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Chrysanthemum morifolium Ramat.: an *in vitro* Plantlets Regeneration Protocol

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Abstract

Studies on *in vitro* propagation of *Chrysanthemum morifolium* demonstrated that shoot apices from mature garden grown plants of yellow flower producing cultivar are capable of forming multiple shoots. Proliferation of explants was obtained on MS (Murashige and Skoog) basal medium containing different concentrations and combinations of cytokinins and auxins. Largest number of shoots was induced by shoot apices with 2 mg/l BA (6-benzyle adenine). In this medium, 8-14 shoots developed on shoot apices within 4-5 weeks of culture. Transfer of shoot clamps to a medium containing a lower concentration of BA (0.1mg/l) after cutting of some elongated shoots allowed further rapid growth and increased the number of shoots produced per culture. Better rooting was achieved by transferring the individual elongated shoots to MS medium containing 0.5 mg/l IAA (indole-3- butyric acid). Regenerated plantlets were successfully established in soil under natural environment and survival rate was more than 80 percent. *In vitro* mass propagation was achieved from this protocol and successful flower production was happened.

Keywords: *Chrysanthemum morifolium*, Regeneration, Explants.

INTRODUCTION

Chrysanthemum is a various colored flower producing plant belongs to the family Asteraceae. Among the commercially viable horticultural plants, cultivars of *Chrysanthemum morifolium* are most important. *Chrysanthemum* occupies an important place in the flower market of USA, Japan and many European countries as well as in India. The broad spectrum of colors and a good keeping quality seem to be the basic reason for its wide popularity. *Chrysanthemum* is one of the most popular flowers in Bangladesh and it has extensive demand as cut and pot flower throughout the country. It has a good export potential as cut flowers to many countries of the world (Erler and Siegmund 1986). Apart from their ornamental value, the flowers of 52 varieties of *Chrysanthemum morifolium* are also edible (Endo *et al*. 1990).

*Chrysanthemum*s are generally multiplied vegetative by rooting terminal cuttings. This conventional process of propagation is slow and they are susceptible to many diseases as well as environmental hazards that cause gradual degeneration of the cultivars. Moreover, this conventional method of propagation is not very useful when large scale propagation is necessary for commercial purpose. The plants are complex hybrids and if grown from seeds, the population segregates into many diverse flower forms.

Large scale production of *Chrysanthemum* through tissue culture technique has been reported by Levin *et al.* (1988). Bhattacharya *et al.* (1990) also reported, the rapid mass propagation of *Chrysanthemum* through callus derived from stem and leaf explants. Khan *et al.*

(1994), Hoque and Fatima (1995) works on *in vitro* propagation of *Chrysanthemum* which grown in Bangladesh. Seeds of most varieties are not viable and rate of seed germination is very poor, so large number of plantlets production creates difficulties. Use of tissue culture technique may make it possible to obtain a large number of plantlets from single explants. Considering these, the present study was undertaken to develop the mass propagation protocol of yellow cultivar of *Chrysanthemum morifolium* by using shoot apices from mature field grown plants.

MATERIALS AND METHODS

Materials: In the present study, large, compact and yellow colored flower producing cultivar was used. Shoot tips were used as explants. MS medium was used as basal media singly or fortified with growth regulators (BA, Kin, IAA, IBA, and NAA) in different experiments.

Explants preparation: Actively growing shoot tips (5-6 cm) were collected from garden grown plants of *Chrysanthemum morifolium*. After removing the leaves, the apices bearing 1-2 nodes were collected in distilled water, brought to the laboratory and washed under running tap water for about fifteen minutes to remove any dart from the collected materials. The materials were then rinsed with distilled water for several times. Surface sterilization was done with 0.1% $HgCl_2$ for five minutes inside the laminar air flow cabinet. The material was then washed with sterilized distilled water for 10 minutes with 4-5 changes to remove all stresses of $HgCl_2$. The leaf bases were removed and apices were excised and cultured on the medium.

Media and culture condition: In this study, MS medium was used with different concentrations and combinations of growth regulators and pH was adjusted 5.8 before addition of agar and sucrose. Medium were dispensed into culture vessels and then autoclaved at 120^0C for 15 minutes at 1 kg/cm^2 pressure. Studies on the explants establishment and shoot proliferation were done in full strength MS medium with 3% sucrose, 0.8% agar, 0.5-5 mg/l BA, 0.5-5 mg/l Kin with or without auxins. For adventitious rooting, MS medium with 0.7% agar and 0.1-2.0 mg/l IBA and some IAA concentrations was used. All cultures were grown at 24-27^{0}C with 16 hour photo period from white fluorescent tubes.

Hardening: After rooting, plantlets were kept for 4-5 days in room temperature for hardening by openings the caps of the culture vessels. The *in vitro* regenerated shoots were removed from the culture tube, washed thoroughly to remove the agar and nutrients and transferred to poly bag with 1:1 garden soil and sand. To maintain high humidity, transferred plantlets were covered with polythene bags.

Observations: Presented data regarding mentioned parameters were average number with standard deviation, three replications were used and each replication contained ten explants. In shoot elongation response, 1 cm and more length of shoots were only considered.

RESULTS AND DISCUSSION

Shoot regeneration: Shoot apices cultured on MS medium supplemented with different concentrations and combinations of cytokinins and auxins showed their first response by enlargement and emergence of shoot apices. Rapid and early proliferation was observed in media with lower concentrations of cytokinins (Table-1). Shoot development was found to start from the shoot apices within 2-3 weeks of culture. Media having IAA or NAA delayed shoot proliferation. More than 50% of explants responded in media supplemented with only cytokinin. In combination of BA and Kin also showed better response. It was also observed that, the survival percentage of shoots reduced when IAA or NAA was combined with cytokinins.

The number of shoots per explants increased with the increase of cytokinins up to 2 mg/l and declined in more than the concentrations. Maximum number of shoots per explants was achieved where 2 mg/l BA was used followed by 1 mg/l. Cent percent multiple shoot forming cultures were not found in different BA combinations and this findings are disagreement with the findings of Khan *et al.* (1994), it may be happened due to the use of BAP instead of BA. In the present work, highest responding culture (82.35) was found in MS medium containing 1.0 mg/l BA+0.5 mg/l Kin but Bajaj *et al.* (1992) achieved maximum response from meristem tip and stem segment by using MS medium with 2.0 mg/l IAA+0.5 mg/l Kin. Over all performance of cultured explants was better in media enriched with BA than those with kinetin. Addition of IAA or NAA with BA in the media did not improve shoot proliferation and resulted in callus production at the base of the explants, which suppressed the growth of shoots and no of usable shoots also low. Hoque (1995) and Malaure *et al.* (1991) also used NAA with BAP and they got best response, this is the disagreement from the present study, it may be occurred due to the use of different genotype as well as explants. Bhattacharya *et al.* (1990) used IAA with

BAPand obtained desirable morphogenic responses from nodal segments, shoot tips and calli from leaf and stem of *Chrysanthemum*, their findings are disagreement with present findings. Shoot elongation was more or less inverse to the number of shoot and maximum shoot length was observed in 0.5 mg/l kin.

Table-1: Effects of growth regulators on shoot proliferation and multiplication from shoot apices of *Chrysanthemum morifolium* after six weeks of culture with three replications.

Growth regulators (mg/l)	Days taken to bud proliferation	% of culture proliferating	No. of shoots per culture Mean±SD	Shoot length (cm) Mean±SD
BA 0.5	9.34	70.52	5.14±1.229	4.03±0.749
BA 1.0	9.22	64.32	8.92±1.598	1.75±0.688
BA 2.0	12.65	78.64	12.15±1.956	2.55±0.787
BA 3.0	15.52	54.19	6.78±0.658	3.40±1.088
Kinetin 0.5	10.33	56.87	3.53±0.618	4.99±0.936
Kinetin 1.0	9.23	60.55	4.74±0.889	3.36±0.591
Kinetin 2.0	13.31	51.96	6.75±1.289	2.93±0.506
Kinetin 3.0	17.87	54.55	4.14±1.104	3.08±0.631
0.5 BA+0.5 Kin	8.36	62.80	3.33±0.730	3.11±0.995
0.5 BA+1.0 Kin	10.87	58.19	3.80±0.487	3.80±1.114
1 BA+0.5Kin	9.66	82.35	4.32±0.672	4.42±0.672
1 BA+1.0 Kin	13.38	74.31	4.85±0.709	3.54±0.548
Kinetin 0.5	10.38	56.82	3.57±0.793	4.93±0.835
Kinetin 1.0	9.23	60.54	4.75±0.728	3.39±0.970
Kinetin 2.0	13.32	51.95	6.74±1.524	2.94±0.812
Kinetin 3.0	17.83	54.54	4.13±1.164	3.07±0.999
BA 2+NAA0.2	14.54	44.66	11.52±2.007	2.52±0.560
BA 2+IAA 0.2	13.93	43.76	10.35±1.349	2.95±0.679
Kinetin 2+NAA 0.2	13.96	47.85	5.62±0.662	2.47±0.523
Kinetin 2+IAA0.2	15.38	41.94	5.95±1.140	2.67±0.818

Root induction: From proliferated shoot clumps individual shoots (approximately 3 cm length) were excised and transferred to rooting media. Roots could produce in auxin free media but they were thin and number of roots per cutting was low. In the present investigation, full strength of MS Media supplemented with 0.5 mg/l IBA showed improved rooting frequency, rooting quality and number of roots per cutting. All most 100% shoots produced roots with 5-8 roots per shoot in this medium. Das *et al.* (1986), was used IBA at different concentrations with MS and half MS media for root initiation of *in vitro* regenerated shoots. Hoque (1995), observed optimum root induction in MS medium with 0.2 mg/l IBA and Khan *et al.* (1994) reported that, 0.25 mg/l IBA with MS medium showed good root induction which was near to the present findings. IAA was used with MS medium for root induction but IBA expressed better performance. After harvesting of shoot micro cutting for rooting, the stock culture were transferred to fresh medium (MS+0.1 mg/l BA) after removing dead and discolored tissue. In this way stock cultures were maintained for continuous production of shoots for several months.

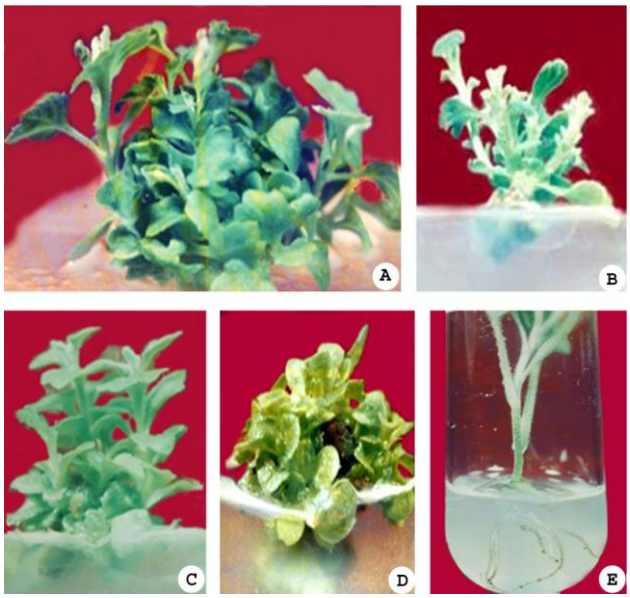

Figure: A-E: *In vitro* plantlet regeneration in *Chrysanthemum morifolium*. **A.** Formation of multiple shoots on MS medium supplemented with 2.00 mg/l BA after five weeks of culture. **B.** Multiplication of shoots on MS medium containing 2.00 mg/l BA + 0.2 mg/l NAA. **C.** Shoot multiplication and proliferation on MS medium with 2.00 mg/l BA + 0.5 mg/l Kin. **D.** Multiple shoot initiation on MS medium supplemented with 2.00 mg/l BA + 0.2 mg/l IAA. **E.** Root induction on MS medium with 0.5 mg/l IBA after four weeks of culture.

After hardening, plantlets were taken from auxin supplemented media survived better (80%) than those were taken from auxin free media (20%). More than 80% of the regenerated plantlets survived and resumed growth. These regenerated plants produced yellow flowers like to their parent stocks.

Table-2: Effects of growth regulators on root induction of *Chrysanthemum morifolium* after five weeks of culture with three replications.

Growth regulators (mg/l)	Days taken to root initiation	% of root induction	No. of roots per culture Mean±SD	Root length (cm) Mean±SD
IBA				
0.1	10-15	90	4.62±0.809	8.85±1.735
0.5	5-8	100	5.20±0.950	8.91±1.522
1.0	5-12	100	4.70±0.856	7.40±0.830
1.5	7-20	80	3.17±0.886	4.37±0.636
IAA				
0.5	10-15	50	2.16±0.777	3.15±0.847
1.0	10-15	50	2.03±0.920	3.16±0.818

The results described in this investigation showed that rapid clonal propagation of *Chrysanthemum morifolium* through *in vitro* culture of shoot apices is possible. This protocol can be helpful to propagate the superior variety of *Chrysanthemum* and overcome the problem of conventional propagation method.

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