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Original Paper

Direct adventitious shoot organogenesis and plant regeneration from cotyledon explants in *Neolamarckia cadamba*

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Abstract *Neolamarckia cadamba* is a fast-growing and deciduous tropical hardwood with anatomical, morphological, and chemical characteristics that make it suitable for building materials, pulp production, and medicine raw materials. In this study, a protocol for direct adventitious shoot organogenesis and plant regeneration from the aseptic cotyledons of *N. cadamba* was established. The cotyledons with petioles from 3-week-old seedlings were used for adventitious shoot induction in DCR medium containing 22.20 μM 6-benzyladenine (BA) and 0.27 μM α-naphthaleneacetic acid (NAA). The frequency of adventitious shoot induction was 54.2%. Micro-shoots were then transferred to MS medium containing 4.44 μM BA and 0.25 μM indole-3-butyric acid (IBA) for shoot propagation. Available shoots per explant reached 5.9. The highest rooting percentage (98.3%) was obtained when shoots were transferred to half-strength MS medium supplemented with 0.27 μM NAA and 0.25 μM IBA. The rooted plantlets could be successfully acclimatized to a greenhouse with more than 95% survival, and the regenerated plants showed the same morphological characteristics as those of the control plants in fields. Histological observations revealed that the adventitious shoots only originated from the epidermal tissue around the edge of the cut zone of the cotyledonary petiole.

Key words: Adventitious shoots, cotyledon, *Neolamarckia cadamba*, organogenesis, regeneration.

Neolamarckia cadamba is a fast-growing and deciduous tropical hardwood with anatomical, morphological, and chemical characteristics that make it suitable for building materials, furniture, and pulp production (Lal et al. 2010). The "Ayuredo," an Indian ancient medical reference, discusses treating diseases such as dysentery, fever, and snake bites using this species (Banerji and Dutta 1976). Its bark, leaves, and flowers can be used to extract monoterpenoid, triterpenoid saponin, alkaloid, and ethylene glycol (Ahmed et al. 2011; Banerji 1978; Sahu et al. 1999; Shibli et al. 2001). In addition, it is a high-quality landscape tree and benefits natural ecosystems because it is medium to large sized, attaining a height of 20–45 m and a girth of about 2–2.5 m. The rounded crown is umbrella-shaped and the branches are characteristically arranged in tiers. The leaves are simple and 13–32 cm long. Hence, it is known as the "miracle tree" or "gems tree."

As a sensitive plant, it is distributed only in India, Nepal, Thailand, China, and eastward in the Malaysian Archipelago to Papua New Guinea, and has been introduced successfully to Africa (Flora of China Editorial Committee 2011). At the same time, because leaves and buds of the juvenile plant are rich in nutrition, it can be injured by some Lepidoptera and Coleoptera insects, such as *Dianhania glauculelis* and *Acalolepta cervina* (Danida Forest Seed Centre 2000). Since it is a woody plant and perennial in nature, improvement of this tree is slow. Consequently, a rapid clonal multiplication technique such as micropropagation could be applied to *N. cadamba*, which will supplement conventional culturing methods and lead to rapid improvement of the tree. Although micropropagation in *N. cadamba* has been attempted using explants of both

Abbreviations: MS, Murashige and Skoog; DCR, DCR medium; BA, 6-benzyladenine; 2,4-D, 2,4-dichlorophenoxyacetic acid; TDZ, thidiazuron; 2-ip, N^6-(2-isopentenyl)adenine; NAA, α-naphthalene acetic acid; IBA, indole-3-butyric acid; PGRs, plant growth regulators.

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juvenile (seedling) and mature tree origins, regeneration has been only moderately successful in juvenile-origin explants due to contamination and heavy leaching of phenolics (Zhan 2010). These factors contribute to the poor in vitro responses from mature trees and limit biotechnological improvement of this tree, particularly using genetic modification.

The low hardiness, weaker insect resistance, lower natural propagation, increasing demands for timber and natural medicine, and the narrow habitable range of *N. cadamba* could result in its removal from natural ecosystems. Hence, it is essential to preserve the natural resources of *N. cadamba* using biotechnological methods. Thus, we attempted to regenerate plants from cotyledons and hypocotyls of *N. cadamba*.

Materials and methods

Plant materials and seed germination

N. cadamba seeds were collected from a 13-year-old plus tree of Guangxi Botanical Garden of Medicine Plants (Nanning, Guangxi, China) and stored at 4°C in the dark. In order to improve seed germination, seeds were immersed in water and incubated at 40°C for 24 h on a thermostat shaker set at 120 rpm (under 12-h photoperiod of 90 μmol m^{-2} s^{-1}). Seeds were then surface-sterilized in 20% bleach (5.0% sodium hypochlorite) solution for 15 min, followed by three rinses with sterile distilled water. Disinfected seeds were inoculated on Murashige and Skoog (MS, Murashige and Skoog 1962) basal medium without any growth regulators at 25±2°C and maintained under a 12-h photoperiod of 90 μmol m^{-2}·s^{-1} irradiance provided by cool-white fluorescent tubes (MW1-Y36, Foshan Electrical and Lighting Co., Ltd.) with a relative humidity of 70%.

Adventitious shoot induction

Cotyledons (2–3 mm in length) and hypocotyls (3–4 mm in length, without cotyledonary node) were dissected from 3-week-old in vitro cultured seedlings and cultured on DCR (Gupta and Durzan 1985) basal medium supplemented with 0.00, 4.44, 8.88, 13.32, 17.76, 22.20, 26.64, or 35.52 μM 6-benzyladenine (BA) and either 0.23 μM 2,4-dichlorophenoxyacetic acid (2,4-D) or 0.27 μM α-naphthalene acetic acid (NAA) for adventitious shoot induction. Cotyledons were placed with the abaxial side downwards, and hypocotyls were placed parallel on the surface of the medium. All medium was supplemented with 3.0% (w v^{-1}) sucrose and 0.7% (w v^{-1}) agar (Beijing Dingguo Changsheng Biotechnology Co., Ltd.). The medium pH was adjusted to 5.8 with 1.0 M NaOH prior to autoclaving at 121°C for 20 min. The medium was aliquoted into sterile 250-ml glass culture vessels (25 ml each). There were 12 explants per treatment, and four replications were conducted. Culture conditions were the same as described above. Adventitious shoot induction frequency, defined as the percentage of explants with adventitious shoots, as well as the number of shoot per explant was recorded after 4 weeks of culture. All plant growth regulators were purchased from Beijing Dingguo Changsheng Biotechnology Corporation, Limited.

Shoot proliferation

Micro-shoots were sub-cultured in hormone-free MS basal medium for 2 weeks to eliminate any carry-over effects of the basic hormone (Shibli et al. 2001). For shoot proliferation, micro-shoots approximately 1 cm in length were sub-cultured in MS basal medium supplemented with 0.25 μM indole-3-butyric acid (IBA) and BA at 0.00, 0.89, 2.22, 4.44, or 8.88 μM. For each replicate, 25 ml of the medium was dispensed into 250-ml culture vessels. Each treatment contained 10 micro-shoots and was repeated four times. Culture conditions were the same as described above. After 2 weeks of culture, number of available shoots and micro-shoot growing status were recorded.

Rooting of adventitious shoots

For in vitro root formation, micro-shoots about 3–4 cm in length were transferred to half-strength MS basal medium supplemented with 0.27 μM NAA in combination with 0.00, 0.25, 0.49, or 0.98 μM IBA. Four replications with eight explants per treatment were conducted. Micro-shoots on root induction medium were cultured in the dark for 2 days at 25±2°C prior to being cultured under a 12-h photoperiod (90 μmol m^{-2} s^{-1}). After 10 days of culture, the root formation frequency, root number, root length, and number of lateral roots were determined.

Acclimatization of in vitro-rooted plantlets

Plantlets in the culture vessels were transferred from the culture room to the greenhouse for 3–4 days. Plantlets with well-developed shoots and roots were then removed from the agar medium and potted in 10-cm plastic pots containing a soil-less substrate (peat moss and loam in a 2 : 1 volumetric ratio). Agar was removed from the roots thoroughly prior to transplanting. The pots were covered with polythene films to provide a high relative humidity, and the films were gradually opened after 5 days in the greenhouse to allow plantlets to acclimatize to ambient conditions. Plants were watered every 2–3 days until the films were fully opened, and then watered as required.

Histological observation

To trace the early ontogenic stages of direct adventitious shoot regeneration, cotyledons cultured on initiation medium were collected at 1-day intervals until the adventitious buds were discernible with the naked eye.

Semi-thin sections

Specimens were fixed in the mixture of 2.5% paraformaldehyde and 3.0% glutaraldehyde in 0.1 M phosphate buffer solution (PBS, pH 7.2) for 4 h at 4°C, then washed three times with the same buffer, followed by post-fixation in 1% osmium tetroxide

for 2 h at room temperature and three rinses using the same buffer. Specimens were dehydrated in a graded ethanol series and embedded in Epon812 (SPI Supplies Division of Structure Probe Inc., West Chester, PA, USA). Polymerization was performed for 24 h at 40°C followed by 24 h at 60°C. Specimens were cut to a thickness of 1 μm on a Leica RM2155 microtome (Leica, Inc. Germany), and then the semi-thin sections were affixed to slides with a drop of water. Then, the slides was placed on a slide warmer set a 70°C, stained with 0.5% toluidine blue, covered with a cover slip in place with help of a thin coating of Neutral Balsam, and dried at 38°C for 48 h.

Paraffin sections

Specimens were fixed in FAA solution (formalin (37%) : glacial acetic acid : ethanol (50%), ratio 5 : 5 : 90 in volume) (Jensen 1962). Samples were progressively dehydrated in a graded ethanol series (70–100%), then embedded in paraplast and mounted on block-holders. Samples were sectioned in 8-μm slices with a Reichert 820H Histostat rotary microtome (Warer-Lambert Tech. Inc., USA). The paraffin sections were affixed to slides, stained with a combination of safranin and fast green, covered with a cover slip in place with help of a thin coating of Neutral Balsam, and then dried at 38°C for 48 h (Jensen 1962). All sections were observed and photographed with a Leica DMLB microscope (Leica Microsystems, Germany).

Statistical analysis

Data were analyzed using one-way ANOVA. Duncan's multiple range test was applied at the 0.05 probability level to compare individuals within a treatment (Gomez and Gomez 1984) using the SPSS ver. 19.0 software.

Results

Adventitious shoot formation

For adventitious shoot induction, cotyledons (Figure 1a) and hypocotyls from in vitro-germinated plants were used as explants. Organogenesis from cotyledon explants was induced directly at the cut edges of the cotyledonary petioles (Figure 1b). Adventitious shoots were mainly developed from the basipetal portion around the cotyledonary petioles after 3.5 weeks of culture (Figure 1c), and its development continued until the fourth week, when the results were recorded (Figure 1d). Significant differences in the rates of organogenesis were observed in cotyledon explants (F=626.174, p<0.001). Higher rates (42.12% and 54.17%) were obtained at moderate concentrations of BA (17.76 and 22.20 μM) in combination with 0.27 μM NAA, which yielded 2.86 and 4.42 shoots per cotyledon explant, respectively (Table 1). For treatments of BA combined with 2,4-D, the highest adventitious shoot formation rate was 26.14% induced by 22.20 μM combined with 0.23 μM 2,4-D, which yielded 2.73 shoots per cotyledon explant. Moreover, with increasing concentrations of BA in combination with 0.23 μM 2,4-D, the callus formation frequency decreased gradually in all treatments. In general, poorer organogenesis was the result of low (0.00 or 4.44 μM)

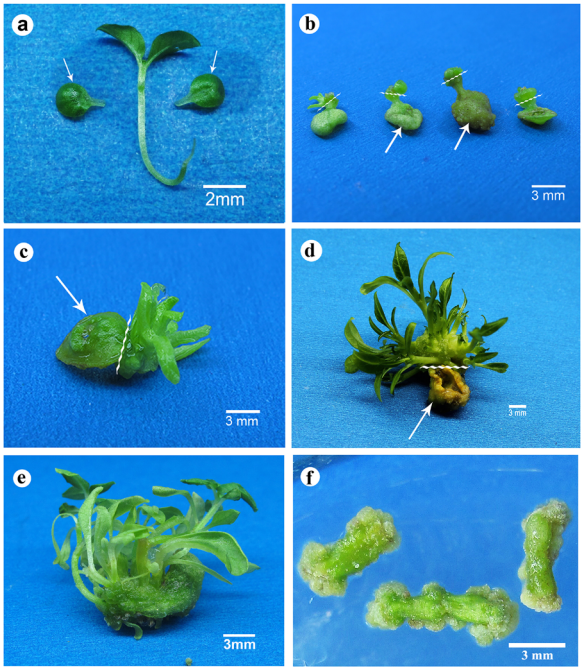

Figure 1. Adventitious shoot formation from in vitro-cultured cotyledons and callus developed from hypocotyls of *N. cadamba*. a. Cotyledons (arrow) with petioles used as explants. b–d. Adventitious shoots developed from the cut zone of cotyledonary petioles (arrow points to the cotyledon) in DCR medium containing 22.20 μM BA and 0.27 μM NAA. e. Vitrification in DCR medium containing 35.52 μM BA and 0.27 μM NAA. f. Only callus developed from hypocotyls after 4 weeks of culture in medium supplemented with 35.52 μM BA and 0.27 μM NAA. Bar (a) 2 mm; (b–f) 3 mm.

Table 1. Effect of PGRs on adventitious shoot induction in *N. cadamba* after 4 weeks of culture.

PGRs (μM)			Frequency of shoot formation (%)	No. of shoots per explant
BA	2,4-D	NAA		
0.00	0.00	0.00	0.00 i	0.00 e
0.00	0.23	0.00	0.00 i	0.00 e
4.44	0.23	0.00	8.20±0.33 gh	1.50±0.29 d
8.88	0.23	0.00	9.91±0.30 f	1.75±0.25 d
13.32	0.23	0.00	15.81±0.80 e	1.75±0.44 d
17.76	0.23	0.00	23.27±0.35 d	2.68±0.27 bc
22.20	0.23	0.00	26.14±0.95 cd	2.73±0.19 bc
26.64	0.23	0.00	22.34±1.45 de	1.42±0.25 d
35.52	0.23	0.00	8.36±0.28 gh	1.13±0.23 d
0.00	0.00	0.27	7.16±0.21 h	1.25±0.25 d
4.44	0.00	0.27	21.95±0.45 de	1.96±0.14 cd
8.88	0.00	0.27	26.86±0.30 cd	2.84±0.30 b
13.32	0.00	0.27	29.96±0.55 c	2.85±0.36 b
17.76	0.00	0.27	42.12±0.92 b	2.86±0.45 b
22.20	0.00	0.27	54.17±2.51 a	4.42±0.44 a
26.64	0.00	0.27	28.62±0.56 c	2.56±0.17 bc
35.52	0.00	0.27	7.10±0.45 h	1.50±0.29 d

Values are means±SE. Means within a column with the same letter are not significantly different based on Duncan's multiple-range test at the 0.05 probability level.

or high (35.52 μM) BA concentrations. When the BA concentration was less than 8.88 μM (in combination with 2,4-D) or 4.44 μM (in combination with NAA), or BA concentrations were higher than 35.52 μM, *N. cadamba* adventitious shoot formation decreased (Table 1). When BA concentrations were greater than 26.64 μM, the adventitious shoots exhibited mild vitrification and the leaf size decreased and became involute (Figure 1e). On the other hand, no adventitious shoots were formed on hypocotyl explants (Figure 1f).

Shoot proliferation

Micro-shoot proliferation was achieved by all treatments. However, the greatest proliferation occurred in MS medium containing 4.44 μM BA and 0.25 μM IBA, which stimulated *N. cadamba* shoot elongation and proliferation most effectively. The number of available shoots per explant peaked at 5.92 (Table 2). The shoots were robust and healthy, exhibiting a dark green color (Figure 2a). Shoots grew rapidly in this medium and nodal segments were sub-cultured every 3 weeks.

Micro-propagation is amenable to in vitro culture on *N. cadamba*. In hormone-free MS medium, the number of available shoots per explant was 2.45. The number of adventitious buds increased in all treatments with increasing concentrations of BA and in combination with 0.25 μM IBA; however, the number of available shoots per explant decreased to 3.83 when BA concentrations exceeded 4.44 μM (Table 2). Moreover, shoots produced at higher concentrations of BA were pale green, and were often distorted, with a light glassy appearance.

In vitro rooting

Roots became evident on the micro-shoots after 7 days of culture in root induction medium (Figure 2b), and developed directly from the base of the stems with minimal callus formation (Figure 2c). Exogenous auxin had a significant effect on root induction rates and the number of roots, compared with the control treatment

Figure 2. In vitro propagation, rooting, acclimatization, and afforestation of *N. cadamba*. a. Shoot propagation. Root formation in root initiation medium (containing 0.25 μM IBA and 0.27 μM NAA) after 7 days (b) and 10 days (c). d. Acclimatization of plantlets in the greenhouse. e. 3-months plantlets in large plastic pots. f. 7-months plantlets in the field. Bar (a–c) represents 3 mm.

Table 2. Effects of BA and IBA concentration on shoot proliferation of *N. cadamba* after 2 weeks of culture.

PGRs (μM)		No. of available shoots per explant	Micro-shoot growth status
BA	IBA		
0.00	0.00	2.45±1.64 d	Slim stem, a few slender roots
0.00	0.25	2.49±1.28 d	Robust stem, a little callus, a few roots
0.89	0.25	2.93±1.92 d	Robust stem, a little callus
2.22	0.25	5.17±1.43 b	Robust stem, more adventitious shoots
4.44	0.25	5.92±0.89 ab	Robust stem, elongated well, more adventitious shoots
8.88	0.25	3.83±1.84 c	Most adventitious shoots, but stem weakness

Values are means±SE. Means within a column with the same letter are not significantly different based on Duncan's test at the 0.05 probability level.

Table 3. Effects of IBA and NAA on in vitro root formation of *N. cadamba*.

PGRs (μM)		Frequency of root formation (%)	Mean No. of roots per shoot	Mean root length per shoot (cm)	Mean No. of lateral roots per shoot	Root status
IBA	NAA					
0.00	0.00	87.2 b	3.7±1.8 c	1.11±0.88 ab	0.2±0.0 c	Slender
0.00	0.27	98.1 a	6.5±2.1 ab	1.45±1.13 a	1.9±0.2 a	Slender
0.25	0.27	98.3 a	5.2±1.5 b	1.22±1.01 ab	1.4±0.6 ab	Short, thick
0.49	0.27	95.4 a	6.0±1.3 ab	0.89±0.78 b	1.2±0.3 b	Shorter, thicker
0.98	0.27	97.9 a	7.3±0.9 a	0.52±0.32 c	0.3±0.1 c	Dense and shorter, a few aerial roots

Values are means±SE. Means within a column with the same letter are not significantly different based on Duncan's test at the 0.05 probability level.

(hormone-free medium) (Table 3). Excluding the control treatment, the root induction rate was more than 95.4% and at least 5.21 roots were formed. The highest rooting percentage (98.3) was induced by 0.25 μM IBA combined with 0.27 μM NAA. However, the highest number of roots (7.32) was obtained with half-strength MS medium containing 0.98 μM IBA and 0.27 μM NAA.

IBA decreased the root length and number of lateral roots in *N. cadamba*, and there was partial antagonism between NAA and IBA in their effects on root length and the number of lateral roots (Table 3). With increased IBA concentrations, the root lengths decreased sharply from 1.45 to 0.52 cm and number of lateral roots decreased from 1.89 to 0.28 (Table 3). Nevertheless, the root thickness increased, which is consistent with previous studies of pomegranate (*Punica granatum* L.) (Patil et al. 2011). However, the IBA concentration did not significantly influence root induction rates.

Additionally, NAA had a positive effect on root length and the number of lateral roots. In culture medium containing only 0.27 μM NAA, both root length and the number of lateral roots were the best (1.45 cm and 1.89 cm, respectively) (Table 3). Therefore, regarding in vitro root formation of *N. cadamba*, NAA likely promoted root and fibrous root induction, while IBA promoted root diameter enlargement.

Ex vitro transplanting

In vitro-rooted plantlets were successfully acclimatized to greenhouse conditions with no less than 95% survival (Figure 2d, e). When the plantlets were 10–20 cm in height, they were transplanted into the field for continued growth and development. After 7 months (Figure 2f), plants reached 2.31 m (average); the maximum ground diameter, length, and width of the mature leaves were 47 mm, 83 cm, and 55 cm, respectively.

Histological observation

Histological studies showed the adventitious shoots derived from epidermal cells (upper area of the vascular elements) of the cut zone of the cotyledonary petiole (Figure 3). The dense contents of the epidermal cell became obvious after 3–4 days of culturing (Figure 3a, b). Domes formed (Figure 3c) between days 10–12 due to periclinal and anticlinal divisions of the epidermal cells, and initiation of shoot buds was observed after 15–30 days (Figure 3d, e, f) of culture. Histological analysis showed that adventitious shoots formed after 4–5 weeks of culture under in vitro conditions.

The emergence of adventitious shoots from the edge of the cotyledonary petioles was observed as domes formed near small meristematic cells, which were restored from epidermal cells with high mitotic activity. As the in vitro plantlets from the *N. cadamba* seeds were cultured in a high humidity and isothermal environment, the epidermal cells of cotyledonary petiole may retain the characteristics of parenchyma cells. Hence, the adventitious shoot buds of *N. cadamba* developed de novo and from the explant epidermal cells as a result of mitotic activity of the epidermal cells in response to appropriate hormone concentrations, but not from preexisting meristems (vascular elements). This emergence is similar to that of the sunflower (Sujatha et al. 2012), but different from *Centaurea ultreiae*, in which meristematic cells arise from the original vascular

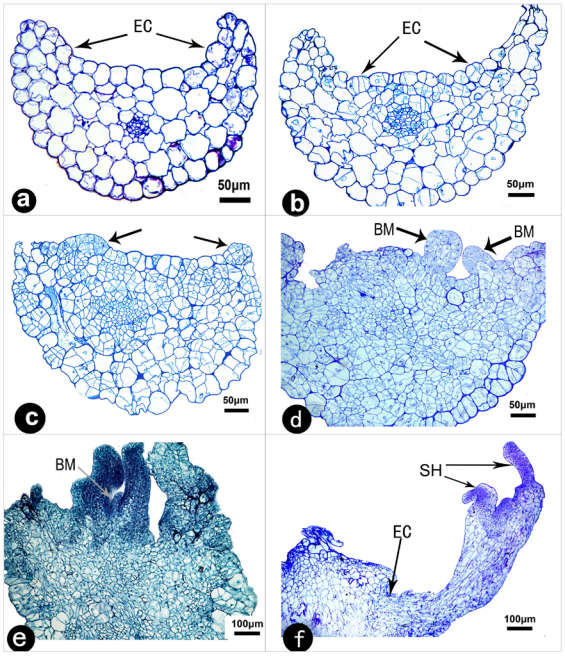

Figure 3. Histological sections of regenerated shoots of *N. cadamba* in adventitious shoot induction medium. The contents of epidermal cells (EC) on cotyledonary petioles increased after (a) 3 days (fewer EC divisions occurring), and (b) 4 days (more EC divisions occurring, arrow points to the divided epidermal cells). c. EC formed a dome (arrow) after 10–12 days. Bud meristem (BM) appeared on the 15th (d) and 20th (e) days. f. Thirty days after culture: longitudinal sections through adventitious shoots (SH) confirm their direct origin from the EC of cotyledonary petioles. Bar (a–d) 50 μm; (e, f) 100 μm. (a–d: semi-thin sections. e, f: paraffin sections).

Table 4. Effects of adventitious shoot formation in different age of cotyledon cultured on regeneration medium after 4 weeks.

Age of cotyledon (days)	20	25	30	35	40	45
Frequency of shoot formation (%)	54.6±1.8 a	51.7±2.6 a	41.6±4.5 b	35.8±1.5 bc	28.3±1.0 c	19.2±2.8 d

Values are means±SE. Means within a row with the same letter are not significantly different based on Duncan's test at the 0.05 probability level. Culture medium (DCR) supplemented with 22.20 μM BA and 0.27 μM NAA.

elements (Mallon et al. 2011).

Discussion

The regeneration of many woody plants using in vitro culture has been accomplished using different explants; e.g., young leaves of *Jatropha curcas* (Tsuchimoto et al. 2012) and *Diospyros kaki* (Yokoyama et al. 2011), buds of *Forsythia* spp. (Morimoto et al. 2011), leafy shoots of *Cryptomeria japonica* (Ishii et al. 2011), shoot explants of *Anacardium occidentale* (Thimmappaiah et al. 2002) and *Eucalyptus* (Hajari et al. 2006), and radicles of the olive (Cerezo et al. 2011), as well as somatic embryogenesis of *Olea europea* (Shibli et al. 2001). In this study, direct adventitious shoot organogenesis and plant regeneration were first established in *N. cadamba* using cotyledons as explants. These results were similar to those of previous studies on yam (Anike et al. 2012), *Solanum aculeatissimum* (Ghimire et al. 2012), olive (Brhadda et al. 2003), *Chrysanthemum morifolium* (Song et al. 2011), *Campanula punctata* (Sivanesan et al. 2011), and *Pulsatilla koreana* (Lin et al. 2011), in which the petioles of the cotyledons or leaves retained sufficient cellular plasticity to achieve plantlet regeneration or somatic embryogenesis. However, a number of parameters are known to influence shoot regeneration. In this study, the most important factors influencing *N. cadamba* adventitious shoot induction included the type and nature of the explants, as well as the ratio and concentrations of plant growth regulators in the culture medium. In vitro cotyledons as explants provided higher adventitious shoot induction frequency than did hypocotyls. Moreover, juvenescent cotyledons (20–25 days) yielded higher adventitious shoot formation frequencies than mature cotyledon explants (30–45 days) (Table 4). Additionally, we found that NAA in combination with the same concentrations of BA was superior to 2,4-D in terms of *N. cadamba* adventitious shoot formation. However, an over-abundance of BA, as for other cytokinins [thidiazuron (TDZ), zeatin or N^6-(2-isopentenyl)adenine (2-ip)], may have negative effects in vitro on cotyledons, such as inhibition of shoot induction and propagation or hyperhydricity, and could limit adventitious shoot formation (Stevens and Pijut 2012).

Nodal sub-culturing in micro-shoot proliferation, which has been successful for some hardwoods and herbaceous plants, many species of oak (Vieitez et al. 2009), *Bixa orellana* (Parimalan et al. 2011), *Senecio cruentus* (Sivanesan and Jeong 2012), and *Piper longum* (Rani and Dantu 2012), was also effective with *N. cadamba* in the present study (data not shown).

It is typically difficult for roots to develop from in vitro woody shoots, while in this study, in vitro *N. cadamba* shoots rooted in medium at a high frequency (87.2–98.3%). This is similar to *Caesalpinia bonduc* (Cheruvathur et al. 2012), *Carica papaya* (Roy et al. 2012), *Santalum album* (Singh et al. 2013), *Gardenia latifolia* (Reddy and Saritha 2013), pumpkin ash (Stevens and Pijut 2012), date palm (Khan and Bi 2012), and *Caesalpinia bonduc* (Kumar et al. 2012). All rooting percentages were at least 70%, and some reached 100%. The significant differences in rooting percentages among woody plants may be due to different levels of endogenous hormones in the explants.

Based on this method, we successfully afforested almost 300,000 plants in Guangdong, Guangxi, and Hainan provinces, China, with total areas of more than 400 ha. The 3-year-old plants exhibited homogeneity and no observable morphological variations were detected among the regenerated and seedling of *N. cadamba* plants.

In conclusion, this is the first study to establish in vitro adventitious shoot induction, rooting, plantlet acclimatization, and field plantation protocols for *N. cadamba* using cotyledons as explants. From these results, it is highly possible that this protocol can be used for rapid mass clonal propagation, afforestation, conservation strategies, germplasm exchange, producing phytomedicines, and that it will be useful for agrobacterium-mediated transformation to introduce useful genes such as virus, insect, and cold resistance genes for increasing resistance of this important species.

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