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Novel strategy for maintenance and mass multiplication of gynoecious line in bitter gourd through micropropagation

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ABSTRACT

This study was undertaken to standardize an efficient protocol for *in vitro* mass multiplication and maintenance of bitter gourd gynoecious line for its use in hybrid seed production. The apical bud gave better response than nodal segment for culture initiation. Murashige and Skoog (MS) basal medium supplemented with 6 benzyl-amino-purine (2 mg/l) + α-Napthalene acetic acid (0.2 mg/l) was found best for *in vitro* survival (81.3%) in plantlets raised through nodal segment, while it was 77.84% in apical bud. The minimum duration (5.53 days) for bud sprouting was recorded for apical bud. Medium combination MS + BAP (1.0 mg/l) + IBA (0.1 mg/l) + GA_3 (0.3 mg/l) was found best earliest shoot proliferation (11.9 &14.62 days for apical bud and nodal segment). The apical bud and nodal segment regenerated 4.77 and 3.56 shoots/ explant, respectively on the same medium. Elongation of micro-shoots was achieved maximum with MS + GA_3 (1 mg/l). The micro-shoots were rooted on full-strength MS medium supplemented with GA_3 (1 mg/l) + activated charcoal (100 mg/l). Minimum days were recorded for rooting (10.81) for apical bud and 11.49 days for nodal segment. The percent survival (81.25%) was maximum in glass jar with PP cap in the shoot tip derived plantlets, which was at par with the nodal segment (80.94%). Rooted plants were acclimatized in the greenhouse and subsequently established in soil. The protocol developed for this study led to an alternative for easy maintenance and use in gynoecious inbred development in bitter gourd.

Key words: Gynoecious line, *in vitro* multiplication, *Momordica charantia*.

INTRODUCTION

Momordica charantia L. commonly known as 'bitter gourd' is an important vegetable crop, containing very high amount of vitamins A and C, iron and other minerals. The F_1 hybrids are gradually gaining popularity in bitter gourd however, inadequate availability of F_1 hybrid seed is the major limitation since it is being produced through hand emasculation and pollination, which is cumbersome and not economical. Bitter gourd is monoecious in nature and the staminate to pistillate flower sex ratio is relatively high (9:1 to 48:1; Dey *et al.*, 2). The availability of genetic mechanism such as gynoecious habit (only pistillate flowers throughout the life cycle) can greatly ease the F_1 hybrid seed production and is highly desirous because it can increases the yield and earliness in bitter gourd (Behera *et al.*, 1). This genetic mechanism is a rare event in nature, which was first isolated in its feral form in *Momordica charantia* var. *muricata*. It was difficult to maintain (*in situ*) since there was no natural maintainer line available and furthermore it was not responsive to sex modifying chemicals. Hence, the tissue culture technique was adopted to maintain the unique gynoecious line.

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The most effective medium for adventitious shoot proliferation from immature cotyledonary node of bitter gourd was 2.0 mg/l BAP in combination with 0.1 mg/l IAA + 2.0 mg/l GA_3 was reported by Sikdar *et al.* (9). Frazier and Kuti (3) found that Murashige and Skoog medium containing 1.0 mg/l kinetin and 0.05 mg/l NAA induced shoots in *Citrullus colocynthis,* when cotyledon was used as explant. Rapid *in vitro* multiplication of two female cultivars (Swarna Alaukik and Swarna Rekha) and one male line of pointed gourd was achieved by culturing shoot tip and nodal explants on MS medium containing IAA (1.0 µM) and IBA (0.2 µM) by Mythili and Thomas (7). In this study, we standardized the protocol for the micropropagation of rare gynoecious bitter gourd plants.

MATERIALS AND METHODS

A gynoecious line DBGy 201 was selected as the experimental material for the present study. Standardization of protocol and multiplication of gynoecious line was carried out at the Central Tissue Culture Laboratory, IARI, New Delhi. Vigorous, disease and pest-free plants were selected as stock plants from the net-house of Vegetable Farm, ICAR-IARI, New Delhi for the experiment. Tender axillary buds (nodal segment) and apical segments derived from healthy vines were used as explants. The explants were excised to 1.0-2.5 cm. The well-prepared explants

were washed in a solution containing detergent [0.1% Teepol®] for 5 min. followed by thorough washing under running tap water for 10-15 min. These explants were then given pre-treatments before *in vitro* inoculation to minimize the microbial contamination. The explants were agitated with carbendazim (0.2%) + 8 HQC (200 mg/l + mancozeb-45 (0.2%) for 1 h followed by three rinses with sterile double-distilled water. The pre-treated explants were surface-sterilized with $HgCl_2$ (0.1%) for 3.0 and 3.5 min. and were rinsed 4 to 5 times with double-distilled autoclaved water.

Explants were cultured on MS (Murashige and Skoog, 6) medium fortified with various concentrations and combinations of BAP, Kin, IAA and NAA for culture initiation; and BAP, NAA, IBA and GA_3 for multiple shoot regeneration. For root induction, elongated shoots were cultured on half-strength of MS medium supplemented with different concentrations of NAA, IBA, IAA and GA_3. For all experiments, the media pH was adjusted to 5.8 ± 0.1 and solidified by 0.8% (w/v) agar-agar. The culture tubes or conical flasks containing media were then autoclaved at 120 ± 1°C at 1.09 kg cm^{-2} pressure for 20 min. Following inoculation, all the cultures were maintained under 16/8 h photoperiod, having light intensity of 47 $\mu mol.m^{-2}s^{-1}$ using white fluorescent lights at regulated temperature of 26 ± 1°C. For acclimatization, agro-peat + vermiculite (3:1, v/v) were used as potting medium moistened with quarter-strength MS salts (macro + micro). Plants after being well established were transferred to the polyhouse and planted for taking observations at different growth stages.

The experiments were laid out in three factorial completely randomized designs (CRD) with three replications comprising 20 to 25 units per treatment. The mean data were analyzed using Duncan's Multiple Range Test (DMRT). The percentage data were subjected to Arc Sin √% transformation before analysis.

RESULTS AND DISCUSSION

Growth regulators are organic compounds naturally synthesized in higher plants, which influence growth and development. The T_7 treatment, *i.e.*, BAP (2 mg/l) + NAA (0.2 mg/l) proved to be the best treatment with high explant survival and successful bud sprouting. Nodal segment had good *in vitro* survival (81.3%), whereas apical bud showed only 77.84% (Fig. 1). The nodal segments had about 93.47% sprouting in comparison to the apical bud with 83.03% in T_7 treatment (Fig. 2). The minimum duration (5.53 days) for bud sprouting was noted for the apical bud with, whereas nodal segment took 5.7 days for sprouting (Table 1). Similar findings have been documented when BAP and NAA are used in different concentrations (Sultana *et al.*, 10). George (5) reported that *in vitro* morphogenesis is regulated by the interaction and balance between the growth regulators supplied in the medium, and the phyto-hormones produced endogenously.

The sprouted buds were excised from the original explants and then sub-cultured on the shoot multiplication medium. The minimum days taken for shoot proliferation from the excised shoot were in T_{10} treatment for apical bud was 11.9 and 14.62 days for the nodal segment. Treatment T_{10} (MS + BAP 1.0 mg/l + IBA 0.1 mg/l + GA_3 0.3 mg/l) yielded maximum sprouts per sub-culture. An average of 3 to 4 shoots was recorded at the 5th sub-culture. The

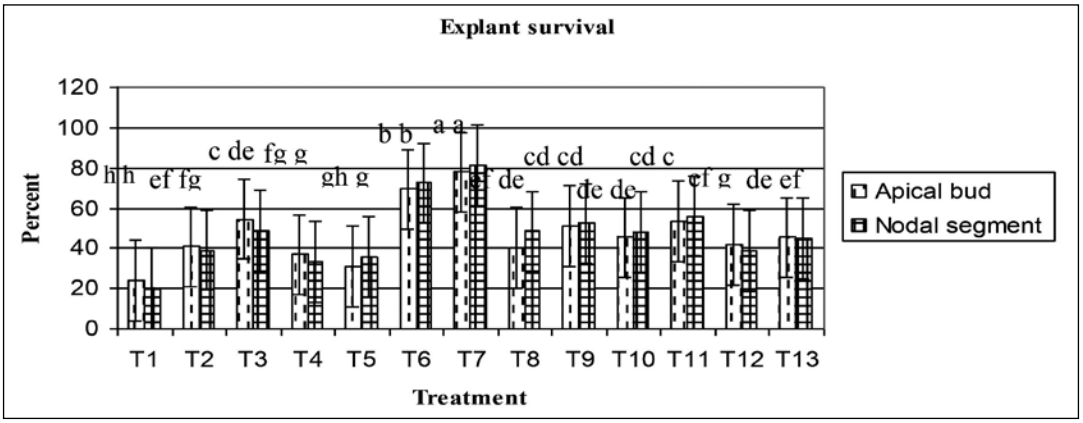

Fig. 1. Effect of different of growth regulators for *in vitro* survival of explants. Each point represents mean ± SE. Means followed by the different letters are significantly different ($P \leq 0.05$).

T1 = MS + No Hormone (control); T2 = MS + BAP (1.0 mg/l); T3 = MS + BAP (2.0 mg/l); T4 = MS + Kn (1.0 mg/l); T5 = MS + Kn (2.0 mg/l); T6 = MS + BAP (1.0 mg/l) + NAA (0.2 mg/l) ; T7 = MS + BAP (2.0 mg/l) + NAA (0.2 mg/L); T8 = MS + BAP (2.0 mg/l) + 1AA (0.2 mg/l); T9 = MS + BAP (2.0 mg/l) + 1AA (0.2 mg/L) ; T10 = MS + Kn (2.0 mg/l) + NAA (0.2 mg/l); T11 = MS + Kn (2.0 mg/l) + NAA (0.2 mg/l); T12 = MS + Kn (2.0 mg/l) + 1AA (0.2 mg/l); T13 = MS + Kn (2.0 mg/l) + 1AA (0.2 mg/l)

Table 1. Standardization of growth regulators for *in vitro* culture establishment in gynoecious bitter gourd.

Treatment	Days required for bud sprouting	
	Apical bud	Nodal segment
T1	11.34 ± 0.55 a	13.32 ± 0.54 a
T2	10.6 ± 0.18 a	11.14 ± 0.15 b
T3	10.92 ± 0.40 a	11.08 ± 0.61 bc
T4	11.02 ± 0.62 a	12.7 ± 0.11 a
T5	10.68 ± 0.14 a	10.86 ± 0.82 bc
T6	6.36 ± 0.27 ef	5.95 ± 0.30 h
T7	5.53 ± 0.20 e	5.7 ± 0.77 h
T8	6.24 ± 0.32 ef	7.48 ± 0.31 g
T9	7.52 ±0.10 cd	8.42 ± 0.07 efg
T10	6.62 ± 0.31 de	7.97 ± 0.10 fg
T11	7.6 ± 0.32 cd	9.39 ± 0.50 de
T12	8.78 ± 0.52 b	9.77 ± 0.28 cd
T13	8.44 ± 0.16 c	8.86 ± 0.29 def
LSD (*P*≤ 0.05)	1.04	1.31
CV(%)	7.17	8.2

T1 = MS + No Hormone (control); T2 = MS + BAP (1.0 mg/l); T3 = MS + BAP (2.0 mg/l); T4 = MS + Kn (1.0 mg/l); T5 = MS + Kn (2.0 mg/l); T6 = MS + BAP (1.0 mg/l) + NAA (0.2 mg/l); T7 = MS + BAP (2.0 mg/l) + NAA (0.2 mg/L); T8 = MS + BAP (2.0 mg/l) + 1AA (0.2 mg/l); T9 = MS + BAP (2.0 mg/l) + 1AA (0.2 mg/L); T10 = MS + Kn (2.0 mg/l) + NAA (0.2 mg/l); T11 = MS + Kn (2.0 mg/l) + NAA (0.2 mg/l); T12 = MS + Kn (2.0 mg/l) + 1AA (0.2 mg/l); T13 = MS + Kn (2.0 mg/l) + 1AA (0.2 mg/l)

apical bud and nodal segment regenerated 4.77 and 3.56 shoots, respectively at the 5th sub-culture (Table 2). A balance between auxin and cytokinin is most often required for the formation of adventitious shoots and roots. Optimum dose of BAP enhances axillary branches and multiple shoot formation Sikdar *et al.* (9).

The well-developed shoots were transferred to the rooting medium. T_8 treatment (MS + GA_3 1 mg/l + 100 mg/l charcoal) proved best for rooting parameters. Minimum time (10.81 days) taken for rooting was for apical bud and 11.49 days for nodal segment. The number of roots on an average per shoot ranged from 0 to 4. The apical bud derived shoots had 4.53, while nodal segment was with 4.06, which were at par (Table 3). Both the explants recorded the longer roots (10.21 and 10.18 cm) in T_8 for apical bud and nodal segment. Apical meristem recorded maximum rooting (92.29%), while in control there was no rooting (Fig. 3). The application of GA_3 in rooting medium not only led to elongation of unidentified micro-shoots but also helped in rooting. Addition of activated charcoal in the rooting medium improved overall rooting capacity. It also proved excellent, in terms of earliness in root induction, root number, and length as well as response to rooting of *in vitro* regenerated micro-shoots (Gantait *et al*., 4). Auxin IBA was better than NAA or IAA along with activated charcoal.

The rooted plants were transferred to glass jars filled with sterile soil, sand, perlite and vermiculite

Table 2. Effect of growth regulators on days required for shoot proliferation and shoot multiplication in gynoecious bitter gourd line.

Treatment	Days required for shoot proliferation		At 5th subculture	
	Apical bud	Nodal segment	Apical bud	Nodal segment
T1	22.93 ± 0.22a	23.32 ± 1.08a	0.71 ± 0.22g	0.34 ± 0.17 g
T2	20.68 ± 0.56b	22.06 ± 0.24a	1.23 ± 0.08f	1.09 ± 0.10 f
T3	19.25 ± 0.13c	19.39 ± 0.12b	1.63 ± 0.12e	1.42 ± 0.10 e
T4	19.51 ± 0.14c	19.77 ± 0.03b	1.73 ± 0.05e	1.57 ± 0.07 de
T5	19.56 ± 0.13c	19.54 ± 0.11b	1.81 ± 0.05e	1.73 ± 0.04 d
T6	17.29 ± 0.04d	17.88 ± 0.38c	2.24 ± 0.03d	2.12 ± 0.06 c
T7	15.7 ± 0.39 e	16.61 ± 0.45cd	2.42 ± 0.02d	2.27 ± 0.04 c
T8	15.19 ± 0.05e	17.54 ± 0.24cd	2.90 ± 0.04c	2.69 ± 0.05 b
T9	15.58 ± 0.18e	16.22 ± 0.60d	3.25 ± 0.02b	2.93 ± 0.03 b
T10	11.9 ± 0.27 f	14.62 ± 0.64e	4.77 ± 0.02a	3.56 ± 0.21 a
LSD (*P*≤ 0.05)	0.78	1.47	4.77 ± 0.02a	0.31
CV(%)	2.59	4.59	0.11	9.4

T1 = MS + No hormone (control); T2 = MS + BAP (1.0 mg/l); T3 = MS + BAP (1.5 mg/l); T4 = MS + BAP (1.0 mg/l) + NAA (0.1 mg/l); T5 = MS + BAP (1.5 mg/l) + NAA (0.1 mg/l); T6 = MS + BAP (1.0 mg/l) + IBA (0.1 mg/l); T7 = MS + BAP (1.0 mg/l) + IBA (0.1 mg/l) + GA_3 (1.0 mg/l); T8 = MS + BAP (1.5 mg/l) + IBA (0.1 mg/l) + GA_3 (1.0 mg/l); T9 = MS + BAP (1.0 mg/l) + 1BA (0.1 mg/l) + GA_3 (0.2 mg/l); T10 = MS + BAP (1.0 mg/l) + IBA (0.1 mg/l) + GA_3 (0.3 mg/l).

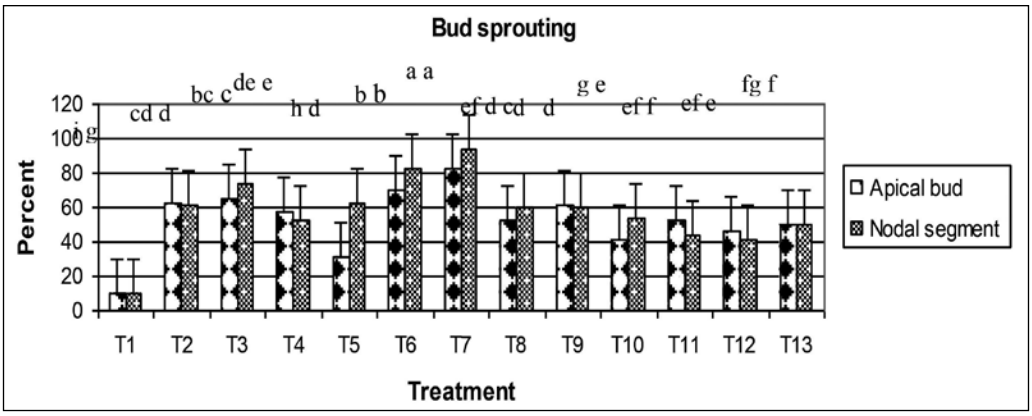

Fig. 2. Effect of different of growth regulators for *in vitro* bud sprouting in gynoecious bitter gourd. Each point represents mean ± SE. Means followed by the different letters are significantly different ($P \leq 0.05$)

Table 3. Standardization of growth regulators for rooting of bitter gourd microshoots.

Treatment	Days to root initiation		No. of roots/ shoot		Root length (cm)	
	Apical bud	Nodal segment	Apical bud	Nodal segment	Apical bud	Nodal segment
T1	24.44 ± 0.05 a	24.77 ± 0.26 a	0 ± 0 f	0 ± 0 f	0 ± 0 e	0 ±0 e
T2	15.54 ± 0.49 c	15.52 ± 0.53 c	1.45 ± 0.09 c	1.44 ± 0.05 c	0.75 ± 0.03 c	0.73 ± 0.05 c
T3	15.05 ± 0.41 c	15.04 ± 0.26 c	2.03 ± 0.21 b	1.95 ± 0.03 b	1.27 ± 0.02 b	1.21 ± 0.02 b
T4	17.74 ± 0.17 b	17.81 ± 0.36 b	0.4 ± 0.52ef	0.4 ± 0.02 e	0.44 ± 0.02 d	0.43 ± 0.02 d
T5	17.21 ± 0.02 b	17.21 ± 0.70 b	0.67 ± 0.03 de	0.67 ± 0.64 e	0.7 ± 0.02 c	0.68 ± 0.03 c
T6	17.07 ± 0.31 b	17.0 ± 0.3b	1.12 ± 0.04 cd	1.13 ± 0.64 d	0.6 ± 0.01 cd	0.57 ± 0.03 cd
T7	17.61 ± 0.50 b	17.3 ± 0.68 b	1.28 ± 0.03 c	1.3 ± 0.04 cd	0.78 ± 0.02 c	0.78 ± 0.02 c
T8	10.81 ± 0.29 d	11.4 ± 0.60d	4.53 ± 0.34 a	4.06 ± 0.27 a	10.21± 0.25 a	10.18 ± 0.22 a
LSD (P<0.5)	1.01	1.328	0.45	0.32	0.27	0.25
CV(%)	3.41	4.467	18.21	13.43	8.57	7.92

T1 = Control; T2 = MS + IBA (0.1 mg/l); T3 = MS + IBA (0.5 mg/l); T4 = MS + IAA (0.1 mg/l); T5 = MS + IAA (0.5 mg/l); T6 = MS + NAA (0.5 mg/l); T7 = MS + NAA (0.1 mg/l); T8 = MS + GA_3 (1 mg/l + 100 mg/l charcoal)

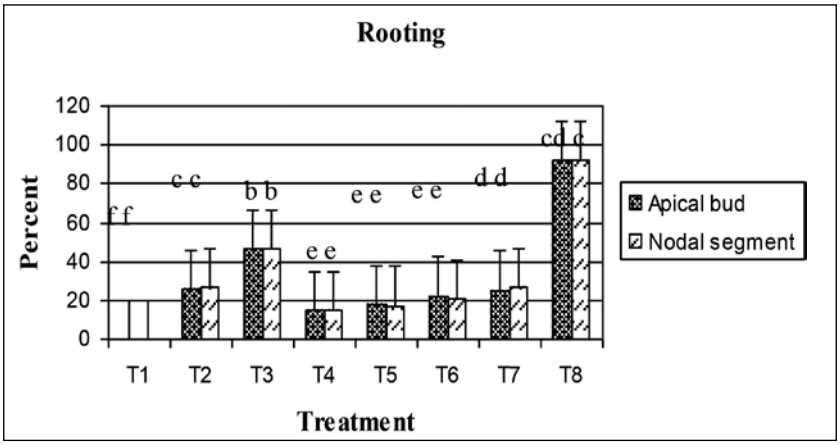

Fig. 3. Effect of different of growth regulators on rooting (mean ± SE). Means followed by the different letters are significantly different ($P \leq 0.05$).

T1 = Control; T2 = MS + IBA (0.1 mg/l); T3 = MS + IBA (0.5 mg/l); T4 = MS + IAA (0.1 mg/l); T5 = MS + IAA (0.5 mg/l); T6 = MS + NAA (0.5 mg/l); T7 = MS + NAA (0.1 mg/l); T8 - MS + GA_3 (1 mg/l +100 mg/l charcoal)

Table 4. Standardization for acclimatization technique for *in vitro* raised bitter gourd plantlets.

	Survival (%)		No. of leaves/ plantlet		Plant height (cm)	
	Apical bud	Nodal segment	Apical bud	Nodal segment	Apical bud	Nodal segment
T1	81.25 ± 0.34	80.94 ± 0.79	7.68 ± 0.22	7.6 ± 0.20	14.82 ± 0.34	14.72 ± 0.19
T2	69.04 ± 0.17	68.02 ± 1.41	5.47 ± 0.44	5.45 ± 0.32	11.83 ± 0.23	11.55 ± 0.36
LSD($P \leq_0$)	1.10	4.62	1.43	1.09	1.18	1.17
CV%	0.63	2.65	9.36	7.18	3.81	3.85

Mean ± SE, T1 = Glass jar with PP cap (Soil + perlite); T2 = Plastic pots with polythene cover (soil + perlite)

mixture (3:1:1:1) after thorough washing with autoclaved water. Two types of acclimatization strategies were used for comparison. The glass jar with PP cap was found to be comparatively better than plastic pot for hardening in respect to survival percentage, number of leaves per plantlet and plant height. The *ex vitro* survival (81.25%) was maximum in glass jar with PP cap in shoot tip derived plantlets, which was at par with the nodal segment (80.94%). The number of leaves per plantlet and plant height was highest in T_1 treatment (Table 4). The jar and the substrate used for hardening the plants during acclimatization phase are the key factors that can be manipulated to optimize plant growth, since the substrate may define the patterns of drainage and development of new roots. The high success in glass jar might be due to high moisture retention and also due to constant maintenance of relative humidity level compared to other strategies. While hardening in plastic pots with polythene covering, the microbial infection was observed as leaves were touching the polythene cover. Superiority of glass jars over pots has been proved earlier also in many crops (Parathasarathy and Nagaraju, 8). After two to three weeks, hardened plants were transferred to earthen pots and kept in shade-net-house.

Efficient protocol has been developed for the multiplication of gynoecious line of bitter gourd through micropropagation, which can pave the way for easy and economical hybrid seed production.

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