African Journal of Biotechnology Vol. 7 (2), pp. 095-101, 18 January, 2008
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB08.826
ISSN 1684–5315 © 2008 Academic Journals

Full Length Research Paper

Micropropagation of *Sterculia urens* Roxb., an endangered tree species from intact seedlings

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Accepted 19 December, 2007

An efficient and reproducible procedure for the large scale propagation of *Sterculia urens* is described. Direct shoot proliferation was induced in aseptic seed cultures of *S. urens* on MS medium (1962) supplemented with 5.0 µM thidiazuron + 1.5 µM GA_3 + 0.1% ascorbic acid. The highest number of shoots (14.0) was formed within 8 weeks of seed culture without root formation on MS medium. Proliferating shoot cultures were established by repeatedly sub culturing mother seedlings (stumps) on fresh medium (MS + 2.5 µM TDZ + 1.5 µM GA_3 + 0.1% ascorbic acid) after excising all newly formed shoots. The shoot forming capacity of seeds was influenced by the cytokinin type and concentration in the medium. Roots formed on excised shoots when they were transferred to quarter strength MS medium containing 9.80 µM indole-3-butyric acid. Plantlets were finally transferred to the soil.

Key words: Intact seedlings, micropropagation, multiple shoots, sterculiaceae.

INTRODUCTION

Sterculia urens is a moderate sized tree belonging to the family Sterculiaceae. It is commonly known as 'gum karaya tree' and it is valued for its gum known as 'Indian tragacanth'. The gum is a complex polysaccharide. It is used as an ingredient in the preparation of emulsions, lotions, denture fixative powders and bulk laxatives. It has a wide application in food, baking and dairy industries. The gum is in great demand both within and outside India. Considerable part of the gum produced in India is exported. Tapping of the gum requires stripping of the bark. As the tree is easily injured, indiscriminate tapping of young tree impairs their viability. Unscientific tapping methods, poor seed viability and meager distribution of this tree are limitations for the availability of the gum. In spite of the rich commercial importance, it grows only as a wild forest plant and is enlisted as an endangered plant species in the Aravalli hills (Anonymous, 1976). Therefore a method to multiply this plant using modern methods is needed.

During the last few years, there has been great interest in propagating economically important tree species (Hossain et al., 1994; Rajasekharan, 1994; Eswara et al., 1998; Kaur et al., 1998; Thakur et al., 1998; Anand et al., 1999; Kulkarni and D'Souza, 2000; Lewu et al., 2006; Moghaieb et al., 2006; Ndiaye et al., 2006; Tefera et al., 2006; Ozyigit et al., 2007). In most of the studies either apical axillary buds or nodal explants taken from field grown plants or shoot tip, hypocotyls, cotyledons from aseptically germinated seedlings or zygotic embryos were used. However, direct germination of seeds on cytokinin supplemented medium also has been used for the regeneration and propagation of plants by multiple shoot induction in *Pigeon pea* (Shiv Prakash et al., 1994), *Muraya koenigii* (Bhuyan et al., 1997), *Dendrocalamus asper* (Arya et al., 1999) and *Litchi chinenisis* (Das et al.,

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Abbreviations: AC, activated charcoal; AS, adenine sulphate; BA, benzyladenine; CH, casein hydrolysate; CM, coconut milk; GA_3, gibberellic acid; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; 2iP, isopentenyl adenine; MS, murashige and skoogs medium; NAA, naphthalene acetic acid; PVP, polyvinylpyrrolidone; TDZ, thidiazuron.

1999). Earlier micropropagation of this tree species via cotyledonary node segments (Purohit and Dave, 1996; Hussain et al., 2007) and somatic embryogenesis (Sunnichan et al., 1998) has been reported. However these protocols involve several stages and are time taking. Here we report a very simple, reproducible and innovative protocol of culturing mature seeds on MS medium containing TDZ for *in vitro* propagation of *S. urens*. To the best of our knowledge, it is the first report of shoot multiplication using intact seedlings in this tree species.

MATERIALS AND METHODS

Establishment of aseptic intact seedlings

Fully ripe and dried follicles were collected from a 15 years old tree of *S. urens* from Tirumala hills in Chittoor district of Andhra Pradesh, India. Healthy seeds removed from follicles were first treated with conc. H_2SO_4 for 1 min, washed thoroughly with running tap water, then rinsed in 5% teepol for 5 min and later treated with cetavlon (a commercial disinfectant containing cetrimide, ICI, UK) for 5 min Then they were surface sterilized by dipping them in 70% ethanol for 1 min and treated with 01% $HgCl_2$ for 20 min and finally given 5 - 6 rinses in sterile distilled water. Disinfected seeds were germinated aseptically in 25 X 150 mm test tubes (Borosil, India) containing 15 ml of germinating and regeneration media [MS medium with 30 g sucrose, 8 g agar; TDZ, BA, 2-iP, zeatin and kinetin were supplemented individually at various concentrations (1.0 – 15 µM). GA_3 (1.5 µM) was added in combination with cytokinins. Growth adjuvants like CH (0.025%), CM (10.0%) and anti-oxidants like activated charcoal (0.05%), PVP (0.1%), ascorbic acid (0.1%) were also used individually with different cytokinins]. Different media like MS, L2, WPM and B5 were also used supplemented with 5 µM TDZ + ascorbic acid (0.1%) and 1.5 µM GA_3. Different concentration of agar added (solid, semi-solid and liquid) to MS medium and supplemented with 5 µM TDZ + 0.1% ascorbic acid + 1.5 µM GA_3. Each treatment consisted of 20 replicates and the experiment was conducted thrice. Cultures were maintained at 25±2°C at 16 h photoperiod of 35 µmol m^{-2} s^{-1} light intensity provided by cool white fluorescent light tube (Phillips, India) and at 50 - 60% relative humidity.

Subculture

Newly formed shoots were excised after 4 weeks of culture and the intact mother seedlings (hereafter referred as stumps) were transferred to medium with MS + 2.5 µM TDZ + ascorbic acid (0.1%) and 1.5 µM GA_3 for subculture for further proliferation of shoots. Sub culturing was performed at 4 week intervals.

Histological analysis

For anatomical structure observation, cotyledonary nodal segments of seedlings after six days of culture were cut into small pieces (0.5 cm^2) and fixed in FAA (formaldehyde : acetic acid : ethanol; 5:5:90; v/v/v) for 24 h. Dehydrated the tissue by passing through the ethanol series (20, 30, 50, 70 and 100%) for 5 min at each stage. The tissue was embedded in paraffin (mp. 58°C) and sectioned longitudinally at 6 – 8 µ thickness on a rotary microtome. The sections were placed on clean microscopic glass slides coated with egg albumin and stained with safranin after bringing it to the aqueous media by processing through the ethanol series in a reverse direction. Sections were mounted with DPX (BDH) and viewed under inverted microscope.

Rooting of shoots

In vitro shoots were transferred to full strength, half strength and quarter strength MS medium containing 3% sucrose and 0.8% agar. All media were supplemented individually with 0.49 to 17.13 µM of IBA, IAA or NAA. Each treatment consisted of 20 replicates and the experiment was conducted thrice.

Acclimatization

Plantlets with well-developed roots were removed from the culture medium, washed gently under running tap water and transferred to plastic pots containing sterile vermiculite (Keltech Energies Ltd., Bangalore, India) under diffuse light (16:8 h photoperiod) conditions. Potted plantlets were covered with a transparent polythene membrane to ensure high humidity and watered every 3 days with quarter -strength MS salt solution for 2 weeks. Polythene membranes were opened after 2 weeks in order to acclimatize plants to field conditions. After 4 weeks, acclimatized plants were transferred to pots containing normal soil and maintained in a greenhouse under normal day length conditions.

Statistical analysis

Frequency of shoot regeneration, mean number of shoots per seedling, mean length of the shoot were analyzed by Tukey test (Steele and Torrie, 1980) using SPSS package, version 7.5 and stand error of the mean was also used to analyze the significance ($P \leq 0.05$) of response induced by each growth hormone at different concentrations. Results of the rooting experiment (frequency of root regeneration, mean number of roots per shoot and mean length of the root) were compared with the standard error of the mean.

RESULTS AND DISCUSSION

Based on thorough literature search, MS medium was selected to carry out the early experiments with growth regulators like TDZ and BA. Seeds of *S. urens* germinated within 6 days of culture on MS medium supplemented with TDZ or BA. The shoot forming capacity of intact seedlings was greatly influenced by the type of growth regulator and its concentration in the medium. However, no shoots were differentiated from the cotyledonary node region of the seedling on MS basal medium. Also addition of different concentrations of 2-iP, zeatin, kinetin and adenine sulphate induced formation of single shoot only from each axillary bud of the cotyledonary nodal region. An average of 9.6 shoots was developed in 4 weeks of culture on MS + 5.0 µM TDZ (Figure 1A; Table 1) and MS +7.5 µM BA produced an average of 7.2 shoots in the same culture period (data has not shown).

Experiments were conducted to scrutinize the effect of growth adjuvants and anti-oxidants on shoot regeneration

Table 1. Effect of growth adjuvants, anti-oxidants and GA_3 in MS medium with 5.0 µM TDZ on shoot regeneration from intact seedlings of *Sterculia urens* in 8 weeks (4 weeks of initial culture + 4 weeks of first sub culture).

TDZ (µM)	Growth Adjuvants	Anti-Oxidants	GA_3 (µM)	Frequency of shoot regeneration	Mean number of shoots per explant	Mean length of the shoot (cm)
5.0	-	-	-	81.7 ±1.0	9.6±0.4	2.4±0.4
5.0	CH (0.025%)	-	-	60.0±1.6	7.2±0.9	0.8±0.3
5.0	CM (10.0%)	-	-	63.3±1.4	7.6±1.2	1.1±0.4
5.0		AC (0.05%)	-	66.7±1.8	9.6±1.6	2.4±0.9
5.0		PVP (0.1%)	-	70.0±1.4	10.2±0.7	2.5±0.6
5.0		Ascorbic acid	-	93.3±0.6	14.0±0.5	2.4±0.1
5.0			1.5	83.3±0.5	9.8±0.2	3.0 ± 0.3

Values represented above are the mean of 3 replicates of 20 explants. ± standard error.

Figure 1

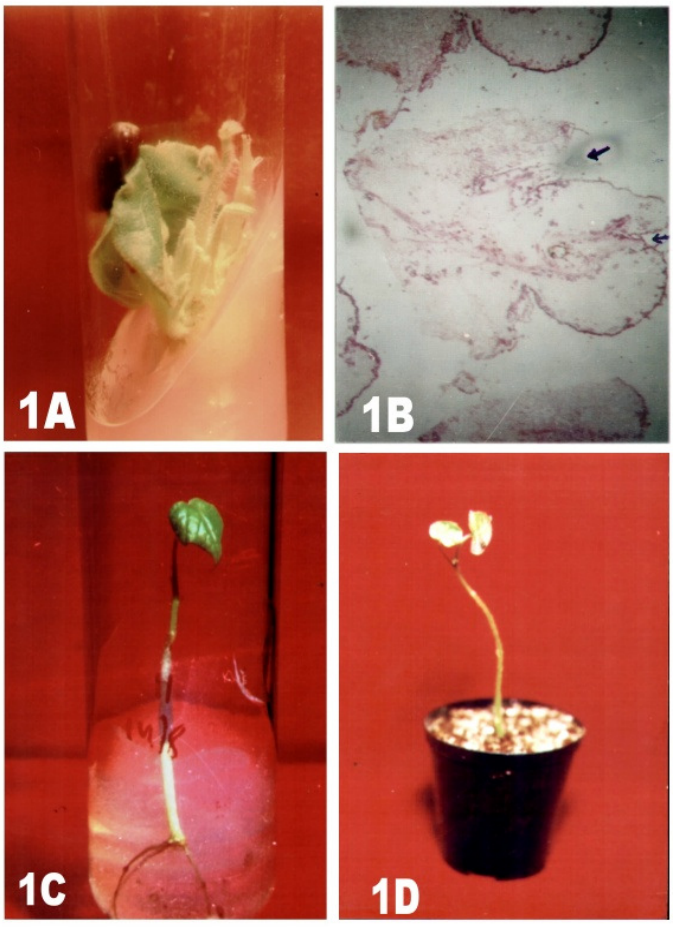

Figure 1. A. Regenerated multiple shoots from the intact seedling on MS + 5.0 µM TDZ. **B.** Histology of cotyledonary node region (arrows indicate regenerated shoot buds). **C.** Rooting of *in vitro* raised shot on quarter strength MS + 9.80 µM IBA after 8 weeks. **D.** Hardened plantlet in plastic pot after 10 weeks.

Table 2. Effect of different concentrations of TDZ, BA and ascorbic acid (0.1%) + 1.5 (µM) GA_3 on the shoot proliferations (4 weeks of initial culture + 4 weeks of first sub culture).

Growth regulator concentrations (µM)		Frequency of shoot regeneration (%)	Mean number of shoots	Mean length of the shoot (cm)
TDZ	BA			
1.0	-	81.7 ^{c} ±1.7	6.8 ^{b} ± 0.1	2.0^{bc} ± 0.2
2.5	-	85.0 ^{d} ±0.5	9.6 ^{d} ± 0.7	2.6 ^{c} ± 0.1
5.0	-	93.3 ^{e} ±0.6	14.0^{e} ± 0.5	3.0^{d} ± 0.4
7.5	-	80.0 ^{bc} ±0.4	7.2^{c} ± 0.4	1.8^{b} ± 0.3
10.0	-	71.1 ^{b} ±1.2	5.8 ^{ab} ± 0.3	1.6 ^{ab} ± 0.5
15.0		65.0 ^{a} ±1.0	4.2 ^{a} ± 1.3	1.5 ^{a} ± 0.9
	1.0	70.0 $^{b c}$ ±0.2	2.4 $^{a b}$ ± 0.2	2.2 ^{b}± 0.1
	2.5	73.8 ^{c} ±1.6	3.0 $^{b c}$ ± 0.5	2.5 ^{c}± 0.3
	5.0	80.0 ^{d}± 0.4	4.2 ^{c}± 0.1	3.0 ^{d} ± 0.5
	7.5	85.0 ^{e} ± 0.1	5.6 ^{d} ± 0.3	3.6 ^{e} ± 0.1
	10.0	66.7^{b} ± 1.2	2.6 ^{b} ± 0.5	2.0 ^{a} ± 0.2
	15.0	58.3 ^{a} ±0.7	2.0 ^{a} ± 1.0	1.6^{a} ± 0.4

Values represented above are the mean of 3 replicates of 20 explants.
Mean values followed by same letter are not significantly different at P≤0.05 (Tukey test).
± Standard Error.

from intact seedlings. Addition of CH (0.025%), CM (10.0%) caused the development of profuse basal callus which in turn reduced the shoot regeneration frequency, shoot number and length considerably (Table 1). Addition of PVP (0.1%), AC (0.05%) proved better than CH, CM but less effective than ascorbic acid (0.1%). Addition of ascorbic acid increased the frequency of shoot regeneration (93.3%) and shoots number (14.0). Therefore, during the later experiments, ascorbic acid was added to the medium along with cytokinins for the culture of different types of explants used. Kaur et al. (1998) also indicated the positive effect of ascorbic acid on micropropagation of *Acacia catechu*.

The addition of 1.5 µM GA_3 to the medium containing BA or TDZ had no stimulatory effect on the frequency of shoot regeneration and the number of shoots per seedling but it enhanced the shoot elongation (Table 1). Similar result has been reported in *Muraya koenigii* (Bhuyan et al., 1997). Shoot length increased by 0.6 cm by the addition of 1.5 µM GA_3 to the medium containing 5.0 µM TDZ. The number of shoots per seedling was greatest (14.0) at the 5.0 µM TDZ + 0.1% ascorbic acid + 1.5 µM GA_3 (Table 2) in 8 weeks and shoots developed on this medium elongated to 3.0 cm. 7.5 µM BA + 0.1% ascorbic acid + 1.5 µM GA_3 produced an average of 5.6 shoots per seedlings. The shoots obtained on MS supplemented with BA + 0.1% ascorbic acid + 1.5 µM GA_3 were longer (3.6 cm) than on MS with 5.0 µM TDZ + 0.1% ascorbic acid + 1.5 µM GA_3 (3.0 cm) supplemented medium (Table 2). BA induced direct shoot regeneration from intact seedlings has also been reported in *Alnus glutinosa* (Perinet and Lalonde, 1983), *Phaseolus* species (Mallik and Saxena, 1992), *Arachis hypogaea* (Saxena et al., 1992), *Murraya koenigii* (Bhuyan et al., 1997), *Litchi chinensis* (Das et al., 1999) and *Dendrocalamus asper* (Arya et al., 1999). At higher concentrations of TDZ or BA (15.0 µM), shoot number decreases significantly (Table 2) and exhibited reduced root development. Similar observation has been reported in *Phaseolus* species (Mallik and Saxena, 1992), *Arachis hypogaea* (Saxena et al., 1992) and *Muraya koenigii* (Bhuyan et al., 1997).

Proliferating shoot cultures were established by repeatedly subculturing the original seedlings (stumps) after harvesting the shoots at every 4 week intervals. First subculture was carried out on the same medium (MS + 5.0 µM TDZ + 0.1% ascorbic acid + 1.5 µM GA_3) which was used for shoot regeneration. But by continuous subculture on the same medium, the stumps showed a decrease in shoot regeneration potential. Further subcultures (from second sub culture onwards) were performed on MS + 2.5 µM TDZ + 0.1% ascorbic acid + 1.5 µM GA_3. Reduction in the concentration of TDZ favored continuous regeneration of shoots. The shoot forming potential of the stumps did not decline even after 8th subculture. Also, the multiplication cycles of shoots did not involve a callus phase, which may lead to a genetic variation.

Among different media used, MS medium supported the better regeneration in terms of shoot regeneration frequency, shoot number and shoot length and it was followed by L2, WPM and B5 (Table 3). And among different concentrations of agar in MS medium tested, MS solid medium supported better regeneration when compared to semi solid and liquid medium (Table 4). Microtomy of cotyledonary nodal segments indicated a clear pattern of shoot buds regeneration in the axil of the explants (Figure 1B).

For the induction of roots, quarter strength MS medium

Table 3. Effect of different media supplemented with 5.0 µM TDZ + 0.1% ascorbic acid +1.5 µM GA_3 on shoot elongation from intact seedlings of *Sterculia urens* in 8 weeks.

Media	Frequency of shoot regeneration	Mean number of shoots per explant	Mean length of the shoot (cm)
MS	93.3±0.6	14.0±0.5	3.0±0.4
L2	71.7±1.4	10.0±0.9	2.2±0.6
WPM	68.3±2.2	8.6±1.2	1.8±0.75
B5	55.0±1.8	6.7±1.6	1.2±0.8

Values represented above are the mean of 3 replicates of 20 explants.
± Standard Error

Table 4. Effect of concentration of agar in MS medium supplemented 5.0 µM TDZ + 0.1% Ascorbic acid +1.5 µM GA_3 on shoot regeneration from intact seedlings (8 weeks).

Medium	Frequency of shoot regeneration (%)	Mean number of shoots per seedling (stump)	Mean length of the shoot (cm)
Solid (0.8% agar)	93.3±0.6	14.0±0.5	3.0±0.4
Semi-solid (0.4% agar)	86.7 ± 0.5	10.6 ± 0.3	2.4 ± 0.2
Liquid (without agar)	80.0 ± 1.0	7.8 ± 0.6	1.6 ± 0.5

± Standard Error

with 9.8 µM IBA was found to be the best concentration resulting in approximately 80% of the shoots forming roots after 2 weeks of culturing without basal callus (Figure 1C). An average of 4.4 roots with an average of 3.8 cm was developed from each shoot within 8 weeks (Table 5). IBA has been reported to have stimulatory effect on root induction in many species including *Alnus glutinosa* (Perinet and Lalonde, 1987), *Morus indica* (Chand et al., 1995), *Balanites aegyptica* (Mansor et al., 2003) and *Bambusa vulgaris* (Ndiaye et al., 2006).

For acclimatization, plantlets were removed from rooting medium 8 weeks after root initiation and transferred to fresh tubes containing autoclaved tap water. After 8 - 10 days, plantlets were subsequently transferred to plastic pots (9 X 9 cm) containing autoclaved vermiculite, covered with perforated polythene bags to maintain humidity and were kept under culture room conditions for about 7 days. Later, polythene bags were removed (Figure 1D) and pots were transferred to the garden, and placed under shade till the new leaf appeared. Then they were planted under normal garden conditions. The transplantation success was about 78%.

Conclusion

In vitro propagation can become an important alternative to conventional propagation for wide range of plant species. Conclusively, a reproducible protocol for the *in vitro* propagation of *S. urens* has been developed in this study. Direct shoot multiplication is preferred for generating true-to-type plants than callus mediated regeneration. This paper supports the rapid multiplication of this commercially important plant by *in vitro* culture technique. This report provides a simple protocol for the mass propagation of this plant from intact seedlings.

We observed that within 10 days following inoculation, shoot buds differentiated in cotyledonary nodal region of the seedlings when grown on MS + 5.0 µM TDZ + 0.1% ascorbic acid + 1.5 µM GA_3. Maximum number of shoots per intact seedling (14.0) was obtained on the same medium in 8 weeks of culture (Table 2) and the shoots were subsequently rooted on quarter strength MS medium containing 19.80 µM IBA (Table 5).

Owing to the differentiation occurring in the intact seedlings, the number of steps required to induce regeneration is reduced to one in comparison to other procedures for the culture of explants. Hence, this method of using intact seedlings for *in vitro* shoot proliferation will simplify micropropagation of this economically important tree species.

ACKNOWLEDGEMENT

Town Mohammad Hussain duly acknowledges the financial assistance provided by the University Grants Commission (UGC), New Delhi, India.

Table 5. Effect of different auxins on rooting of shoots of *S.urens* on quarter strength MS medium after 8 weeks.

Auxin(s) (µM)	Frequency of root formation (%)	Mean number of roots per shoot	Mean length of the root (cm)
0.0	-	-	-
IBA			
0.49	40.0±1.8	1.2±0.2	2.4±0.2
2.46	55.0±0.9	1.6±0.1	3.2±0.2
4.90	65.0±1.7	2.4±0.2	3.4±0.2
9.80	80.0±0.1	4.4±0.2	3.8±0.3
14.70	70.0±1.0	3.8±0.3	2.8±0.2
IAA			
0.57	30.0±3.3	1.0±0.1	1.6±0.1
2.85	35.0±3.3	1.4±0.1	2.0±0.2
5.71	45.0±1.7	2.0±0.1	2.6±0.2
11.42	60.0±5.0	2.4±0.2	2.7±0.2
17.13	55.0±1.0	2.0±0.1	2.2±0.2
NAA			
0.54	40.0±3.3	1.0±0.2	2.0±0.2
2.69	50.0±2.9	1.6±0.2	2.4±0.2
5.37	55.0±1.0	2.0±0.1	2.6±0.3
10.74	70.0±1.0	2.6±0.3	3.0±0.2
16.11	60.0±1.9	2.2±0.2	2.4±0.2

Values represented above are the mean of 3 replicates of 20 explants.
± Standard Error.

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