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Protocol on direct organogenesis in *Chrysanthemum morifolium* Ramat.

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Abstract

An efficient direct organogenesis protocol was developed for *Chrysanthemum morifolium* Ramat., an ornamental herb. The purpose of this study was to determine the effect of different growth regulators on shoot formation and multiplication over the cultural period and mass propagation in large quantities in a short time using tissue culture techniques. Nodal segments, after being sterilized with 0.1% $HgCl_2$ (mercuric chloride) for eight minutes, were inoculated in MS media (Murashige and Skoog, 1962) with different concentrations of BAP (6-benzyl amino purine) singly and combinations with KIN (6-furfural amino purine), Casein hydrolysate (CH), NAA (α-napthalene acetic acid). All of that, shoot growth and development showed better performance in 1.0 mg/l BAP singly, and 2.0 mg/l BAP with 0.5 mg/l KIN. *In vitro* rooting was successfully achieved on MS media supplemented with different concentrations of auxins. Better rooting response was obtained in MS media supplemented with 2.0 mg/l and 2.5 mg/l IBA (Indole-3 butyric acid) and 0.5 mg/l IBA with 1.5 mg/l NAA, respectively. The results from the study will enhance rapid multiplication of high quality planting materials of *Chrysanthemum morifolium* and other valuable plants.

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Introduction

Plant tissue culture is the aseptic culture of cells, tissues, organs and their components under defined physical and chemical conditions *in vitro* (Haberlandt, 1902). Plant tissue culture is widely used to produce clones of a plant in a method known as micropropagation. Organogenesis is a common method of micropropagation that involves tissue regeneration of adventitious organs or axillary buds directly or indirectly from the explants. In direct organogenesis, the tissue undergoes morphogenesis without going through a callus or suspension cell culture stage. The term direct adventitious organ formation is also used for direct organogenesis. So, in which process, firstly shoot formation from explants and later root formation from that, is produced to form a complete plant is called direct organogenesis.

Direct organogenesis protocol was developed for *Chrysanthemum morifolium* Ramat. *Chrysanthemum*, also known as chrysanths or mums, belong to the Asteraceae family and are a variety of flowering perennial plants. Asteraceae family is considered as an advanced family. Chrysanthemum is used in various ways such as ornamental, culinary, environmental, insecticidal and medicinal uses that are known to man since centuries. Chrysanthemum is highly valued as a cut flower worldwide with its diverse floral types and colors (Teixeira da Silva, 2003). It is globally an important cut flower and pot plant species usually cultivated by vegetative cuttings (Jaime and Teixeiria, 2004). It is generally propagated using suckers and terminal cuttings (Rout and Das, 1997). This approach, however, is inadequate to attain fast multiplication rate, as these conventional propagating methods are very slow, time consuming and tiring. Secondly, cuttings obtained repeatedly from mother plants may be subjected to any virus infection and degeneration, thereby increasing production costs (Hahn *et al*., 1998). These problems have been solved by applying micropropagation methods, which are routinely applied to the clonal propagation of a variety of horticultural plants including Chrysanthemum (Ben-Jaacov and Langhans, 1972). *In vitro* culture has become now a viable alternative to the conventional propagation methods.

There are many reports on tissue culture of chrysanthemum from different countries. Khalid *et al*. (1989) described a protocol for regenerated *Chrysanthemum* plants. Bhattacharya *et al*. (1990) reported rapid mass propagation of *Chrysanthemum morifolium* through callus derived from leaf and stem explants. Lu *et al*. (1990) utilized, direct plant regeneration was obtained from fresh chrysanthemum (*Chrysanthemum morifolium* Ramat. cv. Royal Purple) stem segments cultured on Murashige and Skoog's (1962) basal media. Wang *et al*. (2000) investigated a preliminary study on tissue culture of stem apex of *Chrysanthemum morifolium*. Amin *et al*. (2002) reported an efficient protocol for direct regeneration, multiplication and rooting under *in vitro* conditions of *Chrysanthemum*. Vantu (2006) said that, the plants of *Chrysanthemum morifolium* Ramat. (cultivar Romica) have been regenerated from callus cultures, established from stem and leaves explants. Chae (2014) investigate a protocol for improved root organogenesis and micropropagation in *Chrysanthemum morifolium* Ramat. using different media and concentrations. Pant *et al*. (2015) developed an efficient low cost procedure for *in vitro* propagation of *C. morifolium* has been developed with subsequent assessment of antibacterial property of *in vitro* raised plantlets.

Aim of the present study were to identify the growth regulators on shoot formation and multiplication over the cultural period. Besides, mass propagation in large quantities in a short time, especially in off season, minimum cost and minimum energy, which greatly affect the development of economy and the scientific potentiality of the research could be utilized by the governmental institutions, different universities, research organizations of the same, as well by the target community people towards the wider sustainability of the process in the country and in abroad.

Material 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 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

Field grown plant of *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 plants were 2-3 months old, nodal segments were excised from these established plants and used to conduct different experiments.

Sterilization

Shoot segments were collected from field grown plants and 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.

Shoot Multiplication

After surface sterilization, shoot segments were cut into nodal segments with convenient size (3–4cm in length). Then the nodal explants were inoculated on MS media. For optimizing multiple shoot regeneration, various hormonal supplements were added to MS media singly or in combinations, namely 6-furfural amino purine (KIN) concentrations viz. 0.5, 1.0, 1.5, 2.0, 2.5 mg/l which combination with 0.5 mg/l α-napthalene acetic acid (NAA), 6-benzyl amino purine (BAP) singly in 0.1, 0.5, 1.0, 1.5, 2.0 mg/l concentrations, BAP 0.5, 1.0, 1.5, 2.0, 2.5 mg/l which combination with 0.5 mg/l Casein hydrolysate (CH), BAP 0.5, 1.0, 1.5, 2.0, 2.5 mg/l which combination with 0.5 mg/l KIN and BAP 0.5 mg/l combination with 0.1, 0.5, 1.0, 2.0, 2.5 mg/l NAA. At the end of the experiment, percentage of explants produced shoot, mean number of shoot per explant and mean length of shoot (cm) were recorded after 35 days in culture. When the regenerated shoots were 1-3 cm in length they were removed aseptically from the culture vessels and placed on a sterile petridish and cut nodal segments into convenient sizes and again transferred into test tubes containing the same or different hormones supplemented media.

Explants were cultured on solidified MS media. MS supplemented with 30 gm/l sucrose was used for all *in vitro* culture studies. The pH of the medium was adjusted to 5.7 prior to adding 8.5 gm/l agar and autoclaved at 15 lb/inch^2 pressure and at the temperature of 120°C -121°C for 20 min. The cultures were maintained at 27±2°C under the cool fluorescent light intensity varied from 2000-3000 lux. The photoperiod was maintained generally 16 hours light and 8 hours dark.

Rooting

When the proliferated shootlets were 4-5 cm in length they were rescued aseptically from the culture tubes and placed on a sterilized petridish. The shoots cut from the basal stock end of the shoot cultures. Then each of the shoots was inoculated on freshly prepared medium containing MS salts supplemented with IBA singly in 0.5, 1.0, 1.5, 2.0, 2.5 mg/l concentrations and 0.5 mg/l IBA which was combination with 0.1, 0.5, 1.0, 1.5, 2.0 mg/l NAA. In this part of the experiment, percentage of the root formation, mean number of roots per explant, mean length of roots were recorded. Root number and length were recorded after 32 days in culture.

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

Each experiment was repeated three times and each treatment 10 explants were cultured. Recorded data were analyzed statistically 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 and replication. P values < 0.05 were considered as significant and values are the mean ± standard error of mean.

Results

Effect of different concentrations and combinations of KIN and NAA in MS medium on shoot differentiation from nodal segments of Chrysanthemum morifolium

Different concentrations and combinations of KIN and NAA were used for shoot differentiation from nodal segments. In this experiment, MS media having 2.0 mg/l KIN with 0.5 mg/l NAA showed its superiority amongst all the other concentrations and combination levels, in all the parameters studied (Table 1, Figure1). Maximum 60%shoot was initiated and the highest mean number of shoot per culture was (1.2±0.19) and highest mean length of shoot was (2.36±0.25).

Table 1. Effect of different concentrations and combinations of KIN with NAA, BAP singly, BAP with CH, BAP with KIN and BAP with NAA in MS medium on shoot differentiation from nodal segments of *C. morifolium*. In each treatment 10 explants were cultured. Data were recorded after 3-4 weeks of inoculation.

Name of the hormone	Concentration (mg/l)	No. of responsive explants	% of explants produced shoots	Mean no. of shoot per explant	Mean length of shoot (cm)	Degree of callus formation at the base
KIN+NAA	0.5+0.5	0	0	---	---	+++
	1.0+0.5	2	20	0.3±0.22	0.56±0.13	+++
	1.5+0.5	3	30	0.5±0.18	1.00±0.30	++
	2.0+0.5	6	60	1.2±0.19	2.36±0.25	+
	2.5+0.5	4	40	0.7±0.15	1.44±0.43	++
BAP	0.1	6	60	1.5±0.20	4.5±0.29	+
	0.5	8	80	1.9±0.65	4.61±0.28	+
	1.0	10	100	3.1±0.34	7.11±0.20	+
	1.5	9	90	2.1±0.27	5.89±0.16	+
	2.0	5	50	0.6±0.14	2.5±0.11	+
BAP+CH	0.5+0.5	5	50	1.0±0.16	1.45±0.21	+
	1.0+0.5	6	60	1.0±0.25	1.56±0.18	+
	1.5+0.5	8	80	2.0±0.16	3.72±0.29	+
	2.0+0.5	7	70	1.4±0.25	2.91±0.98	+
	2.5+0.5	6	60	1.1±0.28	1.75±0.24	+
BAP+KIN	0.5+0.5	5	50	1.1±0.14	2.54±0.08	-
	1.0+0.5	6	60	1.3±0.23	3.08±0.14	-
	1.5+0.5	8	80	2.0±0.24	4.69±0.13	-
	2.0+0.5	10	100	4.2±0.41	7.8±0.41	-
	2.5+0.5	9	90	3.7±0.43	5.09±0.29	-
BAP+NAA	0.5+0.1	9	90	2.6±0.24	3.82±0.21	-
	0.5+0.5	9	90	2.5±0.26	3.53±0.27	-
	0.5+1.0	8	80	2.1±0	3.46±0.22	-
	0.5+2.0	7	70	2.0±0.15	3.2±0.15	-
	0.5+2.5	5	50	1.8±0.26	2.23±0.18	-

- = No callusing, + = Little callusing, ++ = Moderate callusing; +++ = Massive callusing.

Whereas, the lowest percentage of developing shoots 0% was recorded in MS media having 0.5 mg/l KIN with 0.5 mg/l NAA. Massive callus formation at the base of explants in media with 0.5 mg/l KIN + 0.5 mg/l NAA and 1.0 mg/l KIN + 0.5 mg/l NAA. Little callus at the base of explants was found in 2.0 mg/l KIN + 0.5 mg/l NAA. This might be due to the fact that NAA is not usually considered to be a shoot proliferation growth regulator rather than root promoting regulator.

Table 2. Statistical analysis (ANOVA) of no. of shoot per explant and length of shoot of *Chrysanthemum* sp.

No. of shoots per explants.

Source of variation	df	SS	MS	F	Comment	LSD
Plant Hormone	4	34.9452	8.73631	14.9596	*	0.15573
Replication	2	0.69158	0.34579	0.59212	ns	
Error	68	39.7115	0.58399			
Total	74	75.3483				

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

Length of shoots.

Source of variation	df	SS	MS	F	Comment	LSD
Plant Hormone	4	155.7	38.9251	23.5416	*	0.44092
Replication	2	0.69158	0.34579	0.20913	ns	
Error	68	112.436	1.65346			
Total	74	268.828				

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

Effect of different concentrations of BAP singly in MS medium on shoot differentiation from nodal segment explants of C. morifolium

MS medium supplemented with different concentrations of BAP were used to observed the response of shoot differentiation in chrysanthemum. The result revealed that an intermediate level of BAP 1.0 mg/l showed its supremacy amongst all the other treatments, in all the parameters (Table 1, Figure 2). Maximum shoot initiation percentage 100% and the highest mean number of shoot per culture was (3.1±0.34) and highest mean length of shoot (7.11±0.20) was recorded in MS media supplemented with 1.0 mg/l BAP.

Table 3. Effect of different concentrations of IBA singly and combination with NAA in MS medium on root development of *in vitro* grown shoots of *C. morifolium*. In each treatment 10 explants were cultured. Data were recorded after 32 days of culture.

Name of the hormone	Concentration (mg/l)	Days taken to root initiation	% of roots formation	Mean no. of roots per explant	Mean length of roots(cm)
IBA	0.5	16-17	70	3.7±0.35	1.86±0.12
	1.0	16-17	80	4.4±0.29	2.40±0.25
	1.5	10-12	90	6.1±0.37	3.19±0.15
	2.0	7-10	100	6.9±0.45	3.40±0.19
	2.5	5-8	100	7.8±0.41	3.62±0.18
IBA+NAA	0.5+0.1	10-13	60	2.3±0.37	1.51±0.12
	0.5+0.5	8-12	80	4.4±0.29	2.39±0.10
	0.5+1.0	8-10	90	5.6±0.37	2.83±0.12
	0.5+1.5	6-8	100	7.2±0.43	3.54±0.18
	0.5+2.0	6-9	70	4.2±0.36	2.13±0.12

The next best performance was noted in media containing 1.5 mg/l BAP in which 90% of explants produced shoots and in this concentration, the mean number of shoot per explants was (2.1±0.27) and mean length of shoot was found in (5.89±0.16). Little callus at the base of explants was found. The lowest percentage of shoot induction rate (50%) was found in MS media having 2.0 mg/l BAP.

Effect of different concentrations and combinations of BAP and CH (Casein hydrolysate) in MS medium on shoot differentiation from nodal segments of C. morifolium

BAP along with CH (Casein hydrolysate) also play a vital role in shoot regeneration in chrysanthemum. The result showed that out of different hormonal combinations, 1.5 mg/l BAP + 0.5 mg/l CH showed superiority over all the other combinations (Table 1). The highest percentage of shoot induction rate (80%) was recorded in MS media having 1.5 mg/l BAP + 0.5 mg/l CH, highest mean number of shoot per explants (2.0±0.16) and highest mean length of shoot (3.72±0.29) was found in this media and lowest percentage of shoot induction rate (50%) was recorded in MS media having 0.5 mg/l BAP + 0.5 mg/l CH. Little callus at the base of explants was found.

Table 4. 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
Plant Hormone	1	8.112	8.112	2.83	ns
Replication	2	0.968	0.484	0.17	ns
Error	26	74.46	2.86385		
Total	29	83.54			

ns= not significant.

Length of roots.

Source of variation	df	SS	MS	F	Comment
Plant Hormone	1	1.28547	1.28547	2.47488	ns
Replication	2	0.288	0.144	0.27724	ns
Error	26	13.50456	0.51941		
Total	29	15.07803			

ns= not significant.

Effect of different concentrations and combinations of BAP and KIN in MS medium on shoot differentiation from nodal segments of C. morifolium

In the present study, MS medium supplemented with different concentrations and combinations of BAP with KIN to observe the response of nodal segments (Table 1). All of the concentrations and combinations of BAP with KIN, maximum percentage (100%) of shoot induction was recorded in MS media having 2.0 mg/l BAP + 0.5 mg/l KIN and the highest mean number of shoot per explants (4.2±0.41) and highest mean length of shoot (7.8±0.41) was found in this combination (Figure 3). The lowest percentage (50%) of shoot induction was recorded in MS media containing 0.5 mg/l BAP + 0.5 mg/l KIN. No callus at the base of explants was found.

Effect of different concentrations and combinations of BAP and NAA in MS medium on shoot differentiation from nodal segments of C. morifolium

BAP combinations with NAA were used for shoot regeneration in chrysanthemum. MS media having 0.5 mg/l BAP + 0.1 mg/l NAA (Figure 4), 0.5 mg/l BAP+ 0.5 mg/l NAA showed its superiority amongst all the other concentration levels, in all the parameters studied (Table 1) and highest percentage

(90%) of shoot induction rate was recorded in both medium. On the other hand, lowest percentage (50%) of shoot induction rate was recorded in MS media having 0.5 mg/l BAP + 2.5 mg/l NAA. The highest mean number of shoot per explants (2.6±0.24) and highest mean length of shoot (3.82±0.21) was found in MS media supplemented with 0.5 mg/l BAP + 0.1 mg/l NAA. The next best performance was noted in media containing 0.5 mg/l BAP + 0.5 mg/l NAA in which mean number of shoot per explants was (2.5±0.26) and mean length of shoot was found in (3.53±0.27). No callus at the base of explants was found.

Fig. 1. Direct shoot regeneration from nodal segments of field grown plant in MS media supplemented with 2.0 mg/l KIN + 0.5 mg/l NAA.

MS media supplemented with 0.5 mg/l BAP+0.1 mg/l NAA.

Analysis of variance (Table 2) shows that, except replication, plant hormones were significantly different at 5% level for number of shoot per explant and length of shoot.

Effect of different concentrations of IBA singly and combination with NAA in MS medium on root induction of in vitro grown shoots of C. morifolium

Effect of different concentrations of indole butyric acid (IBA) singly and combination with naphthalene acetic acid (NAA) in MS medium on the rooting of *in vitro* grown shoots of chrysanthemum from nodal segment explants showed a good performance for all the parameters (Table 3). A very highly competitive and encouraging result was observed regarding root initiation percentage as affected by different concentrations of auxins such as IBA and NAA. Maximum (100%) rooting was observed in media having 2.0 mg/l, 2.5 mg/l IBA and 0.5 mg/l IBA + 1.5 mg/l NAA (Figure 5). Besides, lowest percentage of root formation rate 70% was found in media having 0.5 mg/l IBA and 60% was found in media having 0.5 mg/l IBA + 0.1 mg/l NAA. The highest mean number of roots per shoot was (7.8±0.41) and highest mean length of root (3.62±0.18) was found in MS media supplemented with 2.5 mg/l IBA. Second highest mean number of roots per shoot was (7.2±0.43) and second highest mean length of root was (3.54±0.18) cm recorded in 0.5 mg/l IBA + 1.5 mg/l NAA and third highest mean number of roots per shoot was (6.9±0.45) and third highest mean length of root was (3.40±0.19) cm recorded in 2.0 mg/l IBA. On the other hand, lowest mean number of roots per shoot was (2.3±0.37) in 0.5 mg/l IBA + 0.1 mg/l NAA, (3.7±0.35) in 0.5 mg/l IBA and lowest mean length of root was (1.51±0.12) in 0.5 mg/l IBA + 0.1 mg/l NAA and (1.86±0.12) in 0.5 mg/l IBA. So, it has been seen

that IBA singly and combination with NAA, both were showed their superiority in rooting. In number of roots per shoot and length of roots, analysis of variance (Table 4) shows that there were no significant difference at 5% level in plant hormones and replication.

Discussion

Direct Organogenesis of Shoots

The study aimed at identifying the best media for shoot proliferation and rooting in chrysanthemum. The results of the investigations indicated that an efficient *in vitro* propagation method could be achieved for *C. morifolium*. In the present investigation different concentrations of cytokinin alone or in combination with auxin were used to test the response of shoot regeneration from nodal segments. In this part of experiment, KIN+NAA, BAP singly, BAP+CH, BAP+KIN and BAP+NAA media combination were used.

Fig. 2. Shoot regeneration from nodal segment of field grown plant in MS media supplemented with 1.0 mg/l BAP.

Firstly, for the establishment of primary culture, the explants were cultured on to MS media supplemented with different concentrations and combinations of KIN and NAA. At 2.0 mg/l KIN with 0.5 mg/l NAA in MS medium, 60% shoot initiation from nodal segments was recorded. In this experiment, KIN+NAA combination did not show suitable response and massive callus found at the base of explants.

The effect of various concentrations of BAP alone was studied on MS medium. MS media with 1.0 mg/l BAP was found to be best for shoot differentiation from nodal segments. The highest 100% nodal segment explants of *Chrysanthemum* sp. produced multiple shoots in this medium. The highest mean number of shoot per explants and the highest mean length of the longest shoot were recorded also in this same medium. Roset and Bokelmann (1975) obtained similar results at 1.0 mg/l BA. Karim *et al.* (2002) reported that the frequency of multiple shoot regeneration response was 91%, for shoot tips, when cultured on the medium containing MS + 1.0 mg/l BAP. Karim *et al.* (2003), Waseem *et al.* (2011) reported that maximum frequency of explants produced auxillary shoot and the highest number of shoots per explants were obtained when MS medium was fortified with 1.0 mg/l BAP. Khan *et al.* (1994) obtained the results at 2.0 mg/l BA and Hoque (1995) also got the results at 4.0 mg/l BA. Gul (2001) also

reported that in *Chrysanthemum*, maximum shoot regeneration was observed at 0.5 mg/l BAP. Karim *et al.* (2002) described 1.0 mg/l BAP as the best BAP concentration as it had produced maximum shoot initiation in *Chrysanthemum* while using shoot tips as explant. Similar results were also reported by Ali *et al.* (2008) who also reported that when BAP was used alone at 1.0 mg/l concentration, highest number of shoots was obtained in all the culture for carnation. By increase in BAP concentration the rate of shoot multiplication was decreased. Hodson *et al.* (2008) stated that the presence of BAP in the culture medium was necessary for the shoot regeneration, although concentrations higher than 4.44µM reduced the shoot regeneration frequency.

Fig. 3. Shoot regeneration from nodal segment of field grown plant in MS media supplemented with 2.0 mg/l BAP+0.5 mg/l KIN.

Our investigation are also being supported by Ali *et al.* (2008) and Waseem *et al.* (2011) who also recommended 1.0 mg/l BAP as the most optimum BAP concentration for the regeneration of plantlets and by any increase or decrease in its concentration caused a decrease in multiplication rates. Similar results were also reported by a number of other scientists (Gul, 2001; Karim *et al.*, 2003) stating higher percentage of *Chrysanthemum* plantlets formation in MS media supplemented with 1.0 mg/l BAP.

In the present study, different concentrations and combinations of BAP with CH on shoot regeneration from in vitro grown nodal explants were studied on MS medium. MS medium 1.5 mg/l BAP+0.5 mg/l CH was found to be best for maximum shoot proliferation from axillary bud carrying nodal segments but little callus formation was found at the base of explants. The highest 80% nodal explants of *Chrysanthemum* sp. produced multiple shoots in this medium.

The highest mean number of shoot per explants and highest mean length of shoot were recorded also in this same medium. In this experiment, the result was enough good.

The media containing BAP in combination with KIN were found to be satisfactory for shoot differentiation from nodal segments. MS medium 2.0 mg/l BAP+0.5 mg/l KIN was found to be best for multiple shoot induction. The media 2.5 mg/l BAP+1.0 mg/l KIN and 1.5 mg/l BAP+0.5 mg/l KIN was also found to be better for multiple shoot induction in

Chrysanthemum.

The effect of BAP and NAA on multiple shoot regeneration from nodal explants were studied on MS medium. A maximum of 90% multiple shoots regenerated when nodal explants were cultured on to MS medium supplemented with 0.5 mg/l BAP+0.1 mg/l NAA and 0.5 mg/l BAP+0.5 mg/l NAA but shoot growth was low. Hoque and Fatema (1995) regenerated maximum number of multiple shoot from shoot tip explants when 1.0 mg/l BAP+1.0 mg/l NAA were added to MS medium.

Fig. 4. Shoot regeneration from nodal segment of field grown plant in MS media supplemented with 0.5 mg/l BAP+0.1 mg/l NAA.

Tripepi (1997) used various combinations of BAP and NAA for the induction of adventitious shoots as they are among the growth regulators used most often for the shoot organogenesis. Hoque *et al*. (1998) obtained best response towards multiple shoot regeneration on MS medium containing 1.0 mg/l BAP and 0.5 mg/l NAA. Akhter (1998) also obtained best response to multiple shoot regeneration using 0.5 mg/l each of BA and NAA. Chakrabarty *et al*. (2000) regenerated ray florets of *C. morifolium* cv. Colchi Bahar shoots on MS medium supplemented with 0.2 mg/l NAA and 1.0 mg/l BAP. Radojevic *et al*. (2000) also reported that MS medium supplemented with 0.5 mg/l NAA+1.0 mg/l BAP was the most suitable medium for 13 cultivars of *Chrysanthemum*.

Kumari and Varghese (2003) reported that regeneration of shoots took place on MS media fortified with various combinations of BAP and NAA. Shanti *et al*. (2005), Misra and Datta (2007) reported that shoot tip explants of *Chrysanthemum* inoculated in MS media supplemented with 2.0 mg/l BAP and 0.5 mg/l NAA gave better performance for plantlet regeneration. Trifunovic *et al*. (2006) achived induction of morphogenesis in stem segment culture on MS medium supplemented with 0.5 mg/l NAA and 1.0 mg/l BAP. Nahid *et al*. (2007) found 58% shoot initiation in *Chrysanthemum* petals inoculated on MS media supplemented with 1.0 mg/l BAP and 0.1 mg/l NAA. Waseem *et al*. (2011) reported that MS media supplemented with lower concentrations of NAA (0.5 and 1.0 mg/l) and BAP (1.0 mg/l and 1.5 mg/l) showed better results for all the growth parameters.

The findings of the present study indicate that more or less same concentration and combination of BAP and NAA were suitable for the regeneration of multiple shoots. The difference of the results from other reporter may be due to the genotypic difference of the working materials.

In *Chrysanthemum* was able to regenerate multiple shoots using very low concentrations of BAP (0.5µm/l) and NAA (0.01µm/l) in modified Gamborg's (G_5) medium. However, in the present investigation maximum response was observed on MS medium supplemented with 0.5 mg/l BAP and 0.1 mg/l NAA. The difference between the results of the present investigation with that of Choudhary (1992) may be due to use of different basal media. Several reports are also available where MS medium with different concentrations and combinations of BAP and NAA were equally effective for multiple shoot regeneration obtained from different explants of *Chrysanthemum* (Kaul *et al.*, 1990, Lu*et al.*, 1990, Donato and Perucco, 1984, Prasad and Chaturvedi, 1988, Hoque *et al.*, 1991). This difference in shoot regeneration as observed in the present work may be due to different species and genotype as well as different types of explants used.

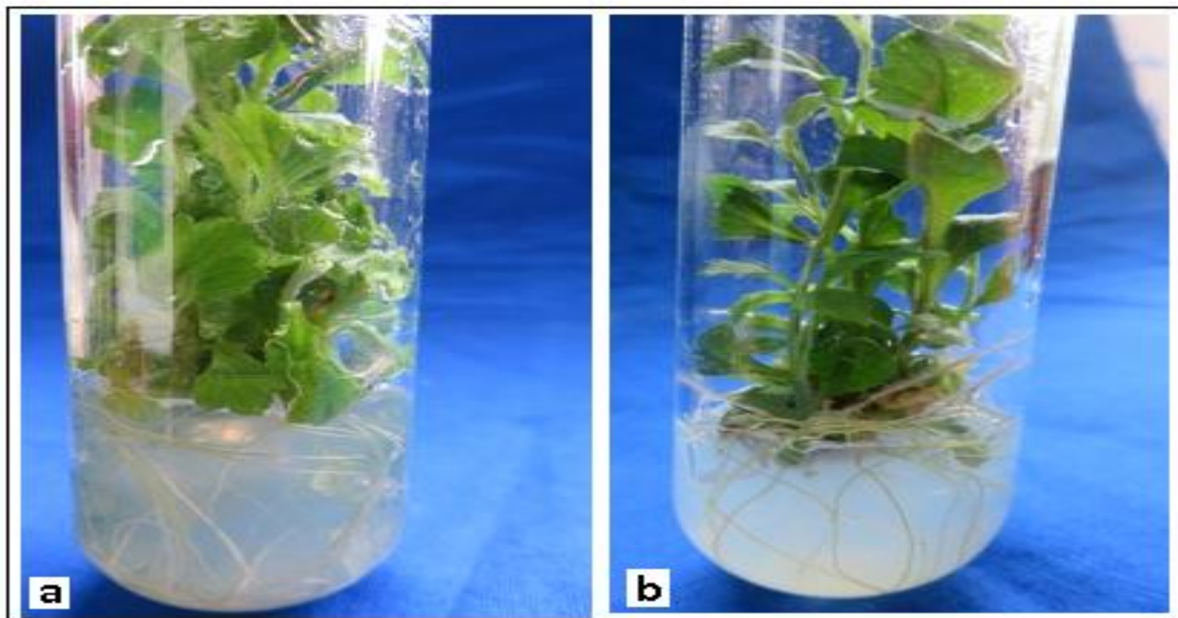

Fig. 5. Induction of roots from *in vitro* regenerated shoots of *Chrysanthemum morifolium* in MS medium supplemented with (a) 2.5 mg/l IBA and (b) 0.5 mg/l IBA+1.5 mg/l NAA.

Induction of Roots

The shoot development as well as the rooting of regenerated shoots is especially important for establishing tissue culture derived shoots (Moss *et al.*, 1988). Shoot regenerated from nodal segments were rooted to develop complete plantlets. Most plants required the presence of auxins for root induction. Different experiments were conducted with MS basal medium supplemented with different types of auxins singly or in combination. Gautheret (1939) suggested first the importance of auxin in root induction. IBA was found to be best for root induction and maximum 100% of the cultured shoots induced roots at 2.0 mg/l IBA and 2.5 mg/l IBA media concentrations. 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). 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 as the best auxin for root induction and development. Khan *et al*. (1994) reported that days taken to root induction were earlier in ½ MS. They also described that root induction was delayed when activated charcoal was added in the medium and average number of roots per shoot and root length were higher in ½ MS with 0.25 mg/l IBA in the medium, while it was less when activated charcoal was added. In the present investigation, activated charcoal and ½ MS media were not used, average root number and root length were higher in MS media supplemented with IBA. This difference may due to the genotypic difference of the plant materials used in the present investigation.

Using IBA with NAA, maximum root induction percentage and highest average number of roots were observed in 0.5 mg/l IBA+1.5 mg/l NAA. Using NAA at different concentrations on MS medium several workers (Earle and Langhansm, 1974) obtained root induction in *Chrysanthemum*.

Conclusion

Clonally propagation by *in vitro* culture using shoot proliferation from different type of culture provides a useful method for obtain of genetically uniform plants. In future, following the direct organogenesis protocol of our investigation, it can help mass propagation in large quantities in a short time, especially in off season, minimum cost and minimum energy, which greatly affect the development of economy. The developed protocol on direct organogenesis systems of *Chrysanthemum morifolium* offers a great potential for large scale commercial multiplication for producing of the studies in *in vitro* conditions. *Chrysanthemum* tissue culture have been exploited for a variety of purposes including studies on the growth in culture of cells and protoplasts, their nutrition, physiology and secondary product metabolism. In *Chrysanthemum* tissue and organ cultures are used both for the production of pathogen free materials and for micropropagation. 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.

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