5.97±0.32
0.5	0.3	46.66	4.93±0.41	4.25±0.27
1.0	0.1	93.33	21.73±1.19	6.43±0.31
1.0	0.2	90	9.40±0.62	4.85±0.31
1.0	0.3	80	6.87±0.42	4.67±0.29
2.0	0.1	86.66	7.07±0.38	4.75±0.23
2.0	0.2	70	5.93±0.33	4.40±0.24
2.0	0.3	56.66	5.60±0.32	4.03±0.22

Results are Mean ± SE of 15 replications.



Figs. 1-4. *In vitro* regeneration of chrysanthemum from nodal explants, (1) Induction of multiple shoots on MS+ 1.0 mg/l BAP+0.1 mg/l IAA after three weeks of culture, (2) Multiple shoots after two-three subculture on the same medium, (3) Rooting of *in vitro* regenerated shoots on half MS + 0.2 mg/l IBA and (4) Acclimatization of *in vitro* grown plantlet in a small plastic pot containing soil and compost (1:1).

Root formation from the *in vitro* grown shoots is an integral part of complete plants. In some cases several roots developed spontaneously while *in vitro* shoot regeneration. But they were found to be inadequate for transplantation and therefore adequate root induction is necessary for transplantation.

Table 3. Effects of IBA, IAA and NAA on *in vitro* root induction in regenerated shoots of *Chrysanthemum morifolium* Ramat. on half strength MS.

MS strength	Growth regulators (mg/l)			Rooted shoots (%)	Days required for rooting	No. of roots/ shoot	Length of roots (cm)
	IBA	IAA	NAA	Mean		Mean±SE	Mean±SE
½ MS	0.1			85	20-22	9.60±0.82	5.27±0.18
½ MS	0.2			98	18-20	12.27±0.53	6.65±0.30
½ MS	0.3			86	20-22	7.87±0.54	4.04±0.16
½ MS		0.1		80	20	7.60±0.81	4.01±0.23
½ MS		0.2		88	17-18	9.13±0.40	4.50±0.29
½ MS		0.3		75	21-22	7.07±0.30	3.87±0.17
½ MS			0.1	82	19-20	8.20±0.31	4.46±0.25
½ MS			0.2	92	18-21	10.93±0.54	5.35±0.20
½ MS			0.3	80	22-23	7.60±0.28	4.17±0.24

Results are Mean ± SE of 15 replications.

Direct root initiation and development was observed onto half-strength MS medium supplemented with different concentrations of IBA, IAA and NAA (0.1-0.5 mg/l) within three weeks (Table 3). The maximum rooting response (98%), the highest number of roots per shoot (12.27 ± 0.53) and root length (3.96 ± 0.17 cm) were recorded on half-strength MS + 0.2 mg/l IBA (Fig. 3). The initiation of roots became visible after six to seven days of culturing, however well-developed healthy roots were achieved after three to four weeks (Table 3, Fig. 3). This finding is more or less similar with earlier reports where 100% root induction was recorded on the same medium [11, 13]. Among the three different auxins IBA showed best response towards root formation. Several earlier workers [16, 19, 20] reported root induction in various ornamental plants by using IBA in MS and modified MS.

After sufficient development of roots, the plantlets were successfully transplanted into small plastic pots containing soil and compost (Fig.4). Following proper acclimatization the plantlets were transferred to experimental field.

4. Conclusion

The result presented here a suitable *in vitro* regeneration protocol for *Chrysanthemum morifolium* Ramat. using nodal explants. Effective media compositions for better shoot and root generation as well as acclimatization were described. The protocol can be exploited for large scale multiplication of Chrysanthemum and production of healthy plant for better floriculture.

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