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In vitro propagation of *gerbera* and assessment of clonal fidelity of the mericlones

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ABSTRACT

Rapid *in vitro* shoot multiplication on Marashige and Skoog basal medium supplemented with 0.7 mg l^{-1} thidiazuron was induced in *Gerbera jamesonii* var. Pink Star. Healthy shoots were developed in large number bearing deep green leaves. Maximum shoot elongation was discernible on the same medium in comparison to other treatments. Cent percent rooting of the shootlets was observed on MS supplemented with 0.5 mg l^{-1} NAA. Addition of 10 µM $AgNO_3$ in combination with 0.5 mg l^{-1} NAA and IBA, respectively, showed development of fewer roots but displayed excellent plant health. RAPD analysis involving decamer primers confirm true to-type-nature of the micropropagated plantlets at genomic level. Profuse microtillering and subsequent whole plant development as gleaned from this study offers ample scope to develop large number of clones from a stock of choice for *in vitro* conservation, characterization and screening for diverse characters.

Key words: Micropropagation, thidiazuron, *gerbera*, RAPD.

INTRODUCTION

Gerbera is the latest sensation in Indian floriculture. It has gained wide acceptance among the growers, traders and flower lovers. However, non availability of uniform planting materials from commercially important strains and varieties in large quantity time is a serious constraint creating hindrance in mass cultivation. Gerbera grows across the world in a wide range of climatic conditions. The plants are found to be ideal for beds, borders, pots and rock gardens. It is one among the few accomplished flowers having very high market demand as a cut flower in the domestic and international markets owing to its eye soothing beauty. This long stalked flowers available are in varieties of colour (yellow, orange, cream white, pink, brick-red, scarlet, maroon, terracotta and various other shades) and size under long day high temperature conditions so that quality blooms are obtained under open range at medium elevations only. Gerbera can be propagated by both sexual and asexual methods. Seed propagation, however, has not always been found satisfactory, since impurity of strain produces a great deal of variation (Schaivo, 21). Although the propagation of gerbera conventionally takes place either through seed or by division of clumps, these methods are found to be not compatible for commercial production of large number of uniform plants. In contrast, *in vitro* micropropagation provides manifold increase of elite stocks through very fast propagation. It involves rapid multiplication of explants by repeated subculturing. Shoot tips (Murashige *et al*., 12), capitulum (Pierik *et al.,* 16; Pierik *et al*., 17) and inflorescence buds, flower heads etc. were employed extensively as explants for micropropagation. To meet year round demand of the flower in the market, protected cultivation has been advised and introduced for gerbera in many countries. Two significant gaps in this context are discernible, the prime being the non availability of appropriate technology for cost effective protected cultivation to suit Indian condition and secondly non availability of plant materials of improved varieties with superior traits to satisfy the consumers demand. Cyclic interruption and limited rate of multiplication are other serious constraints encountered in gerbera cultivation.

In assessing genetic uniformity, PCR has previously been used in conjunction with randomly amplified polymorphic DNA (RAPD) primers to assess the genetic stability of micropropagated grape plantlets (Khawale *et al*., 6; Singh *et al*., 22), MM 106 apple rootstock (Modgil *et al*., 10), peach (Hashmi *et al*., 5) and strawberry (Boxus *et al*., 4). Some reports have suggested the use of more than one DNA amplification technique as being advantageous for evaluating genetic stability of micropropagated plantlets in several crops like kiwifruit (Palombi and Damiano, 14), almond (Martins *et al*., 9), grape (Alizadeh and Singh, 1; Khawale *et al.,* 6) and banana (Lakshmanan *et al*. 7; Ray *et al*., 20). The clonal fidelity of micropropagated gerbera plantlets using ISSR markers has recently been reported (Bhatia *et al.,* 3). Keeping all these in view the present investigation was undertaken to develop an efficient *in vitro* propagation protocol for mass multiplication of gerbera to produce genetically uniform planting material.

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MATERIALS AND METHODS

Gerbera var. Pink Star was obtained from ICAR Research Complex for NEH Region, Shillong, Meghalaya. It was grown in an experimental net house at Garacharma Farm of Central Agricultural Research Institute, Port Blair (lat. 11°41' 13.04" N; long. 92°43 30.16 E) during November-January. Explants were collected from the growing plants at active vegetative phase and then washed thoroughly under running tap water till the adhered dirt removed completely. The rhizomes with shoot buds were dipped in 5% aqueous Teepol solution for 5 min. and rinsed thoroughly with distilled water. Subsequently those were disinfected with 1% solution of Bavistin (carbendazim 5% w/v) for 10 min. and rinsed with distilled water repeatedly. Surface sterilization was done with 0.1% freshly prepared aqueous $HgCl_2$ solution for 10 min. and washed with sterile distilled water thrice under laminar air-flow bench. Rhizomes were dried with sterile tissue paper and were cultured in 10 ml of shoot induction medium consisting of MS (Murashige and Skoog, 11) with 3% sucrose 0.8% agar in 25 × 15 ml culture tubes (Borosil) with different concentrations of cytokinins *viz.,* 6-Benzylaminopurine (BAP), kinetin and 1-phenyl -3- (1,2,3-thiadiazol-5-yl) urea [(Thidiozuron - TDZ)] used singly in varied concentrations. The cultures were incubated under 16/8 h light/dark period. Light intensity amounting 60 µmol $m^{-2}s^{-1}$ irradiance from cool-white fluorescent tube (Philips, India) was maintained during the entire culture period. After 15 to 20 days small leaflets started emerging out and tiny shoots were observed at the side of the rhizomes. Within a month time those shoots were separated out and shifted to micropropagation medium for further multiplication. The shootless were subsequently shifted to the rooting medium consisting of MS with different combinatorial treatments of auxin *viz.,* Indole-3-acetic acid (IAA), indole-3-butyric acid, α-Naphthaleneacetic acid (NAA) and $AgNO_3$. The plantlets were allowed to grow within the culture tube till they attained 4-6 cm height with appreciable good health. The complete plantlets were finally transferred to experimental net house directly into soil in cement pots without hardening. Clonal fidelity of the *in vitro* micropropagated plantlets (which were established under nethouse conditions) was performed through RAPD analysis.

Eight arbitrary decamer primers (Operon Technologies, Kit D) *viz.,* were employed for PCR amplification. Genomic DNA was extracted from young leaves (~1 g fresh weight) from gerbera plantlets developed *in vitro* through micropropagation following standard CTAB method (Murray and Thompson, 13). DNA was purified following Sephaglas™ band prep Kit and quantity of DNA sample was checked through electrophoresis with known standards (Hind III digested λ DNA from Pharmacia-Biotech). Further, quality and quantity of DNA was also re-checked spectrophotometrically from the absorbance data of sample DNA at 260 nm (1 OD 260 = 50 µg of DNA).

Random amplification was performed following a modified PCR method (Williams *et al.*, 23). The reaction was carried out in a thermal cycler (MJ Research, Model No. PTC-200). Each 20 µl of PCR reaction mixture was consisting of thermostable DNA polymerase (Ampli *Taq* DNA polymerase) dNTPs (0.4 mM each) and PCR buffer, primer and 50 ng template DNA was performed in a thermal cycler. After initial heat denaturation of the DNA at 94°C for 1 min., thermal cycling was performed for 30 times following the temperature regimes 94°C for 1 min., 40°C for 2 min., followed by 72°C for 3 min. The final extension step at 72°C for 5 min. was followed by cooling to 8°C to complete the PCR. The amplified products were size fractioned by gel electrophoresis in 0.8% agarose. Photographs were taken by using image analysis software, Bio-profile (Vilber-Lourmat, France). DNA profiling of the *in vitro* developed microtillers was observed in the form of scoreable banding pattern and compared with parent to monitor any changes at the genomic level.

Table 1. Details of oligonucleotide decamer primers used to asses clonal fidelity of gerbera mericlones.

Primer No.	Sequence (5′-3′)	Molecular wt. (Da)	No. of amplicons
OPD-1	ACCGCGAAGG	3384	5
OPD-2	GGACCCAACC	3304	4
OPD-3	GTCGCCGTCA	3326	4
OPD-4	TCTGGTGAGG	3421	4
OPD-5	TGAGCGGACA	3399	5
OPD-7	TTGGCACGGG	3406	2
OPD-8	GTGTGCCCCA	3326	3

RESULTS AND DISCUSSION

Three hormones (*viz.,* TDZ, kinetin and BAP) and their different combinations were deployed to induce maximum shoot multiplication. Rapid and very high rate of multiple shooting were observed on MS supplemented with 0.7 mg l^{-1} TDZ (28.71) followed by 1 mg l^{-1} BAP (24.55) and 1 mg l^{-1} BAP + 2 mg l^{-1} kinetin. Shootlets multiplied in presence of TDZ showed excellent shoot health with maximum number of leaves (5.12) and plant height (6.0 cm). Palanco *et al.* (19); Malik and Saxena (8) and Polanco and Ruiz (18) also observed similar results. Out of three treatments with TDZ, maximum shoot multiplication rate/ shoot were observed with 0.7 mg l^{-1} (28.71) endowed with excellent shoot health followed by 0.8 mg l^{-1} (22.11) and 0.6 mg l^{-1} (20.95). Treatment 2 mg l^{-1} kinetin (22.32) was found to be most promising among the three kinetin treatments (Table 2). Among different concentrations of BAP, 1 mg l^{-1} BAP showed maximum multiplication rate /shoot (24.55) followed by 2 mg l^{-1} BAP (20) and 4 mg l^{-1} BAP (12.42) with moderate health and length (Parthasarathy and Nagaraju, 15; Polanco and Ruiz, 18; Malik and Saxena, 8). Treatment 1 mg l^{-1} BAP in combination with 2 mg l^{-1} kinetin also showed better performance (24) with moderate health. In general, shoot health was found to be excellent to poor across treatments. However, higher concentration of BAP, kinetin and TDZ were found to exert inhibitory effects on shoot growth of gerbera.

Attempt was made to induce and optimize rooting of *in vitro* micropropagated gerbera shootlets. *In vitro* raised shoots measuring 4-6 cm were separated and medium cultured individually on MS basal supplemented with 12 different concentrations and combinations of IBA, IAA, NAA, and $AgNO_3$. Ten shoots were cultured in each treatment with three replicates in each. Observations were made in respect of average number of roots per shoot culture, rooting percentage and plant health after 30 days of culture.

Cent percent rooting with excellent health, length and diameter was observed. Concomitant shoot elongation was also observed. Vigorous growth and maximum number of roots / plant were observed on MS supplemented with 0.5 mg l^{-1} NAA followed by 1 mg l^{-1} IBA (96.61%), 0.5 mg l^{-1} NAA + 10 µM $AgNO_3$ (94.11) and 92.85% in 1 mg l^{-1} NAA. This corroborates with the observations made by Bais *et al.* (2) that $AgNO_3$ enhances rooting efficiency. In some cases it was observed that average number of roots/ plant was less but plantlets were found to have excellent health (Table 3).

Among eight decamer primers used for RAPD analysis except one seven showed amplifications in the form of discernible bands on the 1.2% agarose gel. Maximum five bands were observed in case of OPD-1and OPD-5, whereas minimum two bands were observed with OPD-7 (Table 1). The banding pattern for all the primers in respect of parent and microtillers showed no variation (Fig. 1) indicating true to parent nature of the micropropagated plantlets developed *in vitro*. In the present study DNA profiling using RAPD markers proved to be an effective and rapid technique for assessing the molecular stability of plants of gerbera at genomic level. It is concluded that clonal fidelity and lack of somoclonal variation confirms the utility of micropropagation technique for multiplication of gerbera at commercial scale to produce uniform plantlets. However, other markers like Inter Simple

Table 2. *In vitro* micropropagation of gerbera under the influence of synthetic hormones.

Treatment	Multiplication rate/ shoot	No. of leaves/ plant	Plant height (cm)	Shoot health (1-6) scale**
0.6 mg l^{-1} TDZ	20.95	3.58	5.50	1
0.7 mg l^{-1} TDZ	28.71	5.12	6.00	1
0.8 mg l^{-1} TDZ	22.11	4.48	5.42	1
1 mg l^{-1} kinetin	16.98	2.25	5.40	2
2 mg l^{-1} kinetin	22.32	3.62	5.02	2
4 mg l^{-1} kinetin	18.33	2.52	4.91	5
1 mg l^{-1} BAP	24.55	3.95	4.41	3
2 mg l^{-1} BAP	20.00	3.21	4.05	4
4 mg l^{-1} BAP	12.42	2.12	4.38	3
1 mg l^{-1} BAP + 2 mgl^{-1} kinetin	24.00	3.72	4.12	3
2 mg l^{-1} BAP + 1 mgl^{-1} kinetin	9.95	2.25	3.50	4
2 mg l^{-1} BAP + 2 mgl^{-1} kinetin	12.75	2.95	2.97	6

*Basal medium contains MS salts

**1 = Excellent; 2 = Very good; 3 = Good; 4 = Moderate; 5 = Poor; 6 = Very poor.

Table 3. Root induction in gerbera under *in vitro* culture conditions.

Treatment	Rooting (%)	No. of roots/ plant	Plantlet health (1-6 scale)**
0.5 mgl^{-1} NAA	100.0	2.07	1
1 mgl^{-1} NAA	92.85	1.30	3
0.5 mgl^{-1} IAA	80.00	2.00	2
1 mgl^{-1} 1AA	90.00	2.00	3
0.5 mgl^{-1} 1BA	92.85	1.38	2
1 mgl^{-1} IBA	96.61	1.32	1
0.5 mgl^{-1} NAA + 10 µM $AgNO_3$	94.11	1.56	1
0.5 mgl^{-1} IBA + 10 µM $AgNO_3$	90.00	1.88	1
0.5 mgl^{-1} IAA + 10 µM $AgNO_3$	60.00	1.33	3
0.5 mgl^{-1} NAA + 0.5 mgl^{-1} IAA	81.48	1.13	2
0.5 mgl^{-1} NAA + 0.5 mgl^{-1} IBA	78.84	1.46	2
0.5 mgl^{-1} IAA + 0.5 mgl^{-1} IBA	75.00	1.33	4
Control (MS only)	63.63	1.71	2

*Rooting medium was consisting of MS salts

**1 = Excellent; 2 = Very good; 3 = Good; 4 = Moderate; 5 = Poor; 6 = Very poor.

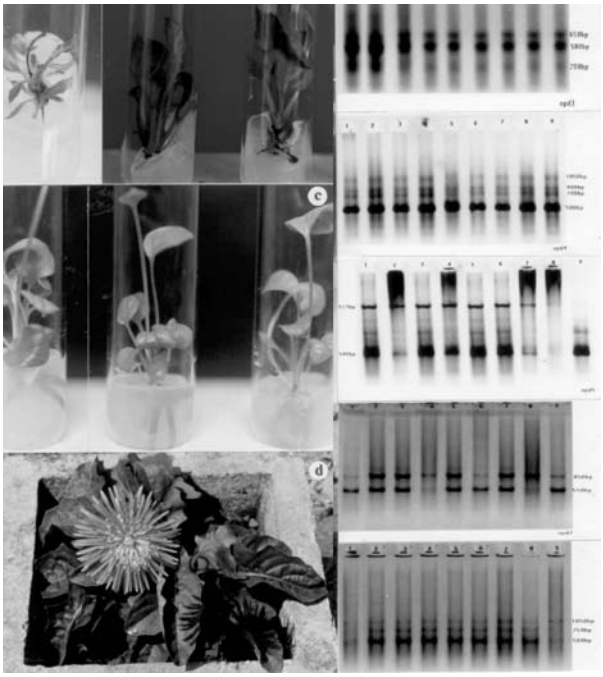

Fig. 1. *In vitro* micropropagation of *Gerbera jamesonii* var. Pink Star a) Miniature shootlets on the multiplication medium. b) Multiplying shootlets on MS supplemented with TDZ. c) Induction of roots on MS containing 0.5 mgl^{-1} NAA. d) A mericlone showing flowers alike parent Pink star. e) Amplicon profile involving decamer primers (OPD-3, OPD-4, OPD-5, OPD-7 and OPD-8). Legends: M- Molecular size marker, 1-9 represent individual plants developed through *in vitro* micropropagation.

Sequence Repeats (ISSR) may also be explored in this endeavour in future. The mericlones developed may be used for large-scale cultivation for commercial purpose, which is expected to be boosting the gerbera based flower industry in Andamans and elsewhere.

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