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Callus induction and indirect organogenesis in *Chrysanthemum morifolium* Ramat.

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Abstract

A procedure for callus induction and indirect organogenesis of *Chrysanthemum morifolium* Ramat., using leaf segments as explant has been developed. For callus induction, leaf segments were cultured in MS medium (Murashige and Skoog's, 1962) supplemented with 2,4-D singly (0.1, 0.5, 1.0, 1.5, 2.0 mg/litre) and IAA (0.5 mg/litre) in combination with BAP (0.1, 0.2, 0.5, 1.0, 1.5 mg/litre). Effective callus induction and growth were obtained in the medium of MS supplemented with 2.0 mg/l 2,4-D and 0.5 mg/l IAA + 0.2 mg/l BAP. 90% callus induced in both medium. Induced callus was transferred to the regeneration medium for effective shoot regeneration in 1.5 mg/l NAA + 1.0 mg/l BAP combination. The shoot regeneration frequency reached 90%. In order to induce roots, the shoots were transferred to the root induction media, and 1.0 mg/l IBA was recorded for maximum (100%) number of root induction on calli regenerated shoots. So, the indirect organogenesis of *Chrysanthemum morifolium* Ramat. was achieved through callus cultures and it may be possible to regenerate a plant from genetically variable cells of the callus tissue.

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Introduction

Chrysanthemum (*Chrysanthemum morifolium* Ramat.) is one of the most important commercial cut flowers. Chrysanthemum belonging to the Asteraceae family, was initially classified as a Compositae (Salinger, 1991). The name originally came from two Greek words, '*chrysos*' meaning golden and '*anthemon*' meaning flower which originated in China and where it has been cultivated for over 3000 years. It is a perennial plant and is an important herb in both Japanese and Chinese traditional medicine. It is now cultivated in Europe, the United States and many other countries because of the great demand for its flowers. *Chrysanthemum* occupy an important position among floral crops in the world and display a wide range of variability for economic floral traits among its cultivars that is hardly surpassed by any floral crop. These plants have a number of ornamental, culinary, environmental, insecticidal, and medicinal uses that are known to man since centuries. They can be propagated vegetatively either through root suckers or terminal cuttings; this conventional process of shoot cutting is very slow (Nhut *et al*., 2005). However, clonal propagation through *in vitro* culture can enhance the multiplication rates (Sauvaire and Galgy, 1978). For modern and industrialized horticulture, the cut flower industry, perhaps different from any other industry, is always in demand and in need of new varieties to routinely attend the continuous flower consumer demands. Consumer preferences change and show new and sometimes uncommon features. Therefore, the priority of the flower and ornamental plant biotechnology segments should be the generation of novel plant and flower types (Hutchinson *et al*., 1982). Callus culture in Chrysanthemum is extremely useful for producing a huge number of explants in a short time. The whole plant can be regenerated in large number from callus tissue through manipulation of the nutrient and hormonal constituents in the culture medium. This phenomenon is known as indirect organogenesis or plant regeneration. So, callus tissue means an unorganized proliferative mass of cells produced from isolated plant cells, tissues or organs when grown aseptically on artificial nutrient medium in glass vials under controlled experimental conditions. Callus tissue is good source of genetic or karyotype variability, so it may be possible to regenerate a plant from genetically variable cells of the callus tissue. Callus culture is very useful to obtain commercially important secondary metabolites. If a bit of tissue from a medicinally important plant is grown *in vitro* and produced callus culture, then secondary metabolites or drug can be directly extracted from the callus tissue without sacrificing the whole plant. So, this alternative technique helps the conservation of medicinal plants in nature. Several biochemical assays can be performed from callus culture.

There are some reports on callus culture of chrysanthemum from different countries. Bhattacharya *et al*. (1990) reported rapid mass propagation of *Chrysanthemum morifolium* through callus derived from leaf and stem explants. Hitmi *et al*. (1999) evaluated a procedure for rapid mass propagation of *Chrysanthemum cinerariaefolium* from young flower heads, which was developed to compare the ability of callus, *in vitro* shoots and rooted plants and original plants to synthesize pyrethrins. The ability to synthesise all six pyrethrin components increased with differentiation. Ben-Jaacov and Langhans (1972), Earle and Langhans (1973) described *in vitro* chrysanthemum micropropagation from shoot tips and shoot initiated callus. Vantu (2006) said that, the plants of *Chrysanthemum morifolium* have been regenerated from callus cultures, established from stem and leaves explants. Callus cultures induced from stems had a greater shoot differentiation than those obtained from leaves. Despite the great opportunity of genetic variation in callus cultures, the regenerated plants differ not in their external appearance from the normal plants. Wankhede *et al*. (2000) studied callus induction and shoot regeneration in chrysanthemum cultivars Zipri and Shyamal Dark Pink.

The main objectives of the present study were to determine the effect of different growth regulators on callus induction, shoot formation and multiplication

over the cultural period. Besides, the regeneration of the whole plants from callus that have been genetically modified. The latter is extensively used for the commercial production for many cultivars. The impact of the techniques of plant biotechnology have not to date, being utilized in the development of new cultivars and improved varieties of *Chrysanthemum* sp.

Materials and methods

Medium preparation

MS basal media was used in the present experiment supplemented with different kinds of hormones either in singles or in combinations for callus induction, shoot regeneration, shoot multiplication and root induction of *C. morifolium*, as well as their acclimatization under *in vivo* condition. The media containing full strength of MS basal salts were used. For the preparation of media, stock solutions were prepared at the beginning and the respective media were prepared from the stock solutions.

Plant material

Chrysanthemum morifolium was used as experimental materials for the present investigation. The sucker was collected from a local nursery and planted in the Research Field. When the plant was 2-3 months old, explants were collected. Immature leaf segments were excised from these established plants and used to conduct different experiments.

Sterilization

For surface sterilization, the explants were cleaned thoroughly under running tap water for several times to reduce the dust and then taken in a conical flask containing distilled water adding with detergent (washing agent) and then add few drops of savlon and washed for 10 minutes with constant shaking. It was then followed by second washing with gradual change of distilled water until all traces of above chemicals are removed. The cleaned explants were finally treated with 0.1% (w/v) $HgCl_2$ with occasional agitation inside the laminar airflow cabinet. After sterilization, the materials were rinsed 5-6 times with sterilized water to remove $HgCl_2$ and placed on sterilized filter paper to remove excess of water before inoculation.

Callus induction

To induce callus, leaf segments were cultured in MS (Murashige and Skoog, 1962) medium supplemented with different callus inducing substances. MS medium was supplemented with five 2,4-dichlorophenoxy acetic acid (2,4-D) concentrations viz. 0.1, 0.5, 1.0, 1.5 and 2.0 mg/l singly and 0.5 mg/l Indole acetic acid (IAA) which combination with benzyl amino purine (BAP) 0.1, 0.2, 0.5, 1.0, 1.5 mg/l and sugar was used as sources of carbon (30 gm/l) for massive callus induction.

Subculture of Callus for maintenance, shoot regeneration and root induction

After 28-32 days of culture initiation the calli induced in different media were rescued aseptically on a sterile petridish and were cut into convenient sizes (0.5-0.6 cm diameter) by a sterile sharp scalpel. These were inoculated to a freshly prepared medium supplemented with the same or different hormonal combinations for the maintenance of callus. For shoot differentiation, calli were inoculated to a freshly prepared MS medium supplemented with napthalene acetic acid (NAA) concentrations viz. 0.5, 1.5 mg/l in combination with benzyl amino purine (BAP) 0.5, 1.0 mg/l. During the subculture programme, inoculation and all aseptic manipulations were carefully carried out in front of lamina airflow cabinet and all the necessary instruments were sterilized before use. When the regenerated shoots were 2-3 cm in length, then each of the shoots were inoculated on freshly prepared MS medium containing Indole-3-butyric acid (IBA) concentrations of 0.5, 1.0, 1.5, 2.0, 2.5 mg/l for root induction.

Well developed plantlets were rinsed thoroughly with sterile water to remove residuals and potted with a sterile soil and sand mixture in the ratio of 1:1, covered with transparent polythene bags to prevent sudden desiccation. The interiors of the polythene bags were sprayed with water at everyday to maintain high humidity around the juvenile plants. The

polythene bags were gradually perforated to expose the plants to the outer environment. After 25-30 days, the fully acclimatized plantlets were finally established in the soil under natural environment.

Statistical analysis

Data were statistically analyzed using analysis of variance technique (ANOVA), Least Significant Difference (LSD) test was used to speculate further if there was a significant difference within various plant hormones, concentrations and replication. P values < 0.05 were considered as significant and values are the mean ± standard error of mean. Each experiment was repeated three times and each treatment 10 explants were cultured.

Results

The present investigation was carried out for callus induction and plant regeneration from leaf segments from *C. morifolium*. Explants were cultured on MS basal media with different concentrations of 2,4-D singly and IAA in combination with BAP in order to find out the most suitable culture media formulation to induce the explants to develop maximum callus.

Table 1. Effect of different concentrations of 2,4-D singly and IAA in combination with different concentrations of BAP in MS medium on callus induction from immature leaves of *C. morifolium*. Data were recorded after 5 weeks of culture.

Name of the hormone	Concentration (mg/l)	No. of explants inoculated	% of explants induced callus	Callus color	Degree of formation of callus
2,4-D	0.1	10	30	LG	+
	0.5	10	40	LG	+
	1.0	10	50	LG	++
	1.5	10	80	G	+++
	2.0	10	90	G	+++
IAA+BAP	0.5+0.1	10	70	G	+++
	0.5+0.2	10	90	G	+++
	0.5+0.5	10	50	G	++
	0.5+1.0	10	50	G	++
	0.5+1.5	10	40	G	+

+ = Little callusing; ++ = Moderate callusing; +++ = Massive callusing; LG = Light green; G = Green.

Callus induction

Effect of different concentrations of 2,4-D singly and IAA in combination with different concentrations of BAP in MS medium on callus induction from immature leaves of C. morifolium

Data on production of callus, derived from immature leaf segments explant of chrysanthemum were recorded after five weeks of incubation. MS media having 2.0 mg/l 2,4-D showed its superiority amongst all the other concentration levels, in all the parameters studied (Table 1, Figure 1; a to c). Maximum number of explants (90%) induced callus were found from immature leaf segments in media having 2.0 mg/l 2,4-D. The next percentage was noted in media containing 1.5 mg/l 2,4-D which 80% of explants induced callus. Massive callusing was found in 2.0 mg/l 2,4-D and 1.5 mg/l 2,4-D while moderate callusing was found in 1.0 mg/l 2,4-D. The callus color were green in 1.5 mg/l and 2.0 mg/l 2,4-D and light green in 0.1, 0.5 and 1.0 mg/l 2,4-D.

IAA along with BAP also play a vital role in callus induction in chrysanthemum. The result showed that out of different hormonal combinations, 0.5 mg/l IAA with 0.2 mg/l BAP showed superiority over all the other combinations (Table 1). The highest percentage (90%) of callus induction was recorded in MS media having 0.5 mg/l IAA with 0.2 mg/l BAP (Figure 2; a to c). Where 70% of explant induced callus was noted in media containing 0.5 mg/l IAA with 0.1 mg/l BAP. Massive callusing were recorded in 0.5 mg/l IAA with

0.2 mg/l BAP and 0.5 mg/l IAA with 0.1 mg/l BAP. The callus color was green in all concentrations.

Shoot regeneration from callus

Effect of different concentrations and combinations of NAA with BAP in MS medium on shoot regeneration from callus

MS medium supplemented with different concentrations and combinations of NAA (0.5 and 1.5 mg/l) with BAP (0.5 and 1.0 mg/l) were used to observe the response of shoot induction from callus (Table 2). Only 2.0 mg/l 2,4-D containing medium derived calli were produce maximum (80%) number of shoots and highest mean number (2.3±0.43) of shoots per callus in combination with 1.5 mg/l NAA + 1.0 mg/l BAP (Figure 1; d to e).

Table 2. Effect of different concentrations and combinations of NAA and BAP in MS medium on shoot formation from callus. Data were recorded after 6 weeks of culture.

Callus induction medium (mg/l)	Growth regulators in regeneration media	% of callus produced shoots	Mean no. of shoots per callus
2,4-D	NAA+BAP		
1.0	0.5+0.5	---	---
	0.5+1.0	---	---
	1.5+0.5	10	0.1±0.18
	1.5+1.0	20	0.3±0.22
1.5	0.5+0.5	---	---
	0.5+1.0	20	0.6±0.44
	1.5+0.5	30	1.2±0.63
	1.5+1.0	50	1.5±0.63
2.0	0.5+0.5	20	0.6±0.44
	0.5+1.0	30	0.9±0.31
	1.5+0.5	60	1.6±0.38
	1.5+1.0	80	2.3±0.43
IAA+BAP	NAA+BAP		
0.5+0.1	0.5+0.5	20	0.5±0.22
	0.5+1.0	30	0.9±0.31
	1.5+0.5	50	1.3±0.28
	1.5+1.0	60	1.9±0.37
0.5+0.2	0.5+0.5	40	0.8±0.25
	0.5+1.0	50	1.4±0.26
	1.5+0.5	70	2.0±0.28
	1.5+1.0	90	2.9±0.34
0.5+0.5.3	0.5+0.5	---	---
	0.5+1.0	30	1.1±0.48
	1.5+0.5	20	0.7±0.22
	1.5+1.0	50	1.4±0.26
0.5+1.0	0.5+0.5	---	---
	0.5+1.0	---	---
	1.5+0.5	20	0.5±0.29
	1.5+1.0	40	1.0±0.18

While 0.5 mg/l IAA with 0.2 mg/l BAP containing medium derived calli were produced maximum percentage (90%) of shoots and highest mean number (2.9±0.34) of shoots per callus in combination with 1.5 mg/l NAA + 1.0 mg/l BAP (Figure 2; d to e). The analysis of variance (Table 3) indicated that, for number of shoots per callus, 2,4-D, IAA with BAP and NAA with BAP all were significantly different at 5%

level.

Root induction from callus derived regenerated shoots

Induction of roots are essential for successful establishment of the plantlets in soil. For root induction from regenerated plantlets of *C. morifolium* were cultured in different concentrations of IBA.

Effect of different concentrations of IBA on root induction from calli regenerated plantlets

Callus derived shoots of *C. morifolium* were used as explants. For root induction, highest percentage (100%) of rooting, mean number of roots (6.0±0.43) and mean length of roots (3.54±0.28 cm) were recorded in media containing 1.0 mg/l IBA within 8-9 days (Table 4 and Figure 1-f; Figure 2-f).

Table 3. Statistical analysis (ANOVA) of no. of shoots per callus of *Chrysanthemum* sp.

Source of variation	df	SS	MS	F	Comment	LSD
2,4-D	2	2.375833	1.187917	9.610112	*	0.201651
NAA+BAP	3	3.151667	1.050556	8.498876	*	0.151238
Error	6	0.741667	0.123611			
Total	11	6.269167				
IAA+BAP	3	4.525	1.508333	18.34459	*	0.092993
NAA+BAP	3	4.205	1.401667	17.0473	*	0.092993
Error	9	0.74	0.082222			
Total	15	9.47				

*= difference in 5% significant level (P<0.05).

Table 4. Effect of different concentrations of IBA singly in MS medium on rooting from *in vitro* grown shoots of *C. morifolium*. Data were recorded after 40 days of culture. Values are the mean ± standard error of mean.

Concentration of IBA (mg/l)	No. of shoots inoculated	Days taken to root initiation	% of roots formation	Mean no. of roots per shoot	Mean length of roots(cm)
0.5	10	16-17	70	2.1±0.36	1.82±0.11
1.0	10	16-17	100	6.0±0.43	3.54±0.28
1.5	10	12-14	90	4.6±0.49	3.16±0.23
2.0	10	8-9	80	3.1±0.46	1.92±0.14
2.5	10	10-12	60	2.4±0.28	1.4±0.06

While lowest percentage (60%) of rooting, mean number of roots (2.4±0.28) and mean length of roots (1.4±0.06 cm) were in media having 2.5 mg/l IBA within 16-17 days. For number of roots per shoot and length of roots, the statistical analysis (Table 5) shows that, except replication, concentrations were significantly different from each other at 5% level.

Discussion

In the present investigation, important cut flower plant *Chrysanthemum morifolium* of Bangladesh was used for callus culture. The simplest tissue in culture media, is a callus, composed of irregular masses of tissue. An exogenous supply of growth regulators is often recommended to initiate callus from different explants. The present study on callus induction was conducted with sources of explants immature leaves. There have been several reports of callus culture in Chrysanthemum. Bhattacharya *et al.* (1990) used 2,4-D for callus induction in *Chrysanthemum* sp. Similarly, Lindiro *et al.* (2013) reported that callus induction from leaf segments in MS medium containing 1.5 mg/l 2,4-D was found to be 100%.

In the present study, different concentrations of 2,4-D were used for callus induction. Among all 2,4-D concentrations, 2.0 mg/l 2,4-D showed the best performance for callus induction and maximum (90%) callus formation was found in this concentration.

Table 5. Statistical analysis (ANOVA) of no. of roots per shoot and length of roots of *Chrysanthemum* sp.

No. of roots per shoot.

Source of variation	df	SS	MS	F	Comment	LSD
Concentration	4	45.236	11.309	63.066	*	0.27567
Replication	2	0.3252	0.16259	0.90668	ns	
Error	8	1.43456	0.17932			
Total	14	46.9957				

ns= not significant, *= difference in 5% significant level (P<0.05).

Length of roots.

Source of variation	df	SS	MS	F	Comment	LSD
Concentration	4	10.4963	2.62408	40.6184	*	0.09932
Replication	2	0.17344	0.08672	1.34235	ns	
Error	8	0.51683	0.0646			
Total	14	11.1866				

ns= not significant, *= difference in 5% significant level (P<0.05).

Besides, at IAA+BAP, immature leaf segments were used and maximum (90%) callus formation was found at 0.5 mg/l IAA +0.2 mg/l BAP. More or less similar observation was noted by Bhattacharya *et al.* (1990). They obtained desirable morphogenic response from nodal segments, shoot apices, and leaf and stem calli of *C. morifolium* on MS containing 0.1 mg/l IAA + 0.2 mg/l BAP. Multiple shoot formation was observed from almost all the combinations.

Shoot formation and multiplication rate and growth varied according to the media composition. NAA with BAP combination played an important role in shoot formation & multiplication. In the present investigation, callus induced at 2.0 mg/l 2,4-D, which was sub cultured at NAA+BAP. In 1.5 mg/l NAA+1.0 mg/l BAP showed best response (80%) on multiple shoot regeneration. Again, callus was induced at 2.5 mg/l 2,4-D+0.75 mg/l BAP, which was sub cultured at NAA+BAP also and this concentration proved to be most effective, showed the best response (90%) on multiple shoot regeneration when they were cultured on MS containing 1.5 mg/l NAA+ 1.0 mg/l BAP. More similar observation was noted by Bhattacharya *et al.* (1990) and Nahid *et al.* (2007).

Bhattacharya *et al.* (1990) used 0.1 mg/l NAA+ 0.2 mg/l BAP for shoot regeneration from callus and Nahid *et al.* (2007) reported that the highest (96%) callus formation obtained in MS medium supplemented with 2 mg/l BA and 0.1 mg/l NAA.

Besides, Bajaj *et al.* (1992) reported that calli obtained from various explants on different media produced shoots when they were sub-cultured on MS containing NAA 0.2 mg/l + KIN 2.0 mg/l. Hoque (1995) reported that nodal explants on MS containing 0.5 mg/l each of IAA and BAP showed best response (100%) in multiple shoot regeneration.

In the present investigation, IBA was found to be best for root induction and maximum rooting (100%) was found in 1.0 mg/l IBA. After rooting obtained complete plantlets. Gautheret (1939) suggested first the importance of auxin in root induction. Bhojwani and Razdan (1983) reported the common range of NAA or IBA is 0.1 to 1.0 mg/l. Following the report of Das *et al.* (1986) and Hoque (1995) IBA was used at different concentrations in MS medium for root induction on *in vitro* regenerated shoots. Hoque (1995) observed optimum root induction in MS medium with 0.2 mg/l IBA. Similar results on *Chrysanthemum* were obtained by Long *et al.* (2006) and Karim *et al.* (2002).

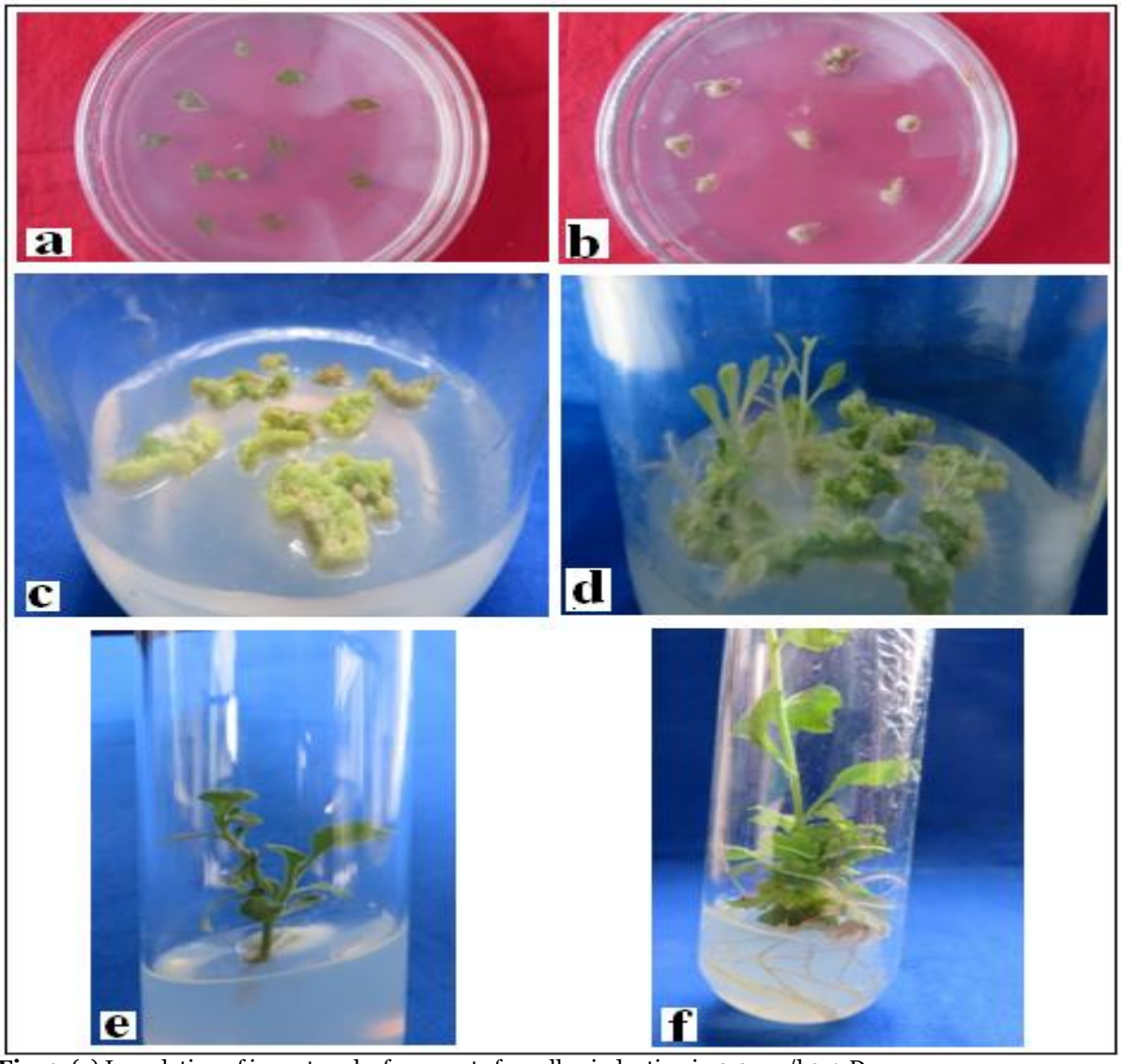

Fig. 1. (a) Inoculation of immature leaf segments for callus induction in 2.0 mg/l 2,4-D.
(b) Callus initiation from leaf segments in 2.0 mg/l 2,4-D.
(c) Proliferation of callus derived from immature leaf segment in 2.0 mg/l 2,4-D.
(d) Primary shoot initiation from callus in 1.5 mg/l NAA + 1.0 mg/l BAP.
(e) Subculture in 1.5 mg/l NAA + 1.0 mg/l BAP.
(f) Induction of roots on *in vitro* regenerated callus derived plantlets in 1.0 mg/l IBA.

Whereas Long *et al.* (2006) reported that highest number of roots where obtained when *Chrysanthemum* microshoot cutting where treated with IBA. Karim *et al.* (2002), who reported that 0.2 mg/l IBA produced the highest root length.

Shatnawi *et al.* (2010) reported that maximum root length was obtained by using 0.2 mg/l IBA or NAA. IBA is considered as the most effective auxin in root induction (Litz and Jaiwal 1990). Komalavalli and Rao (2000), Sarker and Shaheen (2001), Munshi *et al.* (2004), Awal *et al.* (2005), Din *et al.* (2005), Rajani and Patil (2009) suggested indole butyric acid (IBA) as the best auxin for root induction and development.

From the overall experiment, it can be concluded that 2,4-D singly and IAA with BAP were most effective for callus induction, besides, NAA with BAP was most effective for the miximum shoot formation and multiplication and full strength of MS medium supplemented with IBA was best for rooting.

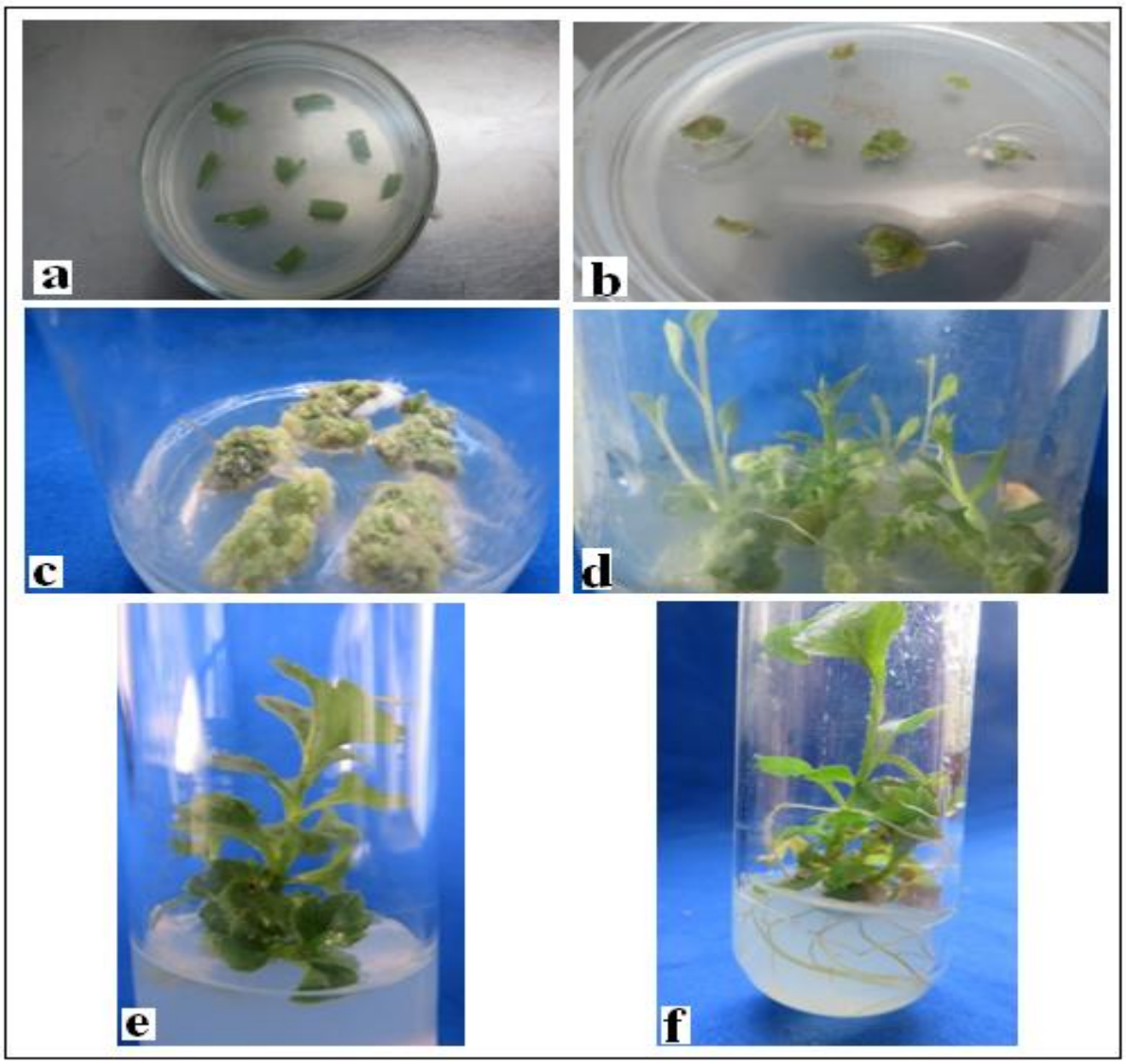

Fig. 2. (a) Inoculation of immature leaf segments for callus induction in 0.5 mg/l IAA + 0.2 mg/l BAP.
(b) Induction of callus from leaf segments in 0.5 mg/l IAA + 0.2 mg/l BAP.
(c) Proliferation of callus derived from immature leaf segment in 0.5 mg/l IAA + 0.2 mg/l BAP.
(d) Primary shoot initiation from callus in 1.5 mg/l NAA + 1.0 mg/l BAP.
(e) Subculture of shoot in 1.5 mg/l NAA + 1.0 mg/l BAP.
(f) Induction of roots on *in vitro* regenerated callus derived plantlets in MS 1.0 mg/l IBA.

Conclusion

Following the protocol of our investigation in future, it is possible to bring somaclonal variation which helps to develop new genotype by callus culture.

The regeneration of the whole plants from callus that have been genetically modified. The developed protocol on callus culture systems of *Chrysanthemum morifolium* offers a great potential for large scale commercial multiplication for producing of the studies in *in vitro* condition. For commercially large scale production of shoot and root using bioreactor or green house. In conclusion, the protocol described in this investigation can be used for the efficient production of *Chrysanthemum morifolium* and Callus culture is very useful to obtain commercially important secondary metabolites.

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