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Clonal propagation of *Chrysanthemum morifolium* ramat using various explants obtained from field grown plants

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Abstract

A reliable and rapid large scale micropropagation method has been established from the node, shoots tip and leaf explant of *Chrysanthemum morifolium* growing in field condition. Experiments were conducted to standardize the culture media with plant hormone for multiple shoot proliferation and rooting for obtaining plantlets with uniform characteristics like mother plant in terms of growth and habits. Different concentrations and combinations of auxins (IAA) and cytokinins (BAP, Kin) were used in MS for the above purpose. Maximum shoot regeneration was found in MS treated with 2.0 mg/l BAP both in node and shoot tip explants. In the above combination, nodal explants produced 16 initial shoots. Shoot tip explants produced 12 shoots and leaf segment produced 07 shoots. For *in vitro* rooting, different concentrations of IBA and NAA were used. Higher rooting percentage was recorded on MS fortified with 1.5 mg/l IBA. The rooted plantlets were hardened and successfully established in the soil. About 90% of the regenerated plantlets survived in the natural environment.

Keywords: Clonal propagation; Chrysanthemum; Plant Tissue culture; Nodal Segments; Suckers

1. Introduction

Chrysanthemum morifolium commonly known as autumn queen belongs to the family Composaetae (*Asteraceae*) [1] is a perennial herb. It also known as "florist's Chrysanthemum "or "mum". It is the world's second most economically important floricultural crop following the rose [2]. Chrysanthemum was first cultivated in China as a flowering herb and now they are native to Asia and north-eastern Europe. Most species originate from East Asia. They are economically significant worldwide as there are a great number of cultivars for cut flowers, pot flowers and garden flowers [3]. Chrysanthemum is grown throughout the world both for cut flowers and as potted plant. It is popular for flower arrangements due to its long vase life.

Chrysanthemums generally propagated vegetatively with Shoot cuttings and root suckers. This conventional process is very slow [4]. Apart from this, since cuttings are obtained repeatedly from mother plants, they can be subjected to virus infection and degeneration, thereby increasing production costs [5]. Hence, there is a need to standardize a quicker method of propagation. These problems have been solved by applying *in vitro* propagation techniques, which are routinely applied to the clonal propagation of a variety of horticultural plants including Chrysanthemum [6]. Clonal propagation through *in vitro* culture can enhance the multiplication rates [7]. Clonal propagation of a selected elite variety can provide true-to-mother type of plants as quality planting material, which otherwise is very difficult to make available [8]. In recent years numerous studies on *in vitro* propagation of different ornamental plants have shown that

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this technique is a solution for rapid propagation of selected plant species [9, 10 and 11]. Their having several reports regarding *in vitro* propagation of Chrysanthemum using various explants [12, 13 and 14]. Chrysanthemum created a great demand for the promotion of floriculture in Bangladesh. The climatic condition and soil of Bangladesh is very favorable for commercial cultivation of Chrysanthemum. But no significant works have been done yet on Chrysanthemum for its improvement either in agronomy or in *in vitro* aspects. Though few efforts were made earlier for commercialization of this plant using *in vitro* techniques in Bangladesh but it was not adequate. Considering it importance and to overcoming the mentioned constrains there is a need to establish a sustainable protocol for the commercial propagation of Chrysanthemum using tissue culture techniques. Therefore, the present study aims at developing a simple, rapid, and efficient high frequency regeneration techniques for potential application in large scale propagation of Chrysanthemum.

2. Material and methods

Young *Chrysanthemum morifolium* saplings were collected from local nursery. They were planted and conserved in the experimental garden at natural environment for explants source. Micro shoots of *C. morifolium* were collected from these plants whenever necessary. Different type of explants viz. shoot tip, nodal segment, leaf segment etc. were excised from these micro shoots. Explants were washed under running tap water for fifteen minutes and then cleaned by liquid soap mixed with Tween 20 and finally washed by distilled water for several times. Explants were treated with 70% ethanol for 30 seconds and dipped in 3.5% sodium hypochlorite for 20 minutes subsequently. Finally, excess detergent was removed by rinsing them in sterilized distilled water four times each for five minutes. The explants were then made into convenient size and put in a sterile conical flask for surface sterilization. Surface sterilization was performed in laminar air flow cabinet by dipping the explants in 0.1% $HgCl_2$ solution (w/v) for 15 minutes followed by several washes with sterilized distilled water. Several washes were necessary to remove traces of $HgCl_2$ completely. After surface sterilization the explants were laid on the sterile Petri dish using sterile forceps and a few mm. lengths of the explants that had been in contact with $HgCl_2$ was removed by cutting with a sterile scalpel. These types of explants were cultured onto MS containing various hormonal supplements viz. BAP and Kin (0.5 to 3.0 mg/l) singly or in combination with IAA (0.5 mg/l) for shoot-bud formation as well as multiple shoot regeneration. Urea (25mg/l-200 mg/l) were also used for growth and development of multiple shoots. MS containing standard salts and vitamins, 3% sucrose and 0.8% agar were used. P^H was adjusted 5.8 before autoclaving. The culture vessels containing medium were then autoclaved at 121°C and at 1.05 kg/cm^2 pressure for 20 minutes. Cultures were incubated in the growth room under 16/8 hours light/dark cycle at 25 ± 2 °C. For induction of roots, *in vitro* regenerated shoots were separated from the clusters of multiple shoots and transferred to ½MS supplemented with various concentrations of IBA (0.5 mg/l to 3.0 mg/l). Data were recorded in different stages viz. number of shoots per culture, Growth and development, days to initiating rooting, survival percentage of rooted plants at natural condition etc. The regenerated plantlets after developing sufficient root system in the *in vitro* condition were deemed ready to transfer to soil. The plantlets were carefully removed from the culture vessels. The roots of the plantlets were gently washed under running tap water to remove agar attached to the root zone. Immediately after washing, they were transferred to small polythene bags containing a mixture of soil, sand and compost in 1:1:1 ratio. The plantlets in the poly bags were covered with thin polythene bags to check sudden desiccation. The inner sides of these bags were sprayed with water at every 24 hours to maintain high humidity around the plantlets. The polythene bags were gradually perforated to expose the plantlets to the outer normal environment and subsequently removed after seven days. By this time the plantlets were become established in to the soil. They were finally transferred to the experimental field.

3. Results and discussion

In the present investigation attempts were made to standardize a reproducible protocol for *in vitro* clonal propagation of *Chrysanthemum morifolium*. The experiment was conducted by using three different types of explants namely shoot tip, nodal segment and leaf segment obtained from field grown plants.

3.1. Development of multiple shoots

For the development of multiple shoots three types of explants mentioned above was culture on to MS fortified with different concentration of cytokinins (BAP and Kn) as well as combination with a single concentration of IAA (0.5 mg/l). Among the various growth regulators used best response towards multiple shoot regeneration were observed from the nodal segments on MS supplemented with 2.0 mg/l BAP (Table.1). In this combination hundred percent explants were regenerated and average of 16 shoot bud were regenerated from nodal segments (Fig. 2).

Table 1 Effects of various concentrations and combinations of auxins and cytokinins on multiple shoot regeneration from shoot tip nodal and leaf segments from field grown chrysanthemum plants

Treatments (mg/l)		**Shoot tip**		**Nodal segment**		**Leaf segment**	
		% of explants	**Shoots/explants (no.)**	**% of explants**	**Shoots/explants (no.)**	**% of explants**	**Shoots/explants (no.)**
BAP	0.5	90	9	90	12	80	4
	1.0	90	9	95	12	80	4
	1.5	95	10	95	13	85	5
	2.0	**100**	**12**	**100**	**16**	**90**	**7**
	2.5	90	9	95	13	90	7
	3.0	85	9	90	11	85	5
Kn	0.5	85	5	85	10	70	2
	1.0	85	7	90	10	70	4
	1.5	90	8	95	11	75	4
	2.0	**95**	**10**	**95**	**12**	**80**	**6**
	2.5	85	9	85	12	75	5
	3.0	80	8	80	10	75	3
BAP + IAA	0.5+0.5	80	4	80	6	70	4
	1.0+0.5	85	5	85	8	75	4
	1.5+0.5	85	7	90	9	75	5
	2.0+0.5	**90**	**8**	**90**	**8**	**85**	**7**
	2.5+0.5	85	7	90	07	85	6
	3.0+0.5	80	7	80	6	80	6
Kn + IAA	0.5+0.5	75	2	75	4	70	-
	1.0+0.5	80	2	80	5	75	2
	1.5+0.5	85	3	80	6	75	2
	2.0+0.5	**90**	**6**	**95**	**8**	**80**	**3**
	2.5+0.5	90	4	95	5	90	2
	3.0+0.5	80	2	80	3	80	-

Hundred percent regeneration was also achieved in 2.0 mg/l BAP using shoot tip explants but the number of shoot bud formation is comparatively low (Fig.1). Leaf explants also produced lower number of shoots in this combination. Two cytokinins (BAP and Kn) combination with IAA (0.5mg/l) separately were used for the initiation of multiple shoots but response was not satisfactory (Table.1). In the present investigation 2.0 mg/l BAP showed comparatively better performance in case of explants regeneration as well as multiple shoot regeneration. Performance also comparatively low when more concentrations of BAP were used for multiple shoot induction.

Figure 1-6 *In vitro* regeneration of plants in *Chrysanthemum morifolium* Ramat. 1. Multiple shoot regeneration from shoot tip explants. 2. Multiple shoot regeneration from nodal segments. 3. Increase of multiple shoot derived from nodal segment after subculture. 4. Growth and elongation of multiple shoots. 5. Induction of roots from the regenerated shoots. 6. Plantlets established on soil

Several investigators got optimum results when they cultured nodal segments in the BAP enriched MS media singly in different concentrations for multiple shoot induction [15, 16 and 17]. The present investigations are in agreement with their findings. Earlier a significant number scientists [18 and 19] obtained better results for the development of *in vitro* multiple shoots in Chrysanthemum using either shoot tip or nodal segment in MS fortified with different concentration and combination of cytokinins (BAP and Kn) and auxins (IAA and NAA). In the present study we also used same hormonal supplements, which proved the justification for using these types of hormonal combinations for obtaining multiple shoot regeneration. Though 2.0 mg/l BAP showed best performance for multiple shoot regeneration but the frequency was low in higher as well as lower concentration. This indicates that a certain level of hormonal concentration is necessary for obtaining maximum results. Lower concentrations cannot stimulate shoot bud for regeneration and on the other hand higher concentrations are inhibitory because plant has also capability to produce hormone by its own naturally. BAP has been considered to be one of the most effective cytokinins for the induction of shoot regeneration in plant tissue culture [20]. A few studies showed that BAP is more effective than Kn in enhancing on shoot multiplication on Chrysanthemum as well as several plant species [21, 22 and 23]. Multiple shoots thus obtained were sub-cultured and maintained in the optimum media composition for obtaining adequate number of *in vitro* shoot stalks. Shoot bud was increased huge in numbers after two to three sub-cultures (Fig. 3).

3.2. Growth and development of multiple shoots

In the present study, though huge number of multiple shoots were developed in the BAP enriched MS basal media but the *in vitro* grown shoots were stunted to dwarf in size and not suitable for further sub culture leading to *in vitro* rooting. So prior to *in vitro* rooting, proper growth and development of these micro shoots are necessary. To overcome this constrain, multiple shoots were sub-cultured in the BAP fortified MS basal media adding with different concentrations of urea (25 mg/l to 200 mg/l) for obtaining satisfactory apical growth. Their length was increased in adequate size within three to four weeks (Fig. 4 and Fig. 7) when 125mg/l urea were used. BAP enriched MS basal media adding with urea in different concentrations are suitable for plant's apical elongation and it is proved by several investigators earlier [24 and 25]. The present investigations are in agreement with the earlier findings.

3.3. *In vitro* root induction

Multiple shoots growing *in vitro* were devoid of roots. Root formation is important for plant's adaptation as well survival in the natural condition. Plant uptakes nutrients and water by roots. So root formation is crucial for completely development of *in vitro* derived plantlets. For that purpose, well developed micro shoots were properly excised from the cluster of multiple shoots and transferred to different root inducing media for root induction. Half strength MS adding with different concentrations IBA were used for root formation.

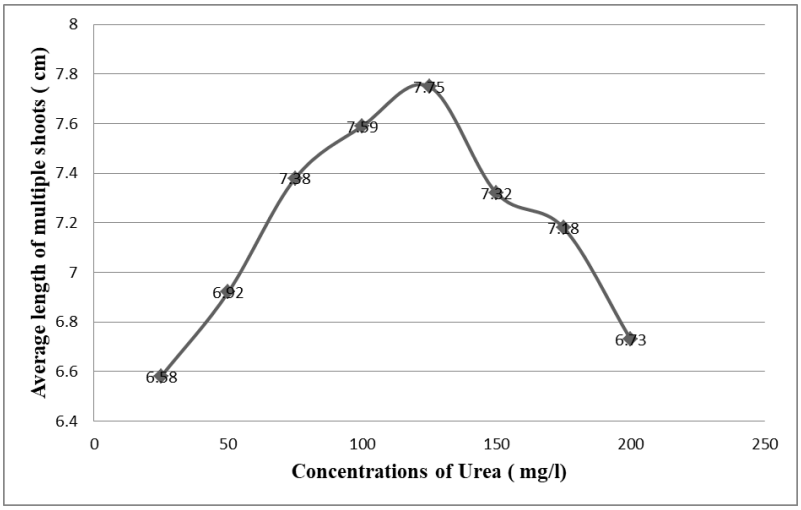

Figure 7 Effects of Urea for the growth and development of Chrysanthemum's multiple shoots growing *in vitro*

Table 2 Effects of Different concentrations of IBA in MS on root induction of *in vitro* grown Chrysanthemum micro shoots

Treatments (mg/l)	IBA			NAA		
	shoots implanted (no.)	Roots/shoot. (no.)	Root induction (Days)	shoots implanted (no.)	Roots/shoot. (no.)	Root induction (Days)
0.5	15	6	40	15	05	40
1.0	15	11	35	15	09	35
1.5	**15**	**14**	**35**	**15**	**11**	**35**
2.0	15	12	40	15	10	40
2.5	15	8	40	15	06	40
3.0	15	7	40	15	07	40

Root initiation was started within seven days and about 35-40 days were required to get healthy and mature roots. Best root development was occurred in ½ MS fortified with 1.5 ml/l IBA (Fig. 5). *In vitro* grown roots were satisfactory in numbers (Table. 2). Several previous investigators reported efficient *in vitro* root formation in different Chrysanthemum species using various type of auxins and among them IBA showed better performance [26]. On the basis of previous report, IBA was singly used in the present study and it showed better response for root induction (Fig. 5 and Table 2). It has been reported that IBA is the suitable auxin for adventitious root initiation and its performance is better rather than IAA or NAA ([27 and 28J.F. Hutchinson 1981 and R.E. Litz 1990]. After development adequate number of healthy and mature roots the plantlets were transferred to small pots containing 1:1:1 non- sterilized garden soil, sand and coco-peat and gradually adapted to field conditions (Fig. 6) l. About ninety per cent of the plantlets were survived in natural conditions. The protocol which achieved in the present study can be helpful for large scale propagation of these top demanding ornamental plants in Bangladesh and it can be an alternative solution for the floriculture extension in the country.

4. Conclusion

From the present investigation it can be concluded that this protocol will be performed as alternative pathway for the floriculture of this studied plant. This established protocol will be helpful to mitigate the increasing demand of ornamental plants not only Bangladesh but also around the world.

Compliance with ethical standards

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Disclosure of conflict of interest

We declare that there is no conflict of interest in connection with this paper.

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