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IN VITRO PROPAGATION OF *OCHREINAUCLEA MISSIONIS* (WALL. EX G. DON), AN ETHNOMEDICINAL ENDEMIC AND THREATENED TREE

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SUMMARY

Biotechnology has offered a nonconventional method of plant propagation and has been intensively applied as a conservation strategy for sustaining biodiversity for rare plants. *In vitro* conservation through micropropagation of *Ochreinauclea missionis*, a rare, endemic and medicinal tree species of Western Ghats in Karnataka region of India is reported. Multiple shoots were initiated from nodal explants on Murashige and Skoog (MS) medium supplemented with 8.8 μ*M* 6-benzylaminopurine (BA) and 0.3% (w/v) activated charcoal. Shoots were elongated in MS medium with a combination of 2.2 μ*M* BA and 5.3 μ*M* α-naphthaleneacetic acid (NAA) or growth regulator-free medium. Individual shoots with a minimum of one node were excised and rooted *in vitro* on MS medium with 0.3% activated charcoal or *ex vitro* rooted by treatment with 49 μ*M* indole-3-butyric acid (IBA) for 30 min. Regenerants acclimated in Soil-rite exhibited 65% survival in the greenhouse.

Key words: micropropagation; *Ochreinauclea missionis*; medicinal; plant conservation; forest tree.

INTRODUCTION

Anthropogenic factors, mainly deforestation for agriculture and agricultural practices, have led to a decline of plant species in the wild. About 34 000 plant species or about 12.5% are globally threatened with extinction due to extensive clearing of land (Anonymous, 1990). To circumvent further deterioration of species it is necessary to vegetatively propagate them by application of *ex situ* methods. While *ex situ* techniques involving conventional propagation through natural regeneration and vegetative cuttings may be slow and cumbersome, in contrast *in vitro* culture offers a sustainable and viable tool for rapid propagation and storage of germplasm. Plant tissue culture has already been effectively applied to mass multiply diverse, rare and endangered medicinal tree species (Lynch, 1999).

Ochreinauclea missionis, a member of the Rubiaceae family, is an evergreen tree found growing along the banks of rivers and streams. Fragmented populations of this plant occurring in separate discrete localities are endemic to Central and Southern Western Ghats of Peninsular India. Degradation of natural forest cover for agricultural purposes and exploitation of the species at the individual level are the causes for its rarity. Besides being phytogeographically significant, it represents a monotypic endemic genus with medicinal properties. Locally known as 'Jalamdasa', *Ochreinauclea missionis* is very much exploited by the local people for its purported medicinal values (Nayar and Sastry, 1990). The bark is used locally for curing rheumatism, leprosy, and ulcers (Kirtikar and Basu, 1975). The tree is also known to yield moderately hard light wood and exhibits potential for bioprospecting. Short seed viability period, rare occurrence, endemism, and exploitation indicate the need for its conservation.

This report describes the clonal propagation of *O. missionis* through nodal explants and the subsequent establishment of plantlets in the field. The results constitute the first reported data on *in vitro* propagation of *O. missionis*.

MATERIALS AND METHODS

Tender branches and woody stem cuttings were excised from 8- to 10-yr-old mature trees growing in the wild along the river bank of Sitanadhi, Udupi district during different seasons (summer, spring, winter, and monsoon) to determine the most suitable time for culture establishment. Tender branches were kept in running tap water for 45 min. A few drops of Tween 20 were added followed by fungicide treatment with 0.3% (w/v) bavistin (carbendazim 50% WP), a systemic fungicide, for 2 h. The branches were cut to give 1.5–2-cm long shoot tip or nodal explants. These explants were disinfected with 0.6% (w/v) mercuric chloride for 7 min for nodal explants and 5 min for shoot tip explants, followed by thorough rinsing in sterile distilled water for at least four or five times. The explants were trimmed further and transferred to MS (Murashige and Skoog, 1962) medium with or without growth regulators. The cytokinins 6-benzylaminopurine (BA; 2.2–22.1 μ*M*) and kinetin (KN; 2.3–23.2 μ*M*) were used alone or in combination to examine their effect on axillary bud break. Activated charcoal (AC; 0.3% w/v; LR, Ranbaxy, India) or soluble polyvinylpyrrolidone (PVP; 40, 0.1% w/v; Sigma Chemical Co., St. Louis, MO, USA) was incorporated into the media to control phenolic oxidation and blackening of the explants. Three percent sucrose was added before adjusting the pH to between 5.7 and 5.8, and the media were gelled with (0.8%, w/v) agar (Regular grade, SRL, Bombay, India). Media (20 ml) were dispensed into bottles (10.5 × 6.5 cm) prior to autoclaving at 121°C at 105 kPa for 20 min. All cultures were maintained at 25 ± 2°C under a 16-h photoperiod provided by cool-white fluorescent tubes (50 μmol m^{-2} s^{-1}). Twelve replicates were used for each treatment.

The efficacy of both *ex vitro* and *in vitro* rooting were compared. For *in vitro* rooting, microshoots from second subcultures measuring between 1.5

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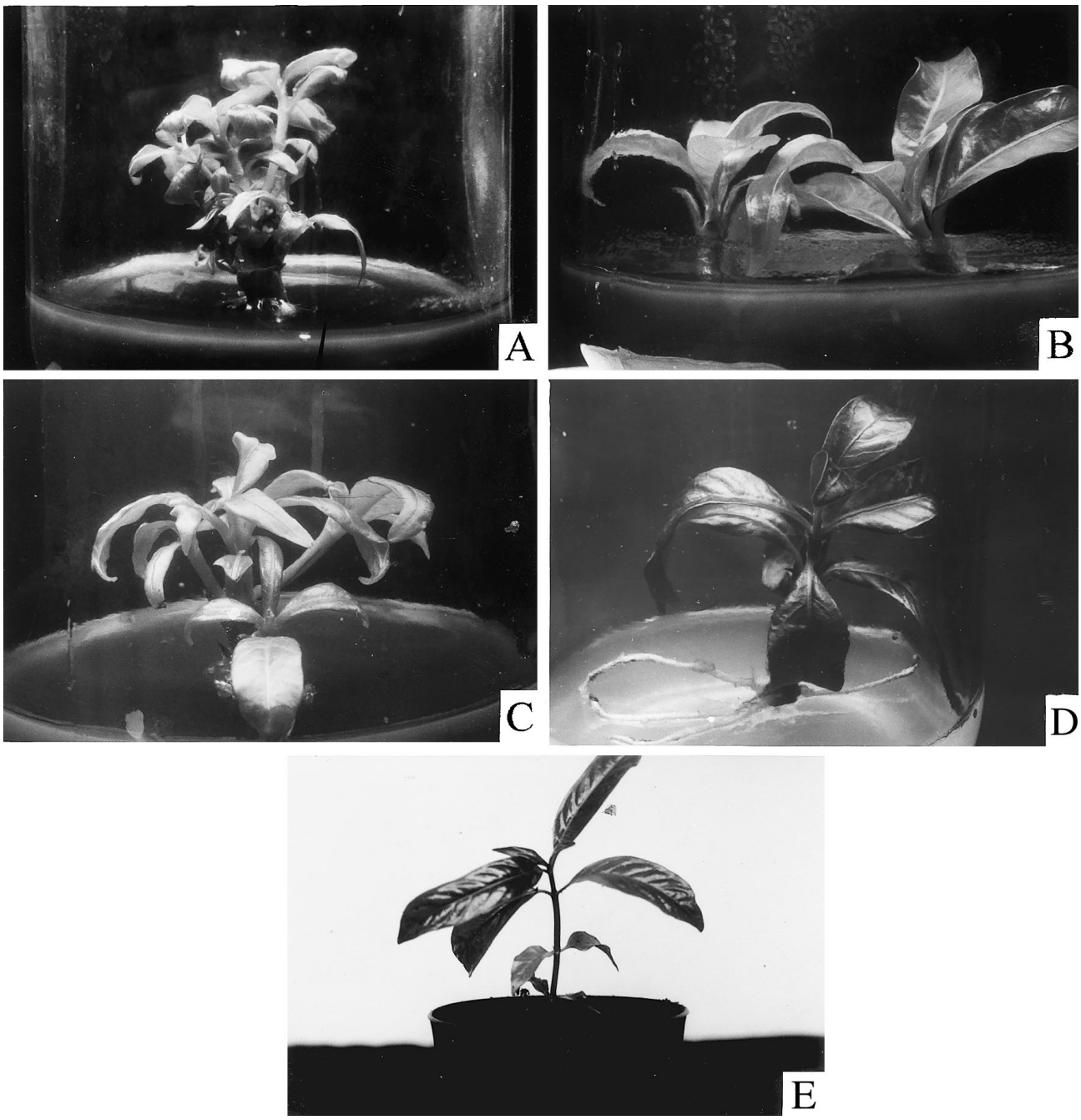

FIG. 1. Micropropagation of *Ochreinauclea missionis* through shoot tip and nodal explants. *A*, Multiple shoots initiated from nodal explant in BA (8.8 μ*M*). *B*, Axillary shoots sprouting from nodal exlants in MS supplemented with activated charcoal. *C*, Multiple shoots induced in shoot tip explant on MS + BA (4.4 μ*M*) + KN (4.6 μ*M*) + activated charcoal. *D*, Initiation of rooting in MS basal medium. *E*, Regenerant subjected to hardening in Soil-rite.

and 4 cm were cultured on either full-strength solid MS basal medium with 0.3% (w/v) activated charcoal and sucrose (3% w/v) or MS liquid medium supplemented with auxins indole-3-acetic acid (IAA, 5.7 μ*M*) and indole-3-butyric acid (IBA; 4.9 μ*M*), and sucrose (3% w/v). For *ex vitro* rooting, the microshoots were washed with sterile water to remove traces of agar adhering to the shoot followed by immersing in 0.3% bavistin solution for 5 min. The base of the shoot was dipped for 30, 60, or 120 min into concentrated solutions (49 and 490 μ*M*) of IBA and immediately transferred into plastic pots containing sterile Soil-rite (equal proportions of decomposed coir and peat moss, Karnataka Explosives, Bangalore, India). The pots were covered with polythene bags, having been punched with holes, and maintained in the growth chamber at 80% relative humidity. After 5 wk the number and length of roots were recorded.

Statistical analysis. Twelve explants were used per treatment on each multiplication and rooting medium. Experiments were repeated twice by employing a completely randomized design. Collected data were analyzed by ANOVA and variations among means were compared using post hoc Duncan's multiple range test at $P < 0.05$.

Results and Discussion

Nodal explants were more responsive compared to shoot tips in terms of rapid bud break. The frequency and the rate of multiplication depended on the type of explant, cytokinin, and its concentration either alone or in combination. Enlargement and subsequent break of axillary buds was the initial response of nodal explants cultured on MS media supplemented with various levels of different cytokinins. The highest frequency of nodal explants responding (83.3%) was on medium supplemented with 8.8 μ*M* BA and 0.3% activated charcoal (Fig. 1*A*; Table 1). New shoots (six to eight) developed in this medium attained a mean length of 1 cm and two to three nodes within a span of 30 d. A maximum of two shoots per nodal explant was produced from 92% of the cultures on growth regulator-free medium after 40 d (Fig. 1*B*).

Shoot tips with at least one node (2 cm long) were used as explants to initiate multiple shoots. However, elongation of the shoot tip was observed in all media irrespective of the type and concentration of the cytokinin used. The shoot tip meristem exerted a strong apical dominance with consequent inhibition of axillary buds, as observed in *Ixora coccinea* (Lakshmanan et al., 1997).

A few cultures growing on medium containing BA and KN initiated lateral shoots in the axils of lower leaves of the shoot tip (Fig. 1*C*).

Plant material collected during the winter and rainy seasons gave the maximum response as measured by percentage of explants with axillary bud break (Fig. 2). The winter and rainy seasons correspond with the timing of the most active growth phases. Under natural conditions, fresh sprouts appear vigorously in winter, i.e. December and January, and new shoots are seen emerging and growing rapidly during the monsoon season (June through August), although the plant growth is comparatively less in other seasons. Type of explant, season of explant collection (Tisserat, 1985), and physiological conditions (Thorpe and Biondi, 1984) have been shown to be critical factors in establishment of responsive cultures.

All newly produced shoots in high concentrations (>8 μ*M*) of cytokinin augmented media exhibited characteristic stunted growth. Shoot length was significantly improved by sub-culturing the entire explant into MS basal medium or by incorporating 5.3 μ*M* NAA into the medium. Shoot elongation due to the presence of auxin in cytokinin-supplemented media has been reported by Siril and Dhar (1997).

Activated charcoal was observed to be more effective than PVP in reducing phenolic exudation. About 67% of the explants

TABLE 1

RESPONSE OF NODAL EXPLANTS OF *OCHREINAUCLEA MISSIONIS* ON DIFFERENT CONCENTRATIONS OF CYTOKININS AFTER 5 WK

Growth regulators (μ*M*)	Shoot formation (%)	No. of shoots per shoot tip explant
BA (8.8)	83.3	6.6 ± 0.91 a
BA (17.7)	33.3	1.5 ± 0.64 b
KN (9.2)	25.0	0.8 ± 0.44 b
KN (18.5)	16.6	0.5 ± 0.35 b
BA (4.4)+KN (4.6)	58.3	1.3 ± 0.43 b
BA (13.3)+KN (13.9)	50.0	2.3 ± 0.34 b

The means±SE are shown. Means followed by the same *letter* are not significantly different at 0.05 level (Duncan's multiple range test).

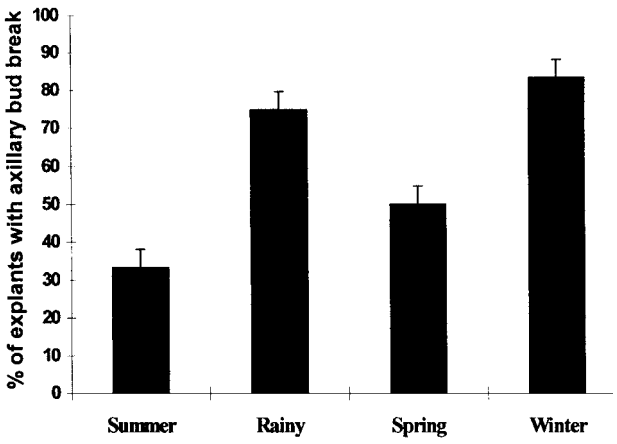

Fig. 2. Influence of season on sprouting of axillary buds of nodal explants of *Ochreinauclea missionis* on MS + BA (8.8 μ*M*) + activated charcoal (0.3% w/v). Each mean was an average of 12 explants (*bars* show±SE).

inoculated in medium supplemented with PVP exhibited browning after incubating cultures for more than 45 d. Better growth responses of plant tissues have been associated with addition of activated charcoal as it removes inhibitory substances from the media produced either on autoclaving (Weatherhead et al., 1978) or by the tissue itself (Fridborg et al., 1978).

Apical necrosis of shoots and vitrification were two major constraints encountered during incubation of *O. missionis* cultures for a long duration. It is known that nutritional stress enhances the activities of polyphenol oxidases (George and Sherrington, 1984) thus increasing the release of phenols from cells into the culture media, which can also contribute to the rise in media pH. Such effects may have been involved in the browning and death of tissues as observed in *Ocotea* cultures (Moura-Costa et al., 1993) which were kept for long periods in the same medium. Transfer of cultures exhibiting initiation of browning into the same medium composition prepared freshly, with a low concentration (2.2–4.4 μ*M*) of cytokinin and incorporation of 24.6 μ*M* IBA or 26.8 μ*M* NAA, controlled apical necrosis. The effect of IBA-supplemented medium on reduction of explant necrosis in *Hibiscus* and *Rhododendron* hybrids and NAA on decreasing browning of *Ribes uva-crispa* was observed by Preece and Compton (1991).

Vitrification was controlled to a certain extent by subculturing into half-strength MS basal medium. All these cultures were incubated for a period of 2–3 wk before re-culturing into their previous media composition.

Microshoots that attained a height of 3 cm with at least one node were individually excised from the shoot cluster and selected for rooting. *In vitro* rooting was efficiently obtained in 83.3% of the cultures within a period of 25–30 d in MS medium with 3% (w/v) sucrose and 0.3% (w/v) activated charcoal (Table 2). Roots were 3–4 cm long, white and slender (Fig. 1*D*). The effect of activated charcoal on rooting has also been reported in other tree species (Liew et al., 1999). Rooting in liquid MS medium with either (4.9 μ*M*) IBA or (5.7 μ*M*) IAA was preceded by the formation of friable callus at cut ends of the shoot in contact with the medium, through which brittle roots were formed.

Ex vitro rooting proved to be superior to *in vitro*, with 80%

TABLE 2

ROOTING OF SHOOTS OF *OCHREINAUCLEA MISSIONIS* IN DIFFERENT MEDIA AFTER 5 WK

Medium	No. of roots	Root length (cm±SE)	Rooting (%)
In vitro method			
MS+0.3% AC+3% sucrose+0.8% agar	4.3 ± 0.62 a	3.4 ± 0.46 ab	83.3
Liquid MS+IBA (4.9 μ*M*)	4.3 ± 1.98 a	0.3 ± 0.11 d	33.3
Liquid MS+IAA (5.7 μM)	0.5 ± 0.34 b	0.1 ± 0.12 d	16.6
Ex vitro method			
IBA (49 μ*M*), 30 min	5.0 ± 0.48 a	3.8 ± 0.40 a	91.6
IBA (49 μ*M*), 60 min	4.9 ± 0.69 a	2.8 ± 0.40 b	83.3
IBA (49 μ*M*), 120 min	7.5 ± 1.03 a	1.6 ± 0.31 c	83.3

Values followed by the same *letter* within *columns* are not significantly different at 5% level when subjected to Duncan's multiple range test.

transplantation success. The application of *ex vitro* techniques have been reported to be useful and economical for several woody species (Augustine and D'Souza, 1997). IBA is generally known to induce rooting in plant cuttings either *in vitro* or *in vivo*, but IBA had negative effects on root growth *in vitro* as roots disintegrated during hardening. However, pulses of 30 min with 49 μ*M* IBA were adequate to stimulate rooting from the basal portion of the shoot in 92% of the cultures after 5 wk (Table 2). Shoots exposed to an IBA concentration of 490 μ*M* did not survive, as necrosis set in after 2–3 d. All *ex vitro*-rooted shoots exhibited vigorous root systems. An incubation of 60 d in the growth chamber at 25 ± 2°C and 80% RH improved the survival rates of the regenerants to 65% after their establishment in the greenhouse. No morphological abnormalities were visible in the transplanted plants.

We report a feasible protocol for micropropagation of *O. missionis* through nodal explants from mature trees. Thus we were able to establish 150 plants in the greenhouse. Therefore, *in vitro* techniques can also be applied as an alternative method of propagation to conserve this species.

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