Plant Growth Regulation **17**: 115–120, 1995.
© 1995 *Kluwer Academic Publishers. Printed in the Netherlands.*

A simple and efficient method for clonal propagation of *Casuarina sumatrana* (de Vriese) L. Johnson

C. J. Goh, P. Lakshmanan, C. L. Lee, C. S. Loh & M. Tanaka[1]
*Department of Botany, National University of Singapore, Lower Kent Ridge Road, Singapore 0511 (*author for correspondence: Department of Horticulture, Kagawa University, Miki-cho, Kagawa-ken, Japan)*

Received 22 November 1994; accepted 20 February 1995

Key words: auxin, *Casuarina sumatrana*, clonal propagation, Neoflon film, rooting, trees

Abstract

A new and efficient method for clonal propagation of *Casuarina sumatrana* by rooting stem cuttings is described. High percentage (about 60–70%) of rooting was achieved with mature softwood stem cuttings. A quick-dip of 5 s in NAA (1–10m*M*) solution followed by sand culture under high humidity were required for a high rate of survival and rooting of stem cuttings. A simple, closed chamber propagation system, using fluorocarbon polymer (tetrafluoroethyleneperfluoroalkyl vinyl ether) film (Neoflon PFA film), was successfully developed for the rooting of stem cuttings without mist. Rooted cuttings inoculated with *Frankia* were easily transplanted and established in field conditions with very low (about 3%) mortality. The significance of these findings for mass clonal propagation of *C. sumatrana* is discussed.

Abbreviations: IBA = indole-3-butyric acid; NAA = α-naphthaleneacetic acid.

1. Introduction

Species of the genus *Casuarina* (Casuarinaceae) are fast-growing evergreen trees with a wide natural distribution in tropical and subtropical regions of the world [7]. They are extensively used for afforestation, establishment of shelter belts, fuel wood production, dune stabilization and landscaping [7, 8, 9].

Casuarinas are propagated largely by seeds. Seeds of many *Casuarina* species are recalcitrant and seed propagation usually results in stands exhibiting great variation in growth and form [14]. For commercial exploitation of genetically superior clones as well as for tree breeding programmes, it is necessary to propagate selected clones vegetatively. Research on vegetative propagation of *Casuarina*, however, is limited. Conventional vegetative propagation methods, including air layering [4], rooting of stem cuttings [11] and root suckers [5], are inefficient with low yield. The only *in vitro* method reported for propagation of *Casuarina* [2] is not suitable for large-scale production due to a low rate of shoot multiplication and poor rooting of microcuttings. In this paper we describe a simple, efficient and inexpensive method for the mass propagation of *C. sumatrana*, a species used for afforestation and landscaping, by rooting of stem cuttings.

2. Materials and methods

2.1 *Plant material*

Mature softwood stem cuttings were obtained from the side branches of 10–12 year-old-trees of *C. sumatrana* (de Vriese) L. Johnson grown on the campus of the National University of Singapore. Each stem cutting (15–20 cm long) was stripped of lateral green shoots except for the terminal 6–8 cm. Cuttings were processed within 2 h after harvest.

2.2 *Plant growth regulator treatment*

The cuts ends of stem cuttings (about 1 cm length) were re-cut at an acute angle immediately before treatment.

Two methods of application were used; a) soaking: about 4 cm of the basal portion of cuttings were soaked in 1.0 m*M* IBA for 15, 30, 45 and 60 min or in 1.0, 2.5, 5.0 and 10.0 m*M* IBA for 4 h; b) quick-dip method: basal ends of cuttings were dipped in 1.0, 5.0 and 10.0 m*M* IBA or NAA for 5 s. Both IBA and NAA were used as potassium salts and were prepared in 50% ethanol for the quick-dip method. After IBA and NAA application, the basal portions (4 cm) of all the cuttings were briefly dipped in 0.06%(w/v) benlate suspension and then planted in the rooting medium.

2.3 *Growth conditions*

Stem cuttings were rooted in plastic troughs (50 × 30 × 30 cm) containing a 10–12 cm deep sand bed, vermiculite or Oasis foam (Smithers-Oasis Floral Products, USA). In order to maintain high humidity around the cuttings, the plastic troughs, kept inside the green house, were covered with a transparent polyethylene cover (0.13mm thick) supported by a metal frame. Plastic troughs (except those used for the closed chamber propagation units) were perforated at the bottom at two places (1 cm diameter holes) to drain excess water from the rooting medium. All rooting media were sterilized by autoclaving at 1.1 kg cm^{-2} for 20 min at 120 °C and moistened with tap water before setting the cuttings. During the experiment, the light intensity inside the greenhouse averaged 220 μmol m^{-2} s^{-1} (12h photoperiod) with a minimum and maximum day temperature range of 24 to 30 °C. Relative humidity and mean day temperature inside the plastic troughs with polyethylene cover were 96% and 26 °C respectively. Stem cuttings were watered once daily unless otherwise specified.

2.4 *Development of a closed chamber propagation unit*

The closed chamber system consisted of plastic troughs (50 × 30 × 30 cm), containing moistened sand, and covered tightly with a fluorocarbon polymer (tetrafluoroethyleneperfluoroalkyl vinyl ether) film (Neoflon PFA film; Daikin Industries, Ltd., Osaka, Japan) which permits free exchange of gases but retains moisture. Stem cuttings were treated with 10 m*M* NAA and then planted in these troughs. The elative humidity and mean day temperature inside the closed chamber were 99% and 27 °C, respectively. The closed chambers were not opened (and not watered) for two months.

2.5 *Preparation of Frankia culture, inoculation of rooted cuttings and transplanation*

During transplanting, rooted stem cuttings were inoculated with pure cultures of *Frankia* strains Br and Cj (isolated from *C. equisetifolia*) obtained from the microbiological collection of the Botany Department, National University of Singapore. All *Frankia* isolates were cultured in the dark in a modified propionic acid medium (1) containing (g L^{-1}) $CaCl_2$. $2H_2O$, 0.01; $MgSO_4$. $7H_2O$, 0.05; NH_4Cl. 0.27; propionic acid, 0.48; ethylenediaminetetraacetic acid (Fe-Na salt), 0.01 and the following trace elements and vitamins (mg L^{-1}) : H_3BO_3, 2.9; $CoSO_4$. $7H_2O$, 0.001; $CuSO_4$. $5H_2O$, 0.08; $MnCl_2$. $4H_2O$, 1.8; Na_2MoO_4. $2H_2O$, 0.025; $ZnSO_4$. $7H_2O$, 0.22; thiamine hydrochloride, 0.01; nicotinic acid, 0.5; pyridoxine monohydrochloride, 0.05; biotin, 0.02; folic acid, 0.05 and calcium pantothenate 0.05. The medium was added with 1*M* KH_2PO_4–K_2HPO_4 buffer (10 mL L^{-1}), pH 6.7, before autoclaving at 1.1 kg cm^{-2} for 20 min at 120 °C. Cultures were maintained in 250 mL Erlenmeyer flasks containing 100 mL liquid medium at 25 °C and agitated continuously on a gyratory shaker at 130 rpm.

To prepare the inoculum, 4–5-week-old cultures of *Frankia* strains Br and Cj (about 25 colonies each) were washed thrice with distilled water, homogenized and then resuspended in 500 mL distilled water. Inoculation was performed by immersing roots of the cuttings in *Frankia* suspension for 60 min before transplanting the cuttings to a mixture of top soil and sand (1:1 v/v) in pots. After transplantation, plants were kept in the greenhouse for two weeks and then transferred to field conditions. Plants were watered once daily.

2.6 *Experimental design and statistical analysis of data*

Experiments were carried out in randomized design and repeated at least once. The data presented are means of two or three experiments with 30–50 stem cuttings per treatment. Assessment of rooting was carried out eight weeks after treatment and only cuttings with at least 0.5 cm long roots were scored as rooted. Cuttings were considered dead when the side branches were shed and their stems were either dried or blackened. Data were subjected to one-way analysis of variance (ANOVA) and Tukey's test, using STATGRAPHICS software (Statistical Graphics Corp., Rockville, MD).

Table 1. Effect of indole-3-butyric acid (IBA)(4th soaking) on survival of *C. sumatrana* stem cuttings after 30 days in sand medium

Treatment IBA (m*M*)	Number of cuttings	% Survival ±S.E.
0.0	30	47 ± 8a
1.0	30	13 ± 3b
2.5	30	7 ± 3b
5.0	30	0 ± 0
10.0	30	0 ± 0

In each column, the mean followed by the same letter are not significantly different as indicated by Tukey's test ($P = 0.05$).

Table 2. Effect of indole-3-butyric acid (IBA) (1 m*M*) treatment on survival and rooting of *C. sumatrana* stem cuttings after 60 days in sand medium

Time (min) of IBA application	Number of cuttings	% Survival ±S.E.	% Rooting ±S.E.
0	30	53±7a	0±0a
15	30	60±6a	17±6b
30	30	47±9a	13±7b
45	30	23±9b	10±6b
60	30	20±10b	14±9b

In each column, the mean followed by the same letter are not significantly different as indicated by Tukey's test ($P = 0.05$).

3. Results

3.1 *Effect of IBA concentration and application time on survival and rooting of stem cuttings*

Preliminary experiment showed that stem cuttings of *C. sumatrana* required a highly humid environment for their survival. All the cuttings in plastic troughs not covered with polyethylene sheets died within 12 days. Therefore, covered plastic troughs were employed for all subsequent experiments.

Survival of stem cuttings was affected by both the concentration (Table 1) and duration (Table 2) of IBA treatment. Blackening of the stem and abscission of side branches were observed in nearly 90% of the cuttings treated for 4 h with 1.0 and 2.5 m*M* IBA, and all with 5.0 and 10.0 m*M* IBA, within 12 days. These cuttings all died subsequently (Table 1). However, with lower concentrations of IBA and shorter duration of application (15–30 min), the survival rate of IBA-treated stem cuttings was comparable to that of the control (Table 2).

Root development occurred in nearly 17% of the cuttings treated with 1 m*M* IBA for 15 min but a further increase in the time of application of IBA treatment had little effect on the proportion of cuttings that rooted (Table 2). All the cuttings soaked in IBA solution developed large amounts of callus at the basal portion of the stem. Root development did not occur in untreated control cuttings.

3.2 *Effect of IBA and NAA applied by quick-dip method on survival and rooting of stem cuttings*

The potential of survival and rooting of stem cuttings increased considerably when IBA was applied by a quick-dip method. With 1 m*M* IBA treatment, about 62% of stem cuttings survived and half of them were rooted. No significant increase in the rate of survival or rooting was observed with further increases in IBA concentration (Table 3).

Table 3. Effect of indole-3-butyric acid (IBA) and α-naphthalene-acetic acid (NAA) application by quick-dip (5s) on survival and rooting of *C. sumatrana* stem cuttings after 60 days in sand medium

Treatment	% Survival ±S.E.	% Rooting ±S.E.	Mean no. of roots per cutting ±S.E.	Mean root length (cm) ±S.E.
IBA (m*M*)				
0	60 ± 12a	0 ± 0a	0.0 ± 0.0a	0.0 ± 0.0a
1	62 ± 11a	30 ± 7b	1.5 ± 0.2b	7.6 ± 1.2b
5	68 ± 6a	34 ± 5b	1.7 ± 0.2b	6.4 ± 0.8b
10	77 ± 11a	38 ± 11b	2.7 ± 0.4c	5.8 ± 1.0b
NAA (m*M*)				
1	76 ± 12a	57 ± 9c	2.4 ± 0.4c	6.3 ± 1.0b
5	80 ± 6a	63 ± 3c	3.6 ± 0.5c	7.9 ± 0.9b
10	84 ± 12a	70 ± 6c	5.1 ± 0.6d	7.4 ± 0.7b

In each column, the mean followed by the same letter are not significantly different as indicated by Tukey's test ($P = 0.0.5$).

Treatment with NAA stimulated more rooting than IBA (Table 3). When equimolar concentrations of these two auxins were tested, NAA at all concentrations caused almost twice as many stem cuttings to root as IBA. Rooting occurred within two weeks in NAA-treated cuttings as compared to four weeks required for IBA-treated cuttings. NAA also considerably increased the number of roots per cuttings, but the mean root length was about the same (Table 3, Fig. 1).

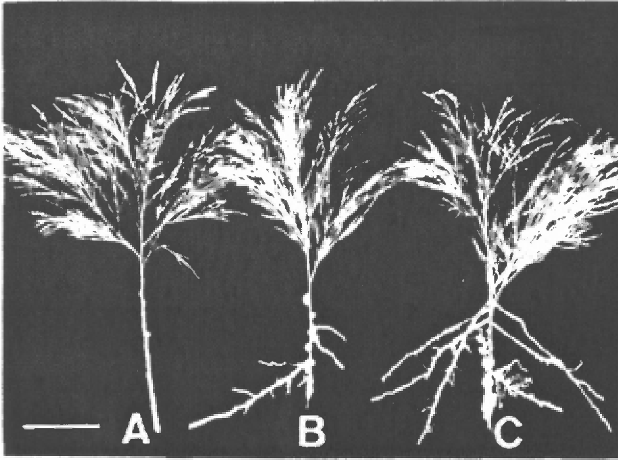

Fig. 1. Effect of 10mM indole-3-butyric acid (IBA) or α-naphthaleneacetic acid (NAA) on rooting of stem cuttings of *C. sumatrana* after 60 days in sand medium. A – control; B – 10mM IBA; C – 10mM NAA. *Bar* = 3 cm.

3.3 *Effect of different rooting media on survival and rooting of stem cuttings*

Of the three rooting media (sand, vermiculite and Oasis) used, sand was found to be the most suitable medium for root formation although stem cuttings survived equally well in both sand and vermiculite (Table 4). About 30% of the stem cuttings, irrespective of the concentration of IBA used, rooted in a sand medium. This was as much as three times more than that obtained in vermiculite (Table 4). In Oasis, all the cuttings died within two weeks.

3.4 *Use of closed chamber propagation system on survival and rooting of stem cuttings*

As much as 70% of the stem cuttings treated with 10 mM NAA survived in a closed chamber system compared to 84% survival in troughs loosely covered with polyethylene sheets (Table 5). Nonetheless, the rooting ability was not significantly affected by the type of the enclosure, with both treatments inducing 60–70% of the stem cuttings to produce an average five roots per cutting.

3.5 *Frankia inoculation and transplantation*

All the roots developed from the stem produced many laterals. *Frankia* inoculation was not necessary for successful establishment and continued growth of rooted cuttings in pots. Both *Frankia* inoculated and uninoculated rooted cuttings produced root nodules within four weeks after transplanting and grew well under field conditions. Only 3% of the rooted cuttings died following transplanting.

Table 4. Effect of rooting medium on survival and rooting of *C. sumatrana* stem cuttings treated with indole-3-butyric acid (IBA). Data were collected after 60 days

Rooting medium	IBA (mM)	% Survival ±S.E.	%Rooting ±S.E.	Mean no. of roots per cutting ±S.E.
Sand	0	60 ± 12a	0 ± 0a	0.0 ± 0.0a
	1	62 ± 11a	30 ± 7b	1.5 ± 0.2b
	5	68 ± 6a	34 ± 5b	1.7 ± 0.2b
	10	77 ± 11a	38 ± 11b	2.7 ± 0.4c
Vermiculite	0	67 ± 15a	0 ± 0a	0.04 ± 0.0a
	1	63 ± 9a	13 ± 3c	1.5 ± 0.3b
	5	72 ± 14a	10 ± 6c	1.5 ± 0.5b
	10	53 ± 12a	23 ± 9c	1.9 ±0.3b
Oasis	0	0	0	0
	1	0	0	0
	5	0	0	0
	10	0	0	0

In each column, the mean followed by the same letter are not significantly different as indicated by Tukey's test (P = 0.05).

Table 5. Comparison of open and closed chamber propagation systems on survival and rooting of *C. sumatrana* stem cuttings treated (quick-dip, 5 s) with 10 mM NAA. Data were collected after 60 days

Propagation system	% Survival ±S.E.	% Rooting ±S.E.	Mean no. of roots per cutting ±S.E.
Open chamber*	84 ± 12a	70 ± 6a	5.1 ± 0.6a
Closed chamber	70 ± 10a	63 ± 8a	4.7 ± 0.5a

* Plastic troughs loosely covered with polyethylene cover, allowing free movement of air and moisture.

In each column, the mean followed by the same letter are not significantly different as indicated by Turkey's test (P = 0.05).

4. Discussion

Casuarinas, like many hardwood tree species, are considered difficult to root [17] and successful vegetative propagation methods are documented only for a few species of this genus [6, 13]. The present results

clearly indicate that *C. sumatrana* can be multiplied by rooted stem cuttings with appropriate manipulation of the rooting environment and exogenous application of NAA.

The frequency of rooting and successful transplantation was very low in most of the methods described previously for the vegetative propagation of *Casuarian* species [13]. A relatively high percentage of rooting of stem cuttings in *C. sumatrana* was achieved in the present study. With 10 m*M* NAA, using a quick-dip method, as many as 80% of the cuttings were rooted within eight weeks. This high percentage of rooting could be due in part to three factors: a) use of mature softwood cuttings, b) the method of auxin application and c) provision of a highly humid environment during rooting.

Many previous studies on woody plants [3] showed that juvenile cuttings are more responsive to plant growth regulator treatments than mature shoots, for juvenile tissues normally have more young, less differentiated and actively dividing cells and tend to contain negligible amounts, if any, rooting inhibitors [3, 10]. The poor root initiation observed in some of the previous *Casuarian* rooting experiments [13] could be due to the use of mature cuttings. However, in the present study, a high percentage of rooting was obtained even when using mature cuttings. This indicates that with proper plant growth regulator treatment and manipulation of the rooting environment, even mature stem cuttings could be used as propagules for casuarina propagation with high success.

From our results it appears that the method of auxin application also plays an important role in root production. Administration of auxin by a quick-dip method was superior to that of soaking stem cuttings in an auxin solution for extended periods, the most common method employed for *Casuarina* rooting by previous workers [6, 13]. The exact reason for this difference in rooting performance is not understood at present.

An important and novel finding of the present study is the applicability of a closed chamber system for the vegetative propagation of *C. sumatrana*. The closed chamber system with the use of Neoflon film which maintains a highly humid atmosphere around the stem cuttings without any gas or temperature build up proved to be an efficient plant propagation system. The propagation unit requires little attention following the initial planting of the cuttings and produces high yield. The use of Neoflon film to develop cheap and disposable vessels for commercial tissue culture has already been demonstrated [12]. Its potential in improving conventional propagation methods, as shown in this study, is to be further explored.

The method described here is simple, efficient and inexpensive for clonal propagation of *C. sumatrana* by rooting of stem cuttings. This rapid propagation method, particularly the quick-dip method of auxin application and the use of closed chamber system, will be of considerable practical importance for the large-scale production of clonal planting material of not only *C. sumatrana* and other casuarinas, but also various other economically important tree species. Experiments with tropical rain forest tree species are now in progress in our laboratory.

Acknowledgements

The following students, Zulaiha Shireen, F. F. Yong, W. L. Wee, H. C. Wong and L. S. Wong participated in some of the experiments presented in this paper. The *Frankia* cultures were kindly provided by our colleague, Dr. T. K. Tan.

References

1. Burggraaf AJP and Shipton WA (1982) Estimates of *Frankia* growth under various pH and temperature regimes. Plant Soil 69: 135–147
2. Duhoux E, Sougoufara B and Dommergues Y (1986) Propagation of *Casuarian equisetifolia* through axillary buds of immature female inflorescences cultured *in vitro*. Plant Cell Rep 3: 161–164
3. Hartmann HT, Kester DE and Davis FT (1990) Plant Propagation. Principles and Practices, Fifth edition, p 645. New Jersey: Prentice-Hall International Editions, New Jersey
4. Hussain S (1957) A note on vegetative multiplication of *Casuarina cnninghammi*. Indian For 83: 226–227
5. Husain AMM and Ponnuswamy PK (1980) Propagation of *Casuarina junghuhniana* by planting shoots and root suckers. Indian For 106: 298–299
6. Lundquist R and Torrey JG (1984) The propagation of *Casuarina* species from rooted stem cutting. Bot Gaz 145: 378–384
7. Midgley SJ, Turnbull W and Johnston RD (eds) (1983) *Casuarina* Ecology, Management and Utilization, p 286. Melbourne: CSIRO
8. National Academy of Sciences (1980) Firewood Crops, p 237. Washington, DC: National Academy Press
9. National Academy of Sciences (1984) Casuarinas: Nitrogen-fixing Trees for Adverse Sites, p 118. Washington, DC: National Academy Press
10. Paton DM, Willing RR, Nichols W and Pryor LD (1970) Rooting of stem cuttings of *Eucalyptus*: a rooting inhibitor in addult tissue. Aust J Bot 18: 175–183
11. Somasundaram TR and Jagadees SS (1977) Propagation of *Casuarina equisetifolia* Forst. by planting shoots. Indian For 103: 737–738

12. Tanaka M (1991) Disposable film culture vessels. In: Bajaj YPS (ed) Biotechnology in Agriculture and Forestry, Vol 17 High-Tech and Micropropagation 1, pp 212–228. Berlin: Springer-Verlag
13. Torrey JG (1983) Root development and root nodulation in *Casuarina*. In: Midgley SJ, Turnbull W and Johnston RD (eds) *Casuarina* Ecology, Management and Utilization, pp 180–192. Melbourne: CSIRO
14. Turnbull JW and Martensz PN (1983) Seed production, collection and germination in Casuarinaceae. In: Midgley SJ, Turnbull W and Johnston RD (eds) *Casuarina* Ecology, Management and Utilization, pp 126–132. Melbourn: CSIRO Melbourn