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DEVELOPMENT OF *NEOLAMARCKIA CADAMBA* (KELEMPAYAN) TISSUE CULTURE TECHNIQUES FOR SUSTAINABLE SUPPLY OF PLANTING MATERIALS FOR COMMERCIAL PLANTATION

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Abstract

Neolamarckia cadamba (kelempayan) is a multipurpose and fast growing timber species. The tree is grown for timber, paper-making and as ornamental plant. It is reported that its barks and leaves possesed medicinal values and its flowers are used in perfumes. The species is also known to be suitable for plywood, packing case, toys and short-fibred pulp. Therefore, mass production of high quality planting material of *N. cadamba* is important to support plantation program of this species. Here we presented mass production of *N. cadamba* through tissue culture techniques. Nodal segments derived from *in vitro* germinated seeds were used and induced direct organogenesis to produce shoots and roots using MS media (1962) and plant growth regulators (BAP and IBA) that are relatively cheaper than previously used methods. The tissue culture technique of *N. cadamba* developed may help in ensuring supply of planting materials that are feasible for commercial plantation purposes.

Keywords: Neolamarckia cadamba, tissue culture technique, planting materials

1.0 INTRODUCTION

Neolamarckia cadamba is a fast-growing timber species which belongs to Rubiaceae family. This light hardwood timber is locally known in Malaysia as 'kelempayan' or 'laran' [1] which can also be found distributed in neighboring countries such as India, Nepal, China and Thailand [2]. Due to its fast-growth properties, it is one of the recommended species for forest plantation which able to attain height of 20 to 45 m and a girth of 2.0 to 2.5 m [3, 4]. Its anatomical and morphological characteristics make this species suitable for building materials, furniture and pulp production [5]. In addition, *N. cadamba* can grow very well in exploited and denuded areas especially in logged-over areas for reforestation plantation programmes. Furthermore, the leaves and buds of juvenile *N. cadamba* trees are known for its medicinal properties to treat diseases such as dysentery, fever and snake bites [6], injuries from insects such as *Dianhania glauculelis* and *Acalolepta cervina* [7]. The bark, leaves and flowers were discovered to contain monoterpenoid, triterpenoid saponin, alkaloid and ethylene glycol [8, 9, 10, 11]. Recent studies showed alkaloids extracted from the bark and leaves of *N. cadamba* can be use as eco-friendly corrosion inhibitors [1]. In Malaysia, *N. cadamba* is planted mainly in the states of Perak,

Pahang Sabah and Sarawak [1, 4]. Most of the commercial plantations were established from wildling collected and grown in nurseries [1]. With the increasing demands of timber and natural medicine, it is crucial to preserve and conserve the resources of *N. cadamba* as well as supporting the demand from timber industry and timber-based products through commercial plantation. Therefore, mass propagation of *N. cadamba* is needed as an alternative to support the program. Previous studies using explants originated from seedlings and mature tree had showed little success due to heavy leaching and phenolic compounds [13]. On the other hand, high successful rates reported using cotyledon explants [8]. These techniques showed to be useful in mass production of *N. cadamba* clones however, improvements are needed to sustain the planting materials through large scale production for plantation programmes. Therefore, this study was conducted to improve the available method of *N. cadamba* plantlets mass production *via* tissue culture technique using nodal segments on simplified media formulations.

2.0 EXPERIMENTAL

2.1 Plant materials and Seed Germination

N. cadamba seeds were obtained from different sources (mother tree) in Peninsular Malaysia (Langkawi, Kedah-Ky1; Manong, Perak-Ky2; Lancang, Pahang-Ky3; SPF Jeli, Kelantan-klon Ky4). The seeds were immediately disinfected in different concentrations of commercial clorox (ranges from 50 to 100%) coupled with Tween20 at different exposure time (min) to determine the most suitable surface-sterilization protocol (Figure 1). At the end of the surface-sterilization protocol, the seeds were cultured in test tubes containing 10 mL of Murashige and Skoog (MS, 1962) basal medium without plant growth regulators, PGRs (Figure 1a). The disinfected seeds were grown under 16 hours photoperiod of cool-white fluorescent tubes (2000 lux) at 22 ± 2 °C with 50% relative humidity for 4 weeks. Seeds with the least contamination and highest germination rates will be nominated as the best surface sterilization protocol for *N. cadamba* seeds. Germinated seeds were left to elongate for at least 3 nodes from each seed obtained (Figure 1b).

2.2 Shoot Multiplication and Plant Height

Single nodal segments were excised from 4 weeks old germinated seeds and cultured on MS medium supplemented with different concentrations of 6-benzylaminopurine, BAP (ranges from 0.01 to 0.2 mg/L) for shoot induction and multiplication (Figure 1c). All media was supplemented with 3.0% (wv^{-1}) sucrose and 0.3% gelrite. The media pH was adjusted to 5.8 with 1.0 M NaOH prior to autoclaving at 121 °C for 15 min. The experiment was carried out in 4 replications and 5 replicants in each replication. Culture conditions were the same as described above in Plant Materials and Seed Germination. The shoot induction data, defined as number of shoots per explants was recorded after 4 weeks of culture as well as plant height data were recorded simultaneously. Well developed and healthy shoots were further selected for rooting.

2.3 Rooting Induction

Selected shoots were transferred to ½ strength MS basal medium supplemented with different concentrations of indole-3-butyric acid, IBA (0.1 to 1.0 mg/L IBA). Culture conditions were the same as described above. After 3 weeks in culture, the number of shoots with root formation was determined. Plantlets were selected for acclimatization process.

2.4 Acclimatization of *in Vitro*-rooted Plantlets

In vitro-rooted *N. cadamba* plantlets were removed from the agar medium and carefully discarded the agar medium under running tap water. There were 3 replicants in each jar and 3 replications were conducted. Then the plantlets were briefly soaked in fungicide solution (Thiram 80), blotted dry and transplanted in potting medium (Jiffy-7). Jiffy-7 containing the plantlets were then arranged in glass container and covered with plastic sheet to ensure 90 to 100% humidity and temperature of 22 ± 2 °C during the acclimatization. Plantlets were watered (mist) twice a day for the 3 weeks incubation. Mortality rates of *N. cadamba* plantlets were determined at the end of the 3rd week and those that survived were transferred into polybags containing mixture of sand and cocopeat (1:1). *N. cadamba* plantlets were maintained at least for 2 months in the greenhouse.

2.5 Statistical Analysis

Data were analyzed using one way ANOVA and Duncan's multiple range tests (P=0.05) was applied using SAS9.1 software. The successful rates of surface sterilization, shoot induction and root induction were determined based on the percentages of clean cultures and roots developed from *in vitro* explants.

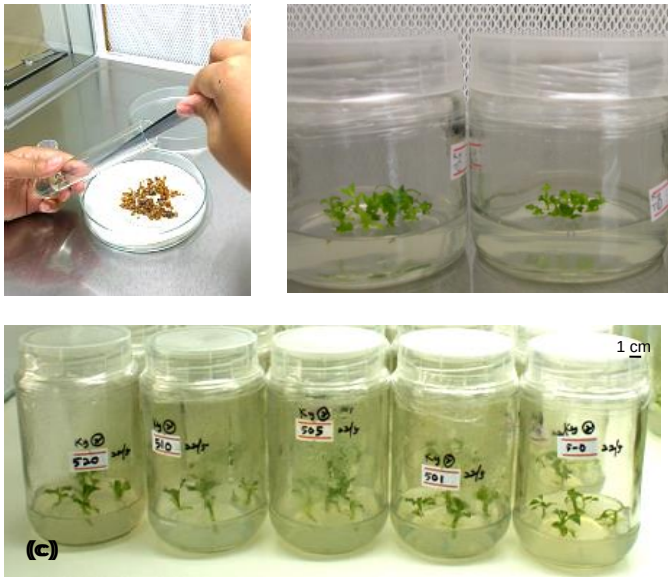

Figure 1(a) surface sterilized seeds cultured into MS PGR-free medium, (b) *N. cadamba* seeds germination and nodal elongation, (c) selected nodal segments for shoot induction.

3.0 RESULTS AND DISCUSSION

3.1 Seeds Surface Sterilization Protocol of *N. cadamba*

The surface sterilization protocols were carried out on different sources of *N. cadamba* seeds to determine the best seeds disinfectant treatment. The protocol not only should be able to disinfect the seeds but also the capability of the seeds to germinate *in vitro* after surface sterilization treatments. The results showed that seeds from different mother trees gave different responses to different treatments (Table 1). The most suitable surface sterilization method was with 100% Clorox at 60 minutes exposure time for all clones compared to other treatments applied. The results showed that Ky2 mother tree gave the highest percentages (92%) of disinfected seeds which were able to germinate, followed by Ky1 with 85% germination. The seeds from Ky3 and Ky4 however did not respond very well to the treatments, with only 56-67% survival rates using the same method. Therefore, only Ky2 clone was used for further shoot induction and multiplication purposes.

Table 1 Surface sterilization of *N. cadamba* seeds using various concentrations of Clorox and exposure time

Clone:	[Clorox], (%):	Exposure time, (min):	Percentages of disinfected seeds and germinated (%):
Ky1	100	20	48 ± 0.02
		40	49 ± 0.01
		60	85* ± 0.01
	70	20	47 ± 0.02
		40	53 ± 0.04
		60	55 ± 0.02
	50	20	44 ± 0.03
		40	39 ± 0.01
		60	43 ± 0.01
Ky2	100	20	50 ± 0.02
		40	63 ± 0.01
		60	92* ± 0.01
	70	20	41 ± 0.02
		40	45 ± 0.02
		60	46 ± 0.01
	50	20	21 ± 0.01
		40	28 ± 0.03
		60	32 ± 0.02
Ky3	100	20	32 ± 0.01
		40	51 ± 0.01
		60	67 ± 0.02
	70	20	45 ± 0.04
		40	52 ± 0.02
		60	65 ± 0.02
	50	20	46 ± 0.01
		40	57 ± 0.02
		60	63 ± 0.02
Ky4	100	20	46 ± 0.01
		40	48 ± 0.01
		60	56 ± 0.01
	70	20	20 ± 0.04
		40	24 ± 0.02
		60	46 ± 0.02
	50	20	15 ± 0.01
		40	25 ± 0.01
		60	32 ± 0.02

(asterix * - best surface sterilization method to obtain highest percentages (%) of germination of disinfected seeds).

3.2 *N. cadamba* Shoot Induction and Multiplication

For shoot induction, nodal segments from *in vitro* germinated seeds were used as explants. It was reported that plant regeneration using *in vitro* culture has been accomplished using different explants in many woody plants, such as young leaves in *Jatropha curcas* [14] and shoots explants in *Anacardium occidentale* [15]; compared to mature tree origins or juvenile-origin explants such as *N. cadamba* [12]. In this study, direct organogenesis was induced using nodal segments of *N. cadamba* with a significant difference in the rates of organogenesis between the different concentrations of the BAP used (P<0.05) (Figure 2a). After 4 weeks,

shoots and plant heights data were collected. The results showed that the number of shoots increased as the concentrations increases and reached the highest at 0.1 mg/L of BAP, before the number decreased at 0.2 mg/L of BAP. High shoot multiplication rates were obtained using concentrations of BAP ranges from 0.05 and 0.1 mg/L, which yielded 26.2 to 26.4 shoots per explants, respectively (Figure 2b). However, plants' heights were best induced in nodal segment with 0.05 mg/L BAP followed by 0.1mg/L (Figure 2c). The lowest (0.1 mg/L) and highest (2.0 mg/L) BAP concentrations resulted in 9.8 and 16.8 per explants, respectively. It was observed that even in control cultures (without exogenous cytokinin, BAP) the explants were able to induce high number of shoots, but was the least compared to others, with 8.4 shoots per explants. Previous study using two explants (cotyledons and hypocotyls) showed that shoots were only able to be derived from cotyledons, and none from hypocotyls [3]. Both cotyledons and hypocotyls needed to go through 'cell dedifferentiation' pathway before 'cell redifferentiation' to develop into shoots. This process took longer time to develop while nodal segments can directly produced shoots. Nodal sub-culturing in micropropagation, has been successful for some hardwoods and herbaceous plants, such as oak species [16], *Bixa orellana* [17], *Senecio cruentus* [18] and *Piper longum* [19].

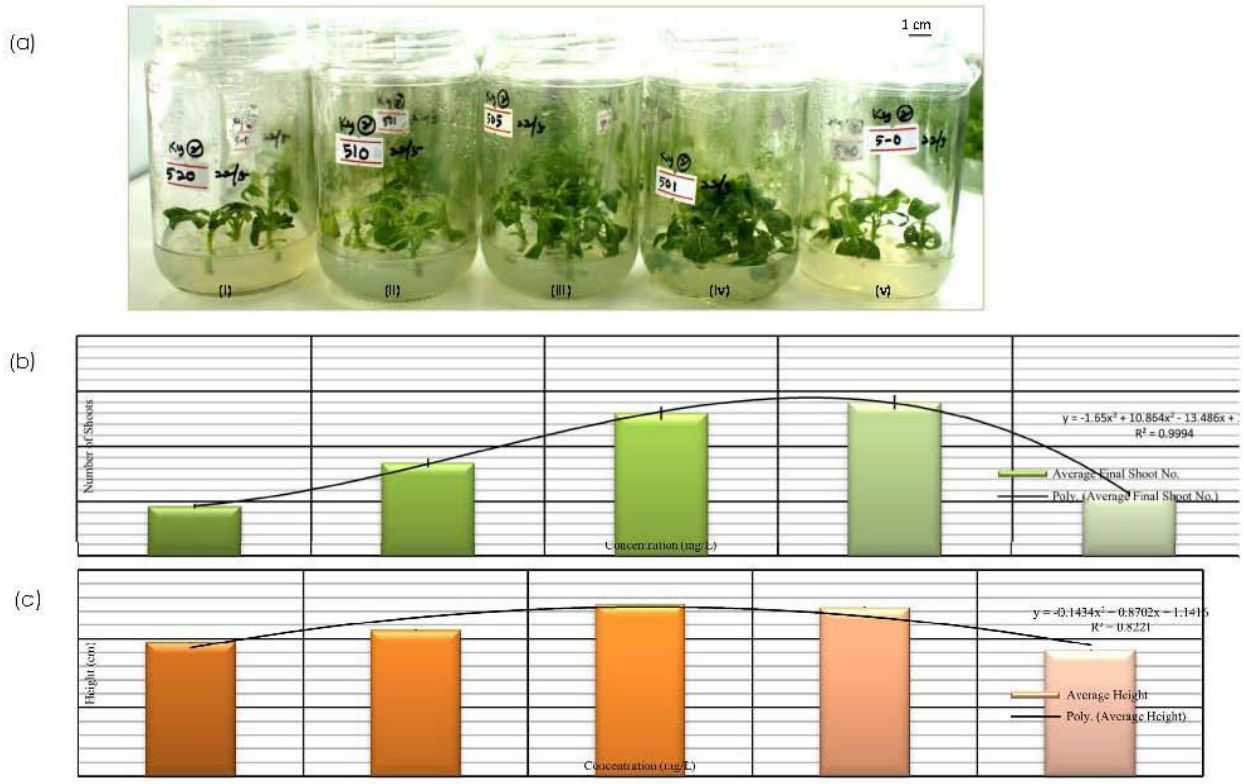

Figure 2 (a) Development of *N. cadamba* shoots in different concentrations of BAP: (i) control (PGR-free medium; (ii) 0.01mg/L, (iii) 0.05 mg/L, (iv) 0.1 mg/L and (v) 0.2 mg/L; (b) Graph showed that 0.1mg/L had the highest shoot multiplication rates followed by 0.5 mg/L with 26.4 to 26.2 shoots per explants, respectively; (c) 0.05 mg/l showed the best plant height induction followed by 0.1 mg/L

3.3 In Vitro Rooting

Healthy shoots were selected for further root induction experiment. Roots were observed to develop after 2 weeks in culture using different concentrations of IBA. Callus proliferation was observed growing at minimal amount at the base of the stem. The root development showed morphological differences in respond to the concentrations of IBA used. Interestingly, control cultures (without any auxin) were able to produce roots, thus similar to *Caesalpinia bonduc* [20], *Carica papaya* [21]. Media supplemented with 0.5 mg/L IBA showed the best results in terms of root development, produced broader leaves and stimulate plant heights most effectively compared to other concentrations. The *N. cadamba* was observed to produce more vigorous roots at IBA concentrations higher than 0.5 mg/L, with dense and thick roots, elongated but yet smaller leaves and exhibited mild

vitrification. Whereas, lower concentrations than 0.5 mg/L IBA induced slender and longer roots and broad dark leaves. Therefore, exogenous auxin has a significant effect on root induction rates and the number of roots compared with the control treatment (Figure 3).

3.4 Ex vitro transplanting

Overall, all plantlets had a high successful acclimatization rates, except for one from ½ strength MS medium supplemented with 0.5 m g/L IBA (Table 2). The results showed that there were differences in terms of plantlets development and adaptation originated from different rooting treatment. For example, plantlets from ½ strength MS hormone-free medium were observed to have 100% survival rates, and the number decreased to 95% in plantlets from ½ strength MS medium supplemented with 0.1 mg/L IBA. The number decreased again 65% in plantlets from ½ strength MS medium supplemented with 0.5 mg/L IBA. The survival rate however, increased to 85% in plantlets originated from ½ strength MS medium containing 1.0 mg/L IBA. Therefore, ½ strength MS medium supplemented with lower levels of IBA increased the chances of the plantlets to survive after transplanting. For feasible and economical reasons it is suggested to use ½ strength MS medium supplemented with less than 0.1 mg/L of IBA for the most suitable medium to ensure high survival rates of *N. cadamba* after transplanting (Figure 4).

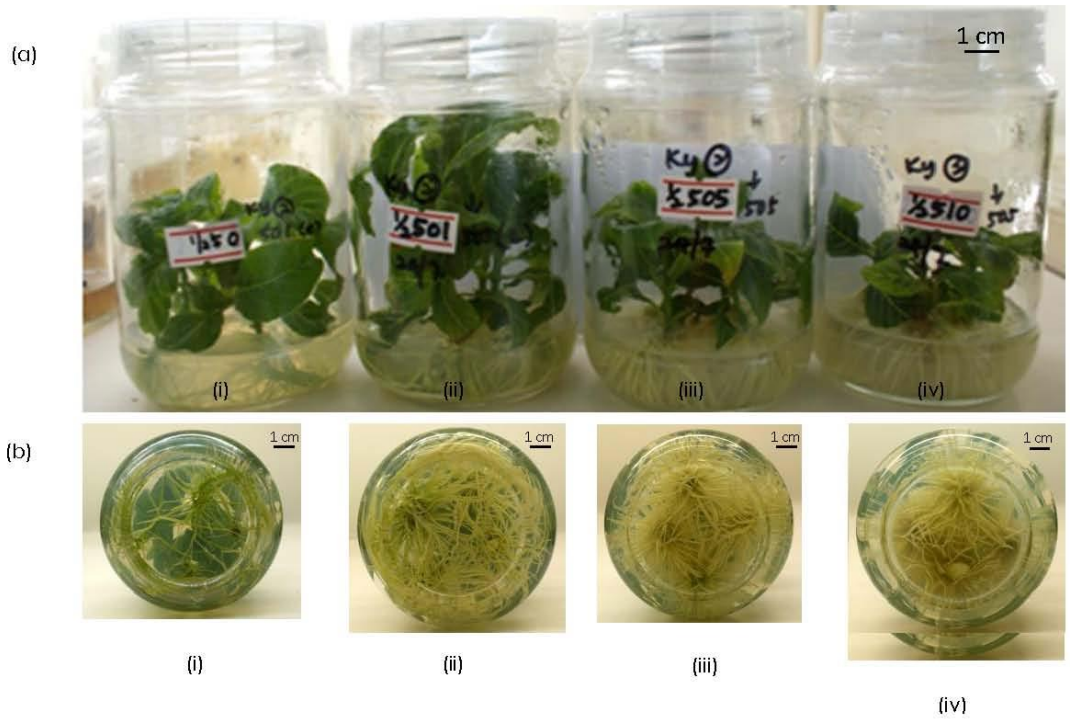

Figure 3 (a) *N. cadamba* plant development during root induction with ½ MS medium supplemented with different concentrations of IBA (mg/L): (i) 0 mg/L (control), (ii) 0.1 mg/L, (iii) 0.5 mg/ and (iv) 1.0 mg/L. Results had showed that the best plant development (root induction and plant height) was from (ii) 0.1 mg/L IBA ($P<0.05$); (b) Morphological observations of the roots showed higher levels of IBA (iii and iv) formed dense and thick roots whereas lower concentrations induced slender and longer roots (i and ii).

Table 2 Survival rates after acclimatization of *N. cadamba* from different treatments during root development using tissue culture techniques

Treatments:	Survival rates (%):
½ MS hormone free medium	100
½ MS + 0.1 mg/L IBA	95
½ MS + 0.5 mg/L IBA	60
½ MS + 1.0 mg/L IBA	85

Values are mean ± SD of triplicates test on the survival rates in percentages (%).

Figure 4 *N. cadamba* plantlets after 3 weeks acclimatization process and ready to be transferred into greenhouse for 2 months.

4.0 CONCLUSION

For *N. cadamba* the nodal segments were found suitable for shoot induction and multiplication with minimal concentrations of the BAP. This study *N. cadamba* showed very high root frequency for woody species which allowed rapid transplanting of *N. cadamba* plantlets. In conclusion, protocol developed could be very useful in the production of *N. cadamba* for commercial plantation programme of this species.

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