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Plant Regeneration from Callus and Cell Suspension Cultures of *Gerbera jamesonii* cv. 'Diablo'

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Summary

Plant regeneration was achieved from callus and cell suspension cultures derived from disc floret, ray floret and petal explants of *Gerbera jamesonii* cv. 'Diablo'. The highest callus induction from these explants was achieved with 1.5 mg l^{-1} NAA, 2 mg l^{-1} NAA and 2 mg l^{-1} 2, 4-D, respectively. The highest number of calli producing shoots and highest average number of shoots per callus was recorded on MS basal medium supplemented with 2 mg l^{-1} BA + 0.5 mg l^{-1} IAA. The calli derived from all explants cultured in media containing 2 mg l^{-1} 2, 4-D were highly friable, had poor regeneration potential and were selected for cell suspension studies. Cell suspensions were initiated with liquid MS medium containing 2 mg l^{-1} 2, 4-D and sub-cultured weekly to fresh medium to obtain maximum cell density. The cultures were allowed to grow into micro-colonies in liquid medium and subsequently into calli on semi-solid and solid MS medium. Calli differentiated into adventitious shoots on MS medium containing 2 mg l^{-1} BA and 0.5 mg l^{-1} IAA. Plantlets were obtained from *in vitro* derived shoots with 56–60 % survival after 30 days of their transfer to pots.

Zusammenfassung

Regeneration von *Gerbera jamesonii* der Sorte 'Diablo' aus Kallus- und Suspensionskultur. Kallus- und Zellsupensionskulturen aus Scheiben- und Strahlenblüten sowie aus Blütenblättern der *Gerbera jamesonii*-Sorte 'Diablo' konnten zu Pflanzen regeneriert werden. Die höchsten Kallusinduktionsraten wurden bei diesen Explantaten mit 1.5 mg l^{-1} NAA, 2 mg l^{-1} NAA bzw. 2 mg l^{-1} 2, 4-D erzielt. Die größte Anzahl sprossbildender Kalli und die größte durchschnittliche Sprosszahl wurde auf dem MS-Grundmedium mit 2 mg l^{-1} BA + 0.5 mg l^{-1} IAA erzielt. Die Kalli aller untersuchten Explantate waren in Medien mit 2 mg l^{-1} 2, 4-D stark krümelig, wiesen nur geringes Regenerationspotenzial auf und wurden für die Untersuchungen mit Zellsuspensionskulturen ausgewählt. Zellsuspensionen wurden zunächst in flüssigem MS-Medium mit 2 mg l^{-1} 2, 4-D kultiviert und dann wöchentlich umgesetzt, um eine möglichst hohe Zelldichte zu erreichen. Die Kulturen konnten sich in dem flüssigen MS-Medium zu Mikrokolonien und dann schrittweise auf halbfestem und festem MS-Medium zu Kallus entwickeln. Die Kalli differenzierten auf MS-Medium mit 2 mg l^{-1} BA und 0.5 mg l^{-1} IAA Adventivsprosse. 56–60 % der *in vitro*-gebildeten Pflänzchen waren 30 Tage nach Überführung in Erde vital.

Key words. adventitious shoot – disc floret – growth regulator – petal – plantlet – ray floret

Introduction

Gerbera is commonly used worldwide as a cut flower. Dwarf hybrid lines also exist which are suited for potted or bedding plants (ROGERS and TIJA 1990). The single flowers are borne on a non-branching scape. Flowers are white, yellow, red, pink, orange, or scarlet. Since the genetic variability within the Gerbera genus is relatively limited, breeding potential for new flower colours, and patterns as well as resistance to biotic or abiotic stresses is also limited (ORLIKOWSKA et al. 1999). Gerbera can be propagated by both sexual and asexual methods. The multiplication through divisions of clumps was the most common method used for several decades, but plant multiplication by this method was too slow to be commercially practical. Now *in vitro* propagation is commonly used for rapid and large-scale multiplication (PIERIK et al. 1975; ROGERS and TIJA 1990; ERWIN et al. 1991; PALAI et al. 1998; ASWATH and CHOUDHARY 2002; ZHANG 2002). Gerbera has been regenerated through callus using various explants (MAIA et al. 1983; SCHUM and BUSOLD 1985; BARBOSA et al. 1994; PARTHASARATHY et al. 1997; RADICE and MARCONI 1998; LE et al. 1999; POSADA et al. 1999; KUMAR et al. 2004). Hardly any report is available on regeneration from petal, disc and ray floret explants through callus and cell suspension cultures. The adventitious shoots are needed in mutation breeding to induce genetic variability. In the present investigation, an attempt was made to develop the regeneration protocol from callus and cell suspension cultures in *G. jamesonii* using petal, disc and ray floret as explants in order to make the breeding work more efficient.

Materials and Methods

Callus culture

Plants of commercial cut flower gerbera (*Gerbera jamesonii* cv. 'Diablo') were procured from a commercial nursery, Solan (H.P.) and allowed to grow in glasshouse. Floral buds (0.8–1.0 cm of dia) were removed and dissected to excise disc florets, ray florets and petals. The explants (2–3 mm) were surface sterilized with 0.1 % $HgCl_2$ for 3–4 min and washed thoroughly with sterile distilled water to remove sterilants. The explants were subsequently cultured on MURASHIGE and SKOOG (MS, 1962) medium supplemented with 1, 1.5 and 2 mg l^{-1} each of

Table 1. Effect of different concentrations of growth regulators alone and in combinations on per cent callus induction, growth and type of callus in *Gerbera jamesonii* cv. 'Diablo'.

Treatment	(mg l^{-1})	Callus induction (%)*			Growth of callus			Type of callus		
		Disc floret	Ray floret	Petals	Disc floret	Ray floret	Petals	Disc floret	Ray floret	Petals
Control	0.0	0.0 [0.00]	0.0 [0.00]	0.0 [0.00]	–	–	–	–	–	–
2, 4-D	1.0	91.3 [72.8]	90.1 [71.7]	84.0 [66.5]	++++	++++	++++	F	F	F
	1.5	90.2 [71.7]	93.5 [75.3]	85.5 [67.6]	++++	++++	++++	F	F	F
	2.0	92.1 [73.8]	91.5 [73.0]	87.2 [69.0]	++++	++++	++++	F	F	F
NAA	1.0	92.0 [73.6]	93.2 [74.9]	76.0 [60.6]	++	+	+	N	N	N
	1.5	93.2 [74.9]	90.5 [72.0]	77.5 [61.6]	++	+	+	N	N	N
	2.0	91.5 [73.1]	95.0 [77.1]	79.5 [63.0]	++	+	+	N	N	N
BA	1.0	20.2 [26.6]	15.5 [23.1]	4.9 [12.6]	+	+	+	F	F	F
	1.5	20.3 [25.6]	22.9 [28.3]	6.5 [14.7]	+	+	+	F	F	F
	2.0	31.5 [34.1]	14.5 [22.3]	9.2 [17.6]	+	+	+	F	F	F
2, 4-D+BA	1.0+1.0	67.0 [54.9]	57.5 [49.3]	50.2 [45.1]	++	+	+	F	F	F
	1.5+1.0	68.2 [55.6]	60.0 [50.7]	52.5 [46.4]	++	+	+	F	F	F
	2.0+1.0	70.5 [57.1]	62.5 [52.2]	51.2 [45.6]	+++	+	+	F	F	F
NAA+ BA	1.0+1.0	44.0 [41.5]	39.5 [38.8]	31.0 [33.8]	+	+	+	N	N	N
	1.5+1.0	30.0 [33.2]	28.0 [31.9]	31.2 [33.9]	+	+	+	N	N	N
	2.0+1.0	30.5 [33.5]	35.5 [36.5]	28.0 [31.9]	+	+	+	N	N	N
$CD_{0.05}$		6.3	3.8	2.0						

* Mean of 30 explants
–: No callus formation
Growth of callus: +: Fair callus growth; ++: Good callus growth; +++: Very good callus growth; ++++: Excellent callus growth
Type of callus: F: Friable; N: Nodular
Figures in the square parenthesis are arc sine transformed values

2,4-dichlorophenoxyacetic acid (2,4-D), naphthalene acetic acid (NAA) and benzylaminopurine (BAP) alone and in combinations for callus initiation (Table 1). Three replications were maintained for each treatment and ten explants in each treatment were evaluated. The cultures were kept in a culture room at 25±2 °C under dark conditions. The cultures without growth regulators served as control. One-month-old calli (4x6 mm dia) were transferred to shoot regeneration medium comprising of MS salts with cytokinins alone and in combinations with auxins (Table 2). The data were recorded on per cent callus induction, growth and type of callus after 30 days of incubation and on per cent calli producing shoots and average number of shoots per callus after 60 days of incubation. The data recorded for different parameters were subjected to completely randomised design (GOMEZ and GOMEZ 1984). The statistical analysis based on mean values per treatment was made using analysis of variance technique for CRD.

Cell suspension culture

Suspension cultures were obtained using the most friable calli derived from petal, disc or ray floret. Thirty Erlenmeyer flasks (100 ml) containing 0.5 to 1.0 g callus per 20 ml liquid MS medium supplemented with 2 mg l^{-1} 2, 4-D were maintained for each treatment. The cultures were placed on a orbital platform shaker at 100 rpm under dark condition at 25 °C. The cultures were sub-cultured at weekly intervals to obtain maximum cell density.

The contents were filtered through autoclaved double-layered muslin cloth to separate debris from cells and cells from microcalli. The cultures were further maintained by regular sub-culturing in fresh liquid medium by transferring approximately 5 ml of suspension into 20 ml liquid medium for maintenance of stock suspension. The viability of the isolated cells was examined using Evan's blue dye. The density of the isolated cells (1–3x10^4 cells ml^{-1}) was adjusted with the help of a haemocytometer by diluting the cell suspension with culture medium. The behaviour of freely suspended cells was examined using a microscope (45X) for observation and photographic records. 2 ml of the suspension was added to Petri-dishes (4 cm dia) containing 10 ml of semisolid and solid MS medium supplemented with 2 mg l^{-1} 2,4-D. The Petri-dishes were swirled to ensure proper mixing of cells in the agar medium. The Petri-dishes were sealed with parafilm and incubated in dark for cell growth and differentiation. The regeneration of calli was carried out on MS medium supplemented with 2 mg l^{-1} BA + 0.5 mg l^{-1} IAA.

The rooting of *in vitro* shoots was achieved by quick dipping the base of the shoot in 2000 mg l^{-1} IBA solution. The shoots were subsequently transferred to pots (10 cm dia) containing a mixture of farmyard manure:sand mixed in the ratio 1:1. Initially the plantlets were covered with jars to maintain high relative humidity. After 15–20 days, when the shoots showed initial signs of establishment, the jars were temporarily removed daily for few hours and plants were sprayed with water. After one month jars were removed completely and the plants were kept in glasshouse and gradually exposed to the normal conditions. The per cent survival of the plants was observed 30 days after transfer to pots.

Table 2. Effect of different concentrations of growth regulators on per cent calli producing shoots and mean number of shoots per callus.

Treatment	(mg l^{-1})	Calli producing shoots (%)*			Mean number of shoots per callus		
		Disc floret	Ray floret	Petals	Disc floret	Ray floret	Petals
Kinetin+IAA	5.0+0.5	3.2	7.2	0.0	0.7	1.7	0.0
		[8.7]	[13.5]	[0.0]			
Kinetin+NAA	5.0+0.1	3.7	5.0	0.0	1.0	1.5	0.0
		[9.5]	[10.9]	[0.0]			
Kinetin+2, 4-D	5.0+0.1	0.0	0.0	0.0	0.0	0.0	0.0
		[0.0]	[0.0]	[0.0]			
BA+IAA	1.0+0.1	11.7	54.0	46.0	3.2	4.5	3.5
		[17.2]	[47.3]	[42.6]			
	2.0+0.5	67.5	70.0	53.0	3.5	4.7	5.0
		[56.7]	[56.8]	[46.7]			
BA+NAA	1.0+0.25	16.2	44.5	25.0	2.2	3.5	2.7
		[23.5]	[41.8]	[29.9]			
	1.5+0.25	63.7	43.2	32.0	2.2	2.5	3.5
		[52.7]	[41.0]	[34.4]			
BA+NAA+IBA	0.75+1.0+0.75	6.0	5.2	2.7	1.5	1.0	0.7
		[12.7]	[11.4]	[6.7]			
$CD_{0.05}$		7.5	6.2	3.6	3.3	3.0	2.0

* Mean of 30 explants
Figures in the square parenthesis are arc sine transformed values

Results

The explants failed to induce callus on medium without growth regulators. The maximum callus induction of 93 and 95 % was observed on medium containing 1.5 and 2 mg l^{-1} NAA on disc florets and ray florets, respectively and of 87 % on medium containing 2 mg l^{-1} 2,4-D on petal explants. The best callus growth was recorded with 2 mg l^{-1} 2,4-D in all the explants (Table 1). The calli derived from MS medium supplemented with 1, 1.5 and 2 mg l^{-1} NAA were found to have maximum shoot regeneration potential. About 70 % of the calli derived from ray florets produced shoots on MS medium supplemented with 2 mg l^{-1} BA + 0.5 mg l^{-1} IAA followed by 54 % on the medium with 1 mg l^{-1} BA + 0.1 mg l^{-1} IAA (Fig. 1b). The average number of 4–5 shoots per callus was observed with these treatments (Table 2). Shoots were not observed on medium with 1, 2, 3 and 5 mg l^{-1} BA or kinetin when used alone. Similarly, highest percentage of calli producing shoots i.e., 67.5 and 53 % were recorded on MS medium with 2 mg l^{-1} BA + 0.5 mg l^{-1} IAA when disc florets and petals, respectively were used as explants (Figs. 1a and 1c). The mean number of 3.5 shoots per callus from disc floret and 5 shoots per callus from petals was observed with 2 mg l^{-1} BA + 0.5 mg l^{-1} IAA (Table 2).

The cells were isolated after seven days of inoculation on a orbital platform shaker. The cell density was increased by sub-culturing suspension into fresh liquid medium at weekly intervals and the maximum cell density was found after two months of inoculation. Cell survival was determined by Evan's blue dye. The first division in cells occurred within a week of suspension culture, the dividing cells changed to elongate to kidney shaped and showed bulge at one position and septum in the culture before the formation of two cells. Two dividing cells also showed papillae indicating cell growth and their preparedness for cell division to form four-celled stage (Fig. 2a). 8-celled colonies were emerged within one week of occurrence of first cell division (Fig. 2b). Cells were allowed to divide in the liquid medium till the formation of micro-colonies of more than 32 cells (Fig. 2c) followed by their culturing on same but semi solid (0.6 % agar) or solid medium (0.8 % agar). Brown spherical microcalli were observed within 4 weeks of culturing on solid medium (Fig. 2d), which were allowed to increase in size to form macro-callus (Fig. 2e). The macro-calli were allowed to increase subsequently in size for 3–4 weeks to form callus on MS medium containing 2 mg l^{-1} 2,4-D. The calli were subsequently transferred to MS medium supplemented with 2 mg l^{-1} BA + 0.5 mg l^{-1} IAA and differentiated into adventitious shoot buds (Fig. 2f). These buds were cultured on the same medium and shoots were obtained after 2–3 weeks of culture (Fig. 2g). The complete plantlets were obtained after *in vivo* rooting of shoots with 56–60 % survival 30 days after transfer to pots (Fig. 2h).

Discussion

The callus induction of all the used explants was most abundant on the medium with 1.5, 2 mg l^{-1} NAA and 2 mg l^{-1} 2,4-D. PARATHASARATHY et al. (1997) reported the best callus growth from fully expanded leaves on MS medium with 1 mg l^{-1} each of NAA, BA and IBA in *G. jamesonii*. The calli differentiated into shoots on medium containing 2 mg l^{-1} BA + 0.5 mg l^{-1} IAA and 1 mg l^{-1} BA + 0.1 mg l^{-1} IAA. ARELLO et al. (1991) found best translucent callus and regeneration with 2 mg l^{-1} BA + 0.5 mg l^{-1} IAA from capitulum explants of *G. jamesonii*. The regeneration from floral buds or capitulum cultures, has also been reported in gerbera by many workers (PIERIK et al. 1975; BARBOSA et al. 1994; RADICE and MARCONI 1998; CHEN and CHEN 2002). In our study, about 70 % of the calli differentiated into 4.7 shoots per callus in ray floret and 67.5 and 53 % with 3.5 and 5.0 shoots per callus in disc floret and petal explants, respectively on MS medium containing 2 mg l^{-1} BA + 0.5 mg l^{-1} IAA. KUMAR et al. (2004) reported 11.0 and 14.7 shoots per explants from leaf and petiole explants with 3 mg l^{-1} BA. PARTHASARATHY and NAGARAJU (1999) reported mean number of 6.8 and 14.2 shoots per callus in cvs. 'SWM' and 'Dilmaya', respectively, of *G. jamesonii* Bolus with BAP. ORLIKOWSKA et al. (1999) observed 2.6, 2.4 and 4.9 shoots per callus in cvs. 'Rebecca', 'Boy' and 'Mariola', respectively with 2.2 µM BA + 0.3 µM IAA. The variation in the number of shoots may be due to the difference in the cultivar and type of explants used. In the present study, calli from petal, disc or ray floret showed almost same organogentic potential. The best callus growth from these explants was observed on medium

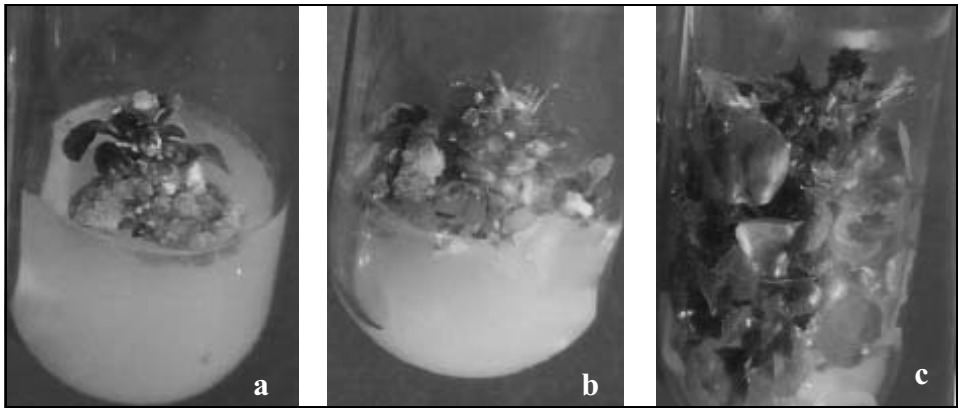


Fig. 1a-c.Adventitious shoot regeneration on MS medium supplemented with 2 mg l^{-1} BA and 0.5 mg l^{-1} IAA on calli derived from disc florets (a) ray florets (b) and petals (c).

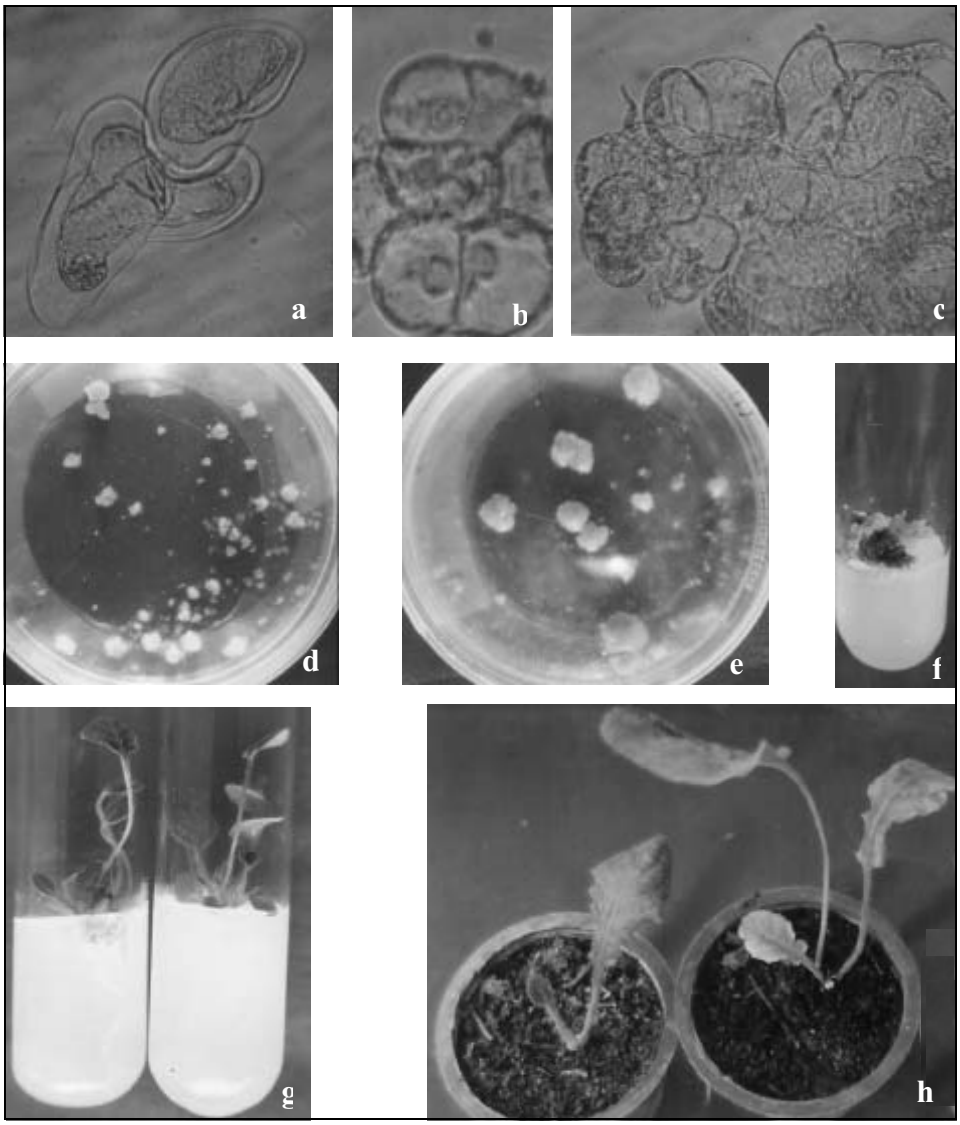


Fig. 2a-h.Photomicrographs illustrating cell growth and their preparedness for cell division to form four celled stage (a) 8 celled stage (b) microcolony of more than 32 cells in liquid MS medium supplemented with 2 mg l^{-1} 2,4-D (c). Formation of microcalli (d) macrocalli (e) on solid MS medium supplemented with 2 mg l^{-1} 2,4-D. Differentiation of calli derived from cell suspension culture into adventitious shoot buds on MS medium supplemented with 2 mg l^{-1} BA and 0.5 mg l^{-1} IAA (f). Adventitious shoot regeneration from shoot buds on MS medium supplemented with 2 mg l^{-1} BA and 0.5 mg l^{-1} IAA (g). Hardened plants after 2 months in potting mixture of sand and farmyard manure (h).

with 1, 1.5 and 2 mg l^{-1} 2, 4-D, the callus produced was friable, did not regenerate shoots and used for cell suspension studies. Cell suspension cultures consisted of single cells and cell aggregates in a moving liquid medium. Hardly any report is available on regeneration from disc florets, ray florets and petals through callus and cell suspension cultures. RUFFONI and MASSABO (1991) studied only cellular features of liquid cultures in *Gerbera jamesonii* hybrida but our work encompasses the differentiation and complete plant regeneration from cell suspension derived calli. The optimum concentration of 2 mg l^{-1} 2,4-D was found to be best for isolation and separation of cells in liquid medium when kept on shaking speed of 100 rpm in darkness. REMOTTI (1995) also established the cell suspension cultures from friable callus derived from cormel slices of gladiolus with 2 mg l^{-1} 2,4-D. In our investigation, the optimum quantity of callus for suspension culture was found to be 0.8–1.0 g per 20 ml of liquid MS medium containing 2 mg l^{-1} 2, 4-D. These observations were in agreement with FAMELAER et al. (2000) who inoculated 0.3 g of callus from *Tulipa praestans* per 5 ml of liquid MS medium. In the present investigation, the optimum plating cell density was adjusted to 1 to $3x10^4$ cells ml^{-1}. It was also observed that single cell showed poor division whereas cell aggregates of 2–5 cells showed enhanced divisions leading to micro-callus formation. SCOTT et al. (1988) reported that single cells of *Shorea roxburghii* failed to form colonies whereas small clusters of 5–6 cells did divide to form callus. This may be attributed due to the reason that the single cells may be damaged during isolation or were unable to adapt to the phase change from liquid to solid medium where the material was less readily available and inhibitory metabolites build up around cells (TORREY 1957; BERGMAN 1960; TORREY and REINERT 1961).

The shoots regenerated from the calli as well as cell suspension derived calli were rooted and the plantlets were hardened in a potting mixture containing sand and farmyard manure mixed in 1:1 ratio. The establishment of plants regenerated from suspension culture provide an additional tool for efforts at varietal improvement in gerbera.

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