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Research Article
Validating the Efficacy of an Established Micropropagation Protocol for Commercial Propagation of *Neolamarckia cadamba*

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Abstract

Background and Objective: Micropropagation is an efficient technique for mass-producing superior clones used in establishing planted forests. However, there is a lack of comprehensive reports on the effectiveness and reliability of the established micropropagation protocol for *Neolamarckia cadamba*. The aim of this study was to demonstrate the effectiveness and reliability of the established micropropagation protocol for mass propagating true-to-type *N. cadamba* clones. **Materials and Methods:** Two selected candidates plus trees of *N. cadamba* were cultured in B5 media supplemented with 0.8 mg L^{-1} BAP for shoot multiplication and in ½ B5 media supplemented with 0.1 mg L^{-1} PBZ for root regeneration. The growth performance, the presence of phytopathogens and morphological differences were investigated. The collected data were subjected to a two-tailed t-test ($p<0.05$). **Results:** The results showed no significant variation ($p<0.05$) in the number of shoots regenerated from each explant compared to the reference clone N5 (B39 = 4.6, B42 = 4.3 and N5 = 4.8). Moreover, the rooting patterns of the investigated clones (B39 = 14.5 and B42 = 9.4) significantly outperformed clone N5 (6.9), with over 90% successful root regeneration. Phytopathogen analysis using ERIC-PCR assay confirmed that the *in vitro* regenerants were free of any phytopathogens. Additionally, histological examination revealed no significant differences between the stock plants and *in vitro* regenerants. **Conclusion:** This study successfully ascertained the effectiveness and reliability of the established micropropagation protocol for mass propagating true-to-type *N. cadamba* clones.

Key words: Micropropagation, ERIC-PCR, *in vitro* culture, phytopathogen, kelampayan, planted forest

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Competing Interest: The authors have declared that no competing interest exists.

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

INTRODUCTION

Neolamarckia cadamba, also known as Kelampayan locally in Sarawak, is an essential source of plywood in Malaysia. It is a fast-growing plantation tree species for planted forest development and is proven able to reach a height of 45 m in 8 years[1-3]. The maturation period and rotation cycle are relatively short, only five to ten years, compared to some other timber species. Due to its ability to grow in exploited and denuded land, especially in logged-over areas, *N. cadamba* plantations are known to be able to reduce the logging pressure of natural forests, which results in increasing demand in the wood-based industry[4,5]. It also poses a self-pruning ability, leaving a branchless bole of more than 25 m. To meet the increasing demand for the species, *in vitro* micropropagation of *N. cadamba* was introduced. Several protocols for micropropagation of *N. cadamba* were established using different regeneration pathways and types of explants. These included the direct somatic embryogenesis from internodes[6], direct shoot induction and plant regeneration using apical bud and nodal explants obtained from mature trees[7], direct adventitious shoot multiplication and plant regeneration through the aseptic cotyledons of *N. cadamba in vitro* seedlings[8] and optimized direct organogenesis from nodal segments of *in vitro* seedlings[9]. However, some studies showed that even though the micro-propagated plants are from the same species, they might respond differently to the same protocol because they have different genotypes. Thus, ascertaining the protocol is essential.

One of the significant advantages of micropropagation is that this technique can produce disease-free plants or is also known as axenic cultures[10,11]. Hamill *et al.*[12] reported that vegetative propagations are troubled by systemic diseases, which often carry the pathogen in the new plants. They mentioned that micropropagation serves as an effective technique to eliminate pathogens from mother plants, producing germ-free cultures. Only a few studies have addressed verifying micro-propagated cultures, specifically identifying phytopathogenic bacteria. Currently, molecular techniques applied in identifying phytopathogenic bacteria are Enzyme-Linked Immunosorbent Assay (ELISA) and Polymerase Chain Reaction (PCR)[13]. ELISA is dependent on the antigen-antibody reaction and it is reported that there is a high possibility of false positive or negative results due to insufficient blocking of immobilized antigens and antibody instability[14]. The PCR techniques, meanwhile, are primarily gene-specific. This causes the identification process to be less efficient from the aspects of labour and costs[15]. Thus, a technique that can identify multiple bacteria species is recommended.

Numerous studies have shown the efficacy of enterobacterial repetitive intergenic consensus (ERIC) PCR technique for identifying bacteria[16-18]. The ERIC sequences are 127 bp imperfect palindromes that Hulton *et al.*[19] first identified in the genomes of vibrios and enteric bacteria in 1991. According to their findings, the sequences are only found in transcribed regions of the genome, either in untranslated regions upstream or downstream of open reading frames or in intergenic regions of polycistronic operons. Although the chromosomal locations of ERIC sequences vary between species, their nucleotide sequence conservation is high, making the primer universal. The ERIC-PCR method has been used to identify numerous bacterial species[20-22].

The specific objective of this study was to ascertain the efficiency and reliability of the micropropagation protocol established previously[9] for *N. cadamba*. The morphological and growth performance of micro-propagated cultures derived from two selected *N. cadamba* candidates plus trees (i.e., B39 and B42) was comprehensively analyzed. In addition, the ERIC-PCR technique was employed to reaffirm the absence of contaminants in the micro-propagated *N. cadamba* cultures.

MATERIALS AND METHODS

This study was carried out from July, 2019 to June, 2022. The experiments were carried out at the Laboratory of Forest Genomics and Informatics (fGiLab), University of Malaysia Sarawak (UNIMAS), Malaysia.

***In vitro* germination and propagation of *Neolamarckia cadamba*:** Dried preserved fruits were procured from the chosen *N. cadamba* plus trees in the Kelampayan planted forest in Kanowit (N02E00.780'E and 112E03.877'), Sarawak, Malaysia. About 100 seeds were first isolated and pre-treated with a water bath at 35°C for 24 hrs to break seed dormancy. Pre-treated seeds were sterilized in 20% commercial Clorox for 15 min and 70% ethanol for 30 sec. Sterilized seeds were cultured on basal B5 media in Petri dishes. The micropropagation protocol established previously[9] was applied in this study. Multiplication media (B5+0.8 mg L^{-1} benzylaminopurine (BAP)+2% (w/v) sucrose) was used to obtain and multiply nodal segments and subculture was conducted every three weeks until the