Acta Bot. Croat. 61 (2), 125–134, 2002

CODEN: ABCRA 25
ISSN 0365–0588

Rapid plant regeneration from *Gerbera jamesonii* Bolus callus cultures

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A high frequency shoot organogenesis and plant establishment protocol has been developed for *Gerbera jamesonii* from *ex vitro* leaf derived callus. The optimal callus was developed on Murashige and Skoog (MS) basal medium supplemented with 0.4 mg L^{-1} 6-benzylaminopurine (BAP), 4.0 mg L^{-1} α-naphthalene acetic acid (NAA) and 3% (w/v) sucrose. Two callus types differing in their structures and growth rates were observed. A friable and non-chlorophyllous callus with high growth rate appeared at the cut surfaces of the explant, and a compact chlorophyllous callus. The rate of shoot bud regeneration was positively correlated with the concentration of growth regulators in the nutrient media. The explants were highly responsive (83.3%) in a medium containing 2 mg L^{-1} NAA and 1 mg L^{-1} BAP after 3 weeks of callus transfer to a medium. Regenerated plantlets were transferred to soil where they grew normally with a survival rate of 95%. This protocol offers rapid build up of selected clones and opens up prospects for using biotechnological approaches for gerbera improvement.

Key words: 6-benzylaminopurine, compact callus, leaves, friable callus, *Gerbera jamesonii*, alpha-naphthalene acetic acid, organogenesis

Introduction

The gerbera (*Gerbera jamesonii*) is a valuable ornamental species grown as a potted plant and for cut flowers. Because genetic variability within the *Gerbera* genus is limited, the breeding potential for new flower colors and patterns such as resistance to biotic or abiotic stresses is also limited. However, cultivar improvement for these and other traits through genetic transformation looks promising. ELOOMA et al. (1993) used Agrobacterium tumiefaciens mediated transformation to introduce an antisense gene for chalcone synthase into 'Terra Regina'. NOWAK et al. (1997) introduced the *Gus* and *NPTII* marker genes into five gerbera cultivars.

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In the previous work, adventitious gerbera shoots were generated primarily from shoot tips, flower buds of greenhouse grown plants, the pith of rhizome, *in vitro* leaves and *in vitro* petioles.

One necessary pre-requisite for obtaining genetically transformed plants is an effective and reliable protocol for long-term regeneration from single cells. Callus establishment and regeneration of shoots were reported by RUFFONI and MASSABO (1991) in shoot tip, MIYOSHI and ASAKURA (1996) from the ovule, ORLIKOWSKA et al. 1999 from *in vitro* petioles, HUANG et al. (2001) from the shoot tip, stem and *in vitro* petiole. They found frequent browning of the media with slow and unstable regeneration in few cultivars. However, there has so far been no report on callus regeneration and subsequent recovery of the plants from *ex vitro* leaves.

For successful transformation, it is important that regeneration be maintained over a relatively long period, in which organogenetic calli are first produced from single transformed cells, after which shoots can be regenerated from the calli. In the gerbera, directly regenerated shoots were either not transformed or not stably transformed (ORLIKOWSKA and NOWAK 1997). Therefore the use of an effective long-term protocol for indirect regeneration increases the chances of obtaining transgenic gerbera plants. This work documents the effects of growth regulators and long term shoot regeneration from the calli obtained from *ex vitro* leaves of two gerbera cultivars.

Materials and Methods

Plant material

Tissue culture plants of AV101 and AV108 were obtained from Shri Ramco Biotech, Bangalore. These plants were grown under polyhouse in the Indian Institute of Horticultural Research, Bangalore. AV101 is a cut flower variety, which produces flowers with long stalks of 60 cm and a diameter of 13 cm, where as AV108 is a dwarf potted variety, produces a 40 cm flower stalk and has a diameter of 9 cm. The explants size 5mm × 5mm, were cut transversely to the mid rib from mature leaves (3^{rd} leaf from top). They were washed thoroughly with water for 5 minutes, disinfected with 70% ethanol for 30 s, and surface sterilized with filtered 0.1% sodium hypochlorite. After 10 minutes explants were rinsed five times with sterilized distilled water, blotted on sterile filter paper and 15 explants were plated with adoxial surface in contact with the medium (25ml) in 100-mm petri dishes.

Culture media and experimental conditions

Callus were regenerated on MURASHIGE and SKOOG (1962) (MS) basal medium supplemented with 3% sucrose, 1% agar-agar (Bipinchandra and Co., Mumbai) and different concentrations (0, 0.2, 0.4, 1.0 and 4.0 mg/L) of α-naphthaleneacetic acid (NAA) and 6-benzylaminopurine (BAP). The culture media were adjusted to pH 5.8 and sterilized at 120 °C for 20 minutes. After the positioning of the explants, the petri dishes were sealed with cell-o-frais plastic film to minimize water loss. The cultures were maintained at 23 ± 1 °C under 60 μmol m^{-2} s^{-1} fluorescent illumination with a 16 hour photoperiod.

After 4 weeks, the initiated calluses were placed on MS medium supplemented with different concentrations (0, 1, 2 and 4 mg L^{-1}) of NAA and BAP for initiation and prolifera-

tion of shoots. Shoot cultures were raised in culture tubes (1.5 cm diameter × 15 cm length) containing 10 mL medium. After 8 weeks the shoot height was measured (from the base to the highest point of the upper leaf) and then transferred to NAA, IAA or hormone-free MS medium for rooting.

Transfer to soil

Agar was carefully washed from the regenerated plantlets with well-established root system before transfer to pots (15 cm) filled with a mixture of cocopeat and compost (1:1 v/v). Plantlets were maintained under high relative humidity (80 ± 10%) for 3 weeks. Acclimatized plants were kept under a natural photoperiod and a temperature of 25 ± 2 °C.

Observation of cultures

Twenty petri dishes/tubes were used per treatment and each experiment was repeated three times. All cultures were examined periodically and visual observations of any morphological changes were recorded. The data pertaining to the percentages of cultures responding to callusing, percentage of organogenetic calluses per culture, mean number of shoot bud/ culture, mean percentage of rooting and number of roots/shoot were statistically analyzed by the post-Hoc Duncan's multiple range test (MARASCUILO and MC SWEENEY 1977). The average figures followed by the same letter were not significantly different at $p < 0.05$ levels

Results and discussion

The classical findings of SKOOG and MILLER (1957) reported that organogenesis in tissue culture governed by the balance of auxin and cytokinin in the medium cannot be demonstrated universally due to the explant sensitivity or the original content of the endogenous growth regulators. It is strongly advocated here that such reports constitute a basic work for a more complete characterization of callus induction and early organogenesis. The development processes of gerbera callus required both cytokinins and auxins. No callus was induced when explants were cultured on BAP or NAA free medium. Callus formation from explants was observed 3 weeks after culture initiation in the cut edges of the explant. The frequency of callus formation is shown in Table 1. The frequency of callus induction reached 60–95% at high concentrations of NAA and low concentrations of BAP. Two callus types differing in their structures and growth rates were observed. A friable and non- chlorophyllous callus (Fig. 1) with high growth rate appeared at the cut surfaces of the explant and a compact chlorophyllous callus (Fig. 2) with low growth rate was formed directly from whole explants. Only this second type of callus contained adventitious shoot primordia. However, such cultures showing initial stages of differentiation did not develop further on the same medium. Fewer compact calluses than friable calluses were initiated on all the media. A high frequency of callus initiation was obtained only in the MS medium supplemented with 1 or 4 mg L^{-1} NAA; the highest callus initiation was obtained after culture in MS medium with 4 mg L^{-1} NAA and 0.4 mg L^{-1} BAP (95%). Direct formation of short shoots was observed in this medium at low frequency (3%). Total growth of the callus did not vary with different cultivars; however, the type of callus varied with the cultivars.

The cv. AV101 produced more friable non-chlorophyllous callus and a less compact chlorophyllous callus than AV108. Our results agreed with those of PIERIK and SEGERS (1973), showing that the induction of callus-formation by cytokinin was promoted by the addition of an auxin, especially by indole–3 butric acid, the most effective cytokinin being BAP. Further it is also supported by HUANG et al. (2001) where they found MS+ BA 1.0 mg L^{-1} + IBA 0.05 mg L^{-1} was suitable for the induction of callus from shoot tip, stem and petiole.

Tab. 1. Initiation frequency of friable and compact calluses from *ex vitro* leaves of gerbera on MS medium with NAA and BAP

Growth hormones (mg/L)		Initiation frequency of calluses (%)*					
		Av101			AV108		
NAA	BAP	Total calluses	Friable calluses	Compact calluses	Total calluses	Friable calluses	Compact calluses
0	0 to 4	0	0	0	0	0	0
0.2	0	0	0	0	0	0	0
0.2	0.2	2.7	2.7	0	3.1	1.5	1.6
0.2	0.4	5.4	5.4	0	6.6	4.8	1.8
0.2	1.0	16.2	13.5	2.7	15.6	12.5	3.1
0.2	4.0	15.3	12.2	4.1	15.4	9.1	6.3
0.4	0	0	0	0	0	0	0
0.4	0.2	28.4	21.6	6.8	26.6	18.1	8.5
0.4	0.4	25.7	18.9	6.8	23.9	15.2	8.7
0.4	1.0	16.3	12.2	4.1	15.4	9.3	6.1
0.4	4.0	8.1	8.1	0	9.9	7.6	2.3
1.0	0	0	0	0	0	0	0
1.0	0.2	58.1	33.8	15.2	53.7	28.1	25.6
1.0	0.4	50	31.1	18.9	56.9	28.6	28.3
1.0	1.0	28.4	20.3	8.1	38.7	20.3	18.4
1.0	4.0	14.9	14.9	0	17.2	12.0	5.2
4.0	0	0	0	0	0	0	0
4.0	0.2	60.8	40.5	20.3	63.5	33.2	30.3
4.0	0.4	94.6	60.8	33.8	95.0	57.2	37.8
4.0	1.0	43.2	31.1	12.1	52.3	29.7	22.6
4.0	4	4.1	4.1	0	16.4	12.1	4.3

* Data represents mean of 20 replicates/treatment: repeated three times

Other than this, different types of callus like a high thickness with a cluster of 15–20 cells, very high thickness and clusters of medium size 30–50 cells, high thickness with clusters of medium size of 15 cells and single cells; medium thickness with big clusters of more than 50 cells were observed in different treatments (data not shown), however their frequency was low. RUFFONI and MASSABO (1991) also observed this type of different calli in suspension cultures of gerbera with growth hormones like BAP, 2ip, 2,4-D, pCPA and IBA.

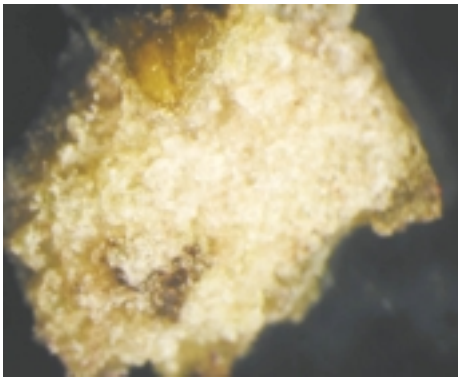

Fig. 1. A friable and non-chlorophyllous callus

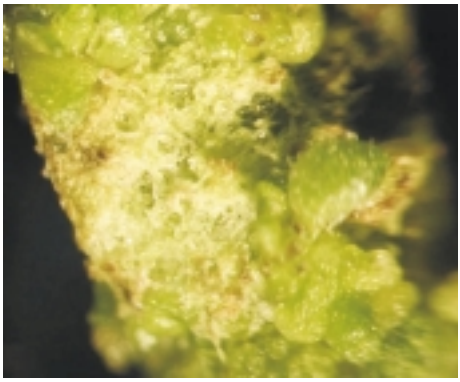

Fig. 2. A compact and chlorophyllous callus

To induce shoot formation the compact callus obtained after culture in MS medium with 4 mg L^{-1} NAA and 0.4 mg L^{-1} BAP was transferred to MS media with different concentrations of NAA and BAP. The frequency of shoot formation and the numbers and heights of developed shoots are given in Table 2. Shoots appeared to develop directly from

Tab. 2. Formation, multiplication rate and height of shoot formed from compact calluses of gerbera.

Growth hormones (mg/L)		Shoot characteristics*					
		Shoot frequency (%)		Number of shoots/callus		Shoot height (cm)	
NAA	BAP	AV101	AV108	AV101	AV108	AV101	AV108
0	0	0	0	0	0	0	0
0	1	12.5 a	15.3 a	1 ± 0.3 a	2.1 ± 0.2a	0.5 ± 0.1a	0.35 ± 0.1a
0	2	12.5 a	17.6 a	1 ± 0.3 a	1.4 ± 0.1a	0.75 ± 0.2a	0.4 ± 0.1a
0	4	12.5 a	23.1 b	1 ± 0.2 a	2.3 ± 0.2a	0.6 ± 0.1a	0.5 ± 0.1a
1	0	0	0	0	0	0	0
1	1	41.7 e	41.7 d	5.1 ± 0.7 c	3.2 ± 0.2b	4.5 ± 0.3c	3.1 ± 0.2c
1	2	29.1 d	49.1 e	3.9 ± 0.7 b	5.1 ± 0.3c	4.1 ± 0.7c	2.1 ± 0.2b
1	4	16.7 b	26.7 b	1.6 ± 0.6 a	4.1 ± 0.2c	3.2 ± 0.4b	2.0 ± 0.1b
2	0	0	0	0	0	0	0
2	1	83.3 h	63.1 f	9.9 ± .0.1 e	11.1 ± 0.9e	4.9 ± 0.6c	2.1 ± 0.1b
2	2	66.7 g	60.2 f	7.5 ± 1 d	14.3 ± 0.5e	3.7 ± 0.4c	2.7 ± 0.2c
2	4	58.3 f	63.3 f	5.9 ± 0.8 c	7.2 ± 0.3d	3.7 ± 0.6c	2.9 ± 0.2c
4	0	0	0	0	0	0	0
4	1	12.5 a	41.5 d	2 ± 0.7 a	4.0 ± 0.2c	1 ± 0.3a	1.5 ± 0.1b
4	2	20.8 c	36.0 c	2.8 ± 0.5 b	6.0 ± 0.3d	2.1 ± 0.4b	1.7 ± 0.2b
4	4	33.3 d	37.2 c	2.6 ± 0.7 b	3.5 ± 0.2b	2.5 ± 0.3b	1.2 ± 0.1b

* Data represents mean of 20 replicates/treatment: repeated three times

a–g mean having the same letter in a column were not significantly different by Post- Hoc Multiple Comparison test $p < 0.05$ level.

adventitious primordia already formed in the compact callus. The media containing only BAP induced translucent short shoots with a lower frequency. With up to 2 mg L^{-1} NAA in the medium, BAP had a negative effect on shoot development, multiplication rate and the height of the shoots. In contrast, increasing the BAP concentration in the medium had a positive effect when combined with a high NAA concentration (4 mg L^{-1}). JERZY and LUBOMSKI (1991) observed the highest number of shoots from 8–11 on medium with 10 mg L^{-1} BAP from *in vitro* leaf, but the shoots were frail, concise and showed vitrification symptoms. On a medium with lower levels of BA (1 and 2 mg L^{-1}) they observed one to three shoots. BARBOSA et al. 1993 reported the establishment of shoots *in vitro* from a young capitulum in 3 mg L^{-1} BAP, while, HENRIQUE et al.1994 got the best results from a capitulum at 3 and 9 mg L^{-1} BAP. However, in contrast to these studies VARDJA and VARDJA (2001) observed the tendency of vitrified shoots in high concentrations of BAP and recommended not using BAP in gerbera multiplication. The highest percentage of multiple shoot formation (83%) occurred 3 weeks after AV101 callus transfer on a medium with 2 mg L^{-1} NAA and 1 mg L^{-1} BAP. After 4 weeks of culture, a complex mass of multiple shoots was formed. After 8 weeks the shoots, when separated from the callus and transferred to the same medium, multiplied more rapidly and the cycle was repeatable. No appreciable difference in the shoot multiplication rate was observed during several repeated cycles.

No shoot development was obtained from the friable callus. BLAKESLEY and LENTON (1987) reported that cytokinins were usually added to tissue culture media to stimulate axillary or adventitious shoot development. The type and concentration of cytokinin had profound effects on shoot multiplication. Rates of multiplication of gerbera shoots *in vitro* are much slower in the presence of natural zeatin (Z) than the synthetic cytokinin (6 BAP). These differences in growth response may be related to the rate of metabolism of zeatin and BAP observed in callus cultures. The highest regenerability rates of 83.3 and 63.3% were obtained in AV101 and AV108, respectively. The number of shoots regenerated per explant was highest for cv. AV108, ranging from 1 to 14.3 shoots while regeneration was lower for cultivar AV101 with 1 to 10.6 shoots per explant; this difference may be due to the high production of compact chlorophyllous callus by cv. AV108. Overall shoot height was more in AV101 than in AV108. The difference in multiplication rate and shoot height may be related to the dwarf genetic nature of the cultivar AV108. Our results agreed with those of JERZY and LUBOMSKI (1991), REYNOIRD et al. (1993) and ORLIKOWSKA et al. (1999), showing that gerbera regenerability is genotype-dependent. Although different cultivars showed varying results, our method was effective for these two cultivars

The effect of NAA or BAP on callus induction was pooled and plotted in graphical form (Figs. 3 a, b). The performance of callus cultures initiated and maintained on medium supplemented with NAA and BAP showed different responses. The concentration of NAA had a profound effect on both friable and compact callus, and as the concentration increased, the amount of callus also increased (Figs. 3 a, b). This is the first report in which NAA was used for the induction of callus in gerbera in leaves. However a reverse trend was observed in case of BAP. As the concentration of BAP increased the amount of friable and compat callus decreased and was found to be lethal at 4 mg L^{-1} (Figs. 3a, b). HUANG et al. (2001) in shoot tip and ORILKOWSKA et al. (1999) in petioles also found a low concentration of BAP (1.0 mg L^{-1}) with IBA and IAA, respectively, was best for shoot proliferation.

The effect of NAA or BAP on shoot proliferation was pooled and plotted in graphical form (Figs. 3c, d, e). When BAP was used as the sole growth regulator in the culture me-

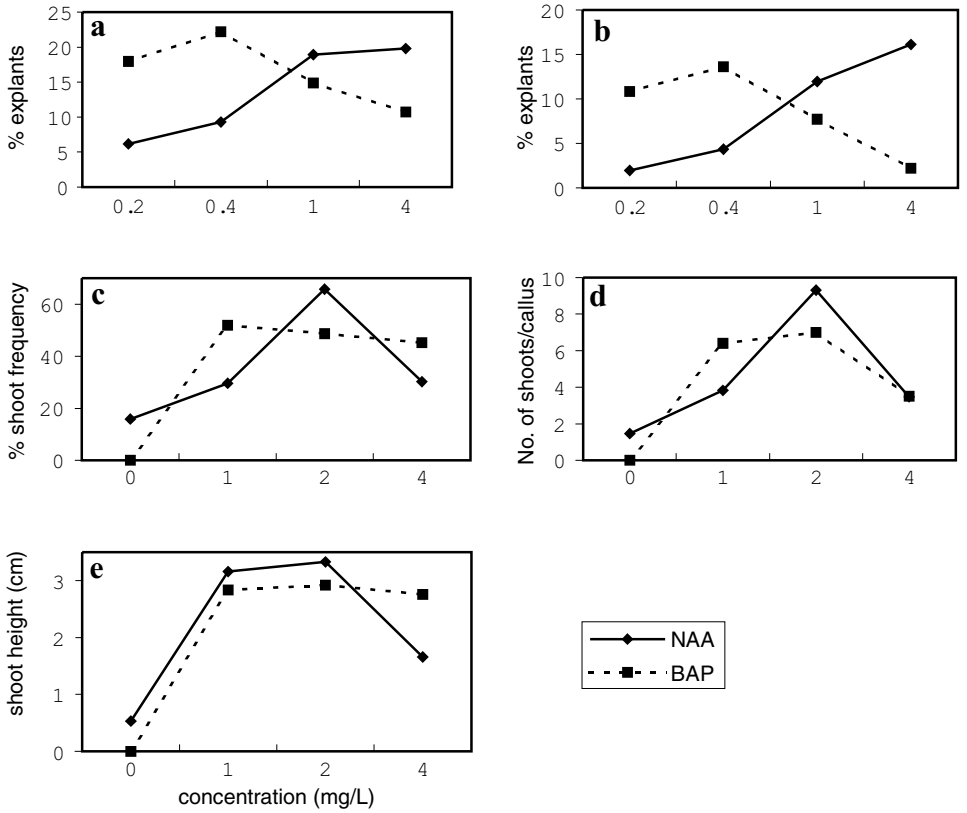

Fig. 3. Cumulative effect of NAA and BAP on induction of friable callus (a), compact callus (b), shoots frequency from callus (c), number of shoots/callus (d), shoot height from callus (e)

dium without the interaction of NAA, a limited shoot proliferation were observed in few explants, while NAA alone as the sole growth hormone did not initiate any shoot proliferation. Shoot frequency, the number of shoot/callus and shoot height was highest in 2 mg L^{-1} NAA and the number of shoots decreased when the level of NAA was increased from 2 to 4 mg L^{-1}.

To induce rooting, individual shoots from a multiple shoot complex originated from the callus were separated after 8 weeks of shoot initiation and transferred to MS medium free of hormones or containing only NAA and IAA (Tab. 3). In both IAA and NAA the frequency of rooting was 100%. In all media, the first roots appeared after 1–2 weeks of culture and after 4–5 weeks, the root system was well developed. On medium without NAA and IAA, we observed the initiation of single root with secondary root formation, whereas in media with NAA and IAA the formation of multiple adventitious roots without secondary roots was observed. IAA was slightly better than NAA in inducing roots in both the cultivars. Even though we observed the difference in shoot height between cultivars there was no difference found as far as root number is concerned. The greatest shoot height (about 6.8 cm) was observed on medium with 2 mg L^{-1} NAA. BARBOSA et al. (1992) also found a better root system in gerbera sprouts at all levels of IAA (0.5, 1.0, 2.0 and 4.0 mg L^{-1}).

After 4 weeks of culture, the whole plants obtained were removed from the medium, transferred to soil, acclimatized for 3 weeks and subsequently cultivated in a glass house. The success rate of this transplantation was 95%. After 3 months, rooted shoots had grown to a height of more than 20 cm. We observed no difference in any of the phenotypic characters including flowering among regenerated plants in either of the cultivars.

Tab. 3. Rooting from multiple shoots of gerbera

Growth hormones (mg/L)	Characteristics of plantlets*			
	Average number of roots /shoot		Shoot height (cm)	
	AV101	AV108	AV101	AV108
0	1.3	1.3	3.8 ± 0.6	2.9 ± 0.4
NAA 1	3.6	4.1	5.6 ± 0.3	3.3 ± 0.3
2	3.8	3.9	6.8 ± 0.3	4.5 ± 0.4
3	4.8	3.8	4.2 ± 0.4	6.2 ± 0.3
4	4.0	4.2	4.1 ± 0.3	4.1 ± 0.3
IAA 1	4.1	4.3	3.9 ± 0.3	3.1 ± 0.4
2	4.8	4.9	4.2 ± 0.2	3.5 ± 0.2
3	5.2	6.1	6.2 ± 0.4	4.3 ± 0.2
4	5.1	4.2	4.1 ± 0.1	4.1 ± 0.1

* Data represents mean of 20 replicates per treatment in three repeated experiments.

Adventitious shoot formation in gerbera was reported by HEDTRICH (1979). Using *in vitro* techniques she obtained a small number of adventitious shoots from the leaf blades of only one cultivar. JERZY and LUBOMSKI (1991) initiated shoot regeneration from petiole and there is a tendency of *in vitro* petioles to form vitrified shoots at a higher concentration of BAP; in addition, there is a possibility of getting only one to three shoots at a lower concentration. The capability of gerbera *ex vitro* leaf explants to regenerate 9–11 shoots at a lower concentration of growth hormones in the present paper is the first report, and the method seems to be more effective in inducing non-vitrified shoots.

The present experiment has shown that *ex vitro* leaves are able to give a callus. From this callus, healthy plants developed, with a high survival rates when transplanted. The protocol is simple easy to carry out and can produce large number of plants for transformation or mutagenesis or for mass propagation. However the risk of somoclonal variation should be ascertained before using it for mass propagation. This ability also opens up the prospects of using biotechnological approaches for gerbera improvement

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