



In vitro shoot regeneration system from leaves wrapped by bud scales of a multipurpose tree (*Neolamarckia cadamba*)

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Abstract

Neolamarckia cadamba (*N. cadamba*) is an evergreen tree species known for its rapid growth, remarkable wood properties, and significant value in medicine, feeding, and landscape. In order to clone a *N. cadamba* individual with excellent genotype, a plant regeneration protocol was successfully established with leaves wrapped by bud scales as explants. The optimal sterilization method for the leaves was 0.1% Mercury Chloride (HgCl₂) treatment for 1 min before culturing on Murashige and Skoog's medium (MS) supplemented with 3.0 mg/L Thidiazuron (TDZ), 0.1 mg/L 2–4 Dichlorophenoxyacetic acid (2-4D), 0.05 mg/L α -Naphthaleneacetic acid (NAA) and 1 mL/L Plant Preservative Mixture (PPM) to induce calluses. The medium containing 1 mL/L PPM could effectively inhibit explant contamination without an unfavorable impact on the final induction rate of callus from the leaves. Three types of calluses were induced from the leaves cultured on the above medium. Among them, only the Type II callus, which was green and nodular, had few particle masses, could differentiate into adventitious shoots on the MS medium supplemented with 1.5 mg/L 6–Benzylaminopurine (6-BA) and 0.05 mg/L NAA, with the induction rate of 78.89% and adventitious shoot number per callus of 11.67. The adventitious shoots were proliferated on the MS medium supplemented with 1.0 mg/L 6-BA and 0.05 mg/L Indole-3-butyric acid (IBA) with the proliferation coefficient of 3.37. And the micro-shoots developed roots in the MS medium supplemented with 0.05 mg/L NAA and 0.05 mg/L IBA. The regeneration protocol can be used in the propagation and large scale production of seedlings with the same genotype as an excellent individual of *N. cadamba* in the field.

Key message

A plant regeneration protocol was successfully established with leaves wrapped by bud scales in the field, furthermore it can be used in the propagation and large scale production of seedlings with the same genotype as an excellent individual of *N. cadamba*.

Keywords *Neolamarckia cadamba* · Tissue culture · Explant sterilization · Plant hormone · Plant preservative mixture (PPM) · Contamination

Buye Li and Qingmin Que contributed equally to this work.

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Abbreviations

<i>N. Cadamba</i>	<i>Neolamarckia cadamba</i>
HgCl ₂	Mercury Chloride
MS	Murashige and Skoog's medium
TDZ	Thidiazuron
2-4D	2–4 Dichlorophenoxyacetic acid
NAA	α -Naphthaleneacetic acid
PPM	Plant Preservative Mixture
6-BA	6–Benzylaminopurine
IBA	Indole-3- butyric acid

Introduction

Neolamarckia cadamba is a tropical evergreen tree species belonging to Rubiaceae. *N. cadamba* has been widely used as an ideal raw material for industries such as artificial fiber, fiberboard, plywood and pulp, and also in garden landscape (Ouyang et al. 2016). As recorded in the “Ayurvedo”, an indian traditional medical reference, the bark of *N. cadamba* is used to treat snake bites and dysentery (Kumar et al. 2015). Various biologically active compounds have been isolated from *N. cadamba* and most of them have multiple desirable therapeutic effects, including antipyretic, antioxidant, anti-microbial and antilipidemic activities (Pandey and Negi 2016). Furthermore, it has the advantages of fast growth, high biological yield, rich secondary metabolites, high protein content and low water content in leaves (He et al. 2018), suggesting that the leaves can be used as woody forages. Interestingly, the meat quality of golden pompano (*Trachinotus ovatus*) has been improved by adding the leaves to forages (Chen et al. 2018).

Plant tissue culture technology has been widely used in seedling production and scientific researches (Loyola-Vargas and Ochoa-Alejo 2018). However, there are some problems in the technology, such as contamination, browning and vitrification (Huang and Wang 2016). Among them the contamination is the most toughest problem during the cultural process. The contamination is a phenomenon that tissue materials are infected by fungi, bacteria or other microorganisms during tissue culture, so a large number of microorganism plaques grow in the culture container, eventually leading to tissue materials growth failure or even death (Leelavathy and Sankar 2016). Some researchers have achieved in vitro regeneration of *N. cadamba* with stem segments and leaves from sterile seedlings as explants (Wee et al. 2021; Li et al. 2019b). However, there has not been any reported success in regenerating *N. cadamba* growing in the field. This is because it is extremely challenging to sterilize the explants of *N. cadamba* collected from field. The tissue culture with explants from *N. cadamba* in the field has been hindered by contamination, which is difficult to overcome

due to a large number of endophytic microorganisms in them. At present, production of *N. cadamba* seedlings still only depends on seed seedlings including sterile seed seedlings (Huang et al. 2014). However, the seed seedlings have the defect of poor genetic stability due to the gene separation from a single outstanding individual. To propagate the excellent individuals of *N. cadamba*, explants from those individuals in the field should be used in asexual reproduction, particularly through tissue culture with the advantage of rapid propagation (Li et al. 2019b).

There are many factors that will affect the sterilization effect for explants, including temperature, relative humidity, photoperiod, light intensity, irrigation and fertilization, as well as age, genotype, sampling season, type and size of explants, and type, treatment time and concentration of disinfectant (Teixeira Da Silva et al. 2016). Disinfectants popularly used encompass HgCl₂ (0.1–0.2%, M/V), ethanol (70–75%, V/V), sodium hypochlorite (NaClO) (2–10%, M/V), hydrogen peroxide (H₂O₂) (3–10%, V/V) and so on (Cardoso and Teixeira, 2012). Additionally, explants can also be sterilized with sodium lauryl sulfate, n-hexane solution, chlorine dioxide gas, nanomaterials or Plant Preservative Mixture (PPM, Plant Cell Technology, Washington, US) (Allahbakhsh et al. 2016; Stubblefield et al. 2014). Among them, PPM is a broad-spectrum antibiotic that can efficiently inhibit contamination during plant tissue culture (Niedz 1998), and can be added into media prior to autoclaving due to the superior thermal stability (Nascimento et al. 2018). Therefore, its utilization is very convenient, compared with other antibiotics that will be ineffective after autoclaving. The contamination rate of cultural materials can be significantly reduced by adding a suitable concentration PPM in media (Silveira et al. 2016). Furthermore, as a disinfectant, PPM also has a better sterilization effect than HgCl₂ and NaClO in some cases (Ahmad et al. 2011; Boncaldo et al. 2010).

We successively used stems and leaves growing openly as materials, trying to obtain clonal seedlings of *N. cadamba*, but failed because of the inability to obtain sterile materials. We also tried to refer to the method of successful in vitro regeneration of *Acer saccharinum* (silver maple), *Fraxinus pennsylvanica* (green ash), *Carya illinoensis* (pecan) and *Tectona grandis* (teak) using forced growth of softwood from various lengths of large stem segments that have been removed from intact trees and shrubs as materials (Aftab and Preece 2007; Akram and Aftab 2016), to obtain sterile materials of *N. Cadamba*. However, stem and branch segments of *N. cadamba* rapidly decayed after being buried in the soil and no shoots were produced.

In this study, the leaves wrapped by bud scales of *N. cadamba* in the field were selected as explants. We analysed the effects of HgCl₂ treatment time, as well as sampling season

and type of explant and PPM concentration on sterilization, studied the main influencing factors of callus induction, and optimized the medium for inducing adventitious buds from calluses, in order to overcome the problem of incomplete sterilization for explants from *N. cadamba* in the field and provide technical support for the large-scale asexual propagation for excellent individuals of *N. cadamba* in the field.

Materials and methods

Plant materials

The materials were collected from Qilinnan nursery at South China Agricultural University (113°21' E, 23°9' N). The buds on robust branches of *N. cadamba* were collected and taken back to the laboratory (Fig. 1A) and the scales of branch buds were peeled off in an ultra-clean bench (Fig. 1B), followed by excising the young leaves wrapped by the bud scales as explants (Fig. 1C).

Sterilization with HgCl

The branch buds (Fig. 1A) of *N. cadamba* were collected in autumn (September - November) and were gently scrubbed with a soft-bristled brush under running tap water for 10 min to remove the dust particles and the microbial load. All subsequent operations were carried out in an ultra-clean bench. The scales of branch buds were peeled off (Fig. 1B), followed by excising the young leaves wrapped by the bud scales (Fig. 1C). To determine the optimal treatment time with 0.1% (w/v) HgCl₂, the leaves (Fig. 1C) were surface sterilized with 0.1% (w/v) HgCl₂ for 30, 60, 90, 120 or 150 s, followed by rinsing thoroughly with sterile double distilled water for 5 times to remove the traces of HgCl₂ before inoculation. After the surface-sterilized leaves were cut to get two wounds on sterile plates (Fig. 1D), they were implanted on Murashige and Skoog's medium (MS, Murashige and Skoog 1962) with the abaxial side in contact with the medium (If no otherwise specified).

Inhibitory effect of PPM on endophytic microorganisms

The leaf explants (Fig. 1C) were treated for the optimal HgCl₂ sterilization time determined above, and then the leaves with two wounds (Fig. 1D) were cultured on the MS medium supplemented with 0, 0.5, 1.0, 2.0 or 3.0 mL/L PPM to determine the optimal inhibitory concentration of PPM on microorganisms.

Effect of sampling seasons on explant sterilization

The explants were sampled at stage 1 from December, 2021 to February, 2022, and stage 2 (March–May), stage 3 (June–August) and stage 4 (September–November) in 2022, representing the four seasons in Guangzhou, winter, spring, summer and autumn, respectively. The leaf explants were surface-sterilized with 0.1% (w/v) HgCl₂ for the optimal time determined above, followed by implanting the leaves with two wounds (Fig. 1D) on MS medium containing the optimal PPM concentration determined above to determine the optimal sampling season. In this and previous parts, data were scored after culturing for both 15 d and 30 d following the leaf sterilization, Contamination rate (%) = (Number of contaminated leaves/Number of cultured leaves) × 100%, mortality rate (%) = (Number of dead leaves/Number of cultured leaves) × 100% and survival rate (%) = (Number of survival leaves/Number of cultured leaves) × 100%.

Callus induction

The surface-sterilized leaf with two wounds (Fig. 1D) was transferred to MS medium containing different plant growth regulators (NAA, 2-4D, TDZ). Their concentration combination was orthogonally designed, with 5 levels for each plant growth regulator, and contained a total of 25 treatments (Table 1). In addition, all the media were supplemented with the optimal PPM concentration determined above. The media were changed with the same fresh media every 30 d. The induction rates of callus were scored after incubating 60 d in the callus-inducing medium, callus induction rate (%)

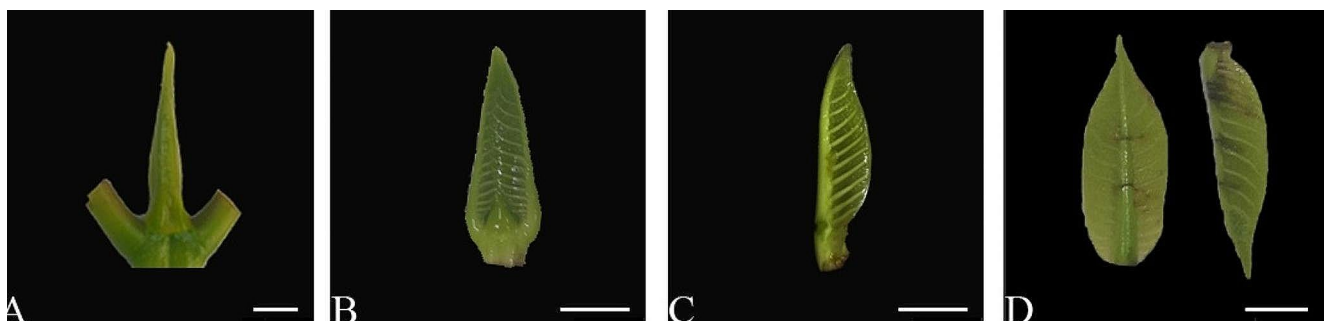


Fig. 1 Explants of *N. cadamba*. **A**, Bud; **B**, Bud without bud scale; **C**, Leaf wrapped by bud scales; **D**, Sterilized leaves; Bar = 1 cm

Table 1 Formulation of callus induction medium

Medium	TDZ (mg/L)	2-4D (mg/L)	NAA (mg/L)	Medium	TDZ (mg/L)	2-4D (mg/L)	NAA (mg/L)
1	3.0	0.1	0.05	14	4.0	0.1	0.20
2	3.0	0.05	0.10	15	4.0	0.05	0.25
3	3.0	0.2	0.15	16	2.5	0.3	0.05
4	3.0	0.3	0.20	17	2.5	0.4	0.10
5	3.0	0.4	0.25	18	2.5	0.1	0.15
6	3.5	0.05	0.05	19	2.5	0.05	0.20
7	3.5	0.2	0.10	20	2.5	0.2	0.25
8	3.5	0.3	0.15	21	2.0	0.4	0.05
9	3.5	0.4	0.20	22	2.0	0.1	0.10
10	3.5	0.1	0.25	23	2.0	0.05	0.15
11	4.0	0.2	0.05	24	2.0	0.2	0.20
12	4.0	0.3	0.10	25	2.0	0.3	0.25
13	4.0	0.4	0.15				

= (Number of leaves forming callus/Number of cultured leaves) $\times$ 100%.

Adventitious bud induction

Adventitious buds could be only induced from the type II callus which was green and nodular, induced from the leaves of the sterile seed seedlings, in MS medium supplemented with 0.5 mg/L 6-BA and 0.05 mg/L NAA (Li et al. 2019b). The differentiation ability of calluses induced from the explants in the field may be weaker than those induced from the leaves of sterile seed seedlings, leading to that it may be more difficult to induce adventitious buds with leaves wrapped by bud scales in the field as explants in this study. So the higher concentration of 6-BA (0.5, 1.0, 1.5 or 2.0 mg/L) was added in the induction medium of adventitious buds, in combination with 0.05 mg/L NAA, to achieve the purpose of more rapidly producing more adventitious buds with the calluses obtained in the study. Adventitious buds were scored after incubating 60 d of callus in the adventitious bud-inducing medium, adventitious bud induction rate (%) = (Number of calluses forming adventitious buds/Number of cultured calluses) $\times$ 100%.

Shoot proliferation and root formation

The adventitious buds were transplanted into MS medium containing 1.0 mg/L 6-BA + 0.05 mg/L IBA for shoot proliferation according to the previous method (Huang et al. 2014), and the shoots with height of 3 cm were excised and implanted in the MS medium supplemented with 0.05 mg/L IBA + 0.05 mg/L NAA for root formation, following the previously described method (Li et al. 2019b). Proliferation coefficient of adventitious bud and rooting rate of shoot were scored both after incubating 15 d in the proliferative medium and rooting medium, respectively. Proliferation

coefficient = Number of shoots induced from adventitious buds/Number of inoculated adventitious buds, rooting rate (%) = Number of rooting shoots/Number of inoculated shoots.

Culture conditions

Unless otherwise specified, all media based on MS basal medium in this study were adjusted to pH 5.8 with 1 N NaOH or 1 N HCl, and supplemented with 25 g/L sucrose and 5 g/L agar, prior to autoclaving at 121 °C for 20 min. All the leaf explants after sterilization were incubated on the optimal callus induction medium determined above in darkness for 2 d, followed by maintaining at 23–25 °C under a 12/12 h (day/night) photoperiod illuminated with light provided by cool white fluorescent lamps at an intensity of 30 $\mu\text{mol m}^{-2} \text{s}^{-1}$, which was also adopted in the adventitious bud induction, proliferation and root formation of shoots.

Data statistics and analysis

Unless otherwise specified in this study, in each treatment, 30 explants (one explant per tissue culture bottle) were used and all experiments were repeated 3 times. Data was analyzed statistically using one-way ANOVA. Statistical differences among the means were analyzed by Duncan's multiple range test using the SPSS 22.0.

Results

Explant sterilization with HgCl_2

Leaf explants collected in autumn were sterilized with 0.1% (w/v) HgCl_2 for different time, and it was found that there were significant differences in sterilization effect among

Table 2 Effects of treatment time of HgCl₂ on leaf explants

Cul- ture days	Treatment time(s)	Contamination rate (%)	Mortality rate(%)	Survival rate (%)
15	30	85.56 ± 5.09 g	4.44 ± 1.93a	10.00 ± 3.33c
	60	57.78 ± 5.09e	16.67 ± 3.33b	25.56 ± 3.85d
	90	41.11 ± 1.93bc	45.56 ± 5.09c	13.33 ± 1.93c
	120	37.78 ± 3.33b	53.33 ± 6.67c	8.89 ± 1.93bc
	150	22.22 ± 1.93a	77.78 ± 6.94d	0.00 ± 0.00a
30	30	95.56 ± 1.93 h	4.44 ± 1.93a	0.00 ± 0.00a
	60	74.44 ± 1.93f	16.67 ± 3.33b	8.89 ± 1.93bc
	90	50.00 ± 3.33de	45.56 ± 5.09c	4.44 ± 1.93ab
	120	46.67 ± 6.67 cd	53.33 ± 5.09c	0.00 ± 0.00a
	150	22.22 ± 1.93a	77.78 ± 6.94d	0.00 ± 0.00a

the different treatment time (Table 2). Generally, with the increase of treatment time, the contamination rate gradually decreased, but the mortality rate gradually increased. However, there was another trend for the survival rate increasing first and subsequently decreasing. When the explants were sterilized for 60 s, the survival rate of leaves was the highest with 25.56% among all leaves cultured after 15 d, and it was also the highest among all leaves cultured after 30 d although with only 8.89%. Furthermore, mortality rate was the same over culture time for each sterilization time (Table 2; Fig. 2). So the leaf explants sterilized with HgCl₂ for 60 s were adopted in the all subsequent experiments.

Values represent the mean (± SD) of three independent experiments. Different lowercase letters within the same column indicate statistically significant differences ($P < 0.05$) among the different sterilization times after a certain culture time (15 d or 30 d) on MS medium.

Effect of PPM on the inhibition of contamination

The PPM was chosen as an antibiotic to inhibit the contamination of explants of *N. cadamba* collected in autumn, due to its excellent antimicrobial effect in many studies (Nascimento et al. 2018; Genna et al. 2015; Orlikowska et al. 2012). The culture media supplemented with PPM all achieved ideal antimicrobial effect of reducing the contamination rate and increasing the survival rate (Table 3). After 15 d of inoculation, the leaf explant survival rate was the highest with 72.22% on the medium supplemented with 3 mL/L PPM, while not significantly higher than that on the medium with 1 mL/L or 2 mL/L PPM. Even with the

Table 3 Effect of PPM on the inhibition of contamination

Cul- ture days	Level(mL/L)	Contamination rate (%)	Mortality rate (%)	Survival rate (%)
15	0	57.78 ± 5.09d	16.67 ± 3.33a	25.56 ± 3.85b
	0.5	40.00 ± 5.77c	18.89 ± 1.93a	41.11 ± 5.09c
	1	13.33 ± 5.77ab	17.78 ± 1.93a	68.89 ± 5.09d
	2	11.11 ± 1.93ab	18.89 ± 7.70a	70.00 ± 8.82d
	3	7.78 ± 1.93a	20.00 ± 5.77a	72.22 ± 5.09d
30	0	74.44 ± 1.93e	16.67 ± 3.33a	8.89 ± 1.93a
	0.5	43.33 ± 3.33c	18.89 ± 1.93a	37.78 ± 5.09c
	1	15.56 ± 3.85b	17.78 ± 1.93a	66.67 ± 3.33d
	2	13.33 ± 3.33ab	18.89 ± 1.93a	67.78 ± 5.09d
	3	8.89 ± 1.93ab	20.00 ± 3.33a	71.11 ± 1.93d

increase of culture time, the survival rates for each concentration PPM did not decrease significantly, respectively. Furthermore, there was no significant difference in the survival rates among the three media containing the three different PPM concentrations (1 mL/L, 2 mL/L and 3 mL/L) after the same days of inoculation, even with the increase of culture time. However, the survival rates of explants on the three media were all significantly higher than that on the medium with 0 mL/L or 0.5 mL/L PPM (Table 3). Therefore, when the leaf explants were used for tissue culture, 1 mL/L PPM should be added into the medium, and was the enough concentration for antimicrobial effect.

Values represent the mean (± SD) of three independent experiments. Different lowercase letters within the same column indicate statistically significant differences ($P < 0.05$) among the different PPM concentrations after a certain culture time (15 d or 30 d) through the same sterilization time (60 s).

Effect of sampling season on sterilization of explants

There were different sterilization effects on leaf explants of *N. cadamba* collected in different seasons with the same sterilization treatment of 0.1% (w/v) HgCl₂ for 60 s (Table 4). The highest contamination rate was 38.89% for leaf explants collected from March to May, whereas the lowest contamination rate was 22.22% from September to November, after 30 d of inoculation. There were also significant differences in the survival rate among the explants collected in the different seasons. The survival rates of explants

Fig. 2 Leaves sterilized with 0.1% HgCl₂. **A**, Leaf explant without contamination (15 d); **B**, Leaf explant without contamination (30 d); **C**, Contaminated leaf explant (6 d); **D**, Dead leaf explant (9 d). Bar = 1 cm



Table 4 Effect of sampling season on sterilization of explants

Cul- ture days	Sampling time	Contamination rate (%)	Mortality rate (%)	Survival rate (%)
15	Mar. - May.	32.22 ± 1.93d	7.78 ± 1.93a	60.00 ± 3.33b
	Jun. - Aug.	23.33 ± 3.33ab	4.44 ± 1.93a	72.22 ± 5.09c
	Sep. - Nov.	18.89 ± 1.93a	7.78 ± 1.93a	73.33 ± 3.33c
	Dec. - Feb.	25.56 ± 3.85bc	22.22 ± 6.94b	52.22 ± 6.94ab
	Mar. - May.	38.89 ± 3.85e	7.78 ± 1.93a	53.33 ± 5.77ab
30	Jun. - Aug.	26.67 ± 3.33bc	4.44 ± 1.93a	68.89 ± 3.85c
	Sep. - Nov.	22.22 ± 1.93ab	7.78 ± 1.93a	70.00 ± 3.33c
	Dec. - Feb.	28.89 ± 1.93 cd	22.22 ± 3.85b	48.89 ± 5.09a

collected in summer (Jun. - Aug., 68.89%) and autumn (Sep. - Nov., 70.00%) were significantly higher than those in winter (Dec. - Feb., 48.89%) and spring (Mar. - May., 53.33%) after 30 d of inoculation, with the similar results after 15 d of inoculation. So, in order to reduce contamination rate and increase survival rate, the optimal sampling seasons of leaves wrapped by bud scales are summer (Jun. - Aug.) and autumn (Sep. - Nov.) in Guangzhou, China.

Values represent the mean ($\pm$ SD) of three independent experiments. Different lowercase letters within the same column indicate statistically significant differences ($P < 0.05$) among the different seasons after a certain culture time (15 d or 30 d) on MS media containing 1 mL/L PPM through the same sterilization time (60 s).

Callus induction

The leaf explants of *N. cadamba* were inoculated into 25 media based on an orthogonal experimental design, resulting in the induction of three distinct types of callus, which could be distinguished by their surface color and texture (Fig. 3; Table 5). Type I callus, rigid and light-green, bearing small granular structures on the surface (Fig. 3B), and Type II callus, green and nodular, had few particle masses (Fig. 3A),

similar to the previous report (Li et al. 2019b). Type III callus, Loose mixture callus of yellow-white (Fig. 3C). However, the ratio of these types in induction results among the media was significantly different. According to Duncan multiple comparison (Table 5), the media with the highest callus induction rate were treatment 1 (MS + TDZ 3.0 mg/L + 2-4D 0.1 mg/L + NAA 0.05 mg/L) and treatment 13 (MS + TDZ 4.0 mg/L + 2-4D 0.4 mg/L + NAA 0.15 mg/L), both with the total induction rate of 88.89%. The medium with the lowest callus induction rate was treatment 19, with only 12.22%. In orthogonal experimental analysis, the K avg (K average) value represents the average value of experimental data at one certain level for one certain factor in the experiment. It is generally believed that the higher the value, the better the effect of the factor at one certain level. So, the optimal combination scheme can be obtained by combining each factor level with the highest value of K avg (Zhou et al. 2013). R (Range) value indicates the differences between the maximal and minimal value of all levels for each factor, which can reflect the important order of each factor. The greater the R value, the greater the effect of the factor on experimental results (Zhou et al. 2013). According to K avg value, when TDZ, 2-4D and NAA concentrations were 3.0 mg/L with the corresponding K avg value of 62%, 0.4 mg/L with the corresponding K avg value of 81.56% and 0.05 mg/L with the corresponding K avg value of 60.89%, respectively, the callus induction rate should be the highest, because K avg value was the highest for the each factor level in analysis of the orthogonal experiment results (Table 5). However, this optimal concentration combination of TDZ, 2-4D and NAA was not one of the 25 treatments in the experimental design (Table 5), so this medium formula should be experimentally validated. In fact, this optimal combination in theory did not perform well, showing that induction rate of type II callus was significantly lower than that of treatment 1, the induction rate of type C callus was significantly lower than that of treatment 13, and total induction rate was also significantly lower than that of both treatment 1 and treatment 13 (Table 6). Therefore, to induce callus from leaf explants of *N. cadamba*, the medium containing the combination of plant growth regulators in treatment 1 or 13 should be selected. In range analysis (Table 5), the R values of

Fig. 3 Callus induced from *N. cadamba* leaves wrapped by bud scales. (A) Type II callus, green and nodular, had few particle masses. (B) Type I callus, rigid and light-green, bearing small granular structures. (C) Type III callus, Loose mixture callus of yellow-white. Bar = 1 cm

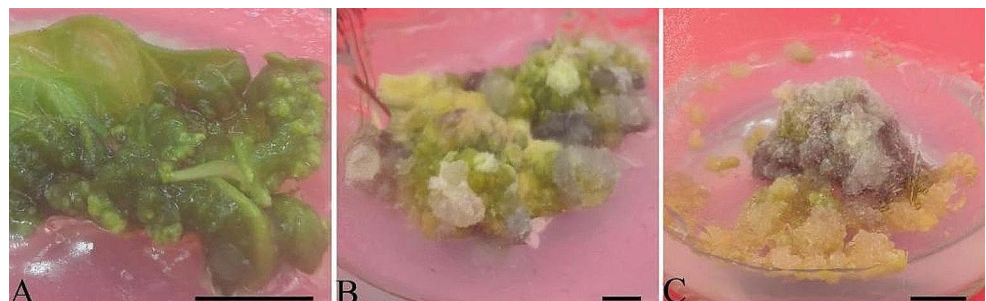


Table 5 Callus formation in the orthogonal experiment

Treatment	Plant growth regulator(mg/L)			Callus type(%)			Total induction rate (%)
	TDZ	2-4D	NAA	II	I	III	
1	3.0	0.1	0.05	77.78 ± 5.09j	8.89 ± 1.93ab	2.22 ± 3.85a	88.89 ± 1.93k
2	3.0	0.05	0.10	10.00 ± 1.93 cd	11.11 ± 5.09abc	0.00 ± 0.00a	21.11 ± 6.93ab
3	3.0	0.2	0.15	27.78 ± 3.85 g	14.44 ± 3.85abcd	8.89 ± 1.93ab	51.11 ± 1.93de
4	3.0	0.3	0.2	11.11 ± 5.09de	14.44 ± 3.85abcd	37.78 ± 6.94d	63.33 ± 5.77fgh
5	3.0	0.4	0.25	0.00 ± 0.00a	16.67 ± 3.33bcd	68.89 ± 5.09 fg	85.56 ± 3.85jk
6	3.5	0.05	0.05	4.44 ± 1.93abc	24.44 ± 8.39de	0.00 ± 0.00a	28.89 ± 5.09bc
7	3.5	0.2	0.1	42.22 ± 5.09 h	15.56 ± 3.33abcd	8.89 ± 1.93ab	66.67 ± 6.67ghi
8	3.5	0.3	0.15	22.22 ± 1.93ab	14.44 ± 1.93abcd	44.44 ± 5.09de	61.11 ± 3.85efg
9	3.5	0.4	0.2	0.00 ± 0.00a	21.11 ± 5.09cde	62.22 ± 5.09f	83.33 ± 6.67jk
10	3.5	0.1	0.25	17.78 ± 3.33f	15.56 ± 3.33abcd	0.00 ± 0.00a	33.33 ± 8.82c
11	4.0	0.2	0.05	51.11 ± 5.09i	5.56 ± 1.93a	5.56 ± 1.93a	62.22 ± 5.09efg
12	4.0	0.3	0.1	0.00 ± 0.00a	21.11 ± 5.09cde	47.78 ± 5.09e	68.89 ± 9.62ghi
13	4.0	0.4	0.15	0.00 ± 0.00a	15.56 ± 1.93abcd	73.33 ± 5.77 g	88.89 ± 5.09k
14	4.0	0.1	0.2	16.67 ± 1.93ef	28.89 ± 5.09e	0.00 ± 0.00a	45.56 ± 11.70d
15	4.0	0.05	0.25	1.11 ± 1.93ab	21.11 ± 3.85cde	0.00 ± 0.00a	22.22 ± 5.09abc
16	2.5	0.3	0.05	2.22 ± 1.93ab	16.67 ± 3.33bcd	30.00 ± 6.67c	48.89 ± 5.09d
17	2.5	0.4	0.1	0.00 ± 0.00a	11.11 ± 1.93abc	63.33 ± 6.67f	74.44 ± 5.09hij
18	2.5	0.1	0.15	0.00 ± 0.00a	17.78 ± 1.93bcd	14.44 ± 3.33b	32.22 ± 5.09bc
19	2.5	0.05	0.2	0.00 ± 0.00a	12.22 ± 5.09abc	0.00 ± 0.00a	12.22 ± 5.09a
20	2.5	0.2	0.25	0.00 ± 0.00a	10.00 ± 3.33ab	42.22 ± 5.09de	52.22 ± 8.39def
21	2.0	0.4	0.05	0.00 ± 0.00a	12.22 ± 1.93abc	63.33 ± 6.67f	75.56 ± 5.09ij
22	2.0	0.1	0.1	1.11 ± 1.93ab	17.78 ± 3.85bcd	7.78 ± 1.93ab	26.67 ± 5.77bc
23	2.0	0.05	0.15	8.89 ± 3.33 cd	15.56 ± 5.09abcd	1.11 ± 1.93a	25.56 ± 8.39bc
24	2.0	0.2	0.2	7.78 ± 1.93bcd	64.44 ± 5.09f	8.89 ± 1.93ab	81.11 ± 5.09jk
25	2.0	0.3	0.25	1.11 ± 1.93ab	18.89 ± 3.85bcd	48.89 ± 6.94e	68.89 ± 7.70ghi
K avg 1	62.00%	45.33%	60.89%				
K avg 2	54.67%	22.00%	51.56%				
K avg 3	57.56%	62.67%	51.78%				
K avg 4	44.00%	62.22%	57.11%				
K avg 5	55.56%	81.56%	52.44%				
R	18.00%	59.56%	9.33%				

Table 6 Validation of the optimal medium in theory

Medium	Callus type(%)			Total induction rate(%)
	II	I	III	
X	51.11 ± 5.09b	11.11 ± 1.93a	15.56 ± 3.33b	77.78 ± 5.09a
1	77.78 ± 5.09c	8.89 ± 1.93a	2.22 ± 3.85a	88.89 ± 1.93b
13	0.00 ± 0.00a	15.56 ± 1.93b	73.33 ± 5.77c	88.89 ± 5.09b

callus induction rate for TDZ, 2-4D and NAA were 18.00%, 59.56% and 9.33%, respectively, indicating that the strength order of effect of the three plant growth regulators on callus induction was 2-4D > TDZ > NAA.

Values represent the mean ($\pm$ SD) of three independent experiments. Different lowercase letters within the same column indicate statistically significant differences ($P < 0.05$) among treatments.

The K avg values in the table represent the average values of total callus induction rate at each level for each plant hormone.

The R values in the table mean the differences between the maximal and minimal value of total callus induction rate at the five levels for each plant hormone. The values = max(K avg) - min(K avg).

“X” in the table indicates the optimal culture medium according to the K avg. Values represent the mean ($\pm$ SD) of three independent experiments. Different lowercase letters within the same column indicate statistically significant differences ($P < 0.05$) among media.

For the three plant growth regulators, the range analysis of induction rate was conducted on the induction results of callus type I, II and III, respectively (Table 7), and the

Table 7 Range analysis for induction rate of each type callus in the orthogonal experiment

Callus type	K avg	Plant growth regulator		
		TDZ (%)	2-4D (%)	NAA (%)
II	K1	25.33	22.67	27.11
	K2	17.33	4.89	10.67
	K3	13.78	25.78	11.78
	K4	0.44	7.33	7.11
	K5	3.78	0.00	4.00
	R	24.89	20.89	23.11
I	K1	13.11	17.78	13.56
	K2	18.22	16.89	15.33
	K3	18.45	22.00	15.56
	K4	13.56	17.11	28.22
	K5	25.78	15.33	16.45
	R	12.67	6.67	14.66
III	K1	23.56	4.89	20.22
	K2	23.11	0.22	25.56
	K3	17.27	14.89	20.38
	K4	30.00	41.78	21.78
	K5	26.00	58.15	32.00
	R	12.73	57.93	11.78

strength order of effect of plant growth regulators on formation of callus type II, I and III was TDZ>NAA>2-4D(R values were 24.89%, 23.11% and 20.89%, respectively.), NAA>TDZ>2-4D(R values were 14.66%, 12.67% and 6.67%, respectively.), and 2-4D>TDZ>NAA(R values were 57.93%, 12.73% and 11.78%, respectively.), respectively. So TDZ, NAA and 2-4D played a dominant role in the formation of the three types of callus, respectively.

The K values in the table represent the average of induction rate of one type callus at each level for each plant hormone. The R values in the table mean the differences between the maximal and minimal value of one type callus at the five levels for each plant hormone, the values = max (K avg) - min (K avg).

Adventitious bud induced from callus

In the study, there was significant difference in adventitious bud formation among different callus types. Adventitious buds could only be induced from the type II callus (Fig. 4A; Table 8), while type I callus would die or grow adventitious

Table 8 Adventitious bud formation

6-BA concentration (mg/L)	Callus type	Adventitious bud number per callus	Induction rate (%)
0.5	II	3.67 ± 1.15b	22.22 ± 5.09b
	I	0.00 ± 0.00a	0.00 ± 0.00a
	III	0.00 ± 0.00a	0.00 ± 0.00a
1.0	II	7.67 ± 1.53c	47.78 ± 3.85c
	I	0.00 ± 0.00a	0.00 ± 0.00a
	III	0.00 ± 0.00a	0.00 ± 0.00a
1.5	II	11.67 ± 2.52d	78.89 ± 5.09e
	I	0.00 ± 0.00a	0.00 ± 0.00a
	III	0.00 ± 0.00a	0.00 ± 0.00a
2.0	II	7.33 ± 1.53c	54.44 ± 5.09d
	I	0.00 ± 0.00a	0.00 ± 0.00a
	III	0.00 ± 0.00a	0.00 ± 0.00a

roots (Fig. 4B, C), as well as type III callus would die (Fig. 4D) with the increase of culture time. The adventitious buds were not induced well from type II callus on the previous medium MS + 0.5 mg/L 6-BA + 0.05 mg/L NAA medium (Li et al. 2019b), with the number of adventitious bud per callus and induction rate of adventitious buds of only 3.67 and 22.22% in this study, respectively (Table 8). In combination with 0.05 mg/L NAA, when the concentration of 6-BA was increased to 1.5 mg/L, the two values increased to 11.67 and 78.89%, respectively (Table 8). However, when the concentration of 6-BA was 2.0 mg/L, the two values both decreased. So, the optimal concentration of 6-BA was 1.5 mg/L, in combination with 0.05 mg/L NAA, with both of the adventitious shoot induction rate for type II callus and the number of adventitious shoots produced per type II callus much higher than those of the other treatments.

Values represent the mean (±SD) of three independent experiments. Different lowercase letters within the same column indicate statistically significant differences ($P < 0.05$) among adventitious bud number or induction rate of three callus types in MS media supplemented with the difference 6-BA concentration, in combination with 0.05 mg/L NAA.

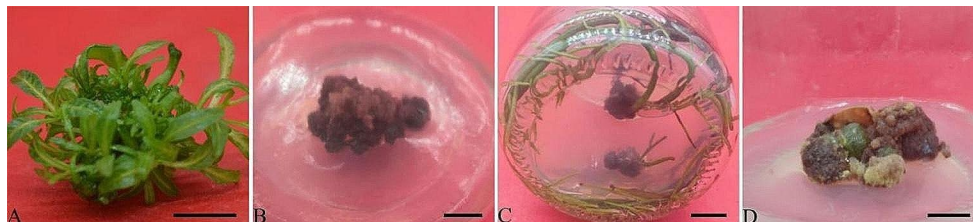


Fig. 4 Results of adventitious bud induction. **A**, The adventitious buds induced from type II callus in the MS medium supplemented with 1.5 mg/L 6-BA + 0.05 mg/L NAA; **B** and **C**, The died type I callus and

adventitious roots induced from type I callus, respectively; **D**, The died type III callus. Bar = 1 cm

Table 9 Effects of different concentrations of PPM on callus induction

Culture days	PPM(mL/L)	Callus type(%)			Total induction rate(%)
		II	I	III	
30	0	8.89 ± 1.93bc	1.11 ± 1.93ab	1.11 ± 1.93a	11.11 ± 1.93bc
	0.5	7.78 ± 1.93bc	0.00 ± 0.00a	0.00 ± 0.00a	7.78 ± 1.93abc
	1	4.44 ± 1.93ab	0.00 ± 0.00a	0.00 ± 0.00a	4.44 ± 1.93ab
	2	3.33 ± 0.00ab	0.00 ± 0.00a	0.00 ± 0.00a	3.33 ± 0.00ab
	3	0.00 ± 0.00a	0.00 ± 0.00a	0.00 ± 0.00a	0.00 ± 0.00a
60	0	65.56 ± 5.09 g	8.89 ± 1.93d	4.44 ± 1.93bc	78.89 ± 5.09f
	0.5	41.11 ± 1.93e	8.89 ± 1.93d	1.11 ± 1.93a	51.11 ± 1.93de
	1	35.56 ± 1.93de	5.56 ± 1.93c	4.44 ± 1.93bc	45.56 ± 1.93d
	2	32.22 ± 3.85d	3.33 ± 3.33bc	7.78 ± 1.93d	43.33 ± 6.67d
	3	12.22 ± 3.85c	2.22 ± 1.93ab	0.00 ± 0.00a	14.44 ± 5.09c
90	0	70.00 ± 5.77 g	8.89 ± 1.93d	4.44 ± 1.93bc	83.33 ± 5.77f
	0.5	67.78 ± 6.94 g	10.00 ± 0.00d	4.44 ± 1.93bc	82.22 ± 8.39f
	1	63.33 ± 5.77 g	8.89 ± 1.93d	6.67 ± 3.33 cd	78.89 ± 9.62f
	2	64.44 ± 5.09 g	5.56 ± 1.93c	8.89 ± 1.93d	78.89 ± 5.09f
	3	48.89 ± 6.94f	5.56 ± 1.93c	2.22 ± 1.93ab	56.67 ± 6.67e

Effect of PPM on callus formation

Since only type II callus can produce adventitious buds (Table 8; Fig. 4), the callus induction medium (MS + TDZ 3.0 mg/L + 2-4D 0.1 mg/L + NAA 0.05 mg/L, Table 5) with the highest ratio of type II callus was used to test whether PPM would inhibit the dedifferentiation of the leaf explants of *N. cadamba* in the field, while improving the survival rate (Table 3). The results showed that the induction rates of type II callus for different PPM concentrations were significantly different both after 30 d and 60 d of inoculation, respectively, but there was no significant difference among them after 90 d of inoculation, except the medium containing 3 mL/L PPM (Table 9). It can be concluded that there was no significant difference in the callus induction rate between 60 d and 90 d after inoculation without PPM in the medium. When PPM was added into the medium, except 3 mL/L PPM, it took 90 d to achieve the similar induction rate of type II callus to that after 60 d of inoculation without PPM. When the concentration of PPM was 3 mL/L in the medium, the callus induction rate was still significantly lower than that in the medium containing the other concentration PPM, even after 90 d of inoculation. Generally, in the medium containing the higher concentration of PPM, the longer time was required for callus induction. Therefore, PPM can impact on the speed of callus induction, but will not eventually change the callus induction rate.

Values represent the mean ($\pm$ SD) of three independent experiments. Different lowercase letters within the same column indicate statistically significant differences ($P < 0.05$) among callus induction rate for different PPM concentrations after one certain day of inoculation (30, 60 or 90 d).

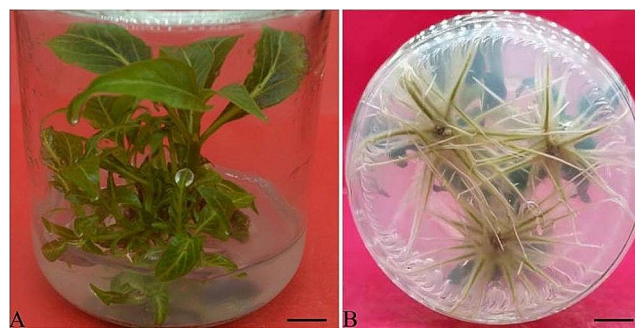


Fig. 5 Regenerated bud proliferation. **A**, The proliferation result of adventitious buds; **B**, Roots formation. Bar = 1 cm

Shoot proliferation and Root formation

According to the previous method of adventitious buds proliferation and rooting (Huang et al. 2014), the adventitious buds were proliferated in the MS medium supplemented with 6-BA 1.0 mg/L + IBA 0.05 mg/L. After 15 d of inoculation, the proliferation coefficient of buds from callus was 3.37 (Fig. 5). The shoots higher than 3 cm were excised, were implanted into MS medium supplemented with 0.05 mg/L IBA + 0.05 mg/L NAA and cultured for 2 weeks to induce roots (Fig. 5B).

Discussion

Inhibitory effect of PPM on endophytic microorganisms

Contamination has always been a serious problem in plant tissue culture. If a sterile system cannot be established, subsequent work cannot be carried out. There are many factors affecting the sterilization effect of explants, including

the tissue type, age, sampling time, pretreatment method, and type, treatment time and concentration of disinfectant. The explant sterilization of some plants is relatively easy, such as Carnation (*Dianthus caryophyllus* L.) (Gautam et al. 2019), *Bambusa tulda* (Bhadrawale et al. 2017). The young tissues with less microorganisms are selected as explants during continuous sunny day in appropriate season and used for sterilization with alcohol, HgCl_2 , NaClO or/and CaClO . As long as the concentration of disinfectant and sterilization time are appropriate, the ideal sterilization effect could be obtained. However, there are also many plants containing a variety of endophytic microorganisms, so the sterile system should be established not only by surface sterilization, but also using antibiotics to inhibit increase of endophytic microorganisms (Julie et al. 1991; Leelavathy and Sankar 2016). In the study, there were a large number of endophytic microorganisms in explants of *N. cadamba* in the field, and many fungi were visible after 3 day of inoculation, most of which were white and some were black. Bacteria were visible after 3–7 d of inoculation, and they were pink, red or light yellow in addition to white. Due to the contamination from endophytic microorganisms, it is extremely difficult to completely sterilize explants of *N. cadamba* in the field. In the pre-experiment, the inhibitory effects of common antibiotics such as rifampicin, gentamicin, streptomycin sulfate and fluconazole, on increase of endophytic microorganisms in the leaf explants of *N. cadamba* were not ideal (data not shown), but PPM showed a certain inhibitory ability. Therefore, PPM was selected in the study to inhibit increase of endophytic microorganisms during the culture.

The inhibitory effect of PPM on endophytic microorganisms in explants generally depends on plant type, explant type and PPM concentration. When the medium contained PPM, the survival rate of leaf explants wrapped by bud scales of *N. cadamba* significantly increased after sterilizing with HgCl_2 for 1 min. When the medium contained 2 mL/L or 3 mL/L PPM, the survival rate was little higher than that in the medium with 1 mL/L PPM after the same day of inoculation, whereas there was no significant difference in survival rate among them (Table 3). Additionally, the survival rate in the medium with 1 mL/L PPM was significantly higher than that in the two media supplemented with 0 mL/L and 0.5 mL/L PPM, respectively. So 1 mL/L PPM was enough to inhibit increase of endophytic microorganisms in the leaf explants. Furthermore, we also tried to use PPM to inhibit the contamination of other explant types of *N. cadamba*, such as stem segments and leaves not wrapped by bud scales, but did not obtain sterile explants (data not shown). These may be due to that they were long-term directly exposed to air and might contain more microorganisms in vivo and more pollutants on surface, so sterilization was more difficult (Cassells 1991). However,

in the study, the leaves wrapped by bud scales did not have direct contact with the outside world due to the protection by bud scales.

Additionally, the androgenesis of tobacco was not affected in the medium supplemented with 10 mL/L PPM, in contrast, the number of adventitious buds of *Petunia* was reduced in the medium containing only 2 mL/L PPM (Michael and Jennifer 2001). So whether there is an unfavorable influence of PPM on tissues or organs induction also depends on plant type, explant type and PPM concentration. In our study, PPM had no unfavorable influence on the final induction rate of callus from the leaf explants wrapped by bud scales of *N. cadamba*, but only delayed the formation of callus to a certain extent (Table 9). So the appropriate PPM concentrations should be selected according to plant species and explant type, and 1 mL/L PPM should be selected to add into the callus induction medium to inhibit growth of endophytic microorganisms in the study.

Regeneration of *N. cadamba* growing in the field

In tissue culture, plants can be regenerated through organogenesis or somatic embryogenesis with plant growth regulators (Gaspar et al. 1996). Plant growth regulators commonly used in plant tissue culture include auxin and cytokinin. Among them, auxins include IAA, IBA, NAA, 2-4D, etc. (Jiménez 2016), and cytokinins include 6-BA, ZT, KT, TDZ, etc. (Süreyya and Emine 2007). The direction of cell differentiation can be regulated by controlling the ratio and concentration of hormones. It is generally believed that adventitious buds are easily generated when the cytokinin concentration is higher than auxin, adventitious roots are easily generated when the cytokinin concentration is lower than auxin, and callus is easier to form when auxin and cytokinin at a certain concentration ratio are added into the medium (Zhao et al. 2008). In this study, TDZ, NAA and 2-4D were used as the hormones for callus induction. The results showed that different combinations of hormone concentration all could induce callus, but the status of callus was different (Table 5; Fig. 3). The three types of calluses were induced in the study, type I, type II and type III. The type I and type II callus, was similar to type I and type II in the previous study with the leaves of the sterile seed seedling of *N. cadamba* as explants in MS medium supplemented with TDZ, 2-4D and NAA (Li et al. 2019b). However, type III callus, Loose mixture callus of yellow-white was different from previous study. In addition, the type II callus could differentiate into both embryogenic callus and adventitious buds in the same callus clumps in the medium for callus induction in that study. However, in our study, adventitious buds could not be directly induced from leaves wrapped by bud scales like the previous study

(Li et al. 2019b), but only by inducing callus first in callus induction medium and subsequently inducing adventitious buds from the callus in adventitious bud induction medium. This may be that because there were great differences in the physiological state between the sterile leaf explants in the previous study and the leaf explants in the field, particularly which may be damaged during the sterilization in this study.

6-BA plays an important role in adventitious bud induction in many plant tissue cultures (Paula et al. 2019; Shimada et al. 2007). There were significant differences in the induction of adventitious buds of *N. cadamba* with different 6-BA concentrations, in combination with 0.05 mg/L NAA. When MS medium contained 0.5 mg/L 6-BA and 0.05 mg/L NAA, the number of adventitious buds regenerated per callus was only 3.67, and the frequency of adventitious bud induction was only 22.22% in the study, both significantly lower than those in the previous study (Li et al. 2019b). When the 6-BA concentration was increased to 1.5 mg/L, the adventitious bud number and induction frequency reached the highest value, with 11.67 and 78.89%, respectively. This may be that because the callus in this study was generated from the explants collected from field individuals with the different physiological state from leaves of sterile seed seedlings or the internal tissue of callus induced from the leaves of sterile seed seedlings is different from that with limited differentiation ability in this study. Therefore, higher concentration of 6-BA was needed to induce adventitious buds (Zhang et al. 2020).

Some researchers have already reported the in vitro regeneration of *N. Cadamba* by utilizing leaf or stem segments from sterile seed seedlings (Mok and Ho 2019; Wee et al. 2021), which require the initial step of seed sterilization and germination and the subsequent growth of the seedlings under sterile conditions. In contrast, our research innovates by directly using plant materials sourced from mature trees growing in the nature. This method not only avoid the seasonal constraints of seed collection but also addresses the practical challenges associated with harvesting seeds from tall trees. Furthermore, our method ensures the preservation of the superior genetic traits of the parent trees, which may not be guaranteed when working with seeds due to potential genetic variability.

Conclusion

In this study, an efficient plant regeneration protocol was established for *N. cadamba* in the field using leaves wrapped by bud scales as explants. To our knowledge, the plant regeneration from the leaf explants in the field in tissue culture has not been reported previously for *N. cadamba* in the wild. During the process of tissue culture, PPM showed a

very good inhibitory effect on increase of endophytic microorganisms, but did not cause an unfavorable impact on the final induction rate of callus. The regeneration method could be applied to preservation and large-scale breeding for a *N. cadamba* individual with excellent genotype, and could also provide a reference for the development of tissue culture technology for other tree species with many endophytic microorganisms, difficult sterilization and easy pollution.

Author contributions All authors contributed to the study conception and design. B.L. and C.L. performed the experiments, data collection and analysis were performed by B.L. and Q.Q., the first draft of the manuscript were performed by L.B. W.Z., L.Z., K.D., Q.X., W.C., M.Z., X.C., Z.Z., X.H., K.O. supervised the writing of the manuscript. All the authors have read and approved the manuscript. All authors read and approved the manuscript.

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Data availability The data that support the findings of this study are available on request from the corresponding author, Ouyang Kunxi, upon reasonable request.

Declarations

Competing Interests The authors have no relevant financial or non-financial interests to disclose.

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