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Research Article
Rapid *in vitro* Propagation and Efficient Acclimatisation Protocols of *Neolamarckia cadamba*

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Abstract

Background and Objective: *Neolamarckia cadamba* is a fast-growing commercial timber tree species with considerable economic returns to the growers on a rotation period of 4-10 years. The present study was aimed to establish an efficient micropropagation protocol for *N. cadamba* through direct organogenesis by using nodal explants. **Materials and Methods:** The nodal explants from *in vitro*-germinated seedlings were cultured on B5 medium supplemented with various BAP concentrations. The *in vitro* shoots were then rooted in 1/2 B5 medium added with growth regulators such as IBA, NAA and PBZ. Three types of potting media were tested for transplantation efficiency. **Results:** The B5 medium supplemented with 1.0 mg L^{-1} BAP provided the most suitable medium for shoot induction from nodal explants with a mean of 5.4 shoots per explant. The subculture interval could be shortened by proliferating the regenerated axillary shoots on B5 medium supplemented with 0.8 mg L^{-1} BAP. Half-strength B5 medium enriched with 0.1 mg L^{-1} PBZ was able to induce root growth with 100% of root formation and resulted in more than 95% survival during acclimatisation stage. **Conclusion:** This micropropagation protocol could pave the way for mass production of quality *N. cadamba* seedlings for industrial tree plantation development in order to assure the local timber industries to meet the global demand for wood. Therefore, this could reduce the reliance on natural forests for wood production.

Key words: *Neolamarckia cadamba*, direct shoot organogenesis, *in vitro* culture, shoot proliferation, paclobutrazol (PBZ), acclimatisation

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Neolamarckia cadamba (Roxb.) Bosser belongs to the Rubiaceae family, which is commonly known as Kelampayan in Sarawak or Laran in Sabah. It is native to South Asia and Southeast Asia, such as Malaysia, Indonesia, China, India, Thailand, Vietnam and Papua New Guinea. It is classified as lightweight hardwood and it is likely to be selected for plantation programmes due to its fast-growing properties[1-3]. The tree is harvested for matches and pulpwood after 4-5 years of planting under optimal maintenance[4]. Meanwhile, *N. cadamba* on 7-10 years rotation was suitable for wood production[5]. It can grow on several varieties of soils as well as in dry areas with about 200 mm rain/year and tolerate flooding[6]. It is used as a shade tree for coffee and tea plantations as the trees have no allelopathic effect on plants under its canopy[7]. Besides harvested as the essential raw materials for plywood, hardboard, paper and furniture[8], *N. cadamba* also have medicinal values as its dried barks are used to relieve fever[9]. Its extracts have significant antibacterial and antifungal activities against various microorganisms and have strong healing capacity[10,11]. Therefore, it is known as the miracle tree.

The rising demand for wood coupled with the depletion of natural forests due to over-exploitation has urged the need to find an alternative timber resource by promoting the establishment of planted forests recently. The planting material of a fast-growing tree species such as *N. cadamba* is much needed to be mass propagated for planted forests as well as for reforestation programme. The seed viability of *N. cadamba* is variable, which ranged from 32-63% and the germination rate is generally low[12] at about 25%. A study by Aminah *et al.*[13] showed that the survival rate of *N. cadamba* cuttings was generally low, which ranged at about 26-63%. Poor seed germination and slow conventional propagation methods by cuttings had resulted in depletion of *N. cadamba* natural populations[14]. According to Huang *et al.*[15], low hardiness, weak insect resistance and lower natural propagation of *N. cadamba* could result in depletion of this tree species from the natural ecosystem. Therefore, it is crucial to propagate elite germplasm of *N. cadamba* through plant tissue culture to overcome the shortage of clonal planting stocks for planted forest establishment.

Propagation of tree species from conventional methods such as grafting, layering and vegetative cutting is slow and less productive for production on a commercial scale[16,17]. Mass propagation of elite germplasm that has high economic values and returns in a short duration is the primary interest of most commercial forest plantations and timber industries. Plant micropropagation technology had emerged during the 1900s and continuously evolving until today, which encompass rapid means of producing clonal planting stock that free from virus infection[18-20]. Besides, plants of superior quality, high yield and well-adapted genotypes are able to be cloned and produced in large scale rapidly by micropropagation technology that acts as an alternative way of asexual propagation of plants[21].

Micropropagation of *N. cadamba* has been attempted through direct adventitious shoot induction from cotyledon[15] and shoot regeneration from callus clumps[22,23]. However, direct regeneration method by using apical and nodal explants is considered as a reliable method for clonal propagation of selected germplasm since the shoots are induced from pre-existing meristems[24,25]. Shoot culture has been preferred for *in vitro* clonal propagation and conservation of various economic important as well as endangered plants[26-29]. It is usually referred to as cultures that are established from explants with intact shoot meristem, which results in direct shoot formation and proliferation from the explants[30]. A study on the mass production of *N. cadamba* plantlets via direct shoot organogenesis had been reported[31], but the data on the shoot and root induction was not detailed. The high mortality rate in the initial stage of acclimatisation is the bottleneck on the micropropagation of tree species[32]. Therefore, more studies are required to improve the transplantation rate of *N. cadamba* to substrate media.

Based on the background above, the focus of this study was to present an efficient and simple protocol for *N. cadamba* regeneration through direct organogenesis by using node from *in vitro*-germinated seedlings, which produce compact plantlets with high survival rate during acclimatisation stage. The effects of different types and concentrations of plant growth regulators (PGRs) to shoot induction, shoot multiplication and root induction were evaluated in the present study. The establishment of an efficient protocol for mass propagation of selected elite clones of *N. cadamba* with desired traits such as wood quality, disease resistance and short rotation period could pave the way to improve the development of industrial tree plantations and reforestation programmes in the tropics.

MATERIALS AND METHODS

Plant materials and explant establishment: Seeds were harvested from the selected candidate plus tree of *N. cadamba* at the Kelampayan planted forest in Kanowit (N02°00.780'E 112°03.877'), Sarawak, Malaysia. The processed seeds were stored at 4°C under dark condition. The seeds were soaked in sterile distilled water and incubated at 45°C for 30 min. The seeds were then surface sterilised in 15% commercial bleach solution added with 3 drops of Tween 20 for 15 min and rinsed 3 times with sterile distilled water.

Finally, the seeds were immersed in 70% ethanol solution for 30 sec, followed by 4 rinses with sterile distilled water. The seeds were then cultured on petri dish containing Gamborg's B5 medium[33] without plant growth regulator (PGR) to provide clean stock plants. Seedlings of 90 days old after culture with at least four nodes were selected for the experiments. The single-node segments were excised as the explants for the establishment of nodal cultures.

Shoot induction and multiplication: Gamborg's B5 medium[33] supplemented with 3.0% (w/v) of sucrose and 0.8% (w/v) of Phyto agar were tested for the efficiency of shoot induction and multiplication. The medium was supplemented with various concentrations of BAP (0.5, 0.8, 1.0 and 2.0 mg L^{-1}). Medium with basal nutrients devoid of PGR was used as a control. The B5 medium was adjusted to pH 5.5±0.1 before autoclaving at 121°C for 20 min. Each petri dish contained 4 explants with 5 replicates for each treatment. The experiment was repeated twice. After 2 weeks in the induction medium, the explants were transferred into culture bottles containing medium of the same composition for further shoot proliferation in another 2 weeks. Data were recorded after 4 weeks of culture. For the subsequent subcultures, the microshoots (height ≤1.0 cm) were separated from the explants and subcultured to B5 medium supplemented with 0.8 mg L^{-1} of BAP at 3-week intervals for shoot proliferation.

Rooting of the regenerated shoots: The healthy and well-grown shoots with about 1.0 cm height were detached individually from the shoot clumps and treated with rooting medium that containing half-strength of Gamborg's B5 basal nutrients incorporation of NAA or IBA (0.1 mg L^{-1}) and PBZ (0, 0.1, 0.5 and 1.0 mg L^{-1}). Each treatment contained 5 microshoots and with 5 replicates. The experiment was repeated twice. After 2 weeks of cultures, the percentage of shoots that formed roots, number of roots per shoot, changes in plant height (cm), root length (cm) and root width (mm) were recorded.

Acclimatisation of plantlets: After 4 weeks in the rooting media, the rooted plants (height ≥2.5 cm) were subjected for acclimatisation and hardening. Rooted plantlets were removed from the rooting medium and washed under running tap water to wash off the remaining agar. The plantlets were transplanted into plastic pots containing autoclaved potting media, i.e., soil, soil and peat mixture (3:1) and peat pellet. The survival rate of the plantlets was recorded after 4 weeks of transplanting to potting media. Each treatment contained 20 plantlets and repeated 4 times.

Statistical analysis: The data were collected and analysed through one-way Analysis of Variance (ANOVA) and Duncan's Multiple Range Test (DMRT) ($p<0.05$) using SPSS statistical software (Version 22). The multiplication medium with significant growth of shoots is defined as an effective shoot induction medium; meanwhile, rooting medium with a higher percentage of root induction and healthier plantlets is denoted as an optimum rooting medium.

RESULTS AND DISCUSSION

Effects of BAP on shoot induction and multiplication: Direct shoot formation was successfully initiated in the present study (Fig. 1). The microshoots were propagated with B5 medium supplemented with various concentrations of BAP (0.5, 0.8, 1.0 and 2.0 mg L^{-1}). The effects of different concentrations of BAP were observed in terms of the number of shoots multiplied and morphological appearances of microshoots (Table 1). With the enlargement of axillary meristem regions, the first axillary buds were sprouted within the 1st week of inoculation on all types of shoot induction media (Fig. 1a). At the 2nd week, the axillary shoots were elongated while swellings at the basal portion of explants also observed (Fig. 1b). The following week, elongated axillary shoots were started to produce new shoots from the nodal segments and huge swellings or compact calli were grown at the basal of explants (Fig. 1c). Finally, about 2-10 microshoots were proliferated per explant after 4 weeks of culture (Fig. 1d).

Table 1: Effects of B5 Gamborg medium with various BAP concentrations on shoot induction and multiplication from nodal explants of *N. cadamba* seedlings with age of 90 DAC

Medium+BAP concentration (mg L^{-1})	Shoot induction (%)	Mean No. of shoots/explant±SD	Mean plant height (cm)±SD	Callus induction (%)	Type of callus formed at the basal
B5+0	100.0^{a}	1.725±0.452^{d}	0.422±0.450^{c}	0.0^{c}	-
B5+0.5	100.0^{a}	2.875±1.362^{c}	0.978±0.694^{b}	87.5^{a}	Compact, solid/loose
B5+0.8	100.0^{a}	4.475±2.562^{b}	1.428±0.657^{a}	82.5^{a}	Compact, solid/loose
B5+1.0	100.0^{a}	5.400±2.240^{a}	1.145±0.395^{b}	85.0^{a}	Compact, solid
B5+2.0	97.5^{a}	2.900±1.549^{d}	0.625±0.393^{c}	62.5^{b}	Compact, solid

Data were recorded after 4 weeks of culture, means within a column with the same letter are not significantly different based on Duncan's test at the 0.05 probability level

Fig. 1(a-k): *In vitro* direct shoot organogenesis from nodal explants of *in vitro*-germinated *N. cadamba*, (a) Nodal explants were inoculated in media supplemented with BAP and axillary buds were sprouted within the 1st week of inoculation, (b) The axillary shoots were elongated at the 2nd week of cultures, (c) New axillary shoots were continued to sprout from elongated shoots and huge swellings were observed at the cut ends of nodal explants, (d) Shoot clump that contained 2-10 microshoots was obtained in B5 medium fortified with 1.0 g L^{-1} of BAP, (e) No shoot multiplication was observed from explants that cultured in all control media, (f) Hyperhydricity in medium supplemented with high BAP, (g, h) Microshoots with height $\geq$1.0 cm were successfully rooted in 1/2 B5 medium+0.1 mg L^{-1} PBZ, (i) Plantlets were transferred to potting medium that containing soil (3): peat (1) and successfully acclimatized, (j) *In vitro*-developed roots from plantlets were elongated, penetrated and continued to spread within the substrates and (k) Acclimatised plants are ready to be transferred to greenhouse

Although shoot induction was observed from the control medium without BAP, no shoot multiplication was observed (Fig. 1e). Moreover, most of the shoots were stunted after 1-2 weeks cultured in the control medium.

Shoot proliferation was accompanied by the formation of calli at the cut ends of explants. The highest percentage of callus formation was observed from the explants that cultured on B5 medium supplemented with 0.5 mg L^{-1} BAP and the lowest when explants were inoculated on 2.0 mg L^{-1} BAP. In the control medium, no callus formation was found. This formation of callus was similar to the reports by other researchers, which depicted the effect of BAP treatment[34,35]. According to Ndoye *et al.*[36], species with strong apical dominance has a higher frequency of callus formation at the basal cut ends of node segments that cultured on cytokinin enriched medium. Moreover, the formation of callus at basal of the explants was probably due to the exogenous supply of plant growth regulator that distorted the polarity and subsequently led to the callus formation[37]. Besides that, accumulation of auxin at the basal cut ends of the nodal explants may induce cell proliferation when growing on medium enriched with cytokinins[38].

Cytokinin is one of the important plant growth regulators which play a role in plant development, especially on the control of shoot formation and multiplication besides promoting the cell growth[39]. It promotes shoot proliferation by releasing the lateral shoot buds from dormancy. Among the cytokinins, BAP is preferably selected for *in vitro* shoot proliferation of a wide variety of plants due to its effectiveness[40,41]. In the present study, high shoot induction percentage was recorded in B5 medium supplemented with 0.5, 0.8 and 1.0 mg L^{-1} of BAP, with 100% of shoot induction including the controls (Table 1). However, the frequency of shoot induction declines when the concentration of BAP increases. The average number of shoots produced per explant in B5 medium was significantly increased from control to 0.8 mg L^{-1} of BAP and the highest at 1.0 mg L^{-1} with 5.4 of shoots but decreased at 2.0 mg L^{-1} with 2.9 of shoots/explant. At 1.0 and 2.0 mg L^{-1} of BAP, the incidence of explants necrosis was frequently observed when compared with a lower concentration of BAP. Necrosis in shoot tips of regenerated shoots also was observed in B5 medium enriched with 1.0 and 2.0 mg L^{-1} of BAP after 2-3 weeks in culture.

The B5 medium added with 1.0 mg L^{-1} of BAP was capable of inducing and proliferating higher shoot number in the present study. However, healthier and greener shoots with higher shoot length were observed in B5 medium with 0.8 mg L^{-1} (Table 1). Moreover, the high concentration of cytokinins may promote somaclonal variations among the plantlets after a long period of subcultures[42-44]. Hence, a reduction in the concentration of cytokinins in subsequent subcultures is required to minimise the frequency of somaclonal variants as well as to produce healthier shoots that are favourable for rooting. In the present study, B5 medium fortified with a lower concentration of BAP (0.8 mg L^{-1}) was used for continuous subcultures of the microshoots at 3-week intervals, up to 3 subcultures with shoot number ranging from 3.50-5.40/explant (Table 2). The number of shoots was generally observed with a trend of inclined with subculture passages from S1-S3. This observation was similar to the reports on other species which usually shown the increment of shoot multiplication rate from the first passage to 4 or 5th passage[38,24,45].

The regeneration pathway selected in the current study was direct shoot organogenesis from pre-existing shoot meristems as the nodal segments were used as the explants. Therefore, high shoot induction percentage (100%) was reported in the present study when compared to the previous study where 54.2% of shoot regeneration from cotyledon explants was reported when cultured on medium added with

Table 2: Three cycles of direct shoot regeneration through subcultured of microshoot explants (B5+0.8 mg L^{-1} BAP)

Stock plants	Subculture	Mean number of shoots/explants±SD	Highest number of shoots/explants observed
1	S1	3.500±0.577[a]	4
	S2	4.296±2.072[a]	11
	S3	4.410±1.599[a]	10
2	S1	3.500±1.000[a]	4
	S2	5.423±2.318[a]	12
	S3	4.921±1.761[a]	9
3	S1	3.500±1.000[a]	4
	S2	4.593±2.062[a]	12
	S3	4.918±1.998[a]	11
4	S1	4.250±1.258[a]	6
	S2	4.222±1.944[a]	12
	S3	4.326±1.426[a]	8
5	S1	4.000±1.414[a]	6
	S2	4.000±2.023[a]	10
	S3	4.267±2.083[a]	12

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

Table 3: Effects of half strength B5 Gamborg medium with various auxins and growth retardant concentrations on root induction of *N. cadamba*

Treatments (mg L^{-1})	Root induction (%)	No. of roots induced±SD	Increase in plant height (cm)±SD	Root length (cm)±SD	Root width (mm)±SD	Callus induction (%)
1/2 B5	74.0^{b}	3.360±1.675^{f}	0.358±0.121^{ab}	3.491±1.119^{a}	0.579±0.137^{e}	0.0^{c}
1/2 B5+0.1 IBA	100.0^{a}	7.920±3.374^{cd}	0.296±0.256^{bc}	1.847±0.766^{c}	0.553±0.192^{e}	36.0^{ab}
1/2 B5+0.1 NAA	96.0^{a}	6.540±2.880^{de}	0.280±0.229^{c}	2.259±1.611^{b}	0.567±0.135^{e}	14.0^{c}
1/2 B5+0.1 PBZ	100.0^{a}	6.880±4.153^{de}	0.362±0.253^{a}	3.303±0.988^{a}	0.705±0.154^{d}	0.0^{c}
1/2 B5+0.5 PBZ	100.0^{a}	6.160±2.461^{e}	0.236±0.103^{c}	2.282±0.792^{b}	0.917±0.204^{c}	4.0^{c}
1/2 B5+1.0 PBZ	96.0^{a}	5.620±3.422^{e}	0.250±0.169^{c}	1.565±0.691^{cd}	1.153±0.318^{a}	22.0^{bc}
1/2 B5+0.1 IBA+0.1 PBZ	94.0^{a}	11.420±4.463^{b}	0.232±0.132^{c}	1.428±0.491^{d}	0.570±0.119^{e}	54.0^{a}
1/2 B5+0.1 IBA+0.5 PBZ	100.0^{a}	14.400±5.962^{a}	0.116±0.093^{d}	1.791±0.823^{cd}	0.749±0.157^{d}	12.0^{c}
1/2 B5+0.1 IBA+1.0 PBZ	100.0^{a}	11.340±4.336^{b}	0.102±0.074^{d}	1.054±0.416^{e}	1.029±0.199^{b}	16.0^{bc}
1/2 B5+0.1 NAA+0.1 PBZ	96.0^{a}	8.840±3.710^{c}	0.230±0.122^{c}	2.324±0.704^{b}	0.623±0.138^{e}	2.0^{c}
1/2 B5+0.1 NAA+0.5 PBZ	100.0^{a}	7.020±3.140^{de}	0.144±0.095^{d}	2.235±1.095^{b}	0.993±0.295^{bc}	4.0^{c}
1/2 B5+0.1 NAA+1.0 PBZ	92.0^{a}	6.140±2.871^{e}	0.148±0.105^{d}	1.054±0.950^{e}	1.029±0.292^{b}	6.0^{c}

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

a high concentration of BAP[15]. Although no data on shoot regeneration frequency was available from the previous studies[22,23], higher shoot numbers (6-26 shoots/explant) were reported from the studies on *N. cadamba* shoot regeneration through leaf explants[15,22,23] and nodal explants[31]. The shoot regeneration frequency from leaf explants is generally low since the shoot formation from differentiated cells involves complicated gene regulations and expressions that also affected by genotypes[46-48]. Moreover, the applications of auxins and high concentration of cytokinins for shoot induction from differentiated tissues are known to involve in abnormal regulations within cells that may lead to somaclonal variations[49,50]. The regeneration pathway from pre-existing meristem is noted as the rapid and straight forward way for vigorous production of genetically identical clones and therefore is adopted for mass propagation of elite germplasm[30].

Hyperhydricity in microshoots was observed in media added with BAP (Fig. 1f). Higher BAP concentration (1.0-2.0 mg L^{-1}) resulted in shoots with pale green colour, smaller shoot stem diameter and often with symptoms of vitrification. As reported in many studies[51,15], microshoots regenerated from high BAP concentrations showed a mild hyperhydricity, smaller leaf with the glassy appearance and often distorted. Hyperhydric shoots were observed with thick, brittle and translucent appearance and lower regeneration efficiency. Lack of chloroplasts and other subcellular organelles in hyperhydric shoots was suggested to influence the regeneration ability of the plantlets[52,53].

Effects of types and concentrations of PGRs on root induction: Exogenous auxins promote *in vitro* rooting of regenerated shoots[38]. No roots formed from regenerated shoots in the media without auxins[38]. According to Suarez *et al.*[54], rooting percentage of regenerated shoots cultured on media without IBA was the lowest (22%) in comparison to media with IBA (80-100%). Likewise, on the micropropagation of a tropical tree, *Morinda citrifolia* recorded the lowest percentage of rooting on the controls as compared to the media supplemented with IBA[35]. These results were in agreement with the different responses of shoots that cultured on controls and medium supplemented with hormones in the present study. Lower rooting percentage and root number were recorded by shoots cultured in half-strength B5 medium devoid of PGRs which are consistent with the study by Huang *et al.*[15]. Rooting percentages that ranged from 84-100% were observed when IBA, NAA and PBZ were added into both of the media singly or in combinations. Only shoots with length more than 1 cm were preferably selected for root induction since small shoots rooted very poorly or no rooting response was observed (Fig. 1g, h). A summary of the effects of media and the concentration of IBA, NAA and PBZ on root induction is shown in Table 3.

The roots were observed generally within 1 week after cultured on the half-strength B5 medium, which emerged within a shorter period of time when compared to the previous studies on rooting of *N. cadamba* shoots that attempted through half-strength MS medium[15,31]. Similar to the previous study by Huang *et al.*[15], the incorporation of 0.1 mg L^{-1} IBA into the rooting medium had resulted in shorter root length when compared with the control and rooting medium added with 0.1 mg L^{-1} NAA. Since the concentration of IBA and NAA adopted in the present study was considerably low (0.1 mg L^{-1}), no significant difference was observed in the root thickness. As mentioned by Huang *et al.*[15], high concentration of IBA had resulted in thicker roots. The root thickness in the present study was generally increased when the concentration of PBZ was increased, both alone in the rooting medium or in combination with IBA or NAA.

Formation of callus was observed at the basal of shoots from the medium that augmented with various concentrations of IBA, NAA and PBZ. The percentage of callus formation was simultaneously increased from lower to higher concentrations of IBA, NAA and PBZ, except for the combination of 0.1 mg L^{-1} IBA and 0.1 mg L^{-1} PBZ. Addition of PBZ into the media supplemented with IBA, NAA or PBZ had induced callus formation at the basal portion of shoots in the range of 2-54%. Similar observation also had been reported by Huang *et al.*[15] and Rahman *et al.*[31] when the regenerated shoots were inoculated in rooting medium that supplemented with various concentrations of IBA and NAA. According to Shekhawat *et al.*[55], plantlets with more callus and compact roots were poorly developed during hardening. Therefore, the culture media that resulted in optimum root number, high rooting percentage and lower callus formation should be selected. Regarding the *in vitro* root formation of *N. cadamba* in the present study, half-strength B5 medium supplemented with 0.1 mg L^{-1} PBZ had attributed to the high rooting frequency with the reasonable number of roots, plant height, root length and root thickness. Besides that, no callus formation was observed on plantlets under this treatment. Therefore, this treatment had been selected as a suitable rooting medium in this study.

The efficiency of PBZ on rooting of *N. cadamba in vitro* shoots has not yet been reported. Paclobutrazol (PBZ) is a triazole growth retardant that usually applies in the last stage of micropropagation in order to promote the plantlets to withstand the stress associated with acclimatisation[56]. The number of roots induced from shoots was significantly enhanced in the current study when PBZ was added to the rooting medium compared to the medium with auxin alone (Fig. 1f). However, the addition of PBZ also reduced the growth of shoots in contrast with the controls and medium with auxins alone (Table 3). This reduction in plant height is due to the inhibition of gibberellins activity of PBZ[57-58]. Besides that, the addition of PBZ in the present study had shown improvement in the root diameter, especially at the root apex in tandem with the increment of PBZ concentrations that is suggested due to the enlargement in cortical cells and meristematic zone as reported by Wen *et al.*[59]. This significant effect of PBZ on the root diameter had also been reported by Burrows *et al.*[60] and Tsegaw *et al.*[61]. Although plantlets with larger root diameter may good for acclimatisation, the roots are fragile and easily detached from the shoots during removal from agar medium and transplantation. The PBZ may also enhance the sugar accumulation in treated plantlets and provide energy for active growth in the root apex[59]. However, the root length was decreased with the increase of PBZ concentration, which was similar to a study by Rieger and Scalabrelli[62].

Besides that, the incorporation of PBZ to the rooting medium in the present study had resulted in the following positive effects, such as the increased in the thickness of leaves, improvement of rooting and the increment in the thickness of stems as reported in many studies on other species[63-65]. Moreover, the *N. cadamba* plantlets cultured on PBZ-supplemented rooting medium were healthy and observed with dark green leaves. As stated in studies on other species, the increment of leaf thickness in *N. cadamba* plantlets may due to the increment in epicuticular wax, increase in palisade and spongy mesophyll tissue layers[66-67]. In addition, Nizam and Te-chato[67] reported that PBZ-treated plantlets with dark green leaves contained the highest chlorophyll content when compared to the control. Impaired regulation of transpiration rate among tissue cultured-derived plantlets is one of the critical factors attributed to the high mortality rate of plantlets after transferred to *ex vitro* environment. The PBZ is reported to improve the resistance of plantlets to desiccation by reducing the stomatal apertures and improving stomatal activity in many plant species[66,68]. These beneficial effects had attributed to the effective and successful transplantation of PBZ-treated *N. cadamba* plantlets under minimum maintenance with high survival rate even though a simple potting medium such as soil is used in the current study.

Survival rate of *in vitro*-derived plantlets in *ex vitro*: The process of acclimatisation is a critical step in plant tissue culture, which is a determining step for the success of plant micropropagation and the quality of regenerated plantlets. Attempts had been made to acclimatise the PBZ-untreated plantlets. However, none of the plantlets was survived after 1-2 weeks of transplantation, mainly due to the leaf desiccation and fungus infection. Therefore, only *N. cadamba* plantlets rooted in half-strength B5 medium+0.1 mg L^{-1} PBZ were used in this study. The survival rate of plantlets transplanted to peat pellets recorded the highest survival rate up to 96.55%. No significant difference was detected among all of the potting media (Table 4). Various investigations had revealed that peat pellet was suitable for plantlet hardening with a lower mortality rate compared to the soil mix and hence, the former is suitable for the early stage of acclimatisation[69-72].

Table 4: Survival rate of plantlets after transplanted to different potting media at week 4

Potting medium	Survival rate (%)
Soil	94.38±6.575[a]
Jiffy-7	96.55±4.541[a]
Soil:peat moss (3:1)	96.25±4.787[a]

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

The higher ability of peat pellet in maintaining moisture may affect the high survival rate of plantlets in *ex vitro*[71]. The higher survival and growth performance of plantlets in peat pellet also could be resulted from its low pH, high water retaining ability and greater porosity for new roots formation and elongation and hence greater accessibility and absorption of nutrients from the substrate[70]. A previous study by Rahman *et al.*[31] also reported the successful of *N. cadamba* plantlets acclimatisation with 95-100% of survival rate by initial hardening of plantlets with peat pellet. However, the costs and availability of peat pellet are the hindrances for large-scale applications. The soil:peat moss (3: 1) medium in the present study was able to show a high survival rate and no significant difference was observed among them. Hence, soil:peat moss (3:1) is determined as an optimum potting media for acclimatisation and hardening of *N. cadamba*. *In vitro*-developed roots from plantlets transplanted in all types of potting media in the present study were able to elongate, penetrate and continue to spread within the substrates (Fig. 1j). The plantlets were able to form stronger root systems with root hairs. After 4-5 weeks of hardening, the plants were transferred to the greenhouse (Fig. 1k).

CONCLUSION

As a conclusion, the shoot regeneration and root induction were significantly influenced by the type and concentration of the PGRs. High frequency of shoot induction and proliferation were observed in B5 medium supplemented with the optimum concentration of BAP. The PBZ was successfully used for rooting of *in vitro* shoots and resulted in high survival rates of plantlets during acclimatisation and hardening.

SIGNIFICANCE STATEMENT

This study developed a method for *N. cadamba* micropropagation with rapid shoot multiplication by using nodal explants. This is the first report on the effects of paclobutrazol on rooting of *N. cadamba in vitro*-derived shoots. As the results, a high frequency of shoot proliferation and high survival rate of *N. cadamba* plantlets were achieved. Thus, this micropropagation protocol can be utilised for large-scale propagation of this economically important tree species within a relatively short period of time.

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