Plant Cell Reports (1998) 17: 951–956

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Micropropagation of gum karaya (*Sterculia urens*) by adventitious shoot formation and somatic embryogenesis

Received: 26 November 1997 / Revision received: 14 April 1998 / Accepted: 11 May 1998

Abstract Nodal explants from selected trees of gum karaya (*Sterculia urens* Roxb.) in the adult growth phase cultured on Murashige and Skoog (MS) medium supplemented with 6.62 μM N^6-benzylaminopurine (BAP) produced an average of six adventitious shoots in 30 days. Shoots were rooted in vitro on 1/4-strength MS medium containing 9.82 μM indole-3-butyric acid. Nodulated callus was produced from hypocotyl explants cultured on MS medium supplemented with 4.52 μM 2,4-dichlorophenoxyacetic acid and 8.90 μM BAP. Somatic embryos developed when the nodulated callus was transferred to MS medium containing 0.45 μM thidiazuron (TDZ). TDZ treatment for 2 days gave the optimum response. Over 30% of the somatic embryos developed into plantlets when transferred to 1/4-strength MS basal medium without any growth regulators. Plantlets produced from adventitious shoots and somatic embryos were acclimatized to ex vitro conditions and established in the field.

Key words Gum karaya · Somatic embryogenesis · *Sterculia urens* · Thidiazuron · Tree tissue culture

Abbreviations *BAP* N^6-Benzylaminopurine · *2,4 D* 2,4-Dichlorophenoxyacetic acid · *IBA* Indole-3-butyric acid · *Kn* Kinetin · *MS medium* Murashige and Skoog medium · *TDZ* Thidiazuron · *WP medium* Woody plant medium

Introduction

Sterculia urens Roxb. is a medium-sized tree of the family Sterculiaceae that grows in the deciduous Indian forests of Andhra Pradesh, Madhya Pradesh, Rajasthan, and Uttar Pradesh. It is the commercial source of 'gum karaya' (tragacanth type), which is important economically, particularly to tribals and rural poor living in or close to forests. The gum has numerous industrial applications and is used in adhesives, confectionery, foods, pharmaceuticals, and cosmetics (Anon 1976; Gautami and Bhat 1992). The gum is in great demand both within and outside India. Secretion of gum requires tree wounding; however, destructive tapping methods and poor regeneration have resulted in a marked decline in the populations of forest trees, necessitating a ban on tapping in Madhya Pradesh for over 10 years (Nair et al. 1995). Over-exploitation, poor seed germination, and low seedling survival are major constraints in the sustainable availability of gum karaya. Following unsuccessful attempts to propagate *S. urens* by stem cuttings, we conducted research aimed at in vitro propagation.

In vitro adventitious shoot formation and somatic embryogenesis have been employed extensively for many horticultural species (Gupta et al. 1993). Most investigators working with trees have found that seeds and juvenile tissues are more amenable for in vitro manipulations than explants taken from trees in the adult growth phase. In vitro propagation of *S. urens* using seedling explants has been reported recently (Purohit and Dave 1996). However, because of self-incompatibility and heterozygosity (V. G. Sunnichan, unpublished data), it is desirable to use explants from trees in the adult growth phase for in vitro studies. Thus, we describe effective methods for propagating *S. urens* by in vitro adventitious shoot formation using nodal explants from mature trees and somatic embryogenesis using hypocotyl-derived callus. The latter might be extended to explants from trees in the adult growth phase.

Communicated by R. N. Trigiano

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Materials and methods

Plant material

Trees growing naturally in a forest near Village Ghatti, 30 km from Gwalior, Madhya Pradesh, India served as sources of nodal explants and seeds.

Table 1 Effect of BAP and Kn on adventitious shoot multiplication of nodal explants of *Sterculia urens* after 30 days. Means within a column followed by the same letter are not significantly different from each other at $P=0.05$ according to Fisher's least significant difference test

Cytokinin (μM)		Percentage of nodal explants producing shoots ($n=72$)	Number of shoots per explant (±SE)
	0.0	80 c	1.1 ± 0.1 f
BAP	0.44	85 b	2.9 ± 0.3 d
	2.22	85 b	3.3 ± 0.3 d
	4.40	88 a	4.1 ± 0.5 b
	6.62	89 a	5.9 ± 0.4 a
	8.90	90 a	5.5 ± 0.3 a
	17.60	81 c	3.1 ± 0.3 e
Kn	0.46	85 b	2.8 ± 0.2 e
	2.32	86 b	3.1 ± 0.3 e
	4.60	88 a	3.5 ± 0.1 c
	6.92	86 b	3.8 ± 0.2 bc
	9.20	80 c	3.1 ± 0.1 e
	18.40	73 d	3.1 ± 0.1 e

Nodal explants from mature trees

Terminal shoots (ca 12 cm long) were excised from adult trees (tested as good yielders of gum) in August and September 1995, when they had put out fresh vegetative shoots. After removing the apical bud and leaves (1 mm of petiole was retained) from the shoots, the third and fourth nodes were cut (each explant was 0.8–1.0 cm in length and consisted of equal portions of the internode above and below the nodal region) and washed first in running tap water for 30 min and then in a solution containing four to six drops of Cetavlon (a commercial disinfectant containing cetrimide, ICI, UK) followed by dipping in 70% ethanol for 10 s. The explants were then washed in running tap water for 30 min and surface-sterilized with 0.1% mercuric chloride for 5 min. After four or five washings with sterile distilled water, the explants were surface-dried on sterile filter paper and cultured on Murashige and Skoog (1962) (MS) medium with 0.8% agar. Unless otherwise stated, all media contained 3% sucrose. In some treatments, the strength of organic and inorganic components of the MS medium (except sucrose) were reduced to 1/2 or 1/4 strength.

For induction of adventitious shoot buds and somatic embryos, media were supplemented individually or in combination with N^6-benzylaminopurine (BAP) at 0.44–17.60 μM, kinetin (Kn) at 0.46–18.40 μM, indole-3-acetic acid (IAA) at 0.57–17.1 μM, indole-3-butyric acid (IBA) at 2.46–14.70 μM, 2,4-dichlorophenoxyacetic acid (2,4-D) at 2.26–4.52 μM and thidiazuron (TDZ) at 0.22–0.92 μM. The pH of all media was adjusted to 5.8 before autoclaving at 121°C for 18 min in 150×25 mm glass culture tubes, each containing 20 ml of medium.

Hypocotyl explants

Seeds were harvested from mature fruits in May 1995. They were washed and surface-sterilized as described for nodal explants and cultured on 1/2-strength MS medium alone or in combination with the aforementioned growth regulators. Seed cultures were maintained at 24±1°C with a 16-h photoperiod of 60 μmol m^{-2} s^{-1} (measured at culture level with Sunfleck PAR Ceptometer, Decagon Devices, USA) provided by cool-white fluorescent tubes. Hypocotyl cuttings, 1 cm long, from 7-day-old seedlings were cultured for callus induction. The resulting callus was subcultured on 1/2- and full-strength MS, B_5 (Gamborg et al. 1968) and woody plant (WP) medium (Lloyd and McCown 1980) with the intention of inducing somatic embryogenesis. Somatic embryos at the cotyledonary stage were isolated and transferred to culture tubes containing 1/4-strength MS medium without growth regulators to promote plantlet development. The cultures were maintained under the same conditions as described above for seed cultures. Twenty-four cultures were raised for each treatment. All treatments were repeated three times. A completely randomized block design with three replications was used and the data were subjected to analysis of variance by Fisher's least significant difference (LSD) test at the 5% level of significance ($P=0.05$) (Panse and Sukhatme 1985). Data percentage responses were arcsin transformed before analysis.

Plantlets produced from nodal explants and from somatic embryos were taken at the four- to six-leaf stage, washed with tap water to remove all traces of medium and transferred to plastic cups (8 cm diameter) containing 1:1 (by vol) autoclaved vermiculite:soil (2 parts of soil mixed with 1 part of leaf mould). Pots were maintained in a polythene chamber in the culture room and irrigated every other day with a solution of 1/10-strength MS inorganic salts. After 4 weeks, plantlets were transferred to the field or transplanted to 15-cm-diameter earthen pots for further growth in a greenhouse.

Table 2 Effect of IBA on adventitious rooting of microcuttings of *S. urens*. Medium = 1/4-strength MS + 1% sucrose; growth period = 21 days, $n=72$. Means within a column followed by the same letter are not statistically significant at the 5% level by Fisher's least significant difference test

Concentration (μM)	Percentage of cultures producing roots	Mean number of roots per shoot (±SE)	Mean root length (cm) (±SE)
0.0	0.0	0.0 ± 0.0	0.0 ± 0.0
2.46	45 d	1.1 ± 0.1 c	2.8 ± 0.1 b
4.90	51 d	1.4 ± 0.1 c	2.9 ± 0.1 b
7.36	58 c	2.8 ± 0.3 b	2.7 ± 0.3 b
9.82	75 a	3.9 ± 0.4 a	3.1 ± 0.5 a
12.28	68 b	3.9 ± 0.3 a	2.9 ± 0.3 a
14.70	65 b	3.7 ± 0.2 a	2.6 ± 0.4 b

Results and discussion

Nodal explants

In the absence of BAP or Kn, a single shoot emerged from the axil in about 80% of the cultures (Table 1). However, three to six supernumerary shoots developed from the leaf axil on medium supplemented with BAP or Kn. The frequency and number (and length) of shoots regenerated were highest on medium with 6.62 μM BAP (Table 1, Fig 1 A). At higher concentrations, BAP and Kn reduced markedly the number of shoots. In general, Kn was less effective than BAP in inducing shoots. There was no enhancement in shoot number on medium containing BAP+Kn.

Individual shoots with four or five leaves were excised and cultured on full- or 1/4-strength MS medium (with sucrose reduced to 1%)+IBA or IAA to induce rooting. Treatment with IAA failed to stimulate rooting at any concentration; however, IBA was effective (Table 2). Up to 75% of the shoots rooted on IBA (9.82 μM)+1/4-strength MS medium in 7 days (Fig. 1B). Full-strength MS medium supplemented with 9.82 μM IBA induced callus at the cut end of the shoots. Subsequently, roots developed from the callus as revealed by free-hand sections of rooted callus. The

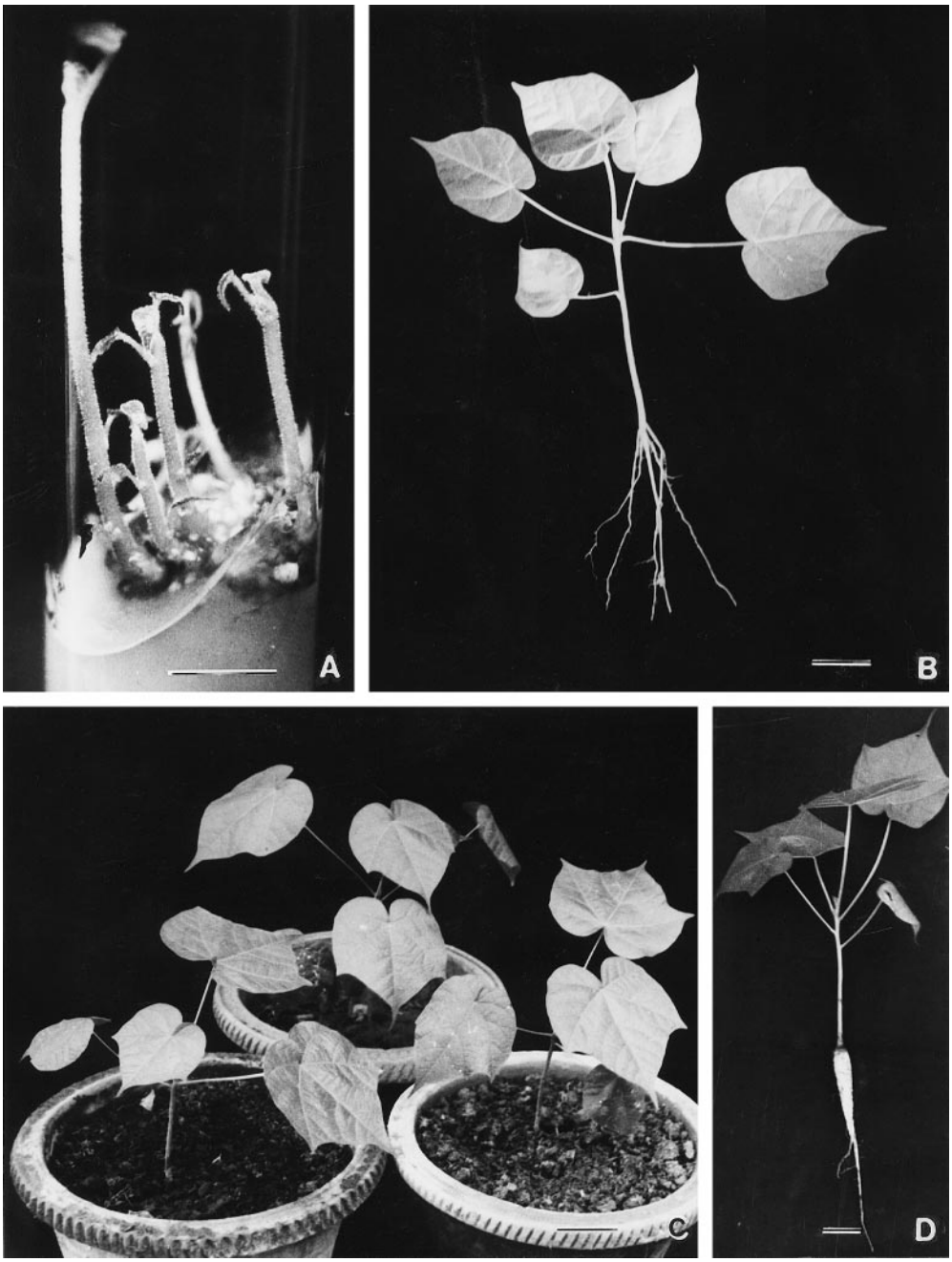

Fig. 1 **A** Formation of multiple shoots from a nodal explant of *Sterculia urens* cultured on MS medium supplemented with 8.90 μM BAP (*bar* 1 cm). **B** Regenerated plantlet (*bar* 1 cm). **C** Potted plants 4 months after transfer from culture tubes (*bar* 4 cm). **D** A plant showing the formation of a fusiform root tuber. Older leaves have abscised (*bar* 2 cm)

promotive effect on rooting of shoots by low salt and reduced sugar concentration has been reported by Ghosh and Mohan Ram (1986) in carnations (*Dianthus caryophyllus* L.) and by Purohit and Dave (1996) in *S. urens*. In the present study, rooted shoots were later transferred to 1/4-strength MS medium without IBA and sucrose to improve root growth. After 2 weeks, plantlets were transferred to soil. Of 86 plantlets transferred, 68 survived with susequent establishment (Fig. 1C). To the best of our knowledge, this is the first report of multiple adventitious shoot formation from mature explants and their subsequent rooting and acclimatization.

Seeds and hypocotyl segments

Seeds germinated in 7 days on MS medium regardless of the presence or absence of growth regulators. Up to 90% germination, and normal seedling growth were observed on a medium containing 0.89 μM BAP and 0.57 μM IAA. Higher concentrations of BAP reduced seedling growth.

Hypocotyl explants (1 cm long) were excised from 7-day-old seedlings and cultured on MS, B_5 and WP basal media. None of these media promoted callus formation from the explants in the absence of growth regula-

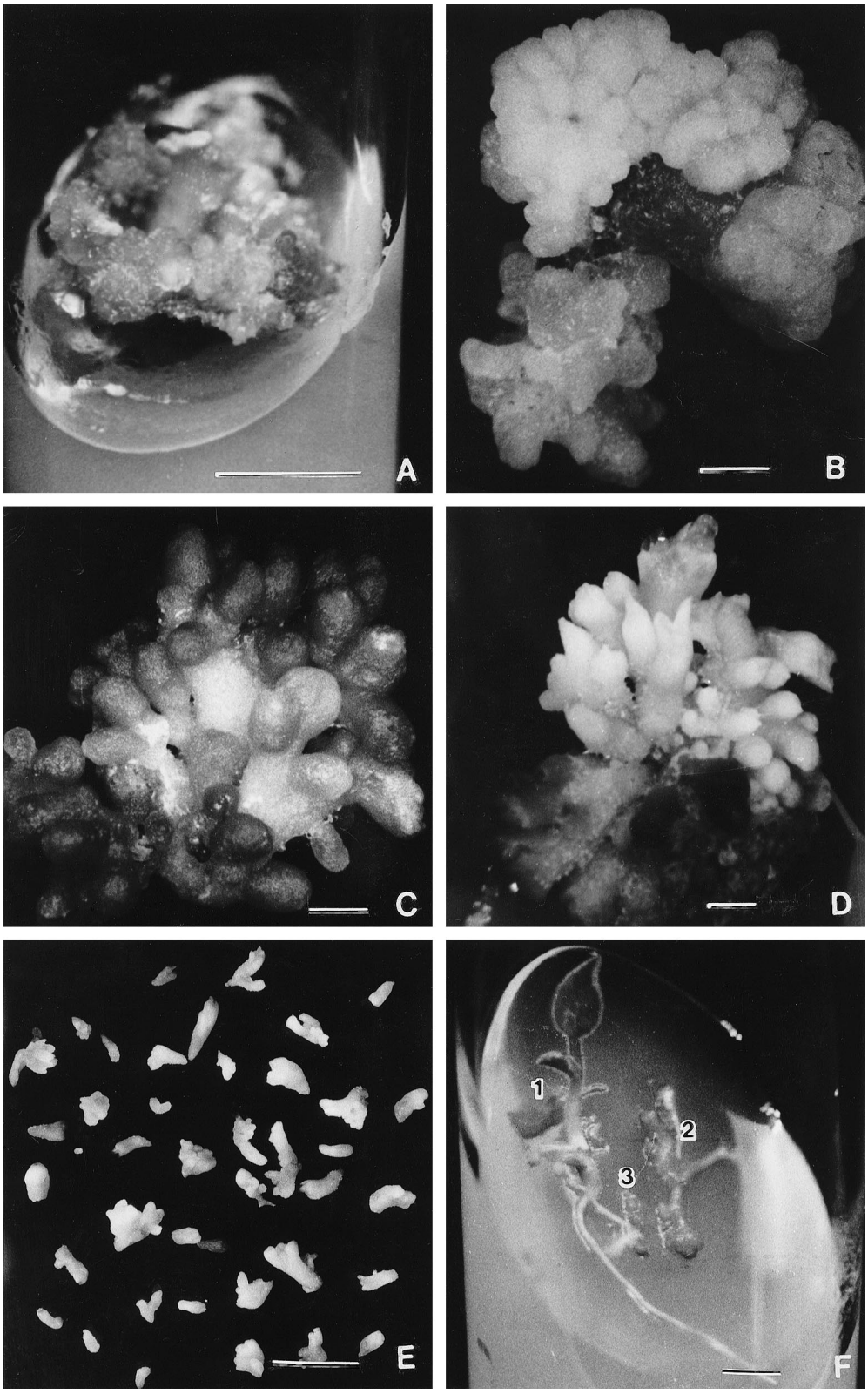

Fig. 2A–F Development of somatic embryos from callus derived from hypocotyl explants of *S. urens*. **A** Friable callus formed on MS medium supplemented with 4.4 μM BAP and 2.85 μM IAA (*bar* 1 cm). **B** Nodulated callus formed on MS+8.90 μM BAP+4.52 μM 2,4-D (*bar* 1 mm). **C, D** Development of numerous globular embryo-like structures (**C**) and torpedo-shaped and dicotyledonous embryos (**D**) differentiated from nodulated callus on MS medium containing 0.22 μM TDZ (*bar* 1 mm). **E** Embryos with variant developmental pattern when the nodular callus was grown continuously on TDZ-supplemented medium for 4 weeks (*bar* 1 mm). **F** Plantlets regenerated from somatic embryos on 1/4-strength MS basal medium. Plantlet *no. 1* has produced two roots and three leaves (*bar* 1 cm)

tors. However, with addition of growth regulators, profuse callus growth was observed. Initially, callus development was confined to the cut ends of the explants and it subsequently spread to the entire surface. In most of the treatments, friable callus was obtained (Fig. 2A). However, on a medium containing 8.90 μM BAP and 4.52 μM 2,4-D, nearly 50% of the cultures produced a compact and distinctly nodulated callus (Fig. 2B). Organogenesis or embryogenesis did not occur in the friable or the nodulated callus.

Table 3 Effect of nutrient medium containing 0.22 μM TDZ on somatic embryogenesis from nodulated callus. Data were recorded 4 weeks after transferring the nodulated callus (induced on BAP+ 2,4-D) on to different media containing TDZ. Means within a column followed by the same letter are not significantly different from each other at P=0.05 by Fisher's least significant difference test

Medium	Cultures with embryogenesis (%)	Cultures with embryos with two cotyledons (%)	Average number of embryo/culture		
			Heart-shaped	Torpedo	Dicotyledonous
1/2 MS	78 a	71 a	27 a	23 a	24 a
MS	71 b	66 a	22 bc	18 b	19 b
WP	41 c	23 b	18 c	14 b	15 b
B_5	32 d	11 c	14 c	13 b	14 b

Somatic embryogenesis

When nodulated callus was transferred to a medium supplemented with 0.45 μM TDZ, somatic embryogenesis was observed (Table 3). However, somatic embryos failed to differentiate from the friable callus even on TDZ-containing medium.

TDZ elicited the best response when used with 1/2-strength MS medium followed by full-strength MS, WP or B5 (Table 3). The nodules developed into globular, embryo-like structures in about 3 weeks (Fig. 2C), subsequently passing through the heart-shaped and cotyledonary stages (Fig. 2D). Culturing of nodulated callus for 2 days on TDZ-supplemented medium followed by transfer to basal medium yielded the most satisfactory results. Thus, the development of somatic embryos is a two-stage process. The first involves formation of nodular structures on medium containing 2,4-D+BAP and the second consists of differentiation of nodular structures into somatic embryos. TDZ was essential for the second stage. Formation of somatic embryos in two stages has been observed in other plants (Eapen and George 1993; Kysley et al. 1987).

The somatic embryos formed a loose aggregate such that individual embryos could be separated easily. When somatic embryos were maintained continuously on TDZ-containing medium, many developed abnormal cotyledons (fusion, multiple cotyledons), and multiple shoots with a single radicle and formation of secondary embryos (Fig. 2E).

TDZ is one of several substituted phenyl ureas and is active at lower concentrations than purine-based cytokinins (Mok et al. 1982, 1987). TDZ is effective in inducing caulogenesis and/or somatic embryogenesis in several herbaceous plants (Malik and Saxena 1992) and a few trees (Bates et al. 1992; Sankhla et al. 1994). In some species, TDZ can replace the requirement of auxin and/or cytokinin for induction of somatic embryogenesis (Gill and Saxena 1992, 1993). In the present study, nearly 40% of the somatic embryos transferred individually to 1/4-strength MS medium without growth regulators developed into plantlets within 14 days (Fig. 2F). Expansion and greening of cotyledons occurred about 6 days after transfer. Sixty plantlets were acclimatized and transferred to the soil, of which 47 have survived.

Plantlets (derived from adventitious shoots as well as somatic embryos) transferred to soil followed the same pattern of development as those propagated in soil by seed. The shoot grows for 3–4 months followed by senescence, leaving a perennating tuber in the soil. The next year, the tuber produces a new shoot during June–July. The shoot grows for 3–4 months and the size of the tuber increases further. In the 3rd year, the tuber puts out a perennial shoot which develops into a young tree. Plantlets transferred from the cultures also produced tubers during the 1st year (Fig. 1D) and a new shoot the 2nd year.

Propagation by somatic embryogenesis has several advantages over organogenesis. The success of regeneration by adventitious shoots depends on adventitious rooting which may be a constraint in many tree species. The multiplication rate is generally faster with somatic embryos and this method has proved valuable in the propagation of some forest trees (Gupta et al. 1993). Somatic embryos are also amenable for production of synthetic seeds (Attree and Fowke 1993). In our work, somatic embryos were obtained from juvenile material. Attempts are continuing to produce them from explants taken from high-gum-yielding trees in the adult growth phase.

Acknowledgements We gratefully acknowledge financial assistance from the University Grants Commission, New Delhi (to V. G. S.) and the Ministry of Environment and Forests, Government of India for granting a project entitled "Biology of some gum and gum-resin yielding forest trees" (no. 14/4/93-MAB/RE). We extend our deep appreciation to the officials of the Madhya Pradesh Forest Department (Gwalior Range) for providing necessary permission and help in conducting research. HYM thanks the Indian National Science Academy for the support received.

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