Plant Biotechnology 36, 13–19 (2019)
DOI: 10.5511/plantbiotechnology.18.1119a

Original Paper

High frequency plant regeneration from leaf culture of *Neolamarckia cadamba*

Jingjian Li[1,2,3], Deng Zhang[1], Kunxi Ouyang[1,*], Xiaoyang Chen[1,2,3,**]

[1] College of Forestry and Landscape Architecture, South China Agricultural University, Guangzhou 510642, China; [2] State Key Laboratory for Conservation and Utilization of Subtropical Agro-bioresources, Guangzhou 510642, China; [3] Guangdong Key Laboratory for Innovative Development and Utilization of Forest Plant Germplasm, Guangzhou 510642, China
* E-mail: Kunxi_123@163.com Tel: +86-20-8528-0001 Fax: +86-20-8528-2693
** E-mail: calljone@163.com Tel: +86-20-8528-0001 Fax: +86-20-8528-2693

Received September 3, 2018; accepted November 19, 2018 (Edited by T. Kobayashi)

Abstract *Neolamarckia cadamba* is a miracle tree species with considerable economic potential uses as a timber wood, woody forage and traditional medicine resource. The present study aimed to establish a highly efficient and robust protocol of plant regeneration for *N. cadamba*. Greenish callus was induced from very young leaf explants of sterile *in vitro* plantlets cultured on Murashige and Skoog's (MS) medium supplemented with 3 mgl^{-1} thidiazuron (TDZ), 0.1 mgl^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.05 mgl^{-1} α-naphthaleneacetic acid (NAA). The callus could differentiate into nodular embryogenic structures or adventitious shoots, and these two regeneration pathways often occurred in the same callus clumps. The micro-shoots developed roots in MS supplemented with 0.05 mgl^{-1} NAA and 0.05 mgl^{-1} indole-3-butyric acid (IBA), while the nodular embryogenic structures germinated directly and developed into plantlets on induction medium contained with 0.5 mgl^{-1} (or 1 mgl^{-1}) 6-benzyladenine (6-BA) and 0.05 mgl^{-1} NAA. The rooted plantlets could be successfully acclimatized to a greenhouse with more than 92.0% survival. This regeneration protocol can be used in large scale cultivation needs and may be useful for future genetic modifications of *N. cadamba*.

Key words: adventitious shoots, *In vitro* culture, leaf explants, *Neolamarckia cadamba*, plant regeneration.

Introduction

Neolamarckia cadamba is a tropical evergreen tree which grows mainly in South China and South Asia. It has been recently introduced to South Africa, Costa Rica, Puerto Rico, Surinam, Venezuela and other tropical and subtropical countries (Orwa et al. 2009). As a fast-growing tree with anatomical, morphological, and chemical characteristics, *N. cadamba* have tremendous economic and ecological value in furniture, pulp, forage and pharmaceutical production (Lal et al. 2010; Zayed et al. 2014). In Indian traditional formulations, *N. cadamba* recorded as a common herbal medicine and used clinically for the treatment of various diseases such as sour throat, cough, fever, infections and inflammation (Pandey and Negi 2016). In south China, *N. cadamba* not only served as one of the best landscape tree for urban greening and forest rehabilitation, but also used for furniture manufacturing and woody forage (Ouyang et al. 2013; Wang et al. 2017). Owing to these utilizable economic value, it is affectionately known as the "miracle tree." In the past few years, *N. cadamba* has increasingly attracted the attention of research groups especially in phytochemical and biomolecular field (Chaubey et al. 2015; Li et al. 2017; Ouyang et al. 2016; Zhao et al. 2014). Phytochemical studies have revealed various biologically active compounds from root, bark, leaves and fruits of *N. cadamba*, including alkaloids, flavonoids, terpenoids and saponins, etc (Acharyya et al. 2013; Chandel et al. 2012; Pandey and Negi 2018). Among these, triterpenoid saponins have been identified as the important active components in medicinal plants (Augustin et al. 2011). Recent study found that higher abundant transcripts related to triterpenoid saponin and phytosterol biosynthesis were existed in *N. cadamba* (Ouyang et al. 2016). Medicinal properties of *N. cadamaba* might be due to the presence of these bioactive compounds. However, little is known about the control point and biochemical or genetic cross-talk within and between pathways which will facilitate the engineering of existing metabolic targets of *N. cadamba*.

The successful application of genetic transformation techniques for any wood plant mainly depends on the availability of efficient protocol for callus induction and plant regeneration (Kumar et al. 2010; Ribas et al. 2011; Stevens and Pijut 2014). Besides, the establishment

This article can be found at http://www.jspcmb.jp/
Published online March 16, 2019

of tissue culture protocols for *N. cadamba* could also be usefully applied to various aspects of biotechnology such as micropropagation, germplasm conservation, and production of secondary metabolites. However, despite being environmentally friendly and economically important, *N. cadamba* tissue culture has not received much progress due to endophytic fungus contamination and heavy leaching of phenolics. Until now, only one report is available on adventitious shoot induction from cotyledon for *N. cadamba* (Huang et al. 2014), but no information is available regarding callus induction and somatic embryogenesis from *in vitro* plantlets of this species. By the development of callus induction and plant regeneration protocol, *N. cadamba* may be improved genetically through transformation techniques.

Materials and methods

Plant material

Mature seeds of *N. cadamba* were collected from a 10-year-old plus tree in South China Agricultural University (Guangzhou China), and stored at 4°C in the dark until used. Seeds were immersed in water and incubated at 40°C overnight on a thermostat shaker set at 120 rpm, then surface sterilized using 75% alcohol for 60 s, followed by three rinse with sterile distilled water, additionally immersed in *ca.* 10% sodium hypochlorite for 10 min followed by three rinses in distilled water. The surface-sterilized seeds were blotted dry on sterile filter paper and implanted on Murashige and Skoog (MS, Murashige and Skoog 1962) basal medium without any growth regulators. This basal medium contained 3% sucrose and 0.7% agar. The pH of MS media was adjusted to 5.8 prior to autoclaving at 121°C for 20 min. Cultures were maintained at 25±2°C under a 16/8 h (day/night) photoperiod illuminated with light provided by cool white fluorescent lamps at an intensity of 30 μmol $m^{-2} s^{-1}$ with a relative humidity of 70%. These culture conditions were the same for all experiments, unless indicated otherwise.

Induction of callus from leaf cultures

Young leaves (*ca.* 1 cm length) from 2-months-old sterile seedlings were dissected using a surgical knife, and inoculated on MS basal medium with the abaxial side in contact with the medium. The culture medium was supplemented with different plant growth regulators (PGRs) to induce callus and adventitious shoot. In each treatment, 30 explants were used and all experiments were repeated three times. Cultures were observed weekly and callus induction was expressed as a percentage response. After culturing for 3 weeks, callus induction was investigated.

Recovery of nodular embryogenic structures

Nodular embryogenic structures were cultured and germinated on medium supplemented with 0.0, 0.1, 0.5, 1.0, 1.5, or 2.0 $mg l^{-1}$ 6-BA and 0.05 $mg l^{-1}$ NAA. After 4 weeks of culture, shoot buds induction was investigated. After rooting, forty plantlets were transplanted to pots with a mixture of nutrient soil and vermiculite at a 2 : 1 ratio (v/v). To ensure high humidity, the pots were covered with transparent polythene covers and the covers were gradually opened one week later. The pots were placed in a growth chamber and irrigated with tap water once a day. Plant survival (%) was assessed after 2 weeks.

Shoot proliferation, root formation and acclimatization

When shoots reached 1 cm in height, they were cut off at the base and transferred to MS medium supplemented with 1 $mg l^{-1}$ 6-BA and 0.05 $mg l^{-1}$ IBA for shoot proliferation. When the shoots grow to a 3 cm in height, they were excised and implanted individually into MS medium containing 0.5 $mg l^{-1}$ NAA and 0.05 $mg l^{-1}$ IBA to induce roots (Huang et al. 2014). After 2 weeks of culture, root formation was investigated. The plantlets with more than 5 roots were removed from the culture bottles and agar was gently removed by rinsing water. The plantlets were transplanted to pots in the growth chamber and the culture condition were the same as describe above.

Statistical analyses

All experiments were repeated three times. Each experimental treatment contained six explants per culture bottles and five bottles per treatment. The number of regenerated buds from each explant was counted, and the number of the roots was measured in the rooting cultures of the isolated buds. Data was analyzed statistically using one-way ANOVA. Statistical differences among the means were analyzed by Duncan's multiple range test using the SPSS ver 7.5 (Snedecor and Cochran 1989).

Results and discussion

Callus induction from leaf explants

In preliminary experiments, several cytokinins and auxins were used to test their induction effects on the leaf explants of *N. cadamba in vitro*. When the leaf explants were cultured on MS media supplemented with different concentrations of PGRs, alone or in combination, the surface of leaf became swollen and callus started to form after one week. These calluses could be distinguished during the culture stage into three types based on their surface color and texture (Table 1 and Figure 1). Type I callus, which was rigid and light-green, bearing small granular structures on the surface (Figure 1A), was most commonly induced when the culture media supplemented with 3 $mg l^{-1}$ (or 5 $mg l^{-1}$) 6-BA and 0.05 $mg l^{-1}$ NAA. The callus response was higher than 77%. However, none of these structures regenerated into plantlets, and they turned brown and became necrotic after 12 weeks of culture. Type II callus, which was green and nodular, had few particle masses, was induced on

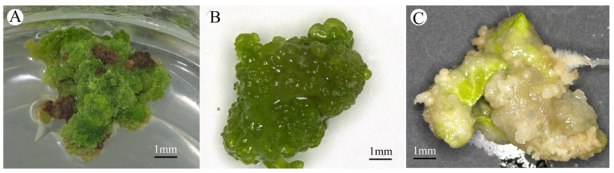

Figure 1. Callus induction from leaf explants of *N. cadamba*. (A) Type I callus, rigid and light-green, bearing small granular structures induced on induction medium containing 5 mg l^{-1} 6-BA and 0.05 mg l^{-1} NAA. (B) Type II callus, green and nodular, turned to embryogenic callus which was induced on MS medium containing 5 mg l^{-1} TDZ and 0.05 mg l^{-1} NAA. (C) Type III callus, compact and whitish, commonly observed in induction medium containing 1 mg l^{-1} 2,4-D.

Table 1. Callus induction from *N. cadamba* leaf explants on MS media supplemented with different concentrations of PGRs.

PGRs (mg l^{-1})	Callus types	Callus formation (%)
6-BA (3)+NAA (0.05)	I	77.13±3.46c
6-BA (5)+NAA (0.05)	I	86.47±1.28b
TDZ (5)+NAA (0.05)	II	85.67±2.73b
TDZ (5)+2,4-D (0.1)+NAA (0.05)	II	86.77±1.30b
2,4-D (1)	III	95.83±0.68a
6-BA (5)+2,4-D (0.1)+NAA (0.05)	I and III	90.17±3.90ab

Type I callus was rigid and light-green, bearing small granular structures. Type II callus was green and nodular, turned to embryogenic callus which was induced on MS medium containing TDZ. Type III callus was compact and whitish, commonly observed in induction medium containing 2,4-D alone, or in combined with 6-BA and NAA. The percentage of callus induced was evaluated after 3 weeks of culture. Callus response values are mean±standard error. Means followed by the different letters in the same column are significantly different from each other at $p \leq 0.05$ level, according to Duncan's multiple range test.

media supplemented with TDZ and auxins (2,4-D and/or NAA). Callusing response was higher than 85% (Figure 1B). This callus type proliferated slowly compared with type I and type III callus. Four weeks later, type II callus entered one of two types of morphogenic routes: shoot organogenesis and nodular embryogenic structures. It seems that these two regeneration pathways often occurred in the same callus clumps. Embryogenic callus or somatic embryogenesis is considered to be one of the most efficient regeneration and transformation system for plants (Ceasar and Ignacimuthu 2011; Koetle et al. 2015; Parimalan et al. 2011). Type III callus, which was compact and whitish (Figure 1C), was commonly observed in medium supplemented with 2,4-D alone, or in combination with 6-BA and NAA. This callus proliferated rapidly, and the callus induction percentage was higher than 90%. However, Type III callus gradually became brown and necrotic in medium supplemented with 2,4-D alone, while some root-like structures were observed after 8 weeks of culture (Figure 1C). In addition, we found that type I and type III callus could not convert to type II callus, i.e., it is an irreversible callus type.

Effect of PGRs on shoot-bud regeneration

In addition to the type of PGRs, the dosage is also the key factors controlling the morphogenetic response of plant cells *in vitro*. In order to get the information with regard to the best effective concentration of the PGRs on shoot-bud induction, leaf explants were placed on MS medium containing different concentration of cytokinin TDZ, auxins 2,4-D and NAA. The induction results are shown in Tables 2–4. In the regeneration progress under appreciate *in vitro* culture conditions, a small amount of callus was first formed along the cut surface after two weeks, and then numerous nodular embryogenic structures appeared in another two weeks (Figure 2). Although quite large numbers of embryogenic structures were induced, only a few of them elongated and became shoots. It is clear that cytokinin TDZ was necessary for embryogenic structure formation, but not yet efficient for shoot bud induction. However, the frequency of differentiation varied with the different concentration of cytokinin and auxins in the media (Tables 2–4). Even though 2,4-D was more effective than NAA for callus induction from leaves, we found that these hormones could not work effectively alone and the induction efficiency was greatly improved when they were combined. The greatest efficiency was achieved when all three PGRs were present. The best tested groups can give rise to the greatest mean shoot number (7.47, 8.71 or 8.63) from one leaf explant, obtained from media supplemented with 5 mg l^{-1} (or 4 mg l^{-1}, or 3 mg l^{-1}) TDZ, 0.1 mg l^{-1} 2,4-D and 0.05 mg l^{-1} NAA, respectively. There were no significant differences in frequency of organogenesis between these high concentrations of TDZ (Table 2), i.e., redundant TDZ did not seem to enhance the induction effects further. Moreover, shoots produced at higher concentrations of TDZ were distorted, and exhibited mild vitrification.

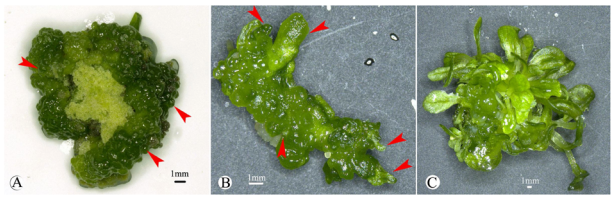

Figure 2. Shoot organogenesis and regeneration via nodular embryogenic structures from embryogenic callus. (A) Greenish and smooth embryogenic structure formation (red arrowheads) from embryogenic callus on MS medium containing 3 mg l^{-1} TDZ, 0.1 mg l^{-1} 2,4-D and 0.05 mg l^{-1} NAA. (B) Embryogenic structures that was cultured on MS medium containing 0.5 mg l^{-1} 6-BA and 0.05 mg l^{-1} NAA for 2 weeks to induce shoot buds (red arrowheads). (C) Shoot bud elongation on the same MS medium in (B) after another 2 weeks of growth.

Shoot-buds inducted from nodular embryogenic structures

Nodular embryogenic structures formed on the surface of embryo-like callus (type II callus) after four weeks culture on the media supplemented with 3 mg l^{-1} TDZ,

Table 2. Effect of TDZ on the formation of shoots and embryogenic structures.

TDZ ($mg l^{-1}$)	Number of shoots per explant	Number of nodular embryogenic structures per explant	Callus formation (%)
0	0.00±0.00e	0.00±0.00d	30.87±1.89d
1	2.19±0.19d	10.87±1.59c	45.50±1.50c
2	5.29±0.35c	17.90±0.40b	76.23±1.33b
3	8.63±0.27a	21.53±2.06ab	85.73±2.13a
4	8.71±0.34a	25.80±3.04a	86.97±2.00a
5	7.47±0.30b	23.40±0.86a	88.13±1.63a

Each value represents the mean±SE of three replicates, each with 30 explants. All the media contained 0.1 $mg l^{-1}$ 2,4-D and 0.05 $mg l^{-1}$ NAA. The different letters behind data mean significantly different from each other at $p \leq 0.05$ level, according to Duncan's multiple range test.

Table 3. Effect of 2,4-D on the formation of shoots and embryogenic structures.

2,4-D ($mg l^{-1}$)	Number of shoots per explant	Number of nodular embryogenic structures per explant	Callus formation (%)
0.00	1.44±0.15e	10.77±0.38c	69.40±0.39d
0.01	3.70±0.56cd	13.30±1.06bc	77.80±0.74c
0.05	6.08±1.11ab	22.70±2.74a	76.47±1.04c
0.10	7.60±0.78a	25.47±0.78a	86.07±0.69b
0.15	5.23±0.08bc	16.77±0.66b	86.47±1.42b
0.20	2.43±0.65de	10.37±0.55c	89.80±0.80a

Each value represents the mean±SE of three replicates, each with 30 explants. All the media contained 3 $mg l^{-1}$ TDZ and 0.05 $mg l^{-1}$ NAA. The different letters behind data mean significantly different from each other at $p \leq 0.05$ level, according to Duncan's multiple range test.

Table 4. Effect of NAA on the formation of shoots and embryogenic structures.

NAA ($mg l^{-1}$)	Number of shoots per explant	Number of nodular embryogenic structures per explant	Callus formation (%)
0.00	0.00±0.00c	3.87±0.85d	71.27±2.60c
0.01	5.17±0.11b	17.57±0.81b	75.77±5.67bc
0.05	7.82±1.06a	24.70±2.16a	84.77±2.84ab
0.10	4.80±0.78b	20.47±3.00ab	88.20±0.70a
0.15	1.44±0.11c	11.33±1.07c	87.93±1.71a
0.20	0.00±0.00c	6.77±0.44cd	90.47±1.85a

Each value represents the mean±SE of three replicates, each with 30 explants. All the media contained 3 $mg l^{-1}$ TDZ and 0.1 $mg l^{-1}$ 2,4-D. The different letters behind data mean significantly different from each other at $p \leq 0.05$ level, according to Duncan's multiple range test.

Table 5. Effects of 6-BA on the secondary differentiation from nodular embryogenic structures.

6-BA ($mg l^{-1}$)	Number of shoots per explant	Number of nodular embryogenic structures per explant	Callus formation (%)
0.0	0.00±0.00d	0.00±0.00d	0.00±0.00c
0.1	4.79±0.63c	6.54±1.12c	72.02±2.11b
0.5	12.27±1.10a	15.94±0.41a	88.08±0.64a
1.0	11.63±0.23a	16.00±1.66a	86.14±0.86a
1.5	8.38±1.48b	11.30±1.00b	80.72±5.62ab
2.0	4.96±0.15c	4.48±1.37c	80.31±2.90ab

Embryo-like callus (type II callus) was used as the starting material, which was induced on the media supplemented with 3 $mg l^{-1}$ TDZ, 0.1 $mg l^{-1}$ 2,4-D and 0.05 $mg l^{-1}$ NAA after four weeks of culture. Each value represents the mean±SE of three replicates, each with 30 explants. All the media contained 0.05 $mg l^{-1}$ NAA. The different letters behind data mean significantly different from each other at $p \leq 0.05$ level, according to Duncan's multiple range test.

0.1 $mg l^{-1}$ 2,4-D and 0.05 $mg l^{-1}$ NAA. They were green globular structures with a smooth shiny surface, and most of them were globular with no visible appendages (Figure 2A). Nodular embryogenic structures could be individually isolated to repeatedly subcultured to create a repetitive cycle of proliferation, but they could not be differentiated into shoot buds with prolonged culture time. Interesting, when nodular embryogenic structures were cultured on MS media containing 0.5 $mg l^{-1}$ (or 1 $mg l^{-1}$) 6-BA and 0.05 $mg l^{-1}$ NAA, secondary differentiation took place on the surface (Table 5). Initially, there was some green swelling on the smooth surface of nodular embryogenic structures after 2 weeks in culture (Figure 2B), then shoot buds developed on the surface after another 2 weeks in culture (Figure 2C). 71.4% of shoot buds could germinate and develop into normal plantlets in which the plumular end had formed a shoot, allowing isolated individuals to be cultured in the elongation and proliferation medium.

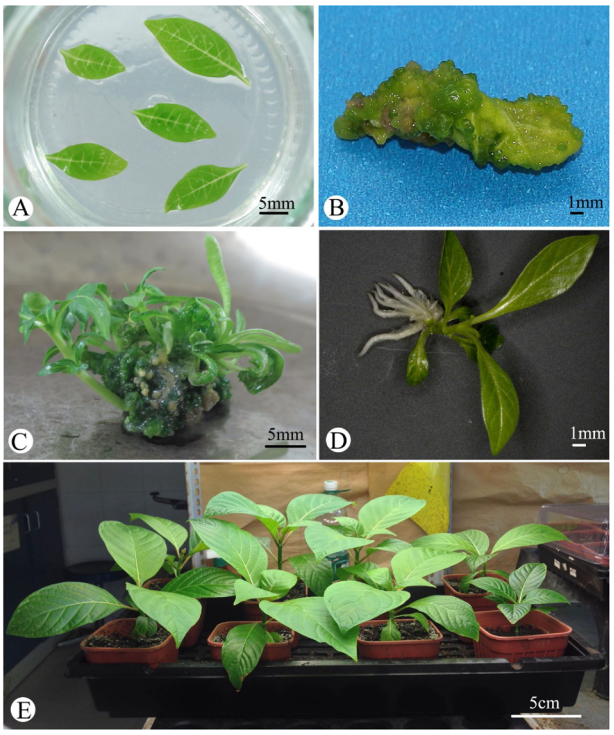

Figure 3. Simplified flow diagram of the pathways of plant regeneration from *N. cadamba* leaf. (A) Young leaves were cultured on MS medium containing 3 mg l^{-1} TDZ, 0.1 mg l^{-1} 2,4-D and 0.05 mg l^{-1} NAA. (B) Embryo-like callus was induced after three weeks of culture. (C) Shoots induction from embryogenic structures on MS media containing 0.5 mg l^{-1} 6-BA and 0.05 mg l^{-1} NAA after four weeks of culture. (D) Roots formed in MS with 0.05 mg l^{-1} NAA and 0.05 mg l^{-1} IBA. (E) Acclimatized plantlet after one month transplantation.

Shoot proliferation, root formation and acclimatization

The shoot buds could develop into multiple shoots after 3 weeks of culture on improved MS medium containing 1 mg l^{-1} 6-BA and 0.05 mg l^{-1} IBA, which has been proved to be the optimal conditions for adventitious shoot buds and plumule proliferation (Huang et al. 2014). Although some calluses were occasionally visible at the cut surface of the shoot base, the shoots grew healthy in this medium, and nodal segments can be sub-cultured every 3 weeks to create a repetitive cycle of proliferation. To achieve normal growth following shoot proliferation, healthy micro-shoots about 3 cm height were transferred to medium containing 0.05 mg l^{-1} IBA and 0.05 mg l^{-1} NAA induce root growth. After two week, more than 92% of the shoots had 5 roots at least, which were enough to be transferred into greenhouse conditions (Figure 3).

The hormonal controls behind plant regeneration

Plant *in vitro* regeneration is influenced by many factors, including medium, plant growth hormones, and explant sources. Most studies relating induction of plant regeneration have been focused on effects of plant growth hormones (Farhadi et al. 2017; Li et al. 2018; Zhang et al. 2017). In general, cytokinin alone or a high cytokinin-to-low auxin ratio was found to be necessary for the induction of callus and adventitious shoots in plant regeneration. Among the plant growth hormones, cytokinin such as 6-BA combined with auxin NAA have been widely used for callus induction and shoot proliferation in various plants (Kim et al. 2016; Kwapata et al. 2010; Zhang et al. 2017). In our previous study, we have established *in vitro* adventitious shoot induction protocol (5 mg l^{-1} 6-BA +0.05 mg l^{-1} NAA) for *N. cadamba* using cotyledon as explant. However, this protocol can not induce callus formation or direct shoots formation from leaf explant. Fortunately, we found that the addition of TDZ, 2,4-D and NAA to the culture medium are well suited for callus induction, shoot initiation and subsequent multiplication using leaf as explant. Unlike the regeneration studies conducted by Collado et al. (2013) and Lee and Chen (2014), TDZ when used alone with 2,4-D or NAA did not respond well in our study (Table 1). On the contrary 2,4-D and NAA exhibited synergistic effect on organogenesis from leaf explant here, resulting in the embryogenic callus induction or direct adventitious shoots regeneration. This synergistic effect was seen upto a certain limit (3 mg l^{-1} TDZ, 0.1 mg l^{-1} 2,4-D and 0.05 mg l^{-1} NAA) and further increase in the cytokinin or auxin levels indicated decrease in regeneration. Such kind of response may attribute to the toxicity produced in the cells, especially when supplemented with higher concentrations of 2,4-D. In early period, high concentration of 2,4-D can stimulate callus formation, but may also results in suppressing plant recovery (Tables 1, 3). As shown in the preliminary experiments, the callus structures induced in the high concentration of 2,4-D were hardly differentiate into adventitious shoots or somatic embryos and they turned brown and became necrotic after 8 weeks of culture (data not shown). In our study, embryogenic callus was induced by strongly active cytokinins TDZ combined with weakly active auxins, 2,4-D and NAA, and this embryogenic callus could finally differentiate into nodular embryogenic structures or adventitious shoots. Furthermore, we found an optimum dosage of 6-BA combined with NAA was effective for shoot differentiation of nodular embryogenic structures (Figure 3). Besides, the use of nodular embryogenic structures could provide a cyclic process of regeneration for *N. cadamba*. This culture cultures could be an effective alternative method for the micropropagation of *N. cadamba* in the future.

Conclusion

In this study, an efficient plant regeneration protocol was established for *N. cadamba* using leaf explants. To our knowledge, the plant regeneration from leaf explants

in tissue culture has not been reported previously for *N. cadamba*. The regeneration technique based on an abundant source of leaf material is an efficient and rapid way for large-scale production of *N. cadamba* plants in a relatively short period and with high multiplication rate, which propagates commercial cultivars. Plantlets produced using the protocol were uniform, healthy and had high survival rates after transplanting to soil. In the future, this protocol will also facilitate us to produce transgenic *N. cadamba* plants with resistance characteristics (i.e., virus, insect, and cold resistant) and higher content of pharmaceutical compounds or a modified secondary metabolites profile.

Acknowledgements

This work was supported by National Natural Science Foundation of China (grant number 31600525), Science and Technology Program of Guangdong, China (grant number 2017B020201008), Science and Technology Program of Guangzhou (grant number 201607020024), Extension and demonstration project of forest science and technology from China state financial budget (2018GDTK-08) and Guangdong natural science foundation (2015A030313419).

Conflict of interest statement

The authors declare that they have no conflicts of interest.

Author contribution statement

Jingjian Li and Xiaoyang Chen designed the experiments. Jingjian Li, Deng Zhang and Kunxi Ouyang conducted the experiments. Jingjian Li wrote the manuscript.

References

Acharyya S, Dash GK, Abdullah MS (2013) Antihyperglycemic and antilipidemic activity of *Anthocephalus cadamba* (roxb.) Miq. roots. *Eur J Exp Biol* 3: 116–120

Augustin JM, Kuzina V, Andersen SB, Bak S (2011) Molecular activities, biosynthesis and evolution of triterpenoid saponins. *Phytochemistry* 72: 435–457

Ceasar SA, Ignacimuthu S (2011) *Agrobacterium*-mediated transformation of finger millet (*Eleusine coracana* (L.) Gaertn.) using shoot apex explants. *Plant Cell Rep* 30: 1759–1770

Chandel M, Sharma U, Kumar N, Singh B, Kaur S (2012) Antioxidant activity and identification of bioactive compounds from leaves of *Anthocephalus cadamba* by ultra-performance liquid chromatography/electrospray ionization quadrupole time of flight mass spectrometry. *Asian Pac J Trop Med* 5: 977–985

Chaubey N, Singh VK, Savita Quraishi MA, Ebenso EE (2015) Corrosion inhibition of aluminium alloy in alkaline media by *Neolamarkia cadamba* bark extract as a green inhibitor. *Int J Electrochem Sci* 10: 504–518

Collado R, Veitía N, Bermúdez-Caraballoso I, García LR, Torres D, Romero C, Rodríguez Lorenzo JL, Angenon G (2013) Efficient *in vitro* plant regeneration via indirect organogenesis for different common bean cultivars. *Sci Hortic (Amsterdam)* 153: 109–116

Farhadi N, Panahandeh J, Azar AM, Salte SA (2017) Effects of explant type, growth regulators and light intensity on callus induction and plant regeneration in four ecotypes of Persian shallot (*Allium hirtifolium*). *Sci Hortic (Amsterdam)* 218: 80–86

Huang H, Li JC, Ouyang KX, Zhao XH, Li P, Liao BY, Chen XY (2014) Direct adventitious shoot organogenesis and plant regeneration from cotyledon explants in *Neolamarckia cadamba*. *Plant Biotechnol* 31: 115–121

Kim MJ, An DJ, Moon KB, Cho HS, Min SR, Sohn JH, Jeon JH, Kim HS (2016) Highly efficient plant regeneration and *Agrobacterium*-mediated transformation of *Helianthus tuberosus* L. *Ind Crops Prod* 83: 670–679

Koetle MJ, Finnie JF, Balázs E, Staden JV (2015) A review on factors affecting the *Agrobacterium*-mediated genetic transformation in ornamental monocotyledonous geophytes. *S Afr J Bot* 98: 37–44

Kumar N, Anand KGV, Pamidimarri DVNS, Sarkar T, Reddy MP, Radhakrishnan T, Kaul T, Reddy MK, Sopori SK (2010) Stable genetic transformation of *Jatropha curcas* via *Agrobacterium tumefaciens*-mediated gene transfer using leaf explants. *Ind Crops Prod* 32: 41–47

Kwapata K, Sabzikar R, Sticklen MB, Kelly JD (2010) *In vitro* regeneration and morphogenesis studies in common bean. *Plant Cell Tissue Organ Cult* 100: 97–105

Lal M, Dutt D, Tyagi CH, Upadhyay JS, Upadhyay S (2010) Characterization of *Anthocephalus cadamba* and its delignification by kraft pulping. *Tappi J* 9: 30–37

Lee PL, Chen JT (2014) Plant regeneration via callus culture and subsequent *in vitro* flowering of Dendrobium huoshanense. *Acta Physiol Plant* 36: 2619–2625

Li JC, Hu XS, Huang XL, Huo HQ, Li JJ, Zhang D, Li P, Ouyang KX, Chen XY (2017) Functional identification of an EXPA gene (NcEXPA8) isolated from the tree Neolamarckia cadamba. *Biotechnol Biotec Eq* 1000: 1–10

Li P, Shang YY, Zhou W, Hu XS, Mao WM, Li JJ, Li JC, Chen XY (2018) Development of an efficient regeneration system for the precious and fast-growing timber tree *Toona ciliata*. *Plant Biotechnol* 35: 51–58

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol Plant* 15: 473–497

Orwa C, Mutua A, Kindt R, Jamnadass R, Anthony S (2009) Agroforestry tree database: A tree reference and selection guide version 4.0

Ouyang K, Li J, Zhao X, Que Q, Li P, Huang H, Deng X, Singh SK, Wu AM, Chen X (2016) Transcriptomic analysis of multipurpose timber yielding tree *Neolamarckia cadamba* during xylogenesis using RNA-Seq. *PLoS One* 11: e0159407

Ouyang KX, Liu MQ, Pian RQ, Liu SS, Chen XY (2013) Isolation and analysis of alpha-expansin genes in the tree *Anthocephalus chinensis* (Rubiaceae). *Genet Mol Res* 12: 1061–1073

Pandey A, Negi PS (2016) Traditional uses, phytochemistry and pharmacological properties of Neolamarckia cadamba: A review. *J Ethnopharmacol* 181: 118–135

Pandey A, Negi PS (2018) Phytochemical composition, *in vitro* antioxidant activity and antibacterial mechanisms of *Neolamarckia cadamba* fruits extracts. *Nat Prod Res* 32: 1189–1192

Parimalan R, Venugopalan A, Giridhar P, Ravishankar GA (2011) Somatic embryogenesis and *Agrobacterium*-mediated transformation in *Bixa orellana* L. *Plant Cell Tissue Organ Cult* 105: 317–328

Ribas AF, Dechamp E, Champion A, Bertrand B, Combes MC, Verdeil JL, Lapeyre F, Lashermes P, Etienne H (2011) A

grobacterium-mediated genetic transformation of *Coffea arabica* (L.) is greatly enhanced by using established embryogenic callus cultures. *BMC Plant Biol* 11: 92–107

Snedecor GW, Cochran WG (1989) *Statistical Methods, 8th ed*. Iowa State University, Ames City

Stevens ME, Pijut PM (2014) *Agrobacterium*-mediated genetic transformation and plant regeneration of the hardwood tree species *Fraxinus profunda*. *Plant Cell Rep* 33: 861–870

Wang SN, Liu GB, Li YK, Cui ZL, Zhou DE, Liu DW, Sun BL (2017) Effect of different proportion silage *Anthocephalus chinensis* substitute silage whole plant corn on growth performance, slaughter performance and meat quality of Lezhi Black goat in fattening period. *Siliao Gongye* 38: 37–44

Zayed MZ, Zaki MA, Ahmad FB, Ho WS, Pang SL (2014) Comparison of mimosine content and nutritive values of *Neolamarckia cadamba* and *Leucaena leucocephala* with medicago sativa as forage quality index. *Int J Sci Technol Res* 3: 5

Zhang JJ, Yang YS, Lin MF, Li SQ, Tang Y, Chen HB, Chen XY (2017) An efficient micropropagation protocol for direct organogenesis from leaf explants of an economically valuable plant, drumstick (*Moringa oleifera* Lam.). *Ind Crops Prod* 103: 59–63

Zhao X, Ouyang K, Gan S, Zeng W, Song L, Zhao S, Li J, Doblin MS, Bacic A, Chen XY, et al. (2014) Biochemical and molecular changes associated with heteroxylan biosynthesis in *Neolamarckia cadamba* (Rubiaceae) during xylogenesis. *Front Plant Sci* 5: 602