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In vitro propagation of *Citrus indica* Tanaka—An endangered progenitor species

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A method for *in vitro* propagation of *Citrus indica* Tanaka by shoot organogenesis from leaf-derived callus was developed. Regenerative calli were induced on MS medium supplemented with 0.01 mg L^{-1} TDZ and 0.1 mg L^{-1} NAA. Shoots were regenerated on WPM medium supplemented with 0.5 mg L^{-1} BAP, 0.25 mg L^{-1} TDZ and 0.25 mg L^{-1} NAA. Regenerated shoots were rooted on MS medium supplemented with 1.0 mg L^{-1} NAA. Sixty per cent of the rooted plantlets were acclimatized successfully under *ex situ* conditions.

Keywords: *Citrus indica* endangered, *ex situ* adaptibility, leaf-derived callus, progenitor, shoot organogenesis

Introduction

Citrus indica Tanaka is one of the most primitive species of citrus and is considered to be the progenitor of cultivated citrus[1]. It is endemic to the Tura range of Garo Hills of Meghalaya[2,3]; this area falls within the buffer zone of Nokrek Biosphere Reserve. Villagers, belonging to the Garo tribe, who live around the biosphere reserve, attribute medicinal and religious values to *C. indica*. The fruit is used to treat jaundice and stomach diseases of humans and domestic animals. Powder made from the fruit as well as raw fruits is taken as a cure for small pox. Fruits are placed on dead bodies during the last rites with the belief that it will ward off ghosts of the departed[4].

Absence of organised cultivation and destruction of natural habitat by deforestation and jhum activities have severely depleted the population of *C. indica*. The endemic nature of this species has further contributed to its decline. It is essential to prevent the extinction of *C. indica* for its taxonomic and ethnobotanical importance as well as for the fact that it can be used as rootstock for propagation of commercial citrus species for being hardy and free from pests and diseases in its natural habitat[4]. The buffer zone comprising the natural habitat of *C. indica* has been established as Citrus Gene Sanctuary[1]. However, this *in situ* conservation effort has had limited impact on halting the decline in population. It is, therefore, necessary to establish *ex situ* conservation methods like micropropagation as supplemental measures. The present *in vitro* propagation study on *C. indica* was taken to develop a method for multiplication of this endangered progenitor species.

Materials and Methods

Plant Material

Fruits of *C. indica* were collected during January, 2004 from plants growing around the Tura Peak, which is situated inside the Nokrek Biosphere Reserve, at 1,412 m above MSL. The fruits (Fig. 1a) have orange coloured outer covering and bear one to three monoembryonic seeds (Fig. 1b) in each fruit segment. The seeds take up most of the space in a segment. Due to scarcity of plant material, the work was started with only 20 seeds.

Seeds were surfaced sterilized by soaking in 5% (w/v) calcium hypochorite for 20 min. The surface sterilant suspension containing the seeds was kept agitated during the duration of treatment. The calcium hypochlorite suspension was then decanted, seeds shifted to a fresh container and washed five times with sterilized distilled water to remove all traces of the surface sterilant. Surface sterilized seeds were germinated on 0.7% agar, gelled in distilled water and sterilized by autoclaving at 15 psi for 18 min. Seedlings were considered as a genetic variants and were marked as V (Variant) 1 to V 20.

Leaf explants were prepared 6 d after germination when seedlings were 5-7 cm long. Petiole was removed from a leaf and 1 cm^2 portion of lamina comprising the midrib was excised. Throughout the study, the marking scheme as described above was

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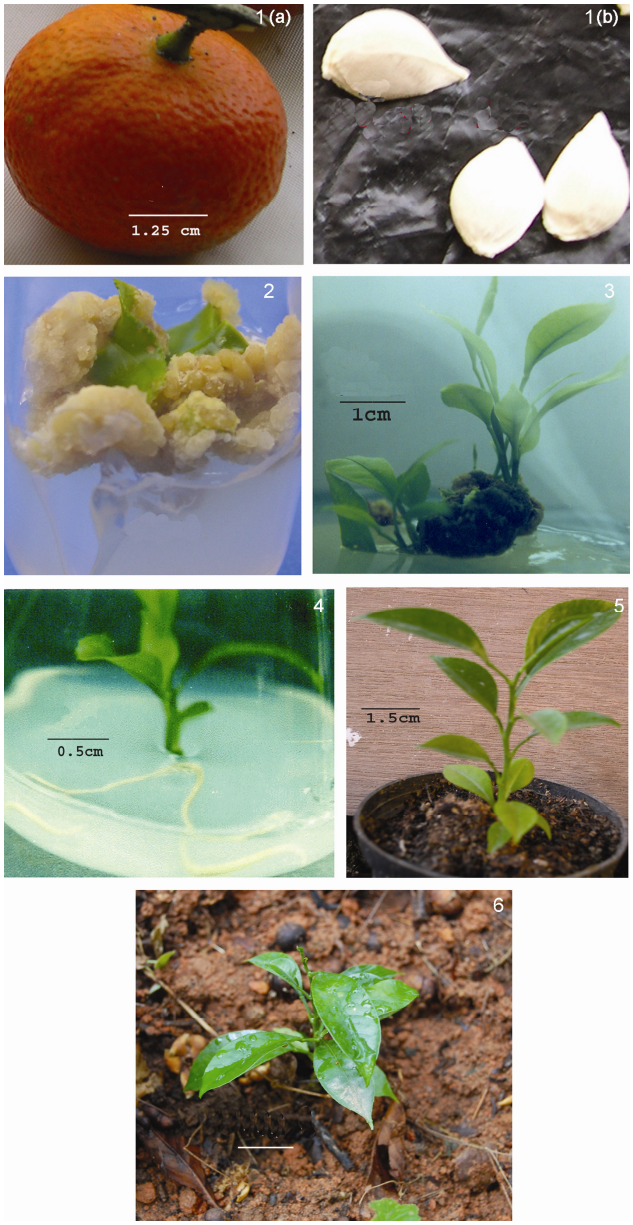

Figs (1-6)—1: a. Ripe Fruits, b: Seeds; 2: Leaf-derived callus; 3: Shoot organogenesis; 4: Root development; 5: Rooted plantlet in pot; and 6: acclimatized plant.

used to maintain the identity of leaf explants, calli and regenerants that originated from each variant.

Media

To induce callusing form leaf explants, the constituents of MS[5] and WPM[6] media were supplemented with NAA (α-napthalene acetic acid), 2,4-D (2,4-dichlorophenoxy acetic acid) and TDZ (thidiazuron). NAA was used at concentrations from 0.2 to 1.25 mg L^{-1}, 2,4-D at concentrations from 0.1 to 1 mg L^{-1} and TDZ at concentrations from 0.005 to 0.1 mg L^{-1}. NAA and TDZ were used alone and in various combinations, while 2,4-D was used alone. The regenerative ability of calli was tested by inoculating them on MS or WPM media supplemented with BAP (6-benzyl amino purine), Kn (kinetin), TDZ and NAA singly and in various combinations. BAP was used at concentrations from 0.1 to 1 mg L^{-1}, Kn at concentrations from 0.05 to 0.5 mg L^{-1}, TDZ at concentrations from 0.0025 to 0.05 mg L^{-1} and NAA at concentrations from 0.25 to 0.5 mg L^{-1}. To induce multiple shoots from axillary buds, shoots regenerated from leaf-derived calli were excised into nodal segments bearing one axillary bud and inoculated on MS- or WPM-based media supplemented with growth regulators mentioned above. To induce rooting from regenerated plantlets, MS- or WPM-based media supplemented with IBA (indole 3-butyric acid) or NAA, both at concentrations from 0.25 to 1 mg L^{-1} were tested. The *p*H of all media was adjusted to 5.8 before autoclaving at 15 psi for 18 min. Media, except those tested for root induction, were gelled with 0.7% agar. Media used for testing root induction were gelled with 0.6% agar.

Rooted plantlets were removed from culture vessels. The roots were washed under tap water to remove adherent media and planted in plastic pots containing river sand, leaf-mould compost and garden soil in the ratio, 3:1:1. The potted plantlets were kept in the shade covered with perforated polythene bags. The plantlets were irrigated with tap water. The irrigation schedule and volume of water was calibrated to keep the pot mixture saturated and prevent flooding. The covers were removed after 10 d.

Physical Parameters

The source of light was cool fluorescent tubes. Germination of seedlings and the experiments on callus induction and rooting were done under 200 lux luminance and 12 h photoperiod. Regeneration experiments were done under 400 lux luminance and 12 h photoperiod. The temperature inside the culture room was maintained at 25±1°C during all stages of the study.

Data Analysis

Experiments were set in the completely randomized block design (CRD). A single leaf explant inoculated on 10 mL of callusing media contained in a culture tube (25 cm × 1.5 cm) formed a replicate; 15 such replications were provided for

every variant. Similarly, 10 replications were provided during the trials on shoot organogenesis from leaf-derived calli, axillary shoot proliferation and on root induction from shootlets. However, media in these cases were dispensed in Erlenmeyer flasks of 150 mL capacity and 30 mL of a medium was poured in each flask.

The parameters studied were percentage of explants that underwent callusing, average number of shoots regenerated from each callus, mean shoot length, average number of shoots developed per axillary bud, percentage of shoot from which roots developed, average number of roots that developed per shoot and mean root length. Data were subjected to Analysis of Variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT), using SPSS 10.0.

Results and Discussion

Sixty per cent, i.e., 12 out of 20, seeds of *C. indica* germinated in 7-10 d. Thus, there were 12 seedlings or variants from the leaves, of which induction of callus (Fig. 2) occurred. The seedlings were used as sources of explants. Since seedlings are the products of sexual reproduction and since cross-pollination exists in citrus, care was taken to maintain separate identities of leaf explants as well as the calli and regenerants derived from each seedling. Calli were induced in 2,4-D or NAA and TDZ-supplemented MS and WPM media. NAA when used alone induced callus growth at concentrations 0.5 to 1.25 mg L^{-1}; however, these calli were hard and did not undergo shoot regeneration. Similarly, 2,4-D when present in media at concentrations 0.125 to 1.0 mg L^{-1} induced calli that were hard and non-regenerative. Friable (Fig. 2) and regenerative calli were obtained from leaf explants of all 12 variants on MS medium supplemented with 0.01 mg L^{-1} TDZ and 0.1 mg L^{-1} NAA (Medium Ci7, Table 1). The same levels of NAA and TDZ supplements in WPM medium (Table 1) induced hard non-regenerative calli. Callus initiation, irrespective of the medium, took 18-21 d. The percentage of leaf explants from which callus initiated on Ci7 medium varied between 80 to 93% among the twelve variants (Table 1). Callus induction percentage was recorded 7 d after initiation and calli were transferred to regeneration media on the same day. In the present study, combination of an auxin, NAA and a cytokinin, TDZ was necessary to induce initiation of regenerative calli. Similar findings were also reported in *C. grandis*[7] and *Suaeda nudiflora*[8]. TDZ has been used earlier in callus induction[9,10]. It is believed that TDZ had a crucial role in initiating regenerative response in the calli. Other investigators have made similar observations[10,11]. WPM medium supplemented with the same level of NAA and TDZ as in Ci7 medium (Table 1) failed to induce regenerative calli. The lower concentrations of ammonical nitrogen, potassium and calcium in the WPM medium as compared to concentrations of these in the MS medium, and also the absence of nitrate nitrogen, are probably the reasons for the failure. High levels of ammonium and nitrate are generally preferred for callus initiation[12].

WPM medium supplemented with 0.5 mg L^{-1} BAP, 0.25 mg L^{-1} Kn and 0.25 mg L^{-1} NAA (CMM1-2b medium, Table 2) induced shoots (Fig. 3) from leaf-derived calli of *C. indica*. Regeneration of shoots was observed from 100% of the calli of variants V4, V7, V9 and V11; regenerative calli of the rest of the variants were in the range of 91-92% of the calli

Table 1—Induction of callus from leaf explants of *C. indica*

Medium				Variants	%	Nature of
No.	Basal composition	PGR (mg L^{-1})			Callus	callus
		TDZ	NAA			
				V1	86.67	
				V2	93.33	
				V3	80.00	
				V4	93.33	
				V5	93. 33	
Ci7	MS	0.01	0.10	V6	86.67	Friable and regenerative
				V7	93.33	
				V8	86.67	
				V9	86.67	
				V10	80.00	
				V11	93.33	
				V12	93.33	
				V1	73.33	
				V2	80.00	
				V3	80.00	
				V4	86.67	
				V5	86.67	
Ci10	WPM	0.01	0.10	V6	80.00	Hard and non-regenerative
				V7	73.33	
				V8	86.67	
				V9	73.33	
				V10	66.67	
				V11	93.33	
				V12	86.67	

All media had 3% sucrose; PGR= Plant growth regulator

induced (Table 2). Initiation of shoot regeneration was noticed 12-16 d after transfer of calli to CMM1-2b medium. Comparative study between the twelve variants done 15 d after initiation on the mean number of shoots that regenerated from a callus revealed differences, some of them significant (Table 2). The highest mean shoot induction frequency, 4.9±0.23 shoots/callus, was observed in calli of V1. This was significantly higher from the mean shoot induction frequency in calli of all other variants, except V2 (4.7±0.37 shoots/callus) and V12 (4.6±0.27 shoots/callus). It was necessary to supplement the WPM basal medium with the cytokinins (BAP, Kn) and the auxin (NAA) to induce shoot regeneration. The combination of BAP, Kn and NAA was used for the regeneration of plants from the callus cultures of *S. nudiflora*[8]. As in the present study, other workers have reported the use of combination of cytokinins and auxins for inducing shoot regeneration where the concentrations of cytokinins were higher than that of auxins[13].

Significant differences also existed among the variants in the average length of shoots measured 20 d after induction (Table 2). The highest mean shoot length was 4.63±0.19 cm recorded from V3 regenerants. This value was significantly different from the mean shoot length recorded from all other regnerents, barring V1 regenerants (4.42±0.22 cm). The frequency of shoot organogenesis and the mean length of shoot did not show similar trends. For example, the regeneration frequency from V4 calli (2.3 ±0.21) was among the lowest but the average shoot length of these shoots (4.11±0.19) was among highest (Table 2). On the other hand, regeneration frequency from V2 calli (4.7 ±0.37) was insignificantly different from the highest value (4.9 ±0.23 recorded from V1 calli) but the mean shoot length of these shoots was 2.92 ±0.15, comparable to the lowest mean value (2.78 ±0.13) recorded from V9 shoots.

Proliferation of axillary shoot was achieved in three different media (Table 3), but the best results were recorded on the same medium (CMM1-2b) in which shoot organogenesis was also induced, i.e., WPM basal medium supplemented with 0.5 mg L^{-1} BAP, 0.25 mg L^{-1} Kn, 0.25 mg L^{-1} NAA. The mean number of shoots regenerated per axillary bud of variants V1 to V11 was significantly higher in CMM1-2b medium than that in either CMM1-2a or CMM1-2d media (Table 3). Frequency of shoot proliferation from V12 axillary buds was a little different; the mean number of shoots regenerated per axillary bud on CMM1-2b was significantly higher (5.1±0.31) than that in CMM1-2d (1.2±0.2); but there was no significant difference between the proliferation obtained in CMM1-2b and CMM1-2a media (Table 3). The time required for initiation of multiple shoot was the same (8-10 d) on both CMM1-2a and CMM1-2b but was more (18-21 d) on CMM1-d. As in the present study, use of the same medium for shoot regeneration and also for proliferation of axillary shoots has been reported in *Capparis*

Table 2—Regenertion of plantlets from leaf-derived callus of *C. indica*

Medium					Variants	*% regenerative callus	Shoots regnerated/callus	Shoot length (cm)
No.	Basal composition	PGR (mg L^{-1})						
		BAP	Kn	NAA				
					V1	92.31	4.9 ±0.23d	4.42 ±0.22cd
					V2	92.86	4.7 ±0.37cd	2.92 ±0.15a
					V3	91.67	3.4 ±0.16b	4.63 ±0.19d
					V4	100.00	2.3 ±0.21a	4.11 ±0.19bc
					V5	92.86	2.1 ±0.41a	2.98 ±0.09a
CMM1-2b	WPM	0.50	0.25	0.25	V6	92.31	3.9 ±0.23bc	2.94 ±0.06a
					V7	100.00	3.4 ±0.22b	3.93 ±0.2b
					V8	92.31	1.7 ±0.21a	2.8 ±0.09a
					V9	100.00	1.5 ±0.17a	2.78 ±0.13a
					V10	91.67	1.8 ±0.2a	3.04 ±0.13a
					V11	100.00	1.9 ±0.23a	2.95 ±0.07a
					V12	92.86	4.6 ±0.27cd	3.02 ±0.05a

All media had 3% sucrose; *Response obtained only from callus induced on Ci7 medium (vide text & Table 1); Means ± S.E. within columns followed by the same letters are not significant according to DMRT (p=0.05); PGR= Palnt Growth Regulator

spinosa[14], *Terminalia bellerica*[15] and in *Chrysanthemum*[16]. In the present study, the WPM basal medium was found better than the MS basal medium for shoot proliferation. Similar results have also been reported in *Tinospora cordifolia*[17] and in *Quercus semecarpifolia*[18]. Axillary shoot proliferation was poor in CMM1-2d when compared to that in CMM1-2b, both in terms of mean number of shoots regenerated per axillary bud and the time required for initiation of multiple shoots, due to lower concentrations of BAP, Kn, and NAA (Table 3). Studies on *in vitro* propagation of other *Citrus* species[19,20] also reported similar findings.

Table 3—Proliferation of axillary shoots of *C. indica*

	*CMM1-2a medium		CMM1-2b medium		CMM1-2d medium	
Variants	Shoots/bud	[1]DI	Shoots/bud	DI	Shoots/bud	DI
V1	3.6 ±0.43g		6.3 ±0.42a		1.2 ±0.2h	
V2	4.0 ±0.26fg		5.1 ±0.28cde		1.2 ±0.13h	
V3	3.6 ±0.22g		4.9 ±0.43cdef		1.4 ±0.16h	
V4	3.8 ±0.2g		5.8 ±0.42abc		0.9 ±0.23h	
V5	3.5 ±0.31g		5.1 ±0.31cde		1.0 ±0.21h	
V6	3.7 ±0.3g	8-10 d	5.0 ±0.47cde	8-10 d	1.3 ±0.15h	18-21 d
V7	4.1 ±0.32efg		5.7 ±0.42abc		1.1 ±0.23h	
V8	4.2 ±0.25efg		5.3 ±0.3bcd		1.2 ±0.13h	
V9	4.5 ±0.37defg		5.5 ±0.5abc		1.0 ±0.26h	
V10	4.0 ±0.26fg		5.3 ±0.3bcd		1.0 ±0.21h	
V11	4.4 ±0.43defg		6.1 ±0.38ab		1.1 ±0.23h	
V12	4.2 ±0.33defg		5.1 ±0.31cde		1.2 ±0.2h	

*CMM1-2a: MS + 0.5 mg L^{-1} BAP + 0.25 mg L^{-1} Kn + 0.25 mg L^{-1} NAA; CMM1-2b: WPM+ 0.5 mg L^{-1} BAP + 0.25 mg L^{-1} Kn + 0.25 mg L^{-1} NAA; CMM1-2d: WPM+ 0.25 mg L^{-1} BAP + 0.1 mg L^{-1} Kn + 0.05 mg L^{-1} NAA
All media had 3% sucrose
[1]DI: Days required for initiation of multiple shoots
Means ± S.E. followed by the same letters across rows & columns are not significant according to DMRT (p= 0.05)

MS medium supplemented with 1.00 mg L^{-1} NAA and gelled with 6% agar (CCM3 medium, Table 4) induced roots (Fig. 4). NAA has previously been used for induction of roots from *in vitro* regenerated shoots of citrus[7,21,22] and other species[23]. Root induction was observed 10-12 d after transfer of shoots to CCM3 medium; observations as detailed below were recorded 10 d after induction of roots (Table 4). In V7, 92.86% of shoots had root growth; this was the highest incidence of rooting. The lowest percentage of root regeneration was 71.43%, recorded in V11. The variation in the average number of roots developed per shoot was not very wide. The highest average value (2.5±0.17 roots/shoot) was recoded in V7 shoots. This frequency of root induction was not significantly different from the values recorded from shoots of other variants. However, significant differences were present in the mean length of roots. The longest roots were recorded from the shoots of V6; the mean value, 3.87 ±0.08 was not significantly different from mean values recorded from the shoots

Table 4—Rooting from plantlets of *C indica*

Medium			Variants	% plantlets from which rooting occurred	Roots/shoot	Root length (cm)
No.	Basal composition	PGR (mg L^{-1})				
		NAA	V1	83.33	1.9 ±0.17a	3.8 ±0.17d
			V2	76.92	1.9 ±0.06a	3.24 ±0.06b
			V3	81.82	2.2 ±0.25a	2.83 ±0.07a
			V4	85.71	1.9 ±0.18a	3.15 ±0.06bc
			V5	76.92	1.7 ±0.21a	3.56 ±0.01cd
CCM3	MS	1.00	V6	83.33	2.0 ±0.21a	3.87 ±0.08d
			V7	92.86	2.5 ±0.17a	3.28 ±0.06bc
			V8	75.00	1.9 ±0.18a	3.23 ±0.07bc
			V9	84.62	2.2 ±0.13a	3.57 ±0.14cd
			V10	90.91	2.1 ±0.18a	2.98 ±0.12ab
			V11	71.43	2.3 ±0.26a	3.78 ±0.1d
			V12	92.31	1.6 ±0.22a	3.61 ±0.12d

All media had 3% sucrose; Means ±S.E. within columns followed by the same letters are not significant according to DMRT (p=0.05); PGR= Plant growth regulator

of V1, V5, V9, V11 and V12, but significantly higher than that recorded from the shoots of rest of the variants (Table 4).

Sixty per cent of the rooted plantlets were acclimatized (Fig. 5) to the ambient conditions of Shillong. A hardened plant of *C. indica* maintained in the Botanical Garden of St Anthony's College, Shillong is shown in Fig. 6.

The significant differences observed for some of the parameters as described above might be manifestations of genetic differences between the seedlings. The authors are developing DNA-based markers to evaluate the genetic homogeneity of intra- and inter-group regenerants. Nonetheless, the present report provides a method for *in vitro* multiplication of *C. indica* and also demonstrates its adaptability to *ex situ* conditions. Both aspects will have useful impact on the conservation efforts of this species and also on its use as a rootstock for propagation of commercial varieties of citrus, given its hardy and pest resistant qualities.

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References

1 Singh B, *Establishment of first gene sanctuary for Citrus in Garo hills* (Concept Publishing Company, New Delhi), 1981.
2 Upadhyay R C & Sundriyal R C, Crop gene pools in the Northeast Indian Himalayas and threats, in *Managing agrobiodiversity-farmers changing perspective and institutional responses in the Hindu Kush-Himalayan Region*, edited by T Pratap & B Sthapit (ICIMOD & IPGRI, Kathmandu) 1998, 167-173.
3 Singh I P & Singh S, *Exploration, collection and mapping of citrus genetic diversity in India* (National Research Centre for Citrus, Nagpur) Technical Bull No. 7, 2003.
4 Malik S K, Chaudhury R, Dhariwal O P & Kalia R R, Collection and characterization of *Citrus indica* Tanaka and *C. macroptera* Montr.: Wild endangered species of northeastern India, *Genet Resour Crop Evol*, 53 (2006) 1485-1493.
5 Mursahige T & Skoog F, A revised medium for rapid growth and bioassay with tobacco tissue culture, *Physiol Plant*, 15 (1962) 473-497.
6 McCown B M & Llyod G, Woody plant medium: A mineral nutrient formulation for microculture of woody plant species, *Hortic Sci*, 16 (1981) 89.
7 Begum F, Amin I S, Azad M A K & Rehman M M, *In vitro* plant regeneration from cotyledon-derived callus of three varieties of Pummelo [*Citrus grandis* (L.) Osb.], *Online J Biol Sci* (*Pak*), 3 (2003) 751-759.
8 Singh A, Chikara J & Pandya J B, Plant regeneration from callus cultures in *Suaeda nudiflora* (Wild.) Moq., *Indian J Exp Biol*, 3 (2004) 454-456.
9 Zhang C G, Li W, Mao Y F, Zhao D L, Dong W *et al*, Endogenous hormonal levels in *Scutellaria baicalensis* calli induced by thidiazuron, *Russian J Plant Physiol*, 52 (2005) 345-351.
10 Ipekci Z & Gozukirmizi N, Indirect somatic embryogenesis and plant regeneration from leaf and internode explants of *Paulownia elongata*, *Plant Cell Tissue Organ Cult*, 79 (2005) 341-345.
11 Shankala D, Davis T D & Shankala N, Thidiazuron-induced *in vitro* shoot formation from roots of intact seedlings of *Albizzia julibrissin, Plant Growth Regul*, 14 (1994) 267-272.
12 Vasil I K & Thorpe T A, Plant cell and tissue culture (Kluwer Academic Publishers, Dordrecht, The Netherlands) 1994, 6-7.
13 Vidya S M, Krishna V, Manjunatha B K & Shankarmurthy K, Micropropagation of *Entada pursaetha* DC—An endangered medicinal plant of Western Ghats, *Indian J Biotechnol*, 4 (2005) 561-564.
14 Chalak L & Elbitar A, Micropropagation of *Capparis spinosa* L. subsp. *rupestris* Sibth. & Sm. by nodal cuttings, *Indian J Biotechnol*, 5 (2006) 555-558.
15 Mishra P & Dutta S K, Standardization of *in vitro* protocol in *Chrysanthemum* cv. Madame E. Roger for development of quality planting material and to induce genetic variability using γ-irradiation, *Indian J Biotechnol*, 6 (2007) 121-124.
16 Rathore P, Suthar R & Purohit S D, Micropropagation of *Terminalia bellerica* Roxb. from juvenile explants, *Indian J Biotechnol*, 7 (2008) 246-249.
17 Raghu A V, Geetha S P, Martin G, Balachandran I & Ravindran P N, *In vitro* clonal propagation through mature nodes of *Tinospora cordifolia* (Willd.) Hook. F. & Thoms.: An important ayurvedic medicinal plant, *In Vitro Cell Dev Biol* (*Plant*), 42 (2006) 584-588.
18 Tamta S, Palni L M, Purohit V K & Nandi S H, *In vitro* propagation of brown oak (*Quercus semecarpifolia* Sm.) from seedling explants, *In Vitro Cell Dev Biol* (*Plant*), 44 (2008) 136-141.
19 Hassanein A M & Azooz M M, Propagation of *Citrus reticulata* via *in vitro* seed germination and shoot cuttings, *Plant Cell Tissue Organ Cult*, 47 (2004) 173-177.
20 Beloualy N, Plant regeneration from callus culture of three citrus rootstocks, *Plant Cell Tissue Organ Cult*, 24 (2004) 29-34.
21 Normah M N, Hamidah S & Ghani F D, Micropropagation of *Citrus halimii*—An endangered species of South-east Asia, *Plant Cell Tissue Organ Cult*, 50 (1997) 225-227.
22 Begum F, Amin, Islam S & Azad M A K, A comparative study of axillary shoot proliferation from the nodal explants of three varieties of Pummelo (*Citrus grandis* [L.] Osb.), *Biotechnology*, 3 (2004) 56-62.
23 Siddique I & Anis M, Thidiazuron induced high frequency shoot bud formation and plant regeneration from cotyledonary node explants of *Capsicum annuum* L., *Indian J Biotechnol*, 5 (2006) 303-308.