

Induction of morphogenic callus and multiple shoot regeneration in *Momordica cymbalaria* Fenzl.

T D Nikam^{1*}, S G Ghane¹, J N Nehul² and R B Barmukh³

¹Department of Botany, University of Pune, Pune 411 007, India

²Dada Patil Rajale College, Adinathnagar (Dist Ahmednagar) 414 505, India

³Modern College of Arts, Science and Commerce, Shivajinagar, Pune 411 005, India

Received 9 July 2008; revised 2 February 2009; accepted 5 April 2009

An *in vitro* propagation protocol for a wild vegetable and an ethnomedicinal plant, *Momordica cymbalaria* Fenzl., has been developed. The influences of 0.0-5.0 μM 6-BA, 0.0-20.0 μM Kn alone and in combination with 2.5- 5.0 μM IAA, 2.5-5.0 μM NAA and 2.5-5.0 μM 2,4-D on *in vitro* multiple shoot production from node, internode and leaf explants was studied. The maximum number of indirect (callus interspersed) regeneration of multiple shoots (9.0 ± 0.5 shoots per explant) was achieved from leaf explants on MS medium enriched with 2.5 μM BA alone. Further, large-scale shoot formation (35 ± 3.4 shoots per culture) was achieved by repeated subculturing of leaf-callus on shoot regeneration medium (MS+2.5 μM BA). The capacity of large-scale shoot regeneration remained constant in the callus over a period of 2 years. The best root induction (100%) and survival (88%) was achieved on hormone free half strength MS medium. Addition of IAA or NAA in rooting medium induced callus formation in the shoots. Field established plants showed uniform growth and were morphologically identical to the parental stock.

Keywords: Callus, ethnomedicinal, *in vitro* propagation, *Momordica cymbalaria*, vegetable

Introduction

Momordica cymbalaria Fenzl. (Cucurbitaceae) is a perennial herbaceous climber, either allowed to trail on the ground or to climb on supports with the aid of tendrils¹. It is widely distributed in the tropical regions of Africa and India. Its fruits are used as a vegetable. The minimum requirement of vegetable consumption is 280 g/d². However, in many developing countries, vegetable consumption is far below the normal requirement, which may be attributed to the low availability or unaffordable cost. The feasible alternative is to explore additional sources of food and to minimize the load on production of conventional food plants. The fruits of *M. cymbalaria*, consumed as vegetable, are rich in crude fibre, calcium, potassium, sodium and vitamin C as compared to bitter melon¹. The plant is disease resistant and its fruits have medicinal value³. The tuber is used as an abortifacient⁴. The plant is used as hypoglycemic, antidiabetic and hypolipidemic⁵. Its crude fibre decreases the absorption of cholesterol from the gut. It delays the digestion and conversion of

starch into sugars. Such attributes would be desirable for diabetic patients⁶.

The major constraints in growing *M. cymbalaria* are the non-availability of standardized propagation method and low yield. A limited number of perennial tubers survive in soil and produce single plant in the next season. The population is reduced by consumption of tubers by animals and drying or deterioration of tubers in drought and water logging conditions, respectively. The fruit has few seeds that remain dormant and low germination rate further curtails the propagation through seeds.

In vitro propagation technique is an important method to recover unique variants induced either spontaneously or by mutagenesis, protoplast fusion or DNA uptake. The regenerated variant plant(s) could be used to complement existing plant breeding programmes. In addition, *in vitro* propagation technique could be a valuable alternative to conventional propagation methods for mass multiplication as well as gene conservation. To achieve this goal, the first important step is to establish a reliable tissue culture system for plant regeneration. To date, there has been no report on tissue culture system of *M. cymbalaria*. In this study, we describe an

*Author for correspondence:

Tel: 91-20-25601439; Fax: 91-20-25690498

E-mail: tdnikam@unipune.ernet.in

efficient method for *in vitro* propagation and mass multiplication of *M. cymbalaria*.

Materials and Methods

Source of Explants and Sterilization

The tuber derived seedlings of *Momordica cymbalaria* were collected from agricultural fields of Barshi Taluka, Solapur district, Maharashtra State of India and maintained in the Botanic Garden, Department of Botany, University of Pune. The seedlings were used as plant material for initiating the *in vitro* cultures. The nodes, internodes and leaf explants were washed 5 times with sterile distilled water. The explants were surface sterilized with 0.1% (w/v) Sovistin (a commercial broad spectrum fungicide) for 4 min followed by 0.1% HgCl₂ for 7 min. The explants were then washed 4 times with sterile distilled water to remove traces of HgCl₂.

Callus Induction, Culture Medium and Conditions

The node (10 mm), internode (10 mm) and leaf (10 mm²) explants were cultured for callus induction and shoot regeneration in 60×120 mm glass bottles and 25×150 mm borosilicate test tubes containing Murashige and Skoog medium (MS)⁷ supplemented with 30 g L⁻¹ sucrose, 7 g L⁻¹ agar and various concentrations of cytokinins such as 6 benzyl adenine (BA) and kinetin (Kn) and auxins such as indole-3-acetic acid (IAA), α -naphthalene acetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2, 4-D), either alone or in combinations as described in Tables 1 and 2. Control cultures initiated on MS medium without growth regulators. The pH was adjusted to 5.8 prior to the addition of agar. Media were autoclaved at 1.06 kg cm⁻² (121°C) for 15 min. Cultures were incubated at 25±2°C and relative humidity of 60±10%. An 8 h photoperiod (16 h dark) with light intensity of 20-30 $\mu\text{molm}^{-2}\text{s}^{-1}$ was provided by cool day light fluorescent tubes.

The calli were isolated from the explants and transferred on fresh medium of the same composition (Table 2). The explants responding for callus and shoot regeneration, number of shoots produced per explant and length of the shoots was measured after 4 wks of initiation of cultures. The calli were subcultured on fresh medium at an interval of 4 wks under similar conditions.

Rooting and Acclimatization

An individual shoot regenerated either from explants or from calli was separated and subcultured on half or

full strength MS medium without auxin or enriched with different levels of IAA or NAA (0.25-5.0 μM). After 4 wks of root initiation, plantlets were taken out of the culture vessels and rinsed with tap water to remove the nutrient medium. Plantlets transplanted in earthen pots containing garden soil were kept inside the glass chamber for 1 wk (light 60 $\mu\text{mol m}^{-2}\text{s}^{-1}$, temperature 25±2°C and humidity ~70%). During the first wk, the lid of glass chamber was removed daily for 1 h in the morning and 1 h in the evening and this exposure time was gradually increased during the second wk. Little water was added near the base of the plantlets every day in the morning. After 2 wks of hardening, the potted plantlets were exposed to field conditions for further growth.

Callus Maintenance and Large-scale Shoot Regeneration

Large-scale propagation was achieved by repeated subculturing of the callus obtained from nodes, internodes and leaf explants on fresh parental shoot regeneration medium for five subcultures at an interval of 4 wks. The regenerating calli were subcultured on fresh medium at an interval of 4 wks and maintained on the parental medium over a period of two years.

Statistical Analysis

The experiments were conducted using the completely randomized design. All experiments were repeated at least thrice with minimum 21 explants. Data were subjected to statistical analysis for analysis of variance (ANOVA) and means of different experiments were compared using Duncan's Multiple Range Test at the 5% level⁸.

Results and Discussion

Effect of BA and Kn on Callus Induction and Shoot Regeneration

Leaf, internode and nodal explants on MS basal medium did not exhibit morphogenesis but showed little swelling at the cut ends. The per cent frequency of explants responding for shoot regeneration, mean number of shoots per explant and length of shoot and callus formation in explants were affected by the inclusion of different levels and combinations of BA and Kn (Tables 1 & 2). Low levels of BA (2.5 μM), when present alone, induced little callus and callus interspersed multiple shoot regeneration from nodal, internodal and leaf explants. Incorporation of higher levels of BA (5.0 μM) promoted induction and proliferation of callus from all over the surface of explants. The callus was initially soft and completely

Table 1—Influence of BA and Kn on *in vitro* callus formation and shoot regeneration in *M. cymbalaria* after four weeks of cultures

Cytokinin (μM)	Nodal explants		Internodal explants		Leaf explants	
	% of explant responding for shoot regeneration	Mean no. of shoots per explant \pm SE	% of explant responding for shoot regeneration	Mean no. of shoots per explant \pm SE	% of explant responding for shoot regeneration	Mean no. of shoots per explant \pm SE
BA						
0.0	-	-	-	-	-	-
0.62	-	-	-	-	-	-
1.25	69*	2.7 \pm 0.3 ^c	29*	1.2 \pm 0.7 ^d	69*	5.2 \pm 0.8 ^d
2.5	88*	4.0 \pm 0.8 ^a	49*	2.0 \pm 0.9 ^a	93**	9.0 \pm 0.5 ^a
3.75	74*	2.6 \pm 0.1 ^c	33*	1.9 \pm 0.2 ^b	84**	7.2 \pm 0.5 ^b
5.0	41**	2.1 \pm 0.6 ^d	30**	1.5 \pm 0.4 ^c	59*	6.3 \pm 0.4 ^c
Kn						
2.5	55	1.0 \pm 0.4 ^f	-	-	-	-
5.0	63*	2.0 \pm 0.3 ^d	26*	1.4 \pm 0.5 ^c	45*	3.0 \pm 0.2 ^f
10.0	72**	4.0 \pm 0.5 ^a	34**	1.8 \pm 0.2 ^b	59*	3.9 \pm 0.3 ^e
15.0	46*	2.9 \pm 0.6 ^b	32**	1.5 \pm 0.2 ^c	52**	2.7 \pm 0.1 ^g
20.0	24*	1.2 \pm 0.8 ^e	-	-	28*	2.1 \pm 0.2 ^h

* = Slight callus formation at cut end of explants ** = Extensive callus formation from explants.

- = no response of explant for callus formation and shoot regeneration. SE = Standard error

Values are mean of at least three independent experiments.

Means followed by the same letters within columns are not significantly different at the 5% level.

Table 2—Influence of auxins together with cytokinins on callus formation and shoot regeneration in *M. cymbalaria* after 4 wks of culture

Cytokinins (μM)	Auxins (μM)			Nodal explants		Internodal explants		Leaf explant		
	BA	IAA	NAA	2,4-D	% of explant responding for shoot regeneration	Mean no. of shoots per explant \pm SE	% of explant responding for shoot regeneration	Mean no. of shoots per explant \pm SE	% of explant responding for shoot regeneration	Mean no. of shoots per explant \pm SE
2.5	2.5	-	-	-	43*	2.6 \pm 0.9 ^d	48*	1.1 \pm 0.4 ^{de}	68*	4.7 \pm 0.7 ^a
2.5	5.0	-	-	-	46**	2.4 \pm 0.5 ^{ef}	39**	1.0 \pm 0.3 ^{ef}	86**	4.3 \pm 0.4 ^b
2.5	-	2.5	-	-	41**	2.9 \pm 0.4 ^c	45**	1.0 \pm 0.6 ^{ef}	84**	3.9 \pm 0.3 ^c
2.5	-	5.0	-	-	39**	2.4 \pm 0.6 ^{ef}	37**	0.9 \pm 0.2 ^g	81**	3.6 \pm 0.7 ^{ef}
2.5	-	-	2.5	-	48**	2.5 \pm 0.7 ^{de}	41**	0.9 \pm 0.4 ^{fg}	78**	3.7 \pm 0.6 ^{de}
2.5	-	-	5.0	-	44**	2.0 \pm 0.3 ^g	36**	0.3 \pm 0.3 ^{jk}	72**	3.2 \pm 0.4 ^h
Kn										
10.0	2.5	-	-	-	54*	1.8 \pm 0.4 ^h	24*	0.8 \pm 0.4 ^{gh}	54*	3.9 \pm 0.1 ^c
10.0	5.0	-	-	-	49**	1.5 \pm 0.2 ⁱ	18**	0.7 \pm 0.3 ^{hi}	49**	3.4 \pm 0.3 ^g
10.0	-	2.5	-	-	52**	1.7 \pm 0.7 ^h	20**	0.8 \pm 0.2 ^{gh}	43**	4.2 \pm 0.5 ^b
10.0	-	5.0	-	-	43**	1.4 \pm 0.3 ^{ij}	19**	0.6 \pm 0.3 ⁱ	41**	3.8 \pm 0.5 ^{cd}
10.0	-	-	2.5	-	49**	1.3 \pm 0.6 ^j	17**	0.4 \pm 0.2 ^j	44**	2.7 \pm 0.4 ⁱ
10.0	-	-	5.0	-	41**	1.1 \pm 0.4 ^k	14**	0.2 \pm 0.3 ^k	40**	2.5 \pm 0.2 ^j
BA	Kn									
2.5	0.62	-	-	-	44*	2.3 \pm 0.4 ^f	37*	1.2 \pm 0.4 ^{cd}	57*	3.5 \pm 0.5 ^{fg}
2.5	1.25	-	-	-	49**	2.5 \pm 0.5 ^{de}	43*	1.8 \pm 0.6 ^a	64*	3.7 \pm 0.6 ^{de}
2.5	2.5	-	-	-	52**	2.6 \pm 0.2 ^d	48**	1.3 \pm 0.2 ^{bc}	69**	3.8 \pm 0.4 ^{cd}
0.62	10.0	-	-	-	49*	3.7 \pm 0.1 ^b	41*	0.9 \pm 0.6 ^{fg}	52*	3.4 \pm 0.5 ^{ef}
1.25	10.0	-	-	-	55**	3.8 \pm 0.9 ^b	47*	1.4 \pm 0.2 ^b	56**	3.6 \pm 0.4 ^{ef}
2.5	10.0	-	-	-	58**	4.1 \pm 0.6 ^a	48**	1.1 \pm 0.1 ^{de}	49**	3.9 \pm 0.6 ^c

* = Slight callus formation at cut end ** = Extensive callus formation. SE = Standard error

Values are mean of at least three independent experiments.

Means followed by the same letters within columns are not significantly different at the 5% level.

off-white in colour but afterwards some areas on the surface of the callus appeared greenish in colour which resulted in organization of shoots within two wks of culture (Table 1, Figs 1 a & b). The initial swelling and callus formation in the explants on medium containing either BA or Kn and lacking auxins might be possible due to higher endogenous levels of auxins in the explant tissue.

Among the different levels of BA, highest per cent frequency of explants responding for shoot regeneration, mean number of callus interspersed multiple shoots per explant was observed in leaf (9 shoots/explant), internodal (2 shoots/explant) and nodal (4 shoots/explant) explants on MS medium

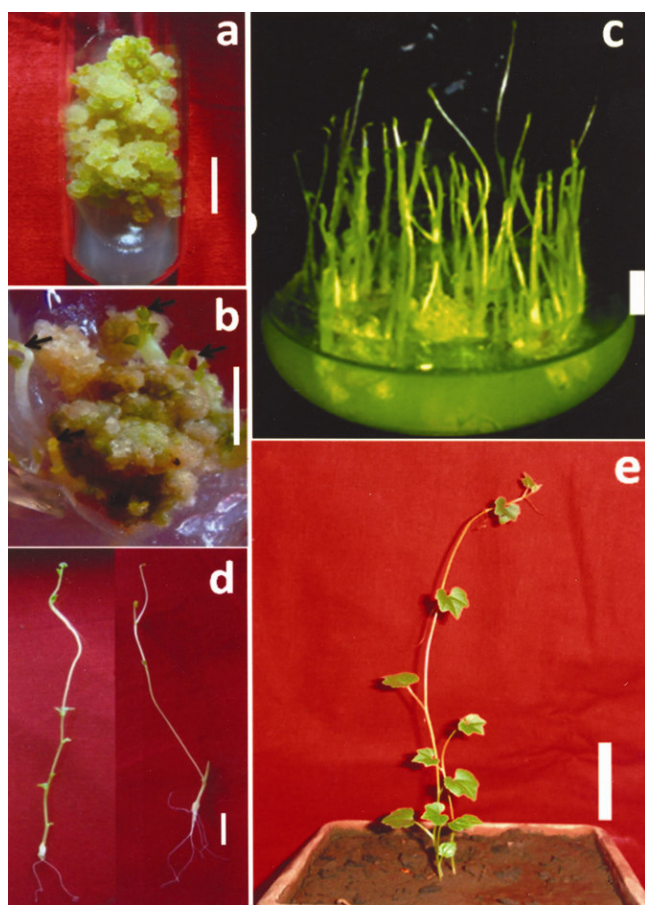


Fig. 1—Micropropagation of *Momordica cymbalaria*: a. Leaf callus on MS medium supplemented with 2.5 μM BA after 2 wks of culture (bar = 1 cm); b. Induction of shoots from leaf callus on MS medium fortified with 2.5 μM BA after 3 wk of culture (bar = 1 cm); c. Multiple shoot induction from leaf callus on MS medium amended with 2.5 μM BA after 4 wk of culture (bar = 1 cm); d. *In vitro* rooting in isolated shoot on half strength MS medium lacking growth regulators (bar = 1 cm); & e. Acclimatized plant to natural conditions (bar = 10 cm).

enriched with 2.5 μM BA. The elongation of shoots was rapid on the same medium and attained height of 5.8 ± 0.4 cm. Inclusion of very high levels of BA (10.0 μM) significantly hampered callus formation and subsequently regeneration of multiple shoots. Inclusion of various levels of Kn in the medium also resulted in callus interspersed shoot formation from the explants. Among the various levels of Kn used with different explants, maximum number of shoots per explant was formed from nodal explants on MS medium supplemented with 10.0 μM Kn (Table 1) but the number of shoots per explant was comparatively less to that of BA (Table 1). BA was reported to be highly effective in multiple shoot induction in cucurbits⁹⁻¹¹. Incorporation of very high levels of Kn (15-20 μM) prevented both callus formation as well as induction of shoots. Combination of BA and Kn slightly improved the callus formation but did not result in improvement of number of shoots regenerating per explant. On the basis of above observations, among various levels of BA (0.0-5.0 μM) and Kn (0.0-20.0 μM) tested, 2.5 μM BA alone was superior for maximum number of callus interspersed shoot formation in all the explants (internode, node and leaf).

Effect of NAA, IAA and 2, 4-D together with BA and Kn on Callus Induction and Shoot Regeneration

The cumulative work on plant tissue culture revealed that the ability of shoot regeneration and callus formation depends on donor tissue and influenced by type of growth regulator and their concentration in the nutrient medium^{12,13}. Similarly, in *M. cymbalaria*, the formation of callus and frequency of shoot bud formation varied depending upon the type of explants, type of combination and concentration of auxins and cytokinins (Table 2). Addition of low levels of auxins (IAA, NAA and 2, 4-D) in combination with BA (2.5 μM) or Kn (10.0 μM) induced callus formation from the cut ends of explants. In the second and third wk of culture, entire surface of explants was covered with callus which generated shoots in the subsequent two wks. The size of callus increased with time (Table 2). All the combinations initially resulted in white coloured soft callus, which subsequently became partly greenish and resulted in shoot regeneration. The best shoot bud induction was obtained on media supplemented with 2.5 μM BA alone. However, the synergistic effect of NAA in conjunction with BA in

the formation of multiple shoots has been reported for other members of cucurbitaceae such as *Citrullus lanatus*¹⁴ and *Momordica charantia*¹⁵.

A combination of 2,4-D and BA or Kn facilitated proliferation of callus but produced less number of shoot buds. Inclusion of higher concentration of IAA, NAA and 2, 4-D (5.0 μM) together with BA (2.5 μM) or Kn (10.0 μM) resulted in extensive proliferation of callus. However, the included auxin counteracted the effect of BA or Kn for shoot bud formation (Table 2).

Effect of Age of Callus and Large-scale Shoot Regeneration

After removal of regenerated shoots, the mother calli from nodal and leaf explants, when subcultured on parental medium (MS+ 2.5 μM BA), resulted in the formation of increased number of shoots per culture for the period of five subcultures. Though the callus proliferation was same, the leaf callus was highly regenerative compared to nodal callus. A maximum of 14 shoots per culture in nodal callus and 35 shoots per culture in leaf callus was recorded (Table 3, Fig. 1c). On repeated subculturing to parent media, the tendency of proliferation of callus accompanied with induction of shoots did not decrease over a period of 2 years (Table 3). This is an important achievement not reported before, and is required for commercial production of this important wild vegetable and medicinal species.

Rooting and Acclimatization

The individual shoots were separated from the regenerative callus mass and transferred to the rooting medium. Basal portion of the shoots in contact with

the full strength growth-regulator-free MS medium showed swelling, followed by the formation of fibrous roots. Addition of 0.25 μM IAA or 0.25 μM NAA to half strength and full strength MS medium induced callus interspersed root formation. The degree of callus formation has been given in Table 4. Such shoots did not survive on transfer to field conditions. However, in *Cucumis sativus* the rooting of microshoots was induced on half strength MS medium containing 1.0 μM NAA¹¹. Likewise in *M. charantia* efficient rooting was achieved on half strength MS medium fortified with 2.5 μM NAA¹⁵ and in *C. lanatus*, on MS medium containing 0.5 μM NAA¹⁴.

In the present investigation, all the shoots rooted well within 2 wks on growth-regulator-free half-strength MS medium (Table 4, Fig. 1d). These well

Table 3—Effect of age of callus on shoot regeneration in *M. cymbalaria*

Age of callus (in wks)	Number of shoots per culture Mean \pm SE	
	Nodal explant derived callus	Leaf explant derived callus
4	6.0 \pm 1.2 ^d	9.0 \pm 1.6 ^e
8	9.0 \pm 1.3 ^c	12.0 \pm 2.2 ^d
12	11.0 \pm 1.5 ^b	18.0 \pm 3.1 ^c
16	14.0 \pm 2.2 ^a	25.0 \pm 3.8 ^b
20	14.0 \pm 1.9 ^a	35.0 \pm 3.4 ^a
72	14.0 \pm 2.1 ^a	35.0 \pm 3.7 ^a
144	14.0 \pm 2.1 ^a	35.0 \pm 3.4 ^a
216	14.0 \pm 2.1 ^a	35.0 \pm 3.3 ^a
288	14.0 \pm 2.1 ^a	35.0 \pm 3.4 ^a

Incubation period 4 wks. Values are mean of 21 replicates. SE = Standard error Means followed by the same letters within columns are not significantly different at the 5% level.

Table 4—Effect of IAA and NAA on rooting and survival percentage in in vitro regenerated shoots in half strength MS medium

Auxins (μM)	Rooted shoots (%)	Mean no. of roots/shoot Mean \pm SE	Mean root length(cm) Mean \pm SE	Degree of swelling and callusing	Survival %
IAA					
00	100	11 \pm 1.8 ^{bc}	1.5 \pm 1.3 ^a	*	88
0.25	100	10 \pm 1.5 ^c	1.3 \pm 1.3 ^b	**	13
1.0	100	08 \pm 1.7 ^d	1.1 \pm 1.2 ^c	***	00
5.0	100	07 \pm 1.6 ^d	0.8 \pm 1.0 ^d	***	00
NAA					
0.25	100	12 \pm 1.5 ^{ab}	1.4 \pm 1.4 ^{ab}	**	00
1.0	100	13 \pm 1.3 ^a	1.3 \pm 1.2 ^b	***	00
5.0	100	13 \pm 1.7 ^a	1.1 \pm 1.5 ^c	***	00

Incubation period 4 wks. Values are mean of 21 replicates. SE = Standard error Means followed by the same letters within columns are not significantly different at the 5% level.

* = slight swelling at base of the shoot followed by rooting.

** = swelling and callusing at base of the shoot followed by rooting.

*** = Swelling and extensive callusing at base of the shoot followed by rooting

rooted shoots, on transfer to soil and maintained in ventilated glass chamber for 1 wk, survived under natural conditions with 88% survival rate. The plants grew well and did not show any distinct morphological abnormality in vegetative and reproductive phase of growth (Fig. 1e). Each plant produced 11-14 fruits which was equivalent to the yield of naturally grown wild plants.

Acknowledgement

The financial assistance provided under UGC-SAP-DRS-II, ASIST and DST-FIST programme of Government of India to Department of Botany, University of Pune is gratefully acknowledged.

References

- 1 Parvathi S & Kumar V J F, Studies on chemical composition and utilization of wild edible vegetable athalakkai (*Momordica tuberosa*), *Plants Foods Hum Nutr*, 57 (2002) 215-222.
- 2 Kalloo G, Vegetable research in India, *Indian J Agric Sci*, 68(8) (1998) 15-26.
- 3 Choudhury B, *Vegetables* (National Book Trust, New Delhi) 1967, 152-154.
- 4 Nadkarni K M, in *Indian materia medica*, vol I, edited by A K Nadkarni, (1994) 775.
- 5 Rao B K, Kesavulu M M & Apparao C, Antihyperglycemic activity of *Momordica cymbalaria* in alloxan diabetic rats, *J Ethnopharmacol*, 78 (2001) 67-71.
- 6 Gopalan C, Rama Sastri B V & Balasubramanian S C, in *Nutritive value of Indian foods*, 2nd edn (National Institute of Nutrition, ICMR, Hyderabad) 1993, 132-133.
- 7 Murashige T & Skoog F, A revised medium for rapid growth and bioassays with tobacco cultures, *Physiol Plant*, 15 (1962) 473-497.
- 8 Duncan D B, Multiple range and multiple F tests, *Biometrics*, 11 (1955) 1-42.
- 9 Srivastava D R, Andrianov V M & Piruzian E S, Tissue culture and plant regeneration of watermelon (*Citrullus vulgaris* Schrad. cv. Melitopolski), *Plant Cell Rep*, 8 (1989) 300-302.
- 10 Compton M E & Gray D J, Shoot organogenesis and plant regeneration from cotyledons of diploid, triploid and tetraploid watermelon, *J Am Soc Hort Sci*, 118 (1993) 151-157.
- 11 Ahmad N & Anis M, *In vitro* mass propagation of *Cucumis sativas* L. from nodal segments, *Turk J Bot*, 29 (2005) 237-240.
- 12 Bhojwani S S & Razdan M K, *Plant tissue culture: Theory and practice*, revised edn (Elsevier, The Netherlands) 1996, 10-90.
- 13 Thorpe T A, History of plant tissue culture, *Mol Biotechnol*, 37 (2007) 169-180.
- 14 Sultana R S, Bari M A, Rahman M M, Siddique N A & Khatun N, *In vitro* rapid regeneration from leaf explants of watermelon (*Citrullus lanatus* Thumb.), *Biotechnology*, 3 (2004) 131-135.
- 15 Sultana R S & Bari M A, *In vitro* propagation of karella (*Momordica charantia* Linn.) from nodal segment and shoot tip, *J Biol Sci*, 3 (2003) 1134-1139.