Tropical Agricultural Research Vol. 2 1990

In Vitro Propagation of Nadun *(Pericopsis mooniana)*

W.M. Abeyaratne[1], D.C. Bandara and Y.D.A. Senanayake

Postgraduate Institute of Agriculture, University of Peradeniya
Peradeniya

ABSTRACT. Nadun trees provide valuable timber for the furniture industry. However, the demand outstrips the supply because Nadun is very rare in our forests. The species is now considered to be endangered.

Since propagation by seed is slow, feasibility of propagation in vitro is being studied. Experiments are being conducted to propagate by callus and shoot tip culture.

Studies were conducted to determine the best explant for callus culture and selection of suitable nutrient media for callus initiation and regeneration . While hypocotyl sections from in vitro grown seedlings were the best explants, MS (revised in 1962) modified with BAP (2.0 mg/l) and 2, 4−D (5.0 mg/l) gave best callus initiation. For regeneration, of plants from callus MS medium modified with AS (40 mg/l), CH (200 mg/l), CW (10%), BAP (2.0 mg/l) and GA_3 (1.0 mg/l) produced roots in callus but shoot regeneration failed.

In multiple shoot culture, establishment of seedling shoot tips were best on MS medium with BAP (2.0 mg/l) or BAP (5.0 mg/l) and NAA (0.1 mg/l) or BAP (5.0 mg/l) and NAA (0.5 mg/l). Among the tested cytokinins (BAP, Kn, 2ip), BAP produced the maximum number of shoots when added to MS medium. BAP (5.0 mg/l) gave the highest number of shoots. When NAA was added to the nutrient media, shoot multiplication was suppressed to some extent the quality of shoots were improved and maximum number of shoots longer than 5 mm was observed from the medium supplemented with BAP (2.0 mg/l) and NAA (0.1 mg/l). Shoot multiplication was improved by adding AS (40 mg/l) to the multiplication medium containing BAP (2.0 mg/l).

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1 Royal Botanical Gardens, Peradeniya

Among the tested nutrient media, 60% of shoots produced roots after 8 weeks in culture when nutrient media contained of NAA (5.0 mg/l).

Key to abbreviations

AC: activated charcoal, AS: adenine sulphate, BAP: 6 benzyl-aminopurine, CH: casein hydrolysate, CW: coconut water, 2, 4-D: 2, 4-dichloro phenoxy acetic acid, GA_3: gibberelic acid, IBA: indole-3-butric acid, 2ip: 2-isopentil adenine, Kn: kinetin, MS:Murashige and Skoog medium, NAA: napthalene acetic acid.

INTRODUCTION

Nadun (*Pericopsis mooniana*) a tropical tree species belonging to the family Leguminosae, provides valuable timber for the furniture industry. Although its demand is increasing, mature trees required for this purpose are rare in the forests and hence it is now thought that nadun could be included among the endangered plant species. Thus efforts at planting this species in suitable localities are much to be desired.

Though nadun could be propagated by seeds, the demand for plant propagules cannot be met, because of its seasonal flowering behaviour and the difficulty of collecting seeds.

Tissue culture is an important technique for rapid multiplication of plants. Application of tissue culture methods has so far been confined mainly to the herbaceous plants (Murashige, 1977, 1978) and these methods are now used commercially to mass produce ornamentals. Although similar advances have not been made on rare forest species or other tree species (Bonga, 1980). The potential usefulness of tissue culture techniques for the propagation of forest trees has long been recognized.

A number of reports covering a wide range of forest trees and fruit trees belonging to the angiosperms have been recently published. These include studies on *Acacia koa* (Skolmen and Mapes, 1976); *Albizzia lebbeck* (Gharyal and Maheshwari, 1983); *Acacia mangium* (Basri *et al.*, 1987); Tectona grandis (Gupta *et al.*, 1980); *Santalum album* (Lakshmi

Sita *et al.*, 1979); *Malus Pumila Mill* (Ochatt and Caso, 1983).

The general objective of this study was to investigate the feasibility of *in vitro* propagation of nadun.

MATERIALS AND METHODS

Nadun seeds collected from the Botanical Gardens, Peradeniya were mechanically scarified, surface – sterilized with 10% clorox added with a few drops of tween – 20, rinsed thrice in sterilized distilled water and grown *in vitro* under aseptic conditions on MS basal medium (Murashige and Skoog, 1962) under a light intensity of 1000 lux.

Sterile explants were obtained from these *in vitro* grown seedlings when 10 – 15 days old. One centimeter long hypocotyl sections and one centimeter long shoot tips were used as the explants.

For the establishment of callus cultures from hypocotyl explants, the basal medium was modified with different concentrations of 2, 4 – D (0 – 5.0 mg/l) and BAP (0 – 2.0 mg/l). Cultures were kept in the dark. Explant survival and callus formation after 30 days in culture were scored on a scale ranging from 1 to 6.

Next regeneration of shoots and roots from callus was tested in the basal medium modified with AS, 40 mg/l; CH, 200 mg/l; BAP, 2.0 mg/l and GA_3, 1.0 mg/l individually or in different combinations. The cultures were kept under light (1500 lux) at 26 $\pm$ 1 C temperature.

For the establishment of shoot tip culture the explants were cultured in the basal medium supplemented with different concentrations of BAP (0 – 5.0 mg/l) and NAA (0 – 0.5 mg/l) and the cultures were kept under light (1500 lux) at 26 $\pm$ 1 C temperature.

After 60 days in culture, all the possible observations were recorded such as number of shoots per explant produced and basal callus formation of the explants.

For shoot multiplication, different concentrations (0 – 5.0 mg/l) of BAP, Kn and 2ip were tested individually. Different concentrations of BAP (0 – 5.0 mg/l) combined with different concentrations of NAA

(0 – 0.5 mg/l) and different amounts of AS (40 – 200 mg/l) combined with BAP 2.0 mg/l were also tested for shoot multiplication. Shoot multiplication was evaluated after 60 days in culture.

Shoots about 1 – 2 cm. long harvested from stock cultures and grown on MS medium supplemented with BAP 2 mg/l were used for rooting experiments.

Rooting media consisting of IBA or NAA (0 – 5.0 mg/l) with or without 0.2% AC were used. After 60 days in culture under 1500 lux light intensity, nutrient media were evaluated for their ability to initiate roots. For root elongation, these shoots were subcultured on 1/2 MS nutrient medium supplemented with 2% sucrose. This medium had no growth regulators.

RESULTS AND DISCUSSION

Establishment of callus culture and plant regeneration

The survival and callus formation capacity of the hypocotyl explants in the callus establishment media shows that all media for callus induction were those modified with 2, 4 – D concentrations between 2.0 and 5.0 mg/l and BAP concentrations between 0.5 and 2.0 mg/l (Table 1). The greatest average callus size (147 mm^2) was obtained from the medium consisting of BAP 2.0 mg/l and 2, 4 – D 5.0 mg/l (Table 2).

Although callus induction was achieved from the explants, plant regeneration failed in all the tested regeneration media. However, a few calli in the medium with AS (40 mg/l), CH (200 mg/l), CW (10%), BAP (2.0 mg/l) and GA_3 (1.0 mg/l) regenerated a few roots. After 5 – 6 weeks, all cultures showed browning and deterioration.

These results are similar with those obtained with trees such as *Eucalyptus* species (Lakshmi Sita, 1981) and *Eugenia grandis* (Kong and Rao, 1981).

In tissue culture of *Eucalyptus* (Lakshmi Sita, 1981) it was revealed that all cotyledon cultures supplemented with zeatin and NAA also rooted, and that callus cultures derived from the hypocotyl did not differentiate, which was confirmed by repeated experiments. In the case

Table 1. Survival and callus formation grades after 30 days in culture.

BAP (mg/l)	2 4-D (mg/l)			
	0	0.5	2	5
0	2	3	2	2
0.5	2	4	6	5
2	2	4	5	6

(Average of 15 replicates)

Grade 1 - explant dead
Grade 2 - explant survived but no callus
Grade 3 - explant slightly enlarged but no callus
Grade 4 - callus formation poor
Grade 5 - callus formation fair
Grade 6 - callus formation good

Table 2. Mean callus size (mm^2) from hypocotyl explants after 30 days on culture. Proportion of callus formation (explants with callus to total number of explants) is given in parenthesis).

BAP (mg/l)	2 4-D (mg/l) 0	0.5	2	5
0	– (0/15)	56.37 ± 26.6 (2/15)	– (0/15)	36.51 ± 9.71 (4/15)
0.5	– (0/15)	42.52 ± 29.33 (9/15)	74.52 ± 45.20 (12/15)	69.27 ± 10.62 (12/15)
2	– (0/15)	52.56 ± 13.25 (10/15)	54.36 ± 13.02 (13/15)	147.28 ± 30.64 (14/15)

Estimation of callus size

Callus area was used as an estimate of callus size following the method of according to Dale and Deambrogio (1979). For each callus, two diameters were measured, the largest and that perpendicular to it. Callus area was estimated as follows;

$$\text{Callus area} = \frac{\text{diameter 1} \times \text{diameter 2} \times \pi}{4}$$

of *Eugenia grandis* (Kong and Rao, 1981), callus developed from internodal explants, but plant regeneration was negative. Flick *et al.*, (1983) pointed out that plant regeneration has been difficult among legumes.

Shoot tip establishment and multiplication

Addition of BAP only at 2 and 5 mg/l induced shoot establishment to yield 5.6 to 5.4 shoots per explant as well as 3.6 to 2.6 shoots longer than 5 mm. Addition of NAA (0.1 or 0.5 mg/l) resulted in increase of BAP requirement for good shoot tip establishment. The best results were obtained from 5 mg/l of BAP added with NAA at 0.1 mg/l or 0.5 mg/l and the resulting yields were 5.8 to 6.6 shoots per explant respectively. However, added NAA resulted in the basal callus development of cultured explants especially at the higher level of BAP (Table 3).

In the shoot tip establishment stage, explants may develop either into single shoot or into multiple shoot musses. However, basal callusing of the explants may be disadvantageous is establishment of shoot tip culture, because callusing may inhibit the further growth of explants.

Among the cytokinins tested for the multiplication of shoots, BAP gave better results than kn or 2ip (Table 4). The maximum number of shoots per explant resulted from the medium supplemented with 2.0 mg/l BAP. When NAA (0 – 0.5 mg/l) was added to the multiplication medium supplemented with BAP (0 – 0.5 mg/l) shoot multiplication slightly better at lower concentration but was suppressed at 5.0 mg/l. However, the quality of shoots such as leaf development appearance (intensity of green colour) was improved by the addition of NAA. Added NAA resulted in basal callusing of shoots even at 0.5 and 1.0 mg/l of BAP (Table 5). In the presence of low amounts of adenine sulphate (40 mg/l) in the multiplication medium (BAP 2.0 mg/l) shoot multiplication was enhanced, but at higher concentration of adenine sulphate it was suppressed (Table 6).

Lakshmi Sita (1981) reported that multiplication of *Eucalyptus* shoot apices showed more vigorous and sturdy shoots, and these resulted when the media was supplemented with NAA and BAP. Her results are similar to the results obtained in shoot multiplication of nadun.

Table 3. Effect of BAP and NAA on shoot tip establishment of nadun after 60 days in culture.

BAP (mg/l)	NAA (mg/l)	Basal callus	Average No. shoot per explant	Shoots > 5mm/explant
0	0	-	0	0
0.5	0	+	2.8	1
1	0	+	4.3	3.3
2	0	+ +	5.6	3.6
5	0	+ +	5.4	2.6
0	0.1	-	0	0
0.5	0.1	+	1.0	0.5
1	0.1	+ +	1.0	0.75
2	0.1	+ +	3.4	2.00
5	0.1	+ + +	5.8	3.00
0	0.5	-	0	0
0.5	0.5	+ +	0.4	0
1	0.5	+ +	1.6	0
2	0.5	+ + +	4.3	2.5
5	0.5	+ + +	6.0	4.4

(Average of 5 replicates)

- \- no callus
- \+ poor callus
- \+ + fair callus
- \+ + + good callus

Table 4. Average number of shoots produced from shoot explants in different cytokinins after 60 days in culture.

Cytokinins	Mg/L	Average shoots/shoot
BAP	0	2
	0.5	3.2
	1.0	3.6
	2.0	4.3
	5.0	3.2
Kn	0	2
	0.5	1.3
	1.0	1.4
	2.0	1.8
	5.0	2.6
2ip	0	2
	0.5	1.4
	1.0	1.9
	2.0	2.0
	5.0	2.9

(Average of 10 replicates)

Table 5. The average number of shoots produced and basal callus formation from shoot explants in different combination of BAP and NAA after 60 days in culture.

BAP (mg/l)	NAA (mg/l)	Shoots/shoot	Shoots > 5mm/ shoot	Callusing
0	0	1.1	1	-
0.5	0	3.5	1.7	-
1.0	0	5.3	3.6	-
2.0	0	7.7	3.4	+ + +
5.0	0	11.7	1.2	+ + +
0	0.1	1.6	0.6	-
0.5	0.1	4.0	1.7	+
1.0	0.1	5.9	2.5	+ +
2.0	0.1	8.5	4.1	+ +
5.0	0.1	7.4	2.4	+ + +
0	0.5	1.6	0.8	-
0.5	0.5	3.9	2.1	+
1.0	0.5	6.0	2.2	+ +
2.0	0.5	6.8	3.7	+ +
5.0	0.5	9.3	1.8	+ + +

- no callus
+ slight callus
+ + fair callus
+ + + good callus

Table 6. Average number of shoots produced and basal callus formation from shoot explants in media containing 2.0 mg/l BAP and different amounts of AS after 60 days in culture.

AS mg/l	Shoots/shoot	Shoots > 5mm/shoot	Basal callus
0	7.3	2.7	+ +
40	8.0	4.7	+ +
80	5.1	3.6	+
100	5.4	2.8	+ +
120	3.7	1.5	+ +
150	3.2	1.4	+
200	2.9	0.6	+ +

(Average od 10 replicates)

\- no callus
\+ slight callus
\+ + fair callus
\+ + + good callus

Since the main objective of the multiplication stage is to produce the maximum number of useful propagule units, the nutrient medium supplemented with 2.0 mg/l of BAP with 0.1 mg/l of NAA can be selected as the best medium for shoot multiplication. This medium gave the highest number of shoots per shoot which are longer than 5 mm (average of 4.1) and the basal callusing also fairly limited. When shoots are long enough to harvest into single shoot, it may be advantageous for rooting stage. Since basal callusing of shoots may suppress the growth of cultures, it is generally accepted that the multiplication medium should not produce more callus at the base of the shoots.

Root regeneration of cultured shoots

Nutrient media supplemented with different concentrations of IBA or NAA with or without AC (0.2%) resulted in different percentages of rooting after 60 days in culture (Table 7). Among the tested auxins NAA promotes root initiation more than IBA. Higher concentration of NAA (5.0 mg/l) resulted in 60% rooting, but average root length was about 1 mm. However, NAA 1.0 mg/l with 0.2% AC resulted in 20% rooting, with average root length was about 3 mm.

AC may absorb toxic substances as well as auxins present in the root induction medium (Heberle - Bors, 1980). Therefore, root formation may be reduced with the presence of AC in the nutrient medium due to insufficient of auxin for root induction.

Among the common auxins, NAA is the most effective auxin for induction of roots in most species grown in vitro (Ancora *et al.*, 1981; Kitto and Young, 1981; Johnson, 1978).

There are three phases involved in rizogenesis; (a) induction, (b) initiation and (c) elongation. Since it is rather difficult to isolate the induction phase in most experiments, this phase has usually been combined into the phase of initiation. It is well known, from the classic work of Skoog and Miller (1957), that *de novo* root initiation depends on a low cytokinin to a high auxin ratio. This phenomenon is similar to what was observed in the root initiation phase of nadun. However, the root elongation phase needs low level of auxin or no auxin in the nutrient medium. In the case of nadun root elongation seems to be .

Table 7. Effect of IBA, NAA and AC on rooting after 60 days in culture.

Growth regulator (mg/l)		% Rooting	Average root/ shoot	Root length (mm.)
IBA	0	0	–	–
	0.5	0	–	–
	1.0	0	–	–
	2.0	0	–	–
	5.0	0	–	–
AC.2% + IBA	0	0	–	–
	0.5	0	–	–
	1.0	20	1	1 mm
	2.0	0	–	–
	5.0	0	–	–
NAA	0	0	–	–
	0.5	0	–	–
	1.0	20	1	1 mm
	2.0	20	1	1 mm
	5.0	60	1	1 mm
AC.2% + NAA	0	0	–	–
	0.5	0	–	–
	1.0	20	1	3 mm
	2.0	0	–	–
	5.0	0	–	–

(All MS basal medium with 3% sucrose).
(Average of 5 replicates)

promising in the nutrient medium free from auxin. This experiment is still in progress.

From the results presented it can be concluded that *in vitro* propagation of nadun through the shoot tip culture method seem to be promising.

ACKNOWLEDGEMENTS

The authors are thankful to Mr. D.B. Sumithraarachchi, Deputy Director, Royal Botanical Gardens, Peradeniya, for suggesting the study and for providing facilities for the research.

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