Direct somatic embryogenesis and plant regeneration from ray florets of chrysanthemum

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Abstract

Direct somatic embryogenesis from ray floret explants of five chrysanthemum cultivars has been obtained within 12 - 15 d on Murashige and Skoog medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-benzyladenine (BA). Scanning electron microscopic observation also confirmed the direct origin of somatic embryos from explants. Somatic embryos developed asynchronously on the adaxial surface of explants. Among the five cultivars tested, Birbal Sahani was best responding (40 % explants responded on 4 mg dm^{-3} 2,4-D and 2 mg dm^{-3} BA supplemented medium). Precocious germination of somatic embryos was noticed on the same medium. The best sucrose concentration in the medium was found to be 60 g dm^{-3} where 70 % explants responded while 55 % embryogenic response was obtained on medium supplemented with 400 mg dm^{-3} inositol. Plants developed from somatic embryos were transferred to soil and produced true-to-type flowers.

Additional key words: 6-benzyladenine, *Chrysanthemum morifolium*, cultivar differences, 2,4-dichloro-phenoxyacetic acid, naphthaleneacetic acid.

Introduction

Flower colour/shape mutations mostly appear as chimeras. Isolation of mutant tissue is possible through conventional methods when entire branch is mutated. It was almost impossible to isolate through available conventional technique when a sector of a flower is mutated. A large number of new flower colour/shape chimeric tissues were being lost because of the unavailability of conventional microtechniques for management of such chimeric tissues. Recently, a novel technique has been standardized in our laboratory for the management of such chimeric tissues through direct shoot organogenesis from flower petals (Chakrabarty *et al*. 1999, 2000, Datta *et al*. 2001). It has solved the chimeric problem and new mutants have been established in chrysanthemum (Chakrabarty *et al*. 1999, 2000, Mandal *et al.* 2000a,b, Datta *et al*. 2001). Shoot bud have multiple cell origin whereas somatic embryo originates from single cell (Haccius 1978, Maheswaran and Williams 1985, Mandal and Dutta Gupta 2003). There is an urgent need to regenerate plants from a single cell, *i.e*. through somatic embryogenesis for management of single cell mutation event. The present paper reports an efficient somatic embryogenesis protocol in chrysanthemum.

Materials and methods

Ray florets of *Chrysanthemum morifolium* Ramat. cv. Purnima were collected from field grown plants. Ray florets were then washed in tap water with liquid detergent (5 %). Then ray florets were dipped in 70 % ethanol for 30 s followed by 0.1 % $HgCl_2$ for 2 min and washed thoroughly in sterile distilled water. Ray florets were cut transversely into two pieces and were used as explants. All the explants were cultured on the Murashige

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Abbreviations: BA - 6-benzyladenine; 2,4-D - 2,4-dichlorophenoxyacetic acid; MS - Murashige and Skoog; NAA - naphthaleneacetic acid.
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and Skoog medium (MS medium, Murashige and Skoog 1962) containing 0.8 % bacto-agar, 3 % sucrose and 2,4-dichlorophenoxy acetic acid (2,4-D, at 0.0, 0.2, 0.5, 2.0, 4.0 and 6.0 mg dm^{-3}) in combination with 6-benzyl-adenin (BA, at 0.0, 0.2, 0.5, 1.0 2.0 and 4.0 mg dm^{-3}). The pH of the medium was adjusted to 5.6 before autoclaving at 121 °C for 15 min. All the cultures were incubated under a 16-h photoperiod (irradiance of 36 μmol m^{-2} s^{-1}) and temperature of 25 ± 1 °C. Each treatment had 10 replications, each replication consisting of two explants, arranged in a completely randomized design.

As combinations of 2.0, 4.0 and 6.0 mg dm^{-3} 2,4-D with 1.0 and 2.0 mg dm^{-3} BA were suitable, these combinations were tested with four other cultivars (cvs. Birbal Sahani, Maghi, Colchi Bahar and Kasturi Bi). Among the five cultivars tested for somatic embryogenesis, cv. Birbal Sahani was found best responding, therefore, further experiments by replacing 2,4-D with naphthalene-acetic acid (NAA, at 1.0, 2.0, 4.0 and 6.0 mg dm^{-3}) and optimization of sucrose (20, 30, 40, 60, 120 and 180 g dm^{-3}) and inositol (50, 100, 200, 400 and 800 mg dm^{-3}) concentrations in the medium were restricted to this cultivar only.

Percent of embryogenesis and number of somatic embryos per responding explant were evaluated after 4 weeks of culture initiation. Precocious germination on the embryo induction medium was observed within 6 weeks of culture initiation. Embryo germination percentage was calculated as (number of germinated somatic embryos/ total number of somatic embryos) × 100.

Rooted shoots of 2 - 3 cm in length were transferred to plastic pots containing mixture of soil and leaf mold (1:3) and kept in hardening chamber at high humidity. After 2 - 3 weeks of hardening, plants were transferred to earthen pots and kept in the field for flowering.

The floret explants with somatic embryos at different developmental stages were fixed in 3 % glutaraldehyde in 0.025 M phosphate buffer at pH 6.8 for 24 h, washed in the same buffer, post-fixed in 1 % osmium tetroxide, dehydrated through graded series of acetone. Dehydrated tissues were then critical point dried and sputter coated with gold. Gold coated samples were observed under scanning electron microscope and photographed using a *JEOL-JEM 35 C* scanning electron microscope at NBRI, Lucknow, India.

Results and discussion

Enlargement of ray floret explants of cv. Purnima were observed within 4 d of culture initiation. Direct somatic embryogenesis was noticed after 12 - 15 d on the adaxial surface and mostly in the cut margin of the explants. Within 30 d of culture initiation, different developmental stages of somatic embryos were detected on the explant surface. Somatic embryo development was asynchronous in nature. At the same time, globular stage (Figs. 1*A*, 2*A*,*C*), heart-shaped stage (Figs. 1*A*, 2*B*), torpedo-stage (Fig 1*B*), early cotyledonary stage (Fig. 2C), and mature cotyledonary-stage (Figs. 1*C*, 2*D*) somatic embryos were observed. Somatic embryos were developed directly on the explant surface without going through callus phase (Fig. 1*A*,*B*,*C*). Direct somatic embryogenesis has been reported in many other plants (*e.g.* Lazzeri *et al.* 1987, Sellars *et al.* 1990, Fambrini *et al.* 1996). In comparison to callus mediated indirect somatic embryogenesis, such direct embryogenesis seems to be associated with greater genetic and cytological uniformity (Maheswaran and Williams 1984). Among different treatment combinations used for somatic embryo induction in cv. Purnima, somatic embryos were induced only at 2.0, 4.0 and 6.0 mg dm^{-3} 2,4-D with 1.0, 2.0 and 4.0 mg dm^{-3} BA (Table 1). Other combinations were failed to induce somatic embryogenesis. Maximum embryogenic response (30 %) with maximum number of somatic embryos per responding explant (15.8 ± 1.2) was observed in 4.0 mg dm^{-3} 2,4-D with 2.0 mg dm^{-3} BA. Increase or decrease of the growth regulator concentration reduced the embryogenic frequency as well as the number of somatic embryos. Four other cultivars were also tested for their embryogenic potentiality on combinations of 2.0, 4.0 and 6.0 mg dm^{-3} 2,4-D with 1.0, 2.0 and 4.0 mg dm^{-3} BA. All these cultivars responded

Table 1. Effect of different concentrations [mg dm^{-3}] of BA and 2,4-D on direct somatic embryogenesis from ray floret explants of chrysanthemum cv. Purnima after 4 weeks. Means ± SE.

BA	2,4-D 2.0 response [%]	number of embryos [explant^{-1}]	2,4-D 4.0 response [%]	number of embryos [explant^{-1}]	2,4-D 6.0 response [%]	number of embryos [explant^{-1}]
1.0	15	4 ± 0.7	15	4.7 ± 1.1	5	5 ± 0.0
2.0	25	11 ± 1.1	30	15.8 ± 1.2	15	7 ± 1.3
4.0	10	4 ± 0.7	10	5.0 ± 0.7	5	3 ± 0.0

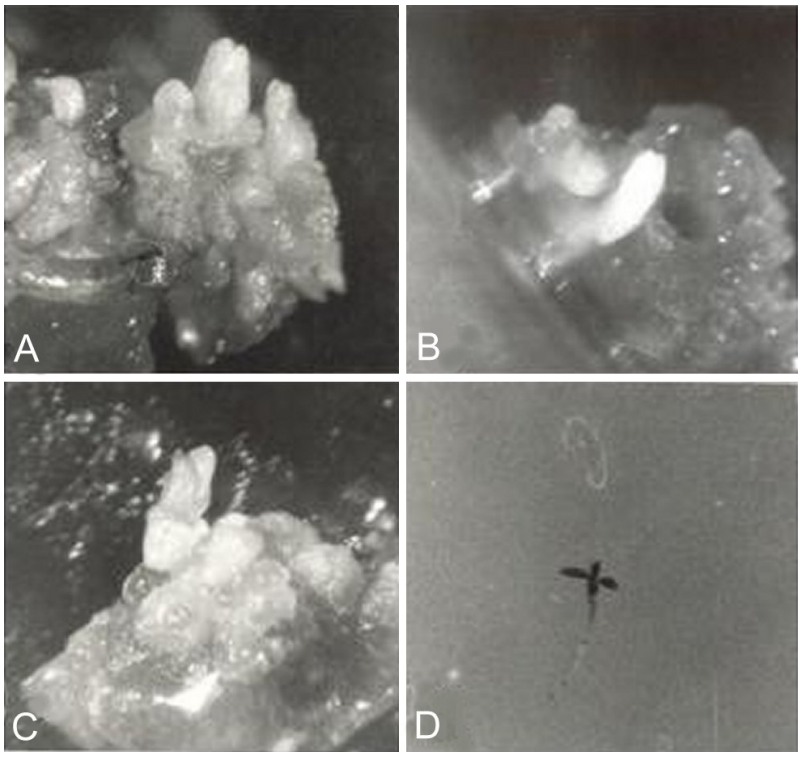

Fig. 1. Stereomicroscopic photographs of somatic embryo development from floret explants of chrysanthemum cv. Purnima cultured on MS medium supplemented with 4 mg dm^{-3} 2,4-D + 2 mg dm^{-3} BA: *A* -globular and heart-shaped stage; *B* - torpedo shaped stage, *C* - cotyledonary stage; *D* - germinated somatic embryo with root and shoot.

Table 2. Effect of genotypes and different concentrations [mg dm^{-3}] of BA and 2,4-D on direct somatic embryogenesis from ray floret explants of chrysanthemum after 4 weeks. Means ± SE.

Cultivar	BA	2,4-D 4.0 response [%]	number of embryos [explant^{-1}]	2,4-D 6.0 response [%]	number of embryos [explant^{-1}]
Birbal Sahani	1.0	25	6.8 ± 1.0	15	3.0 ± 1.24
	2.0	40	7.1 ± 0.7	20	2.7 ± 0.65
	4.0	40	6.7 ± 1.0	20	2.2 ± 0.65
Maghi	1.0	10	1.5 ± 0.35	10	2.5 ± 1.10
	2.0	30	5.0 ± 0.74	20	3.7 ± 0.90
	4.0	30	4.3 ± 0.77	25	3.2 ± 0.71
Colchi Bahar	1.0	10	2.5 ± 1.10	10	3.5 ± 1.77
	2.0	10	3.0 ± 0.70	15	6.3 ± 1.44
	4.0	10	4.0 ± 1.40	5	4.0 ± 0.00
Kasturi Bi	1.0	0	0.0	0	0
	2.0	10	6.0 ± 1.40	0	0
	4.0	0	0.0	0	0

differently in different treatment combinations. Combinations of 2.0 mg dm^{-3} 2,4-D with 1.0, 2.0 or 4.0 mg dm^{-3} BA did not induce somatic embryogenesis in any of these four cultivars. A strong influence of genotypes on somatic embryo induction was noticed (Table 2). Such genotypic influence on somatic embryogenesis has also been well documented in other species (*e.g.* Chen *et al.* 1987, Oziaz-Akins *et al.* 1992, Mandal *et al.* 2001). The best responding cultivars was found to be Birbal Sahani where a maximum of 40 % response with 7.1 ± 0.7 number of somatic embryos was observed and the least responding one to be cv. Kasturi Bi where only 10 % explants responded with 6.0 ± 1.4 somatic embryos. However, the cv. Purnima produced the

Table 3. Effect of different concentrations [mg dm^{-3}] of NAA and BA on direct somatic embryogenesis from ray floret explants of chrysanthemum cv. Birbal Sahani after 4 weeks. Means ± SE.

NAA	BA 1.0 response [%]	number of embryos [$explant^{-1}$]	BA 2.0 response [%]	number of embryos [$explant^{-1}$]	BA 4.0 response [%]	number of embryos [$explant^{-1}$]
1.0	10	5.0 ± 0.7	0	0.0	0	0
2.0	10	4.0 ± 0.7	5	3.0 ± 0	0	0
4.0	20	5.0 ± 0.7	35	6.4 ± 0.8	5	2.0 ± 0

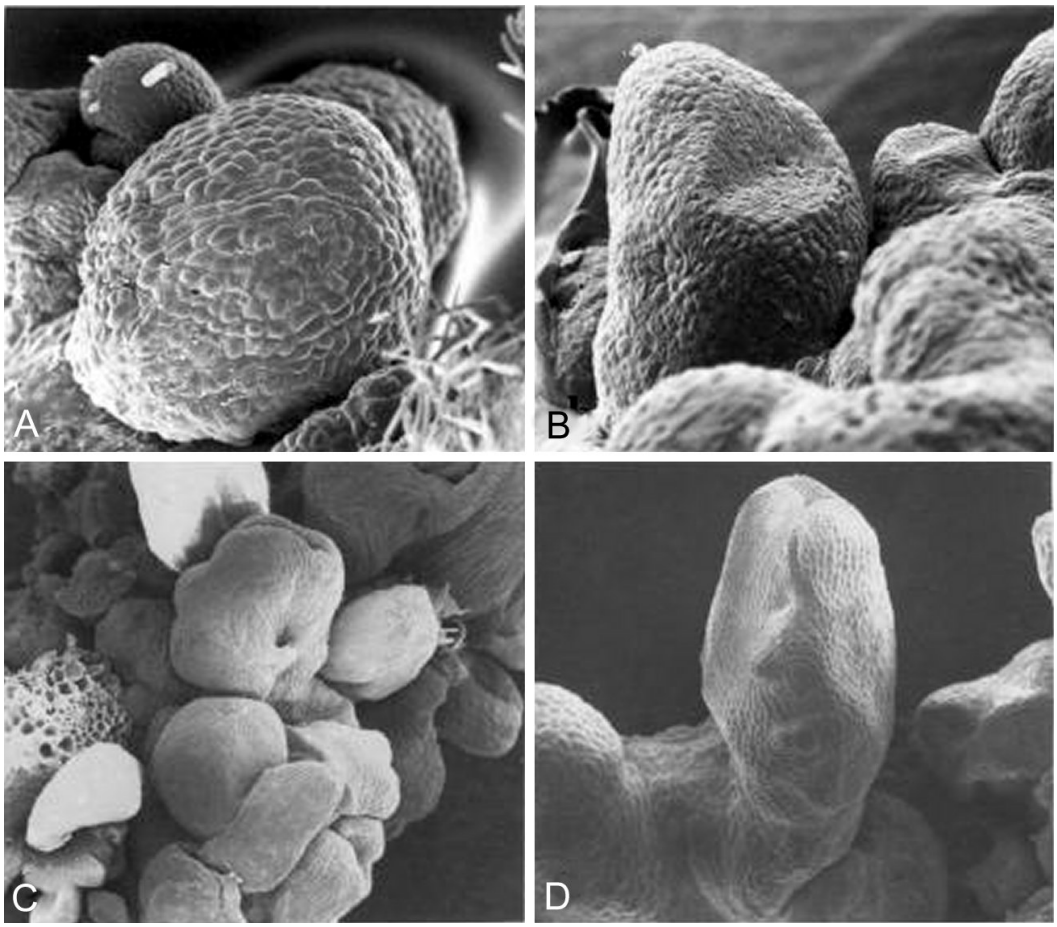

Fig. 2. Scanning electron micrographs of somatic embryo development from floret explants of chrysanthemum cv. Purnima cultured on MS medium supplemented with 4 mg dm^{-3} 2,4-D + 2 mg dm^{-3} BA: *A* - globular stage; *B* - heart-shaped stage; *C* - globular and early cotyledonary stage; *D* - mature cotyledonary stage.

highest number (15.8 ± 1.2) of somatic embryos per responding explant.

The cv. Birbal Sahani was the best responding, therefore, further experiments were restricted to this cultivar only. In the next experiment, 2,4-D was replaced with NAA (1.0, 2.0, 4.0 and 6.0 mg dm^{-3}) in combination with BA (0.5, 1.0, 2.0 and 4.0 mg dm^{-3}). All the treatment concentrations of NAA with 0.5 mg dm^{-3} and all the treatment concentrations of BA with 6 dm^{-3} were failed to induce somatic embryogenesis. Comparing with 2,4-D, NAA was found less effective in induction of somatic embryos in this cultivar (Table 3). A maximum of 35 % of explants responded with 6.4 ± 0.8 number of somatic embryos per explant at 4 mg dm^{-3} NAA + 2 mg dm^{-3} BA. Experiments were conducted to assess the effect of different sucrose and inositol contents on somatic embryogenesis. MS media containing 4 mg dm^{-3} 2,4-D and 2 mg dm^{-3} BA were supplemented with 20, 30, 40, 60, 120 and 180 g dm^{-3} sucrose. Significant effect of sucrose concentrations was noticed. The best response

was obtained at 60 g dm^{-3} sucrose where 70 % of explants responded with 15.4 ± 1.2 somatic embryos per explant (Table 4). At 180 g dm^{-3} sucrose concentration no somatic embryo formation was noticed. High sucrose concentration has been reported to induce somatic embryos in sunflower (Finer 1987). Among different concentrations of inositol tested in the present experiment, the highest embryogenic response was obtained in concentration of 400 mg dm^{-3} where 55 % of explants responded with 14.0 ± 0.8 somatic embryos per responding explant (Table 4). In chrysanthemum, May and Trigiano (1991) also reported induction of somatic embryos at higher inositol treatment.

Table 4. Effects of sucrose and inositol concentrations on direct somatic embryogenesis from ray floret explants of chrysanthemum cv. Birbal Sahani after 4 weeks. Means ± SE.

Treatment	[g dm^{-3}]	Response [%]	Number of embryos [$explant^{-1}$]
Sucrose	20	10	2.0 ± 0.3
	30	35	8.1 ± 0.8
	40	55	12.7 ± 0.6
	60	70	15.4 ± 1.2
	120	20	9.5 ± 0.8
Inositol	50	15	8.0 ± 1.0
	100	30	8.9 ± 0.5
	200	45	10.2 ± 1.1
	400	55	14.0 ± 0.8
	800	50	10.0 ± 0.7

Two percent of somatic embryos were germinated on the same cultural conditions after 6 weeks of culture initiation (Fig. 1*D*). Germinated somatic embryos with shoots were transferred to growth regulator free MS medium where roots were formed (Fig. 1*D*). Rooted shoots were transferred to soil and kept under high humidity condition for the first week for acclimatization. Acclimatized plants were finally moved to the field condition where they grew well and produced true-to-type flowers.

Present results clearly demonstrate the usefulness of somatic embryogenesis in mutation breeding. Present technique will open up a new way for isolating new flower colour/shape ornamental cultivars through retrieval of single mutated cell.

References

Chakrabarty, D., Mandal, A.K.A., Datta, S.K.: Management of chimera through direct shoot regeneration from florets of chrysanthemum (*Chrysanthemum morifolium* Ramat.). - J. hort. Sci. Biotechnol. **74**: 293-296, 1999.

Chakrabarty, D., Mandal, A.K.A. Datta, S.K.: Retrival of new coloured chrysanthemum through organogenesis from sectorial chimera. - Curr. Sci. **78**: 1060-1061, 2000.

Chen, T.H.H., Marowitch, J., Thompson, B.G.: Genotyping effects on somatic embryogenesis and plant regeneration from callus cultures of alfalfa. - Plant Cell Tissue Organ Cult. **8**: 73-81, 1987.

Datta, S.K., Chakrabarty, D., Mandal, A.K.A.: Gamma ray induced genetic manipulation in flower colour and shape in *Dendranthema grandiflorum* and their management through tissue culture. - Plant Breed. **120**: 91-92, 2001.

Fambrini, M., Coinini, G., Pugliesi, C.: Development of somatic embryos from morphogenetic cells of the interspecific hybrid *Helianthus annuus* × *Helianthus tuberosus*. - Plant Cell Tissue Organ Cult. **114**: 205-214, 1996.

Finer, J.J.: Direct somatic embryogenesis and plant regeneration from immature embryos of hybrid sunflower (*Helianthus annuus* L.) on a high sucrose containing medium. - Plant Cell Rep. **6**: 372-376, 1987.

Haccius, B.: Question of unicellular origin of non-zygotic embryos in callus cultures. - Phytomorphology **28**: 74-81, 1978.

Lazzeri, P.A., Hildebrand, D.F., Collins, G.B.: Soybean somatic embryogenesis. Effects of nutritional, physical and chemical factors. - Plant Cell Tissue Organ Cult. **10**: 209-220, 1987.

Maheswaran, G., Williams, E.G.: Direct somatic embryoid formation on immature embryos of *Trifolium repens*, *T. pratense* and *Medicago sativa* and rapid clonal propagation of *Trifolium repens.* - Ann. Bot. **54**: 201-211, 1984.

Maheswaran, G., Williams, E.G. Origin and development of somatic embryos formed directly on immature embryos of *Trifolium repens in vitro*. - Ann. Bot. **56**: 619-630, 1985.

Mandal, A.K.A., Chakrabarty, D., Datta, S.K.: *In vitro* isolation of solid novel flower colour mutants from induced chimeric ray florets of chrysanthemum. - Euphytica **114**: 9-12, 2000a.

Mandal, A.K.A., Chakrabarty, D., Datta, S.K.: Use of *in vitro* techniques in mutation breeding of chrysanthemum. - Plant Cell Tissue Organ Cult. **60**: 33-38, 2000b.

Mandal, A.K.A.**,** Dutta Gupta, S., Chatterji, A.K.: Factors affecting somatic embryogenesis in safflower. - Biol. Plant. **44**: 503-507, 2001.

Mandal, A.K.A., Dutta Gupta, S.: Somatic embryogenesis of safflower: influence of auxin and ontogeny of somatic embryos. - Plant Cell Tissue Organ Cult. **72**: 27-31, 2003.

May, R.A., Trigiano, R.N.: Somatic embryogenesis and plant regeneration from leaves of *Dendranthema grandiflora.* - J. amer. Soc. hort. Sci. **116**: 366-371, 1991.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bio-assays with tobacco tissue culture. - Physiol. Plant. **15**: 473-491, 1962.

Ozias-Akins, P., Anderson, W.F., Holbrook, C.C.: Somatic embryogenesis in *Arachis hypogea* L.: genotype comparison. - Plant Sci. **3**: 103-111, 1992.

Sellars, R.M., Southard, G.M., Phillips, G.C.: Adventitious somatic embryogenesis from cultured immature zygotic embryos of peanut and soybean. - Crop Sci. **30**: 408-414, 1990.