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Full Length Research Paper

High frequency shoot regeneration of *Sterculia urens* Roxb. an endangered tree species through cotyledonary node cultures

Town Mohammad Hussain[1, 3]*, Thummala Chandrasekhar[2] and Ghanta Rama Gopal[3]

[1]Department of Horticulture, Hamelmalo Agricultural College, Keren, P.O. Box no: 397, Eritrea, North East Africa.
[2]Faculty of Biotechnology, Cheju National University, Jeju – 690 756, South Korea.
[3]Department of Botany, Sri Venkateswara University, Tirupati-517 502, Andhra Pradesh, India.

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A protocol is described for rapid and large scale propagation of an endangered, commercially and medicinally important tree species, *Sterculia urens*, by *in vitro* culture of cotyledonary nodes from 15 days old seedlings. Of the four different cytokinins (thidiazuron, isopentenyladenine, zeatin and adenine sulphate) evaluated as supplements to Murashige and Skoog medium (1962), thidiazuron at an optimal concentration of 2.27 µM was most effective in inducing bud break (83.0%). Although, multiple shoot formation was a function of cytokinin activity alone, enhanced frequency of shoot regeneration (93.3%) and number of shoots per explant (19.0) were observed by the addition of ascorbic acid (0.1%). Concentrations of all cytokinins tested above the optimum level reduced the frequency of shoot regeneration and shoot number. A proliferating shoot culture was established by repeatedly sub-culturing the original cotyledonary node on shoot multiplication medium (0.45 µM thidiazuron) after the second harvest of newly formed shoots. Rooting was best induced (80.0%) in shoots excised from proliferating shoot cultures on a quarter strength MS medium fortified with an optimal concentration of indole-3-butyric acid (9.80 µM).

Key words: Cotyledonary nodes, micro-propagation, multiple shoots and sterculiaceae.

INTRODUCTION

Sterculia urens is a moderate sized, deciduous tree belonging to the family Sterculiaceae. It is commonly known as `gum karaya tree' and it is valued for the gum known as `Indian Tragacanth'. This gum is a complex polysaccharide. It has numerous commercial applications. It is used as an ingredient in the preparation of emulsions, lotions, denture fixative powders, bulk laxatives, as a pulp binder in the preparation of thin papers. It has a wide application in food, baking and dairy industries (Anonymous, 1976). The gum is in great demand both within and outside India. Considerable part of the gum produced in India is exported. Tapping of gum requires stripping of the bark. As the tree is easily injured, indiscriminate tapping of young trees impairs their viability. Blazing too deeply or notching and exposing the wood leads to borer attack, which may prove fatal. Unscientific tapping methods, poor seed viability and meager distribution of this tree are major limitations for the availability of the gum. In spite of its rich commercial importance, it grows only as a wild forest plant and is enlisted as an endangered plant species in the Aravalli hills (Sharma, 1993). Therefore, a method to multiply this plant using modern methods is the need of the hour. Micro-propagation through culture of tissues having resident meristem is a powerful option to multiply species

*Corresponding author. E-mail: dr.tmdhussain@yahoo.co.in. Phone: 002911401229 Fax: 002911401589.

Abbreviations: BA, N^6-Benzyladenine; IAA, Indole-3-acetic acid; IBA, Indole3-butyric acid; 2iP, isopentenyl adenine; MS, Murashige and Skoogs medium; NAA, 1-Napthalene acetic acid; TDZ, thidiazuron.

that are difficult to propagate, rare or endangered. Considerable work has been done in the last two decades on the propagation of forest trees by using tissue culture technique (Kulkarni and D'Souza, 2000).

The present paper describes a simple and more efficient procedure for the *in vitro* propagation of *S. urens* by high frequency shoot proliferation from cotyledonary nodes. Micro-propagation of *S. urens* via cotyledonary node segments (Purohit and Dave, 1996), somatic embryogenesis (Sunnichan et al., 1998) has been reported. However, these published protocols resulted in relatively low number of shoots per explant.

MATERIALS AND METHODS

Disinfection and germination of the seeds

A 15 year old elite tree of *S. urens* was selected from Tirumala hills from which the dried and mature follicles were collected. Healthy seeds were removed from the follicles, treated with conc. H_2SO_4 for 1 min, washed thoroughly with running tap water, and then rinsed in 5% teepol for 5 min, followed by treatment 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 0.1% $HgCl_2$ for 20 min and finally given 5 - 6 rinses in sterile distilled water.

Culture conditions

The surface sterilized seeds were inoculated aseptically in 25 x 150 mm test tubes (Borosil, India), each containing 15 ml of water - agar medium (0.8% agar and 3% sucrose). The pH of the medium was adjusted to 5.8. The culture tubes were incubated at 25 ± 2°C under lighting at a 35 µmol m^{-2} s^{-1} irradiance level provided by white fluorescent tubes and 60% relative humidity.

Culture media and shoot multiplication

Fifteen days old seedlings served as the source of explants. Shoot tips, auxiliary buds and cotyledonary nodes were excised and inserted vertically into 25 x 150 mm test tubes and 150 ml conical flasks (Borosil, India) containing various nutrient media: MS (Murashige and Skoog, 1962), WPM (Lloyd and McCown, 1981), B5 (Gamborg et al., 1968) and L2 (Phillips and Collins, 1979) supplemented with 0.1% ascorbic acid. Four plant growth regulators (TDZ, 2-iP, Zeatin and AS) were supplemented to the medium at various concentrations (0.0 - 54.29 µM). The pH of the medium was adjusted to 5.8 before autoclaving at 121°C for 15 min. Agar (0.8%) was added and sterilized at 15 psi pressure for 15 min. The cultures were maintained under conditions similar to those described earlier for seed germination.

Subculture

The original explant, after excising the shoots were sub-cultured on the same medium (2.27 µM TDZ) for one time. After first subculture, further subcultures were carried out on MS + 0.45 µM TDZ. Subcultures were performed at every 4 week intervals. Each treatment consisted of 20 replicates and each experiment was conducted thrice.

Rooting

The shoots formed *in vitro* (4 weeks) were excised and used for rooting. Shoots of 2 - 3 cm in height 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 different concentrations of (0.0 - 17.13 µM) IBA, IAA and NAA. Each treatment consisted of 20 replicates and the experiment was conducted thrice.

Statistical analysis

Data on frequency of shoot regeneration, number of shoots per explant, shoot length were analyzed by Tukey test (Steele and Torrie, 1980) using SPSS package, version 7.5 to analyze the significance ($P \leq 0.05$) of response induced by each plant growth regulator at different concentrations.

RESULTS AND DISCUSSION

Culture establishment

The seeds of *S. urens* showed very poor germination (53.0%) and are viable only up to seven months. Shoot tips, auxiliary buds (except first auxiliary bud) produced only a single shoot when they were cultured on MS medium with any of the cytokinins used. Among the explants used, only cotyledonary node and first auxiliary bud (auxiliary bud immediately above the cotyledonary node) produced multiple shoots. Because of the superior *in vitro* response of cotyledonary nodal explants compared to other explants, they were used in subsequent experiments. The superiority of cotyledonary node in micro-propagation has been documented in *Wrightia tinctoria* (Purohit et al., 1994), Cashew nut (Das et al., 1996), *Dalbergia sissoo* (Pradhan et al., 1998).

Shoot multiplication

Single shoot (Figure 1A) was formed from each axil of the cotyledonary nodal explants when they were inoculated on MS basal medium. Similar result was reported by Dhawan and Bhojwani (1985) in *Leucaena leucocephala*. Hence, the present study showed that cytokinins were indispensable for shoot multiplication and growth. This was supported by the works of Patnaik and Debata (1996) and Aliou et al. (2006). Within 10 days following inoculation, shoot buds differentiated in cotyledonary nodal explants when grown on MS medium with all the four cytokinins tested. It was also observed that the type and concentration of the cytokinin had a significant effect on the frequency of shoot regeneration, shoot number and shoot length as also seen in *Bauhinia vahlii* (Upetri and Dhar, 1996). Though, explants cultivated on all four cytokinins differentiated multiple shoots, yet the high number of shoots per explant recorded on TDZ.

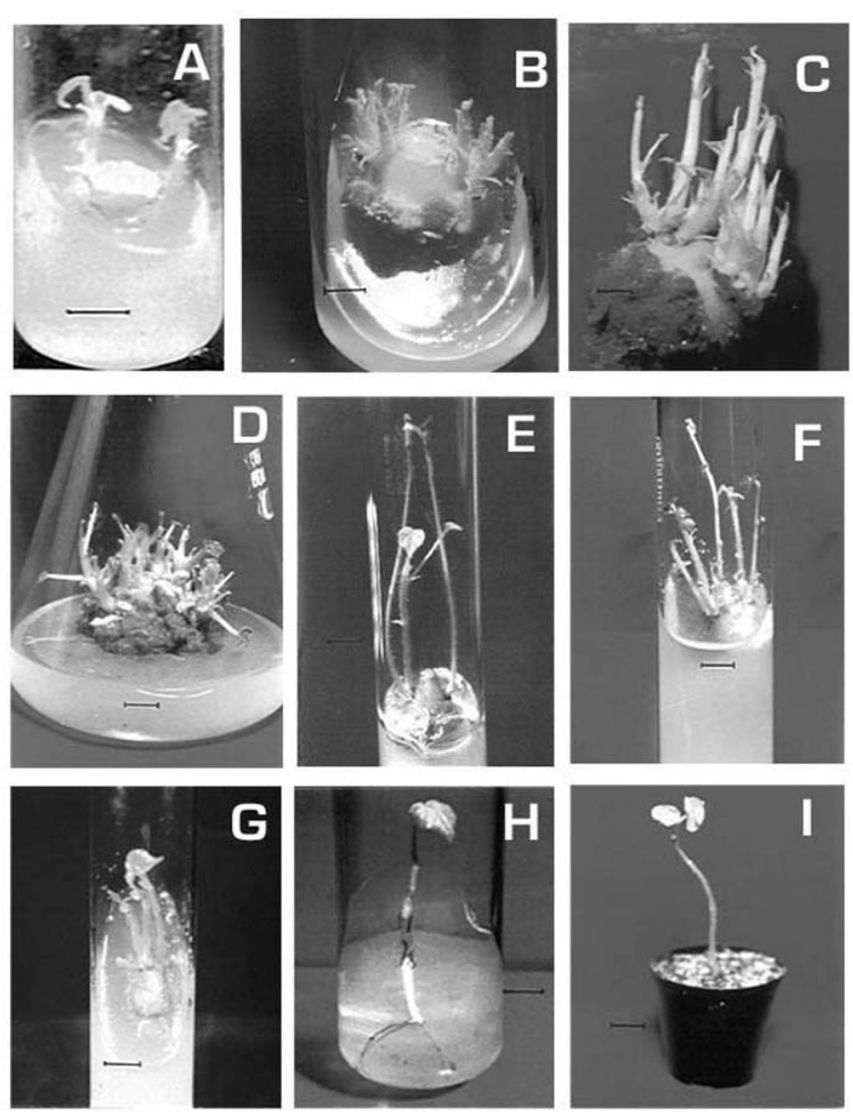

Figure 1. (A) Formation of single shoots from each axil of the cotyledonary node on MS basal medium after 2 weeks. Bar equals 7.1 mm. (B) Elongating shoot buds on MS + 2.27 µM TDZ after 18 days of culture. Bar equals 6.5 mm. (C) Multiple shoots on MS + 2.27 µM TDZ after 4 weeks. Bar equals 1.7 mm. (D) Formation of stunted shoots with profuse basal callus on MS + 45.41 µM TDZ. Bar equals 7.0 mm. (E) Multiple shoots on MS + 4.92 µM 2iP. Bar equals 7.1 mm. (F) Elongating multiple shoots on MS+ 9.12 µM Zeatin. Bar equals 7.1 mm. (G.) Multiple shoots on MS+ 10.86 µM AS. Bar equals 3.7 mm. (H) Rooting of *in vitro* raised shoots on quarter strength MS + 9.80 µM IBA after 8 weeks. Bar equals 5.3 mm. (I) Hardened plantlet in plastic pot. Bar equals 15.0 mm.

TDZ at a concentration of 2.27 µM induced a mean of 17.6 shoot buds with 83.3 percent frequency of shoot regeneration. These buds appeared as small green protuberances (Figure 1B) that elongated into shoots (Figure 1C) in 4 weeks of culture.

Maximum number of shoots per explant (19.0) and frequency of shoot regeneration (93.3%) obtained by the addition of ascorbic acid (0.1%) to the TDZ (2.27 µM) enriched medium in 8 weeks of culture (Table 1). Therefore, ascorbic acid was supplemented to the MS medium with cytokinins. Kaur et al. (1998) also indicated the positive effect of ascorbic acid on micro-propagation of *Acacia*

Table 1. Effect of different cytokinins + 0.1% ascorbic acid on shoot multiplication from cotyledonary nodes of *S. urens* after 8 weeks (4 weeks of initial culture + 4 weeks of first subculture).

Concentration of Plant growth regulators (µM)	Frequency of shoot regeneration (%)	Number of shoots per cotyledonary node	Mean length of the shoot (cm)
TDZ			
0.45	90.0^{d}	14.0^{d}	2.6^{c}
2.27	93.3^{d}	19.0^{e}	3.0^{c}
4.54	85.0^{bc}	12.0^{cd}	2.4^{b}
9.08	80.0^{ab}	10.6^{bc}	2.0^{b}
22.71	78.3^{a}	8.8^{ab}	1.8^{a}
45.41	75.0^{a}	7.0^{a}	1.4^{a}
2iP			
0.49	78.3^{cd}	3.0^{bcd}	4.4^{b}
2.46	80.0^{cd}	3.4^{cd}	5.8^{c}
4.92	81.7^{d}	4.0^{d}	6.0^{c}
9.84	76.7^{bc}	2.8^{abc}	4.2^{b}
24.61	73.3^{ab}	2.2^{ab}	3.6^{b}
49.21	70.0^{a}	1.8^{a}	2.2^{a}
Zeatin			
0.46	63.3^{a}	1.8^{a}	1.6^{a}
2.28	65.0^{ab}	2.6^{abc}	2.5^{b}
4.56	70.0^{bc}	3.0^{bc}	3.2^{c}
9.21	73.3^{c}	3.6^{c}	3.8^{c}
22.81	61.7^{a}	2.2^{ab}	2.3^{b}
45.62	60.0^{a}	1.6^{a}	1.4^{a}
AS			
0.54	50.0^{a}	1.2^{ab}	1.6^{bc}
2.71	51.7^{a}	1.8^{abc}	1.8^{c}
5.43	55.0^{b}	2.0^{bc}	2.0^{c}
10.86	60.0^{b}	2.4^{c}	2.1^{c}
27.14	53.3^{a}	1.6^{abc}	1.2^{ab}
54.29	48.3^{a}	1.0^{a}	1.0^{a}

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

catechu. The number of shoots per explant was highest (19.0) on MS medium with 2.27 µM TDZ followed by L2, WPM and B5 (Table 2). Consequently, in all experiments, only MS medium was used. Addition of auxins (IAA and NAA) to the MS medium failed to improve the shoot regeneration and caused undesirable callus. Garland and Stoltz (1981) also supported that in a number of cases, cytokinins alone are enough for optimal shoot multiplication as also indicated by the works of Amin and Jaiswal (1993), Aliou et al. (2006). The stimulatory effects of TDZ on bud break and shoot regeneration has been reported earlier for *Morus australis* (Pattnaik et al., 1996) and for several woody plant species (Huetteman and Preece, 1993). Comparison of Table 1 indicates that TDZ is a better cytokinin than other cytokinins used in this study for multiple shoot initiation since it is able to induce more number of shoots at much lower concentration. Although, high concentration of TDZ (45.41 µM) induced high amount of basal callus and more number of shoot buds but they failed to elongate (Figure 1D). The formation of stunted shoots on TDZ supplemented medium has been reported for *Rhododendron* sp. (Preece and Imel, 1991).

Inhibition of shoot elongation may be due to the high cytokinin activity of TDZ, whereas the presence of a phenyl group in TDZ may be possible cause of shoot bud fascination (Huetteman and Preece, 1993). The high stability

Table 2. Effect of various media supplemented with 2.27 µM TDZ + 0.1% ascorbic acid on shoot regeneration from cotyledonary nodal explants of *S. urens* after 8 weeks.

Media	Frequency of shoot regeneration (%)	Mean number of shoots per cotyledonary node	Mean length of the shoot (cm)
MS	93.3±0.5	19.0±1.0	3.0±0.1
L2	90.0±0.9	14.2±0.7	2.7±0.2
WPM	83.3±0.5	13.6±1.1	2.3±0.1
B5	60.0±0.4	10.0±0.8	2.0±0.2

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

of TDZ due to its resistance to cytokinin oxidase (Mok et al., 1987) might be a reason for the efficiency.

Multiple shoots were also induced in the cotyledonary nodes on medium containing 2iP, Zeatin, and AS. Although, the mean number of shoots per explant (4.0) low with 2iP (4.92 µM), it is able to produce shoots with highest height (6.0 cm) (Figure 1E) Zeatin at 9.12 µM induced an average of 3.6 shoots per explant (Figure 1F). Adenine sulphate was found to be the least effective of all cytokinins used (Table 1) (Figure 1G).

Subculture

A proliferating shoot culture was established by repeatedly sub-culturing the original explant (stump) after harvesting the shoots at every 4 week intervals. The beneficiary effect of sub-culturing the explant in shoot multiplication was earlier reported in *D. sissoo* (Pradhan et al., 1998). Repeated sub-culturing was said to be one of the methods of maintaining juvenility (Franclet et al., 1987). First subculture was carried out on the same medium (2.27 µM TDZ), which was used for shoot generation. But, continuous subculture on the same medium, explants showed a decrease in shoot regeneration potential (data has not shown). Further subcultures (from the second subculture onwards) were performed on MS + 0.45 µM TDZ. Reduction in the concentration of TDZ favored continuous production of shoots. This result was consonant with those of Amin and Jaiswal (1987) in Guava. This may be due to the change in endogenous plant growth regulator level during the period of culture establishment, which consequently led to the differential sensitivity of the tissue to exogenously supply of plant growth regulators as accounted by Okubo et al. (1991).

Rooting response

Excised shoots failed to produce roots on full strength, half strength and quarter strength MS medium without any growth regulator. This was earlier observed by Rao et al. (1998) in *Excoecaria agallocha*. Of the three auxin supplements used to induce root formulation (IBA, IAA and NAA), IBA at 9.80 µM was most effective when augmented with quarter strength MS medium (Table 3). The beneficiary effect of reducing the salt concentration of MS in *in vitro* rooting of shoots has been described in several reports (Manzanera and Pardos, 1990; Purohit et al., 1994). Quarter strength MS medium containing 19.80 µM IBA induced rooting in 80.0% cultures within 2 weeks. In 8 weeks period, an average of 4.0 roots had formed per shoot with an average of 3.8 cm in length (Figure 1H). The stimulatory effect of IBA on rooting was earlier reported in *E. agallocha* (Rao et al., 1998), *Melia azedarach* (Thakur et al., 1998), *Ficus carica* (Kumar et al., 1998), *Balanites aegyptica* (Mansor et al., 2003) and *Bambusa vulgaris* (Aliou et al., 2006).

Transfer of plantlets to soil

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. After 4 weeks, polythene bags were removed (Figure 1I) and pots were transferred to the garden and placed under shade till the new leaf appeared. Then they were planted under normal garden conditions.

Conclusion

In vitro propagation can become an important alternative to conventional propagation for wide range of plant species. A reproducible protocol for the in *vitro* propagation of *S. urens* has been developed in this study. Direct

Table 3. 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.

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 and provides a simple protocol for the mass propagation of this plant from cotyledonary nodes. Based on the data of the present study we can conclude that the reported regeneration system is repeatable and can be easily used to regenerate *S. urens* plants.

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