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RAPID CLONAL PROPAGATION OF *CHRYSANTHEMUM* THROUGH EMBROYOGENIC CALLUS FORMATION

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Abstract

Chrysanthemum nodal explants were cultured on ½ X MS medium containing different combinations of growth hormones. A reasonable callus formed on explants when MS contained 0.5 mg. cm^{-3} each of BAP and NAA after about 3 weeks. Similar results were obtained on MS + 0.5 mg. cm^{-3} BAP + 0.1 mg. cm^{-3} NAA or 1.0 mg. cm^{-3} BAP + 0.1 mg. cm^{-3} NAA. A maroonish embryogenic callus was formed on MS containing BAP only. This callus, then exhibited various embryonic developmental stages and gave rise to normal seedlings. These were acclimatized and transferred to natural conditions. The plants ultimately flowered exhibiting superior quality and early maturity compared to stock plants.

Introduction

Chrysanthemum is one of the widely grown greenhouse ornamentals. It is a perennial herb that belongs to the family Asteraceae. Florist 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. Chrysanthemum is a complex hybrid which is grown from seed and segregates into many diverse flower forms.

Tissue culture studies on chrysanthemum were first initiated by Morel & Martin in 1952. They used meristem tip culture to obtain virus free plants. Now tissue culture is a necessary first step in the production of virus indexed chrysanthemums. It serves as a nuclear stock for the delivery of high quality cuttings (Debergh, 1994). Much work has been done on callogenesis and organogensis of chrysanthemum. Many workers have obtained shoot organogenesis from floret derived callus (Bush *et al*., 1976; Dejong & Custers, 1986; Malaure *et al*., 1991). Sutter & Langhans (1981) obtained adventitious shoots from callus derived from leaf and shoot tips. Earle & Langhans (1974) obtained multiple shoots and leafy callus in chrysanthemum by using 2.0 mg. cm^{-3} Kn and 0.02mg cm^{-3} NAA. Rapid clonal propagation occurred in stem and leaf derived calli in *Chrysanthemum morifolium* (Bhattacharya *et al.*, 1990).

Somatic embryogenesis through tissue culture technique is playing an important role in improving herbaceous dicots (Brown *et al.*, 1995). Neverthless, production of somatic embryos from cell cultures present opportunity not available to plantlets regenerated by the organogenic routes such as mechanization (Ammirato, 1983). The objective of the present work was to investigate the effect of various growth regulators on callus induction, its proliferation and embryogenesis in *Chrysanthemum cinerariaefolium.*

Materials and Methods

Chrysanthemum shoots were collected from the plants grown in the University of Peshawar. Nodal explants were taken from somewhat hard lower portions of the plants. After removing the leaves, stem pieces were washed with household detergent to remove the impurities and then washed thoroughly under tap water. Further sterilization was done with 70% alcohol for 2 minutes followed by 1% $HgCl_2$ for 2-3 minutes. Nodal cuttings were finally washed with sterilized distilled water for several times. After sterilization

each segment was divided by a longitudinal cut so that each piece received a half of the nodal portion. These pieces were then inoculated onto the Murashige & Skoog's (1962) medium supplemented with various growth regulators, viz., Benzylamino purine (BAP) and Naphthalene acetic acid (NAA). Sucrose was used as a carbon source and the medium was solidified with 0.9% agar. pH of the medium was adjusted to 5.6 by using 0.1 HC1 or NaOH. The medium was autoclaved at 121°C at a pressure of 103.44 Pascal second for 15 minutes. The cultures were incubated in biotrons with 16 hours light period in 24 h cycle at 25 ± 1°C.

Results

Stem nodal segments of Chrysanthemum were inoculated onto the MS medium of half strength supplemented with various combinations of BAP and NAA for callus induction as shown in Table 1.

Callus got initiated within two weeks when the medium was supplemented with 0.5 mg. cm^{-3} each of BAP and NAA. A reasonable amount of callus was formed after another two weeks. This callus was dark green in colour and granular in appearance (Fig. 1). Moreover, 80% of the explants responded to callogenesis. Similarly a reasonable amount of callus was also obtained in another set of experiments when BAP was used at the same level i.e., 0.5 mg. cm^{-3} while NAA was reduced to 0.1 mg. cm^{-3}. This callus was also yellowish green in colour with granular appearance (Fig. 2). Again the callogenic response was excellent roughly being 80% (Table 1). Still further in another set of experiments the concentration of NAA was kept the same (0.1 mg. cm^{-3}) while that of BAP increased to 1.0 mg. cm^{-3} level. This combination was not very effective as only a slight callus was formed on the nodes after 5-6 weeks. Although this callus was brownish white and soft in the beginning, later on it turned brown and died very rapidly. The percentage of explants which responded to callus formation was also low.

Embryogenesis: The call obtained in the above experiments at 0.5 mg/1 each of BAP and NAA and 0.5 mg. cm^{-3} BAP + 0.1 mg. cm^{-3} NAA were isolated and transferred to the regeneration medium containing various levels of BAP (Table 2).

The callus obtained at 0.5 mg. cm^{-3} each of BAP and NAA proliferated further when subcultured at various concentrations of BAP. However it did not exhibit any morphogenesis. The callus formed at 0.5 mg. cm^{-3} BAP + 0.1 mg. cm^{-3} NAA was also transferred to the MS medium containing BAP (0.2 - 3.0 mg. cm^{-3}). In this case, the callus began to change its colour from green to maroonish brown after a culture period of one week. In addition a little proliferation of the callus was also observed. After about 5 weeks small green leaf like structures appeared in this callus. Microscopic studies revealed that the callus was embryogenic in nature. A number of green embryodial masses were embedded in the apparently maroonish granular callus. It was noticed that the number of green cells was highest in the callus when cultured on the medium provided with 0.5 mg. cm^{-3} BAP, while a very few green cells were present in the callus at 3.0 mg. cm^{-3} BAP with rest of the callus being dead. Further microscope studies were made to closely monitor the various developmental stages. During the course of development, the somatic embryos first appeared as simple rounded groups of cells. These were small and densely packed cytoplasmic cells (Fig. 3). Their further development resulted in the formation of characteristic three lobed embryos. They finally gave rise to complete somatic embryos with tiny green leaves at one end and a radical at the other (Fig. 4). These somatic embryos immediately developed into normal seedlings (Fig. 5). However these seedlings did not grow further unless transferred to a fresh MS medium.

Table 1. Callus induction on stem nodal segments of Chrysanthemum inoculated on ½ MS medium supplemented with BAP and NAA.

Growth regulators (mg. l^{-1})		No. of explants inoculated	Culture period in weeks	% age of explants exhibiting regenerable callogenesis	Callogenesis	Remarks
'	NAA					
	0.1	20	3	80	+++	Callus formed was bright green in colour and soft in texture
	0.5	20	3	80	+++	Callus was brownish green in colour and granular in appearance
	0.1	20	6	15	+	Callus was brownish white in colour and soft in texture

nt; ++ = Moderate; +++ = Good callus formation

Table 2. Effect of various concentrations of BAP on embryo development when the callus obtained at 0.5 mg. l^{-1} BAP + 0.1 mg. l^{-1} NAA was transferred to the ½ MS medium.

AP Conc. (g. l^{-1} cm^{-3})	No. of calli cultured	Culture period after which		%age of calli developing embryos
		Callus developed embryos	First green leaves emerged	
0.2	10	4	7	100
0.5	10	3	5	100
1.0	20	3	7	100
2.0	20	4	7	75
3.0	20	4	8	60

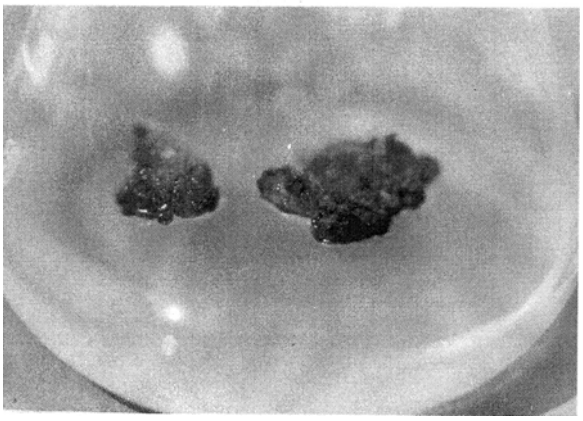

Fig. 1. Callus formation on stem nodal segments of chrysanthemum on ½ MS medium supplemented with 0.5 mg. l^{-1} each of BAP and NAA. The callus was dark green in colour and granular in appearance.

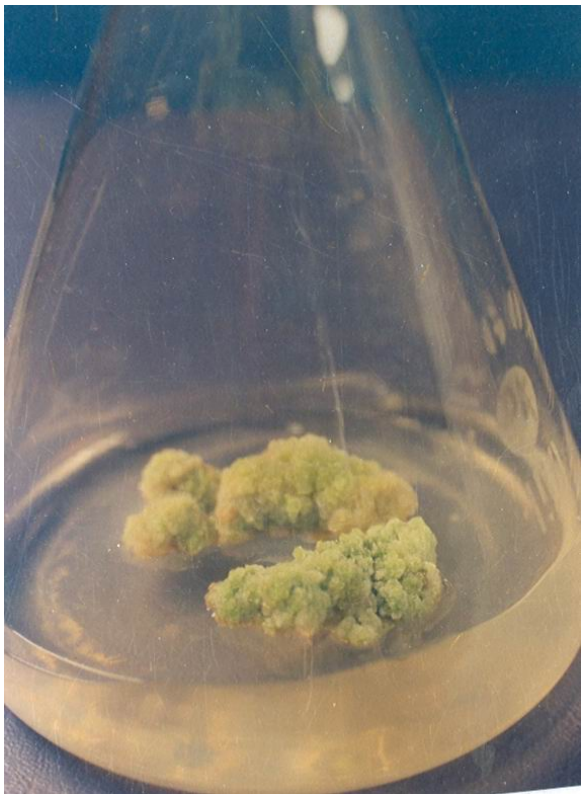

Fig. 2. Yellow green callus formed at 0.5 mg. cm^{-3} BAP and 0.1 mg. l^{-1} NAA. It was granular in appearance.

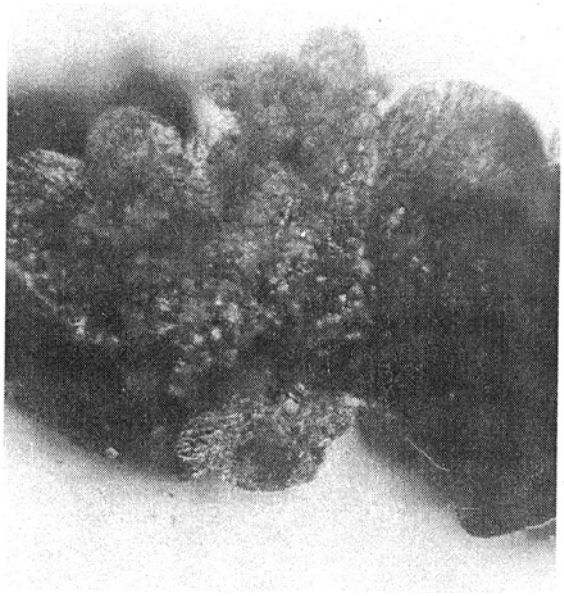

Fig. 3. Green embryoidal masses of cells embedded in the brown callus.

Fig. 4. A small embryo with cotyledonary leaves and radical as seen under SEM

Growth of seedlings into plantlets: The seedlings obtained from embryogenic callus at various concentrations of BAP were transferred to plain 1/2 MS medium for further growth and development. Resultantly these seedlings developed into complete plantlets within 3 weeks. It was also interesting to note that embryogenic callus formation continued at the basal ends of plantlets without affecting the plantlets growth. These callus masses were isolated and transferred to the regeneration medium provided with 0.5mg. cm^{-3} of BAP. Comparable results were again obtained i.e. embryo development followed by plantlet and callus formation. Interestingly this callus retained its potential for more than 10 subcultures, thus resulting into the formation of numerous plantlets.

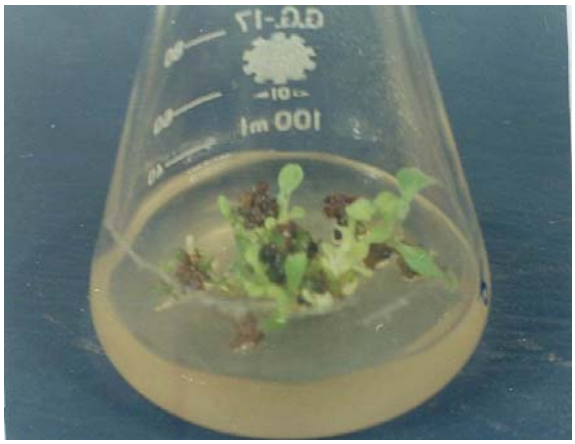

Fig. 5. Somatic embryos grew into seedlings on MS containing 0.5 mg/1 of BAP.

Fig. 6. Blooming of *in vitro* raised plants. Regenerants in full bloom.

Hardening and transfer to the field: Plantlets that had attained the size of 5-7cm were subjected to hardening prior to field transfer. For this purpose they were transferred aseptically to the test tubes containing 2-3 ml of ¼ MS salt solution added with 1% sugar. Test tubes were covered with cotton plugs and kept under room conditions. Cotton plugs were removed after about two weeks and the plantlets were kept as such in the tubes. After 3-4 days plantlets were transferred to the pots containing well drained loamy soil. Pots were then covered with transparent polythene bags to protect delicate plants from excessive dessication. Pots were then uncovered after about 10 days when the plants got established in the soil. The plants rapidly spread their roots in the soil and exhibited a substantial vegetative growth. Moreover survival rate of the plants was 100%. These plants were healthier than the mother plants. They also gave early blooming i.e., in start of October as compared to the control plants which bloomed in November. Furthermore flowers were large and bright coloured proving the tissue culture raised plants better for marketing (Fig. 6).

Discussion

During the present investigation embryogenic callus formation and its regeneration from stem nodal segments of *Chrysanthemum cinerariaefolium* was studied. Murashige & Skoog's (1962) medium was used throughout the experiments. The medium was supplemented with various combinations of BAP and NAA. BAP was used at a range of 0.5-1.0 mg. cm^{-3} in combination with either 0.1 or 0.5 mg. cm^{-3} NAA. The most suitable hormonal combination for callus induction was found to be 0.5 mg. cm^{-3} BAP, alongwith 0.1 mg. cm^{-3} NAA. Regenerable callus was readily induced at this combination in 80% of the explants.

A remarkable decrease in callusing response occurred with a rise in BAP level relative to NAA. Our results are in full agreement with those of Chakrabarty *et al.*, (2000). They also reported callus formation in Chrysanthemum on MS medium supplemented with 1.0 mg. cm^{-3} BAP and 0.2 mg cm^{-3} NAA. Our findings are also in conformation to the results obtained by Hussain *et al.*, (1994) for callus induction in carnation from seedling explants. They obtained embryogenic callus by using various combinations of BAP (0.5 mg. cm^{-3}) and NAA (0.1-5.0 mg. cm^{-3}). The callus obtained at 0.5 mg. cm^{-3} BAP and 0.1 mg. l^{-1} cm^{-3}NAA turned maroonish brown on transfer to the regeneration medium supplemented with BAP. As the calli were embryogenic in nature they gave rise to somatic embryos within four weeks. Concentration of BAP in the medium was responsible for duration of embryo development and number of embryos produced. The most optimal concentration proved to be 0.5 mg. cm^{-3} BAP where earlier and maximum number of embryos differentiated from the callus. Nonetheless embryo and subsequent plantlet development was slow when concentration of BAP was increased in the medium. Furthermore, it was also noticed that the number of embryos was much less compared to the callus mass. Although a large number of cells in the explants or culture generally respond to the inducing treatment, only a limited number of cells actually become embryogenic (Toonen *et al.*, 1996). In dicots somatic embryo development follows the characteristic globular, heart and torpedo stages. Our results revealed that the somatic embryos of *Chrysanthemum* followed the same pattern. The callus was heterogeneous in composition having various stages of embryo development, embryos and seedlings in a single culture. The seedlings eventually grew into plantlets on transfer to fresh MS medium at half strength. Altaf *et al.*, (1985) also reported embryogenesis in the callus of *Citrus reticulata* on MS medium with low levels of BAP (0.01-0.1 µm). These embryos developed into plantlets on transfer to the basal medium. Agudeo-Santacruz *et al.*, (2000) reported regeneration of both organogenic and embryogenic callus of *Bouteleua gracilis* on MS medium at half or full strength of MS containing 1.0 mg. cm^{-3} GA_3.

Chrysanthemum plantlets thus raised through embryogenic callus formation were transferred to the field after hardening. The plantlets successfully acclimatized in the natural environment. They were true to type and superior to the parent plants in their health and vigour.

Conclusion

The results show that plantlet formation through embryogenic callus was more suitable for micro-propagation of chrysanthemum. Plants formed through embryogenesis proved to be superior with regard to flower size and colour. These qualities would probably render them better for floriculture trade.

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