Indian Journal of Agricultural Sciences **82** (9): 768–74, September 2012

In vitro mass multiplication of gerbera (*Gerbera jamesonii*) using capitulum explant

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Received: 5 January 2011; Revised accepted: 25 May 2011

ABSTRACT

An experiment was conducted to develop highly efficient and economically viable protocol for rapid *in vitro* multiplication of gerbera cv. cabana using capitulum as explant during 2005–07. Among the two stages initially used for culture initiation, capitulum explants cultured at immature stage responded better than the mature stage. Best establishment of immature capitulum explants was obtained on modified MS medium supplemented with 10 mg/l BAP and 1 mg/l IAA. The type and concentration of cytokinin also showed profound effect on proliferation. Better proliferation of quality shoots was obtained on BAP supplemented medium than the medium containing kinetin. The maximum numbers of quality shoots were obtained on MS medium containing 1 mg/l BAP and 0.1 mg/l IAA. Half strength MS medium containing 1 mg/l IBA was optimum for root induction. This protocol can produce 85000– 430312 healthy, true-to-the-type plants per year from a single capitulum. Hence, the above protocol can be of high economic interest to the growers opting tissue culture as commercial venture.

Key words: Capitulum, Gerbera, Growth hormones, Micropropagation

Floriculture is increasingly regarded as a viable diversification from the traditional field crops due to increased per unit returns. With the declaration of floriculture as an "extreme focus area" by the Ministry of Commerce and Industry, Government of India, floriculture sector has acquired a special status in the flower basket of India. Many private growers has opted flower growing as commercial venture. Gerbera (*Gerbera jamesonii* Bolus), is one of the choicest flower among the flower growers and traders in India. It has wide applicability in the floral industry as cut flower and potted plant. In India, commercial production of gerberas is centered around Pune and Bangalore, parts of Sikkim, Nagaland, Meghalaya and Uttarakhand, from where flowers are being sent to local and international market. The non-availability of good quality planting material of commercially important strains is a major constraint for its widespread cultivation in India. Its commercial propagation through division of clumps and other conventional methods of propagation is slow and inadequate for the production of large number of uniform propagules (Aswath and Choudhary 2001). Micropropagation is the only viable alternative for large-scale multiplication of gerbera. This method is free of seasonal bonds and enables manifold multiplication of the selected plants. The other advantages are product uniformity, disease-free plants, easy exchange of germplasm and planting material. Furthermore, this technique provides basis for application of different genetic improvement tools, viz. *in vitro* mutagenesis, *in vitro* selection, genetic transformation etc.

The *in vitro* response in gerbera varies with cultivar, explants, and composition of media. Over the years gerbera is being propagated by direct or indirect organogenesis using various explants including stem tips, floral buds, leaf, capitulum etc. The plants are produced from explants of capitulum in red flower gerbera (Pierik *et al.* 1975, Pierik *et al*. 1982), leaves (Kumar *et al.* 2004, Jerzy and Lubomski 1991), floral buds (Mandal *et al*. 2002), floral bracts (Maia *et al*. 1983) and inflorescence (Schum and Busold 1985). Shoot tip culture is by far the most common *in vitro* method for commercial multiplication as shoot tip commences the growth more rapidly and contain more number of axillary bud (Murashige *et al.* 1974). The advantages of the capitulum method over shoot tip are the easier sterile isolation *in vitro*. It is also non-destructive, only inflorescences are used and no shoots are lost from the plant (Pierik *et al.* 1982). The present investigation was undertaken to develop an efficient and economically viable protocol for commercial mass multiplication of gerbera through capitulum explant.

Based on Ph D thesis of the first author submitted to Indian Agricultural Research Institute, New Delhi during 2007.

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MATERIALS AND METHODS

The gerbera cultivar Cabana was grown under polyhouse conditions at Centre for Protected Cultivation Technology, Indian Agricultural Research Institute, New Delhi, during October 2005. All the recommended cultural practices were followed with regular spraying of plant protection chemicals to avoid diseases and pests. The tissue culture experiments were conducted and repeated during the year 2006–07. The capitulum explants were collected at two stages, i e at immature stage (0.5 to 1.0 cm diameter) and at mature stage (1.5 to 2.0 cm diameter). The outer involucral bracts of the capitulum were removed and then it was segmented into 4–8 pieces. Well prepared explants were washed with teepol (0.1%) solution for 5 min. followed by thorough washing under running tap water for 10–15 min. to remove any residue of the detergent. The explants were pre-treated with 0.1% Bavistin ® (Carbendazim) + 0.1% Ridomil ® (Metalaxyl + Mancozeb) + 200 mg/l 8-HQC for two hours. The explants were surface sterilized with 0.1% mercuric chloride for 3 min. in order to minimize culture contamination. Surface sterilized explants were given 4–6 washings with sterile double-distilled water to remove the traces of sterilizing agent(s) immediately after treatment.

The explants were inoculated on basal Murashige and Skoog (1962) medium containing 1 mg/l Thiamine-HCl, 5 mg/l, Pyridoxine-HCl and 5 mg/l Nicotinic acid. The effect of different combinations of 3–10 mg/l BAP and 1–2 mg/l IAA was studied on culture establishment. Medium without any hormone served as control. The *p*H of the medium was adjusted to 5.7 to 5.8 with drop-wise addition of 1 N KOH or 1 N HCl using a digital *p*H meter. The contents were then sterilized in a vertical autoclave at 121ºC for 20 minutes (15 lbs/inch^2). The cultures were maintained at 25±1ºC under fluorescent white light (47 ìmol/m^2/s) at a photoperiod of 16:8 hours light and dark cycles. Twenty explants were inoculated per replication and each experiment was replicated thrice.

The sprouted shoots were separated and multiplied on MS medium supplemented with different combinations of BAP (1, 3 and 5 mg/l), kinetin (1, 3 and 5 mg/l) and IAA (0.1 and 0.2 mg/l). Proliferated micro shoots were transferred to MS medium containing 1.0 mg/l GA_3 for elongation. The shoots proliferation and length was recorded after four weeks of transfer to the shoot proliferation media. A total of 5 conical flasks (250 ml) were subcultured in each treatment and each treatment was replicated thrice.

In vitro rooting of the micro-shoots was obtained on half strength MS medium containing 45 mg/l sucrose and 8g/l Agar. Different concentrations of auxins (IBA & NAA) were tested either individually or in combination for *in vitro* rooting. Fifteen-days-old rooted plantlets were carefully removed from flasks. Roots were rinsed with running water to eliminate residue from the culture media and then soaked in a fungicidal solution (Bavistin 0.1%) for 5 min. Subsequently plantlets were transferred to the hardening media consisting of sterilized peat and soilrite mixture saturated with ½ strength MS medium containing only macro- and micro-salts. For *in vitro* hardening of rooted plants two hardening strategies, viz. glass jars with polypropylene caps and plastic pot with polythene cover were used. The survival rate of the acclimatized plants was recorded three weeks after transplanting.

The data was analyzed employing completely randomized design. COSTAT programme was used for statistical analysis. Data were subjected to analysis of variance (ANOVA) test. The means were compared using Duncan's New Multiple Range test (DMRT).

RESULTS AND DISCUSSION

Effect of explants and induction media on culture establishment

The morphological potential in gerbera varied with the developmental stage of explant. Among the two stages, capitulum explant cultured at immature stage responded better than the mature stage (Table 1). At immature stage, 57.81% explants established, whereas in mature capitulum only 9.73% of explants responded. Shoot emergence was observed after 73.36 days and 88.73 days in immature and mature capitulum, respectively. The number of sprouted shoots per explants was also significantly higher (10.41) in immature capitulum as compared to mature capitulum (1.47). Type of explant, stage, growth, biochemical composition, coupled with the presence of phytohormones, their ratio and level etc. decide the *in vitro* behaviour of explants (Nugent *et al*. 1991). Schum and Busold (1985) also reported quicker and higher *in vitro* shoot production from immature floral buds than that of fully developed inflorescences in gerbera.

When development pattern was studied, two explants behaved differently (Fig 1). In immature capitulum, initially no significant change in the morphology of the floral bud was observed except loosening of florets and drying of outer involucral bracts. Subsequently, growth of florets was seen in the form of swelling and greening of the florets. Later, shoot development occurred directly from these florets. This might be due to the formation of meristematic tissues in segment of immature flower heads (Mandal *et al.* 2002). Shoot development from dormant buds situated in the axils of the bracts surrounding the receptacles of capitulum has also been reported by Pierik *et al.* (1975). The mature capitulum showed normal floral development with the formation of ray and disc florets. Later on the ray and disc florets dried completely and the explant turned brown. In few explants, shoot emergence was seen on the completely dried explant from the base of the receptacles. Schum and Busold (1985) also observed shoot development in the axils of involucral bracts of immature floral buds, while shoots developed from undifferentiated calli in fully developed inflorescence.

Table 1 Effect of explants and induction media on culture establishment in gerbera

*Media	Per cent establishment			Days to shoot emergence			Number of shoots/explant		
	E_1	E_2	Mean	E_1	E_2	Mean	E_1	E_2	Mean
MS + no hormone (Control)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00	0.00	0.00	0.00	0.00	0.00
MS + BAP (3 mg/l) + IAA (1 mg/l)	13.64 (21.63)	0.00 (0.00)	6.82 (10.82)	120.07	0.00	60.03	1.47	0	0.73
MS + BAP (5 mg/l) + IAA (1 mg/l)	84.64 (67.03)	7.73 (15.92)	46.19 (41.48)	77.00	134.00	105.50	13.53	1.27	7.40
MS + BAP (8 mg/l) + IAA (1 mg/l)	79.24 (62.95)	9.71 (18.01)	44.47 (40.48)	67.87	130.73	99.30	17.20	1.87	9.53
MS + BAP (10 mg/l) + IAA (1 mg/l)	80.02 (63.52)	20.51 (26.87)	50.27 (45.20)	61.67	123.60	92.30	22.13	2.47	12.30
MS + BAP (3 mg/l) + IAA (2 mg/l)	16.94 (24.28)	0.00 (0.00)	8.47 (12.13)	105.47	0.00	52.73	2.80	0.00	1.40
MS + BAP (5 mg/l) + IAA (2 mg/l)	81.15 (64.35)	7.52 (15.72)	44.33 (40.03)	82.53	141.40	111.97	8.20	1.87	5.03
MS + BAP (8 mg/l) + IAA (2 mg/l)	80.02 (63.52)	15.36 (23.01)	47.67 (43.27)	75.40	137.27	106.33	12.00	2.47	7.23
MS + BAP (10 mg/l) + IAA (2 mg/l)	85.26 (67.51)	26.70 (31.08)	55.98 (49.30)	70.27	131.53	100.90	16.40	3.33	9.87
Mean	57.81 (48.31)	9.73 (14.51)		73.36	88.73		10.41	1.47	
CD (*P*=0.05)	Explant		1.41	Explant		0.52	Explant		0.25
	Treatment		2.99	Treatment		1.11	Treatment		0. 52
	Treatment × Explant		4.22	Treatment × Explant		1.57	Treatment × Explant		0.74

* Modified MS + 30.0 g/l sucrose + 8.0 g/l agar and *p*H 5.75

E_1, Immature capitulum; E_2, mature capitulum

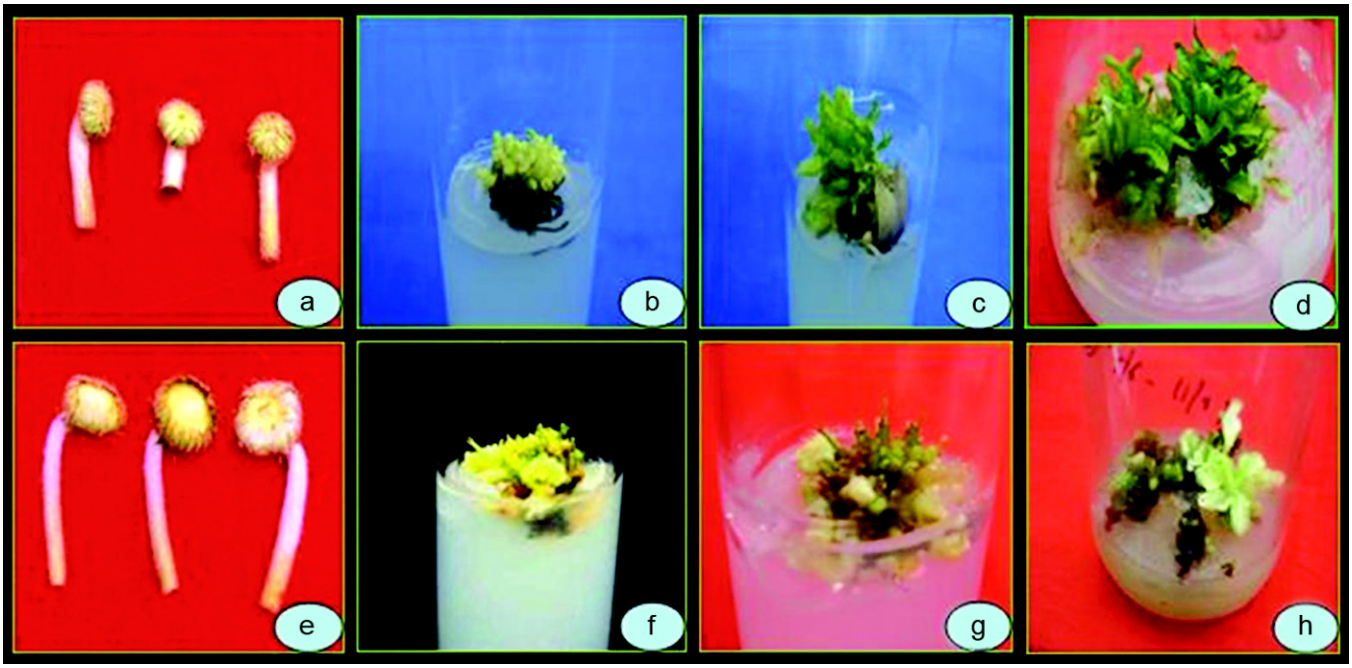

Fig 1 Stages of culture development in immature (a–d) and mature capitulum (e–h). a Immature capitulum explant; b swelling of florets after 25 days of culturing; c elongation and greening of florets after 40 days; d profuse shoot emergence from immature capitulum after 75–80 days of culturing; e mature capitulum explant; f mature capitulum showing flower development; g drying of explant; h shoot emergence in mature capitulum.

Table 2 Effect of different combinations of BAP and IAA on establishment and sprouting of immature capitulum explant in gerbera cv. Cabana

*Treatment BAP	IAA	Establishment (%)	Days to sprouting	Mean number of shoots/explant
-	-	0.00	0.00^{g}	0.00^{e}
3	1	21.63	120.07 ± 4.41^{a}	1.47 ±0.12^{e}
5	1	84.64	77.00 ± 4.61^{cd}	13.53 ±2.91^{bc}
8	1	79.24	67.87 ± 4.82^{ef}	17.20±3.54^{b}
10	1	80.02	61.67 ± 5.68^{f}	22.13 ±3.04^{a}
3	2	16.94	105.47 ± 5.62^{b}	2.80 ±0.40^{e}
5	2	81.15	82.53 ± 5.75^{c}	8.20 ±3.60 ^{d}
8	2	80.02	75.40 ± 5.57^{cde}	12.00 ±2.55^{cd}
10	2	85.26	70.27 ± 4.22^{def}	16.40 ±2.69^{bc}

* Modified MS + 30.0 g/l sucrose + 8.0 g/l agar and *p*H 5.75
** Means followed by different letters within columns are significantly different at *P*=0.05, Duncan test

The induction media had significant effect on initial culture establishment from capitulum explant (Table 1, 2). The capitulum explants failed to establish on medium devoid of growth regulators. However, significant improvement in culture establishment was observed with the addition of growth regulators to the media. Medium containing 10 mg/l BAP and 1 mg/l IAA was optimum for initial establishment. On this media shoot emergence was observed after 61.67 ± 5.68 days of culturing. The number of shoots per explant was also maximum (22.13 ± 3.04) on this media. With the increase in level of BAP, significant reduction in days to sprout and an increase in the sprouted shoot were observed. However, with the increasing level of IAA, shoot emergence was delayed and a reduction in number of shoots was noticed. It might be due to the fact that high concentration of IAA induced callus and indirect regeneration pathway. The role of auxins and cytokinin in micropropagation is well known and the best morphogenetic response can be obtained from synergistic effect of compatible auxins and cytokinin combination (Aswath and Choudhary 2001). The favourable effect of cytokinins on shoot meristem initiation, axillary bud bursting and multiple shoot production have been demonstrated by Pierik *et al.* (1975).

Shoot proliferation

Most of the micro-shoots placed on MS medium devoid of hormones remained as such; however, few shoots proliferated into two or three shoots thus producing on an average of 1.22 shoots per micro-shoot. Significant improvement in shoot proliferation was observed with the use of growth hormones (Table 3). Linear increase in number of shoots was observed with increased concentration of cytokinins (BAP or kinetin); however, elevated level of auxin from 0.1 to 0.2 mg/l significantly decreased the rate of multiplication. Increasing the concentration of IAA to 0.2 mg/l had resulted in profuse callus formation at the base of the shoot, which might have reduced the rate of proliferation. A perfect balance between cytokinin and auxin is essential to obtain maximum proliferation. Elevated level of auxin (IAA) might have disturbed the balance between cytokinin and auxin and thus reduced the proliferation rate as reported by Aswath and Choudhary (2001).

MS medium supplemented with 1 mg/l BAP and 0.1 mg/l IAA was found optimum for proliferation of quality shoots. On this media shoots were normal and healthy with a good proliferation rate (10.53 ± 1.94). The maximum proliferation (20.40 ± 2.71) was observed on MS medium supplemented with 5 mg/l BAP + 0.1 mg/l IAA. However, the shoots developed on this media were lanky and abnormal with linear strap shaped leaves and exhibited vitrification symptoms (Fig 2). Li *et al.* (2003) anticipated that an excess of cytokinins along with the high water potential of the medium were the major reasons for the vitrification of shoots. Though BA is best cytokinin for *in vitro* propagation of gerbera, however, its positive effect on shoot multiplication is related to detrimental effect on their growth. High concentration of BA coupled with high humidity often result in vitrification of *in vitro* raised shoots (Kataeva *et al.* 1991, Jerzy and Lubomski 1991).

The type and concentration of cytokinin also had profound effect on proliferation. Better proliferation of quality shoots was obtained on BAP supplemented medium than the medium containing kinetin. These differences in growth response may be attributed to difference in rate of metabolism of BAP and kinetin as reported by Blakesley and Lenton (1987), where they observed better multiplication of *in vitro* shoots in the presence of BAP than the natural cytokinin (zeatin). Further, they demonstrated that gerbera shoots

Table 3 Effect of various growth regulators on shoots proliferation in gerbera cv. Cabana

*Treatment (mg/l) BAP	IAA	KIN	Mean number of shoots	Average shoot length (cm)
	0.1		1.20 ± 0.20^{g}	2.6 ± 0.52^{abcd}
1	0.1		10.53 ± 1.94^{cd}	3.23±0.52^{a}
3	0.1		16.33 ± 2.01^{b}	2.25±0.58^{bcd}
5	0.1		20.40± 2.71^{a}	1.91±0.42^{cd}
1	0.2		8.33 ± 1.80^{de}	3.06 ±0.54^{ab}
3	0.2		12.80 ± 2.23^{c}	2.07 ±0.63^{cd}
5	0.2		16.60 ± 2.11^{b}	1.7± 0.24^{d}
	0.1	1	3.67 ± 1.03^{fg}	2.53 ±0.44^{abcd}
	0.1	3	6.47±1.92^{ef}	2.79±0.45^{abc}
	0.1	5	5.53 ± 1.22^{ef}	2.67±0.47^{abc}

*MS + 30.0 g/l sucrose + 8.0 g/l agar and *p*H 5.75
**Means followed by different letters within columns are significantly different at *P* =0.05, Duncan test

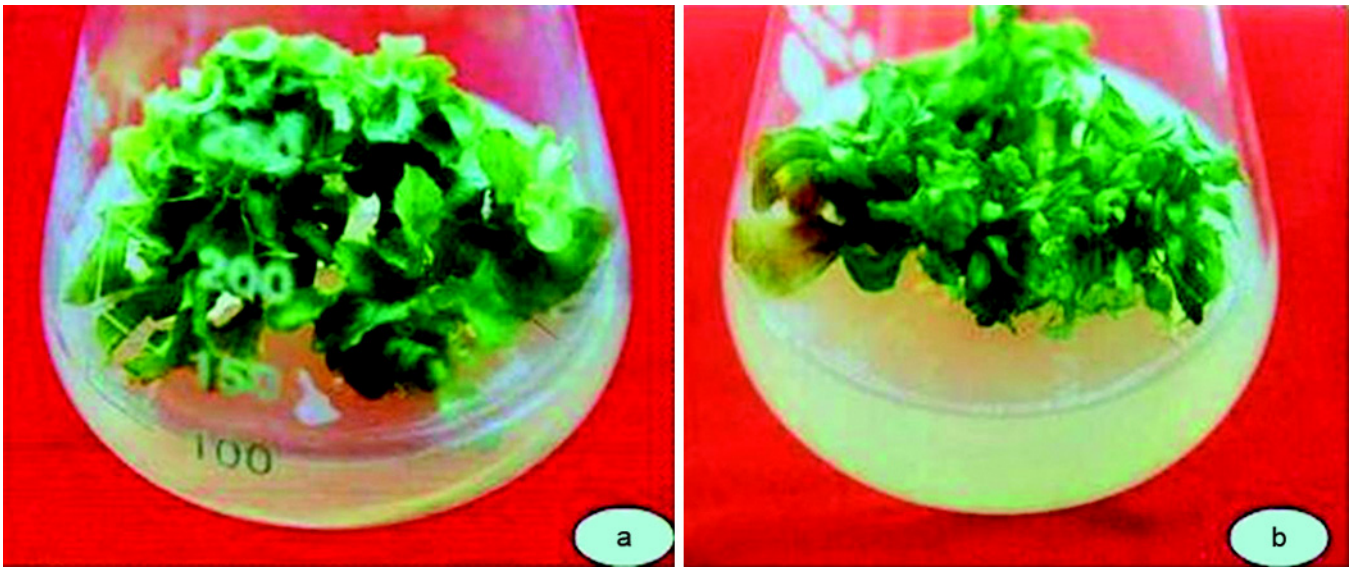

Fig 2 Shoot proliferation in gerbera after one month of subculturing. (a) Healthy shoots proliferated on MS + BAP (1 mg/l) + IAA (0.1 mg/l), (b) vitrification of shoots on MS + BAP (5 mg/l) + IAA (0.1 mg/l)

multiplied *in vitro* in the presence of BAP displayed a higher ability to conjugate BAP.

In vitro *root induction*

Fortification of half strength MS medium with either of NAA or IBA had a profound effect on inducing early rooting (Table 4). The shoots cultured on medium devoid of rooting hormone showed poor rooting (13.33%) and took maximum time (31.13 ± 2.77 days) to root initiation. Rooting was significantly improved with the addition of auxins into the media. Half strength MS medium containing 1 mg/l IBA was optimum for root induction. On this medium root initiation occurred at the earliest (11.33± 2.53 days). The longest roots (6.76 ± 1.09 cm) with good root growth were also recorded on this medium. The root formation is markedly influenced by the presence of auxins and sugars, whereas presence and absence of macro-elements did not influence rooting (Pierik *et al.* 1975). Studies carried out by Palai *et al.* (1998) also indicated that IBA is the best auxin for rooting in gerbera.

The concentration and source of auxin (IBA/NAA) also had significant influence on root initiation and development. Among the two auxins tried, IBA was found better when compared with NAA for inducing good quality roots. Longer and well developed roots with uniformly distributed root hairs were obtained on IBA supplemented media. The maximum number of roots per micro-shoot was recorded on half MS medium supplemented with 1 mg/l NAA; however, the roots were short and swollen, brownish in colour and were without root hairs as reported earlier by Mariska *et al.* (1989). The synergistic effect of IBA and NAA on root induction was also studied. The synergistic effect of was observed for days to root initiation and number of roots per shoot only when both the hormones were used at low concentration. However, rooting was delayed and number of

Table 4 Effect of different concentration of IBA and NAA on root induction in gerbera cv. Cabana

Treatment	Rooting (%)	Days to root initiation	Mean number of roots/shoot	Average root length (cm)
½ MS + no hormone (control)	13.33	31.13 ± 2.77^{a}	1.33 ± 0.12^{e}	1.82 ± 0.09^{e}
½ MS + IBA (0.5mg/l)	98.33	14.27± 2.00^{cde}	4.40 ± 0.62^{c}	5.79 ± 1.14^{ab}
½ MS + IBA (1 mg/l)	100.00	11.33± 2.53 ^{e}	5.73 ± 0.80^{b}	6.76 ± 1.09^{a}
½ MS + IBA (2 mg/l)	53.33	18.67± 3.23 ^{c}	3.27 ± 0.51^{d}	5.17 ±1.05^{abc}
½ MS + NAA (0.5 mg/l)	91.67	15.80± 2.88^{cde}	3.13 ± 0.38^{d}	4.19± 0.97^{bc}
½ MS + NAA (1 mg/l)	100.00	17.67± 3.23^{cd}	7.33 ± 1.00^{a}	3.55± 0.91^{cd}
½ MS + NAA (2 mg/l)	45.00	25.00± 2.51 ^{b}	2.13 ± 0.49^{e}	2.41 ± 0.77^{de}
½ MS + IBA (0.5 mg/l) + NAA (0.5 mg/l)	100.00	12.67± 2.84^{de}	5.33 ± 0.40^{bc}	5.39 ± 1.08^{ab}
½ MS + IBA (1 mg/l) + NAA (1 mg/l)	78.33	18.00± 3.41^{cd}	4.53 ± 0.38^{c}	4.84 ± 0.94^{bc}

*Means followed by different letters within columns are significantly different at P =0.05, Duncan test

roots was reduced when high dose of both the auxins were used in combination. Delayed rooting with few numbers of roots was noticed at higher level of both NAA or IBA or their combination. This is due to the fact that cut ends of micro-shoots initiated callus in the beginning and rhizogenesis occurred at later stage (Palai *et al.* 1998, Parthasarathy and Nagaraju 1999).

Acclimatization of in vitro *raised plants*

Out of the two *in vitro* hardening strategies, better hardening was observed in glass jars with polypropylene cap (Table 5). The per cent survival during hardening in glass jars with polypropylene caps was 93.33 per cent. Whereas, only 76.67 per cent of plants survived when hardening was done in plastic pots covered with polythene cover. High mortality due to desiccation and contamination was observed during hardening in plastic pots covered with polythene covers. This high success in glass jar might be due to high moisture retention and also due to constant maintenance of relative humidity (RH) level compared to other strategies. Superiority of glass jars over pots has been proved earlier in gerbera (Parathasarathy and Nagaraju 1999) and chrysanthemum (Ravindra 2005)

Table 5 Effect of various hardening strategies on acclimatization of *in vitro* raised plantlets of gerbera.

Treatment	Survival (%)	Number of leaves/plant
Glass jars with polypropylene caps	93.33	7.27
Plastic pots with polythene covers	76.67	6.67
SEm±	3.33	0.133
CD (*P*=0.05)	13.08	0.827

Multiplication potential of the developed protocol

A single multiplication cycle in gerbera from proliferation to acclimatization is of 8–12 weeks (Fig 3). Therefore approximately four multiplication cycles are possible per year. Keeping an average of 10 healthy shoots regenerated from a single capitulum explant at establishment stage. As high as high as 85 000–430 312 healthy, true-to-

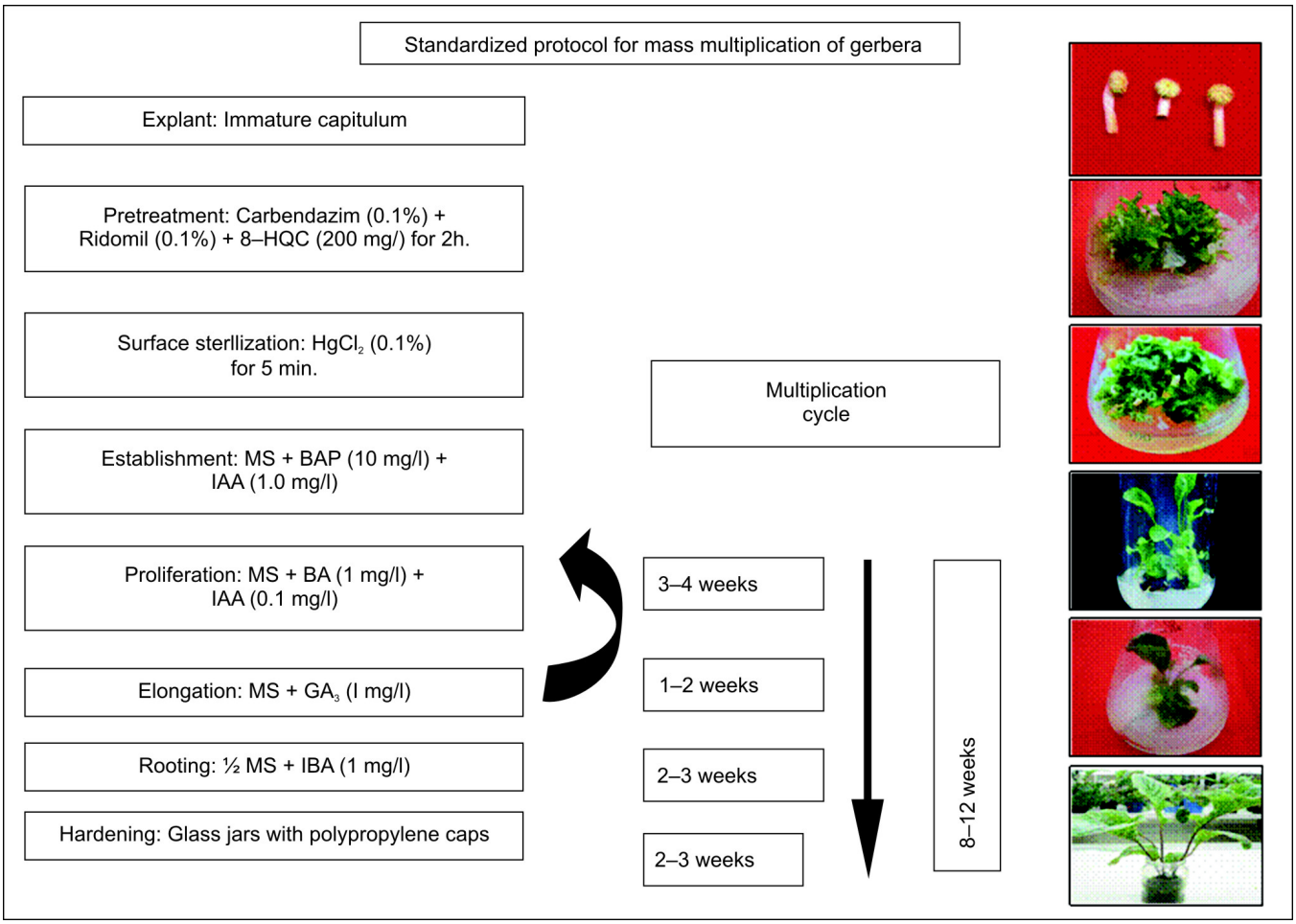

Fig 3 Standardized protocol for large-scale multiplication of gerbera

Proliferation rate	1st cycle	2nd cycle	3rd cycle	4th cycle	*Total
15	150	2 250	33 750	5 06 250	4 30 312
10					
10	100	1 000	10 000	1 00 000	85 000

*After deduction of 15% plants due to mortality/contamination etc.

Fig 4 Multiplication potential of the micropropagation protocol developed in gerbera

the-type plants can be produced per year from a single capitulum with an average proliferation rate of 10–15 (Fig 4). The present study describes an efficient protocol for rapid *in vitro* multiplication of gerbera. The true to the type nature of the *in vitro* raised clones was also confirmed using DNA based markers (Bhatia *et al.* 2011). No variability was detected among the tissue culture raised plantlets. Hence this protocol can be successfully employed for commercial multiplication of gerbera without much risk of genetic instability.

ACKNOWLEDGEMENT

The fellowship provided by Indian Agricultural Research Institute, New Delhi, to the senior author is highly acknowledged.

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