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In Vitro Rapid Multiplication of Wrightia Tomentosa (ROXB.) Roem. and Schultz an Endangered Medicinal Tree Species

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Abstract

The protocol for in vitro multiple shoot development and plantlet formation was developed using cotyledonary node and shoot tip explant in Wrightia tomentosa (Roxb.) Roem. and Schultz an endangered medicinally important forest tree. The explants were cultured on ¼ strength MS medium supplemented with various concentrations of BAP. Multiple shoots were induced from the cotyledonary nodal segments and shoot tip explants in all the concentrations of BAP. More percentage of multiple shoot induction was observed in cotyledonary nodal explants compared to shoot tips. Maximum number of shoots proliferation (12±1.07) with high frequency of shooting response was found in shoot tip explants followed by nodal explants at 3.0 mg/L BAP. The micro-shoots were cultured on ¼ strength MS medium with various concentrations of auxins IAA/IBA for in vitro rooting. Profuse rhizogenesis (16.2±2.01, 12.8±1.29 cm) was observed on ¼ strength MS medium supplemented with 1.0 mg/L IAA/1.5 mg/L IBA. The in vitro rooted plantlets were transferred to polycups containing sterile soil: vermiculite (1:1) and hardened in the culture room. These acclimatized plants were transferred into field and the survival percentage was found to be 75. These in vitro regenerated plants were similar to donor plant.

Keywords: *Wrightia tomentosa,* cotyledonary node, shoot tip, multiple shoots, *in vitro* rooting. Abbreviations: MS – Murashige and Skoog, BAP – 6-Benzyl amino purine, IAA – Indole-3-acetic acid, IBA – Indole-3-butyric acid.

Introduction

Plant based remedies have always been an integral part of traditional medicine throughout the world. The demand for herbal remedies has been increasing significantly[1]. Tissue culture techniques are being increasingly exploited for clonal multiplication and *in vitro* conservation of germplasm valuable indigenous threatened plants. Micropropagation offers a great potential for large-scale multiplication of such useful species and subsequent exploitation[2].

The species *Wrightia tomentosa* (Roxb.) Roem.andSchultz. (Apocynaceae) is an important woody medicinal plant. It is locally called as "Dudhi and Tella pala". Flowers, seeds, roots, bark and leaves of this plant parts are used to treat fever, snake bite, arthritis, rhinitis, tumors. Due to indiscriminate collection and over exploitation for medicinal use and toy making, this species has been disappearing very fast and now it has become an endangered. The plant is placed as category I of Red Data Book.

For *ex situ* conservation of an endangered medicinal plant, propagation through cotyledonary nodal segment and shoot tip culture is an economic way to obtain large number of plants within a short period of time. Micropropagation through culture of explants having pre-existing meristem is powerful options which allow multiplying genetically stable and true-to-type progeny of the species that are rare, endangered and difficult to propagate[3,4]. During past few years, there has been an increasing interest for *in vitro* multiplication and germplasm conservation of rare, endangered, aromatic and medicinal plants[5, 6]. We report here a reliable efficient protocol for rapid *in vitro* propagation of *W. tomentosa* using cotyledonary node and shoot tip explants.

Material and Methods

Plant Material: Pods were collected from the trees growing in the Kakatiya Arboretum, Forest Research and Development Divison, Forest Department Warangal District, Andhra Pradesh, India. Seeds were collected from pods and washed thoroughly under running tap water followed by tween-20 (5%-v/v). These were surface sterilized with 0.1% (w/v) $HgCl_2$ for 6 min. After surface sterilization seeds were dissected, isolated the zygotic embryos in the laminar air-flow chamber aseptically and were placed vertically on various culture media. The seeds were germinated aseptically for 6-8 days. The *in vitro* nodal segments and shoot tip explants were collected from a healthy plant for this study.

Terminal portions (5-7 cms long) of *in vitro* grown plants were selected for shoot tip culture. These shoot tips were dried on sterile tissue paper under aseptic conditions. Shoot tips were trimmed aseptically consisting of 1.0-1.5 cm size with leaf primordia and meristem. Later, these were implanted on ¼ strength MS medium supplemented with different plant growth regulators (PGRs).

For cotyledonary nodal culture, 2-3 weeks of *in vitro* grownplantlets were used as explants. These explants were dried on sterile tissue paper under aseptic conditions. The cotyledonary nodal segments were further cut into one node and inoculated on ¼ strength MS medium supplemented with different concentrations of PGRs.

Culture Media and Culture Conditions: Shoot tip explants were cultured on ¼ strength MS medium containing 7.5 g/L sucrose supplemented without growth regulators (MSO) and also different concentrations of growth regulators (1.0-5.0 mg/L) of BAP/Kn and also TDZ (0.2-1.0 mg/L) individually. For further proliferation and elongation of shoots from all the cultures were shifted on to ¼ strength MS medium fortified with 0.5 mg/L GA_3+ 3.0 mg/L BAP.

The cotyledonary nodal segments containing single node were inoculated on ¼ strength MS medium containing 7.5 g/L sucrose supplemented with different concentrations of growth regulators (1.0-5.0 mg/L) of BAP/Kn and TDZ (0.2-1.0 mg/L) individually and also in combination with 0.5mg/L IBA/IAA/NAA + BAP.

The pH of medium was adjusted to 5.8 with either 0.1N NaOH or 0.1N HCl before adding 0.8% (w/v) Difco-bacto agar. The culture medium was dispensed into different culture tubes (15 ml) and also culture bottles and autoclaved at 121^0C under 15 psi for 15-20 minutes. After inoculation, all the cultures were incubated under cool white-fluorescent lights at an intensity of 40-60 µmol m^{-2} s^{-1} for 16 hrs photoperiod at 25^0C. The cultures were maintained by regular subculturing for every 3 weeks on fresh media with the same concentration and combinations of PGRs.

Elongation of shoots: The shoots developed from *in vitro* cotyledonary nodal segments and shoot tip cultures were excised and cultured individually on ¼ strength MS medium supplemented with 0.5 mg/L GA_3+3.0 mg/L BAP for further proliferation and elongation of shoots.

***In vitro* rooting and plantlet establishment:** The isolated micro-shoots (6cm) were cultured on ¼ strength MS medium augmented with various concentrations of auxins IAA/IBA (0.5-3.0mg/L). The *in vitro* rooted plantlets were washed in sterile distilled water and transferred to plastic cups containing sterile garden soil: soilrite (3:1) covered with polythene bags to maintain the RH (85-90%). These were incubated in the culture for 4 weeks. After 4 weeks the fully acclimatized plants were transferred to the research field and maintained under shady conditions in the research field.

Data Analysis: Data were recorded after 4 weeks of culture. Each treatment consisted of 24 explants and each experiment was repeated at least twice. The percentage of response, multiple shoot induction, average length of shoots, average no. of roots, average length of roots per explants were calculated. The data were analyzed statistically using mean and standard deviation and standard error[7].

Results and Discussion

Morphogenic response of the explants (cotyledonary node and shoot tip) of *W. tomentosa* cultured on ¼ strength MS medium augmented with different concentrations of BAP (tables-1, 2). Explants cultured on ¼ strength MSO medium without PGRs failed to induce shoot proliferation.

Table-1
Effect of BAP on multiple shoot induction from shoot tip explants in *W. tomentosa*, [a]Mean±Standard Error

Type of medium	Concentration of BAP(mg/L)	(%) of response	Average number of multiple shoots(±SE)[a]	Average length (cms) of shoot(±SE)[a]
¼ strength MS medium	1.0	68	3.2±0.27	2.8±0.31
	2.0	64	6.7±0.51	3.2±0.42
	3.0	88	12±1.08	3.2±0.89
	4.0	56	2.2±0.18	3.8±0.22
	5.0	50	1.8±0.10	3.6±0.12

Table-2
Effect of BAP on multiple shoot induction from cotyledonary node explants in *W. tomentosa*, [a]Mean±Standard Error

Type of medium	Concentration of BAP(mg/L)	(%) of response	Average number of multiple shoots(±SE)[a]	Average length(cms) of shoots(±SE)[a]
¼ strength MS medium	1.0	92	10±0.61	3.1±0.81
	2.0	92	8.2±0.53	3.4±0.62
	3.0	96	8.6±0.42	3.4±0.87
	4.0	74	3.7±0.18	4.3±0.22
	5.0	60	2.3±0.12	4.2±0.10

Table-3
Effect of IAA/IBA on *in vitro* rooting of shoots developed from cotyledonary node and shoot tip explants of *W. tomentosa,*
[a]Mean±Standard Error

Type of medium	Concentration of PGRs(mg/L)	(%) of response	Average number of roots(±SE)[a]	Average length of roots(cms)(±SE)[a]
¼ strength MS medium	IAA			
	0.1	68	5.4±0.82	2.2±0.18
	0.5	71	5.8±0.91	2.3±0.26
	1.0	18	8.3±1.02	8.5±0.50
	1.5	90	16.0±2.01	14.0±1.20
	2.0	74	4.6±0.21	6.2±0.14
	IBA			
	0.1	70	2.4±0.26	3.0±0.54
	0.5	76	4.2±0.86	6.5±0.43
	1.0	85	13.0±1.29	12.0±1.28
	1.5	67	6.6±0.40	7.5±1.08
	2.0	60	8.2±0.16	5.7±0.39

MS medium supplemented with different concentrations of BAP (1-5mg/L) resulted in the induction of axillary shoots. The frequency of responding explants and the number of axillary shoots per explants increased with an increased in the concentration of BAP upto 2mg/L. These results indicated that BAP played an important role in the induction of multiple shoots. However, BAP at higher concentrations not only reduced the number of shoots formed but also resulted in stunted growth of the shoots.

Among different cytokinins and auxins alone and combination tested, BAP at 1.0mg/L gave maximum number of shoots at 10±0.529 followed by BAP 2.0mg/L. Further increased concentration of BAP decreased the number of shoots (table-2).

The shoot induction and multiple shoot development were observed in all concentrations (1.0-5.0mg/L) of BAP. Promotion of shoot multiplication by BAP has been reported in other medicinal plants, like *Holostemma ada-kodien*[8], *Macadamia tetraphylla*[9] and *Prunus armenica*[10]. The stimulating effect of BAP on multiple shoot formation has been reported for several medicinal plant species[6, 11-13].

Shoot tips cultured on ¼ strength MS medium supplemented with different concentration of BAP (1.0-5.0mg/L). Maximum numbers of shoots (12±1.08) were obtained from ¼ strength MS medium supplemented with 3mg/L BAP with 68% shooting response (table-1). Similar results were obtained in *Baliospermum montanum*[14] and *Morus niger*[15].

Multiple shoots developed from explants of cotyledonary node and shoot tip they did not elongate further. Therefore shoots were transferred on ¼ strength MS medium supplemented with GA_3 (0.1-2mg/L) to ensure their normal elongation. All the shoots were elongated on the GA_3 treatment but optimum shoot length (7.2cm) was recorded on ¼ strength MS 1mg/L GA_3. GA_3 is known to have stimulatory effect on shoot elongation in some other species also[16, 17].

***In vitro* rooting and plantlet establishment:** Rooting of the developed shoots was usually achieved in auxin containing medium[18]. Elongated shoots were separated and cultured on ¼ strength MS medium fortified with different concentrations (0.5-2.5mg/L) of auxins (IAA and IBA). Lower concentration of auxins (IAA, IBA) enhanced the rooting response (figure-1). Maximum number of roots (16.2 and 12.8) was recorded at 1.5mg/L IAA and 1.0mg/L IBA. These concentrations produced longer roots (14.0cm and 12.0cm). Development of such lengthy rootlets would be very much helpful for the establishment of plantlets in the field. Similar effect of IBA and IAA was also observed on *in vitro* rooting in several medicinal plant species various *Sterculia urens*[19], *Azadirachta indica*[20], *Plucgea lanceolata*[21].

During *in vitro* hardening, shoots elongated, leaves turned greener and expanded and root system become extensive. Such plants also grew more vigorously in the green house. Out of 40 plantlets 30 hardened successfully and over 30 plants were transferred in to field and maintained under shady conditions where they are growing well. The established plants did not show any morphological variations or growth characteristics in comparison to mother plant.

Conclusion

A reproducible protocol for the *in vitro* propagation of *W. tomentosa* has been developed during the present investigations. Thus, this efficient *in vitro* procedure of regeneration from cotyledonary node and shoot tip explants can be used to maintain clonal fidelity of this valuable genotype. This protocol can also be useful for the conservation and mass propagation of this critically an endangered medicinally important forest tree.

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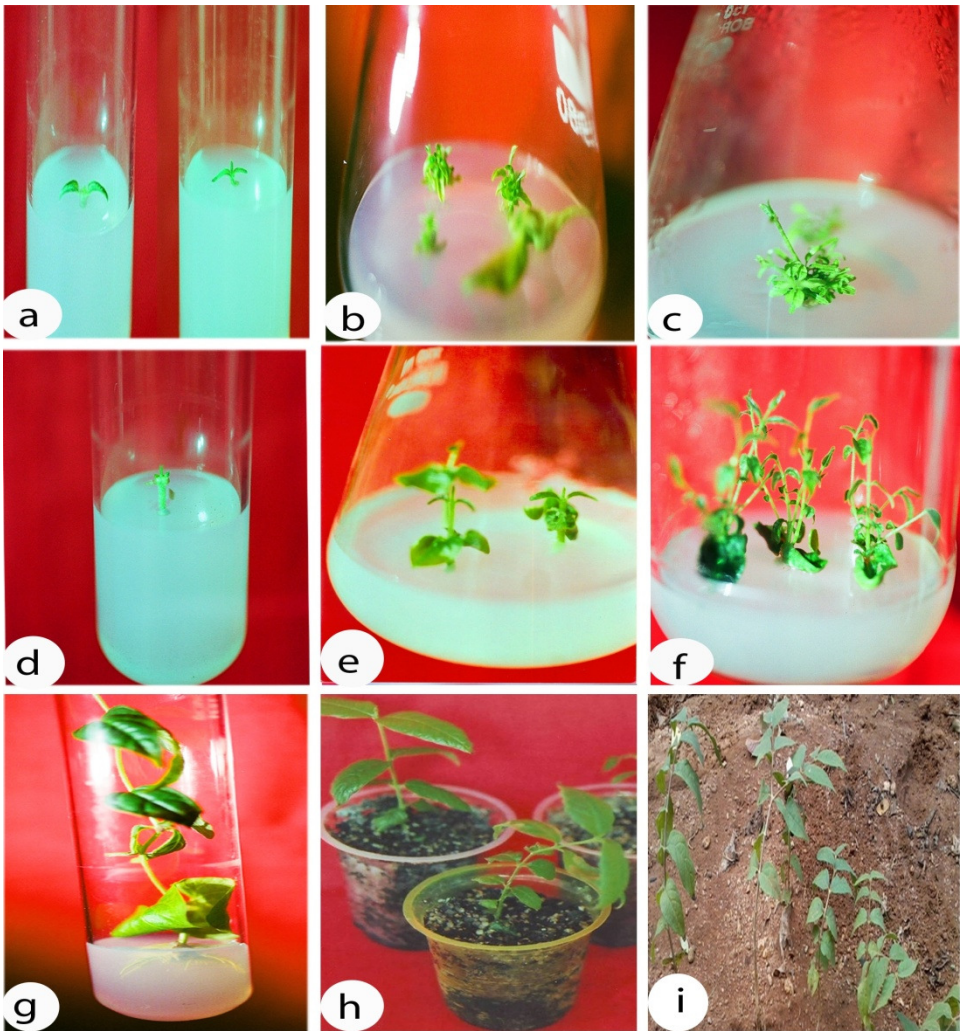

Figure-1

a-i: Shoot tip and Cotyledonary nodal culture on ¼ strength MS + BAP in *W. tomentosa.*

(a) Shoot tip on ¼ strength MSO, (b). Multiple shoots formation at 3.0 mg/L BAP, (c). Multiple shoots with axillary branching at 1.0 mg/L BAP after 4 weeks of culture. (d). Initial stage of cotyledonary node. (e). Axillary node proliferation at 1.0 mg/L BAP, (f). Further proliferation of multiple shoots at 1.0 mg/L BAP after 4th weeks of culture.(g). Rooting on ¼ strength MS + 1.5 mg/L IAA, (h). Acclimatization, (i). Successfully transferred in to field

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