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In vitro propagation of *Wrightia tinctoria*

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Abstract

In vitro method has been developed for propagation of *Wrightia tinctoria* R.Br. using cotyledonary node segments. Murashige and Skoog's (MS) medium supplemented with 5.0 mg dm^{-3} of 6-benzylaminopurine (BAP) and 0.01 mg dm^{-3} of naphthaleneacetic acid (NAA) induced up to eight shoots per explant with an average shoot length of 1.4 cm in 21 d. Three fold multiplication rate was achieved during every subculture of regenerated shoots on the same medium producing an average of 230 shoots per node within 84 d. Reduction in BAP concentration from 5.0 to 1.0 mg dm^{-3} during subculture promoted shoot length without affecting the rate of multiplication. The differentiated shoots could be rooted by a dip treatment into preautoclaved indole-3-butyric acid (IBA - 500 mg dm^{-3} for 5 min) followed by their implantation onto MS medium containing 1/4 salts. Rooting was observed within 8 -10 d in approximately 80 % of shoots inoculated after IBA treatment. 15 d after rooting, the plantlets were transferred to culture bottles containing soil-Soilrite™ (1:1) and liquid nutrient solution comprising 1/4 MS salts. After their partial hardening in these bottles for 10 d they were transferred to pots containing soil-Soilrite (1:1) mixture with 60 % transplantation success. Methods are being standardized to improve the rate of survival and large scale field transfer.

Introduction

Wrightia tinctoria R.Br. (*Apocynaceae*) valued for its wood used for toy making in India has been over-exploited by the people engaged in toy wood industry

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Abbreviations: IBA - indole-3-butyric acid; NAA - napthaleneacetic acid; 2,4-D - 2,4 dichlorophenoxyacetic acid; IAA - indole-3-acetic acid; 2,4,5-T - 2,4,5-trichlorophenoxyacetic acid; BAP - 6-benzylaminopurine; MS - Murashige and Skoog's (1962) medium; B_5 - Gamborg's medium; SH - Schenk and Hildebrandt medium.

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reducing its population considerably. There is therefore strong need for production of a large number of propagules. Traditional multiplication methods of this plant are limited by difficulty in rooting of cutting, high seedling mortality rates and low seed viability period.

Plant tissue culture biotechnology offers an opportunity for rapid clonal multiplication of desired tree species (Biondi and Thorpe 1981, Chalupa 1988, 1990, Ahuja 1991, Shekhawat *et al.* 1993). Rapid *in vitro* asexual multiplication can be achieved by enhanced axillary bud breaking, production of adventitious buds and somatic embryogenesis (Thorpe and Patel 1984). For the purpose of multiplication, the starting explant may be taken from either juvenile or mature trees or tree of any age in between (Aitken-Christie and Connett 1992). Plantlets produced from juvenile tissues of seedlings are considered untested clones unless they have been obtained from a seed produced through controlled pollination (Aitken-Christie and Thorpe 1984). Self pollination reported in *Wrightia tinctoria* (Reddi *et al.* 1979) offers this advantage even if the seedling material is used as starting explant for *in vitro* plantlet regeneration.

Materials and methods

Mature and dry follicles of *W. tinctoria* were collected from an identified superior tree in the forest areas of Kevda, near Udaipur. The seeds were surface sterilized with 0.2 % mercuric chloride for 5 min, washed thoroughly with autoclaved distilled water and inoculated aseptically on 0.8 % water agar for germination. Cotyledonary or epicotyledonary nodes and hypocotyl explants obtained from three week old *in vitro* grown seedlings were cultured on MS basal medium supplemented with various concentrations of cytokinins (0.5 - 8.0 mg dm^{-3} BAP and kinetin) individually and in combination and also with different concentrations (0.1 - 1.0 mg dm^{-3}) of NAA for multiple shoot proliferation. Other media like B_5, SH and White's medium were also screened to optimize the salt requirement by the proliferating explants. Repeated subculturing of the regenerated shoots was done every three weeks on the same or modified medium. During every subculture, cluster of shoots was separated into small groups and transferred onto medium for further proliferation. Elongated shoots were excised carefully and transferred aseptically after proper treatment, wherever necessary, with cut end slightly inserted in the medium, for rooting. Rooting hormones like NAA, IAA, IBA, 2,4-D and 2,4,5-T were used individually in different concentrations (0.1 - 2.0 mg dm^{-3}). A dip treatment of 50 - 2000 mg dm^{-3} of pre-autoclaved IBA for 5 min was also given to excised shoots for root induction. IBA dipped shoots were then implanted onto MS medium containing varying salts (basal, 3/4, 1/2, 1/4 and 1/8) and reduced sucrose level (1.0 %). The pH of the medium was adjusted to 5.8 prior to autoclaving at 1.06 kg cm^{-2} (121 °C) for 15 min. Each treatment consisted of 20 replicates and each experiment was repeated thrice. All the cultures were incubated under a 16 h photoperiod (irradiance of 45 µmol m^{-2} s^{-1}), relative humidity of 60 % and temperature of 26 ± 2 °C.

Fifteen days after rooting, the plantlets were transferred to culture bottles containing soil-Soilrite™ (*Karnataka Explosives Ltd.*, Bangalore, India) mixture (1:1) and fortified with nutrient solution of 1/4 MS salts. Such plants were subsequently shifted to pots containing soil-Soilrite (1:1) mixture and kept under polythene covered chambers which were spray misted to maintain relative humidity of more than 70 %.

Results and discussion

Selection of explant: Among different explants (cotyledonary or epicotyledonary nodes and hypocotyl) tested for shoot proliferation, cotyledonary node segments measuring 2.0 - 2.5 cm were found most suitable. MS medium was found to be the best medium for shoot proliferation followed by B_5, SH and White's media.

Initiation of shoots on explants: The cotyledonary nodes inoculated on MS medium responded differently to various cytokinins (Table 1). In the absence of growth regulators they did not produce any shoot. Average 2.5 shoots (mean length 3.6 cm) per node were obtained within 21 d of inoculation of explants on the medium containing 5.0 mg dm^{-3} of kinetin alone. The other kinetin concentration tested showed poor growth of shoots and reduction in shoot length. Shoots subcultured on medium containing optimum concentration of kinetin showed increase in length upto 4.8 cm but there was no further multiplication. BAP, on the other hand, when used individually (5.0 mg dm^{-3}) produced 7 - 8 shoots per node in 21 d (Table 1).

Table 1. Effect of cytokinins on the multiple shoot induction from the cotyledonary node segments of *W. tinctoria* on MS medium. Data were recorded after 3 weeks, each treatment consisted of 20 replicates and was repeated thrice. Mean ± S.D.

Kinetin [mg dm^{-3}]	BAP [mg dm^{-3}]	Number of shoots	Shoot length [cm]	Remarks
Control		-	-	-
0.5	-	1.3 ± 0.91	2.4 ± 0.16	Callus
1.0	-	1.6 ± 0.54	2.7 ± 0.11	Callus, broad leaf
2.0	-	2.0 ± 0.70	3.0 ± 0.14	Fleshy shoot, broad leaf
5.0	-	2.5 ± 0.84	3.6 ± 0.26	Fleshy shoot, broad leaf
8.0	-	1.7 ± 0.57	2.8 ± 0.15	Fleshy shoot, small leaf
-	0.5	2.1 ± 0.44	2.3 ± 0.15	-
-	1.0	3.4 ± 0.54	2.8 ± 0.08	Little callus
-	2.0	4.6 ± 0.70	2.2 ± 0.13	Moderate callus
-	5.0	7.6 ± 0.54	1.2 ± 0.19	-
-	8.0	5.2 ± 0.54	0.9 ± 0.11	Stunted internode
0.5	0.5	2.2 ± 0.44	2.2 ± 0.11	Moderate callus
1.0	1.0	3.2 ± 0.44	2.4 ± 0.18	Moderate callus
2.0	2.0	3.6 ± 0.54	2.7 ± 0.11	-
3.5	3.5	5.6 ± 0.54	3.3 ± 0.13	-
5.0	5.0	7.5 ± 0.64	3.8 ± 0.28	-

Generally, the shoots on BAP fortified media were shorter as compared to those obtained with kinetin. On the optimum BAP concentration an average of 1.2 cm shoot length was observed. The leaves of shoots regenerated on BAP containing media were smaller in size and showed yellowing. Any variation in BAP concentration in the medium than optimum either reduced the number of shoots or caused suppression of shoot length to a great extent. No concentration of combined cytokinins (BAP and kinetin) in the medium could improve the shoot length as well as proliferation.

Considering BAP at 5.0 mg dm^{-3} as optimum for maximum shoot proliferation, different concentrations (0.1 - 1.0 mg dm^{-3}) of NAA were added in the medium along with BAP. With 0.01 mg dm^{-3} NAA combined with 5.0 mg dm^{-3} of BAP, there was improvement in overall growth of cultures with slight increase in shoot length (Fig. 1). Higher concentrations of NAA caused callusing. It was therefore concluded that a combination of 5.0 mg dm^{-3} of BAP and 0.01 mg dm^{-3} NAA was optimum for shoot proliferation, shoot length and overall growth of the cultures.

The starting tissues or explants for large scale multiplication of plants may be taken from either juvenile or mature trees or trees of any age in between (Aitken-Christie and Connett 1992). In our experiments cotyledonary nodes have been used for multiplication of *Wrightia tinctoria*. Using juvenile material, species of *Betula* (McCown and Amos 1987), *Anogeissus pendula* (Joshi *et al.* 1991) and *Anogeissus latifolia* (Joshi 1991) have been multiplied *in vitro*. It has been generally observed that BAP at higher levels produces a large number of slow growing shoot primordia (Aitken-Christie and Connett 1992). The present investigation also reveals the stunting role of BAP for shoots produced in cultures.

Shoot multiplication and elongation: High frequency multiplication for large scale production of propagules was possible when small groups of proliferated shoots, separated from shoot clusters (obtained through initial culture) were repeatedly subcultured for consecutive passages every three weeks on the same or modified medium (Table 2 and 3). Shoot clusters subcultured on same fresh medium produced 3 fold multiplication rate by which average 230 shoots/node (average length 2.3 cm) could be obtained in 84 d after inoculation (involving 3 subcultures of 21 d each).

Table 2. Effect of repeated subculturing of shoot clusters separated from mother explant on shoot multiplication and elongation of *W. tinctoria* on same medium every 21 d. Mean ± S.D.

BAP [mg dm^{-3}]	After 1st subculture		After 2nd subculture		After 3rd subculture	
	number of shoots	length of shoots [cm]	number of shoots	length of shoots [cm]	number of shoots	length of shoots [cm]
0.5	6.4 ± 0.81	2.8 ± 0.21	18.1 ± 2.6	3.0 ± 0.46	52.4 ± 05.3	3.1 ± 0.68
1.0	8.5 ± 0.96	3.0 ± 0.43	25.6 ± 1.8	3.2 ± 0.56	83.6 ± 05.9	3.4 ± 0.80
2.0	14.5 ± 2.2	2.4 ± 0.48	45.6 ± 2.6	2.6 ± 0.54	125.3 ± 07.5	2.8 ± 0.62
5.0	26.3 ± 3.4	1.6 ± 0.48	78.5 ± 6.8	2.0 ± 0.21	230.7 ± 12.6	2.3 ± 0.26
8.0	17.5 ± 1.6	1.0 ± 0.12	52.4 ± 3.6	1.2 ± 0.11	154.6 ± 08.5	1.5 ± 0.32

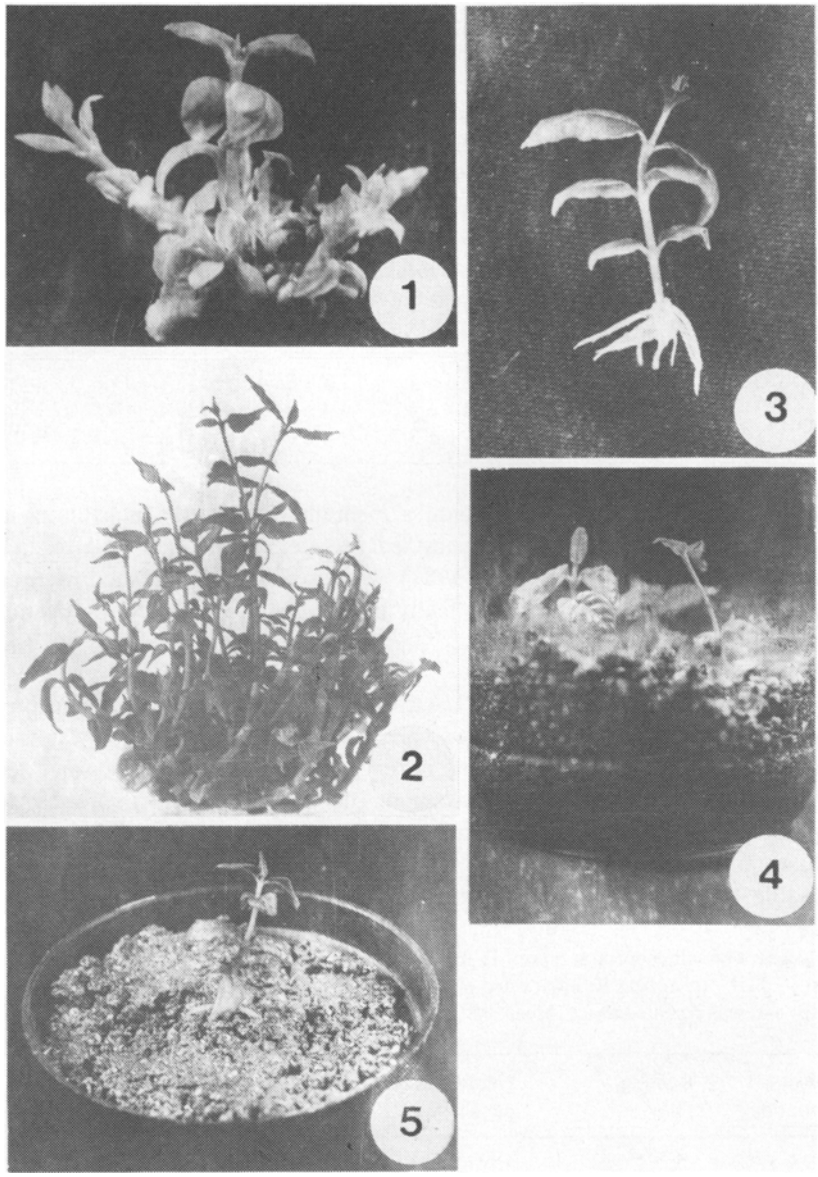

Fig. 1. Multiple shoots from cotyledonary nodal segment on MS medium containing BAP 5.0 mg dm^{-3} + NAA 0.01 mg dm^{-3} after 21 d.

Fig. 2. Multiple shoots obtained after 63 d by subculturing of shoot clusters on MS medium + BAP 1.0 mg dm^{-3} + NAA 0.01 mg dm^{-3}.

Fig. 3. 15 d old rooted plantlet on 1/4 strength MS medium after dip treatment of pre-autoclaved IBA (500 mg dm^{-3}) for 5 min.

Fig. 4. One month old plant in pot containing soil-Soilrite (1:1) mixture.

Fig. 5. 10 d old plantlets in culture botles containing soil-Soilrite (1:1) mixture and 1/4 MS nutrient solution.

Subculture on lower concentration of BAP (1.0 mg dm^{-3}) after initial culture on 5.0 mg dm^{-3} improved shoot length upto 4.2 cm without affecting rate of multiplication (Fig. 2).

Table 3. Effect of lower concentrations of BAP + 0.01 mg dm^{-3} NAA on shoot multiplication and elongation during subcultures after initial culture on MS + 5.0 mg dm^{-3} BAP + 0.01 mg dm^{-3} NAA. Mean ± SD.

BAP [mg dm^{-3}]	1st subculture number of shoots	1st subculture length of shoots [cm]	2nd subculture number of shoots	2nd subculture length of shoots [cm]	3rd subculture number of shoots	3rd subculture length of shoots [cm]
2.0	24.3 ± 2.5	2.5 ± 0.36	72.3 ± 4.3	2.8 ± 0.54	225.6 ± 11.8	3.2 ± 0.54
1.0	24.1 ± 1.8	2.9 ± 0.26	71.8 ± 4.1	3.4 ± 0.89	220.4 ± 10.5	4.2 ± 0.46
0.5	16.5 ± 1.4	2.6 ± 0.41	49.6 ± 3.7	3.0 ± 0.62	154.3 ± 07.8	3.4 ± 0.71

The need for transfer of shoots onto a medium with altered nutritional and or phytohormonal levels has been emphasized for stem elongation and to facilitate rooting later on (Biondi and Thorpe 1981). We have also experienced the need for lowering the BAP concentration drastically in the medium for shoot elongation and healthy growth.

Rooting: Auxins like 2,4-D, 2,4,5-T, NAA, IAA and IBA in different concentrations incorporated into MS medium showed varied effect on rooting. IBA was the only hormone effective in induction of roots in regenerated shoots. IBA incorporated into basal MS medium in different concentrations (0.1 - 2.0 mg dm^{-3}) induced roots but with varied callusing. To further improve the rooting response and to reduce callusing, the salt concentration of MS medium was reduced to 3/4, 1/2, 1/4, 1/8 and zero (Table 4). There was considerable improvement in rooting as about 75 % shoots

Table 4. Effect of salt concentration of MS medium (supplemented with 0.5 mg dm^{-3} IBA) on rooting in shoots of *W. tinctoria*. Results were obtained after 3 weeks, each treatment consists of 20 replicates and was repeated thrice. Mean ± SD.

MS salt concentration	Rooting [%]	Number of roots per shoot	Length of roots [cm]	Callusing
1	48.2 ± 2.4	10.3 ± 2.3	2.4 ± 0.16	Intense
3/4	56.4 ± 3.6	9.4 ± 1.60	2.6 ± 0.12	Moderate
1/2	64.8 ± 3.9	7.6 ± 1.20	3.8 ± 0.81	Little
1/4	75.2 ± 4.6	5.3 ± 0.92	5.2 ± 0.76	Little
1/8	60.2 ± 4.9	3.1 ± 0.46	2.3 ± 0.54	Little
Water-agar	52.0 ± 3.8	2.2 ± 0.89	1.2 ± 0.52	-

could be induced to root on 1/4 MS salt medium containing 0.5 mg dm^{-3} IBA. The problem of callusing still persisted. In order to further improve the rooting conditions, the shoots were given a dip treatment of pre-autoclaved IBA in different

concentrations (50 - 2000 mg dm^{-3}) for 5 min (Table 5). The best rooting response was obtained in shoots treated with 500 mg dm^{-3} of IBA for 5 min implanted on 1/4 MS salt medium (Fig. 3). In such cases 80 % rooting could be obtained in 6 - 7 d with a fairly good length and number of roots per shoot. Other concentrations of dip treatment showed either reduced rooting percentage or different degree of callusing. Rooting by dip treatment of auxin has been recommended by Harry and Thorpe (1991). This has been proved in present case also for better rooting response.

Table 5. Effect of dip treatment of various concentrations of pre-autoclaved IBA on rooting of regenerated shoots of *W. tinctoria*. Each treatment lasting for 5 min followed by shoot implantation on medium containing 1/4 MS salts. Mean ± SD.

IBA [mg dm^{-3}]	Rooting [%]	Number of roots/shoot	Length of roots [cm]	Callusing
50	65.0 ± 3.8	2.8 ± 0.64	1.2 ± 0.20	-
100	72.8 ± 4.6	3.2 ± 0.76	2.9 ± 0.32	-
500	80.2 ± 5.3	4.6 ± 0.52	5.6 ± 0.26	-
1000	62.0 ± 4.1	2.9 ± 0.42	4.1 ± 0.34	Little
2000	21.4 ± 1.2	1.3 ± 0.12	1.2 ± 0.22	Moderate

Hardening and acclimatization: To harden the *in vitro* produced plantlets, 15 d after rooting they were transferred to wide mouth culture bottles (Pickle Jars) containing soil-Soilrite™ (1:1) mixture and liquid nutrient solution comprising 1/4 MS salts (Fig. 5). Such plantlets were kept in these bottles for 10 d and finally removed and established in pots containing soil-Soilrite (1:1) mixture (Fig. 4). There was 60 % survival of plants at the pot level. Direct transfer of plants from culture medium to pots showed high rate of mortality. Further experiments would be necessary to improve the rate of survival to be able to make this protocol viable at field level.

Hardening is most critical factor for achieving success in pot transfer of the regenerated plantlets. We have observed that a gradual shifting of the plants from medium to the culture bottles with low salt concentration without sucrose allowed stress, compelling plants to become partially autotrophic. This step proved useful in achieving more success in hardening. Using the optimum conditions described in this paper a large number of propagules can be obtained in a short period.

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