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Rapid micro-propagation of *Wrightia tinctoria* (ROXB.) R Br: A medicinal tree

*** Meenu R Mridula, Ashalatha S Nair**
Department of Botany, University of Kerala, Karyavattom, Kerala, India

Abstract
A protocol for in vitro production and acclimatization of *wrightia tinctoria* (rox.) R. Br., is reported. Under the regime used in the study, the most critical factor for rooting was activated charcoal (0.05%) along with 6 µM Naphthalene aceticacid. The plantlets hardened with longer photoperiods had better survival percentages when compared with those acclimatised over shorter photoperiods. Hardened plantlets obtained within 45 days, and replanted with 86.8 % survival. This in vitro method can be used for rapid mass clonal propagation of W. tinctoria for conservation strategies, commercial production irrespective of season and gene transformation studies for the production of phytomedicines.

Keywords: *Wrightia tinctoria*, micropropagation, activated charcoal, benzyl aminopurine, napthatene aceticacid, acclimatisation

1. Introduction

Wrightia tinctoria is a medium sized tree in the moist deciduous forests in India. It is chiefly valued for the active principle in its leaves. The plant, known as "Pala Indigo" in commerce is one among the Indigo dye yielding plants in India (Devi *et al.* 2012) [5]. It is a principal indigenous plant, used in the Eastern systems of medicinal practices like Ayurveda and Siddha (Muthu *et al. 2006*) [17]. The bark of the tree possesses proteolytic activity and the compounds isolated from the bark include β-amyrin, lupeol (terpinoid) and β-sitosterol (The Wealth of India, 1976) [2]. The reported constituents from seed pods are alkaloids, terpenes (wrightial and trytanthrins) indole and flavanoids (Ramachandra *et al*. 1993) [22]. The five flavonoid compounds, Indigotin, Indirubin, tryptanthrin, isatin and rutin were isolated and identified from the leaves (Muruganandam *et al*. 2000) [16]. Studies with hexane, methanol and ethanol extract from leaves proved its activity against bacteria and dermatophytic fungi (Sridhar *et al.* 2011; Kannan *et al*. 2006 and Moorthy *et al*. 2010) [26, 10, 13]. Traditionally, the leaf decoction in oil are used to cure dandruff and as a cure for skin diseases. Decoction from seeds are used to treat indigestion (Muthu *et al. 2006*) [17]. It has also found popularity in modern herbal formulations primarily for the treatment of skin ailments, in particular Psoriasis (Kanaujia *et al. 2003*) [9]. The flowers, barks and leaves are proposed to have activity against breast cancer cell lines (Chakravarti *et al. 2012*) [4]. It has been reported that the aqueous extract displayed potent inhibitory activity against both step of HIV-1 IN enzymatic activity (Selvam *et al.* 2009) [23]. Decoction from seeds are used to treat indigestion (Muthu *et al. 2006*) [17]. The seeds are also used as antihelminthic (Khyade and Vaikos 2014) [11]. The good quality white wood is used in carpentry and it is a good agroforestry species as it inter-crops well (Orwa *et al*. 2009) [19]. Within the regions where the plant is found the indigenous population have found uses for the entire plant and/or its parts (Kanaujia *et al.* 2013) [9] making it an important silviculture species. Leaf flushing in this woody species is a periodic phenomenon which is completed around April and May with complete absence of leaf flushing from September to January (Yadav and Yadav 2008) [29]. The seeds are viable for six months and has 46% germinative capacity (Chako *et al.* 2001) [3]. Earlier reports on micropropagtion identified basal callus formation as one of the main constrains in rooting, acclimatization and establishment (Purohit and Kukda 2004) [21]. This study report an effective micro propagation protocol for this tree species.

2. Materials and methods

Plant material and In-vitro culture conditions

In the present study a single field grown ortet was used in order to minimize variations caused by genetic variability and statistical errors on data analysis (Bonga and Patrick 1992) [1]. Nodal regions derived from the fresh flushes of growth from the ortet, two weeks after lopping one major branch served as the explants (Purohit *et al*. 1994) [20]. Explants were rinsed in 2% polysorbitol detergent - Labolene (Fischer Scientific Chemicals) and treated with 0.3% (w/v) carbondazim fungicide (Sigma Aldrich) for 15 min. Cleaned explants were surface sterilized with 0.1% mercuric chloride for 4-5 minutes. Murashige and Skoog (1962) [14] medium with 3% sucrose, 0.75% agar (Sigma Aldrich, A9799 SIGMA) supplemented with auxins and cytokinins were used. Media P^H was set to 5.8 prior to autoclaving (120ºC, 1Kg $cm^{-2}s^{-1}$ for 15 min). Five to seven explants were cultured in 250 ml culture flasks on 50 ml of sterilized medium and incubated at 25±2°C in 40 µmol m^{-2} s^{-1} irradiance to a photoperiod of 8 h and 55±5 % of relative humidity. Full strength MS medium fortified with 2 µM BAP and NAA at 2, 4 or 6 µM was used for initial establishment of explants for 10 days. For subculture, the media was supplemented with 0.05% or 0.10%

activated charcoal (AC) for facilitating for simultaneous shooting and rooting. Two weeks after subculture the shoots with roots were transferred directly to mini pots containing potting mixture (sand and vermiculite 1:1); after 3 minute dip in bavestin (Sigma-Aldrich) at (0.1,0.5 or 1)%, watered and covered with polypropylene bags (Cole-Parmer 1.5 mil). Acclimatization for seven days was ensured with the maximum relative humidity during which the plants were not watered. The temperature was 29 ± 2^0 C during day and 25 ± 2^0 C during night with 2 photo period of 10±2 hours and 12±2.

Data acquisition

A total of ten parameters during two temporal phases vis. at the end of *in vitro* period and at the end of *ex vitro* acclimatization period were recorded. Two weeks after subcultre ie. at the end of in vitro period; 7 parameters were recorded such as: shoot length of longest shoot measured from the basis to shoot apex, number of shoots, root length of the longest root measured from the basis to the root tip, root number, *in vitro* leaves per explant, number of abscised leaf, and pigments(chlorophyll a, chlorophyll b & carotenoids) per unit area of leaf. Two weeks after acclimatization under two photoperiods, 3 parameters were recorded such as: survival percentage, *ex vitro* leaf per plantlet and pigments (chlorophyll a, chlorophyll b & carotenoids) per unit area of leaf.

Data analysis

All experiments were conducted using Randomized Block Design (RBD) and each treatment consisted of 5-7 explants in a culture flask with three replications. Count data was analysed using Poisson regression model and continuous data using multiple linear regression in R. Association of treatments was assessed by the two degree of freedom χ 2 test.

Fidelity testing using ISSR markers

The regenerated plantlets were used to for ISSR analysis. Ten randomly selected micro-propagated plantlets were pooled together and Genomic DNA was isolated using the modified method of Murray and Thompson (1980) [15]. The DNA so obtained was then quantified using the bio photometer (Eppendorf Bio Photometer Plus, Germany). The quantitated DNA was diluted to final concentration of 25 ng/μL in TE buffer (10 mM Tris Cl, 1 mM EDTA, pH 8.0). The diluted DNA was again confirmed for accurate measurement on 0.8% agarose gel (SRL, SISCO, Mumbai). All the diluted DNA samples were ranged from 23–27 ng/μL. Fifteen UBC primers were screened for fidelity analysis using modified method of Jimenez *et al.* 2015. The amplification reactions were performed in a final volume of 25 μL containing 10 mM Tris-HCl at pH 8.0, 15 mM KCl, 2.5 mM $MgCl_2$, 10 mM dNTPs, 1 U Taq DNA polymerase, and 4pM DNA primer. Amplifications carried out in an Eppendorf Mastercycler thermocycler were performed as follows: Initial denaturation at 95°C for 13 min, followed by 40 cycles of denaturation at 94°C for 45 s, annealing temperature using gradient PCR for each primer for 45 s, extension at 72°C for 2 min, and a final extension at 72°C for 5 min at the end of the amplification cycles. The amplification products were separated by electrophoresis under non-denaturing conditions on 1.5% agarose gels (SRL, SISCO, Mumbai). The amplified fragments were detected by Et-Br staining silver-staining and fragment size was determined by the use of a 50-bp step ladder (S7025, Sigma-Aldrich, St. Louis). DNA amplifications with each ISSR primer were repeated at least twice to ensure reproducibility. The bands were considered reproducible and scorable only after observing and comparing them in two separate amplifications for each primer. Clear and intense bands were scored while faint bands against background smear were not considered for further analysis.

3. Results

The explants inoculated on MS medium supplemented with 6 μM NAA and 0.05% AC, produced highest shoot and root number and the longest roots. Maximum shoot length and chlorophyl a content was observed in plants cultured on MS medium supplemented with 6 μM NAA and 0.1 % AC. *In vitro* leaves were maximum on the explants inoculated on MS medium supplemented with 4 μM NAA and 0.1% AC. The shoots generated on MS medium supplemented with 4 μM NAA and 0.05% AC showed maximum leaf fall (Table 1). Highest survival percentage (86.8 ± 2.1) was obtained with the longer photoperiods and 0.5% fungicide treatment (Table 2). Leaf fall was not observed during acclimatization period and leaves generated *in vitro* were retained during acclimatization and further growth stages. In total 15 ISSR primers were screened, of which seven primers resulted in scorable bands and were selected for further study. These seven ISSR primers generated 41 amplicons, ranging from 107.67 to 483.1 bp in size. The number of bands in the selected primers varied from 8 (UBC 808, UBC826) to 3 (UBC 823), with an average of 5.8 bands per ISSR primer (Table 3). All the amplicons obtained from ISSR markers were found to be monomorphic against selected micro propagated plantlets and where similar to mother plant (Fig. 4).

Table 1: Parameters studied during simultaneous shooting and rooting

NAA (µM)	AC (%)	Shoot number	Shoot length (cm)	Root number	Root length (cm)	Number of *in vitro* leaves	Number of abscessed leaves	Chlorophyll a	Chlorophyll b	Carotenoids
2	0	3.8 ± 0.58 ^{ab}	3.44 ± 0.12 ^{c}	0	0	9.3 ± 0.81 ^{abc}	3.1 ± 0.45 ^{a}	0.128 ± 0.002 ^{a}	0.322 ± 0.001 ^{ab}	0.219 ± 0.001 ^{b}
	0.05	3.3 ± 0.58 ^{a}	3.88 ± 0.12 ^{c}	1.50 ± 0.46 ^{ab}	5.00 ± 0.24 ^{ab}	8.4 ± 0.81 ^{ab}	4.1 ± 0.45 ^{a}	0.129 ± 0.002 ^{ab}	0.327 ± 0.001 ^{b}	0.219 ± 0.001 ^{b}
	0.1	6.2 ± 0.58 ^{bc}	2.38 ± 0.12 ^{b}	2.80 ± 0.46 $_{b}$	5.00 ± 0.24 ^{e}	5.8 ± 0.81 ^{a}	3.2 ± 0.45 ^{a}	0.121 ± 0.002 ^{abc}	0.319 ± 0.001 ^{ab}	0.216 ± 0.001 ^{ab}
4	0	6.3 ± 0.58 ^{bc}	2.42 ± 0.12 ^{b}	0	0	9.1 ± 0.81 ^{abc}	3.8 ± 0.45 ^{a}	0.116 ± 0.002 ^{abc}	0.320 ± 0.001 ^{ab}	0.212 ± 0.001 ^{a}
	0.05	4.9 ± 0.58 ^{abc}	1.06 ± 0.12 ^{a}	3.50 ± 0.46 ^{b}	1.61 ± 0.24 ^{bc}	11.0 ± 0.81 ^{bc}	4.6 ± 0.45 ^{a}	0.134 ± 0.002 ^{bc}	0.322 ± 0.001 ^{ab}	0.221 ± 0.001 ^{b}
	0.1	4.5 ± 0.58 ^{abc}	1.79 ± 0.12 ^{a}	2.20 ± 0.46 ^{b}	1.98 ± 0.24 ^{cd}	12.6 ± 0.81 ^{c}	3.6 ± 0.45 ^{a}	0.132 ± 0.002 ^{bc}	0.323 ± 0.001 ^{ab}	0.221 ± 0.001 ^{b}
6	0	5.4 ± 0.58 ^{abc}	3.81 ± 0.12 ^{c}	0	0	10.5 ± 0.81 ^{bc}	3.6 ± 0.45 ^{a}	0.136 ± 0.002 ^{c}	0.323 ± 0.001 ^{ab}	0.221 ± 0.001 ^{b}
	0.05	6.6 ± 0.58 ^{c}	3.56 ± 0.12 ^{c}	3.40 ± 0.46 ^{b}	5.01 ± 0.24 ^{e}	12.2 ± 0.81 ^{c}	4.2 ± 0.45 ^{a}	0.137 ± 0.002 ^{c}	0.323 ± 0.001 ^{ab}	0.221 ± 0.001 ^{b}
	0.1	4.9 ± 0.58 ^{abc}	3.91 ± 0.12 c	2.90 ± 0.46 ^{b}	2.01 ± 0.24 ^{cd}	11.8 ± 0.81 ^{bc}	4.2 ± 0.45 ^{a}	0.138 ± 0.002 ^{c}	0.316 ± 0.001 ^{a}	0.221 ± 0.001 ^{b}

Data represented as mean ± SD. Values with similar superscript are not significantly different at $p > 0.005$

Table 2: Parameters studied during acclimatization

Bavestine (%)	Photoperiod	Survival %	Number of *ex vitro* leaf	Chlorophyll a	Clhorophyll b	Carotenoids
0.1	10	82.94 ± 2.1 ^{b}	2.8 ± 0.23 ^{b}	0.331 ± 0.000 ^{a}	0.565 ± 0.002 ^{a}	0.35 ± 0.005 ^{a}
	12	85.15 ± 2.1 ^{b}	2.4 ± 0.23 ^{ab}	0.3312 ± 0.000 ^{a}	0.5632 ± 0.002 ^{a}	0.354 ± 0.005 ^{a}
0.5	10	85.15 ± 2.1 ^{b}	1.9 ± 0.23 ^{ab}	0.3318 ± 0.000 ^{a}	0.5578 ± 0.002 ^{a}	0.366 ± 0.005 ^{a}
	12	86.8 ± 2.1 ^{b}	2.5 ± 0.23 ^{ab}	0.3318 ± 0.000 ^{a}	0.5578 ± 0.002 ^{a}	0.366 ± 0.005 ^{a}
1	10	48.8 ± 2.3 ^{a}	1.8 ± 0.23 ^{a}	0.331 ± 0.000 ^{a}	0.5632 ± 0.002 ^{a}	0.354 ± 0.005 ^{a}
	12	36.7 ± 2.3 ^{a}	2.4 ± 0.23 ^{ab}	0.331 ± 0.000 ^{a}	0.565 ± 0.002 ^{a}	0.35 ± 0.005 ^{a}

Data represented as mean ± SD. Values with similar superscript are not significantly different at $p > 0.005$

Table 3: ISSR primers used to screen micro-propagated *W. tinctoria* plants and the amplicons generated

Primer code	Primer Sequence (5'-3')	Annealing temperature(°C)	No. of total bands	Approx size range (bp)
UBC808	$(AG)_8C$	45.8	8	483.1-51.35
UBC809	$(AG)_8G$	44.2	4	456.55-107.65
UBC818	$(CA)_8G$	44.2	6	469.85-108.65
UBC820	$(GT)_8C$	41.4	5	461.55-258.9
UBC823	$(TC)_8C$	41.9	3	350.7-232.7
UBC826	$(AC)_8C$	46.5	8	465.95-144.15
UBC827	$(AC)_8G$	42.6	7	467.5-231.5

4. Discussion

The micropropagation strategy developed during this study eliminated a second culture period for rooting as it is achieved alongside shooting. Earlier studies reported rooting in *W. tintoria* in quarter strength MS medium containing 0.02 % AC (Purohit and Kukda 2004) [21]. The micropropagation protocol used in the study may be summarised as -Stage I- Inoculation of nodal explants on full strength MS media supplemented with 2 µM BAP and 6 µM NAA, for *in vitro* conditioning of explants for 10 days; Stage II- *In vitro* simultaneous shooting and rooting on full strength MS media supplemented with 2 µM BAP and 6 µM NAA with 0.05% AC for 14 days (Fig. 1). Stage III- Potting of plantlets in sand and vermiculite (1:1) and acclimatization for 7 days under green house conditions with 12 hr photo-period (Fig. 2). and Stage IV- Growth in green house conditions (Fig. 3). To promote *ex vitro* survival and physiological competence, especially to protect them against water stress and to encourage autotrophy a transitional environment is usually provided as acclimation interval of one to several weeks (Fabbri *et al*. 1986) [7]. Root formation is an energy demanding process and therefore most protocols aim at transforming the plants into autotrophic mode of nutrition (Serret *et al*. 1997) [24]. Thus *ex vitro* rooting- combining both the rooting and hardening phase have been demonstrated in rooting of tissue culture propagules in order to improve rooting quality (Nowak and Pruski 2004) [18].

Multiple comparison ($p > 0.001$) demonstrated that the highest response in terms of shoot and root length, chlorophyll a content and carotenoid was obtained when 6 µM NAA was present in the medium along with AC at 0.05%. The optimum survival was obtained with lower concentration of bavestine (0.1-0.5%). Twelve hour photoperiod was associated with significantly ($p>0.001$) higher number of *ex vitro* leaves generated as well as higher survival percentages. Other variables were not found to be significantly correlated to the independent variables studied. X^2 analysis suggested that NAA and its interaction with AC had a significant effect on root number ($p>0.001$). Further more, NAA concentration is a statistically significant indicator of shoot length, *in vitro* leaves, chlorophyll a ($p>0.001$) and carotenoids ($p>0.05$). Chlorophyll b content was not dependent on the factors studied at any of the concentrations. Higher concentration of NAA in the medium was required to obtain longer microshoots with more number of leaves in the tested treatments. The best rooting results were observed on the medium supplemented with 6 µM NAA and 0.05% AC indicating a significant positive effect of NAA and AC on *in vitro* simultaneous shooting and rooting of *W. tinctoria.*

The role of AC in plant tissue culture has been reviewed by Thomas (2008) [27]. In woody perennials AC promotes or inhibits *in vitro* growth, depending on the species and tissues used (Vengadeshan *et al*. 2002). In the present study AC had a positive and stimulatory effect in rooting of shoots by reducing basal callus. Similar results were reported in *Acacia leucophloea* and *Cinnamomum verum* (Sharma *et al. 2012* and Mathai *et al. 1997*) [12, 25]. The significant positive effect of longer photoperiods on survival and leaf flushing during acclimatization of *W. tinctoria* was also established.. Leaf chlorophyll content is often highly correlated with leaf photosynthetic capacity (Evans, 1983) [6]. In *W. tinctoria* the chlorophyll content during *ex vitro* acclimatization was significantly lower than during *in vitro* conditions. In photomixotrophically grown plants such abrupt decrease in chlorophyll a and chlorophyll b contents during the first week after transplantation followed by a slow increase has been discussed (Kadleček *et al*. 1998) [8]. The work illustrated the effect of NAA on the parameters usually employed as references of plantlet growth and quality and also showed that AC plays a significant effect on parameters directly related to rooting.

The study suggested that according to the scheme employed, true to type hardened plantlets (maximum of three from a single nodal explant) can be obtained as early as 45 days. This system can be used for rapid mass clonal propagation of *W. tinctoria* for conservation strategies, commercial production irrespective of season, gene transformation studies and to produce phytomedicines.

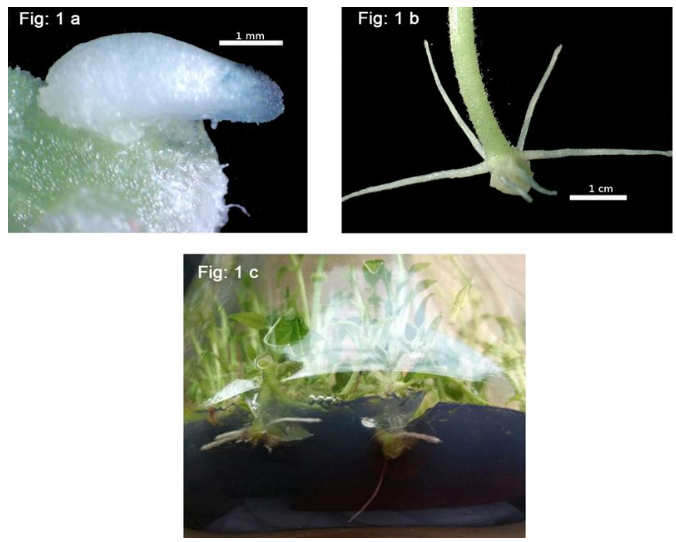

Fig 1: Rooting response in *Wrightia tinctoria (a)* emerging roots 7 days after *in vitro* culture. b- Roots after 14 days. 1c- simultaneous shooting and rooting achieved inside culture flask (14 days).

Fig 2: *Wrightia tinctorial* plantlet during acclimatization period.

Fig 3: Green house grown plant ready for transplantation (30 days).

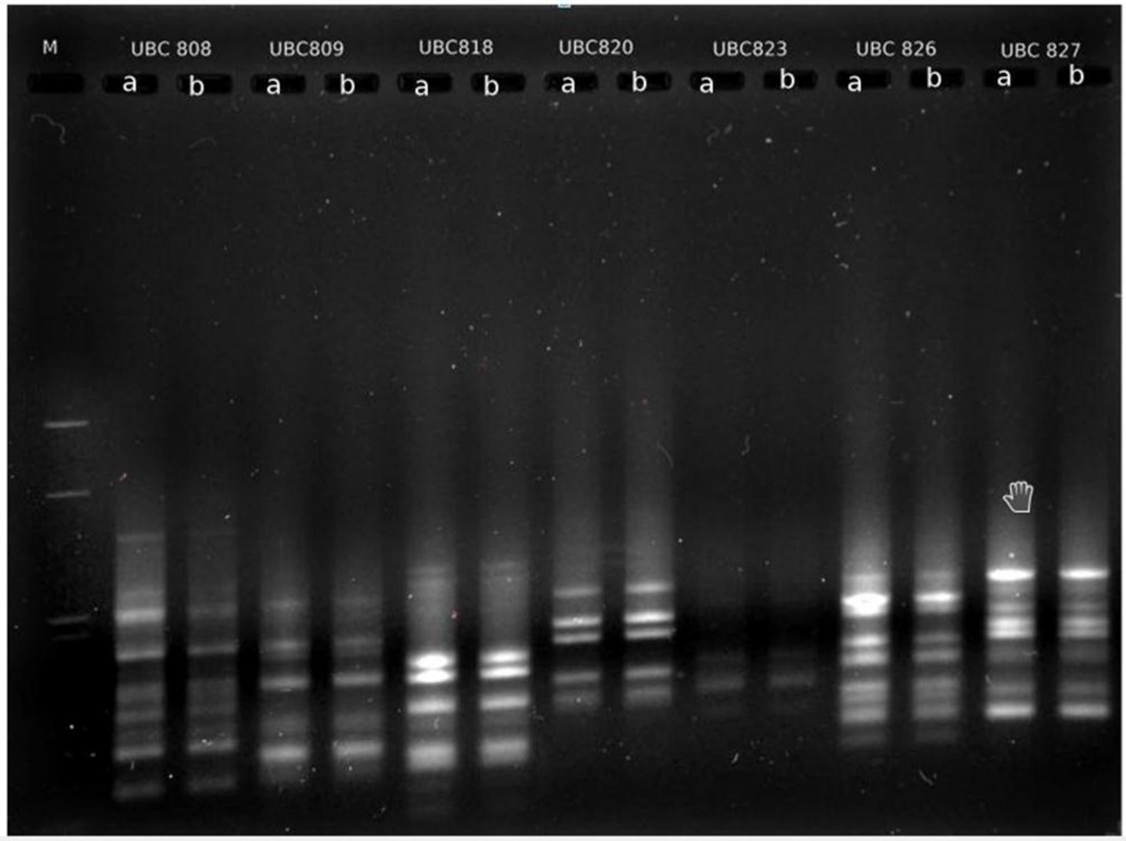

Fig 4: ISSR profiles of mother plant (lanes a) and in vitro shoots (lanes b) at the end of acclimatization period

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