December 2010 ISSN : 0974 - 7435 Volume 4 Issue 4

Trade Science Inc.

BioTechnology

An Indian Journal

FULL PAPER

BTAIJ, 4(4), 2010 [161-167]

Somatic embryogenesis and regeneration in *Chrysanthemum*

Thenmozhi Mani[1], Kalaiselvi Senthil[2]*

[1]Department of Biotechnology, Karpagam University, Coimbatore - 641 021, (INDIA)

[2]Department of Biochemistry, Biotechnology and Bioinformatics, Avinashilingam University for Women, Coimbatore - 641 043, (INDIA)

E-mail : aubio.ptc.kalai@gmail.com

Received: 24th May, 2010 ; Accepted: 3rd June, 2010

ABSTRACT

In genetic improvement schemes, multiplication of elite materials by somatic embryogenesis prevents genetic recombination and the need for long, expensive conventional selection cycles. The objective of the present work was to standardize a protocol for somatic embryogenesis in *Chrysanthemum* through suspension culture. Callus induction from leaf explant in MS medium containing 1.5 mg/L 2,4-D was found to be 100% followed by MS media supplemented with 2.0 mg/L 2,4-D (81%). Callus induction from petal explant in MS medium containing 2.0 mg/L 2,4-D was found to be 100% followed by MS media supplemented with 1.5 mg/L 2,4-D (80%). The best friable calli were transferred to MS media supplemented with 1.0 mg/L BAP for shoot induction. Somatic embryos were obtained when these green calli were subjected to suspension culture in MS media supplemented with 1.0 mg/L BAP. All calli in suspension gave rise to somatic embryos, which were regenerated in MS media supplemented with various concentration of BAP. MS media supplemented with 2.0 mg/L BAP gave the highest number of plantlets (46.3%). The regenerated plantlets were elongated on MS media supplemented with 0.1 mg/L BAP + 2.0 mg/L KIN and rooted on MS basal medium (MS0). This method of somatic embryogenesis could be used as an explant material for transformation studies. Different stages of *Chrysanthemum* were analyzed by HPLC.

KEYWORDS

Callus;
Chrysanthemum;
Regeneration;
2,4-D;
BAP;
Somatic embryogenesis.

INTRODUCTION

Chrysanthemum is a cosmopolitan genus, comprising about 300 species of herbs and under shrubs. Several species of *Chrysanthemum* are ornamentals grown in gardens for their large, showy, multicoloured flowers, and are also important cut flower crop. *Chrysanthemum* is a mild-acting medicine possessing anti-microbial and antiviral properties and shows the mildest anti-oxidation activity. There are various reports on its components such as chlorogenic acid, flavonoids and pentacyclic triterpenes, its clinical applications, its anti-HIV, anti-tumor and anti-mutagenic activities[1]. Its flowers yield an important insecticide, that is, the pyrethrins. Pyrethrins, widely used as natural insecticides, offer all the ad-

FULL PAPER

vantages of chemical compounds, that is, rapidity of action against a broad range of insects, and rapid biodegradability[2].

Even though many reports are available on *in vitro* propagation, the protocols are complicated. Here, we report a very simple economical, rapidly multiplying and highly reproducible protocol for somatic embryogenesisof *Chrysanthemum* through suspension culture.

EXPERIMENTAL

The leaves and petals were collected from the green house (Figure 1) of the Department of Biochemistry, Biotechnology and Bioinformatics. The explants were washed thoroughly under running tap water for 30 min, followed by 0.5% bavistin for 15 min. Bavistin treated explants were washed with sterile distilled water, treated with 5% Tween 20 for 5 min, and washed repeatedly with sterile distilled water. The explants were then surface sterilized with 0.5% mercuric chloride for 2 min and washed with sterile distilled water for 3 times each under the laminar airflow followed by 70% ethanol for 1 min. After washing in sterile distilled water, the explants were inoculated aseptically in MS medium3 (Murashige and Skoog, 1962) containing 30 g/L sucrose and gelled with 8 g/L diffco bacto agar.

Figure 1 : *Chrysanthemum* explant.

Callus induction

MS medium supplemented with the varying concentration of the auxin, 2,4-D was used for callus induction studies. The effect of different concentration of the auxin, 2,4-D on callus induction was studied on two explants namely leaf and petals.

Regeneration from callus

All the calli obtained were subcultured after 30 days and the friable callus obtained from best concentration were transferred to MS medium supplemented with 1.0 mg/L BAP for regeneration. A photoperiod of 16/8 h light and dark was maintained.

Somatic embryogenesis in suspension culture

The calli were maintained in MS medium supplemented with 1.0 mg/L BAP for a month and then were transferred to MS liquid medium supplemented with 1.0 mg/L BAP. A photoperiod of 16/8 h light and dark and the cultures were incubated in the shaker at 75-80 rpm.

Shoot induction

The somatic embryos were subcultured after 20 days and were transferred to the following medium for regeneration. In 10 replicates with 3 explants in each replicate were inoculated.

Elongations and rooting

Thirty day old regenerated plantlets were transferred to MS medium supplemented with 0.1 mg/L BAP + 2.0 mg/L KIN for elongation. A photoperiod of 16/8 h light and dark was maintained. After 30 days the regenerated plants were transferred in to MS0 for rooting.

Hardening

The individual rooted plants were carefully taken out, washed free of agar, and transferred to plastic cups filled with sterile vermiculite and maintained in rectangular glass box inside the growth room under high humidity for initial establishment. After 3- 4 days, they were again hardened in the mist chamber for one week for further growth and establishment.

HPLC analysis

The components present in leaf of *ex vitro* plants, various stages of *in vitro* development namely, callus, green callus and somatic embryos were extracted in petroleum ether and analyzed using HPLC.

Extraction

All the materials were dried in the shade. The dried materials were then powdered. 0.5 g of the samples was taken and extracted with 25.0 ml of petroleum ether and kept on a rotary shaker overnight. Then the aqueous layer was filtered, evaporated and redissolved the residue in 5.0 ml of HPLC grade acetonitrile. Extraction of secondary metabolites from various samples was done[2].

Analysis parameters

A Shimadzu LC 8 A system consisting of a binary pump connected to a fractional collector (FRC – 10 A) and system controller (SCL – 10 A VP) was used to perform HPLC analysis. A reverse phase Varian C18 column (5 µm particle size, 25 cm × 0.25 ?m) was used. The absorbance at 254 nm was measured by a PDA photodiode array variable (SPD – M 10 A VP). Mobile phase solution A consisted of HPLC grade acetonitrile and solution B consisted of 0.1% TFA (Trifluoro acetic acid) in water. Chromatographic separations were achieved by gradient elution using mobile phase 1 and 2 at a ratio of 70:30 over a total run time of 25 min and flow rate of 1.0 ml/min and maximum pressure of 220 lbs.

Statistical analysis

The data generated from the various experiments were subjected to statistical analysis by using the statistical software AGRES, in completely randomized design (CRD). Percentage values were transformed to arcsine values before statistical analysis, wherever necessary. Each experiment had 10 replicates with three explants each.

RESULTS AND DISCUSSION

Effect of 2,4-D concentration on callus induction and proliferation

Effect of four different concentrations of 2,4-D (0.5, 1.0,1.5, and 2.0 mg/L) on callus initiation and callus mass were assessed in leaf and petal explants. Callus initiation and proliferation was observed at weekly intervals. The percentage of callus initiated was recorded 4 weeks after inoculation. The increase in mass as gain in weight was recorded as the proliferation rate of callus after 6 weeks of inoculation. The effect of 2,4-D on callus induction in leaf and petal is summarized in TABLE 1. A significant difference is observed between treatments. A 100% callus induction (Figure 2) was observed for leaf explants in T3 (1.5 mg/L 2,4-D) followed by T4, T2 and T1. The callus induction on MS medium supplemented with 2.0 mg/L 2,4-D (T4) was 81 and 72% in T2 while the callus response was lowest (20%) in T1 (0.5 mg/L 2,4-D). A high callus response of 100% was achieved in MS medium supplemented with 2.0 mg/L 2,4-D (T4) for petal explants. The callus formation was 80% in T3 (1.5 mg/L 2,4-D) and 48% in T2 (1.0 mg/L 2,4-D). The callus induction was found to be lowest T1 (24%) for petal explants. The effect of growth regulators on culture response in *Chrysanthemum* was also studied, the results indicated that MS medium supplemented with 2.0 mg/L 2,4-D was the optimal media for callus induction[4], which is on par with our studies on callus induction in petals. High callus response in *Taxus wallichiana* on half WPM supplemented with 1.0–2.0 mg/L 2,4-D[5] which was in accordance to our results for both leaf and petal explants.

TABLE 1 : Effect of 2,4-D concentration on callus induction

Treatment	Concentration of 2,4-D mg/L	Callus induction Percentage ± SE	
		Leaf	Petals
T1	0.5	20 ± 0.12	24 ± 0.14
T2	1.0	72 ± 0.14	48 ± 0.17
T3	1.5	100 ± 0.26	80 ± 0.22
T4	2.0	81 ± 0.13	100 ± 0.26
SEd		0.19	0.36
CD (0.05)		0.39	0.72

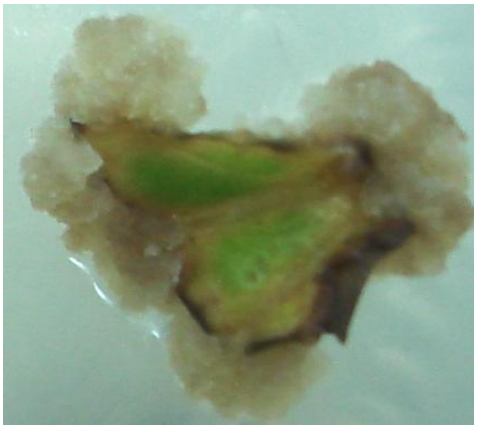

Figure 2 : *Chrysanthemum* callus

FULL PAPER

A range of 2,4-D concentrations (0.1–2.0 mg/L) were used for callus induction from leaf and nodal segments of *Cardiospermum halicacabum* L. Even though high concentrations of 2,4-D (1.5 mg/L) was necessary for callus induction from leaf and nodal cuttings, it adversely affected further growth of the callus[6]. The effect of 2,4-D on callus proliferation is summarized in TABLE 2. A significant difference was observed between treatments. The proliferation rate was found to follow an increasing trend with increase in 2,4-D concentration. At a concentration of 2.0 mg/L 2,4-D (T4),the proliferation of leaf callus was found to be less compared to petal callus. The proliferation rate was minimum in media supplemented with 0.5 mg/L 2,4-D (T1-21 mg) for leaf callus and maximum at 1.5 mg/L 2,4-D (T3-40 mg) supplementation. In case of petal callus it was found that the MS media supplemented with 2.0 mg/L 2,4-D (T2) gave maximum proliferation (43 mg).

TABLE 2 : Effect of 2,4-D concentration on callus proliferation

Treatment	Concentration of 2,4-D mg/L	Callus Proliferation in mg ± SE	
		Leaf	Petals
T1	0.5	21 ± 0.01	18 ± 0.09
T2	1.0	30 ± 0.08	27 ± 0.08
T3	1.5	40 ± 0.11	35 ± 0.09
T4	2.0	26 ± 0.09	43 ± 0.12
SEd		0.09	0.09
CD (0.05)		0.18	0.18

Our results are supported by studies of some authors[7] where the influence of auxins on callus production was significant and good calli were obtained from both leaf and stem segments on MS basal media supplemented with 2.0 mg/L 2,4-D within 2 weeks of culture.

Morphology of callus

The colour of callus varied widely from white to green, brown, depending on the concentration of 2,4-D in the medium. Those explants, which were maintained under light, gave green calli, whereas those maintained under dark gave rise to white friable and brown calli. The texture of callus was found to vary with varying 2,4-D concentration. At low concentration of 2,4-D compact calli was observed in that some of the callus produce root induction along with callus (TABLE 3).

TABLE 3 : Morphology of callus

S.No.	Concentration of 2,4-D mg/L	Mode of Incubation	Colour and Nature of Callus
1.	0.5	Light	Green compact
		Dark	Dark brown compact
2.	1.0	Light	Greenish Friable Callus
		Dark	White friable with roots
3.	1.5	Light	Green friable with roots
		Dark	White friable with roots
4.	2.0	Light	Brownish Green
		Dark	White friable

The occurrence of green colour in callus maintained in a photoperiod of 16/8 can be attributed to the photosynthetic activity and differentiation of the plant cells in the presence of light[8]. Compact callus was observed in high concentration (5.0 mg/L) 2,4-D in *Triticum aestivum* plant[9] (Shah et al., 2003). Pertaining to the texture, two types of callus viz. friable and compact callus were observed. Green callus under light and white callus under dark were obtained in *Dietes Grandiflora* on MS media supplemented with 1.0 mg/L 2,4-D[10].

Shoot induction in callus

The best calli obtained from leaf (1.5 mg/L 2,4-D) and petal (2.0 mg/L 2,4-D) was transferred to MS medium supplemented with 1.0 mg/L BAP (Figure 3). Profuse greening was observed in all the calli.

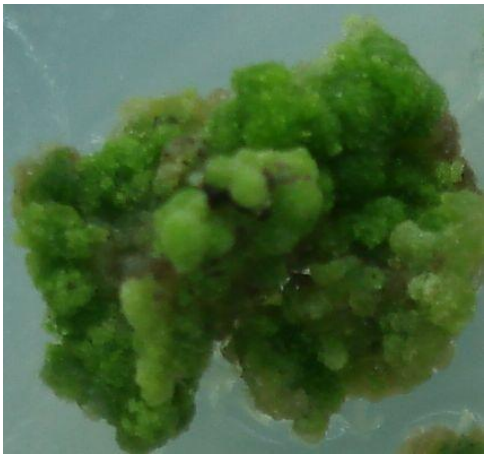

Figure 3 : Green callus.

Induction of somatic embryos through suspension cultures

The friable green callus maintained in 1.0 mg/L BAP was subjected to suspension culture in liquid (without agar) MS medium supplemented with 1.0 mg/L BAP

(Figure 4). This was subcultured every 2 weeks in the same medium. Somatic embryos were induced in all the calli after 2 weeks of inoculation in suspension culture. Cells in suspension show a faster multiplication rate than do cells in callus culture[11]. Somatic embryos of sugar beet in suspension culture in medium supplemented with 0.25 mg/L BAP and 0.25 mg/L 2, 4- D[12] and concluded that increased concentrations of BAP increases the rate of cell division.

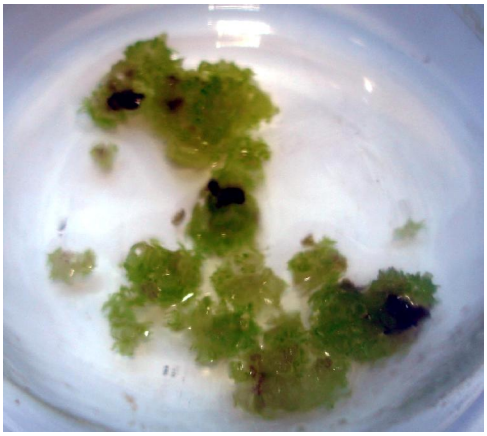

Figure 4 : Callus in suspension culture.

Differentiation of somatic embryos and regeneration

The regenerated somatic embryos differentiating in to globular stage, torpedo stage and cotyledonary stage were observed at various level of sub culturing. The somatic embryos obtained from the suspension culture passed through torpedo and cotyledonary stages (Figures 5 and 6). Early torpedo, late torpedo and cotyledonary stages in somatic embryogenesis *of D. grandiflora*[13]. The regeneration was observed to be 100%. The somatic embryos in the torpedo stage were transferred to MS basal medium for regeneration and cotyledonary stage embryos were obtained. The embryos in the cotyledonary stage were then transferred to MS medium supplemented with varying concentration of BAP. The results were recorded after 3 weeks and summarized in TABLE 4. The largest number of shoots (46.3 ± 0.44) was found in MS medium supplemented with 2.0 mg/L BAP (T4) with significantly high plantlet formation compared to other concentrations. In MS media supplemented with 1.5 mg/L BAP (T3), 27.3 ± 0.36 shoots were observed while with 1.0 mg/L BAP (T2), 16.4 ± 0.37 shoots were observed. The lowest number shoots of 9.4 ± 0.30 were observed in 0.5 mg/L BAP (T1).

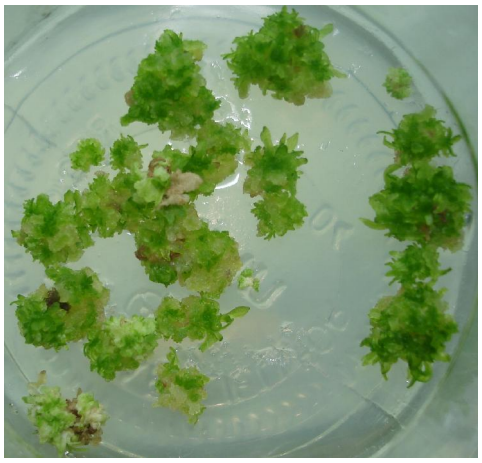

Figure 5 : Torpedo stage.

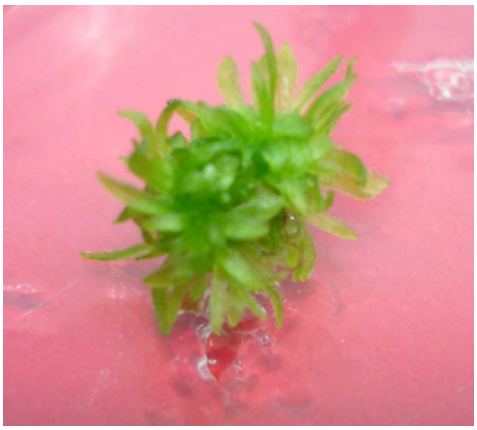

Figure 6 : Cotylendonary stage.

TABLE 4 : Variation of BAP concentration for regeneration

S. No	Media + Concentration of BAP mg/L	Percentage of Number of shoots obtained ± S.E
T0	MS Basal	0.5±0.23
T1	MS + 0.5 BAP	9.4±0.30
T2	MS + 1.0 BAP	16.4±0.37
T3	MS + 1.5 BAP	27.3 ± 0.36
T4	MS + 2.0 BAP	46.3 ±0.44
SEd		0.53
CD		1.07

The effect of BAP was studied on the *in vitro* generated *Chrysanthemum* plantlets, the BAP in low concentrations gave good shoot induction in *Chrysanthemum*[14]. MS media supplemented with 2.0 mg/L BAP, as the optimal media for shoot proliferation on embryo explants of wheat[15], which is comparable to *Chrysanthemum* regeneration in the present study.

Elongation and rooting

The somatic embryo regenerated plants were

FULL PAPER

transferred into elongation medium. The effects of KIN along with BAP were studied for effective elongation of shoot and root and the results were recorded after 1 month. For elongation MS medium supplemented with 0.1 mg/L BAP + 2.0 mg/L KIN was used and the elongation was maximum (19.66 ± 0.34). The effect of BAP and KIN were studied on the *in vitro* generated *Chrysanthemum* plantlets, the combination of AP and IAA low concentrations gave good shoot elongation in *Chrysanthemum*[14]. After elongation the plants were transferred in to MS basal medium (MS0). After 3 weeks profuse rooting was observed in all plantlets (Figure 7).

Figure 7 : Rooting plant.

Hardening

The rooted micro shoots were carefully removed and transferred to presoaked vermiculite for hardening initially inside a rectangular glass box, maintained for 3 days in the culture room and then transferred to the mist chamber for further establishment. Peat-moss vermiculite for hardening and obtained satisfactory acclimatization[16], Good establishment of plants was seen after one week in the mist chamber.

HPLC analysis

Petroleum ether extracts of ex *vitro* and *in vitro* *Chrysanthemum* samples were analyzed by HPLC. Various peaks were eluted between 2-20 min of which the major peaks were considered for analysis. From Figure 8 three major peaks (LP1, LP2 and LP3) were observed for leaf explant. High amount of campesterol and sitosterol components were present in leaf extracts[17]. Also, HPLC analysis of methanolic extracts from *Chrysanthemum* leaf eluted campesterol and sitosterol within 20 min[18]. This suggests that, the major peaks LP1 and LP2 in the present study may be contributed by campesterol and sitosterol. Figure 8 shows a comparative HPLC profile observed in *ex vitro* leaf, callus, green callus and somatic embryos. There is a characteristic change observed in the elution profile of the compounds. The compound contributing to peaks LP2 and LP3 disappears in callus and reappears as differentiation of callus takes place and reaches the same levels as that of leaf explant in the somatic embryo (Figure 8). Further purification and characterization of the compounds contributing to the peaks is essential in order to explain the behavior of these compounds in *in vitro* culture. Peak LP1 is present in all tissues, although varying levels were observed.

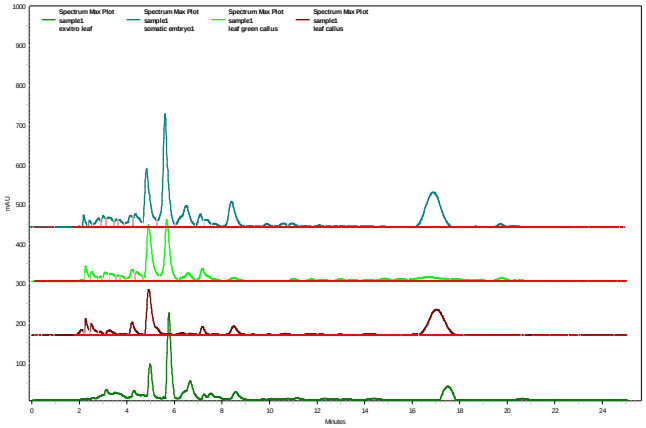

Figure 8 : Comparison of *ex vitro* leaf and leaf derived callus, green callus and somatic embryos.

The result obtained from HPLC studies reveal that certain compounds are synthesized to the same levels in *in vitro* cultures as found in the mature organized tissue structures of plants irrespective of their source of origin.

REFERENCES

[1] K.Chen, W.G.Plumb, N.R.Bennett, Y.Bao; J.Ethnopharmacol., **96**, 201 **(2005)**.
[2] A.Hitmi, A.Coudret, C.Barthomeuf; Critical Reviews in Biochem.Mol.Biol., **35**, 317 **(2000)**.
[3] T.Murashige, F.Skoog; Physiol.Planta., **15**, 472 **(1962)**.
[4] S.D.Obukosia, E.Kimani, K.Wathaika, E.Mutitu, P.M.Kimani; *In vitro* cell Dev.Biol.-Plant, **11**, 162 **(2005)**.
[5] M.M.Datta, A.Majumder, S.Jha; Plant.Cell.Rep., **25**, 11 **(2006)**.

[6] T.D.Thomas, E.A.Maseena; Scientia Horticulturae, **108**, 332 **(2006)**.
[7] P.Bhattacharya, S.Dey, N.Das, B.C.Bhattacharyya; Plant Cell.Rep., **9**, 439 **(1990)**.
[8] A.Slater; 'Plant Biotechnology – The Genetic Manipulation of Plants', Oxford University Press, Noida, **36**, 45 **(2003)**.
[9] M.I.Shah, M.Jabeen, I.Ilahi; Pak J.Bot., **35**, 209 **(2003)**.
[10] J.A.Teixeira da Silva, S.Fukai; Asian Journal for Plant Sciences, **2**, 505 **(2003)**.
[11] G.C.Philips, J.F.Hunstenburger, E.E.Hansen; Plant Cell Tissue and Organ Culture, 67 **(1995)**.
[12] S.Gurel, E.Gurel, Z.Kaya; Turk J.Bot., **26**, 197 **(2002)**.
[13] K.Tanaka, S.Kudo, Y.Kanno, M.Suzuki; Cell Biol.Morphogenesis, **19**, 946 **(2000)**.
[14] S.Bhattacharya, B.Das, T.K.Ghose, S.Bhattacharya; Seed Sci.Technol., **27(1)**, 321 **(1999)**.
[15] H.Alizadeh, M.R.Naghavi, M.Omidi, B.Saatian; Crop Science Society of America, Medison, **44**, 1839 **(2004)**.
[16] P.Castillo, J.Marquez, A.Rubluo, G.Hernandez, M.Lara; Plant Science, **151**, 115 **(2000)**.
[17] A.Kumar, S.P.Singh, R.S.Bhakuni; Current Science, **89**, 1489 **(2005)**.
[18] S.D.Clouse; Ann.Rev.Plant.Physiol.Plant Mol.Bio., **49**, 427 **(1997)**.