



Faculty of Resource Science and Technology

Tissue Culture of *Neolamarckia cadamba* (Roxb.) Bosser

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Tissue Culture of *Neolamarckia cadamba* (Roxb.) Bosser

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DECLARATION

I declare that the work in this thesis was carried out in accordance with the regulations of University Malaysia Sarawak. Except where due acknowledgement have been made, the work is that of the author alone. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.



.....

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ABSTRACT

Neolamarckia cadamba (a.k.a Kelampayan) is one of the fast-growing plantation tree species for reforestation programme and planted forest development in Sarawak. It is one of the best sources of raw material for the plywood industry, while its leaves and bark have medical applications. Hence, this study aimed to establish protocols for *in vitro* seed germination, seedling growth, callus induction, direct and indirect shoot regeneration of *N. cadamba*. Seed propagation of *N. cadamba* is limited due to the lack of viable seed production, thus leading to low germination rate, and the vegetative propagation of *N. cadamba* by traditional means is not successful. The results showed the best media for seed germination, and seedling growth of *N. cadamba* was full-strength Gamborgs (B5) medium. Seedling growth and shoot multiplication of *N. cadamba* were given a positive response to BAP or NAA at 1.0 mg/L, respectively. The combination of plant growth regulators (PGRs) with 10% (v/v) of coconut water (CW) gave the highest percentage of seedling growth under the culture condition 16-h light/ 8-h darkness at room temperature. Also, the combination of PGRs with CW gave a reasonable response to the callus induction, direct and indirect shoot regeneration of *N. cadamba*. *In vitro* seedling of *N. cadamba* grew well under 16-h light/ 8-h dark at room temperature. The good callogenic response was found in a culture that exposed to the 24-h dark treatment (88.9%). However, the 24-h light treatment resulted in a poor callogenic response (22.2%). The good callogenic response was found in B5 medium supplemented with a low concentration of auxins and cytokinins which was 1.0 mg/L of BAP (86.7%) and NAA (80.0%) within eight weeks of inoculation. Indirect shoot regeneration from calli derived from the young leaf explants was successfully initiated by inoculating callus onto B5 medium fortified with combinations of 2.0 mg/L BAP and 0.5 mg/L NAA. BAP 2.0 mg/L added with 10%

coconut water also can help to promote cell regeneration, division and differentiation of meristem cell. *N. cadamba* shoots were actively regenerated when the nodal segments were inoculated on medium added with 1.0 mg/L BAP and 10% CW. About 97.8% of nodal segments were able to regenerate shoots, and an average of 19 shoots per explant was produced after 16 weeks of culture. The present study has successfully established the tissue culture protocol for propagating *N. cadamba*. This newly established protocol could pave the way to overcome the shortage of planting materials for planted forest development in Sarawak, which in turn assures the local timber industries to meet the global demand for wood-related products.

Keywords: *Neolamarckia cadamba*, complex additives, *in vitro* seed germination, seedling growth, callus induction, indirect and direct shoot regeneration, plantlet regeneration.

Kultur Tisu terhadap Neolamarckia cadamba (Roxb.) Bosser

ABSTRAK

Neolamarckia cadamba (atau dikenali sebagai Kelampayan) adalah salah satu spesies pokok perladangan yang tumbuh dengan cepat bagi program penanaman semula dan pembangunan hutan di Sarawak. Ia adalah salah satu sumber bahan mentah terbaik untuk industri papan lapis, manakala daun dan kulit kayu mempunyai aplikasi perubatan. Oleh itu, kajian ini bertujuan untuk menubuhkan protokol untuk percambahan biji benih in vitro, pertumbuhan anak benih, induksi kalus, regenerasi pucuk semula secara langsung dan tidak langsung bagi N. cadamba. Pembiakkan biji benih N. cadamba adalah terhad disebabkan oleh kurangnya pengeluaran biji benih yang berkualiti sehingga mengakibatkan kadar percambahan biji benih yang rendah, dan pembiakan vegetatif N. cadamba secara tradisional tidak berhasil. Keputusan menunjukkan media terbaik untuk percambahan biji benih dan pertumbuhan anak benih N. cadamba adalah Gamborgs (B5) medium berkepekatan penuh. Pertumbuhan anak benih dan penggandaan semula pucuk N. cadamba masing-masing telah memberi respon positif kepada BAP atau NAA pada 1.0 mg/L. Kombinasi PGR dengan air kelapa (10%) memberikan peratusan tertinggi pertumbuhan anak benih di bawah keadaan kultur 16-jam cahaya / 8-jam gelap pada suhu bilik. Selain itu, kombinasi PGR dengan air kelapa memberi respons yang munasabah kepada induksi kalus, penggandaan pucuk dan organogenesis secara tidak langsung terhadap N. cadamba. Anak benih in vitro N. cadamba berkembang baik di bawah cahaya 16-jam/ 8-jam gelap pada suhu bilik. Sementara itu, tindak balas kalogenik yang baik didapati dalam kultur yang terdedah kepada keadaan rawatan 24-jam gelap (88.9%). Bagaimanapun, rawatan 24-jam cahaya menghasilkan tindak balas kalogenik yang lemah (22.2%). Tindak balas kalogenik yang baik didapati dalam medium B5 yang ditambah

dengan kepekatan auxins dan sitokinin yang rendah iaitu 1.0 mg/L BAP (86.7%) dan NAA (80.0%) dalam tempoh lapan minggu inokulasi. Regenerasi pucuk secara tidak langsung dari kalus yang diperolehi dari daun juvenil telah berjaya diinisiasi dengan mengkulturkan kalus diatas medium B5 yang diperkaya dengan kombinasi 2.0 mg/L BAP dan 0.5 mg/L NAA. BAP 2.0 mg/L ditambah dengan 10% air kelapa juga boleh membantu mempromosikan pertumbuhan, pembahagian dan pembezaan sel meristem. Pucuk N. cadamba aktif dihasilkan semula apabila segmen nod dikultur pada medium yang ditambah dengan 1.0 mg/L BAP dan 10% air kelapa. Kira-kira 97.8% segmen nod dapat menghasilkan penggandaan semula pucuk, dengan purata 19 pucuk per eksplan yang dikeluarkan selepas 16 minggu pengkulturan. Kajian ini telah berjaya menubuhkan protokol kultur tisu untuk pembiakkan N. cadamba. Ini dapat membuka jalan untuk mengatasi kekurangan bahan penanaman untuk pembangunan hutan yang ditanam di Sarawak, dan seterusnya menjamin industri balak tempatan untuk memenuhi permintaan global untuk produk berkaitan kayu.

Kata kunci: Neolamarckia cadamba, bahan organik kompleks, percambahan biji benih in vitro, pertumbuhan anak benih, induksi kalus, regenerasi pucuk secara langsung dan tidak langsung, penghasilan semula anak benih.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
B5	B5 (Gamborg et al., 1968)
BAP	6-Benzylaminopurine
BH	Banana homogenate
CW	Coconut water
DAC	Days after culture
DMRT	Duncan's multiple range tests
GA	Gibberellin
GI	Growth index
IAA	Indole-3-acetic acid
KC	Knudson-C
Kn	Kinetin
kPa	kiloPascal
LED	Light emitting diode
MS	Murashige & Skoog (1962)
NAA	α -Naphthalene acetic acid
NR	No response
NS	Not significant
PGR	Plant growth regulator
PPM	Plant Preservative Mixtures
PH	Potato homogenate
SD	Standard deviation

TDZ	Thidiazuron
TTC	2, 3, 5-triphenyltetrazolium chloride
TTZ	Tetrazolium
WPM	Woody plant medium
wks	Weeks
2, 4-D	2, 4-Dichlorophenoxyacetic acid

CHAPTER 1

GENERAL INTRODUCTION

1.1 Background of the Study

Neolamarckia cadamba belongs to the Rubiaceae family. *Neolamarckia cadamba* is a fast-growing and deciduous tropical hardwood with anatomical, morphological, and chemical characteristics that make it suitable for building materials, furniture, and pulp production (Huang et al., 2014). According to Mojiol et al. (2014), *N. cadamba* was formerly synonyms to *Anthocephalus cadamba* and later changed to *Anthocephalus chinensis* and now renamed again by the botanist community as *Neolamarkia cadamba*. *N. cadamba* is known as “Kalempayan” in Peninsular Malaysia, and Kelampayan in Sarawak, “Laran ” in Sabah, “Jabon ” in Kalimantan and “Kadam” in India (Jøker, 2000). Report by Mojiol et al. (2014) stated that *N. cadamba* was established in west and east Java, south and east Kalimantan, across Sumatra and Sulawesi, Sumbawa and Irian Jaya, Indonesia.

In Malaysia, commercial and trial plantings of *N. cadamba* have been established in Sarawak, Sabah, and by the Forest Research Institute of Malaysia (FRIM) (Mojiol et al., 2014). In Sabah, agroforestry trials of inter-cropping oil palm and *N. cadamba* were highly recommended (Mojiol et al., 2014). Meanwhile, in Sarawak, this woody plant is one of the selected plantation tree species either for industrial tree plantation or agroforestry due to its fast-growing characteristics and its silvicultural appearances. The unique features and multiple uses of *N. cadamba* ranging from wood carvings to light construction materials and furniture have become one of the ideal investment choices for timber plantations or

community forestry in Sarawak. *Neolamarckia cadamba* planted in Sarawak has been reported to have a low survival rate, and its growth performance is not as expected.

Neolamarckia cadamba can grow on thick, damp, alluvial sites, generally in the secondary forests along the riverbanks and in the translational zone between moist areas. *Neolamarckia cadamba* is usually growing at below 1000 m altitude within its natural distribution with more than 1,500 mm rain per year (Krisnawati et al., 2011). However, *N. cadamba* also can grow in dry areas with approximately 200 mm rain per year (Jøker, 2000). *Neolamarckia cadamba* tree can grow up to 40 m to 45 m high, and the trunk has a diameter of 100 cm to 160 cm (Krisnawati et al., 2011). The timbers are white with the rough surface due to the presence of large vessels. It is used for light-weight purposes such as veneer, pulp, paper, general utility furniture and plywood (Gunasekharan & Divyakant, 2006). This species is one of the woody plants with a broad conical crown and straight cylindrical bole. *Neolamarckia cadamba* branches arranged in tiers (Figure 1.1 A). For the young trees, the bark is grey, smooth and very light, but trees become rough and longitudinally fissured once developed into old trees (Figure 1.1 B). The leaves utilised as cattle fodder, serviettes, and leaf extract used as a mouth gargle. The dried bark can be applied in the medicinal field as *N. cadamba* can relieve fever and consume as a tonic (Orwa et al., 2009). Also, the root bark can be processed to produce a yellow dye that used as tannin or dyestuff (Patel & Kumar, 2008).

Neolamarckia cadamba has glossy green, opposite, simple sessile to the petiole, ovate to elliptical leaves (Figure 1.1 C). Figure 1.1 D shows the fruits of *N. cadamba* that have fleshy yellow-orange infructescence formed by many small and consists of about 8,000

trigon or irregularly shaped seeds. According to Krisnawati et al. (2011), *N. cadamba* distributed at Malaysia is a seasonal tree that only flowers from June to September and fruits from September to February. The seeds are considered as mature, and fruits can be harvested after the fruit has changed its colour to dark brown (Mojiol et al., 2014). The whole part of the tree can even serve as an ornamental plant and plantation tree (Krisnawati et al., 2011). Irawan and Purwanto (2014) reported that leaves, roots and barks of *N. cadamba* also have high pharmacological values. Besides, the tree also can give many benefits to the human for treating some of the diseases such as treatment of fever, anaemia, diabetes, uterine and liver complaints, menorrhagia, blood and skin diseases, diarrhoea, colitis, stomatitis, dysentery and semen quality improvement by using its leaf, bark and root (Zayed et al., 2014). Patel and Kumar (2008) stated that *N. cadamba* is a miracle tree that is frequently used by the villagers as a traditional medicine in India.



Figure 1.1: *Neolamarckia cadamba*: (A) Four-year-old *Neolamarckia cadamba* trees; (B) The stem bark of young *Neolamarckia cadamba*; (C) Glossy green leaves of *Neolamarckia cadamba*; and (D) Fruits of *Neolamarckia cadamba* (Krisnawati et al., 2011). Scale = 2 cm

According to Jøker (2000) and Mojiol et al. (2014), seed propagation of *N. cadamba* is limited due to the lack of viable seeds production, thus leading to low germination rate. The mature seeds of woody plants species exhibit some degree of dormancy (Baskin & Baskin, 1998). Hence, the seed does not germinate promptly, even when placed under the most favourable environmental conditions. The seeds of most tropical species of woody plants have little or remain dormant for years (Escobar & Torres, 2013). The seed that fails to germinate when exposed to favourable environmental conditions is known as primary dormancy. Meanwhile, seeds exposed to the unfavourable condition may subsequently develop a dormant state is known as a secondary dormancy. Furthermore, the secondary dormancy is induced by specific environmental conditions such as very high or low temperature, burial in soil, anaerobic conditions, or soaking in water (Kozłowski & Pallardy, 1997).

Due to the high demand of *N. cadamba* seedlings, shortage of quality *N. cadamba* seeds, and low *in situ* germination percentage have disheartened local forestry industries from planting *N. cadamba* (Mojiol et al., 2014). In Indonesia, the distribution of *N. cadamba* is limited to areas like Sulawesi, Nusa and Papua (Halawane et al., 2011). Seeds are not suitable for long-term storage due to the nature of the seeds, which decrease the viability of seeds drastically over the storage period. Unfortunately, the *N. cadamba* seeds can lose moisture content and reduce physiological quality during the storage period (Krisnawati et al., 2011). However, the dried seeds of *N. cadamba* stored in airtight containers at moist room will keep viability for about one year (Jøker, 2000). Report by Jøker (2000), stated that the germination rate of *N. cadamba* fresh seeds is generally low at about 25%. However, the seeds of *N. cadamba* potentially germinated at a higher

germination rate (up to 95%) if stored in airtight boxes at 4 °C for 2.5 months (Krisnawati et al., 2011).

According to Escobar and Torres (2013), when seeds of *Alibertia patinoi* (Rubiaceae) were stored at low temperatures (5 °C) for one month, and seed moisture content was high (40.3%), viability was reduced, and 18% of the seeds died during the storage period. Besides, da Rosa et al. (2011) stated that coffee seeds can germinate when they are dried to 12% moisture content, an intermediate moisture level among the levels tolerated by recalcitrant and orthodox seeds.

Neolamarckia cadamba seedlings are susceptible to pests and horticultural disease like damping off disease during or after the germination stage. Damping-off disease is caused by the infestation of soil-borne fungi like *Fusarium* spp., *Rhizoctonia* spp and *Pythium* spp on the roots of the seedling, causing white spots to appear (Islam et al., 2005). Halawane et al. (2011) stated that the damping-off disease was responsible for the first losses of *N. cadamba* seedlings in Indonesian plantations. Besides, Halawane et al. (2011) stated that vegetative propagation of *N. cadamba* by traditional methods is not successful due to the high sensitivity of nodal segments against mechanical injury, poor rooting, readily susceptible to pests in the early growth stage especially nematodes and disease known as 'sudden death of cadamba'. An advanced biotechnology approach via *in vitro* seed germination and micropropagation techniques required for the production of quality *N. cadamba* planting material since the natural distribution of this valuable tree species has become very limited.

Plant tissue culture approach via *in vitro* germination of *N. cadamba* seeds can be applied to provide a controlled environment favouring the germination process of *N. cadamba* seeds by breaking the seed dormancy (Bairu et al., 2009; Baskin & Baskin, 2014). *In vitro* seed germination is one of the well-known tissue culture techniques that can enhance the rate of seed germination, shorten the duration of seedling production and reduce the loss of seedlings due to diseases. *In vitro* seed germination and micropropagation protocol can enhance the timber yield of *N. cadamba* in a short period of time. According to Irawan and Purwanto (2014), the seeds of *N. cadamba* will be germinating about 7–15 days after sowing and would grow into seedlings after 30 days via the conventional method. By using *in vitro* seed germination, the duration of seed germination may decrease, and this will help in the production of high-quality *N. cadamba* seedlings as a genetically and physiologically uniform, developmentally normal and pathogen-free planting material.

The technique of plant tissue culture also can be used for growing isolated plant cells, tissues, and organs under axenic conditions (*in vitro*) to regenerate and propagate entire plants. It is commonly used to describe all types of plant cultures, namely callus, protoplast, cell, anther, meristem, embryo and organ cultures. Micropropagation of woody plants constitutes a noteworthy achievement in the commercial use of *in vitro* cultures which can produce many plantlets from a single individual explant (Mishra et al., 2015; Abrahamsson et al., 2017).

Report by Bairu et al. (2011) and Trajković et al. (2019) stated that plant tissue culture involves cultivating plants outside the natural growth environment and requirements by

optimising various kind of physical, chemical and environmental factors for growth. However, the application of this alternative technique still restrained by some developmental and physiological problems although improved and meticulous efforts have been made in plant tissue culture technique (Ali et al., 2017). Several techniques for *in vitro* plant propagation had been developed, including induction of axillary and adventitious shoots, the culture of isolated meristems and plant regeneration by organogenesis and somatic embryogenesis.

Besides that, another alternative way in tissue culture that usually used is shoot regeneration. According to Agrawal and Sardar (2007), shooting response is affected by the concentration of cytokinin enriched in the culture medium. Cytokinin function as signalling molecules that stimulate totipotent cells of callus for shoot organogenesis whereas, in the present study of direct organogenesis, these molecules activate pre-existing machinery in the case of somatic cells (from cotyledon-derived callus). Many reports on organogenesis involved the *in vitro* formation and development of organs such as shoots, leaflet and roots either directly from explants or indirectly via callus differentiation through wounded parenchyma cells such as findings in *Ocimum sanctum* (Anwar & Siddiqui, 2000), *Santalum album L.* (Singh et al., 2016) and *Arachis glabrata* (Dolce et al., 2018).

The essential factors for rapid *in vitro* germination and micropropagation are light intensity, temperature, source of explant, type of culture media, plant growth regulators and organic compounds (Huang et al., 2014). Photoperiod plays a central role in angiosperm seed to germinate. Oh et al. (2006) stated that angiosperm seeds contain light-sensitive pigments known as phytochromes which regulate the production of

gibberellin (GA) through phytochrome-interacting proteins. When phytochrome-interacting proteins receive red and far-red light signals, these proteins would be disintegrated by proteasome enzymes which in turn triggers the production of GA while inhibiting the degradation of GA (Oh et al., 2014).

Besides light, the temperature affects the germination percentage of endosperm seeds as it is related to the seasonal temperature changes which generally affect the dormancy state of the seeds. According to Welman and Sim (2010), the seeds of *N. cadamba* were well-germinated on culture media by soaking the seeds at 35 °C in a water bath for 24 hours before the surface sterilisation. High-salt basal media like MS and B5 medium are good starting basal media for callus culture of dicot explant tissue as both media contain a high amount of nitrate, potassium and ammonium ions as compared to other basal media. These ions are essential for the regeneration and growth of explant (Chiruvella et al., 2014). Report on Mishra et al. (2013) stated that half strength MS medium improved *in vitro* seed germination of *Pterocarpus marsupium*. They suggested that the half-strength MS medium has less osmotic like sugar and salt which affect the imbibition of the seeds.

Complex organic additives are added into the basal medium as a supplement to substitute the use of plant growth regulators like gibberellin (GA), α -Naphthalene acetic acid (NAA) and kinetin (Kn) for *in vitro* germination and seedling growth of *N. cadamba*. The effects of different basal media and complex additives on *in vitro* seed germination and seedling growth were tested primarily on various woody plants. The biochemical components of organic additive like coconut water (*Cocos nucifera* L.) contain many

cationic minerals (potassium, calcium, phosphorus, sodium, iron and copper), amino acids and vitamins like ascorbic acid and B group vitamins (Gnasekaran et al., 2012). All these additions provided by complex additives are essential in stimulating both the growth and development of plant cells.

Other organic additives such as tomato extract and papaya are creamy with the high amount of natural sugar, high amount of sterols, organic acids and phenolic compounds which are potential growth inhibitors for the plant (Markovic et al., 2006). Even though most organic additives contain beneficial biochemical compounds; they may also include plant growth inhibitors like phenolic compounds. These compounds would accumulate within the culture, causing browning of explant tissue and slowing down the growth of the culture which eventually lead to necrosis of culture (Reigosa et al., 1999).

Another factor that can affect *in vitro* germination and micropropagation is the selection of plant growth regulators in media. Plant growth regulators are synthetic compounds that had established which correspond to the natural organic compounds that synthesised in higher plants, which influence the growth and development of the plants. According to Chawla (2002), there are two main classes of synthetic hormones which are of particular importance in plant tissue culture. These are the auxins and cytokinins, while other regulators for example gibberellins, abscisic acids, and ethylene are of minor importance.

Auxins are commonly used in micropropagation and are assimilated into nutrient media to stimulate the callus proliferation, cell suspensions or organs such as meristem,

shoot or root tips and to control morphogenesis, mainly in aggregation with cytokinins (Anwaar et al., 2002; Kumar et al., 2016). The optimal concentration required depends on the kind of plant growth or development needed; the natural levels of auxins within the explant when it excised, the capacity of the cultured tissues to synthesise auxins naturally and the interaction, if any, between applied synthetics auxins and the natural endogenous substances. The most commonly used auxins are 2, 4-dichlorophenoxyacetic acid (2, 4-D), 1-naphthyl acetic acid (NAA), 3-indoleacetic acid (IAA) and 3-indolebutyric acid (IBA).

Cytokinins such as kinetin (Kn), thidiazuron (TDZ) and BAP (6-benzylaminopurine) are capable of stimulating cell division and inducing shoot formation in tissue culture. Besides, cytokinins frequently act as antagonists to auxins. A high auxin to cytokinin ratio usually favours root formation, while high cytokinins to auxins ratio favours shoot formation, whereas an intermediate ratio favours callus formation.

1.2 Problem statements

Owing to the fast-growing characteristic of *N. cadamba*, the demand for *N. cadamba* seedlings for agroforestry and planted forest development is increasing over the years. However, the availability of quality *N. cadamba* seeds and seedlings for planted forest development is far from enough. Besides, *N. cadamba* seeds have low *in situ* germination percentage discouraged local forestry industries from planting *N. cadamba*. Meanwhile, *N. cadamba* seeds are not suitable for long-term storage due to the nature of seeds which reduces the viability of seeds drastically over the storage time. Furthermore, seed germination percentage and seedling growth of *N. cadamba* are reported to be low by using traditional propagation methods (Krisnawati et al., 2011).

To the best of knowledge, only a limited number of published reports found on the study of *in vitro* seed germination and propagation of *N. cadamba* to date. An advanced biotechnology approach through *in vitro* seed germination and micropropagation techniques is needed to overcome this problem. The development of protocols for *in vitro* seed germination, seedling growth, callus induction, indirect and direct shoot regeneration of *N. cadamba* could pave the way to provide a dependable and fast resource of healthy *N. cadamba* plantlets, where the plantlets are genetically identical, physiologically uniform, developmentally healthy and pathogen-free plantlets, for planted forest development in Sarawak, and in turn, assures the local timber industries to meet the global demand of wood-related products.

1.3 Objective of the Study

The objectives of the present study are:

- a) To identify a suitable medium, organic additives, auxins, cytokinins and culture condition for *in vitro* seed germination and seedling growth of *N. cadamba*;
- b) To determine the best concentration of auxins, cytokinins, combination of hormone, photoperiod and sources of explant derived from young leaf, stem and nodal explants on callus induction;
- c) To determine the suitable concentration of auxins, cytokinins with singly or combination and coconut water on indirect shoot regeneration from young leaf explants; and
- d) To determine the suitable concentration of BAP and NAA at singly or combination, and the effect of BAP and coconut water on direct shoot regeneration from nodal explants.

CHAPTER 2

IN VITRO* SEED GERMINATION AND SEEDLING GROWTH DEVELOPMENT OF *NEOLAMARCKIA CADAMBA

2.1 Introduction

In vitro seed germination and development defined as the process of a new plant developed from the seeds (Aileni et al., 2014). This technique has been applied widely in the process of propagation woody plants such as *Vitellaria* (Iroko et al., 2013), *Pterocarpus santalinus* (Mishra et al., 2013), *Eucalyptus dunii* (Oberschelp, 2015), and *Ceropegia barnesii* (Ananthan et al., 2018). It is an alternative method to propagate plants that are required in high demand and having a problem in seeds viability through conventional plant breeding method. Due to the seedling production of woody species are unsatisfactory mainly forest trees, *in vitro* technique is a practical approach for the continuous supply of quality seedlings to forest plantation programme.

Plant tissue culture approach via *in vitro* seed germination technique of *N. cadamba* can be used to overcome the inability of *N. cadamba* seeds to germinate naturally. This technique can be applied to provide a controlled environment favouring the germination process of *N. cadamba* seeds by breaking the primary seed dormancy. Seed dormancy referred to as a state whereby an intact viable seed prevented from germinating even it is under ideal circumstances of temperature, moisture and oxygen level which are favourable to induce seed germination (Baskin & Baskin, 2004; Ananthan et al., 2018). Both primary and secondary dormancy can inhibit the seed germination and growth.

Several studies had been conducted on *in vitro* growth and development of woody plants (Aparna & Choudhary, 2017; Nyum et al., 2017; Trajković et al., 2019). However, there is still inadequate information regarding the *in vitro* seed germination and growth development of *N. cadamba* seedling to date. The related studies to develop *in vitro* propagation of *N. cadamba* were studied by Huang et al. (2014) on direct adventitious shoot organogenesis and plant regeneration from cotyledon explants, high-frequency plant regeneration from *N. cadamba* leaf culture by Li et al. (2019), and direct organogenesis to produce shoots and roots of *N. cadamba* by Rahman et al. (2015a). Hence, more research is needed to resolve this problem. The results of these studies could pave the way to develop an effective method of *in vitro* propagation of *N. cadamba*.

The *in vitro* seed germination and seedling growth development are influenced by the source of explants, the choice of basal media, type of complex additives, carbon sources (sugar), plant growth regulators and environmental effects such as photoperiod, temperature and relative humidity. According to Arditti (2010) and Atondo-bueno et al. (2016), basal medium plays a significant role in determining the success or failure of plant tissue culture. The basal medium provides water, nutrients, vitamins, and growth regulators for the plant as well as a place for gases exchange and removal of metabolite wastes. A widely known basal medium such as Murashige and Skoog (MS) (Murashige & Skoog, 1962), Vacin and Went, 1949 (VW) and Gamborg's (B5) (Gamborg, 1968) are always be used to start the preparation of most appropriate medium for any new tissue culture system (Arditti, 2010; Gour & Kant, 2011; Bakhshaie et al., 2016).

A suitable organic additives can contribute to the establishment of an efficient and economical *in vitro* propagation protocol of *N. cadamba*. The varieties of organic additives can promote germination and seedling growth to any plant species. Specific growth factors found in organic additives that contain sugar and natural mineral such as acid amino, phosphorus and nitrogen sources. This study was aimed to investigate the effects of different basal media and complex additives on *in vitro* seed germination and seedling growth of *Neolamarckia cadamba*.

2.2 Materials and Methods

2.2.1 Plant materials

Seeds of *Neolamarckia cadamba* used in *in vitro* seed germination study were obtained from the Sarawak Forestry Corporation (SFC), Sarawak, Malaysia (Figure 2.1 A & B). The seeds were kept in a falcon tube and stored in the fridge at 4–8 °C. The three weeks old seedlings with size 2.0 mm length of leaves and 3.0–4.0 mm root length (Figure 2.1 C) were used for the *in vitro* seedling growth and development study.

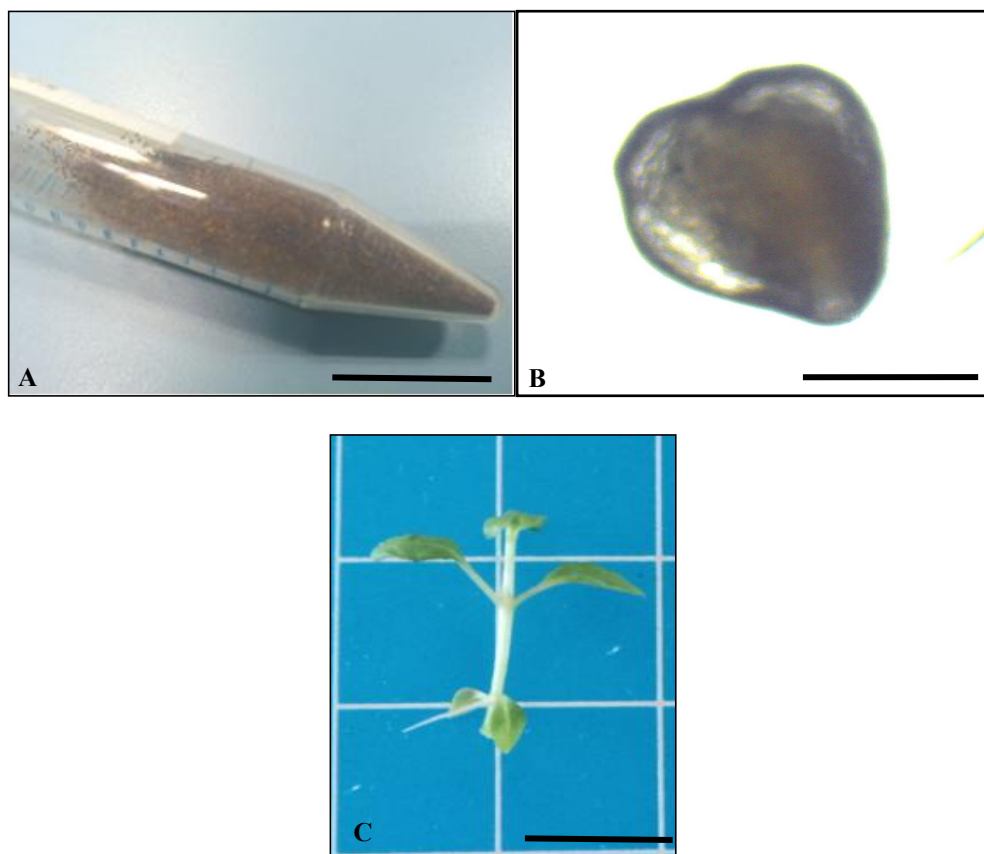


Figure 2.1: (A) Seeds of *Neolamarckia cadamba*; (B) A seed of *Neolamarckia cadamba* under microscope image with magnification 400x; and (C) Three weeks old *in vitro* seedlings of *Neolamarckia cadamba*. Scale A and C = 1.0 cm; and Scale B = 50 mm.

2.2.2 Pre-treatment and surface sterilisation of seeds

The *Neolamarckia cadamba* seeds were immersed in 42 °C (Welman & Sim, 2010) of sterile distilled water for 30 minutes (Figure 2.2 A). Then, the water was discarded, and seeds were air-dried for 2 hours in room temperature at 25 ± 2 °C. The pre-treated seeds were immersed in a 50% bleach solution containing one to two drops of Tween 20 for 10 minutes and agitated gently. The seeds were rinsed three times with sterile distilled water, followed by deep immersed in 70% ethanol for 30 seconds (Figure 2.2 B).

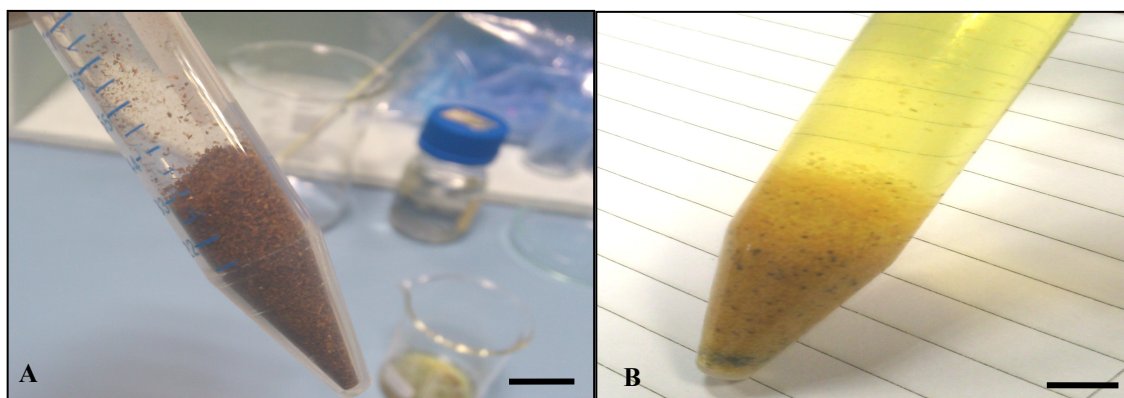


Figure 2.2: (A) Pre-treatment and (B) Surface sterilisation of *Neolamarckia cadamba* seeds. Scale A and B = 0.5 cm.

2.2.3 Media preparation and culture condition

The Gamborg (B5) and the Murashige and Skoog (MS) media were prepared in accordance with the media formulation as shown in Appendix A. Then, the addition of 8.5 gL⁻¹ of Phyto agar (Duchefa, Netherlands) was added in Gamborg (B5) and Murashige and Skoog (MS) media. Then, agar was heated by using microwave (Panasonic) in 2 minutes or until the agar powder adequately melted. Then media Gamborg (B5) and the Murashige and Skoog (MS) were stirred and adjusted to pH 5.5 ± 2 and 5.7 ± 2 , respectively, with 1N NaOH or HCl. The glass bottle culture containing with media was autoclaved at 121 °C and 15 psi for 20 minutes. For Petri dish, the autoclaved media were poured directly and sealed with parafilm to avoid desiccation.

Two separate tissue culture media were used in the present study: first, for *in vitro* seed germination and the second one for the seedling growth development of *Neolamarckia cadamba*. For *in vitro* seed germination culture, the surface-sterilised seeds were inoculated on the MS culture media, either alone or supplemented with complex additives. After three weeks, the seedlings with two leaves and one root were subjected to

standardisation in the B5 basal media for a week. Seedlings were inoculated on the culture media, either alone or supplemented with complex additives. All cultures were placed in a culture room with 70–80% relative humidity; light intensity of 20–50 $\mu\text{mol m}^{-2} \text{s}^{-1}$ provided by light-emitting diode (LED) with duration about at 16-h light/ 8-h dark photoperiod in room temperature at $25 \pm 2 \text{ }^{\circ}\text{C}$.

2.2.4 Effect of different types of basal media on *in vitro* seed germination and seedling growth development

The four basal media tested on *in vitro* seed germination study were the half-strength and the full-strength of Murashige and Skoog (MS) and Gamborg (B5) media. Meanwhile, three basal media were tested for *in vitro* seedling growth development, i.e. the half-strength MS, the half-strength and full-strength B5.

2.2.5 Effect of complex additives on *in vitro* seed germination and seedling growth development

Three complex additives, namely coconut water (CW) (v/v), banana homogenate (BH) (w/v) and potato homogenate (PH) (w/v) with different concentrations at 5%, 10% and 15% were used in this study. Coconut water was obtained from a young green coconut *var.* mawa (8 months) and filtered to remove the impurities. Meanwhile, the ripe banana fruit cultivar *berangan* obtained from the fruit store was used to prepare the banana homogenate. Also the preparation of potato homogenate was used from the fresh potato that bought at Giant supermarket, Kuching. The skin was peeled off and cut into fine pieces, then weighed and blended in a blender (Panasonic, Model, MX-GM1011) according to the concentration of treatment. B5 basal medium was selected as the basal media.

2.2.6 Effect of plant growth regulators on seedling growth development

The effects of plant growth regulators (PGRs) which are auxins and cytokinins were studied at different concentrations on seedling growth development of *N. cadamba*. Besides, the combination of auxins and cytokinins and coconut water on the seedling growth of *N. cadamba* was also investigated.

a. Auxins

The effect of auxins on *in vitro* seedling growth development of *N. cadamba* was studied. In this experiment, auxins such as α -naphthaleneacetic acid (NAA) and 2, 4-dichlorophenoxyacetic acid (2, 4-D) were tested at different concentrations (1.0, 2.0, 3.0, 4.0 and 5.0 mgL⁻¹). This experiment was conducted to see the effect of different concentrations of NAA for determining the optimum NAA concentration to combine with coconut water experiment (see Section 2.2.6 c). B5 media without auxins were used as basal and control media.

b. Cytokinins

The effect of cytokinins (benzylaminopurine (BAP) and thidiazuron (TDZ) was studied on the seedling growth development of *N. cadamba*. Both types of cytokinins were tested at concentrations of 1.0, 2.0, 3.0, 4.0 and 5.0 mgL⁻¹. Beside, this experiment investigated the influence of the various BAP levels in determining the appropriate concentration to be paired with an experiment with coconut water (see Section 2.2.6 c). B5 media without cytokinins were used as a control and basal media in this experiment.

c. Combination of BAP and NAA with coconut water

The optimal concentration of 1.0 mgL^{-1} was chosen for both auxins (NAA) and cytokinins (BAP) from the previous experiments (see Section 2.2.4). B5 media without plant growth regulators (PGRs) were used as a control and basal media. The selected PGRs were combined with coconut water at 10% (v/v) to effect of PGRs and organic additives in enhancing the *in vitro* growth of *N. cadamba* seedling.

2.2.7 Effect of photoperiod on seedling growth development

The effects of photoperiod 24-h light and 16-h light/ 8-h of dark were studied on the seedling growth development of *N. cadamba* (Cardoso, 2018). The culture media used was B5 supplemented with 2.0% (w/v) sucrose (HmvG) and coconut water at 10% (v/v). All cultures placed in a culture room according to the light condition treatment.

2.2.8 Effect of activated charcoal on seedling growth development

The effect of activated charcoal was studied on *in vitro* seedling growth development of *N. cadamba*. B5 media supplemented with BAP 1.0 mgL^{-1} and activated charcoals at 1.0 and 2.0 gL^{-1} , respectively were used in this study. B5 media without activated charcoal were used as a control media.

2.2.9 *Ex vitro* acclimatisation of *Neolamarckia cadamba* seedlings

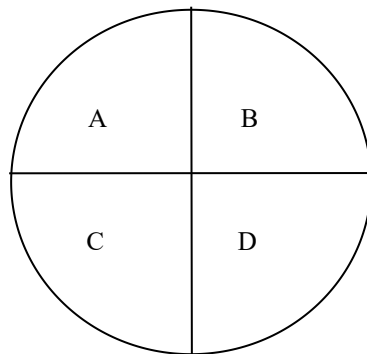
Firstly, the topsoil and organic compost soil were autoclaved at 121°C and 15 psi for 20 minutes. Then, two months old *in vitro* seedlings of *N. cadamba* were selected and removed from the culture bottles. After that, the healthy seedlings were rinsed with distilled water to clean agar that attached to the roots and leaves. Then, seedlings were

immersed in a solution of anti-fungal (Ancom Thiram 80, Malaysia) (0.2%, w/v) for 5 minutes and air-dried for 30 minutes at room temperature (25 ± 2 °C). Further, seedlings planted in pots according to the media treatments, i.e. soil, soil mixed with organic compost (ratio 1:1) and peat moss. The plants were covered with plastic bags at room temperature for one month before transferring to the greenhouse. The number of plants survived under the natural condition were recorded for data analysis.

2.3 Data collection and statistical analysis

In this study, all experiments were carried out in a Completely Randomised Design (CRD). The number of surface-sterilised seeds were distributed randomly and uniformly on the surface of four different basal media. There are five replicates per treatment in this experiment. Each Petri dish was divided into 4 section, which is section A, B, C and D. The number of seeds sown were calculated and recorded. Then, the percentage number of seeds germinated were calculated by using the formulation shown in Figure 2.3. The observation of seeds development and the number of seeds calculated under a light compound microscope (Leica) with magnifying 4/ 10/ 40/ 100 (WF10 x 18).

$$\text{Percentage number of seeds germinated} = \frac{\sum \text{number of seeds germinated}}{\sum \text{number of seeds sowing (A+B+C+D)}^*} \times 100\%$$



*is calculated only for seeds containing embryos

Figure 2.3: The schematic diagram for seeds calculation according to seeds sowing area (Kauth et al., 2008).

Effect of different types of complex additives at different concentrations such as coconut water (CW) at 5, 10, 15% (v/v); banana homogenate (BH) and potato homogenate (PH) at 5, 10 15% (w/v) were investigated on *in vitro* seed germination and seedling growth development of *N. cadamba*. Each treatment was carried out in five replicates. All cultures were observed after five days of culture. The data were collected and calculated at ten days interval up to 30 days on a percentage of seed germination. In seedling growth experiments, all data were collected and calculated at ten days interval up to 60 days after culture by evaluating the height of the plants and the number of leaves, shoots and roots. Percentage number of seedlings survived after transferring to the greenhouse also observed and recorded. All data were analysed either by using T-test sample or one and two ways Analysis of variance (ANOVA) through SPSS software version 22. Subsequently, the post hoc test, Duncan's Multiple Range Test (DMRT) was used to determine significance different among treatment means at $p < 0.05$.

2.4 Results

2.4.1 Developmental stage of *in vitro* seed germination and seedling growth

Neolamarckia cadamba seeds cultured on B5 media supplemented with coconut water (CW) at 10% (v/v) were selected to study the developmental stage of *in vitro* seeds (Figure 2.4). In this study, the embryo was observed after two days of culture and clarified as Stage 0 (Figure 2.4 A). The pre-germination stage identified as Stage 1, whereby embryo become swollen, greenish and begin to germinate after three days of culture (Figure 2.4 B). Stage 2 or germination stage occurred when radicle come out of the seed coat after five days of culture, as shown in Figure 2.4 C.

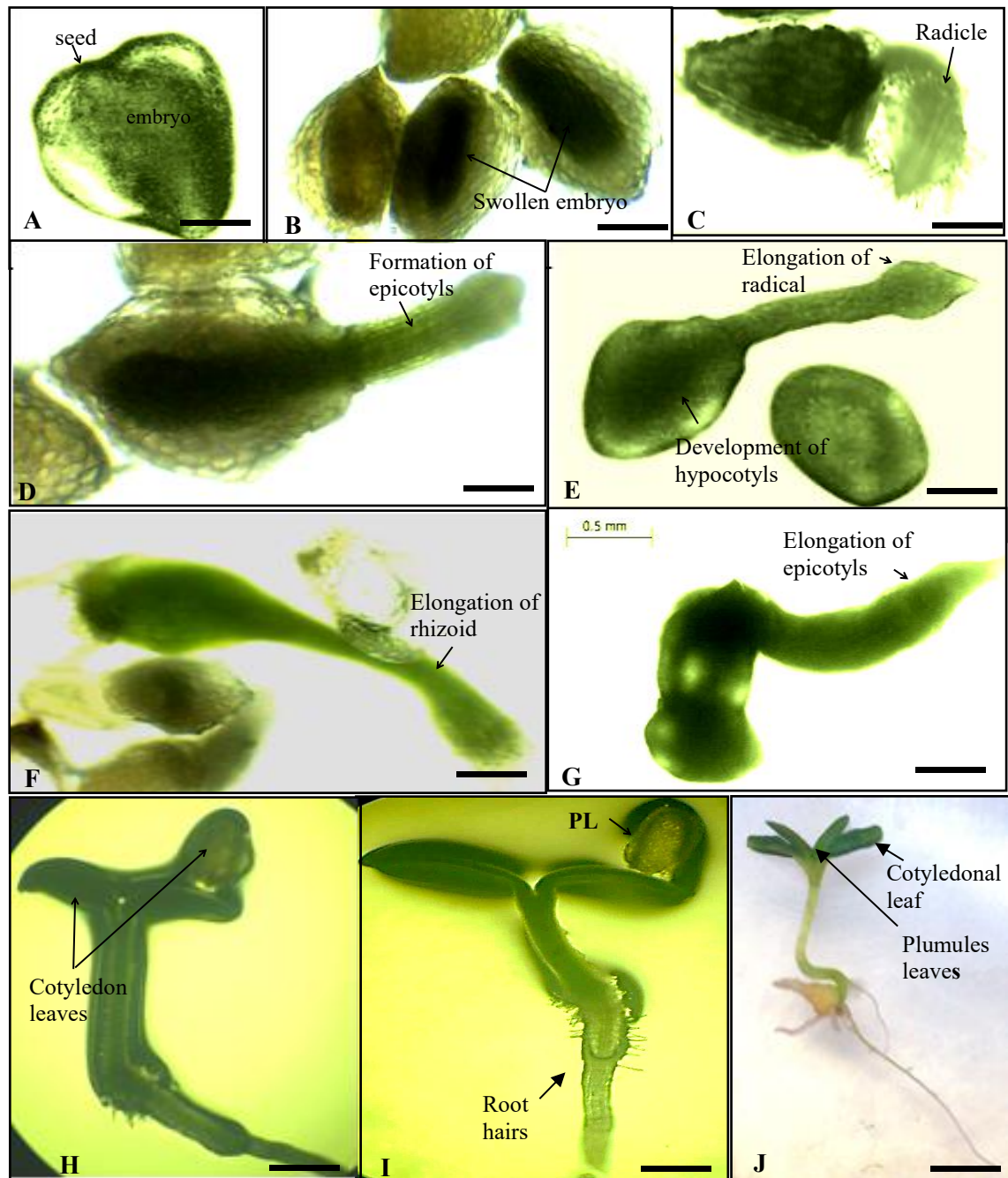


Figure 2.4: Seed development of *Neolamarckia cadamba*. (A) Stage 0: Embryo exist in seed coat; (B) Stage 1: embryo growth, swollen and started to look greens in seed coat; (C) Stage 2: radicle come out of the seed coat show (germination stage) after 5 days of culture; (D) Stage 3: elongation of radicle from the seed occurs; (E) Stage 4: hypocotyl grew longer, and greenish cotyledons pulled out from the seed coat; (F) radicle continue to grow and rhizoids developed; (G) elongation hypocotyl; (H) The cotyledons gradually open outwards with 1.0 mm length of epicotyl; (I) Stage 5: rhizoids and adventitious root buds developed after 3 days of seed germination; (I) stage 6: immature plumule-like-body (PL) appeared after 10 days of seed germination; (J) *N. cadamba* seedling was fully established after 4 weeks. Scale = 0.2 mm in A–B; 0.5 mm in C–F; 0.5 cm; Magnification power: 400× in A–B, 100× in C–F.

After 5–6 days of culture, elongation of radicle from the seed occurred and identified as Stage 3 (Figure 2.4 D). After radicle emerged, the embryo was observed to reach the embryonic leaves in two to three days, which is clarified as stage 4. During this stage, the hypocotyl was also seen to grow longer, and greenish cotyledons would be slowly pulled out from the seed coat (Figure 2.4 E). At the same time, radicles continue to grow, and rhizoids developed along with it (Figure 2.4 F) and elongation of hypocotyl happened at the same time, as shown in Figure 2.4 (G). The cotyledons gradually open outwards to form a pair of embryonic leaves which marked the completion of stage 4 (Figure 2.4 H).

Subsequently, elongation of epicotyl by pushing the pair of plumules upward and then formed the node of *N. cadamba* seedling. Then, plumules would develop to form the primary leaves of the seedling. When the length of epicotyl reached up to 1.0 mm, stage 4 completed. Three days after seed germination, rhizoids and adventitious root buds actively developed in stage 5 and root hair observed, which is classified as the root stage (Figure 2.4 I). The slow growth of roots were observed, which it took up to 5–7 days at this stage. On the 10th day after seed germination, immature plumule-like-body (PL) was observed, indicating the initiation of leaves has occurred and clarified as stage 6 (Figure 2.4 I). Finally, the *in vitro* seedling was fully established and developed into a complete seedling after four weeks of culture (Figure 2.4 J).

2.4.2 *In vitro* seed germination of *Neolamarckia cadamba*

a) Effect of basal media

Based on the result summarised in Table 2.1, the effect of basal media significantly affected seed germination of *Neolamarckia cadamba*. The highest percentage of seed germination was recorded in full-strength B5 medium (89.30%) followed by 48.83% in half-strength B5 and 15.89% in half-strength MS medium after 30 days of culture (Table 2.1). From observation, about 10% of seeds were starting to germinate in half and full-strength B5 medium after five days of culture (Figure 2.5 D & G). Meanwhile, in half and full-strength, MS media did not give a response to germinate after five days of culture. Its start to grow after ten days of culture which was recorded 9% in half-strength MS medium, but no germinated seeds recorded in full-strength MS medium after ten days of culture (Table 2.1 & Figure 2.5 J).

The full-strength B5 medium recorded the highest percentage of seeds germinated (89.3%) after 30 days of culture. Meanwhile, the lowest percentage was half and full-strength MS media that gave 9.89% and 0%, respectively after 20 days of culture (Table 2.1 & Figure 2.5 B & K). The lowest number of seeds germinated (15%) was from half-strength MS medium after 30 days of culture (Table 2.1 & Figure 2.5 C). However, full-strength MS medium was not given any positive respond after 30 days of culture (Table 2.1 & Figure 2.5 L). From the study, the suitable medium that can use to enhance *in vitro* seed germination of *Neolamarckia cadamba* was the full-strength B5 medium.

Table 2.1: The effects of basal media on *in vitro* seed germination of *Neolamarckia cadamba* after 30 days of culture.

Basal media	Seed germination (means $\pm$ SD) %			
	5 DAC	10 DAC	20 DAC	30 DAC
$\frac{1}{2}$ MS	0.0 ^a	9.35 $\pm$ 3.53 ^b	9.89 $\pm$ 3.81 ^b	15.89 $\pm$ 3.43 ^b
MS	0.0 ^a	0.0 ^a	0.0 ^a	0.0 ^a
$\frac{1}{2}$ B5	10.98 $\pm$ 3.29 ^b	39.53 $\pm$ 7.30 ^c	43.13 $\pm$ 4.21 ^c	48.83 $\pm$ 5.45 ^c
B5	10.54 $\pm$ 2.01 ^b	39.72 $\pm$ 6.49 ^c	58.39 $\pm$ 17.30 ^d	89.30 $\pm$ 3.33 ^d

Data analysis was performed from 5 replicates by using one-way ANOVA Means within a column with the same letter are not significantly different based on Duncan's Multiple Range Test at $p < 0.05$.

MS = Murashige and Skoog; B5 = Gamborg's B5

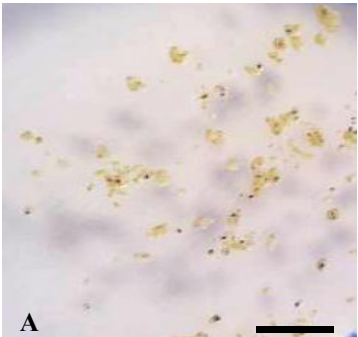
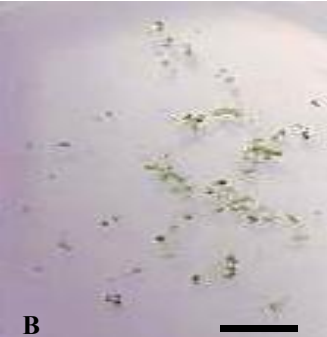
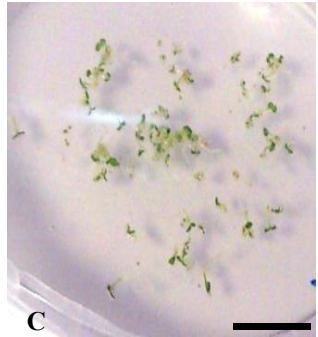
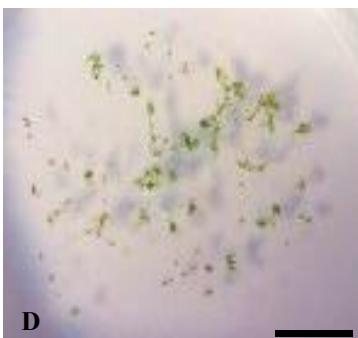
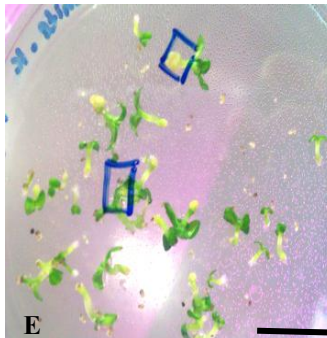
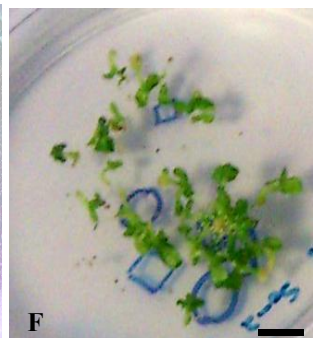
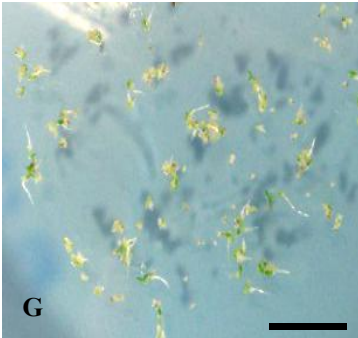
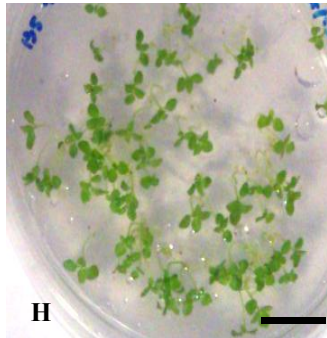
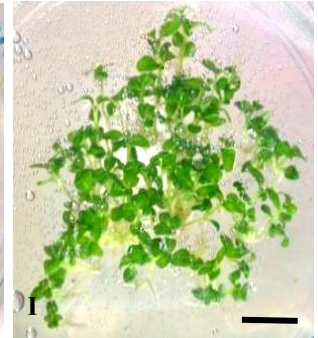
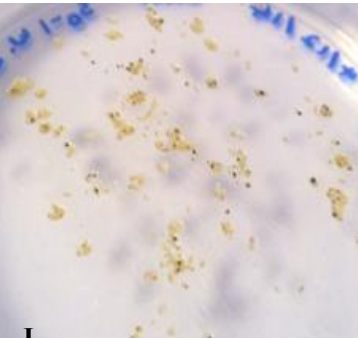
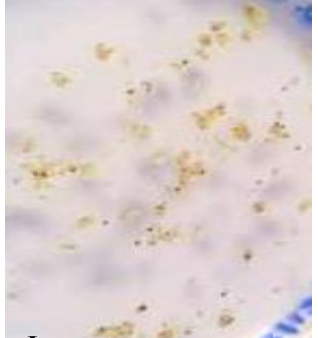
Types of basal media	Days After Culture (DAC)		
	5 DAC	20DAC	30DAC
$\frac{1}{2}$ MS			
$\frac{1}{2}$ B5			
B5			
MS			

Figure 2.5: The effect of different types of basal media on *in vitro* seed germination of *Neolamarckia cadamba*. Scale A–L = 0.2 cm.

b) Effect of complex additives on *in vitro* seed germination

The effect of complex additives on *in vitro* seed germination of *N. cadamba* was significantly different at $p < 0.05$ from 2-way ANOVA (as shown in Table 2.2). From observation, when coconut water, banana, and potato homogenate added into B5 media, the seeds were started to germinate on 5 DAC. Coconut water (CW) at 15% (v/v) recorded the highest seed germination percentage ($44.42 \pm 2.35\%$) after 5-day culture (Table 2.2).

After ten days of culture, media supplemented with CW 15% (v/v) gave the highest percentage of seeds germinated (73%), and then increased to 95.78% at 30 days after culture (Table 2.2 & Appendix B). The media that contained coconut water at different concentrations gave a significant effect to *in vitro* seed germination of *N. cadamba*, where the number of seeds germinated increased over the culture period.

The second highest was potato homogenate at 15% (v/v) that recorded about 38.04% starting from five days after culture. The germination percentage increased to 54.97% at 10 DAC, 69.19% at 20 DAC and 80.74% after 30 days of culture (Table 2.2). Likewise, the number of seeds germinated in potato homogenate was increased over the culture period as shown in Appendix B and Figure 2.6 (D, E & F).

Subsequently, the addition of coconut water by 15% (v/v) significantly increased the number of seeds germinated after 30 days of culture (Table 2.2). Overall, the addition of coconut water, potato and banana homogenate can enhance the number of seeds germinated. However, the selection for the best complex additive and the optimal

concentration was coconut water at 15% (v/v) which gave the highest percentage of *in vitro* seed germination of *N. cadamba* (Figure 2.6 A–J).

Table 2.2: Effect of complex additives on *in vitro* seed germination of *Neolamarckia cadamba* after 30 days of culture.

Treatments	Seed germination (means $\pm$ SD) %			
	5 DAC	10 DAC	20 DAC	30 DAC
Control	7.81 $\pm$ 1.36 ^a	42.59 $\pm$ 3.86 ^{bc}	53.46 $\pm$ 9.12 ^c	64.02 $\pm$ 8.05 ^c
CW 5%	20.02 $\pm$ 3.37 ^b	41.28 $\pm$ 5.13 ^b	46.56 $\pm$ 5.57 ^b	57.43 $\pm$ 7.39 ^{bc}
10%	30.40 $\pm$ 8.79 ^{cd}	59.14 $\pm$ 3.67 ^e	70.72 $\pm$ 2.66 ^e	79.07 $\pm$ 2.84 ^{de}
15%	44.42 $\pm$ 2.35 ^f	73.05 $\pm$ 6.91 ^f	81.91 $\pm$ 3.58 ^f	95.78 $\pm$ 1.97 ^f
PH 5%	25.67 $\pm$ 4.83 ^{bc}	33.22 $\pm$ 4.95 ^a	43.20 $\pm$ 2.91 ^b	52.15 $\pm$ 3.13 ^b
10%	31.24 $\pm$ 5.86 ^{cd}	43.87 $\pm$ 6.89 ^c	55.55 $\pm$ 6.97 ^c	72.99 $\pm$ 4.90 ^d
15%	38.04 $\pm$ 4.40 ^e	54.97 $\pm$ 2.02 ^{de}	69.19 $\pm$ 4.42 ^e	80.74 $\pm$ 6.01 ^e
BH 5%	30.15 $\pm$ 3.04 ^{cd}	36.26 $\pm$ 3.21 ^{ab}	38.89 $\pm$ 3.40 ^a	45.42 $\pm$ 5.50 ^a
10%	32.99 $\pm$ 4.94 ^d	43.61 $\pm$ 2.92 ^c	44.03 $\pm$ 8.76 ^b	58.16 $\pm$ 5.31 ^{bc}
15%	29.35 $\pm$ 2.77 ^c	51.20 $\pm$ 7.59 ^d	60.05 $\pm$ 5.90 ^d	66.98 $\pm$ 4.49 ^c

Data analysis was performed from 5 replicates by using two-way ANOVA. Means within a column with the same letter are not significantly different based on Duncan's Multiple Range Test at $p < 0.05$.

CW = Coconut water; BH = Banana homogenate; and PH = Potatoes Homogenate.

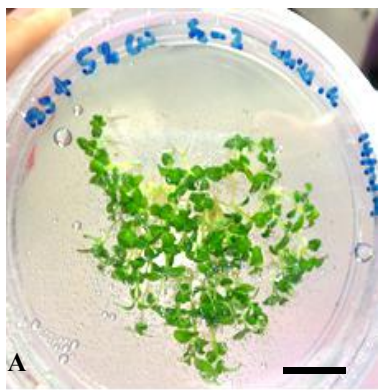
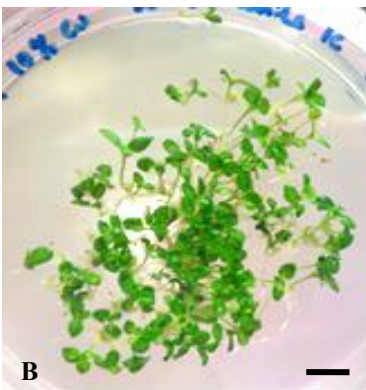
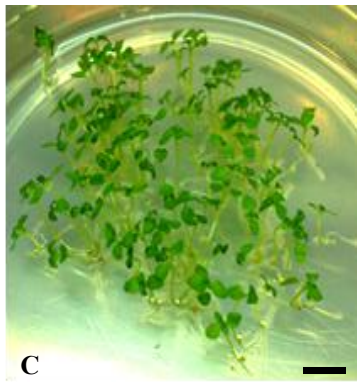
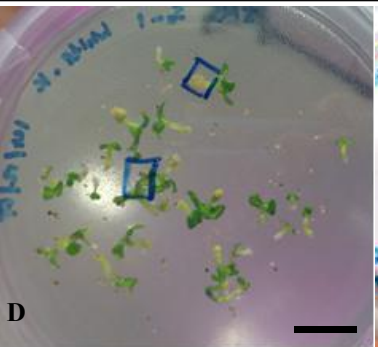
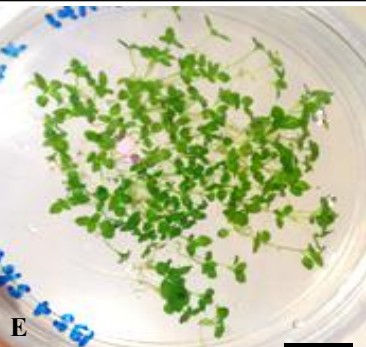
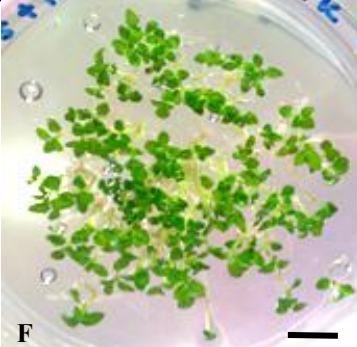
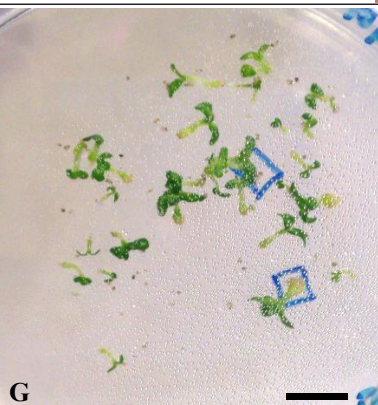
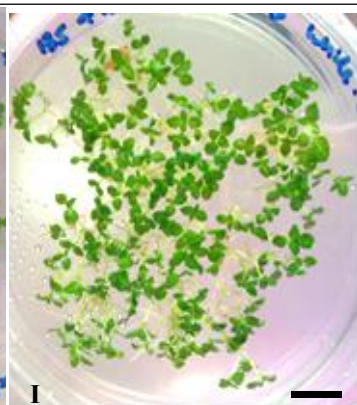
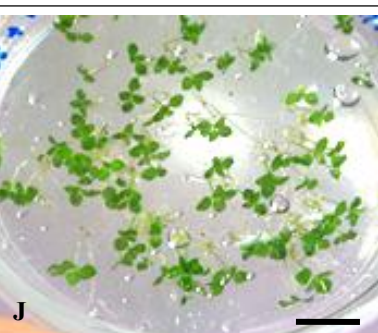
Type of complex additives	Concentration (w/v v/v)		
	5 %	10%	15%
CW			
PH			
BH			
C			

Figure 2.6: Effect of complex additives at different concentrations on *in vitro* seed germination of *Neolamarckia cadamba* after 30 days culture. (Scale: A–J = 0.5 cm); CW = coconut water; PH = potato homogenate; BH = Banana homogenate; C = control.

2.4.3 Effect of basal media on seedling growth and development

The result revealed a significant difference in the number of leaves, roots, shoots and plant height between three basal media used in the present study (Table 2.3 & Appendix C). The highest mean number of roots and shoots observed from B5 medium, which were recorded at 26.60 and 7.20, respectively after 60 days of observation. The highest mean of leaf number (46.60 ± 2.19) recorded on the B5 medium, which was higher than $\frac{1}{2}$ B5 (28.80 ± 3.12) and half strength of MS media (19.80 ± 3.11).

The lowest mean number of shoots (4.00 ± 0.00) was observed on half-strength MS and the second-lowest was half-strength B5 medium (5.20 ± 1.79). After 60 days of culture, B5 medium recorded the highest mean in plant height (7.90 ± 1.60 cm) among the treatments, which was slightly higher than half-strength B5 media (4.34 ± 0.49 cm) and half-strength MS media (2.60 ± 0.55) (Table 2.3 and Figure 2.7). Hence, the best type of basal media can help to increase the *in vitro* seedling growth of *N. cadamba* was full strength B5 media.

Table 2.3: Effect of basal media on seedling growth development of *Neolamarckia cadamba* after 60 days after culture.

Basal media	Means $\pm$ SD			
	Number of Leaves	Number of Roots	Number of Shoots	Plant Height (cm)
B5	46.60 ± 2.19^c	26.60 ± 1.67^c	7.20 ± 0.83^b	7.90 ± 1.60^c
$\frac{1}{2}$ B5	28.80 ± 3.12^b	11.60 ± 2.96^b	5.20 ± 1.79^a	4.34 ± 0.49^b
$\frac{1}{2}$ MS	19.80 ± 3.11^a	6.20 ± 0.84^a	4.00 ± 0.00^a	2.60 ± 0.50^a

Data analysis was obtained from 5 replicates by using one-way ANOVA. Means within a column with the same letter are not significantly different based on Duncan's Multiple Range Test at $p < 0.05$.

MS = Murashige and Skoog; B5 = Gamborg's medium; SD = Standard deviation;

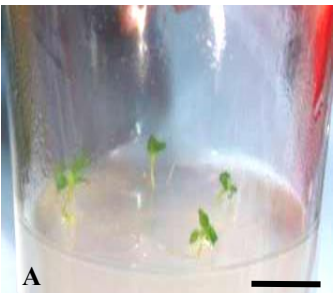
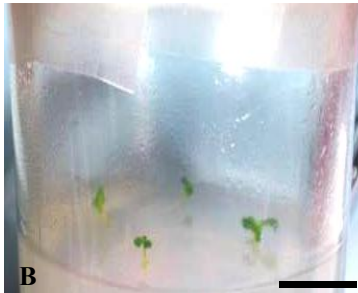
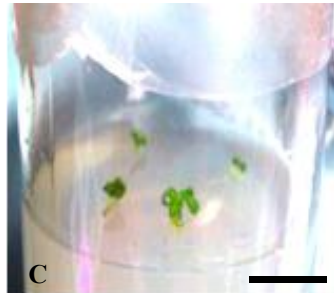
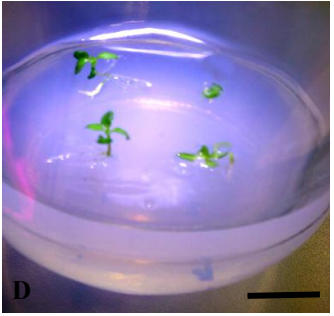
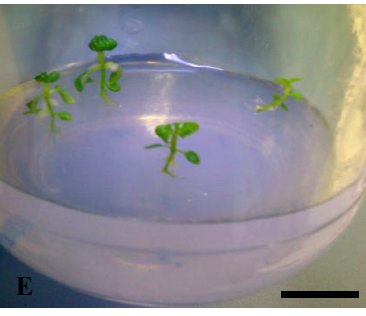
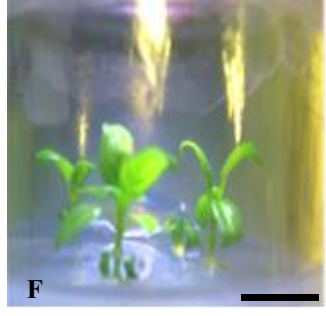
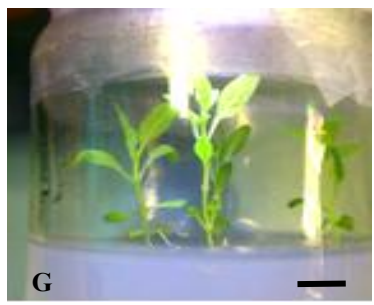
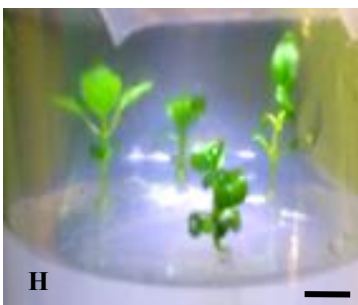
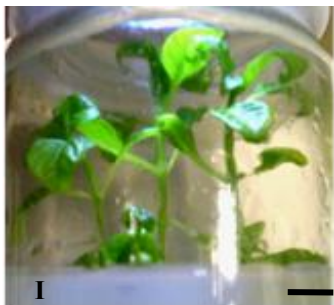
DAC	Basal media		
	$\frac{1}{2}$ MS	$\frac{1}{2}$ B5	B5
0			
10			
60			

Figure 2.7: Effect of basal media on the seedling growth development of *Neolamarckia cadamba* according to days after culture (DAC). Scale A–C = 0.5 cm; D–I = 0.5 cm and J–L = 1.0 cm.

2.4.4 Effect of complex additives on seedling growth and development

In this experiment, there were significantly different ($p < 0.05$) between three type complex additives. Among all the treatments, B5 medium supplemented with 10% coconut water (CW) recorded the highest mean number of leaves (60.6), roots (45.2), shoots (14.2) and plant height (8.18 cm) after 60 days of culture (Table 2.4, Figure 2.8 & Appendix D). However, the lowest mean number of shoots was found on control media (without complexes additive) and the second lowest was media added with banana homogenate or coconut water at 5% (v/v) which recorded 7.60 ± 0.89 and 7.20 ± 0.84 , respectively after 60 days culture.

Table 2.4: Effect of different concentrations of complex additives on seedling growth and development of *Neolamarckia cadamba* at 60 days after culture (DAC).

Complex Additives	Conc. (%)	means $\pm$ SD			
		Number of Leaves	Number of Roots	Number of Shoots	Plant Height (cm)
Control		48.20 $\pm$ 2.28 ^b	34.80 $\pm$ 2.17 ^c	6.60 $\pm$ 0.55 ^a	6.50 $\pm$ 0.46 ^c
CW (v/v)	5	50.20 $\pm$ 3.49 ^{bc}	36.00 $\pm$ 1.58 ^{cd}	7.20 $\pm$ 0.84 ^a	7.02 $\pm$ 0.13 ^{cd}
	10	60.60 $\pm$ 5.41 ^d	45.20 $\pm$ 3.01 ^e	14.20 $\pm$ 1.92 ^d	8.18 $\pm$ 0.64 ^d
	15	55.60 $\pm$ 2.70 ^c	44.80 $\pm$ 3.03 ^{de}	13.20 $\pm$ 1.48 ^d	6.50 $\pm$ 0.41 ^c
BH (w/v)	5	49.20 $\pm$ 2.16 ^b	38.40 $\pm$ 4.39 ^d	7.60 $\pm$ 0.89 ^a	4.16 $\pm$ 0.28 ^a
	10	51.60 $\pm$ 3.44 ^{bc}	50.60 $\pm$ 1.14 ^f	8.40 $\pm$ 0.84 ^{ab}	5.02 $\pm$ 0.34 ^b
	15	55.40 $\pm$ 1.14 ^c	54.80 $\pm$ 1.30 ^e	9.60 $\pm$ 0.90 ^b	6.04 $\pm$ 0.36 ^c
PH (w/v)	5	51.80 $\pm$ 2.59 ^{bc}	14.80 $\pm$ 1.92 ^a	8.80 $\pm$ 1.49 ^{ab}	5.06 $\pm$ 0.24 ^b
	10	57.60 $\pm$ 2.07 ^{cd}	24.60 $\pm$ 2.71 ^b	9.60 $\pm$ 0.89 ^b	5.20 $\pm$ 0.27 ^b
	15	36.60 $\pm$ 2.30 ^a	28.60 $\pm$ 1.52 ^{bc}	11.20 $\pm$ 1.48 ^c	6.14 $\pm$ 0.21 ^c

Data analysis was performed from 5 replicates by using two-way ANOVA. Means within a column with the same letter are not significantly different based on Duncan's Multiple Range Test $p < 0.05$. MS = Murashige and Skoog; B5 = Gamborg's B5; CW = Coconut water; BH = Banana homogenate; PH = Potato homogenate; SD = Standard deviation; Values are means $\pm$ SD.

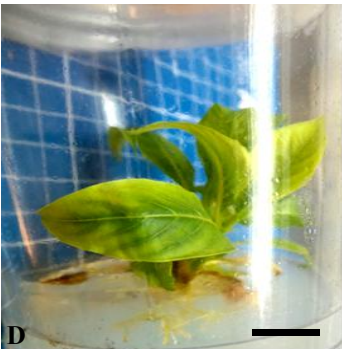
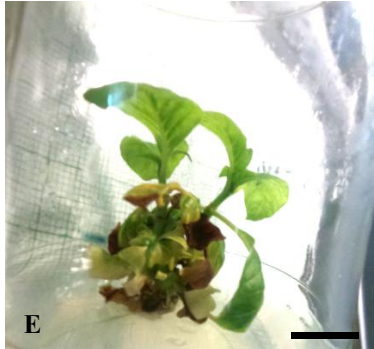
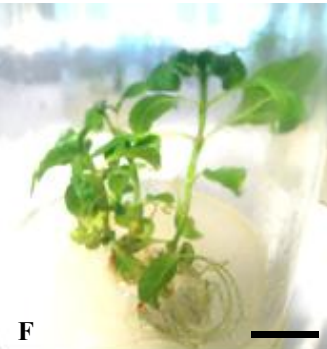
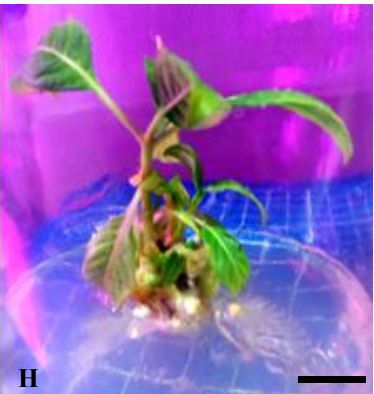
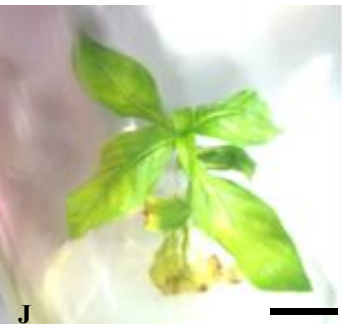
Type of organic additives	Concentration (w/v, v/v)		
	5 %	10%	15%
CW	 A	 B	 C
BH	 D	 E	 F
PH	 G	 H	 I
C	 J		

Figure 2.8: Effect of different complex additive concentrations on seedling growth and development of *Neolamarckia cadamba* after 60 days of culture (DAC). Scale A–J = 1.0 cm. C= control.

2.4.5 Effect of PGRs on seedling growth and development

Plant growth regulators (PGRs) are vital for lipid mobilisation in which lipids used as primary storage in the embryo and seedling growth development. Auxins and cytokinins are always added to the culturing media. NAA (α -Naphthalene acetic acid) and 2, 4-D (2, 4-Dichlorophenoxyacetic acid) meanwhile from cytokinins group; BAP (6-Benzylaminopurine) and TDZ (Thidiazuron) used in this experiment.

a) Effect of auxins

Table 2.5 showed that there were significant differences ($p < 0.05$) between different level of concentrations of 2, 4-D and NAA. From the analysis, B5 media supplemented with NAA at 1.0 mg/L recorded the highest mean number of leaves (44.40), roots (32.20), shoots (6.80) and plant height (5.36 cm) after 60 days of culture (Table 2.5 & Figure 2.9). The second best treatment was media without auxins which recorded 42.60 leaves, 29.40 roots; 6.60 shoots and 5.30 cm in plant height after 60 days culture (Table 2.5 & Figure 2.9 A). B5 media with NAA at 2.0 mg/L also gave a good response of seedling growth and development of *Neolamarckia cadamba* which recorded 35.80 of leaves, 29.80 roots, 6.20 shoots with 4.16 cm height (Table 2.5 & Figure 2.9 H). All treatments with 2, 4-D were given a usual response to seedling growth development but showed positive callogenic respond (Figure 2.9 B & C). This result suggests that *N. cadamba* seedling can grow well on B5 media added with NAA at 1.0 mg/L. However, the best result was NAA 1.0 mg/L, but the control did not vary, except for the number of roots (see Table 2.5).

Table 2.5: Effect of different auxins concentrations on seedling growth and development of *Neolamarckia cadamba* after 60 days of culture (DAC).

means±SD					
Type of Auxins	Conc. (mg/L ⁻¹)	Number of Leaves	Number of Roots	Number of Shoots	Plant Height (cm)
Control		42.60±2.88 ^f	29.40±0.89 ^c	6.60±0.55 ^b	5.30±0.22 ^c
2, 4-D	1.0	17.20±2.59 ^b	6.80±0.84 ^a	5.40±0.54 ^a	2.72±0.19 ^a
	2.0	16.40±1.82 ^b	5.80±0.45 ^a	5.20±0.45 ^a	2.60±0.16 ^a
	3.0	12.80±1.30 ^a	5.40±0.55 ^a	4.80±0.44 ^a	2.20±0.15 ^a
	4.0	12.60±1.14 ^a	5.20±0.84 ^a	4.60±0.55 ^a	2.10±0.07 ^a
	5.0	11.80±0.84 ^a	4.80±0.45 ^a	4.40±0.54 ^a	2.06±0.15 ^a
NAA	1.0	44.40±1.81 ^f	32.20±1.92 ^d	6.80±0.84 ^b	5.36±0.18 ^c
	2.0	35.80±2.68 ^c	29.80±1.91 ^c	6.20±0.83 ^b	4.16±0.15 ^b
	3.0	28.60±2.70 ^d	19.40±1.14 ^b	5.40±0.55 ^a	3.84±0.19 ^{ab}
	4.0	23.80±1.64 ^{cd}	18.40±1.13 ^b	5.20±0.45 ^a	3.40±0.23 ^{ab}
	5.0	20.60±1.14 ^c	16.80±0.84 ^b	5.00 ±0.00 ^a	2.88±0.08 ^a

Data analysis was performed from 5 replicates by using two-way ANOVA. Means within a column with the same letter are not significantly different based on Duncan's Multiple Range Test $p < 0.05$. SD = Standard deviation.

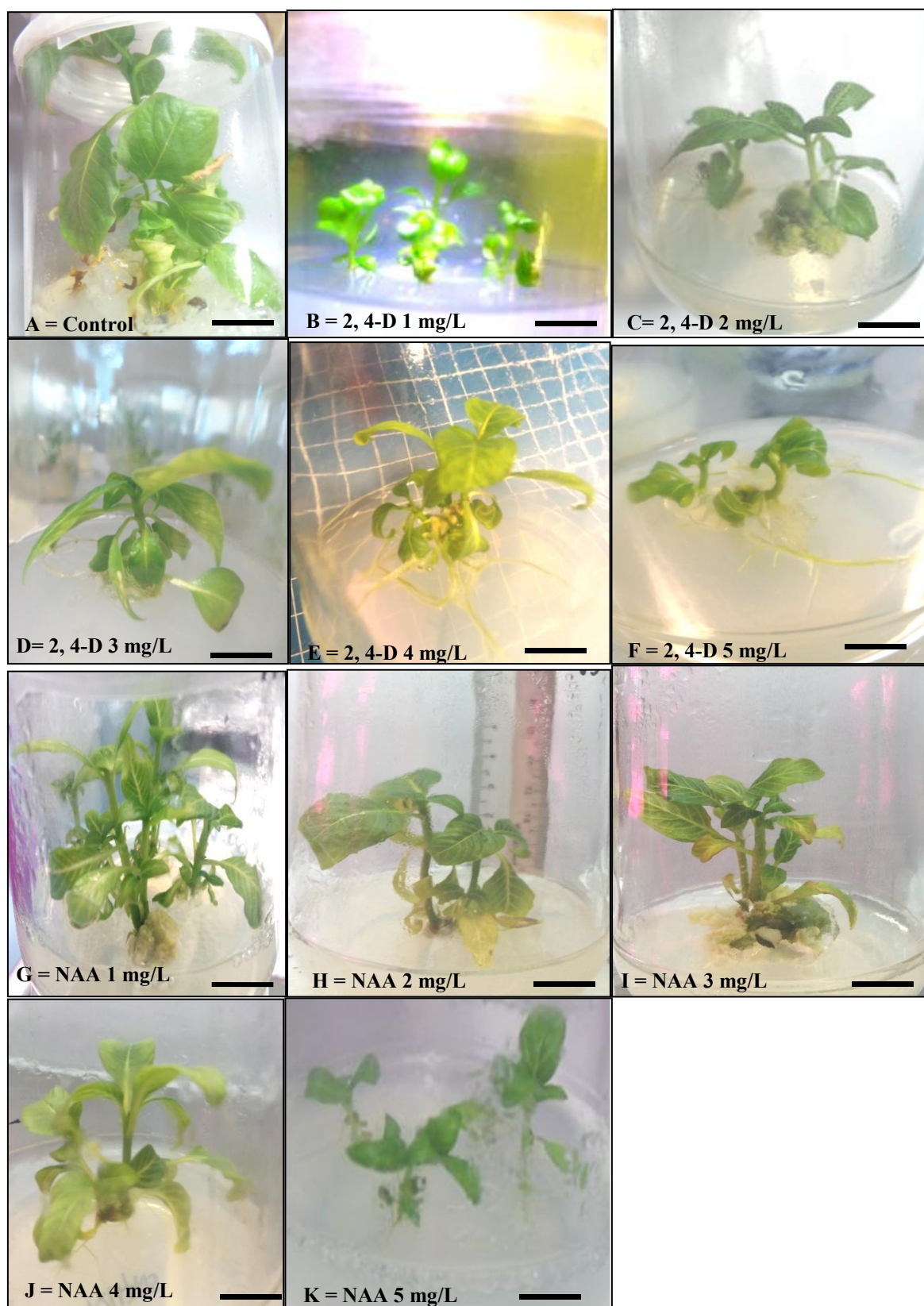


Figure 2.9: Effect of different auxins concentrations on seedling growth and development of *Neolamarckia cadamba* at 60 days after culture. Scale A–K = 0.5 cm.

b) Effect of cytokinins

There were significant differences ($p < 0.05$) between different levels of concentrations of BAP and TDZ used in this study (Table 2.6 & Figure 2.10). From the observation, the highest mean number of leaves, roots, shoots and plant height were observed from B5 media supplemented with BAP at 1.0 mg/L or control media, which was the highest among all the treatments. The mean number of leaves, roots, and shoots recorded in control media were 44.20, 28.60 and 6.40, respectively with 5.08 cm in plant height after 60 days of culture. However, the second-highest mean number of shoots (43.40) was found on B5 media with BAP at 1.0 mg/L with 16.80 roots; 6.60 shoots and 5.14 cm in plant height after 60 days of culture. However, TDZ gave a poor response to seedling growth and development of *N. cadamba*.

The optimum cytokinins level for the development of seedling growth development of *N. cadamba* is BAP 1.0 mg/L since the maximum number of leaves and roots produced has been recorded. The treatment of BAP 1.0 mg/L gave the best result but no difference with the control. However, the colour of *N. cadamba* leaves in BAP 1.0 mg/L culture media is greener than the control that looks green-yellow (see Figure 2.10 A & B). Furthermore, the morphology of the leaves changes from the observation when the number of BAP concentrations increases to 3.0, 4.0 and 5.0 mg/L (see 2.10 D, E & F).

Table 2.6: Effect of different concentrations of cytokinins on seedling growth and development of *Neolamarckia cadamba* after 60 day after culture (DAC).

means $\pm$ SD					
Type of cytokinins	Conc (mg/L ⁻¹)	Number of Leaves	Number of Roots	Number of Shoots	Plant Height (cm)
Control		44.20 $\pm$ 2.17 ^f	28.60 $\pm$ 2.30 ^f	6.40 $\pm$ 0.54 ^c	5.08 $\pm$ 0.08 ^c
BAP	1.0	43.40 $\pm$ 4.03 ^f	16.80 $\pm$ 1.64 ^c	6.60 $\pm$ 0.55 ^c	5.14 $\pm$ 0.16 ^c
	2.0	31.40 $\pm$ 1.67 ^c	8.40 $\pm$ 1.14 ^c	7.20 $\pm$ 0.45 ^d	5.36 $\pm$ 0.27 ^c
	3.0	9.60 $\pm$ 1.34 ^b	5.20 $\pm$ 0.45 ^b	5.00 $\pm$ 1.00 ^b	3.34 $\pm$ 0.15 ^b
	4.0	5.60 $\pm$ 1.31 ^b	4.80 $\pm$ 0.44 ^b	5.20 $\pm$ 0.44 ^b	3.20 $\pm$ 0.21 ^b
	5.0	4.80 $\pm$ 0.84 ^b	4.40 $\pm$ 1.10 ^b	4.20 $\pm$ 0.45 ^b	2.16 $\pm$ 0.11 ^b
TDZ	1.0	18.60 $\pm$ 0.89 ^c	7.20 $\pm$ 0.84 ^{bc}	6.60 $\pm$ 0.55 ^c	4.68 $\pm$ 0.34 ^{bc}
	2.0	28.8 $\pm$ 3.11 ^d	11.60 $\pm$ 2.96 ^d	4.80 $\pm$ 0.83 ^b	3.28 $\pm$ 0.31 ^b
	3.0	17.40 $\pm$ 1.34 ^c	6.20 $\pm$ 0.84 ^b	4.60 $\pm$ 0.54 ^b	3.04 $\pm$ 0.15 ^b
	4.0	0.00 ^a	0.00 ^a	0.00 ^a	0.00 ^a
	5.0	0.00 ^a	0.00 ^a	0.00 ^a	0.00 ^a

Data analysis was performed from 5 replicates by using two-way ANOVA. Means within a column with the same letter are not significantly different based on Duncan's Multiple Range Test $p < 0.05$. SD = Standard deviation

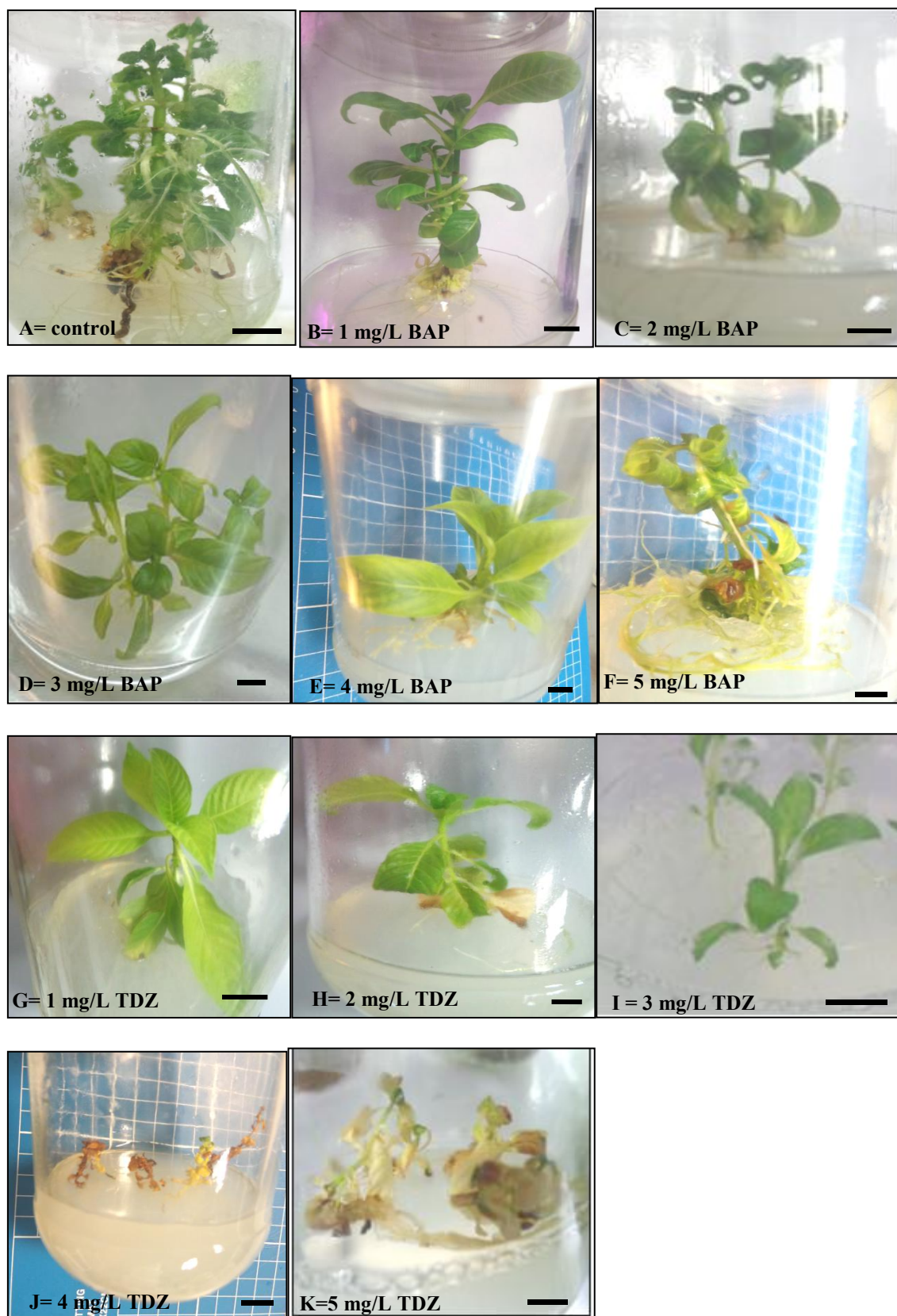


Figure 2.10: Effect of cytokinins on seedling growth and development of *Neolamarckia cadamba* after 60 days of culture. Scale A–K = 1.0 cm.

c) Combination of selected PGRs and CW

The effect of selected PGRs and coconut water (CW) on seedling growth development of *N. cadamba* after 60 days of culture is summarised in Table 2.7 and Figure 2.11. Based on the analysis, there were significant differences among the treatments at $p < 0.05$. The studies found that coconut water at 10% (v/v) was the best treatment for seedling growth and development of *N. cadamba* seedling. After 60 days after culture, the highest mean number of leaves was observed from culture contained coconut water 10% (v/v) and the second-highest was combination BAP at 1.0 mg/L and NAA at 1.0 mg/L, which recorded 58.20 ± 2.17 and 50.60 ± 2.70 , respectively as shown in Table 2.7. A similar observation was also revealed for the mean numbers of roots, shoots and plant height, which recorded 58.20 ± 2.17 , 44.40 ± 3.29 , 14.60 ± 1.52 and 7.94 ± 0.64 cm, respectively from culture medium added with coconut water at 10%. Besides, the low rooting was observed for the treatment of coconut water added with BAP and NAA at 1.0 mg/L, respectively. However, the seedling growth and development with a combination of BAP and NAA at 1.0 mg/L or coconut water added singly gave the best result for rooting (see Table 2.7 & Figure 2.11 B & G).

Table 2.7: Effect of selected PGRs and coconut water (CW) on seedling growth development of *Neolamarckia cadamba* after 60 days of culture (DAC).

Treatments	Means $\pm$ SD			
	Number of Leaves	Number of Roots	Number of Shoots	Plant Height (cm)
Control	45.40 $\pm$ 1.82 ^b	27.00 $\pm$ 1.87 ^c	7.00 $\pm$ 1.00 ^a	5.42 $\pm$ 0.35 ^b
CW10%	58.20 $\pm$ 2.17 ^d	44.40 $\pm$ 3.29 ^c	14.60 $\pm$ 1.52 ^c	7.94 $\pm$ 0.64 ^c
BAP 1.0 mg/L	41.40 $\pm$ 2.88 ^{ab}	11.60 $\pm$ 2.96 ^a	6.20 $\pm$ 1.30 ^a	4.64 $\pm$ 0.40 ^{ab}
NAA 1.0 mg/L	38.00 $\pm$ 1.58 ^a	12.40 $\pm$ 0.89 ^a	6.00 $\pm$ 0.70 ^a	4.30 $\pm$ 0.20 ^{ab}
BAP + CW	35.60 $\pm$ 2.41 ^a	14.00 $\pm$ 0.71 ^{ab}	6.40 $\pm$ 1.14 ^a	3.94 $\pm$ 0.15 ^a
NAA + CW	43.20 $\pm$ 1.30 ^b	30.80 $\pm$ 2.68 ^{cd}	8.20 $\pm$ 0.83 ^{ab}	4.28 $\pm$ 0.19 ^{ab}
BAP + NAA	50.60 $\pm$ 2.70 ^c	38.40 $\pm$ 2.07 ^d	10.40 $\pm$ 0.55 ^b	4.32 $\pm$ 0.18 ^{ab}
BAP + NAA + CW	44.20 $\pm$ 2.16 ^b	15.80 $\pm$ 1.92 ^b	9.20 $\pm$ 1.31 ^b	4.16 $\pm$ 0.17 ^{ab}

Data analysis was performed from 5 replicates by using one-way ANOVA. Means within a column with the same letter are not significantly different based on Duncan's Multiple Range Test $p < 0.05$. SD = Standard deviation

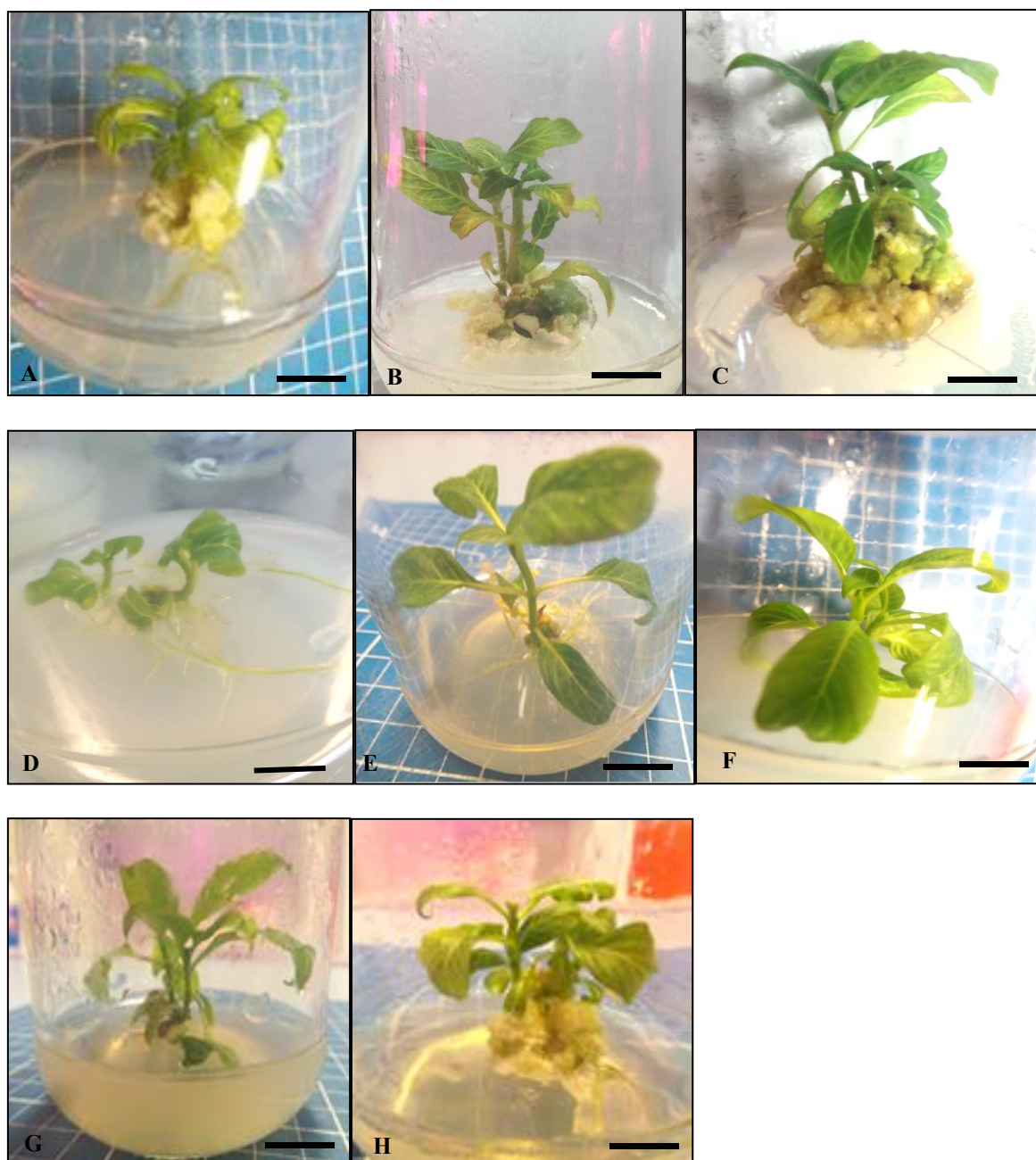


Figure 2.11: Effects of selected PGRs and coconut water (CW) on seedling growth development of *Neolamarckia cadamba* after 60 days of culture. A) Control; (B) CW10%; (C) BAP 1.0 mg/L; (D) NAA 1.0 mg/L; (E) BAP + CW; (F) NAA + CW; (G) BAP + NAA and (H) BAP + NAA + CW. Scale A–H = 1.0 cm

2.4.6 Effect of photoperiod on seedling growth and development

Plant cultures that exposed to 16-h light/ 8-h dark were observed to give a healthy and greener seedling (Figure 2.12) in comparison to those that exposed to 24-h light. There was a significant difference between the two photoperiods used in this study (Table 2.8 & Appendix E). After 60 days of culture, the highest mean number of leaves, roots, shoot and plant height was observed from plant cultures that exposed to 16-h light/ 8-h dark; which recorded 45.40 ± 1.82 leaves, 25.60 ± 1.67 roots, 7.60 ± 1.14 shoots and 7.26 ± 1.30 cm, respectively (Table 2.8 & Appendix D). The change of leaves colour from greener to green-yellow observed when culture exposed to 24-h light up to 40 days (Figure 2.13 B2) and 60 days after culture (Figure 2.13 B3). This experiment found that 16-h light/ 8-h dark is the best culture condition for seedling growth and development of *Neolamarckia cadamba* seedlings.

Table 2.8: Effect of photoperiod on seedling growth and development of *Neolamarckia cadamba* after 60 days after culture (DAC).

Photoperiods	Means $\pm$ SD			
	Number of Leaves	Number of Roots	Number of Shoots	Plant Height (cm)
16-h light	45.40 $\pm$ 1.82	25.60 $\pm$ 1.67	7.6 $\pm$ 1.14	7.26 $\pm$ 1.30
24-h light	26.60 $\pm$ 3.04	10.6 $\pm$ 2.07	4.8 $\pm$ 1.09	4.1 $\pm$ 0.67

Data obtained from 5 replicates. Data analysed by T-test sample.

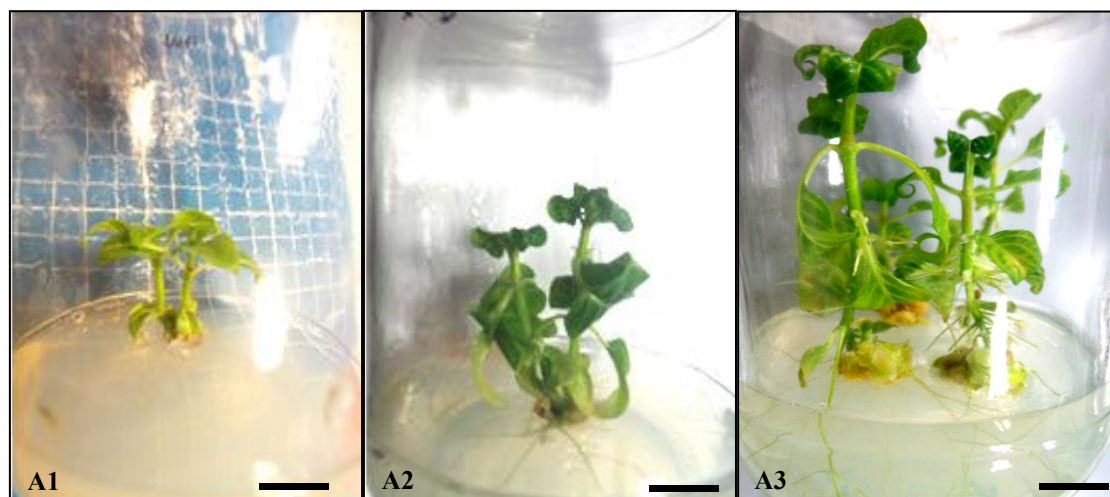


Figure 2.12: Effect of 16-h light/ 8-h dark culture condition on seedling growth and development of *Neolamarckia cadamba* according to days after culture. A1 = 20 days after culture; A2 = 40 days after culture and A3 = 60 days after culture. Scale A1, A2 and A3 = 1.0 cm.

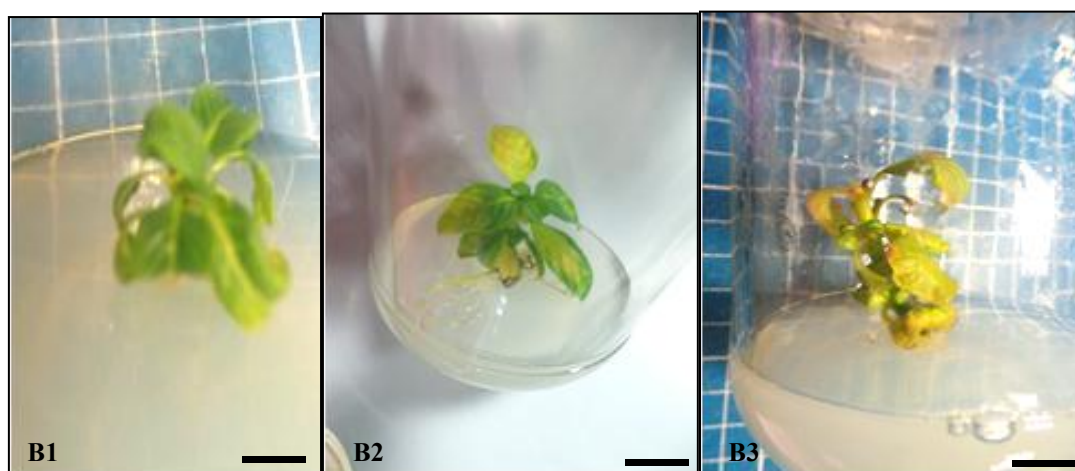


Figure 2.13: Effect of 24-h light on seedling growth and development of *Neolamarckia cadamba* according to days after culture. B1 = 20 days after culture; B2 = 40 days after culture and B3 = 60 days after culture. Scale B1, B2, B3 = 1.0 cm.

2.4.7 Effect of activated charcoal on seedling growth and development

Based on Table 2.9, there were significant differences ($p < 0.05$) between the treatments on the number of roots, shoots and plant height parameters. There was no significant difference observed in the mean number of leaves compared to media control (Table 2.9 and Appendix F). The highest number of leaves, roots and shoots were observed from full-strength B5 media containing 2.0 g/L activated charcoal, which recorded 48.80 ± 1.30 ; 31.20 ± 1.92 , and 8.6 ± 0.55 , respectively after 60 days observation with 7.14 ± 0.65 cm of plant height. Figure 2.14 shows the differences between 2.0 g/L and 1.0 g/L activated charcoal compared with control after 60 days of culture. The addition of 2.0 g/L activated charcoal was observed with the highest number of roots produced when compared to the control and treatment of 1.0 g /L activated charcoal.

Table 2.9: Effect of activated charcoal on seedling growth and development of *Neolamarckia cadamba* after 60 days of culture.

Treatments	means $\pm$ SD			
	Number of Leaves	Number of Roots	Number of Shoots	Plant Height (cm)
Control	44.6 $\pm$ 1.14 (NS)	26.80 $\pm$ 2.28 ^a	8.2 $\pm$ 0.83 ^b	6.46 $\pm$ 0.55 ^b
2.0 g/l	48.80 $\pm$ 1.30 (NS)	31.20 $\pm$ 1.92 ^b	8.6 $\pm$ 0.55 ^b	7.14 $\pm$ 0.65 ^b
1.0 g/l	46.8 $\pm$ 1.31 (NS)	27.00 $\pm$ 1.58 ^a	7.4 $\pm$ 0.54 ^a	5.44 $\pm$ 0.74 ^a

Data analysis was from 5 replicates by using one -way ANOVA. Means within a column with the same letter are not significantly different based on Duncan's Multiple Range Test at $p < 0.05$

SD = Standard deviation; NS = Not significant.

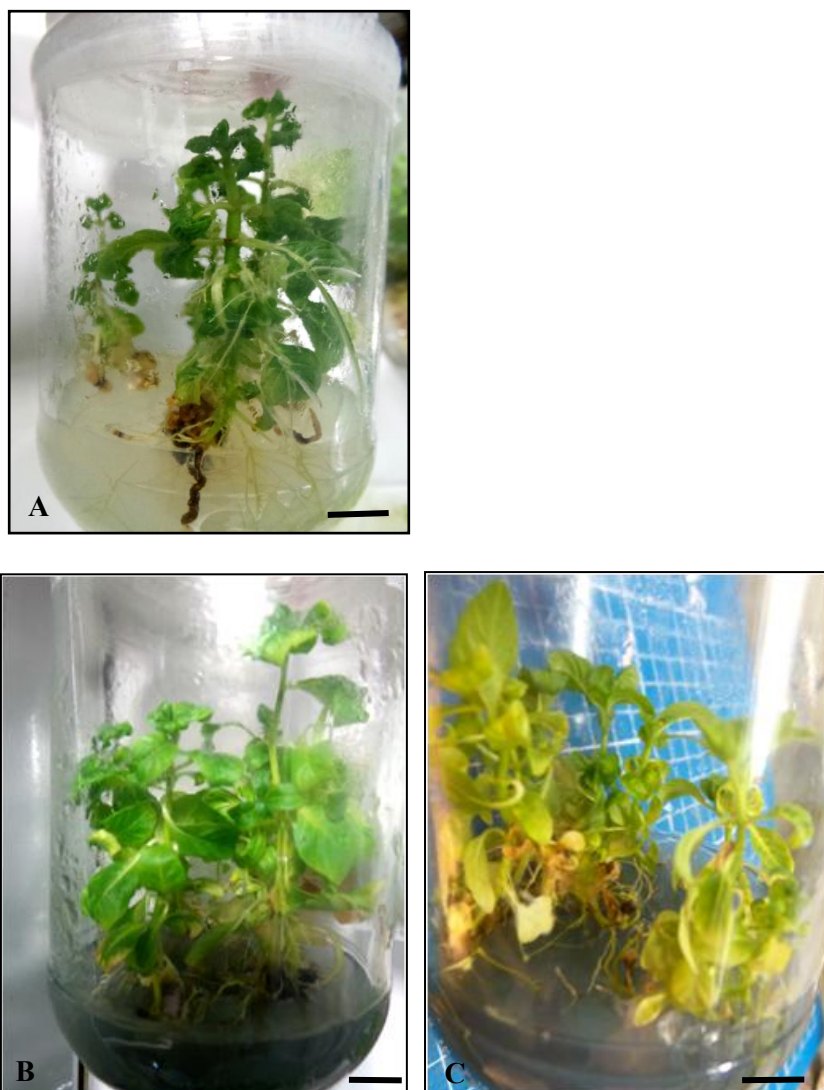


Figure 2.14: Effect of activated charcoal on seedling growth development of *Neolamarckia cadamba* after 60 days of culture. (A) Control; (B) 2.0 g/L activated culture, and (C) 1.0 g/L activated charcoal. B5 media were used as basal media added with coconut water 10% (v/v) and 0.2% sucrose. Scale A–C = 1.0 cm.

2.4.8 *Ex vitro* acclimatisation of *Neolamarckia cadamba* seedlings

Table 2.10 summarises the effect of different potting media for acclimatisation of *Neolamarckia cadamba* seedlings after 60 days. The highest percentage of seedlings survived after transplanting was in media containing soil (80.0%) after three months of planting out. However, only about 20% of transplanted seedlings survived on media containing soil and organic compost. For peat moss media, all seedlings died after five days of planting out (Table 2.10 & Figure 2.15). Figure 2.16 shows the acclimatisation of *in vitro* seedling of *N. cadamba* using soil as planting material.

Table 2.10: Effect of different types of potting media for acclimatisation of *Neolamarckia cadamba* seedling after 60 days.

Treatments	Plant Height (cm)	Number of seedlings survived (%)
Soil	16.0	80.0
Soil + organic compost	7.5	20.0
Peat moss	-	0.0

Data was obtained from 5 planting materials in each treatment.



Figure 2.15: Seedlings of *Neolamarckia cadamba* on different types of potting media after 30 days of transplanting. (A) soil; (B) soil with organic compost; and (C) Peat moss. Scale A–C = 1.0 cm.



Figure 2.16: Acclimatisation of *in vitro* seedling of *Neolamarckia cadamba* on the soil as planting material. (A) *In vitro* seedling, three months old; (B) Pre-treatment before hardening; (C) Two weeks old seedling; (D) Seedling exposed to light after 1-month transplanting (E) Two months old seedling; and (F) Three months old seedling. Scale A–F = 1.0 cm.

2.5 Discussion

2.5.1 Developmental stages of seed and seedling growth of *Neolamarckia cadamba*

The *in vitro* seed germination in B5 media supplemented with 10% (v/v) coconut water was used to examine the embryo development and seedling growth of *N. cadamba*. Based on the result, it took five days to reach from stage 0 (pre-germination stage) to stage 2 (germination stage). Based on the microscopic observation of *N. cadamba* seeds, the *in vitro* seed germination process can be divided into six growth stages, and it is similar to the growth stages of *Canthium Coromandelicum* (Burm. f.) (Ekambe & Surwase, 2016).

The germination pattern of *N. cadamba* seed is classified as epigeal germination due to the cotyledons pulled above the ground. After the seed inoculated in the soil, it will absorb the water and swell that result in increased moisture content. Next, the radicle of seed emerges via the seed coat and subsequently developed into the primary root in the soil. The elongation of radicle will lead to lateral roots emergence and growth of root hairs (Ekambe & Surwase, 2016). From observation, slow root growth was observed at this point, which lasted for up to 5–7 days, whereby according to Kozlowski and Pallardy (1997), normal growth of tree species roots should occur within 2–3 days of the elongation of the radicle.

After seven to ten days of inoculation, the new seedling developed. The hypocotyl starts to elongate and grow upward via the surface of soil together with cotyledon. The hypocotyl may be broken and lead to the death of seedling due to hardness the soil. The cotyledon provides the temporary source of stored food to the seedling. It will fully open

and turn green due to light, and synthesis sugar via photosynthesis process. The epicotyl containing small leaves, buds, and the growing point revealed in the following germination process. After the formation of the first set of rosette leaves, the cotyledons will drop off (Ekambe & Surwase, 2016).

2.5.2 *In vitro* seed germination of *Neolamarckia cadamba*

a) Effect of basal media

The percentage of *in vitro* seed germination of *N. cadamba* was significantly affected by the differences in the composition of organic and inorganic nutrients used in basal media. According to Chiruvella et al. (2014), the composition in basal media used for germination was the primary factor in increasing the number of seeds germinated. In this study, the lowest number of seeds germinated (15%) was observed from half-strength MS medium, and full-strength MS medium was not given any positive respond after 30 days of culture. These findings are similar to a study done by Groll et al. (2002) that salt concentration of full-strength MS and half-strength MS media had no significant effect on the germination percentage and maturation of cassava (*Manihot exculenta*) somatic embryo.

According to Groll et al. (2002), the medium salt concentration in $\frac{1}{4}$ MS could be too low and suboptimal to support the growth and development of the embryo. Therefore, it was presumed that *in vitro* seed germination percentage in full-strength and half-strength MS media was lower as the concentration of osmotica in half-strength MS medium was sub-optimal to support the growth and development of the embryo in the seed. The best medium for *in vitro* seed germination of *N. cadamba* was the full-strength B5 medium as it

contains a high concentration of potassium nitrate (KNO_3) in comparison to half-strength B5 and MS media. The differences in the concentration of potassium nitrate might be the critical factor that contributes to the increased germination percentage in B5 media. Potassium nitrate can break the seed dormancy and enhance the healthiness of seedlings (Gulser et al., 2010). Potassium nitrate also could maintain the hormonal balance and reduce the inhibitors of seed growth, namely abscisic acid.

A study done by Welman and Sim (2010) stated that the different nitrogen source of MS and B5 had a different effect on the *in vitro* germination of *N. cadamba* seeds. Moreover, several Mediterranean species like *Medicago arabica*, *Epilobium hirsutum*, *Epilobium hirsutum* and *Rumex crispus* had higher seed germination percentage when cultured on germination medium with increased nitrogenous compounds (Pérez-Fernández et al., 2006). The findings showed that seeds germinated in early five days after culture in full-strength B5 medium. This could be due to the effect of nitrogen source in the form of nitrate ions that plays an essential role in breaking the seed dormancy. Other studies reported the same findings were *Dactylis glomerata*, *Cistus species*, and *Dittrichia viscosa* (Pérez-Fernández & Rodríguez-Echeverría, 2003).

According to Fenner and Thompson (2005) and George et al. (2008), nitrate ions, NO_3^- was described to enhance germination in weed species by breaking seed dormancy. Based on this theory, the significantly higher seed germination percentage in the B5 medium was presumably due to the presence of higher level of nitrate ions which helps to break the dormancy of *N. cadamba* seeds. The nitrogen source of MS medium contributed from potassium nitrate, KNO_3 and ammonium nitrate ($\text{NH}_4 \text{NO}_3$) while the B5 medium

was KNO_3 and ammonium sulphate (NH_4SO_4). One of the possible reasons that caused the B5 medium to have higher seed germination percentage than MS medium might be due to the preference of *N. cadamba* seed over nitrate ions in the form of ammonium sulphate. This is supported by various published studies elsewhere that different species had shown preference over a different type of nitrogen sources (Pérez-Fernández & Rodríguez-Echeverría, 2003; Pérez-Fernández et al., 2006; Jabeen et al., 2016; Pereira, et al., 2018). For instance, nitrate ions can be assimilated as ammonium nitrate in the pre-germination process by trees such as *Cinchona officinalis* L (Rubiaceae), which improved embryo growth and development (Armijos-González & Pérez-ruiz 2016).

Another possible reason was related to the natural habitat of *N. cadamba* whereby it mostly found nearby riverbanks in valleys (Welman & Sim, 2010). In the phosphorus cycle, phosphorus naturally found in rocks and minerals, erosion of rocks released phosphorus in the soluble form of phosphate ion (Vipranarayana et al., 2012; Shekarriz et al., 2014). Phosphate ion then carried by the river deposited in the soil of riverbanks (Smith, 2013). The higher phosphate level in B5 medium might be similar to the soil composition of its natural habitat which may favour the *N. cadamba* seeds to germinate. Different studies have shown that habitat preferences have affected seed germination patterns (Armijos-González & Pérez-Ruiz, 2016; Cardoso, 2018). No analysis was found to show that phosphate ion affects the germination rate of the seeds. The most habitat-related patterns in germination have been seen in temperate species such as wood anemone, santalum and mountain-big sagebrush (*Artemisia tridentata*), light intensity and the amount of soil nitrate within their natural habitats (Vandelook et al., 2008; Zhang et al., 2016). Due to the higher *in vitro* seed germination percentage, B5 basal medium was

selected as the basal medium of interest for the subsequent studies with natural complex additives.

b) Effect of complex additives

The addition of organic additive also can enhance the seedling growth and development of *N. cadamba*. Complex organic additives are added into the basal medium to substitute the use of plant growth regulators like gibberalene, 2, 4-D, TDZ, NAA and Kinetin for *in vitro* germination and seedling growth of *N. cadamba*. The biochemical components of organic additive like coconut water include various minerals, amino acids and, vitamins like ascorbic acid and B group vitamins. All these supplements are essential in promoting the growth and development of plant cells.

In this study, the addition of coconut water at 10–15% (v/v) enhanced the percentage of seed germination in *N. cadamba*. Coconut water (CW) was first utilised in tissue cultures by enhancing the growth of *Datura stramonium* embryos. The coconut water commonly used in orchids containing carbohydrates, vitamins, amino acids, organic acid, organic ions and enzymes played an integral role in the growth of plant cells, according to earlier studies (Arditti 2010; Jainol & Jualang 2017). It also increases seed germination and seedling growth of many orchid species such as *Encyclia aff. Oncidioides* (Znaniecka et al., 2005) and *Dimorphorchis lowii* (Jainol & Jualang, 2017). There is, however, no study on the use of coconut water as a medium among woody plant species. The coconut water consists of an IAA, gibberalene, 2, 4-D, TDZ, NAA, and Kinetin, which may replace the hormone function, articulate Arditti (2010) on orchid micropropagation.

Similar to CW, potato homogenate (PH) could also be used to enhance seed germination and seedling growth of *N. cadamba*. It contains sucrose, dipotassium phosphate, potassium chloride, ferrous sulphate, nitrogen source, protein, carbohydrate, Vitamin B and C (Bairu et al., 2009; Arditti, 2010). Banana homogenate (BH) had proven to enhance orchid seed germination and seedling growth. It contains growth factors such as cytokinin, auxin and gibberellins, which can act as a buffer in the culture medium (Kalpona et al., 2000; Manawadu et al., 2014). Nonetheless, no previous studies have yet to be conducted on *N. cadamba*. By the addition of complex additives as a nutrient supplement in media, *N. cadamba* gave an excellent response to seed germination compared to conventional propagation. Thus, the use of coconut water, potato homogenate, bananas homogeneous and other complex additives in media may reduce the cost of maintaining culture media and expenditure, particularly on industry tissue culture.

2.5.3 Effect of types basal media on seedling growth and development

Basal media play a significant role in determining the success of plant tissue culture. It provides water, nutrients, vitamins, and growth regulators for the plant, as well as the place for gases exchange and removal of metabolite wastes (Kaur & Bhutani, 2012). In this study, the best basal media composition for seedling growth was full-strength B5 media.

The difference between MS and B5 media might occur due to the calcium and nitrogen concentrations. MS media have eight- and four-fold higher concentration of calcium and nitrogen, respectively as compared to B5 media. Similarly, the salt concentration of B5 media is only half over full strength MS media. Salinity decreased the water uptake of seedling leading to reduced growth and change in metabolism, which

similar to the effect of water stress. Another report also showed that the effect of salinity on Brassica crop by reducing plant height, size and yield plus the quality of the product (Bybordi, 2010).

The high salt concentration in MS medium could inhibit plant growth and result in the death of the plant if salts present in excess. In this research, there is no plant death occurred during the observation. Stressed plants could affect the metabolic process of plant-like, photosynthesis, energy and lipid metabolism, and protein synthesis. According to Reigosa and Pazos-Malvido (2007) and Gulser et al. (2010), photosynthetic mechanisms are affected directly or indirectly attributable to the modified structural organisation and physicochemical properties of thylakoid membranes by salinity stress. The photosynthesis will affect the plant growth since it absorbs and converts light energy into sugar and subsequently utilised in the production of energy for growth and others in respiration.

A widely known basal medium such as Murashige and Skoog basal medium (MS) (Murashige & Skoog, 1962) always be used to start the preparation of most appropriate medium for a new system, so it is selected in the present study (Chiruvella et al., 2014). The MS medium designed for tobacco explants based on its mineral compounds in previous studies (Jha & Ghosh, 2005). Half strengths MS medium had been determined to optimise *in vitro* seed germination of other species such as *Pterocarpus marsupium* (Mishra et al., 2013) and *Psoralea corylifolia* (Pandey et al., 2014). MS medium containing high amounts of both macro- and micronutrients can increase germination and seedling growth development of *Gynochthodes umbellata* (L.) Razafim. and *B. Bremer* (Rubiaceae) (Anjusha & Gangaprasad, 2016).

On the other hand, Gamborg's B5 medium (Gamborg et al., 1968) designed for callus or suspension culture, but it also works as a basal medium for plant regeneration (Perera et al., 2015). A B5 medium was proven that increased seed germination of *Jatropha curcas* (Warakagoda & Subasinghe, 2009). In the cases of *Rhyncostylis retusa*, B5 medium had shown the maximum root length as compared to the other media. Besides, the B5 medium is known as the most likely medium influencing the expression of all the seedling parameters, such as leaf number and size, root length and breadth, fresh weight and dry weight (Basu et al., 2010). Such findings are similar to this analysis in which the best media for the *in vitro* seedling growth and development of *N. cadamba* is the source of Gamborg's B5 media

2.5.4 Complex additives on seedling growth and development

There is some benefit from including small amounts of organic nitrogen in the medium. Complex organic additives are added into the basal medium to substitute the use of plant growth regulators and amino acids for seedling growth development of *N. cadamba*. The biochemical components of organic additive like coconut water (*Cocos nucifera* L.) include various cationic minerals (potassium, calcium, phosphorus, sodium, iron and copper), amino acids, and vitamins like ascorbic acid and B group vitamins (Gnasekaran et al., 2012; Prades et al., 2012). Moreover, coconut water contains amino acids like glutamine, arginine, asparagine, alanine and aspartic acid. All these supplements provided by complex additives are essential in promoting both the growth and development of plant cells (Arditti, 2010). According to Islam et al. (2011), potato extract also can be used as a simulator to enhance the seedling growth and developments on *Vanda* species.

Besides that, organic additive like tomato extract is creamy with a high amount of natural sugar which is up to 2.5 g per 100 g of tomato juice (Markovic et al., 2006). On top of that, the natural sugars present are in the form of glucose, fructose and lycopene which are readily absorbed by the plant cells to enhance cellular proliferation and regeneration (Markovic et al., 2006; Arditti, 2010). Lycopene, together with ascorbic acid and carotene are a potent antioxidant that neutralises free radicals formed by the plant culture, effectively preventing the oxidation of DNA and browning in plant cells. Even though most organic additives contain beneficial biochemical compounds, they may also contain plant growth inhibitors like phenolic compounds. Bansal and Goyal (2005) stated that papaya contains a high amount of sterols, organic acids and phenolic compounds which are potential growth inhibitors for plant explants. These compounds would accumulate within the culture, causing browning of explant tissue and slowing down the growth of the culture, which eventually lead to necrosis of culture. The best complex additives for *in vitro* seedling growth and development of *N. cadamba* is coconut water at 10% (v/v).

2.5.5 Effect of PGRs on seedling growth and development

a) Auxins

Auxins plays a vital role in plant cell division and stimulates root formation and other organs. The presence of auxins also promotes cell division, cell elongation and swelling of tissues, callus, root adventitious and inhibit the formation of shoots. Auxins will induce embryogenesis in suspension culture (Balilashaki et al., 2015). Among the hormones that belong to this group is an acid indoleacetic (IAA), acid indolbutrik (IBA), α -naptalenaasetik (NAA) and 2, 4-dichlorophenoxyacetic (2, 4-D) (Atondo-buenoi et al.,

2016; Bahmankar et al., 2017). Referring to Arditti's report on 2010, a survey of Kano in 1965 managed to promote seed germination and growth of seedlings of the plant by using auxins at a concentration of 0.1 or 1.0 mg/L while the use of auxins exceeds 10.0 mgL⁻¹ were found to exhibit an inhibitory effects on the plants.

According to Armijos-Gonzalezl and Pe´rez-Ruiz (2016), the addition of auxins exceeds 5.0–10.0 mg/L in culture media can cause plant death and mutations in *Anthocephalus cadamba* plant cells. This study found that the addition of NAA and 2, 4-D above 3.0 mg/L to 5.0 mg/L gave an inadequate response to the seedling growth development of *N. cadamba*. This is because auxins such as NAA and 2, 4-D are often used in low concentrations, and quantities required for plant regulators are around 0.01–2.0 mg/L (Nunes et al., 2018). This finding was similar to the study by Anjusha and Gangaparasad (2016) that the addition of lower concentration NAA (1.0 to 1.5 mg/L) could promote the multiplication of shoots of *Gynochthodes* species under Rubiaceae family which is the same family with *N. cadamba*. The outcome has shown that *N. cadamba* seedlings may grow well on the B5 media added with NAA at 1.0 mg/L. However, the result did not differ from the control, except for the number of roots. The roles of NAA in culture media are to promote cell division, cell elongation and swelling of tissues, calluses, root adventitious, and inhibit the formation of shoots (Nunes et al., 2018).

b) Cytokinins

Cytokinins consisting of BA (benzyl adenine), 2ip (2-isopentyl adenine), kinetin, zeatin, thidiazuron (TDZ), Zeatin riboside, N'phenylurea, N–(2-chloro-4-pyridyl) and BAP (Benzilamino purine). Cytokinins act to stimulate cell division, bud growth, cell

specialisation and shoots. If added together, auxins can promote cell division and the formation of callus (Azad et al., 2012; Yong et al., 2014). Besides, cytokinins added to the culture medium fewer than 10.0 mg/L concentrations. Concentrations exceed 10.0 mg/L can inhibit seedling growth and development. The type and concentration of cytokinins are essential for *in vitro* propagation (Bakhtiar et al., 2016; Baskaran et al., 2018).

The seedlings cultured on medium contained with TDZ at 3.0 mg/L to 5.0 mg/L gave a weak response. These seedlings were healthy and green at the beginning but changed to brownish colour after two weeks of inoculation. However, the seedlings were grown well in culture medium supplemented with 1.0 mg/L to 2.0 mg/L of BAP. These findings are similar to Naika and Krishna (2008) and Frank et al. (2000) reported that at high concentrations of cytokinins generally stimulate the formation of shoots but inhibit the formation and growth of roots of the medicinal plant (*Clematis gouriana*) and *Arabidopsis*. According to Bouiamrine and Halimi (2012) and Mohamadi–Nasab et al. (2017), the high concentration of auxins and cytokinins in the range 10.0 mg/L and above can cause somaclonal variation in the regenerated plants from immature embryo callus.

From this study, the treatment BAP 1.0 mg/L showed the best result but not significantly different from the control. Report by Yong et al. (2014), the function of BAP in the shoot growth development, which is to enhance the shoot and bud development, especially in stimulate the cell in leaf cells. Subjectively, this result observed that the colour of leaves in BAP 1.0 mg/L culture media is greener than the leaves observed from the control that looks green-yellow due to the addition of BAP in culture media. According to Naika and Krishna (2008), the morphology of the *Clematis gouriana* leaves gave the

effect to the chloroplast pigment in leaves and then made the colour changes from green to yellow leaf when applying with a high-level concentration of cytokinins.

c) Combination of selected PGRs and coconut water

This study found that culture media supplemented with coconut water without the addition of any plant growth regulators (PGRs) can be used as a substitute for hormone usage during the culture period. Coconut water was found to be the optimum media for seedling growth and development of *N. cadamba* when added singly to the basal media. According to Kalpona et al. (2000) and Arditti (2010), the use of liquid endosperm of coconut was good for *Dendrobium* species grew well in the medium contains coconut water as a nutrient additive. However, the effect of combining coconut water with BAP or NAA in tree species is not reported. Besides, an experiment done by Gautheret in 1985 stated that the use of coconut water was found useful for inducing cell division in quiescent cells of carrot phloem explants when added singly (Prades et al., 2012).

The interaction of PGRs is often complicated, and more than one combination of two substances is likely to produce a high yield. The balance concentration between auxins and cytokinins is required for the formation of adventitious shoots and roots. However, the requisite concentration of each PGR differ significantly according to the type of plants being cultured, the cultural conditions and the compounds used. From this study, the result showed that the combination of coconut water with BAP or NAA at 1.0 mg/L gave poor rooting. However, the seedling growth and development with a combination of BAP and NAA at 1.0 mg/L or coconut water added singly gave the best result for rooting. Thus, the effect of adding more than two substances in culture media will inhibit the rooting of *N.*

cadamba's seedlings. These findings are similar to report by Ahmed et al. (2016), stated that the high-level concentration of cytokinin gave the poor rooting of *Stevia rebaudiana*. The highest percentage of shoot regeneration was obtained from 20% and 40% coconut water which was similar to this study (Ahmed et al., 2016). Ahmed et al. (2016) confirmed that the cytokinin activity in fresh coconut water was recorded in the Kuraishi and Okumura studies in 1961. Some natural cytokinins, such as zeatine and N'diphenyl urea (George, 2008), have been isolated from coconut water, but their concentrations and their specificity in coconut water remain unclear.

2.5.6 Effect of photoperiod on seedling growth and development

Light is essential for *in vitro* plant through the process of photosynthesis. Light requirements needed by the plant are different between species; for example, the use of light intensity for woody plant based on the needs and the type of species (Cardoso, 2018). The results showed that treatment with 16-h light/ 8-h dark is the best photoperiod for seedling growth of *N. cadamba* as indicated by the mean number of leaves, shoots, roots and the height of seedling.

Development and growth of plant cells will be retarded when no light is supplied (Pandey et al., 2014). The effect of light is related to the effects of phytochrome towards photomorphogenesis in a plant which require red and blue photochromic pigment of plants (Kauth et al., 2008). According to Anwar and Siddiqui (2000), the highest level of soluble sugar and starch contents was detected in plant cells that exposed to less than 24-h light. Both substances are essential for the proliferation and growth of seedlings. Cardoso (2018) reported that culture under the 24-h light in the short term or long term would cause

stimulation or inhibition on seedling growth and development of plant cell *Gerbera jamesonii*. Acceptance of light by plants at specific wavelengths also affect gene encoding a protein in morphogenesis, organogenesis and photosynthesis (Anwar & Siddiqui, 2000).

Armijos-Gonzalez¹ and Pe'rez-Ruiz (2016) also stated that 16 hours of light and 8 hours of dark was good for the development and growth of species of *Cinchona officinalis* (Rubiaceae) while 24 hours of light was good for increasing seed germination percentages. Renteria (2002) had reported similar comments in the *C. pubescens* that direct light exposure is required for the germination of small seeds with relatively low nutrient content. This result clearly shows that different photoperiod is required in every stage of plant growth. According to Chandra and Mishra (2003), the reception of light required by the plant is different according to the species plants. In the present study, low- or high-intensity seedling exposes to light will cause the leaves of *N. cadamba* to wilt and eventually the seedling will die. Increasing the time (duration) of plants exposed to light (Renteria, 2002) influenced the potential factors for light to cause these effects. However, plants need a certain amount of darkness to grow properly and should be exposed to light for no more than 16 hours a day (Chandra & Mishra, 2003). According to Armijos-Gonzalez¹ and Pe'rez-Ruiz (2016), the leaves are yellow, often smoking, turning brown and dying when a plant gets too much direct light. These findings are similar to this study when exposure to seedling in the 24 hours light causes the leaves to change colour from greener to green-yellow after 40 days of culture.

Light is an important factor in plant maintenance. The growth rate and the length of time that a plant stays active depend on the amount of light it receives. There are three

areas to consider when assessing the impact of light on plant growth: strength, length and quality. Total of light required by each type of plant is different because depending on the environment of their habitat. Most of the woody plant requires exposure to light at around 65–70%, with the range of light intensity between 1500–4000 lx (Kozlowski & Pallardy, 1997).

2.5.7 Effect of activated charcoal on seedling growth development

Activated charcoal (AC) is carbonised wood which has heated for several hours in steam or air. It possesses strong adsorptive properties and is used in chemistry to absorb both gases and dissolve solids. When in use in tissues cultures, charcoal is commonly thought to remove substances of tissues or origin medium that are growth inhibitory (Thomas, 2008). It is a porous and tasteless material and is distinguished from elementary carbon by removal of all non-carbon impurities and the oxidation of carbon surface. AC has an excellent network of pores, an extraordinarily large surface area and volume that gives it a unique adsorption capacity (Bansal & Goyal, 2005; Karina et al., 2015).

From observation, there were no significant differences ($p < 0.05$) between the use of activated charcoal with a non-charcoal medium on *in vitro* seedling of *Neolamarckia cadamba*. Activated charcoal can maintain the culture from a high percentage of contamination and also can be used as an absorbent to absorb the phenol compound. According to Reigosa et al. (1999) and Arditti (2010), Fridborg in the first experiment in 1978 showed that charcoal only could assist the adsorption of toxic substances in the media during further growth. The beneficial effects of activated charcoal in media are to promote

morphogenesis, root formation, prevented unwanted callus growth and adsorbed unwanted compounds that would inhibit the seedling growth.

Activated charcoal used alone or in combination with an auxin-induced rooting in several plants were recorded. According to Thomas (20008), activated charcoal can enhance the number of roots for pineapple (*Ananas comosus*). WPM medium supplemented with AC, IBA and NAA was confirmed to have successfully rooted the camper tree (*Cinnamomum camphore*) shoots derived from shooting tips, nodal segments. 100% of IBA roots and 2.0 g/L AC medium-containing shoots (Babu et al., 2003).

For *in vitro* rooting, *Harrisonia abyssinica* shoots were held in the darkness for 4 days and shoots were transferred in 0.03 g/L AC to WPM basal medium. The genotype-dependent use of this approach caused 100% rooting (Feyissa et al., 2005). These findings are similar to this study when the treatment of 2.0 g/L activated charcoal gave a higher number of roots produced. Report by Thomas (2008) stated, in particular, the adsorption of inhibitory compounds on the culture medium and the significant reduction of toxic metabolism, phenolic exuberance and brown exudate accumulation can lead to the promotive impact of activated charcoal upon morphogenesis. Feyissa et al. (2005) reported a variety of stimulating and inhibitory activities, including releasing substances which naturally are present in activated charcoal and encourage growth, alteration and darkening of culturally active mediums and adsorption of vitamins, metal ions and plant growth regulators, including abscisic acid and gaseous ethylene, often include activated charcoal.

2.5.8 *Ex vitro* acclimatisation *Neolamarckia cadamba*

The plant generally can grow in various types of planting media. From the observation, soil without any mixture was good for acclimatising *in vitro* seedlings of *N. cadamba*. According to Halawane et al. (2011), the seedlings should be transplanted to different types of soil medium depending on the stages of growth which divided by days. The soil medium for each stage consists of a different composition of sand, soil, coconut fibres, charcoal and compost with ratio 1:1 and 2:1. Irawan and Purwanto (2014) also reported that the best soil medium for seedling growth of Jabon was the mixed soil, cow manure compost and husk charcoal medium with ratio 3:1:1. Hence, these results indicate that the requirements for planting media are different and it depends on the natural habitat and the species type.

As a result, the seedlings of *N. cadamba* were preferred to grow well in only soil planting media, hence from cultivated soil to mixed soil and peat moss treatment. According to Meijer et al. (2011), in the first experiment, indicated that soil texture affects seedling growth of the *Prosopis* seedlings. In contrast to the Yahya et al. (2015) study, peat moss was the best planting material for *Eurycoma longifolia* compared to soil or sand. Also, the report by Hassan (2017), the peat moss is partly decomposed plants of the sphagnum genus, harvested from peat bogs that can improve the *in vitro* date palm plantlet acclimatisation rate. Although peat moss is brown, it has a different texture and weight like soil: it is light and spongy and crumbles into tiny splinters. It is hard to wash, but once it is wet, it retains water like a sponge, minimising surface water drainage. Besides, the peat moss can be added to potting soil as an ingredient to lighten the soil and allow it better to hold the moisture (Hassan, 2017).

According to Ďurkovič and Mišalová, (2008), from a practical point of view, early tree formation (*Sorbus domestica* L.) during *ex vitro* acclimatisation provides brittle plantlets with mechanical support for better handling of deformations and mechanical injuries during subsequent transplantations. In addition, the soil or potting soil mixture is made from the soil with additional ingredients. Other ingredients may include fertilisers, moisturisers for moisture conservation or peat moss (Nizam & Te-chato, 2009).

2.6 Conclusion

In conclusion, full-strength B5 media was the best basal media for *in vitro* seed germination and seedling growth of *N. cadamba*. The complex additives such as coconut water, homogenates bananas, and potato can be used as a nutrient supplement to enhance seed germination and seedling growth via *in vitro* propagation. For seedling multiplication, the best PGRs were BAP at 1.0 mg/L and NAA at 1.0 mg/L. The combination of PGRs with coconut water gave a reasonable response to the seedling growth and development of *N. cadamba*. The seedlings grew well under 16-h light/ 8-h dark under the room temperature. The seedling growth was not affected by the activated charcoal added in the medium. Soil without any mixture of compost or peat moss was found to be the best potting media for acclimatising *in vitro* seedlings of *N. cadamba* in the present study.

CHAPTER 3

CALLUS INDUCTION OF *NEOLAMARCKIA CADAMBA*

3.1 Introduction

Callus are undifferentiated cell masses that developed by plants due to changes in various biotic and abiotic stimulated in micropropagation techniques (Ikeuchi et al., 2013). The plant can regenerate through callus induction by manipulating the growing plant cells, tissues or organs extracted from the sterile plant material ranging from single cells to stem segments under aseptic and suitable culture conditions. It has been recognised to be the most reliable and cost-effective approach for large-scale propagation of identical offspring in Orchideace (Arditti, 2010), sweet potatoes (Michael, 2011), woody perennial species (Smith, 2013), coconut (Sandoval-Cancino et al., 2016), *Dipteryx alata* (Rodrigo et al., 2018) and tree species in *N. cadamba* (Huang et al., 2020).

Callus refers to the enormous growth of cells associated with wounding in early plant studies. Report by Gerszberg et al. (2016) stated that the callus culture has the potential to increase the multiplication rate of elite genotypes and produce improved cultivars. Callus can be induced from a single differentiated cell, and most of the callus cells are totipotent and able to regenerate into complete plantlet (Davey & Anthony, 2010). The callus had a variety of shapes and classified into several subgroups according to their macroscopic characteristics. For instance, callus that shows some degrees of organ regeneration, generally known as shooty, rooty or embryonic callus depending on the

organs they form. According to Awad et al. (2011) and Lone et al. (2016), callus without apparent organ regeneration are called compact or friable callus.

An important factor that affects the *in vitro* callus initiation and plant regeneration is the hormonal balance in the media composition. Genotype and source of the explants also lead to the differences in the production of callus and regenerated plantlet (Li et al., 2002). According to Centibas and Koyuncu (2006), the standard ratio of both auxins and cytokinins will lead to callus induction. The combination of auxins (2, 4-D) and cytokinins (BAP, TDZ), or single concentration is widely used for callus induction and maintenance (Shukla & Shukla, 2014; Trifunovic-momcilov et al., 2016; Coutiño-Cortés et al., 2017).

The organic compound is one of the factors that affect the number of explants to proliferate. Studies on the effect of organic compounds had been reported in plants such as in orchid, Eucalyptus and woody plant species (Acharyya et al., 2010; Michael, 2011). Coconut water (CW) is usually used as a supplement for callus induction. The varieties of coconut cultivar and maturity stages affect the constituents of CW (Prades et al., 2012). According to Michael (2011), coconut water can increase the number of explants to induce callus and then regenerate into plantlet in a short time.

The source of explants that used to multiply callus culture also influences the effectiveness of shoot proliferation (Frank et al., 2000; Borah et al., 2017). The selection of specific tissues as primary explants depends on the ultimate goal of micropropagation, callus production for *in vitro* selection, or a source of protoplasts or cells for cultures. The response of an explant to axenic conditions may vary widely between families, genera,

species, hybrids, clones and genotypes, and even within the same genotype grown under different environmental conditions. Culture conditions such as temperature, light duration, light intensity and relative humidity are essential factors that are affecting callus induction. Therefore, these factors need to be controlled depending on the requirement of the plant. Effect of photoperiod is also determined as an essential factor in increasing the number of the cell to induce callus.

The abiotic and biotic factors are found to be central factors for success in callus induction and plant regeneration. To date, there is a limited report on the callus induction of *N. cadamba*. Hence, this study aimed to determine the optimum condition of callus induction from young leaf, stem and nodal explants of *N. cadamba*. The essential factors investigated in this study were the type of explants, basal media, photoperiod, coconut water usage either in singly or in combination with plant growth regulators (PGRs) at different concentrations.

3.2 Materials and Methods

3.2.1 Plant materials

Figure 3.1 shows the eight weeks old *in vitro* seedling of *N. cadamba* that used as the source of explants. The young leaf was cut into 5.0 mm x 5.0 mm in size and the nodal explants were excised as shown in Figure 3.1 D.

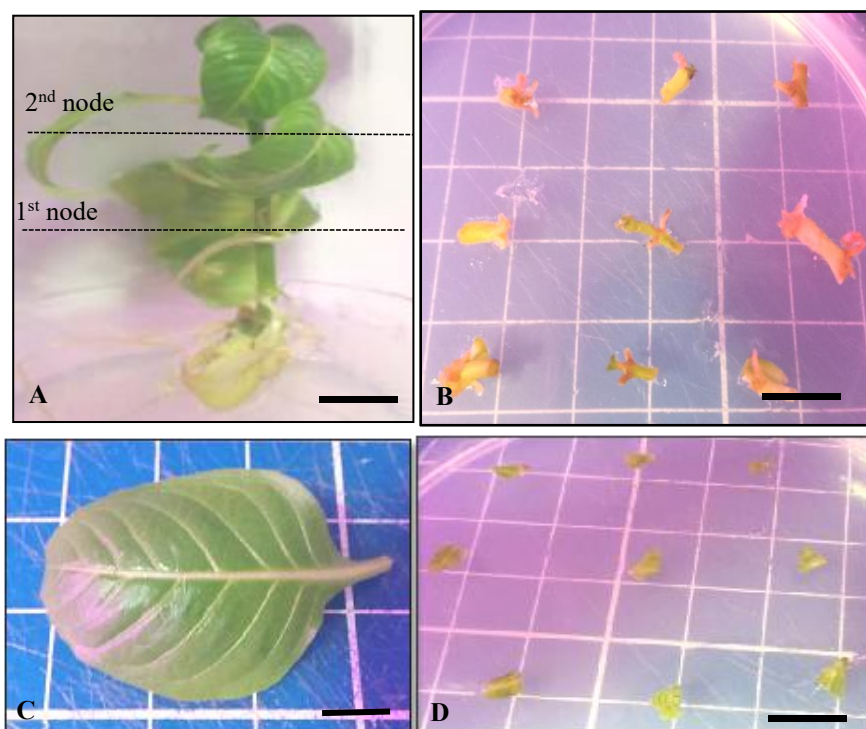


Figure 3.1: Plant material for callus induction: (A) Two months old *in vitro* young seedling of *Neolamarckia cadamba*; (B) Nodal explants with length size 50 mm; (C) *N. cadamba* leaf, and (D) leaf explants with size 5.0 mm x 5.0 mm. Scale A, B, C & D = 1.0 cm.

3.2.2 Medium preparation and culture condition

In this study, the full-strength B5 medium was chosen as the basal media. Culture medium was added with 8.5 gL⁻¹ of agar powder and heated by using microwave (Panasonic) in 2 minutes until the agar melted. Then medium was adjusted to pH 5.5 to 5.7 with 1M NaOH or HCl. The glass bottle containing culture medium was autoclaved at 121 °C and 15 psi for 20 minutes. After that, the autoclaved culture medium was poured into the sterilised Petri dishes, and each Petri dish was sealed with a single layer of para-film to avoid desiccation. All sterilised media were placed at the axenic area under room temperature for 2–3 days before use. All cultures were placed in the dark room for 30 days, and then the cultures were allowed to regenerate and grow at 70–80% relative humidity; light intensity

of 20–50 $\mu\text{mol m}^{-2} \text{s}^{-1}$ provided by red light emitting diode (LED) with duration about at 16-h light and 8-h dark photoperiod in room temperature at 25 ± 2 °C.

3.2.3 Type of explants

Different tissues were used to identify the suitable type of explants for callus induction of *N. cadamba*, such as node, young leaves and stem. All the cultures were cut into the desired size (5.0 ± 0.2 mm x 5.0 ± 0.2 mm). The culture was inoculated in a dark room for 30 days of culture. Then, for shooting and production, the entire cultivation was exposed to 16-h light/ 8-h dark. The B5 medium added with 1.0 mg/L BAP was used as the culture medium.

3.2.4 Type of basal media

The effect of basal media on callus induction of *N. cadamba* was studied. There were four types of basal media tested in this experiment which is half and the full-strength of B5, and MS media added with 1.0 mg/L BAP was used as the culture medium. Young leaves were used as the explants in this study. All cultures were classified in Section 3.2.2 according to the cultural status.

3.2.5 Photoperiod

There are three different types of light duration tested in this experiment, which were 24 hours dark, 16-h light/ 8-h dark and 24-h light. All the light conditions were used to find the best culture condition for callus induction of *N. cadamba*. B5 added with 1.0 mg/L BAP was selected as the culture medium and young leaves were used as the explants in this experiment.

3.2.6 Effect of coconut water

Coconut water (CW) was obtained directly from a young green coconut *var. mawa* (8 months) coconut and filtered to remove the impurities. Then the fresh coconut water was added into B5 medium with different concentrations at 5, 10 and 15 (% v/v). In this experiment, young leaves were used as the explants, and B5 basal medium was used as the control.

3.2.7 Plant Growth Regulators (PGRs)

a) Effect of auxins or cytokinins

Effect of auxins (NAA & 2, 4-D) and cytokinins (BAP & TDZ) alone at a concentration of 1.0, 2.0, 3.0, 4.0 and 5.0 mg/L were studied for the callus induction of *N. cadamba*. B5 medium without any PGRs was used as the control.

b) Combination effect of auxins and cytokinins

The combinations of cytokinin (1.0 mg/L BAP) and auxins which were 2, 4-D and NAA at concentration 0.5 mg/L were tested for callus induction of *N. cadamba*. The best concentration of single PGR from Section 3.2.7 (a) was used in this study.

c) Combination effect of auxins and cytokinins with coconut water

The best PGRs selected from the previous Sections (a) and (b) were 1.0 mg/L BAP and 0.5 mg/L NAA or 2, 4-D were combined with 10% (v/v) coconut water to study the effect for callus induction of *N. cadamba*. In this experiment, B5 medium was used as the basal media.

3.3 Data collection and statistical analysis

The experiments were carried out in a Completely Randomised Design (CRD). The percentage of explants producing callus, necrosis, the intensity of callus formation and time to produce callus were observed and recorded. Qualitative data such as the type of callus texture, colour of callus and morphology variation were observed. Meanwhile, for the nodal segment, number of explants producing the shoot, number of leaves, number of the root, plant height and day start to form shoot were recorded. Any significant morphological variations and changes in callus or shoot were observed.

Each treatment was carried out in five replicates. Each replicate contained nine explants, and the total number of explants was 45 for each treatment. All cultures were observed after five days of culture. Subculturing was carried out at regular intervals. Visual observations of cultures and all the data were collected at 20 days interval up to 60 days on callus proliferation for all treatment. Then, the data were calculated and analysed with Statistical Package Social Science analysis (SPSS) version 22 by using one or two ways Analysis of variance (ANOVA). Subsequently, the post hoc test, Duncan's Multiple Range Test (DMRT) was used to determine the significant difference among treatment means at $p < 0.05$.

3.4 Results

3.4.1 Effect of different types of explant

Based on the observation, the frequency of explants survival and the callogenic response of explants varied with the type of explants. Young leaf explants showed the highest

percentage of explants inducing callus. Hence, young leaves were selected as the explants for subsequent experiments in the present study. Young leaves were started to induce callus after ten days of culture. Within 30 days, 88.89% of the callogenic response was achieved when inoculated in B5 medium added with 1.0 mg/L BAP (Table 3.1). The morphology of callus derived from young leaves of *N. cadamba* was friable and creamy white, as shown in Figure 3.2 B. However, for the node segment, there was no callogenic response observed, but it showed direct shoot organogenesis without forming callus after 1-month of culture (Figure 3.2 A). Meanwhile, the stem explants with appeared callus after 18 days of culture with compact and brownish callus (Figure 3.2 C).

Table 3.1: The effect of different *Neolamarckia cadamba* explants on callus induction after 30 days of culture.

Source of explants	No. and percentage of explants with induced callus (Σ / %)	Necrosis of explant (Σ / %)	The intensity of callus formation	Texture/ color	Time for response (days)	No of shoot regenerated
Node	0 ^a	11/ 24.44 ^b	-	NR	-	21
Young leaves	40/ 88.89 ^c	5/ 11.11 ^a	+++	Friable/ Creamy white	10	0
Stem	12/ 26.67 ^b	33/ 73.33 ^c	+	Compact/ brownish	18	10

Data was obtained from 5 replicates. Each replicate is containing nine explants. Σ No of explant = 45. All data were analysed by using one-way ANOVA. Means within a column with the same letter are not significantly different based on Duncan's Multiple Range Test at $p < 0.05$.

Observation: Profuse callus; +++ (Good callus), ++ (Moderate callus), + (poor callus); - (No response). NR = No response.

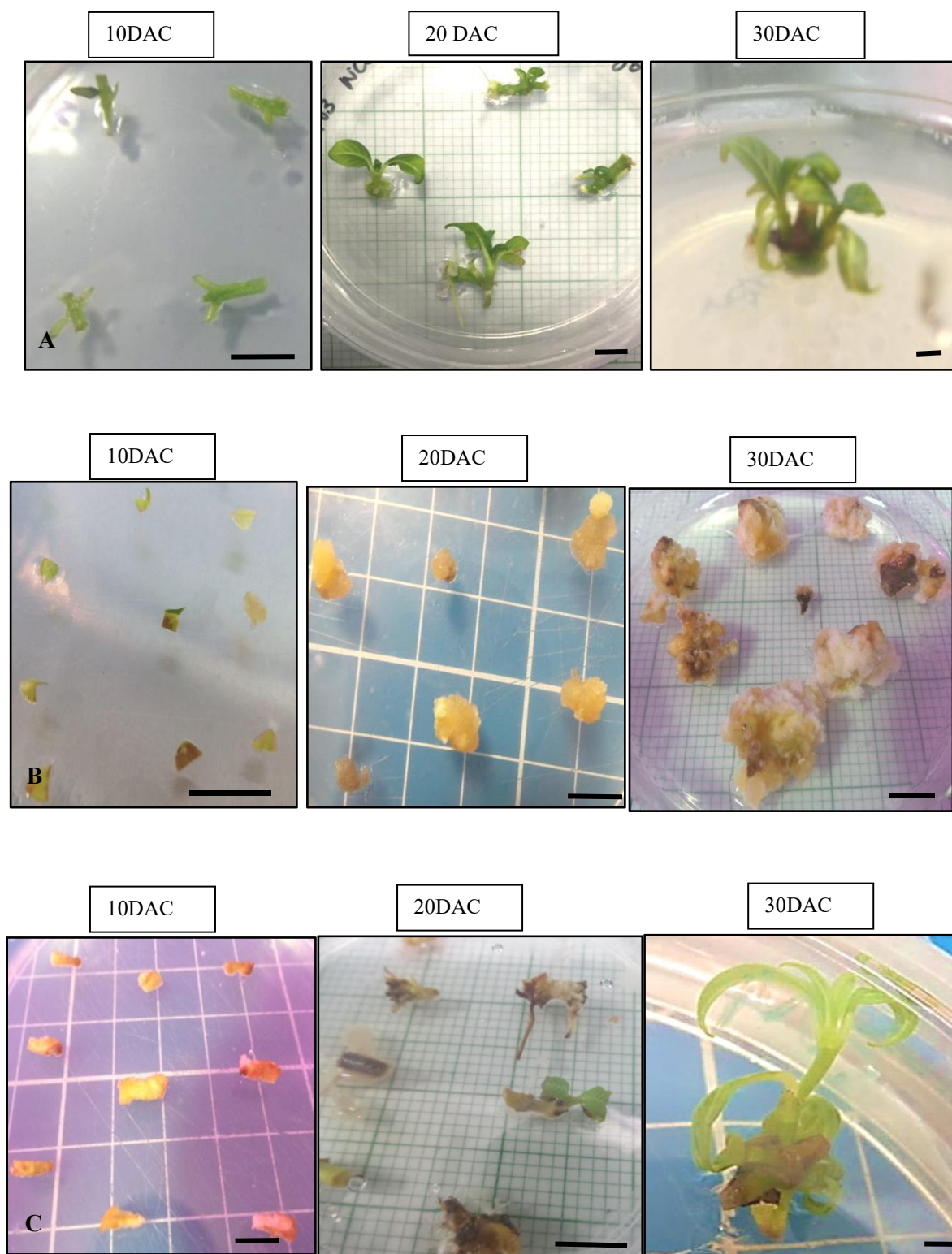


Figure 3.2: Effect of different explants on callus induction of *Neolamarckia cadamba*: (A) node, (B) young leaf; and (C) stem. DAC = days after culture. Scale A–C = 0.5 cm.

3.4.2 Effect of basal media

Four different types of basal media were used to find out the best medium for callus induction of *N. cadamba*. During the subculture, the callus produced a few globular embryo-like structures without any further development on B5 medium (see Figure 3.3 A). After 30 days of culture (DAC), the highest number of explants with induced callus was observed from full-strength B5 medium. Leaf explants started to form a yellowish friable callus at the end of cutting areas on B5 medium after ten days of culture (Figure 3.3 B). After two months of culture, 95.56% of the explants produced callus (Table 3.2). These yellow-white and friable callus were selected, collected in clumps, and transferred onto the fresh B5 medium.

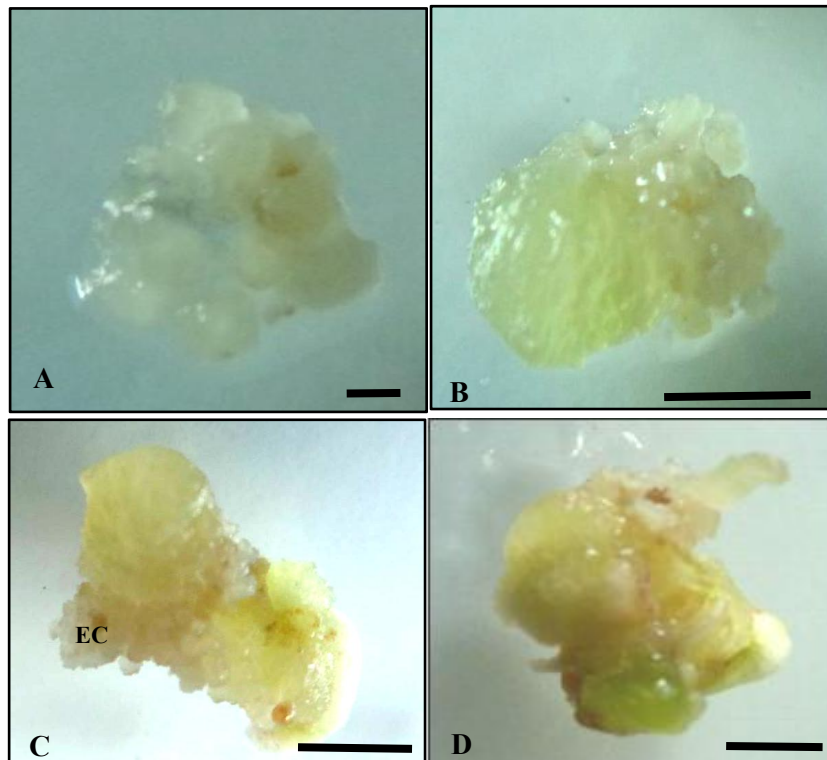


Figure 3.3 A: Morphology of *Neolamarckia cadamba* callus on B5 medium. (A) Initial callus cell; (B) A cluster of callus (C) The embryogenic-like cell (EC) structure and; (D) The globular embryo-like structures without any further development. Scale A = 0.2 cm; and B, C, and D = 0.5 cm.

Table 3.2: The effect of different basal media on callus induction of *Neolamarckia cadamba* young leaf after 60 days of culture.

Types of media	No. and percentage of explants with induced callus (Σ / %)	Necrosis of explants (Σ /%)	The intensity of callus formation	Texture	Colour	Time for response (days)
$\frac{1}{2}$ MS	14/ 31.11 ^a	31/68.89 ^c	⁺	Compact, wet	dark brown	19
$\frac{1}{2}$ B5	22/ 48.89 ^b	23/51.11 ^b	⁺⁺	Soft	off white	15
MS	13/ 28.89 ^a	32/71.11 ^c	⁺	Compact	brownish	18
B5	43/ 95.56 ^c	2/ 4.4 ^a	⁺⁺⁺	Friable	Yellow white	12

Data was obtained from 5 replicates. Each replicate is containing nine explants. Total number of explants is 45 for each treatment. All data were analysed by using one-way ANOVA. Means within a column with the same letter are not significantly different based on Duncan's Multiple Range Test at $p < 0.05$.

Observation: Profuse callus; ⁺⁺⁺ (Good callus), ⁺⁺ (Moderate callus), ⁺ (poor callus); ⁻ (No response). All media were added with BAP 1.0 mg/L + 2% sucrose.

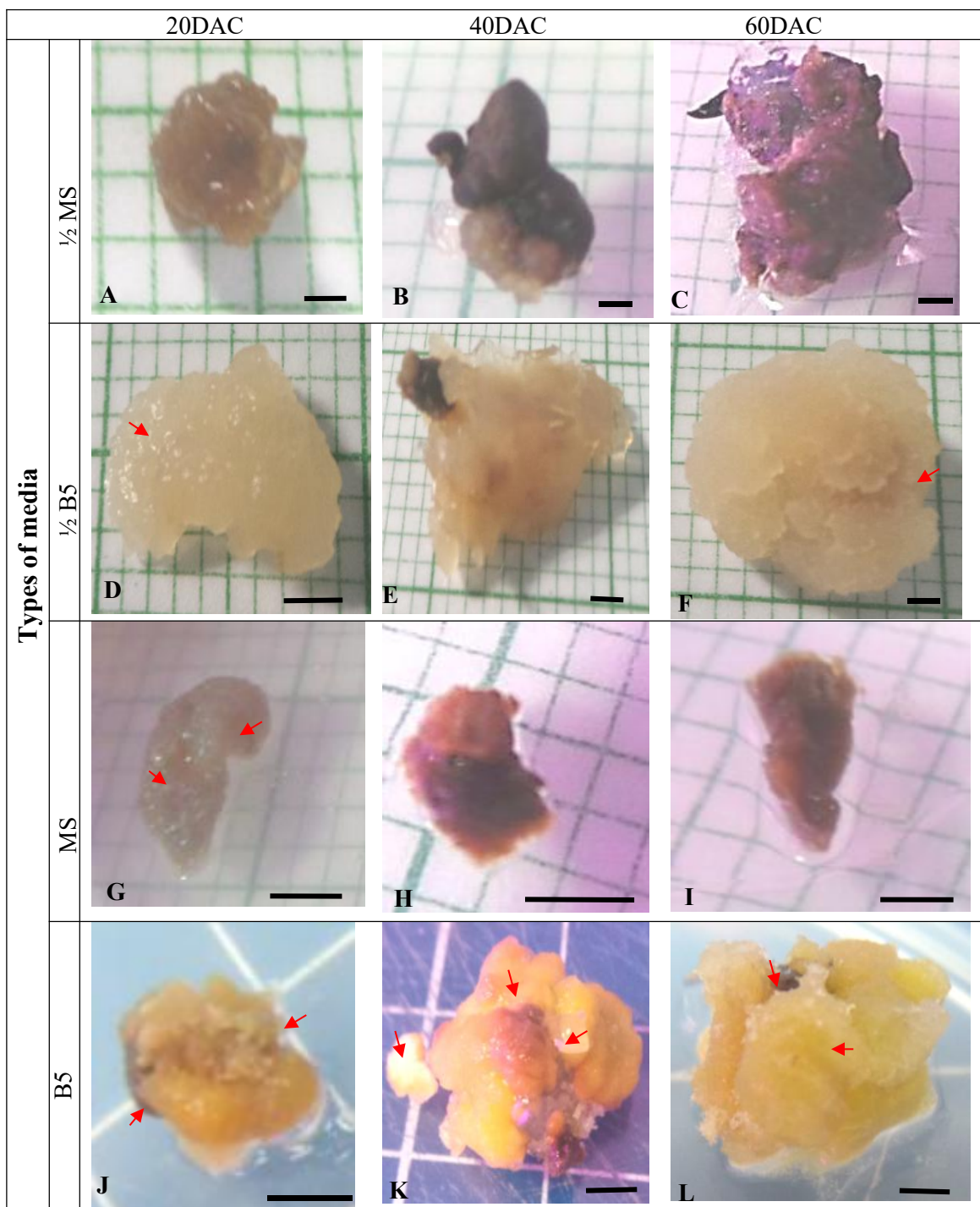


Figure 3.3 B: Effect of different basal media on *Neolamarckia cadamba* callus induction from young leaf at day 20, 40 and 60 of observation. Callus growth; (→). Scale = 0.5 cm.

3.4.3 Effect of photoperiod

The effect of photoperiod was studied in this experiment. Three conditions of light were tested in this experiment. B5 medium was used as the basal medium and supplemented with 1.0 mg/L BAP and 2.0% sucrose. Young leaf explants were started to produce yellowish and loose callus at the end of the cutting areas when cultured in 24-h darkness after ten days of culture (Figure 3.4 A). After 30 days of the culture, the leaf explants were started to form yellowish and compact callus mainly from the cut end that in contact with the B5 medium. After 60 days of culture, the highest number of explants with induced callus was observed in explants cultured in 24-h darkness with 88.89% of explants producing callus (Table 3.3).

The good callogenic response was found in a culture that exposed to the 24 hours dark treatment. The friable and creamy white callus were started to grow at the cut ends of explants that cultured in the darkroom after ten days. 16-h light/ 8h dark treatment had resulted in the moderate response with 66.67% of the explants produced friable and white creamy callus after 15 days of culture. Treatment of 24-h light was observed with a poor callogenic response. All the differences in callus morphology are shown in Figure 3.4.

Table 3.3: Effect of photoperiod on callus induction of *Neolamarckia cadamba* young leaf after 60 days culture.

Photoperiods	No. and percentage of explants with induced callus ($\Sigma/\%$)	Necrosis of explants ($\Sigma/\%$)	The intensity of callus formation	Texture	colour	Time for response (days)
24-h dark	40/ 88.89 ^c	5/ 11.11 ^a	+++	Friable	Creamy white	10
16-h light/ 8h-dark	30/ 66.67 ^b	15/ 33.33 ^b	++	Friable	creamy brown	15
24-h light	10/ 22.22 ^a	35/ 77.78 ^c	+	Compact, wet	brownish	20

Data was obtained from 5 replicates. Each replicate containing 9 explants. Total number of explants is 45 for each treatment. All data were analysed by using one-way ANOVA. Means within a column with the same letter are not significantly different based on Duncan's Multiple Range Test at $p < 0.05$.

Observation: Profuse callus; +++ (Good callus), ++ (Moderate callus), + (poor callus); - (No response). All media were added with BAP 1.0 mg/L + 2% sucrose

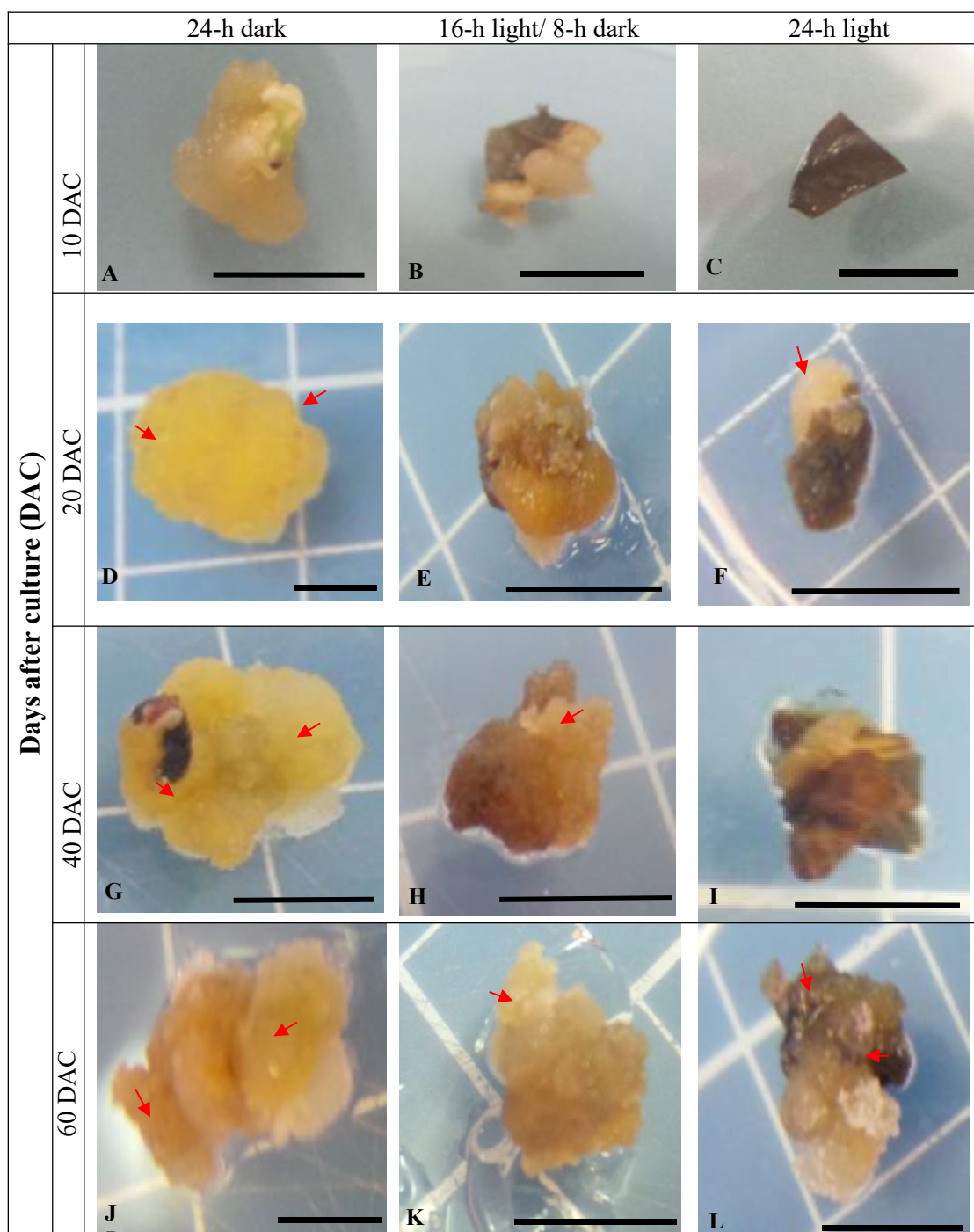


Figure 3.4: Effect of photoperiod on callus induction of *Neolamarckia cadamba* young leaf according to days after culture; h = hours (Scale A–C = 0.2 cm) (Scale D–L = 0.5 cm). (→) = new callus regenerate.

3.4.4 Effect of coconut water

The effect of different coconut water levels which is 5, 10, 15 and 20% (v/v) was carried out in this study. After ten days of culture, young leaf explants were started to form yellowish and loose callus at the end of the cutting area when cultured on a medium that contains 10% and 15% (v/v) of coconut water (Figure 3.5 G). Young leaves were started to induce callus after 14 days of culture on medium added with 10% (v/v) coconut water. Within eight weeks, 66.67% of the callogenic response was achieved when the explants were inoculated on B5 medium added with 10% (v/v) coconut water (Table 3.4). The friable and creamy callus cultured on B5 medium with 10% (v/v) coconut water are shown in Figure 3.5 (G, H, & I).

The lowest number of explants with induced callus was observed when the explants were cultured on medium contained with 5% (v/v) of coconut water, with only about 28.89% of responsive explants after 60 days of culture (DAC) (Table 3.4 & Figure 3.5 M, N & O). However, control medium showed no callogenic response after 60 days of culture (Figure 3.5 A–C).

Table 3.4: Effect of coconut water on callus induction of *Neolamarekia cadamba* young leaf after 60 days of culture (DAC).

Concen tration levels	No. and percentage of explants with induced callus ($\Sigma/\%$)	Necrosis of explant ($\Sigma/\%$)	The intensity of callus formation	Texture	colour	Time for response (days)
Control	0	0	-	-	-	-
5%	13/ 28.89 ^a	32/ 71.11 ^c	+	Loose, wet	creamy yellow	25
10%	30/ 66.67 ^c	15/ 33.33 ^a	+++	Friable	creamy green	14
15%	22/ 48.89 ^b	23/ 51.11 ^b	++	Friable	light Greenish	18
20%	16/ 35.56 ^{ab}	29/ 64.44 ^{bc}	+	Loose & wet	light brown	27

Data was obtained from 5 replicates. Each replicate is containing nine explants. All data were analysed by using one-way ANOVA. Means within a column with the same letter are not significantly different based on Duncan's Multiple Range Test at $p < 0.05$.

Observation: Profuse callus; +++(Good callus), ++ (Moderate callus), +(poor callus) -(No response). All media added with 2% sucrose.

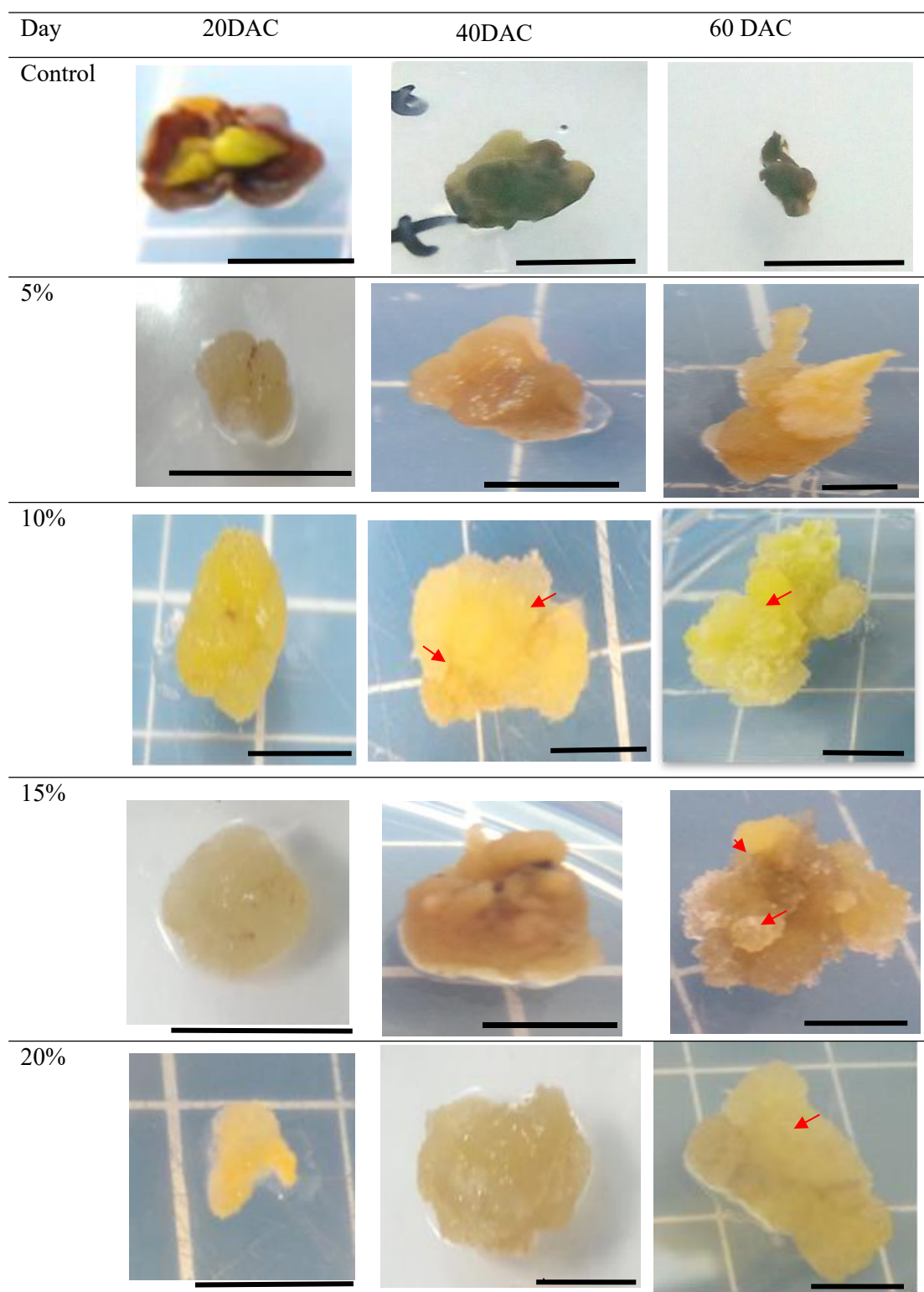


Figure 3.5: Effect of different coconut water concentrations on callus induction of *Neolamarckia cadamba* young leaf at 60 days after culture (DAC); h = hours (Scale = 0.5 cm) (↗) = new callus regenerate.

3.4.5 Effect of Plant Growth Regulators (PGRs)

a) Effect of auxins and cytokinins

The young leaf explants started to induce callus after nine days of culture on B5 medium supplemented with 1.0 mg/L BAP. The good callogenic response was found in B5 medium supplemented with 1.0 mg/L BAP (86.67%) and B5 medium supplemented with 1.0 mg/L NAA (80.0%) within eight weeks inoculation (Table 3.5). Besides, 2, 4-D at 1.0 mg/L also resulted in excellent callogenic response which was about 76%. Callus produced from 1.0 mg/L BAP was friable and greenish (Figure 3.6 C). All the callus obtained from BAP, TDZ, 2, 4-D and NAA have different textures and colour.

TDZ was found not suitable for callus formation, only recorded with about 46.67% of callogenic response from the lowest concentration of TDZ which was 1.0 mg/L (Table 3.5). The high concentrations (3.0, 4.0 and 5.0 mg/L) of TDZ had resulted in a weak response, and the callus formed were loose and wet with off white or dark brown callus (Figure 3.6 L, P & T). All the morphology of callus from the different treatments are shown in Table 3.5 and Figure 3.6.

Table 3.5: Effect of auxins and cytokinins on callus induction of *Neolamarckia cadamba* young leaf at 60 days after culture.

PGRs	Con c. (mg /L ⁻¹)	No. and percentage of explants with induced callus (Σ /%)	Necrosis of explant (Σ /%)	The intensity of callus formation	Texture	colour	Time for respo nse (days)
Control		0 ^a	100 ^f	-	-	-	-
2,4-D	1.0	34/ 75.56 ^{ef}	11/ 24.44 ^{ab}	+++	Friable	creamy green	10
	2.0	32/ 71.11 ^e	13/ 28.89 ^{ab}	+++	Friable	creamy white	12
	3.0	22/ 48.89 ^{cd}	23/ 51.11 ^{bc}	++	Loose	creamy	14
	4.0	15/ 33.33 ^c	32/ 71.11 ^d	+	Loose	creamy	16
	5.0	11/ 24.44 ^{bc}	34/ 75.56 ^d	+	Loose & wet	off white	18
NAA	1.0	36/ 80 ^{ef}	9/ 20 ^a	+++	Friable	Yellowish	10
	2.0	33/ 73.33 ^e	12/ 26.67 ^{ab}	+++	Friable	creamy	12
	3.0	25/ 55.56 ^d	20/ 44.44 ^b	++	Compact	brownish	14
	4.0	18/ 40 ^c	17/ 36.96 ^{ab}	+	Compact	light brown	14
	5.0	14/ 31.11 ^{bc}	21/ 46.67 ^b	+	Loose& wet	black brownish	15
BAP	1.0	39/ 86.67 ^f	6/ 13.33 ^a	+++	Friable	Greenish	9
	2.0	35/ 77.78 ^{ef}	10/ 22.22 ^a	+++	Friable	creamy white	10
	3.0	25/ 55.56 ^d	20/ 44.44 ^b	++	Friable	creamy white	12
	4.0	20/ 44.44 ^{cd}	25/ 55.56 ^{bc}	++	Friable	creamy yellow	14
	5.0	17/ 37.78 ^c	28/ 62.22 ^c	+	Loose &wet	black brownish	15
TDZ	1.0	21/ 46.67 ^{cd}	24/ 53.33 ^{bc}	++	Loose& wet	brown	18
	2.0	16/ 35.56 ^c	29/ 64.44 ^{cd}	+	Loose &wet	brown	20
	3.0	6/ 13.33 ^b	42/ 93.33 ^e	+	Loose & wet	dark brown	21
	4.0	4/ 8.89 ^b	41/ 91.11 ^e	+	Soft & wet	yellowish	25
	5.0	2/ 4.44 ^{ab}	43/ 95.56 ^{ef}	+	Loose &wet	off white	26

All data were analysed by using two-way ANOVA. Means within a column with the same letter are not significantly different based on Duncan's Multiple Range Test at $p < 0.05$.

Observation: Profuse callus; +++ (Good callus), ++ (Moderate callus), + (poor callus), - (No response).

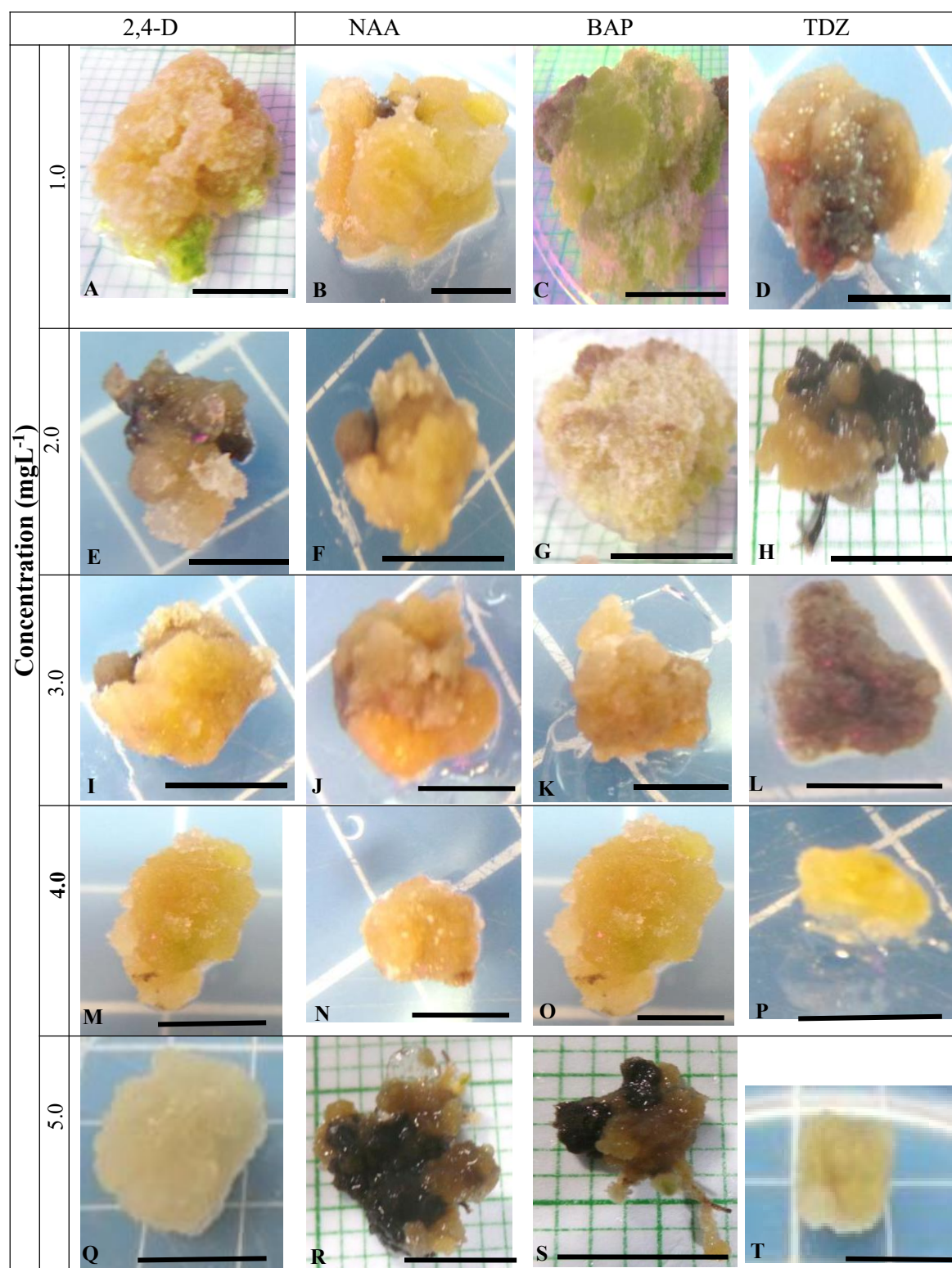


Figure 3.6: Effect of different auxins and cytokinins concentrations on callus induction of *Neolamarckia cadamba* at 60 days after culture. Scale A–T = 0.5 cm

b) Combination effect of auxins and cytokinins

Based on the observation, the frequency of survived explants and the callogenic response of explants were varied with the different type of PGRs used. The changes of explant were started from eight days after cultured on B5 medium added with 1.0 mg/L BAP and 0.5 mg/L NAA. Within eight weeks of inoculation, 95.56% of the callogenic response was achieved when the explants were inoculated in B5 medium added with 1.0 mg/L BAP and 0.5 mg/L NAA (Table 3.6). The callus were friable with greenish coloured (Figure 3.7). The second good callogenic response was found in B5 medium added with 1.0 mg/L BAP and 0.5 mg/L 2, 4-D (88.89%) with friable callus and light green in colour (Table 3.6 & Figure 3.7).

From the observation, the control medium did not give any response to callus induction (Figure 3.7). Plant growth regulators (PGR) used singly in B5 medium (0.5 mg/l 2, 4-D, 1.0 mg/L BAP and 0.5 mg/L NAA) also resulted in good response to callus formation, which recorded 77.78%, 82.22% and 84.44%, respectively (Table 3.6). Overall, the use of PGRs combination was the best medium to optimise the number of explants that producing callus.

Table 3.6: Effect of the combinations auxins and cytokinins on callus induction of *Neolamarckia cadamba* young leaf explants after 60 days of culture.

Conc.	No. and percentage of explants with induced callus	Necrosis of explant (%)	The intensity of callus formation	Texture	colour	Time for response (days)
Control	0 ^a	45/ 100 ^d	-	-	-	-
2, 4-D 0.5	35/ 77.78 ^b	10/ 22.22 ^b	+	Soft	Creamy white	14
NAA 0.5	38/ 84.44 ^b	7/ 15.56 ^b	++	Friable	White-green	13
BAP 1.0	37/ 82.22 ^b	8/ 17.78 ^b	++	Friable	Greenish	12
2, 4-D 0.5 + BAP 1.0	40/ 88.89 ^{bc}	5/ 11.11 ^{ab}	+++	Friable	Light green	10
NAA 0.5 + BAP 1.0	43/ 95.56 ^c	2/ 4.44 ^a	+++	Friable/ compact	Greenish	8

Data was obtained from 5 replicates. Each replicate is containing nine explants. All data were analysed by using one-way ANOVA. Means within a column with the same letter are not significantly different based on Duncan's Multiple Range Test at $p < 0.05$.

Observation: Profuse callus; +++ (Good callus), ++ (Moderate callus), + (poor callus); - (No response). All media added with 2% sucrose.

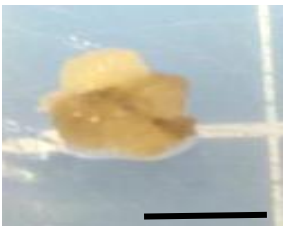
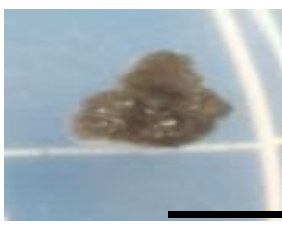
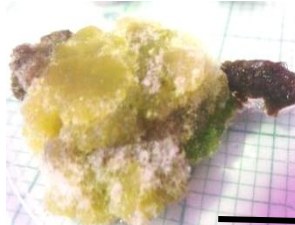
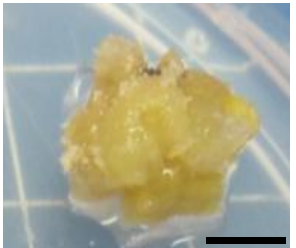
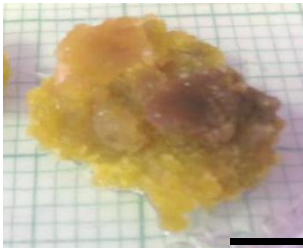
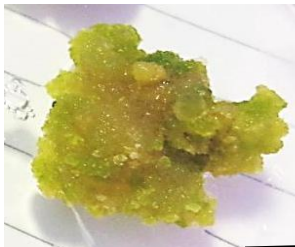
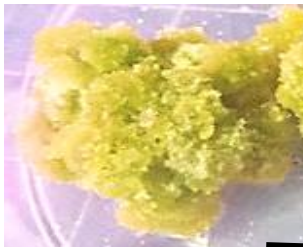
Treatments	20DAC	40DAC	60DAC
Control			
2, 4-D 0.5			
NAA 0.5			
BAP 1.0			
2, 4-D 0.5 + BAP 1.0			
NAA 0.5 + BAP 1.0			

Figure 3.7: Effect of combinations auxins and cytokinins on callus induction from *Neolamarckia cadamba* young leaf explants after 60 days of culture (DAC). Scale = 0.2 cm.

c) Combination effect of auxins and cytokinins with coconut water

In this study, the changes of explant were observed starting from 8 days after cultured on medium added with 1.0 mg/L BAP and 10% (v/v) coconut water. Within eight weeks of inoculation, 86.67% of the callogenic response was achieved when the explants were inoculated on B5 medium added with 1.0 mg/L BAP and 10% (v/v) CW (Table 3.7). The callus were friable with greenish coloured (Figure 3.8). The other good callogenic response was found in B5 medium supplemented with 0.5 mg/L NAA and 10% (v/v) CW (77.78%) with friable and creamy green callus (Table 3.7 & Figure 3.8).

From the experiment, control media did not give any response to the callus induction (Figure 3.8). Overall, the use of auxins or cytokinins in combination with 10% (v/v) coconut water was found to promote callus induction of *N. cadamba*. All the changes and the morphologies of callus from each treatment are shown in Figure 3.8.

Table 3.7: Effect of BAP and NAA in combination with 10% (v/v) coconut water on callus induction of *Neolamarckia cadamba* young leaf explants after 60 days of culture

Treatments	No. and percentage of explants with induced callus (Σ /%)	Necrosis of explant (Σ /%)	The intensity of callus formation	Texture	Colour	Time for response (days)
Control	0 ^a	100 ^d	-	-	-	-
CW	25/ 55.56 ^b	20/ 44.44 ^c	+	Compact	creamy green	15
2, 4-D 0.5 + CW	32/ 71.11 ^c	13/ 28.89 ^b	++	Friable	White green	13
NAA 0.5 + CW	35/ 77.78 ^c	7/ 15.56 ^a	+++	Friable	creamy green	12
BAP 1.0 + CW	39/ 86.67 ^d	6/ 13.33 ^a	+++	Friable	Greenish	8
2, 4-D 0.5 + BAP 1.0 + CW	31/ 68.89 ^{bc}	14/ 31.11 ^b	++	Compact	brownish	14
NAA 0.5 + BAP 1.0 + CW	22/ 48.89 ^b	23/ 51.11 ^c	+	Loose & wet	creamy green	15

Data was obtained from five replicates. Each replicate is containing nine explants. All data were analysed by using one-way ANOVA. Means within a column with the same letter are not significantly different based on Duncan's Multiple Range Test at $p < 0.05$.

Observation: Profuse callus; +++ (Good callus), ++ (Moderate callus), + (poor callus), - (No response).

CW = 10% coconut water (v/v); media added with 2% sucrose

*BAP = 1.0 mg/L

2, 4-D = 0.5 mg/L

CW = 10% (v/v)

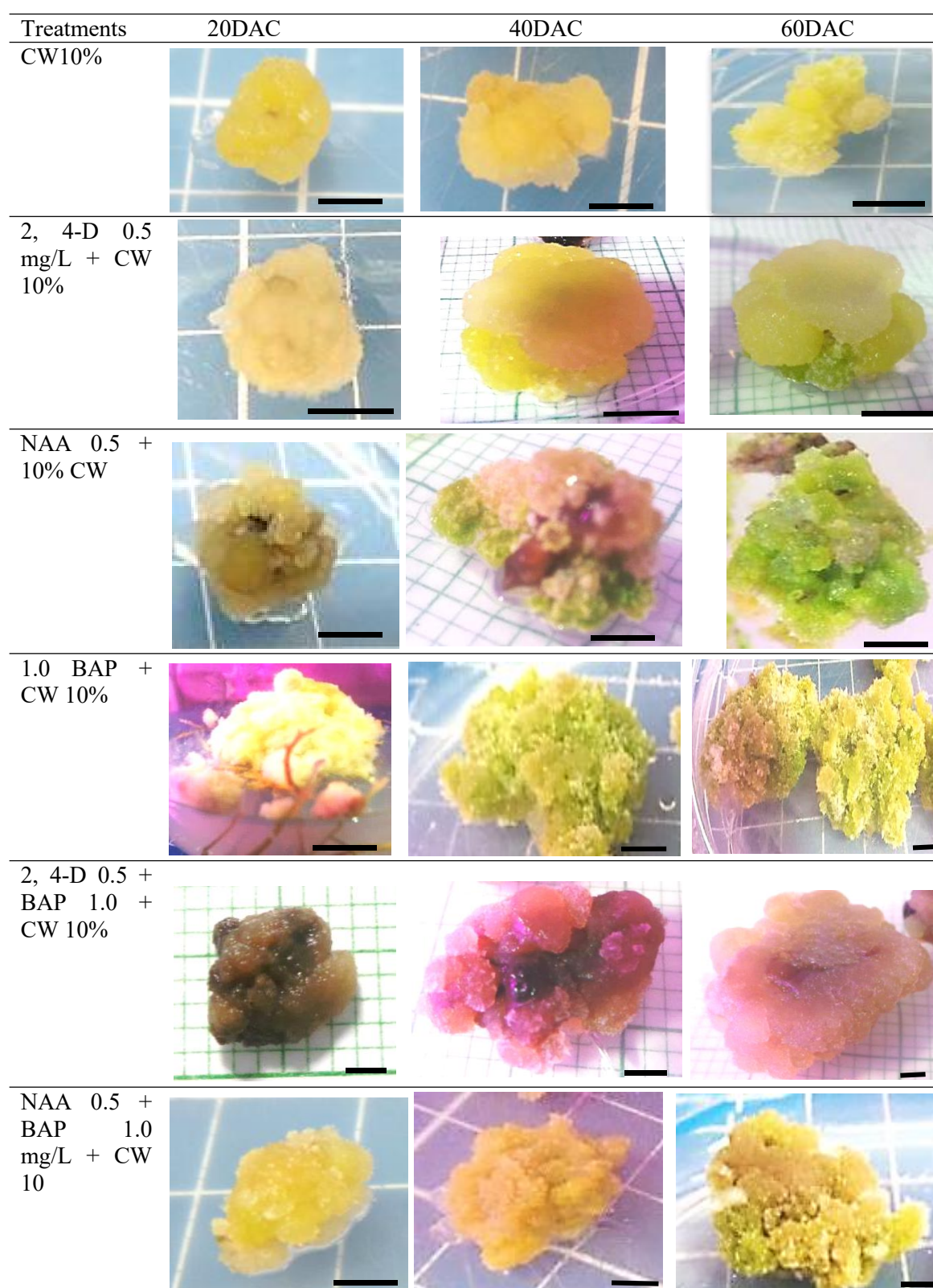


Figure 3.8: Effect of BAP and NAA in combination with 10% (v/v) coconut water for callus induction of *Neolamarkia cadamba* young leaf explants at 60 days after cultured (Scale = 0.2 cm).

3.5 Discussion

3.5.1 Effect of explant types

The types of explants chosen, its size, age and source can affect whether the morphogenesis can induce. According to Sen et al. (2014), different types of explants are good sources of callus induction, morphology analysis as well as indirect plantlets regeneration. This study found that the frequency of survived explants and the callogenic response of explants were varied with the type of explants used, whereby the young leaves explants gave the highest percentage of callus formation when compared with stem and node explants. Besides, stem culture gave a poor callogenic response. However, no callogenic response was observed from the node segment, but it showed direct shoot organogenesis without forming callus after four weeks of culture.

Direct shoot regeneration in *N. cadamba* has been attempted by using explants of both juvenile (seedling) and mature tree origins. Rehabilitation has been only moderately successful in juvenile-origin explants due to contamination and heavy leaching of phenolic (Karina et al., 2015). Micropropagation and regeneration have been reported in several species of woody plants by using different explants; e.g., immature cotyledons and embryos of *Juglans regia* (Cai et al., 2013), young leaves of *Melicope lunu-ankenda* (Rahman et al., 2015b), shoot explants of *Eucalyptus dunii* (Oberschelp et al., 2015), buds of *Hylocereus polyrhizus* (Qin et al., 2017), and leafy shoots of *Phoenix dactylifera* L.-date palm (Mazri et al., 2017). Nodal sub-culturing in shoot proliferation has been successful for some hardwoods and many species of oak (Chavarriaga-Aguirre et al., 2016).

Huang et al. (2014) reported a procedure for adventitious buds induction from the cotyledons of *N. cadamba*. The direct regeneration protocol is ideal for micropropagation in order to obtain a large number of elite seedlings, because buds or shoots originate directly from epidermal cells, passing through a prominent callus stage. In this study, the most critical factors that influenced the *N. cadamba* callus induction are the types of explants used and nature of the explants, as well as the age of seedling. For example, in research conducted by Huang et al. (2020), callus derived from petiole-free cotyledons did not give rise to adventitious buds, suggesting a strong influence of the type of explants and bud formation was also affected by the age of the explants. The tissue culture derived from old explants may also have difficulty distinguishing the cell on the induction medium.

3.5.2 Type of basal media

The selection of basal media in plant tissue culture is significant in determining the success of morphogenesis. According to Huang et al. (2020), media like WPM, MS and B5 are used to provide water, nutrients, acid amino, vitamins, and growth regulators for the tree species plants. In this study, the best medium used for inducing callus from *Neolamarckia cadamba* young leaf explant was full strength B5 medium.

The high salt concentration in the MS medium could inhibit plant growth and results in the death of the plant if salts are present in excess. Stressed plants could affect the metabolic process of plant-like photosynthesis, energy and lipid metabolism, and protein synthesis. According to Gulser et al. (2010) and Shafi et al. (2015), photosynthetic mechanisms are affected directly or indirectly attributable to the modified structural organisation and physicochemical properties of thylakoid membranes by salinity stress.

The photosynthesis will change the plant growth since it absorbs and converts light energy into sugar.

On the other hand, B5 medium is commonly used for callus or suspension culture, but it also works as a basal medium for plant regeneration. In the cases of *Achyranthes aspera* and *N. cadamba*, B5 medium had resulted in the maximum callus proliferation as compared to the other media (Huang et al., 2014; Rahman et al., 2015a). Besides, the B5 medium was known as the most likely medium influencing the cell division and plant regeneration (Sen et al., 2014). A similar finding was also observed in the present study whereby the best media for callus induction and proliferation of *Neolamarckia cadamba* was B5 medium.

3.5.3 Effect of photoperiod

Light is essential for the growth and development of plants. Light requirements are different between the plant species. Photoperiod controls many developmental responses in plants. This is because day length is a reliable indicator of the time of year, enabling developmental events to be scheduled to coincide with particular environmental conditions.

Photoperiodism is the physiological reaction of organisms to the length of day or night. Photoperiodism can also be defined as the developmental responses of plants to the relative lengths of the light and dark periods. The photoperiod is the amount of light and darkness in a daily cycle of 24 hours (Kumar et al., 2018). It is different among plants such as woody plant, herbs, ornamental and palm trees. The present study showed that treatment

with 24-h darkness was the best condition for callus induction of *N. cadamba*. The difference was observed not only in the percentage of explants producing callus, the intensity of profuse callus appearance but also the early response of explants to induce callus. This result also similar to Khan et al. (2002), callus induction frequency of *Bixxa orellana* L. was greater under 24 h dark as compared to 16-h light/ 8-h dark photoperiod or 24-h light photoperiod. These findings may be due to the interaction between light intensity and internal factors which directly affect callus reproduction. However, Li et al. (2019) stated that 16-h light/ 8-h dark photoperiod is the best culture condition to induce high-frequency plant regeneration from leaf culture of *N. cadamba*.

According to Choudhary et al. (2017), shortest light hours (8:16) were insufficient to the proliferation of callus in leaf, shoot bud induction and *de novo* shoot regeneration. However, Aparna et al. (2017) and Zakizadeh et al. (2013) reported that no significant differences among various photoperiods (8:16, 10:14, 12:12) through increasing callus diameter in *Gliricidia* and *Amaryllis*, respectively. Although light is an important factor in micropropagation, reports on the effect of artificial light intensities on plant growth, particularly of woody or tree species are rather scarce. Several aspects, such as wavelength (quality), intensity (quantity) and duration of light, are important factors affecting plant growth (Li et al., 2019). For relatively short time periods, plant performance probably reflects the photosynthetic process. In general, plant cell differentiation, division and regeneration are affected by both internal factors including genotype and plant hormones and external factors such as light, duration, temperature and moisture supply.

Effect of photoperiod on callus induction of *N. cadamba* was also influenced by the genotype and environmental factors (air temperature, relative humidity and oxygen) (Huang et al., 2020; 2014). In addition, Cardoso (2018) stated that culture under the 24-h light in a short or long term would cause stimulation or inhibition on plant cell division and regeneration of *Gerbera jamesonii*. Light shows a significant role for *in vitro* plants for photosynthesis process. The explants expose to light at low or high intensity will wax the leaves of *N. cadamba*, and ultimately the plant cell will not be able to differentiate, divide and regenerate. Amount of light required by each plant species is different depending on the environment of their habitat. Most of the woody plants required the exposure to light at around 65% to 70%, with the range of light intensity between 1500 to 4000 lux (Armijos-González, & Pérez-Ruiz, 2016).

3.5.4 Effect of coconut water

Organic additive enhances the callus induction and shoot multiplication of *Neolamarckia cadamba*. The biochemical components in coconut water include various minerals, amino acids and, vitamins like ascorbic acid and B group vitamins. All these supplements are essential in promoting the division and regeneration of plant cells. In this research, the addition of coconut water at 10% (v/v) can induce callus production and proliferation. A suitable complex additive can contribute to the establishment of an efficient and economical protocol for plant tissue culture. They contain a variety of substances that could promote germination and growth, and not restricted to any species. A specific growth factor is possible to be found in additives, as some of the complex additives used may contain little or no sugar or mineral (Arditti, 2010; Abrahamson et al., 2017).

Coconut water (CW) was first utilised in tissue cultures in order to enhance the growth of *Datura stramonium* embryos. Previous studies had shown that the CW contains carbohydrate, vitamin, amino acid, organic acid, organic ion and enzyme that play an essential role in plant cell development (Jainol & Jualang 2017). The biochemical components of organic additive like coconut water (*Cocos nucifera* L.) include various cationic minerals (potassium, calcium, phosphorus, sodium, iron and copper), amino acids, and vitamins like ascorbic acid and B group vitamins (Gnasekaran et al., 2012; Yong et al., 2014). Moreover, coconut water contains amino acids like glutamine, arginine, asparagine, alanine and aspartic acid. In this study, it is suggested that all of these supplements provided by coconut water at 10% (v/v) are essential in promoting both the differentiation and regeneration of plant cells due to coconut water contained a high amount of natural sugar. Yong et al. (2009) and Arditti (2010) reported that coconut water contains up to 50.0 g per 100 g per one fruit of coconut water. Meanwhile, the natural sugars present are in the form of glucose and fructose that are quickly absorbed by the plant cells for cellular proliferation and regeneration (Sarropoulou, et al., 2016; Haque et al., 2017).

3.5.5 Effect of plant growth regulators (PGRs)

a) Effect of auxins and cytokinins

Auxins is vital to plant cell division, cell elongation and swelling of tissues and callus (Krupa-Malkiewicz & Mglosiek, 2016). Auxins is also involved in embryogenesis in suspension culture. Plant growth regulators (PGRs) that belong to this group is indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), naphthaleneacetic acid (NAA) and 2, 4-dichlorophenoxyacetic acid (2, 4-D). Report by Mateus et al. (2018) had shown that

the addition of auxins exceed 5.0–10.0 mg/L can cause death and mutations in plant cells. It reinforces that all types of auxins in this concentration range will result in inhibition of plant growth, necrosis and finally death. The present study found that the B5 medium with 1.0 mg/L BAP or 1.0 mg/L NAA gave an excellent callogenic response within eight weeks of inoculation. The callus produced from BAP or NAA at 1.0 mg/L were friable with a greenish colour.

According to Dutta et al. (2013) and Pádua et al. (2018), both NAA and 2, 4-D are often used in low concentrations, and quantities required are around 0.01–2.0 mg/L. This observation was in agreement to report by Huang et al. (2014), which had shown that the addition of lower NAA concentration (1.0 to 1.5 mg/L) could promote callus induction from cotyledon leaf explants. BAP is one of the commonly used cytokinins, and it is added to the culture medium at a concentration lower than 10.0 mg/L. Concentrations exceeding 10.0 mg/L can inhibit plant growth and development. The types and concentrations of cytokinins are essential for *in vitro* propagation (Su et al., 2011; Bekheet et al., 2018). Besides, many previous studies had concluded that the use of PGRs in the study of woody plant proliferation singly or combined could enhance the cell division and proliferation of callus, or even in the regeneration of plantlets. Furthermore, according to Raj et al. (2020) study in *Aegle marmelos* callus formation, the highest induction of the shoot buds (4.1) was observed using a woody plant medium with 100% morphogenetic response using 2.0 mg/L BAP. On the Murashige and Skoog medium, a maximum callus induction on leaf explant was observed with 40% morphogenetic response followed by woody plant medium (30%) with 2, 4-D at 2.0 mg/L (Raj et al., 2020).

In this study, TDZ was found to be inappropriate for callus formation of young leaves *N. cadamba*, only recorded at about 46.67% of callogenic response from the lowest concentration of 1.0 mg/L TDZ. The high concentrations (3.0, 4.0 and 5.0 mg/L) of TDZ resulted in a poor response, and the callus developed with white or dark brown callus became loose and wet when continue to expose to high TDZ concentration under 60 days of culture. This observation was similar to Huang et al. (2020) that high concentration (more than 11.35 μ M) of TDZ was harmful to shoot proliferation and development of *N. cadamba*.

b) Combination effect of auxins and cytokinins

Cytokinins include BA (benzyl adenine), 2ip (2-isopentyl adenine), kinetin, zeatin, thidiazuron (TDZ), Zeatin riboside, N'phenylurea, N-(2-chloro-4-pyridyl) and BAP (Benzylamine purine). Cytokinins act to stimulate the cell division, bud growth, cell specialisation and shoots regeneration. If added together with auxin can promote cell division and formation of callus (Aggrey et al., 2003; Adriana et al., 2016). This study showed that 95.6% of the callogenic response was achieved when the explants cultured on B5 medium added with 1.0 mg/L BAP and 0.5 mg/L NAA within eight weeks of inoculation (Figure 3.5). Plant growth regulators (PGRs) used solely in medium gave the second highest percentage of explants that produced callus (0.5 mg/L 2, 4-D, 1.0 mg/L BAP, 0.5 mg/L NAA). Overall, the use of PGRs combination was optimal to increase the number of explant produced callus.

Based on the observation in this study, 95.56% young leaves induced friable and greenish callus within eight weeks of inoculation using B5 medium added with 1.0 mg/L

BAP and 0.5 mg/L NAA. According to Arya and Arya (2015), a combination of 10 μ M 2,4-D with 0.88 μ M BAP in *D. falcatum* (edible bamboo) enhanced the callus proliferation to 2–3 folds. Also, a report by Gao et al. (2019), the combination of 0.25 mg/L BAP and 1 mg/L NAA was the best medium for callus proliferation of *Pinus koraiensis*. In comparison, the mixture of 2, 4-D and BAP also had a more proliferative effect on the callus.

c) Combination effect of auxins and cytokinins with coconut water

This study showed that the addition of 10% (v/v) coconut water with PGRs (BAP or NAA) at 1.0 mg/L given an excellent callogenic response with friable and creamy green callus produced. This result indicated that the use of a combination of PGRs with 10% (v/v) coconut water could optimise the callus proliferation of *N. cadamba*. There is no report yet regarding the usage of coconut water in combination with auxins and cytokinins for callus induction in tree species.

According to Shekarriz et al. (2014), the use of liquid endosperm of coconut as a nutrient additive was good for *Phalaenopsis* species. This report was similar to the present findings, where the seedlings of *Neolamarckia cadamba* were healthy and grown-well after 60 days of inoculation. Besides, many reports showed that the use of coconut water was suitable for inducing cell division in quiescent cells of *Cassia angustifolia* (Agrawal et al., 2007), sweet potato (Micheal, 2011), and orchid species (Arditti, 2010; Sherif et al., 2016; Jainol & Jualang, 2017).

Furthermore, the effects of the combination of auxins and cytokinins are often complicated, and more than one combination of two substances is likely to produce a high yield. However, in this study, the combination of PGRs with coconut water had resulted in a positive response in plantlet growth. Subsequently, the balance between auxin and cytokinin often required for the formation of the adventitious shoot and root meristems (Banasiak, 2011; da Cruz et al., 2014). The requisite concentration of each plant growth regulator differ significantly according to the types of the plant being cultured, the cultural conditions and the compounds use. Finally, the combination of PGRs with coconut water was the potential to promote cell division and callus growth.

3.6 Conclusion

The optimum protocol for callus induction from the young leaf of *N. cadamba* was using full-strength B5 basal media supplemented with BAP at 1.0 mg/L, NAA at 1.0 mg/L and 10% (v/v) coconut water . The good callogenic response for *N. cadamba* was found in a culture that exposed to the 24-h dark culture incubation.

CHAPTER 4

INDIRECT SHOOT REGENERATION OF *NEOLAMARCKIA CADAMBA*

4.1 Introduction

Organogenesis involved *in vitro* formation and development of organs such as shoots and roots either directly from explants or indirectly via callus differentiation through wounded parenchyma. Chen et al. (2016) stated that *in vitro* propagation through organogenesis usually involves three critical stages which are dedifferentiation, induction and differentiation (Figure 4.1). The process of dedifferentiation involves the differentiated cells in explants that dedifferentiate and revert to a less committed and more flexible developmental state that may or may not involve callus tissue.

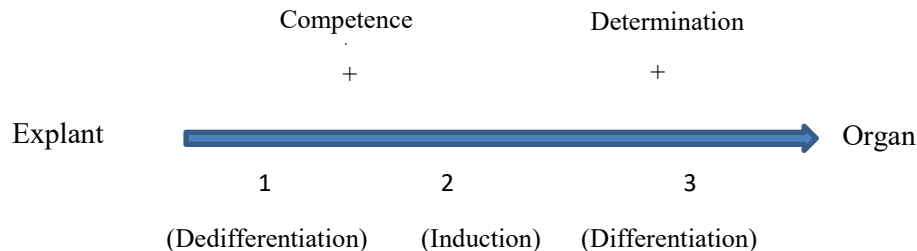


Figure 4.1: Three phases of organogenesis (Adapted from Chen et al., 2016).

In the indirect shoot regeneration, cells from explants dedifferentiate to give rise to callus formation from which the new shoots produced (Pindel, 2017; Dolce et al., 2018). The result of the first phase is that these differentiated cells in explants may become competent and capable of responding to specific inducing signals. Plant growth regulators are one of these inducing signals through which cells become competent. Its followed by induction, which the cells become fully committed to a determined state for the production

of shoots or roots. The morphological differentiation and development of the nascent shoot or root then proceed, eventually resulting in a functional organ (Piyada et al., 2015; Bakhshaie, 2016).

The main objective of this study was to establish an efficient protocol for indirect shoot regeneration of *N. cadamba* from the young leaf explants. The experiment was carried out to find the suitable concentration of BAP (cytokinin) and NAA (auxin) at single concentration or combination. The combination of coconut water in media with the optimum concentration of BAP or NAA was also studied.

4.2 Materials and Methods

4.2.1 Plant materials

Plant materials used in this research was callus cultures from the young leaf explants of *N. cadamba* leaf derived from two months old *in vitro* young seedling. The leaf was cut in size 5.0 mm x 5.0 mm (see Figure 4.2).

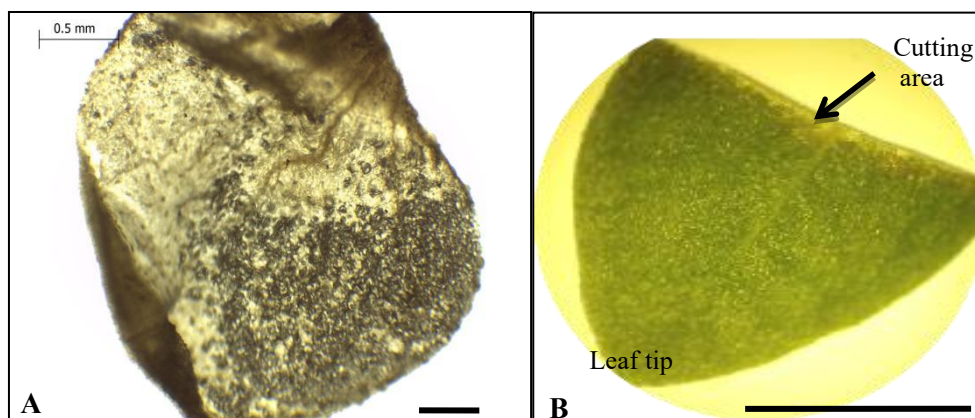


Figure 4.2: (A) Non-cutting leaf of *Neolamarckia cadamba*; and (B) Leaf after cutting in size 5.0 mm x 5.0 mm use as explant on indirect shoot regeneration study (under light microscope with 40x magnification). Scale A = 0.5 mm; and B = 5.0 mm

4.2.2 Media preparation and culture condition

The full-strength B5 medium was selected in this study based on the previous findings. Callus was inoculated on the culture medium, either alone or supplemented with BAP, NAA or combination with coconut water as a complex additive. Callus cultures were placed in the culture room with 24 hours in the dark for four weeks. Then, the cultures were maintained at $25 \pm 2^{\circ}\text{C}$ under 16-h light/ 8-h dark photoperiod at 70–80% relative humidity; light intensity of $20\text{--}50 \mu\text{mol m}^{-2} \text{s}^{-1}$ provided by red light emitting diode (LED).

4.2.3 Indirect shoot regeneration

a) Effect of BAP or NAA

B5 medium added with BAP and NAA at the different levels of concentration such as 1.0, 2.0, 3.0, 4.0 and 5.0 mg/L were studied.

b) Effect of different combinations of BAP and NAA

Explants were inoculated on the B5 media supplemented with the best concentration level of BAP at 2.0 mg/L (from Section 4.2.3 a) and NAA at 0.5, 1.0, 2.0 or 3.0 mg/L. B5 medium used as a basal media and media without PGR was using as a control.

c) Effect of the combinations of BAP and CW

B5 medium supplemented with 2.0 mg/L of BAP mix with coconut water (CW) at 10, 20, 30 % (v/v) and all culture inoculated at culture room.

4.3 Data collection and analysis

Each treatment was carried out in five replicates. The cultures were observed starting from 10 days interval up to 120 days after culture. The percentage of number of adventitious shoot and the mean number of shoot length were observed and calculated. Others parameter observed is the mean number of leaves and roots were calculated. Then, the morphological changes of plants were also observed and recorded. All experiments were conducted in a completely randomised design (CRD). All means were analysed using the software, SPSS by using one-way Analysis of variance (ANOVA) versions 22. Significance differences were assessed using Duncan's Multiple Range test (DMRT) at the 5% probability level.

4.4 Results

4.4.1 Effect of different concentrations of auxins and cytokinins

In this study, the changes in explant were observed starting from 16 days after cultured on medium added with 2.0 mg/L BAP. Within 16 weeks of inoculation, 34.67% of the explants forming adventitious shoot and regenerated into the complete plants on B5 medium added with 2.0 mg/L of BAP. This resulted in plantlets with an average of 2.06 cm of shoot length, 4.20 mean number of leaves and 3.20 average number of roots produced (Table 4.1). The first shoot was started to grow after 16 days cultured on B5 medium added with BAP or NAA at 2.0 mg/L (Table 4.1, Figure 4.3 & Figure 4.4). The B5 medium supplemented with 2.0 mg/L of NAA was recorded with 29.78% of conversion rate, 1.90 cm mean shoot length and 3.60 mean number of leaves (Table 4.1 & Figure 4.4). From the experiment, the control did not give any response to induce shoot. Overall, the

use of single BAP (refer to Figure 4.3) and NAA (Figure 4.4) at 2.0 mg/L can induce the shoot regeneration of the callus cells of *N. cadamba*.

Table 4.1: Effect of BAP and NAA to indirect shoot regeneration of *Neolamarckia cadamba* after 16 weeks of culture.

Treatments	Conc (mg/L)	No. of adventitious shoot (%)	Mean of shoot length (cm)	Mean no. of leaves	Mean no. of roots	Day start to produce shoot (days)
Control		0 ^a	0 ^a	0 ^a	0 ^a	NR
BAP	1.0	10.22 ^d	1.20 ^b	1.40 ^b	0.20 ^b	18
	2.0	34.67 ^f	2.06 ^{de}	4.20 ^c	3.20 ^c	16
	3.0	7.56 ^c	1.18 ^b	1.20 ^b	0 ^a	30
	4.0	0 ^a	0 ^a	0 ^a	0 ^a	-
	5.0	0 ^a	0 ^a	0 ^a	0 ^a	-
NAA	1.0	9.78 ^{cd}	1.10 ^b	1.80 ^b	0 ^a	21
	2.0	29.78 ^e	1.90 ^{bc}	3.60 ^c	0.60 ^b	16
	3.0	2.22 ^b	2.40 ^c	1.20 ^b	0 ^a	35
	4.0	0 ^a	0 ^a	0 ^a	0 ^a	-
	5.0	0 ^a	0 ^a	0 ^a	0 ^a	-

Data analysis was performed from 5 replicates by using two-way ANOVA. Means within a column with the same letter are not significantly different based on Duncan's Multiple Range Test at $p < 0.05$. NR = Not response; Conc = concentration; wks = weeks.

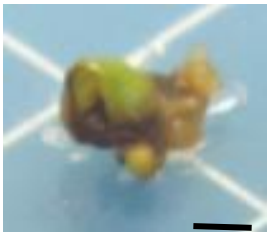
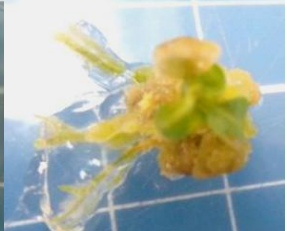
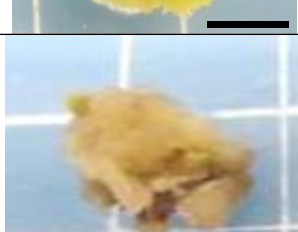
Cytokinins	Conc (mg/L)	2 weeks	4 weeks	16 weeks
BAP	Control			
	1.0			
	2.0			
	3.0			
	4.0			
	5.0			
				

Figure 4.3: Effect of BAP to indirect shoot regeneration of *Neolamarckia cadamba* after 16 weeks of culture. Scale = 0.2 cm.

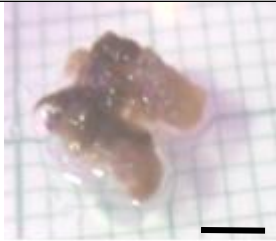
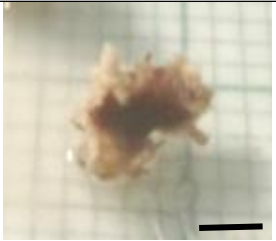
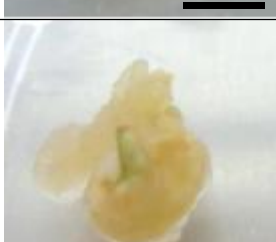
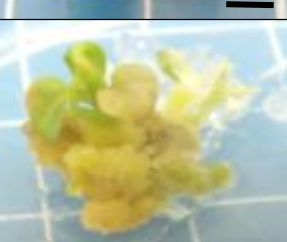
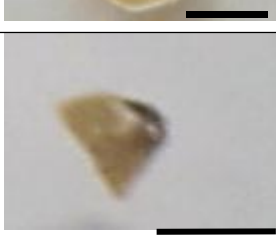
Auxins	Conc (mg/L)	2 weeks	4 weeks	16 weeks
NAA	Control			
	1.0			
	2.0			
	3.0			
	4.0			
	5.0			

Figure 4.4: Effect of NAA to indirect shoot regeneration of *Neolamarckia cadamba* after 16 weeks of culture. Scale = 0.2 cm.

4.4.2 Effect of different combinations of BAP and NAA on indirect shoot regeneration

Shoot regeneration was initiated by inoculating the leaf-derived callus onto B5 medium fortified with different combinations of BAP 2.0 mg/L and NAA at 0.5, 1.0, 2.0 and 3.0 mg/L as shown in Table 4.2. Formation of shoots from the callus was observed after four weeks of culture. A higher percentage of responding culture (57.8%) was observed in the B5 medium added with 0.5 mg/L NAA that resulted in an average 6.40 of leaves and 8.80 roots produced after 16 weeks of culture. All the differences between the treatment and the morphology of callus shown in Table 4.2 and Figure 4.5.

Table 4.2: Effect of the combinations of BAP with different concentration of NAA on indirect shoot regeneration of *Neolamarckia cadamba* after 16 weeks of culture

Treatments	No. of adventitious shoot (%)	Mean of shoot length (cm)	Mean no. of leaves	Mean no. of roots	Days required for shoot induction (days)
Control (BAP)	37.88 ^c	3.40 ^b	4.20 ^b	5.80 ^c	19
BAP + NAA 0.5	57.78 ^c	5.20 ^c	6.40 ^d	8.80 ^d	16
BAP + NAA 1.0	53.33 ^d	4.80 ^c	5.20 ^c	6.40 ^c	21
BAP + NAA 2.0	20.00 ^b	1.80 ^a	2.40 ^a	3.80 ^b	25
BAP + NAA 3.0	13.33 ^a	1.20 ^a	1.80 ^a	2.40 ^a	28

Data analysis was obtained from 5 replicates by using one-way ANOVA. Means within a column with the same letter are not significantly different based on Duncan's Multiple Range Test at $p < 0.05$. BAP = 2.0 mg/L

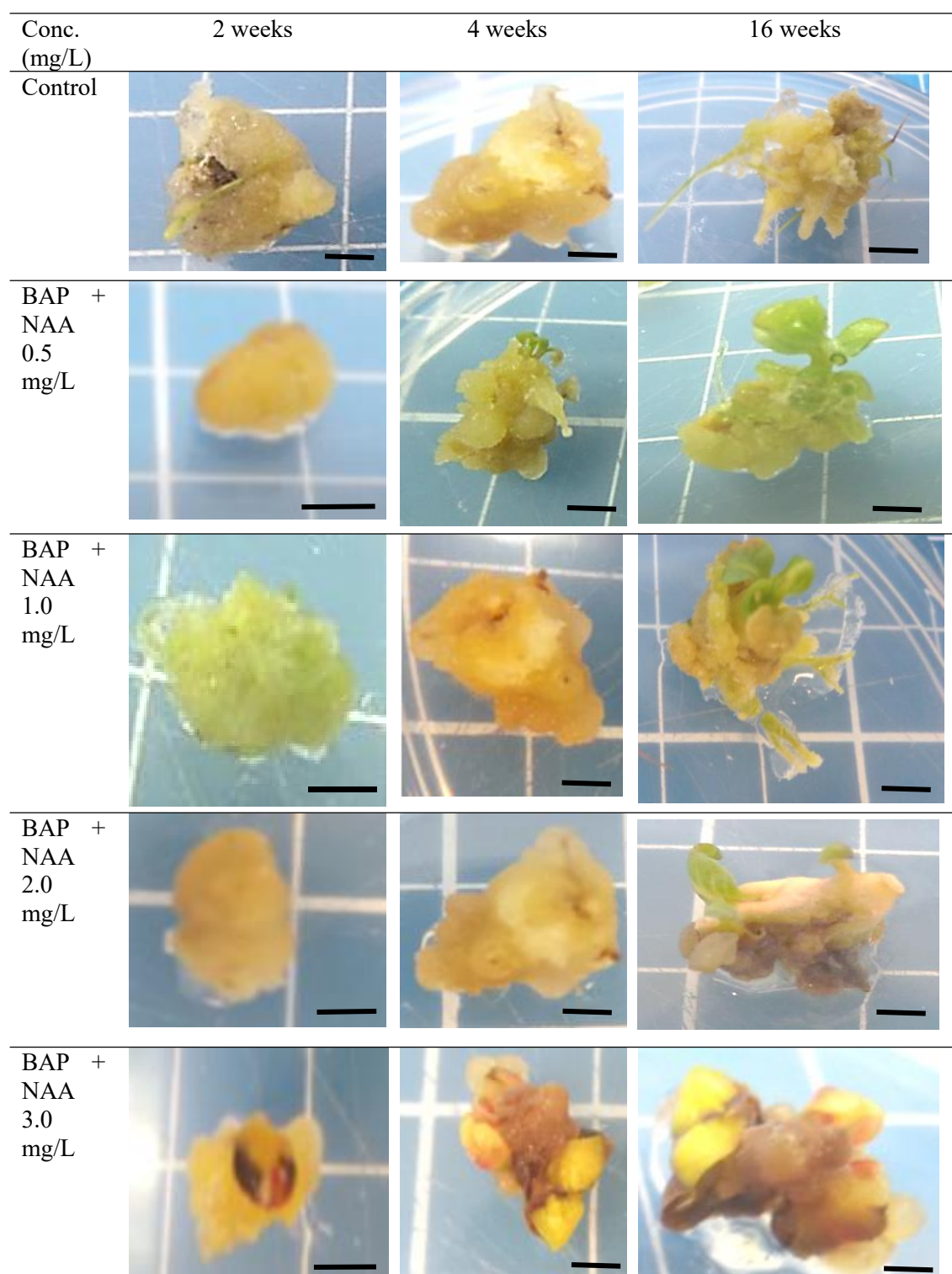


Figure 4.5: Effect of BAP combined with NAA to indirect shoot regeneration of *Neolamarckia cadamba* after 16 weeks of culture. Conc = concentration. BAP = 2.0 mg/L. Scale = 0.2 cm.

4.4.3 Effect of PGRs in combination with coconut water on indirect shoot regeneration

From the previous experiment, two plant growth regulators (PGRs) were selected (BAP and NAA) and combined with three different concentrations of coconut water (CW) to study their effects on shoot regeneration into a complete plantlet. The results showed that the addition of coconut water at 10% (v/v) with 2.0 mg/L of BAP or 2.0 mg/L of NAA was able to give a positive response for the shoot adventitious produced. The changes of explants were observed from 14 days after cultured on medium added with 2.0 mg/L of BAP or NAA combined with 10% (v/v) of coconut water. These had resulted in a high percentage number of adventitious shoot, which recorded at 52.89% and 57.33%, respectively after 16 weeks of culture. Explant produced shoot at 14 days after cultured in both treatments. The BAP combined with 10% CW recorded 2.24 cm of average shoot length, 13.20 leaf number and 3.80 roots (Table 4.3). Meanwhile, NAA and CW treatment recorded an average of 2.14 cm shoot length, 3.20 leaf number and 1.40 roots (Table 4.3). All the changes and the morphology of the callus from each treatment are shown in Figure 4.6.

Table 4.3: Effect of BAP or NAA combined with coconut water on indirect shoot regeneration of *Neolamarckia cadamba* after 16 weeks inoculation.

Treatments	No. of adventitious shoot (%)	Mean of shoot length (cm)	Mean no. of leaves	Mean no. of roots	Day start to produce shoot (days)
Control	0 ^a	0 ^a	0 ^a	0 ^a	NR
BAP + CW 10%	52.89 ^d	2.24 ^c	13.20 ^c	3.80 ^c	16
BAP + CW 20%	34.22 ^c	1.06 ^b	8.20 ^d	1.20 ^b	21
BAP + CW 30%	25.78 ^b	1.42 ^b	4.40 ^c	1.60 ^b	25
NAA+ CW 10%	57.33 ^d	2.14 ^c	3.20 ^c	1.40 ^b	17
NAA+ CW 20%	30.67 ^c	0.98 ^{ab}	1.02 ^b	1.20 ^b	26
NAA + CW30%	20.89 ^b	0.90 ^{ab}	0.96 ^{ab}	0 ^a	28

Data analysis was obtained from 5 replicates by using one- way ANOVA. Means within a column with the same letter are not significantly different based on Duncan's Multiple Range Test at $p < 0.05$. NR = Not response; BAP and NAA = 2.0 mg/L. CW = coconut water

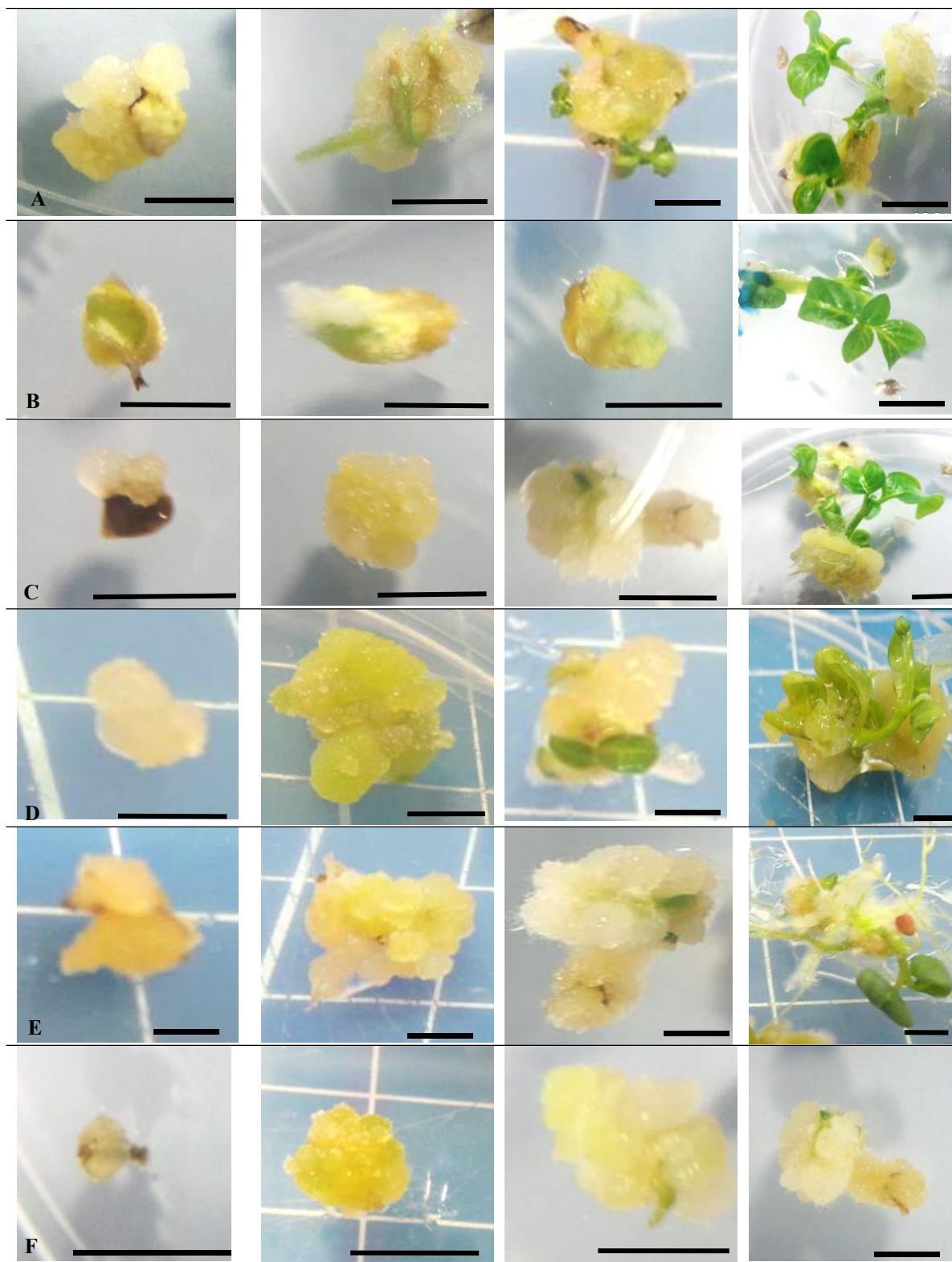


Figure 4.6: Effect of BAP or NAA combined with coconut water on indirect shoot regeneration of *Neolamarkcia cadamba* after 16 weeks of culture. Treatment (A) BAP + CW 10%; (B) BAP + CW 20%; (C) BAP + CW 30%; (D) NAA + CW 10%; (E) NAA + CW 20% and; (F) BAP + CW 30%. Left to right: 4, 8, 12 and 16 weeks. Scale A–F = 0.5 cm. BAP = 2.0 mg/L.

4.4.4 Developmental stages of indirect shoot regeneration from the young leaf

Figure 4.7 illustrates the development of indirect shoot regeneration from young leaf-derived *N. cadamba* callus. The initial callus cultured on B5 medium enriched with BAP 2.0 mg/L, and NAA 0.5 mg/L. Then, the cell divides to give a morphologically-disorganised cluster of callus under seven days of observation on the same media. After two weeks of cultured, the greenish-yellow friable callus was produced from the explants. Shoot buds were initiated from the callus after four weeks of culture on the medium supplemented with 2.0 mg/L of BAP and 0.5 mg/L of NAA (Figure 4.7D). The shoot regenerated after sixteenth weeks of culture on the medium that fortified with 2.0 mg/L of BAP and 10 % (v/v) coconut water, as shown in Figure 4.7E. The new plantlet formation occurred after eighteenth weeks as shown in Figure 4.7F.

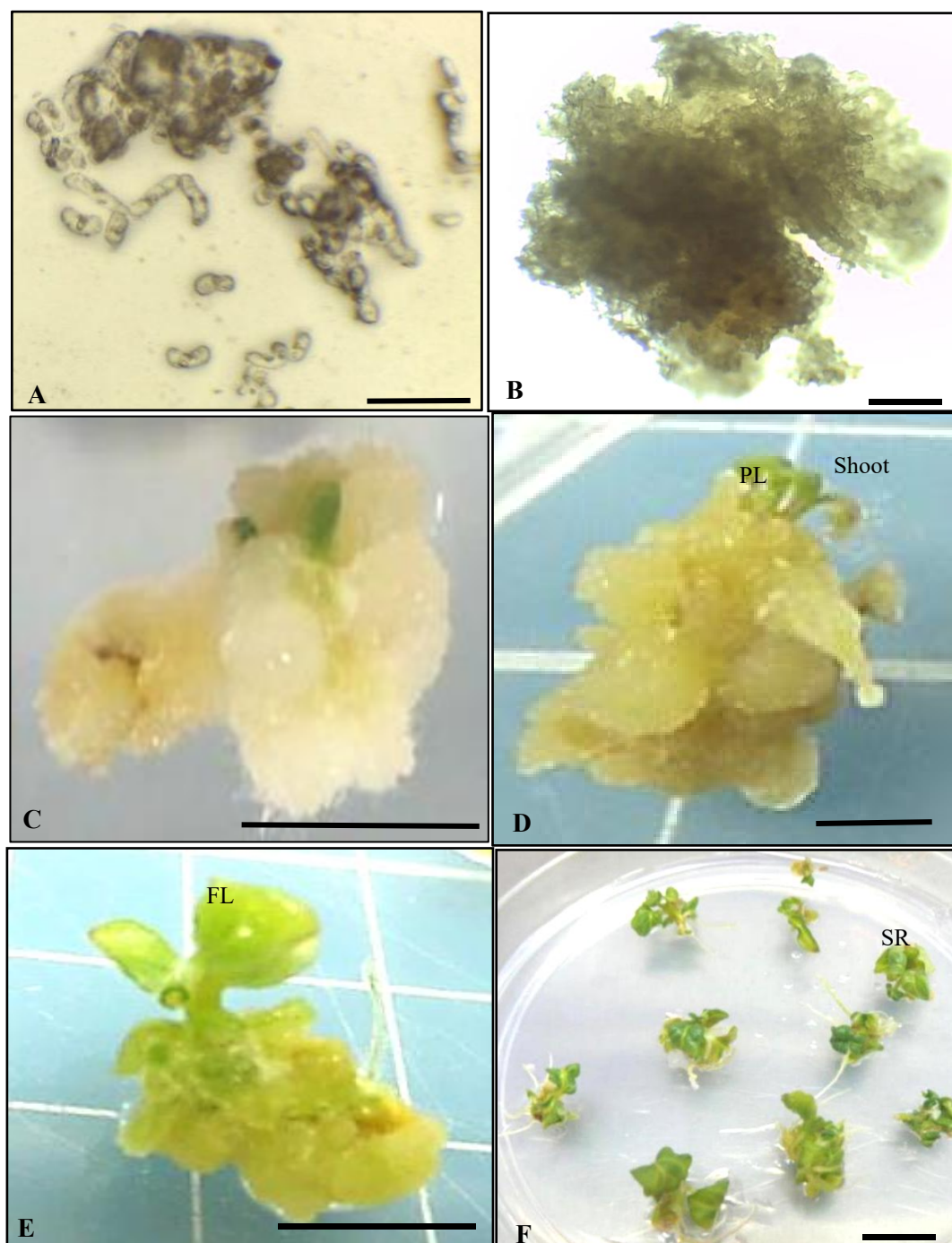


Figure 4.7: Development of indirect shoot regeneration from young leaf-derived callus of *Neolamarckia cadamba*. (A) Initial cell from callus cultured on B5 medium enriched with 2.0 mg/L BAP and 0.5 mg/L NAA; (B) Initial cell divides to give morphologically-disorganised cluster of callus under 7 days of observation; (C) Greenish yellow friable callus were induced from the explants after 2 weeks of cultured (D) callus with initiated shoot buds on the medium that supplemented with 2.0 mg/L BAP and 0.5 mg/L NAA after 4 weeks of culture, (E) shoot regeneration after 16 weeks culture and was formed on the medium that fortified with 2.0 mg/L BAP and 10% (v/v) coconut water; and (F) new regenerated plantlet after 18 weeks. (Scale A–B = 5 mm; C–D = 50 mm; E–F = 1 cm). PL = primordial leaf; FL = first leaf; SR = regenerated shoot.

4.5 Discussion

4.5.1 Effect of auxins and cytokinins

Plant growth regulators (PGRs) do not always behave as typical hormones, i.e. it can exert effects in the cell in which they are produced, and for this reason, many researchers are reluctant to apply the term ‘hormone’ to these substances (Naikawadi et al., 2016; Mohamadi–Nasab et al., 2017). Auxins differ in their physiological activity and to the extent to which they move within the tissue, are bound within cells, or metabolised. Synthetic auxins such as 2, 4-D, NAA and others are widely used for callus induction (Raju et al., 2014; Abrahamsson et al., 2017).

Cytokinins are compounds forming the other class of plant growth substance, which is very important for the regulation of growth and morphogenesis in tissue culture. According to Yumbla-Orbes et al. (2017), the interaction of plant growth regulator with synthetic auxin and cytokinin of somatic embryogenesis and *de novo* shoot organogenesis could be alternatively induced by reactivating pericycle cells in *Lisianthus* (*Eustoma grandiflorum* (Raf.) Shinnery) by using root as an explant.

Cytokinins such as 6-benzylaminopurine (BAP) and kinetin are usually used to reduce apical dominance, and to induce the meristem to form and stimulate sprouts in banana meristematic explants (Rashid et al., 2009; Nakasha et al., 2016). BAP strongly stimulates the growth of axillary, adventitious and foliar buds (Pereira et al., 2018). Although BAP stimulates the shoot proliferation in bananas, if used in high concentrations it can cause high somaclonal variation rates and becoming unviable if used for *in vitro*

seedlings production (Priyanka et al., 2018). The effectiveness of BAP on other cytokinins in the induction of meristem multiplication has also been reported in different banana cultivars (Pereira et al., 2018).

In this study, the use of BAP or NAA at 2.0 mg/L alone in the medium was able to reform adventitious shoot of the *Neolamarckia cadamba* young leaf-derived callus. The study had shown that the callus cells produced from induction medium supplemented with 2, 4-D were highly responsive when transferred to shoot induction medium supplemented with BAP and NAA. Cells will be reverted to a differentiated state and begin to divide when responded to the auxins in *N. cadamba* shoot regenerated (Rahman et al., 2015a; Huang et al., 2020). So, callus tissue initiates meristems cell can only be achieved when plant cells went through the stage of cell differentiation (Naz et al., 2016).

The current findings had supported by a study by Abrahamsson et al. (2017), which reported that the inclusion of 2.0 mg/L BAP in the medium had induced the highest number of shoots per callus explant of meristemoids cell of *Pinus sylvestris* L (Pinaceae family). The meristems structures are observed in callus cell cultures, which eventually develop into complete plantlets. The regulatory mechanisms underlying the organised development of cells or tissues were achieved by balancing the concentration of auxins and cytokinins. However, according to Rahman et al. (2015b), the use of 1.0 mg/L BAP was good for shoot regeneration of *Melicope lunu-ankenda* plantlet.

Many successful studies on the maturation of callus cell or embryos that derived from initial callus cultures for woody plants, such as *Aegle marmelos* L. (Dasa et al., 2009),

Melicope lunu-ankenda (Rahman et al., 2015b); *Vitis vinifera* L. (Maillot et al., 2016); *Dioscorea rotundata* (Uchendu et al., 2016) and *Douglas-fir* (Reeves et al., 2018) had been reported. The ability of callus growth and regeneration are highly variable depending on the types of culture media and plant growth regulators (PGRs) used. As stated by Baskaran et al. (2005) and Mazri et al. (2018), the callus response and regeneration frequency are depended on the callus induction medium, regeneration medium, genotype of the explant as well as the number of *in vitro* regeneration cycles. Therefore, the plant growth regulators (PGRs) must be applied to the explants in an adequate amount under optimal conditions in order to increase the regeneration potential of callus.

4.5.2 Effect of different combinations of auxins and cytokinins

Plant growth regulators (PGRs) added exogenously exert part of their effect by modifying the concentrations of endogenous hormones (Winarto et al., 2015). Plant growth regulators in the tissue culture medium can be manipulated to induce callus formation and followed by the induction of adventitious shoot from the callus cells. The ratio of different plant growth regulators required to induce callus or embryo formation varies with the types of explant. Asymmetrical cell division also seems to be important in the development of somatic embryos, and while failure to form the suspensor cell is lethal to zygotic embryos, it is not lethal for somatic embryos (Vinoth et al., 2014).

In this study, the changes in explant were observed starting from 14 days after cultured on medium added with 2.0 mg/L BAP added with 0.5 mg/L NAA. About 57.7% of the explants forming adventitious shoots within 16 weeks of inoculation, and regenerated into the complete plants on B5 medium added with 1.0 mg/L BAP and 10%

(v/v) of coconut water. This mechanism has also been postulated to explain the regulation of shoot organogenesis by supplying the PGRs partially. However, the addition of NAA at a concentration of 3.0, 4.0 and 5.0 mg/L resulted in the reduction of the percentage of adventitious shoot produce. All the callus derived from each treatment were observed with different textures and colours. According to Akula et al. (2013) and Xia et al. (2017), the maturation, division and differentiation stages in shoot regeneration and somatic embryogenesis of *Azadirachta indica* and *Picea asperata* were affected by the different concentrations of auxins and cytokinins.

According to Vinoth and Ravindhran (2016), modulation of endogenous hormones by exogenous PGRs may occur either directly (through enzyme synthesis) or indirectly (through effectors), as it postulated for auxins. There is evidence for PGRs modifying levels of endogenous hormones belonging, both to the same or to different groups, during organogenesis. Example of the former includes the increase in the contents of IAA as a result of 2, 4-D supply into the culture medium in *Passiflora setacea* cultures (Vieira et al., 2014) and *Clematis gouriana* leaf protoplasts (Naika et al., 2008). Furthermore, Mangena et al. (2015) reported that NAA and indole-3-butyric acid (IBA) treatments promote an increase in the endogenous IAA levels in soybean hypocotyl explants.

Wongsen et al. (2015) reported an increase in the endogenous contents of IAA in immature embryos of sunflower as a consequence of ABA application. This PGR, when added in high quantities, also reduced ethylene contents during division and differentiation plant cells. In another report involving the modulation of ethylene levels, an increase in the synthesis of this gaseous hormone was observed in response to the application of high

amounts of 2, 4-D, which impaired embryo and cell development (Anand et al., 2000; Chang et al., 2003; Yan et al., 2009; Tanaka et al., 2016). A further example of the interaction among PGRs and endogenous plant hormones has been proposed to be the mechanism by which thidiazuron (TDZ) induces adventitious shoot in chrysanthemum florets (Tymoszek & Zalewska, 2014) and somatic embryo in peanut.

4.5.3 Effect of the combination of BAP or NAA with coconut water

Two PGRs, which are BAP and NAA in combination with three different levels of coconut water were used to examine the effects on indirect shoot regeneration into a complete plantlet. The result showed that the addition of coconut water at 10% (v/v) with BAP and NAA gave a positive response to induce adventitious shoots and roots into a complete plantlet.

On the medium supplemented with cytokinins, a various number of adventitious shoots was sprouted from explants and formed shoot clusters. Histological studies showed that the adding of BAP into the embryo and shoot proliferation media has improved the growth of axial shoots and promoted the cells multiplication from the tissues of the explants (Vejsadová et al., 2016). However, Singh et al. (2015) reported that the growth of callus remained stunted and leaves became red and abscised when BAP was used continuously in the medium for every subculture. They had proved that using two different multiplication media contained BAP and kinetin in other passages significantly improved shoot condition and reduction in the deleterious effects seen on the continuous BAP medium.

4.6 Conclusion

In conclusion, this study presents the first report of plant regeneration via indirect shoot organogenesis of *N. cadamba* from young leaf explants. The callus derived from leaf explants produced the higher potential for organogenic differentiation on B5 media supplemented with 2.0 mg/L BAP and 0.5 mg/L NAA. Hence, in the present study, the explants were subcultured to the B5 medium with 2.0 mg/L of BAP and 10% (v/v) coconut water (CW) for subsequent development into complete plantlets. The addition of complex additives such as coconut water at 10% (v/v) remarkably enhanced the *in vitro* seedling growth of *N. cadamba*.

CHAPTER 5

DIRECT SHOOT REGENERATION OF *NEOLAMARCKIA CADAMBA*

5.1 Introduction

Plant regeneration through the nodal segment in tissue culture is a popular technique for woody crops since 1980an (José et al., 2017). Efficient plant regeneration protocols in more than 60 different media have been used for tissues of various woody species. Micropropagation is widely used for commercial purposes as a method of vegetative (asexual) propagation from explants derived from shoots, root tips, leaves, cotyledons, anthers, nodes, meristems or embryos (Aparna & Choudhary., 2017). According to Wang et al. (2016), regeneration of plants by micropropagation could achieve from nodal and internodal segments of seedling, root, and apical meristem from the mature tree.

The success of *in vitro* shooting can be influenced by explant source, basal media types, complex additives and plant growth regulators. Micropropagation media such as the Gamborg (B5), Vacin and Went (VW), Knudson C (KC), Schenk and Hildebrandt (SH) and Murashige and Skoog (MS) medium are widely used. In addition, regulators for plant growth are important elements in media for determining the path of plant cell development.

In tissue culture, auxins and cytokinins are critical to plant regeneration, with cytokinins playing an instrumental role in shoot organogenesis (Huang et al., 2020). Auxins and cytokinins are the most widely used plant growth regulators in plant tissue culture. A high ratio of auxins to cytokinins generally favours root formation, while a high

ratio of cytokinins to auxins favours shooting (Aparna et al., 2017). Type-B response regulators regulate the transcriptional production in response to cytokinins and auxins are essential for the regeneration of plants (Bekheet et al., 2018). Classic regeneration assays have shown that *de novo* shoot meristem formation requires elevated levels of both auxins and cytokinins, with both antagonistic and synergistic roles in these hormones. Ali et al. (2017) stated that the maximum differentiation was observed on 0.5 mg/L NAA + 0.2 mg/L BAP added conditions, and the rate decreased as NAA and BAP treatments for *Coriandrum sativum*. Most plant regeneration methods applied to woody plants require the regeneration of a whole plant from genetically transformed isolated plant cells or tissues (Atondo-bueno et al., 2016). This regeneration is performed *in vitro* so that the environment and medium of growth can be manipulated to ensure high regeneration frequency.

According to Mandal and Laxminarayana (2014), the high concentration of auxins gave a significant redirect effects to the plant cell division to new organ initiation in *Adhatoda vasica*. In addition, cytokinins such as BAP and kinetin have been used in plant tissue culture and found to be able to promote cell division, generation and growth of adventitious buds or shoots and formation of embryonic tissues (Cai et al., 2015). There are several studies regarding the function on combination auxins and cytokinins to induce direct shoot regeneration on tree or woody plant species such in *Magnolia punduana* (Borah et al., 2017), *Tecomella undulata* (Chhajer & Kalia, 2017) and *Eucalyptus polybractea* (Fernando et al., 2016). The ability to regenerate the whole and fertile plant from selected mother plant cells is a key for *in vitro* plant regeneration. This situation is challenging to the horticulturists for the selection of high potential plant to be regenerated.

Many studies had been done for direct shoot regeneration through nodal and internode segments as reported by Warakagoda and Subasinghe (2009) in *in vitro* culture establishment and shoot proliferation of *Jatropha curcas* L., and research by Rahman et al. (2015a) in using cutting internode segment of *N. cadamba* tissue culture techniques for sustainable supply of plantlets. The main objective of this study was to establish an efficient protocol for direct shoot regeneration of *N. cadamba*. Factors such as effects of BAP and NAA either use singly or in combination, and the combination of BAP and coconut water on direct shoot regeneration of *N. cadamba* were also studied to enhance the number of shoots per explant regenerated from the nodal segment.

5.2 Materials and Methods

5.2.1 Plant materials

Plant materials used in this research was obtained from a three-month-old *in vitro* seedling of *N. cadamba*. The young seedling was cut into the desired size (0.5 ± 0.2 cm length) to get the node segment as shown in Figure 5.1.

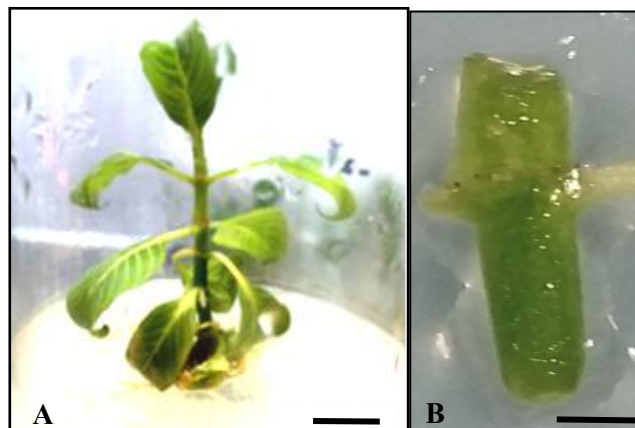


Figure 5.1: (A) Three-month-old *in vitro* seedling of *Neolamarckia cadamba* and (B) Node segment as explant on direct shoot regeneration of *Neolamarckia cadamba* (photo taken by digital camera Samsung with 21 x magnification). Scale A = 1.0 cm; and B = 0.2 cm.

5.2.2 Shoot regeneration through nodal segment

a) Effect of BAP and NAA

B5 medium added with BAP and NAA at different levels of concentration which are 1.0, 2.0, 3.0, 4.0 and 5.0 mg/L were studied.

b) Effect of the combination of BAP and NAA

The best concentration BAP at 1.0 mg/L from Section 4.2.3 (a) was chosen and combined with NAA at 0.5, 1.0, 2.0 and 3.0 mg/L. B5 Medium was used as the basal medium and B5 medium without plant growth regulators (PGRs) was used as the control.

c) Effect of the combination of BAP and coconut water

B5 medium supplemented with 1.0 mg/L of BAP and coconut water (CW) at 5, 10, 15, and 20% (v/v) were also studied.

5.2.3 Media preparation and culture condition

The basal medium used in this study was a full-strength B5 medium. The cultures were maintained under 70–80% relative humidity; light intensity of 20–50 $\mu\text{mol m}^{-2} \text{s}^{-1}$ provided by red light emitting diode (LED) with duration about at 16-h light/ 8-h dark in room temperature at 25 ± 2 °C.

5.3 Data collection and analysis

Each treatment was carried out in five replicates. The cultures were observed starting from 10 days interval up to 120 days after culture. The mean number of shoots per explant

regenerated, and the number of leaves were calculated. Then, the morphological changes of plants were also observed and recorded. All experiments were conducted in a completely randomised design (CRD). All means were analysed using the software, SPSS by using one-way Analysis of variance (ANOVA) versions 22. Significance differences were assessed using Duncan's Multiple Range test (DMRT) at the 5% probability level.

5.4 Results

5.4.1 The growth stages of direct shoot regeneration of *Neolamarckia cadamba*

Direct shoot regeneration from the nodal segment is shown in Figure 5.2. In the first observation, no detectable morphological changes of node explants were found after two weeks of culture. The shoot started to grow from a nodal segment of *N. cadamba* on B5 medium fortified with 1.0 mg/L of BAP after 16 days of culture (Figure 5.2 B). Then, multiple shoots were actively multiplied after four weeks of culture. The elongation of shoots with axillary branching was grown well on B5 media supplemented with 1.0 mg/L of BAP and 0.5 mg/L of NAA after eight weeks of culture (Figure 5.2 D). Subculturing and shoot multiplication of *N. cadamba* was carried out after 12 weeks of culture (Figure 5.2 E). The newly regenerated plantlets of *N. cadamba* were grown well on medium containing 1.0 mg/L of BAP and 0.5 mg/L of NAA after 16 weeks of culture (see Figure 5.2 F).

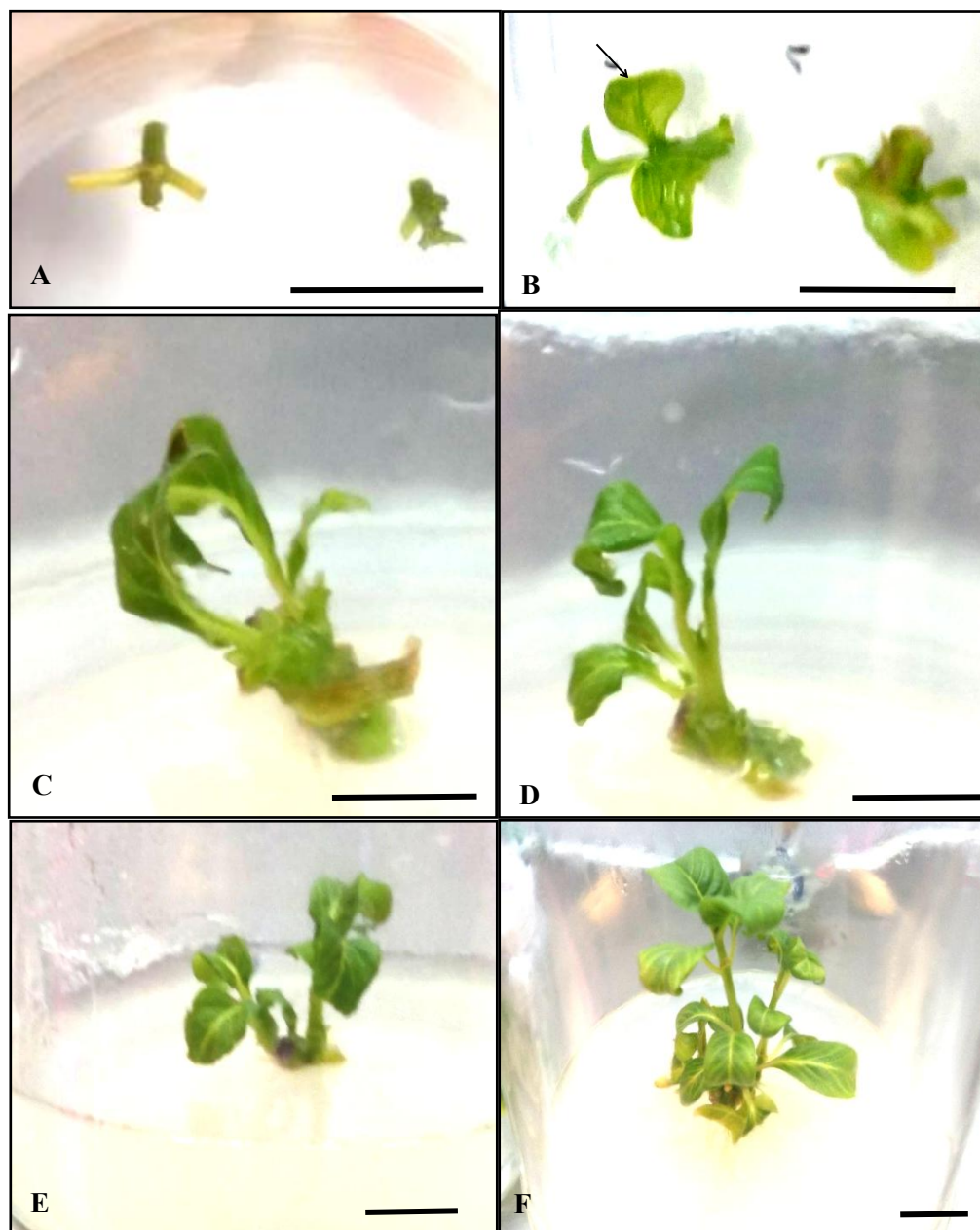


Figure 5.2: Direct shoot regeneration of *Neolamarckia cadamba* from nodal segment. (A) Nodes after 2 weeks culturing; (B) Shoot growing from a nodal segment of *Neolamarckia cadamba* culture on B5 medium fortified with 1.0 mg/L of BAP after 16 days cultured; (C) multiple shoots growing after 4 weeks of culture; (D) good long shoot with axillary branching after 8 weeks; (E) sub culturing and shoot multiplication after 12 weeks of culture; and (F) shoot and root formation in medium containing 1.0 mg/L of BAP and 0.5 mg/L of NAA after 16 weeks of culture. (Scale A–F = 1.0 cm).

5.4.2 Individual effect of BAP and NAA

The highest percentage of shoot regeneration was 73.33% on B5 medium added with 1.0 mg/L of BAP, with 12 shoots per explant after 16 weeks of inoculation (Table 5.1). Multiple shoots were obtained at 16 days of culture, as shown in Figure 5.3 after 8 weeks of culture. The results are presented in Figure 5.3 and Figure 5.4.

At week 8 of culture, BAP at 1.0 mg/L resulted in an average of 6.60 shoots per explant. This amount increased to 9.40 and 12.80 shoots per explant at week 12 and week 16, respectively. The second-highest percentage of shoot regeneration (62%) with 9.80 shoots produced after 16 weeks of culture was observed in B5 medium added with 2.0 mg/L of BAP (Table 5.1).

The results showed that by adding the BAP into the medium is the best option to regenerate shoots from the node segment. In auxins, NAA at a concentration of 1.0 mg/L showed the third-highest percentage of shoot regeneration from the nodal segment (57.78%) with 10 shoots produced after 16 weeks of culture (Table 5.1).

Table 5.1: Effect of BAP and NAA at different levels of concentration for shoot multiplication through the nodal explants.

Treatments (mg/L)	Percentage of responsive explants (%)	Mean number of shoots multiplied per explant			Response time (days)
		8 wks	12 wks	16 wks	
Control	0 ^a	0 ^a	0 ^a	0 ^a	NR
BAP 1.0	73.33 ^h	6.60 ^c	9.40 ^f	12.80 ^g	16
BAP 2.0	62.22 ^g	5.60 ^{de}	7.20 ^e	9.80 ^f	21
BAP 3.0	53.33 ^{ef}	4.60 ^c	5.80 ^d	7.20 ^c	24
BAP 4.0	28.89 ^{cd}	2.60 ^b	4.80 ^c	6.60 ^d	26
BAP 5.0	17.78 ^{bc}	1.60 ^b	2.40 ^b	3.60 ^b	28
NAA 1.0	57.78 ^f	5.20 ^d	7.40 ^e	10.40 ^f	19
NAA 2.0	48.89 ^e	4.40 ^c	5.20 ^d	6.40 ^{cd}	20
NAA 3.0	31.12 ^d	2.80 ^b	4.40 ^c	5.60 ^c	32
NAA 4.0	22.23 ^c	2.00 ^b	2.60 ^b	3.40 ^b	35
NAA 5.0	13.33 ^b	1.20 ^b	1.80 ^b	2.40 ^b	40

Data analysis was obtained from 5 replicates by using one-way ANOVA. Means within a column with the same letter are not significantly different based on Duncan's Multiple Range Test at $p < 0.05$. wks = weeks; NR = Not response.

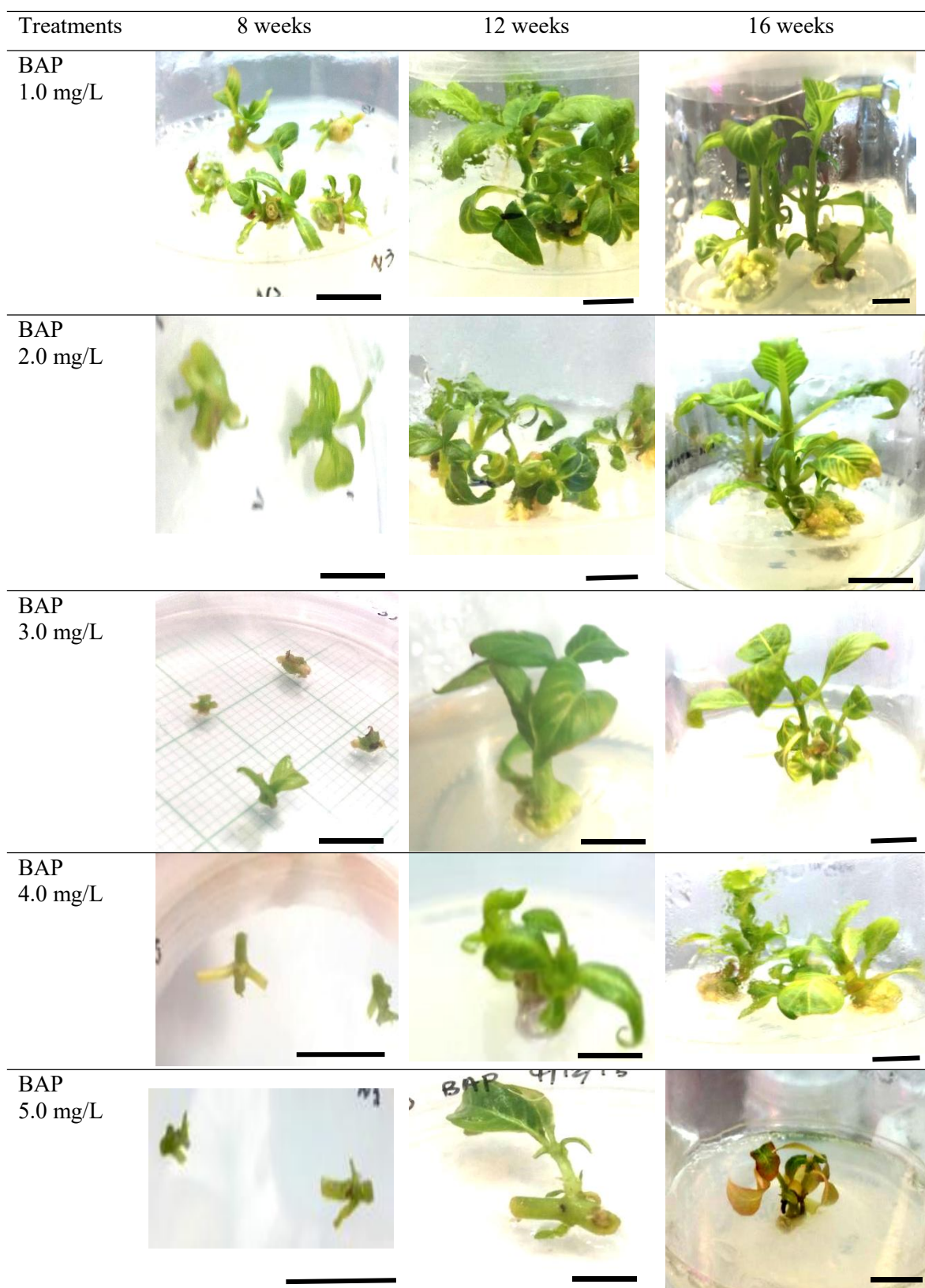


Figure 5.3: Effect of BAP on shoot multiplication of *Neolamarckia cadamba* after 16 weeks of culture. Scale = 1.0 cm.

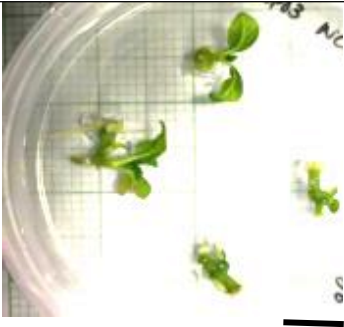
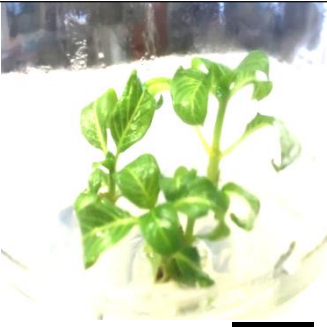
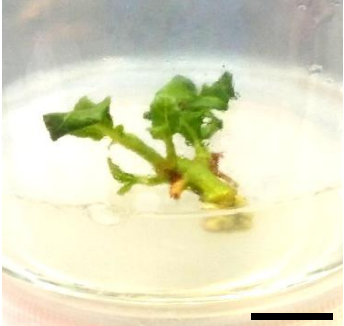
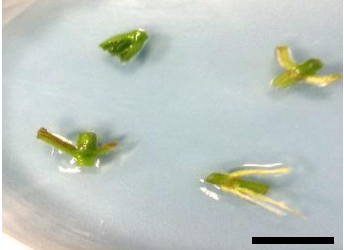
Treatments	8 weeks	12 weeks	16 weeks
NAA 1.0 mg/L			
NAA 2.0 mg/L			
NAA 3.0 mg/L			
NAA 4.0 mg/L			
NAA 5.0 mg/L			

Figure 5.4: Effect of NAA on shoot multiplication of *Neolamarckia cadamba* after 16 weeks of culture. Scale = 0.5 cm.

5.4.3 Effect of BAP and NAA combination

B5 medium was used as a basal medium. The medium with a single concentration of 1.0 mg/L BAP was using as a control. From observation, about 80% of node segments were able to regenerate shoots which produce approximately 13 leaves per explant after 16 weeks of culture on medium supplemented with 1.0 mg/L of BAP and 0.5 mg/L of NAA (Table 5.2).

There was a significant difference between the treatments where the high concentration of NAA in the medium was observed with the lowest percentage number of responsive explants which was around 42% differ from control (71%) after 16 weeks of inoculation. From the results, *Neolamarckia cadamba* shoots were actively regenerated when the node segments inoculated on medium added with 1.0 mg/L of BAP and 0.5 mg/L of NAA (Figure 5.5).

Based on the observation, the shortest duration required for direct shoot regeneration was 14 days when cultured in medium containing 1.0 mg/L of BAP in combination with 0.5 mg/L of NAA. The different morphologies during plantlet formation in different treatments are shown in Figure 5.5. Shoots in medium containing BAP 1.0 mg/L and 0.5 mg/L showed the most greener and healthy plant.

Table 5.2: Effect of BAP and NAA combination at different concentrations on shoots regeneration from the *Neolamarckia cadamba* nodal explants.

Treatments	Percentage of responsive explants (%)	Mean number of shoot per explant			Response time (days)
		8 wks	12 wks	16 wks	
Control (BAP = 1.0 mg/L)	71.11 ^d	6.40 ^c	8.60 ^d	11.60 ^d	16
BAP + NAA 0.5	80.06 ^c	7.20 ^d	9.60 ^c	13.40 ^c	14
BAP + NAA 1.0	64.44 ^c	5.80 ^b	7.60 ^c	9.20 ^c	18
BAP + NAA 2.0	51.11 ^b	4.60 ^{ab}	6.60 ^b	8.20 ^b	21
BAP + NAA 3.0	42.22 ^a	3.80 ^a	4.40 ^a	6.40 ^a	23

Data analyses were obtained from 5 replicates by using two-way ANOVA. Means within a column with the same letter are not significantly different based on Duncan's Multiple Range Test at $p < 0.05$. BAP = 1.0 mg/L. wks = weeks

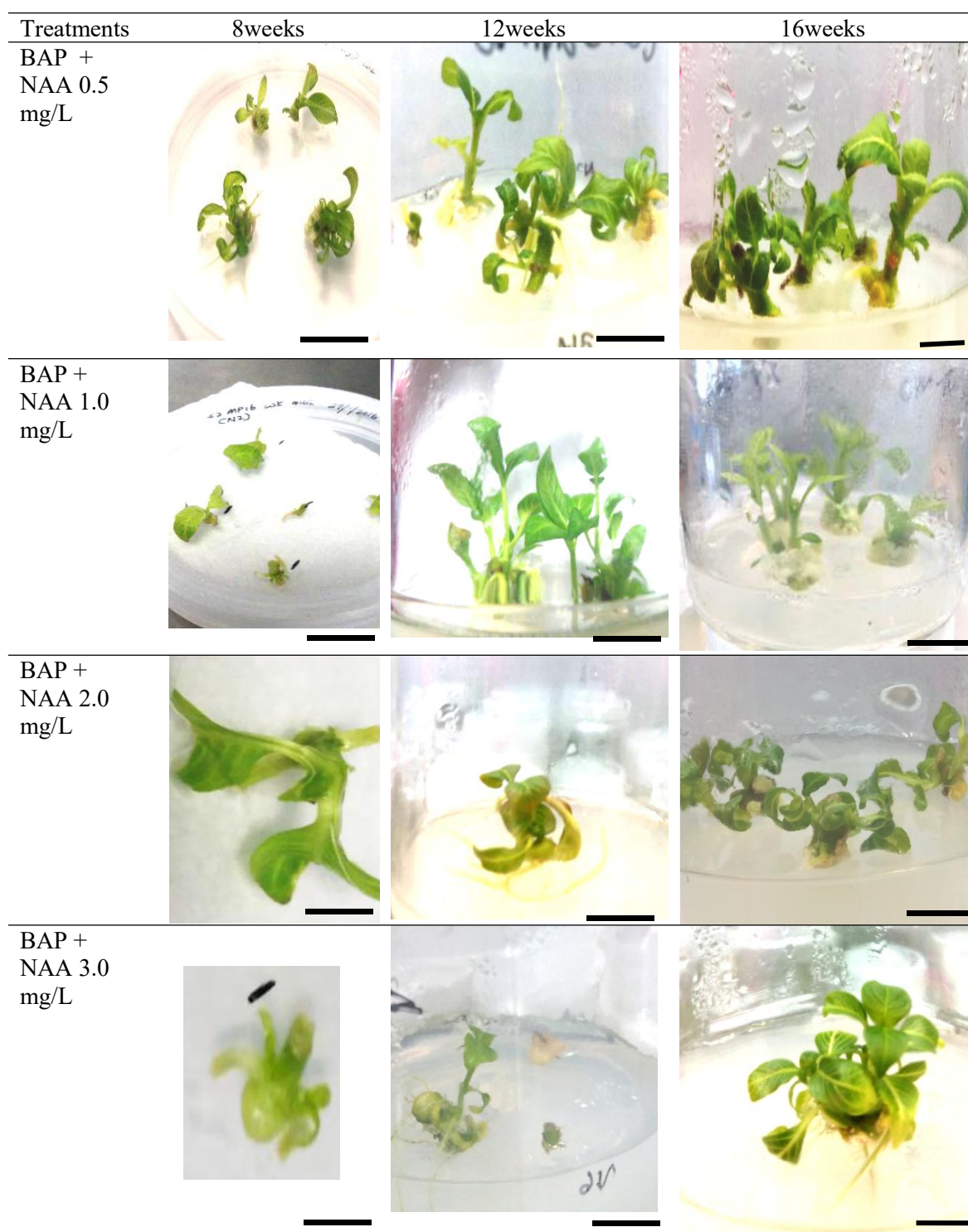


Figure 5.5: Effect of the combination of BAP and NAA on shoot multiplication of *Neolamarckia cadamba* after 16 weeks of culture. BAP = 1.0 mg/L. Scale = 0.5 cm.

5.4.4 Effect of BAP in combination with coconut water

B5 medium fortified with 1.0 mg/L of BAP and 10% (v/v) of coconut water (CW) resulted in a significant difference of shoot number regenerated (Table 5.3). From the observation, about 97.78% of the node segment was induced to produce shoots with an average of 19.20 shoots per explant after 16 weeks of culture (Table 5.3).

From the result, *Neolamarckia cadamba* shoots were actively regenerated when the node segments were cultured on medium added with 1.0 mg/L of BAP and 10% (v/v) CW (Figure 5.6). The shortest duration required to induce shoots was 12 days after cultured in medium containing 1.0 mg/L of BAP in combination with 10% (v/v) CW.

The different morphologies during plantlet formation in different treatments are shown in Figure 5.6. Shoots in medium containing BAP 1.0 mg/L and 10% (v/v) CW showed the most greener and healthy plant. Callus also observed on media fortified with BAP and 10–20% (v/v) of coconut water.

Table 5.3: Effect of BAP in combination with coconut water (CW) on shoot multiplication of *Neolamarckia cadamba* after 16 weeks of inoculation.

Treatments	Percentage of responsive explants (%)	Mean number of shoots per explant			Response time (days)
		8wks	12wks	16wks	
Control, BAP 1.0 mg/L	75.56 ^a	6.60 ^a	8.40 ^a	11.80 ^a	17
BAP 1.0 mg/L + CW 5%	82.22 ^b	7.40 ^b	8.80 ^a	13.20 ^b	14
BAP 1.0 mg/L + CW 10%	97.78 ^d	9.40 ^d	14.80 ^d	19.20 ^d	12
BAP 1.0 mg/L + CW 15%	91.11 ^c	8.20 ^c	11.40 ^c	16.40 ^c	13
BAP 1.0 mg/L + CW 20%	80.00 ^b	7.20 ^b	10.40 ^b	13.60 ^b	14

Data analysis was collected from 5 replicates by using one-way ANOVA. Means within a column with the same letter are not significantly different based on Duncan's Multiple Range Test at $p < 0.05$. CW = coconut water; wks = weeks

Treatments	8 weeks	12 weeks	16weeks
BAP 1.0 mg/L + CW 5%			
BAP 1.0 mg/L + CW 10 %			
BAP 1.0 mg/L + CW 15%			
BAP 1.0 mg/L + CW 20%			

Figure 5.6: Effect of the combination of BAP with different concentrations coconut water (CW) on shoot multiplication of *Neolamarckia cadamba* after 16 weeks of culture. Scale = 0.5 cm.

5.5 Discussion

5.5.1 Effect of single concentration BAP and NAA

According to de Faria et al. (2018), auxin is considered to be the most critical hormone in regulating micropropagation. In the present study, nodal segments showed the highest percentage of shoot regeneration on B5 medium containing 1.0 mg/L of BAP. Meanwhile, NAA had resulted in a moderate response of nodal explants to regenerate shoot at concentration 1.0 to 2.0 mg/L. However, the BAP and NAA at the concentration of 3.0, 4.0 and 5.0 mg/L were observed with the decrease of the percentage of shoot regeneration. Some of the *N. cadamba* nodes became yellowish when 3.0–5.0 mg/L of auxins were used.

According to Gómez-montes et al. (2015), this happened due to the synthesis of carotenoid pigments. This regulation probably occurs through the establishment of an auxin gradient during the induction phase that is essential for bilateral symmetry initiation during plant cell division in dicotyledonous and monocotyledonous. It has been reported that the culture of explants in medium containing NAA, the standard induction treatment for many species, had increased the endogenous auxin levels in the responsive explants (Ghassemi et al., 2015; Du et al., 2015; Balilashaki & Ghehsareh, 2016).

The high concentration of auxins and cytokinins at 5.0 mg/L resulted in a poor response of explant for shoot regeneration. This happened because continuous growth in medium containing auxin and cytokinin does not allow a reduction in endogenous auxin levels and resulting in inhibition of shoot development. Report on Goubitz et al. (2003) and Dutta et al. (2013) stated that evidenced a bidirectional transport of auxins in wheat cell

growing on medium without auxin and an altered distribution pattern of this hormone when external auxin had supplied.

Results showed that nodal explants of *Neolamarckia cadamba* have a greater morphogenetic potential for callus formation and direct organogenesis. However, the response is highly sensitive and directly related to the combinations of exogenous growth regulators in the culture medium. A high ratio of cytokinins to auxins is generally favoured new shoot formation through the equilibrium with endogenous auxins that are manufactured by the apical meristem. The reason behind the increase in the number of shoots is due to cytokinins role in promoting axillary buds growth and ending apical dominance. The present results are in affirmation with the findings of Safdar and Kazemitabar (2010), who reported that multiple shoots from nodal shoot segments from 3 to 6 years old tree, and calli via direct organogenesis in Mose Rose (*Portulaca grandiflora* L.). The results are also in agreement with the results of Mandal and Laxminarayana (2014) in *Adhatoda vasica*, Teshome et al. (2017) research on medicinal plant *Satureja abyssinica*, and Saptari et al. (2017) in *Anthurium adreanum*.

5.5.2 Combination effect of BAP and NAA

Cytokinins are compounds for the other class of plant growth substance which is very important for the regulation of growth and morphogenesis in tissue culture (Silveira et al., 2016). The results in the present study showed that callus was started to regenerate shoot after 16 days cultured on B5 medium added with 1.0 mg/L of BAP and 0.5 mg/L of NAA. However, media containing BAP with NAA at the concentration of 1.0, 2.0 and 3.0 mg/L were observed with a moderate percentage of shoot induction. Besides, when the

concentration of NAA increases, the number of responsive explants was decreased. This may due to some of the aminopurines with cytokinin properties act as reductants in media.

The shoot formation response is affected by the concentration of cytokinins enriched in the culture medium (Ibrahim et al., 2009; Mangena et al., 2015). Cytokinins functions as signalling molecules that stimulate totipotent cells of callus for shoot organogenesis, these molecules also activate the preexisting machinery in the somatic cells (from cotyledon-derived callus) (Gómez-montes et al., 2015). From the experiment, the combination of BAP at 1.0 mg/L with 0.5 mg/L of NAA had found to promote the plantlet regeneration for *Neolamarckia cadamba*. All the plantlets grew from media added with a BAP combined with different concentrations of NAA but resulted in different morphologies of plantlet. This is because of the side effects of PGRs included necrosis, browning of medium and increased the release of ethylene or phenolic acids which impede plant growth and development (Reigosa, 1999; Onuoha et al., 2011; Kaur & Bhutani, 2012). According to Khunchuay and Sompornpailin (2015), the addition of plant growth regulators (PGRs) would cause an adverse effect on explant culture when exposed to the PGRs chemicals for a long incubation period.

5.5.3 Effect of BAP in combination with coconut water

In this study, the coconut water at 10% (v/v) with 1.0 mg/L of BAP can help to promote the shoot multiplication of *Neolamarckia cadamba* and shorten the shoot inducing time to 12 days after culture. However, the percentage and number of shoot multiplied of *N. cadamba* were lowest when the node segments were cultured on media with 1.0 mg/L of BAP (control media). Coconut water has been considered in a nutrient medium to improve

regeneration of plant cells as it provides mainly water (94%) and growth regulating substances that can influence *in vitro* cultures, including vitamins, inorganic ions, amino acids, lipids, organic acids, sugars, sugar alcohols, reduced nitrogen compounds and phytohormones (Yong et al., 2009; Rikiishi et al., 2015).

According to the study of Aktar et al. (2008), the addition of coconut water as a complex organic supplement in the media could enhance the rate of plant cell division and regeneration in orchid tissue culture. According to Arditti (2010) and Tan et al. (2014), the use of coconut water as a complex additive in the medium was good for the proliferation of *Datura* species. Besides, Satish et al. (2015) stated that the addition of coconut water in media was found useful for inducing cell division in quiescent cells of shoot apical meristem of finger millet (*Eleusine coracana* L.) when added singly or combined with a synthetic hormone.

5.6 Conclusion

In this study, *N. cadamba* shoots were actively regenerated from the nodal segments on B5 medium supplemented with 1.0 mg/L BAP and 10% (v/v) coconut water. Approximately 97.8% of node segments were able to regenerate shoots, and after 16 weeks of culture, an average of 19 shoots per explant was produced.

CHAPTER 6

GENERAL DISCUSSION AND CONCLUSION

Neolamarckia cadamba (Kelampayan) is an important tree species for reforestation programme in tropical forests (Na'iem et al., 2014). Recently, *Neolamarckia cadamba* has been widely promoted as one of the potential fast-growing tree species suitable for ideal investment in forest plantation establishment (Huang et al., 2020). It is one of the most frequently planted tree species in Sarawak and approximately 19 000 ha of this species have been planted (Chai et al., 2010). The conventional propagation method is unable to meet the current demand for quality planting materials for planted forest development. Hence, plant tissue culture technique could be considered as one of the alternatives to conventional propagation for large scale production of quality planting materials for this species (Huang et al., 2014; Rahman et al., 2015a). Factors such as culture media, complexes additive, plant growth regulators, photoperiod and sources of explant could affect the seedling growth development, callus induction, callus proliferation, indirect and direct shoot regeneration of *N. cadamba*. The overall findings from this study are summarised as below:

6.1 *In vitro* seed germination and seedling growth development of *Neolamarckia cadamba*

Abdolmohammadi et al. (2014) reported that the selection of basal media plays a significant role in determining the occurrence of *in vitro* seed germination and seedling growth of *Euphytica* species. It provides water, nutrients, vitamins, and growth regulators for the plant, as well as for gases exchange and removal of metabolite wastes. A widely

known basal medium such as Murashige and Skoog (MS) basal medium (Murashige & Skoog, 1962) is always chosen for plant tissue culture, providing that the plant is not sensitive to high salt concentrations. Half-strength MS medium has been used to optimise *in vitro* seed germination of other species such as wood anemone (*Anemone nemorosa* L.) (Mondoni et al., 2011), *Pterocarpus marsupium* (Mishra et al., 2013) and *Psoralea corylifolia* Linn (Pandey et al., 2014).

MS medium contains high amounts of both macronutrients and micronutrients which can increase seed germination and seedling development (Pandey et al., 2014). According to Mostafiz and Wagiran (2018), Gamborg's B5 medium is another tissue culture media used for efficient callus induction, suspension culture and regeneration in selected rice varieties. Huang et al. (2014) reported that B5 media could be used to improve *in vitro* seed germination and seedling growth of *N. cadamba*. Their findings are similar to this study where the present study also demonstrated that B5 medium was the best medium for seed germination and seedling growth of *N. cadamba*. However, the present finding differed from the study by Rahman et al. (2015a), where the half-strength MS medium was the best medium to germinate *N. cadamba* seeds and to induce rooting of *N. cadamba* cultures.

The organic additives such as coconut water, tomato juice and potato extract can promote germination and seedling growth to any plant species (Prades et al., 2012). Results from this study showed that the *in vitro* seed germination and seedling growth of *N. cadamba* were influenced by the type of basal media, plant growth regulators and type of complex additives used. Coconut water (10%) was found to be the optimum media for

seedling growth and development of *N. cadamba* when added singly to the basal media. This study also found that the tissue culture media supplemented with coconut water without the addition of any plant growth regulators (PGRs) can be used as a substitute for hormone usage in *in vitro* seed germination and seedling growth. According to Shekarizz et al. (2014), specific growth factors found in coconut water that contains sugar and natural minerals such as amino acids, phosphorus and nitrogen sources that can be used as a plant nutrient. The use of coconut water as an organic additive had been reported by many previous studies such as in tree *Gleditsia caspica* (Zarinjoei et al., 2014), *Gleditsia caspica* (Ahmed et al., 2017), and *Gossypium arboreum* L. (Yasin & Yasmin, 2018).

The PGRs are usually used singly or combination in seedling multiplication and shoot proliferation such as in studies by Shukla and Shukla (2009); Ncube et al. (2015); Jose and Thomas (2015) and Wesly et al. (2018). The mechanism of action of PGRs is often complicated, and more than one combination of two substances is likely to produce a high yield. The balance between auxin and cytokinin concentrations is required for the formation of adventitious shoots and roots. However, the requisite concentration of each PGR differ significantly according to the type of plants being cultured, the cultural conditions and the compounds used. Such as study by Mazri et al. (2017) and Pérez-Martínez and Castañeda-Garzón (2017) stated that the concentration of inorganic salts showed a crucial function in shoot and root induction. The reduction of MS salts strength to $\frac{3}{4}$ of the original concentration stimulated root formation in date palm tissue culture. According to Jha et al. (2009), the mineral concentration of the culture medium affects rooting characteristics, and some researchers have proposed its reduction to half-strength for rooting improvement.

In this study, seedling growth and shoot multiplication of *N. cadamba* were given a positive response to BAP or NAA at 1.0 mg/L, respectively. The combination of PGRs with coconut water gave the highest percentage of seedling growth under best culture condition 16-h light/ 8-h dark at room temperature. The seedlings were successfully acclimatised by transplanting to the soil without any mixture of compost or peat moss.

6.2 Callus induction of *Neolamarckia cadamba*

Reports by Mokhtari et al. (2015) and Rodrigo et al. (2018) stated that cells derived from competent source tissue are cultured to form an undifferentiated mass of cells called a callus. Plant calli can differentiate into a whole plant, a process called regeneration, through the addition of plant growth regulators in the culture medium. This ability is known as totipotency. Development of organ in callus is depended on the medium component, which is usually required the assistance of plant growth regulators, such as cytokinin and auxin for determining the shoot or root regeneration, respectively. There are many different parts of the plant can be used as an explant for callus induction such as young leaves of buds, leafy shoots, apical shoots, nodes, stems, as well as adventitious shoots and roots (Spahiu et al., 2015; Naz et al., 2016). The present study investigated the callus induction from the young leaf, node and stem. The results showed that young leaf was efficient and suitable explant source to be used in callus induction and proliferation compared to node and stem.

On the other hand, although B5 medium is formulated for callus and suspension cultures, it also works as a basal medium for plant regeneration. B5 medium was proved to increase shoot proliferation of *Jatropha curcas* (Warakagoda & Subasinghe, 2009). In the

cases of *Juglans nigra* the maximum root length was recorded in B5 medium as compared to the other media. Besides, B5 medium was known as the most likely medium influencing the expression of all the seedling parameters, such as leaf number, root length and breadth, fresh weight and dry weight (Steven & Pijut, 2018). However, the factors contributed to these results have yet to be evaluated. For the half-strength B5 medium, it was the most suitable medium for *in vitro* seed germination of *Prunus avium* L.B (Cetinbas & Koyuncu, 2006) and *Vitellaria* (Iroko et al., 2013). B5 media could improve callus induction, shoot organogenesis and plant regeneration from cotyledon explants (Huang et al., 2014; Ouyang et al., 2016). The present study also demonstrated that B5 medium was the best medium for callus induction and plantlet regeneration of *N. cadamba*.

Light requirements needed by the plant are different between the species. The present study showed that treatment with light 16-h light/ 8-h darkness was the best lighting for the seedling growth of *N. cadamba*. For callus induction, explants exposed to dark condition for 24-h dark were the best condition for callus induction. After a month, the culture was exposed to 16-h light/ 8-h dark for callus and shoot multiplication, also for regeneration of plantlets. According to Kauth et al. (2008), the effects of photoperiod on callus induction were influenced by genotype and environmental factors, such as air temperature, relative humidity and oxygen.

Other factors studied in the present study was the effect of coconut water on callus induction and proliferation. The addition of coconut water at 10% (v/v) could induce callus production and proliferation. Coconut water contains amino acids like glutamine, arginine, asparagine, alanine, sugar and aspartic acid. The natural sugars present are in the form of

glucose and fructose that are quickly absorbed by the plant cells for cellular proliferation and regeneration (Sarropoulou et al., 2016; Haque et al., 2017). In this study, all of these supplements provided by coconut water are essential in promoting division, differentiation and regeneration of plant cells.

The good callogenic response was found in B5 medium supplemented with a low concentration of auxins and cytokinins which is 1.0 mg/L of BAP (86.67%) and NAA (80.0%) within eight weeks of inoculation. However, the high concentrations (3.0, 4.0 and 5.0 mg/L) of auxins and cytokinins had resulted in a weak response, and the calli formed were loose and wet with white or dark brown calli. Genotype and source of the explants also lead to the differences in the production of callus and regenerated plantlet (Jesionek et al., 2016; Sampaio et al., 2018).

According to Ozdemira et al. (2015) and Mohamadi-Nasab et al. (2017), the intermediate ratio of both auxins and cytokinins will lead to callus induction. In the present study, the combination of 1.0 mg/L BAP and 0.5 mg/L NAA was used to induce callus of *N. cadamba*. About 95.6% of the callogenic response was achieved within eight weeks of inoculation, and the calli were friable with greenish coloured when inoculated in B5 medium. The combination of auxins and cytokinins, or auxins alone, such as 2, 4-D and BAP, are widely used for callus induction and maintenance (Sandoval-Cancinoe et al., 2016; Nunes et al., 2018). On the other hand, the present study also found that the combination of BAP (1.0 mg/L) with coconut water at 10% (v/v) could also be used to promote callus induction as about 86.7% of explants producing callus within eight weeks of inoculation. The calli were friable with greenish coloured.

6.3 Indirect shoot regeneration of *Neolamarckia cadamba*

Indirect shoot regeneration or organogenesis refers to the *in vitro* formation and development of organs such as roots and shoots either directly from the explants or indirectly through callus differentiation. Yanthan et al. (2017) proposed that organogenesis proceeds *de novo* via the organisation of meristems from unorganised cell masses through patterned cell divisions. The regeneration of shoots from callus can result in somaclonal variation, which depends on genotype, age and type of the callus, culture medium, type of plant growth regulators used and the number of subcultures (Singh et al., 2016; Stevens & Pijut, 2018).

Indirect shoot regeneration from calli derived from the young leaf explants was successfully initiated by inoculating the leaf-derived callus onto B5 medium fortified with different combinations of 2.0 mg/L BAP and 0.5 mg/L NAA that resulted in 57.8% number of adventitious shoot and an average of 8.80 shoots produced after 16 weeks of culture (refer Table 4.2). This finding is similar to report by Li et al. (2019), stated that high frequency plant regeneration from leaf culture of *N. cadamba*. Also similar finding is report by Zarinjoei et al. (2014) that maximum *in vitro* plant regeneration was obtained from leaf-derived callus cultures of leguminous tree *Gleditsia caspica*. The high frequency regeneration protocol was also successfully developed through callus-mediated organogenesis from cotyledon leaf and hypocotyl of *N. cadamba* (Huang et al., 2020).

On the medium supplemented with cytokinins, various numbers of adventitious shoots per explant were sprouted as shoot clusters. The adding of BAP into the shoot proliferation media has improved the growth of axial shoots and promoted the shoots

multiplication from the basal tissues of the *Lycopersicon esculentum* Mill (Sharma & Srivastava, 2014), *Cenchrus ciliaris* (Kumar et al., 2015), *Phalaenopsis* (Winarto et al., 2016), and *Oreomunnea mexicana* (Atondo-bueno et al., 2016) explants.

However, Wongsu et al. (2018) reported that the growth of shoots remained stunted and leaves became red and abscised when BAP was used continuously in the medium for every subculture. They proved that the use of two different multiplication media contained BAP and kinetin in alternate passages significantly improved shoot condition and reduced the deleterious effects as observed on the continuous use of BAP for every subculture. Hence, the explants used in the present study were subcultured to new B5 medium fortified with 10% CW after two weeks of inoculation on the medium with BAP for subsequent development into complete plantlets.

6.4 Direct shoot regeneration through the nodal segment of *Neolamarckia cadamba*

Direct shoot initiation is usually limited to explants obtained from juvenile material; however, several reports have indicated shoot regeneration from mature tree explants as well (Zhang et al., 2015; Kulpa et al., 2018). The phenolic exudations into the medium by explants derived from mature trees can often cause difficulties in establishing *in vitro* cultures and inhibit proliferation of axillary buds (Abrahamsson et al., 2017). In the present study, *Neolamarckia cadamba* shoots were actively regenerated when the node segments were inoculated on medium added with 1.0 mg/L BAP and 0.5 mg/L NAA. About 80.1% of node segments were able to regenerate shoots, and an average of 13 shoots per explant was produced after 16 weeks of culture on medium. This finding is similar to Yumbla-Orbes et al. (2017) which found that the combination of plant growth regulators in

low cytokinins: auxins ratio could promote plantlets regeneration in *Lisianthus* (*Eustoma grandiflorum*).

According to the study of Aktar et al. (2008), the addition of coconut water as a complex organic supplement in the media could enhance the rate of plant's cell division and regeneration in tissue culture. In the present study, 97.8% of node segments were successfully regenerated on medium added with 1.0 mg/L BAP and 10% CW. An average of 19.2 shoots per explant after 16 weeks of culture. Other success studies reported were by Aggrey et al. (2003) on the production, storability, and regeneration of shoot tips of potato (*Solanum tuberosum* L.), Anwaar et al. (2002) for shoot apical meristem in wheat (*Triticum Aestivum* L.), Gómez-montes et al. (2015) on direct shoot regeneration using cotyledonary explants and true leaves from lettuce cv. Romaine (*Lactuca sativa* L.), and Jose and Thomas (2015) in regenerated plants of *Curcuma caesia* Roxb. as an important source of camphor.

Others research on direct shoot regeneration from *in vitro*-derived leaf, stem and root as explants for tree species also had been reported by Mallon et al. (2011) in *Centaurea ultreiae*, *Eucalyptus tereticornis* (Aggarwal et al., 2010), *Eucalyptus polybractea* (Fernando et al., 2016) and *Dipteryx odorata* (Sampaio et al., 2018). Report by Fernando et al. (2016) stated the further shooting efficiencies were naturally correlated with fewer rooting efficiencies in the tree species. Also report by Fernando et al. (2016), the efficiency of the organogenesis protocol for *Eucalyptus polybractea* is relatively high compared to other studies involving explants from adult trees. In addition, the use of cotyledon as a explants were recorded gave the highest rates to mass propagate

Neolamarckia cadamba (Huang et al., 2014; 2020). These techniques have been shown to be useful in mass production of *N. cadamba* clones, improvements are needed to support the planting materials through large-scale planting programs production.

6.5 Conclusion

In conclusion, an efficient protocol for *in vitro* seed germination and propagation of *Neolamarckia cadamba* was achieved by using Gamborg B5 as basal media. The full-strength B5 medium supplemented with plant growth regulators and coconut water was identified as the best medium for *in vitro* seed germination, seedling growth, callus induction, indirect and direct shoot regeneration of *N. cadamba*. The addition of complex additives, i.e. coconut water at 10% (v/v) was remarkably enhanced the *in vitro* seed germination, seedling growth, callus induction and shoot regeneration of *N. cadamba*. Further study is recommended that includes the transplantation of hardened seedlings to the field and genetic fidelity study of tissue culture-derived *N. cadamba* seedlings.

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APPENDICES

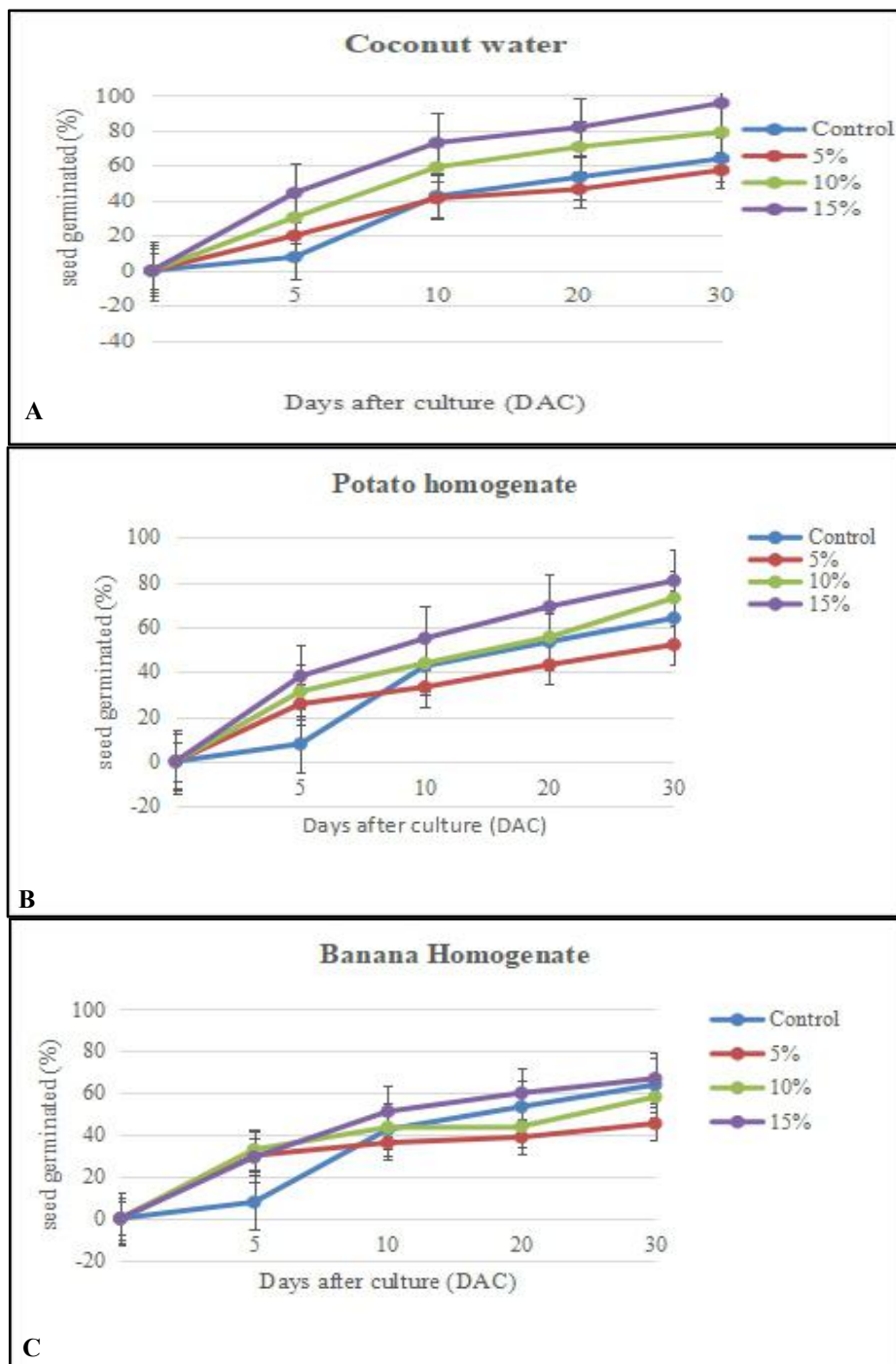
Appendix A

Media composition of MS basal media and B5 basal media.

Constituent		Concentration in medium (mg/L)		Concentration in stock solution (mg/L)	
		MS medium	B5 medium	MS stock solution	B5 stock solution
Macronutrients (10x)					
Ammonium nitrate	NH ₄ NO ₃	1650.0	-	16500.0	-
Potassium nitrate	KNO ₃	1900.0	3000.0	19000.0	30000.0
Magnesium sulfate heptahydrate	MgSO ₄ ·7H ₂ O	370.0	500.0	3700.0	5000.0
Calcium chloride dihydrate	CaCl ₂ ·2H ₂ O	440.0	150.0	4400.0	1500.0
Potassium dihydrogen phosphate	KH ₂ PO ₄	170.0	-	1700.0	-
Monosodium dihydrogen phosphate dihydrate	NaH ₂ PO ₄ ·2H ₂ O	-	150.0		1500.0
Ammonium sulfate	(NH ₄) ₂ SO ₄	-	134.0		1340.0
Micronutrients (100x)					
Boric acid	H ₃ BO ₃	6.2	3.0	620	300
Manganese (II) sulfate tetrahydrate	MnSO ₄ ·4H ₂ O	22.3	-	2230	-
Manganese (II) sulfate monohydrate	MnSO ₄ ·H ₂ O	-	10.0	-	1000
Zinc sulfate heptahydrate	ZnSO ₄ ·7H ₂ O	8.6	2.0	860	200
Potassium iodide	KI	0.83	0.75	83	75
Sodium molybdate dihydrate	Na ₂ MoO ₄ ·2H ₂ O	0.25	0.25	25	25
Copper (II) sulfate pentahydrate	CuSO ₄ ·5H ₂ O	0.025	0.025	2.5	2.5

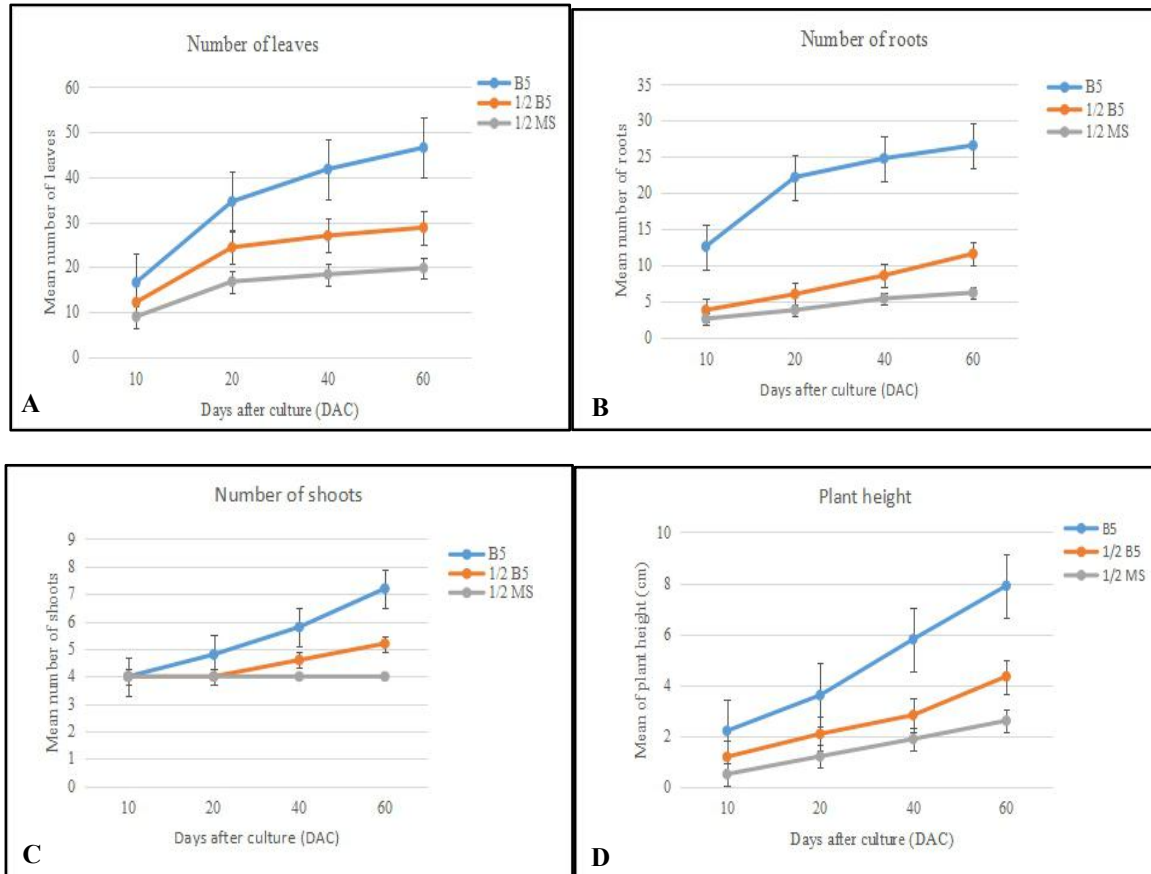
Cobalt (II) chloride pentahydrate	CoCl ₂ .6H ₂ O	0.025	0.025	2.5	2.5
Chelating agents (100x)					
Iron (II) sulfate heptahydrate	FeSO ₄ .7H ₂ O	27.8	27.8	2780.0	2780.0
EDTA disodium salt	Na ₂ EDTA	37.3	37.3	3730.0	3730.0
Vitamins (100x)					
Nicotinic acid		0.5	1.0	50.0	100.0
Thiamine HCl		0.1	10.0	10.0	1000.0
Pyridoxine HCl		0.5	1.0	50.0	100.0
Myo-inositol		100.0	100.0	10000.0	10000.0
Others (100x)					
Glycine		2.0	-	200.0	-

Appendix B



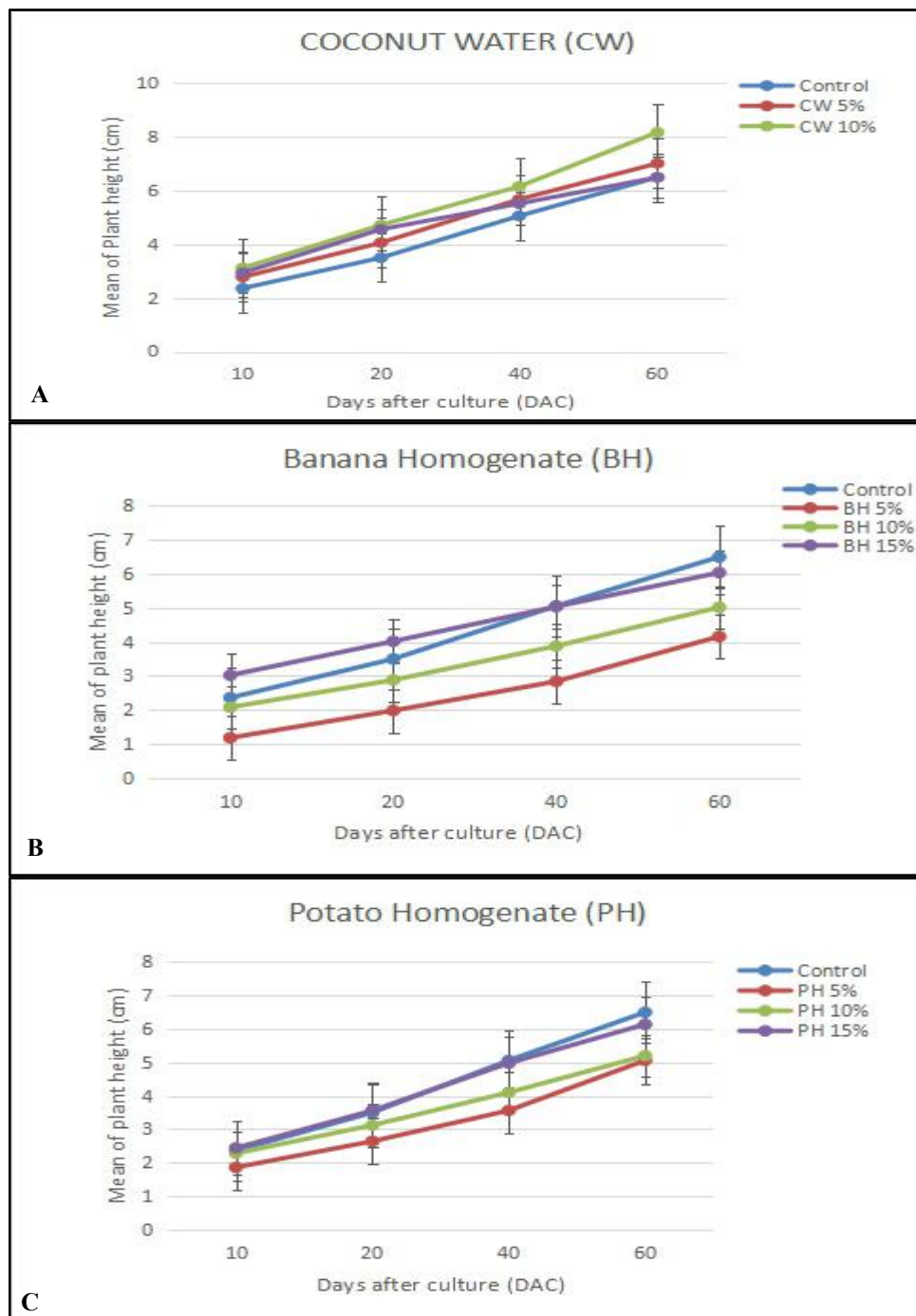
Effect of complex additives: Graft (A) Coconut water; (B) Potato homogenate and (C) Banana homogenate on *in vitro* seed germination of *Neolamarckia cadamba* according to day observation.

Appendix C



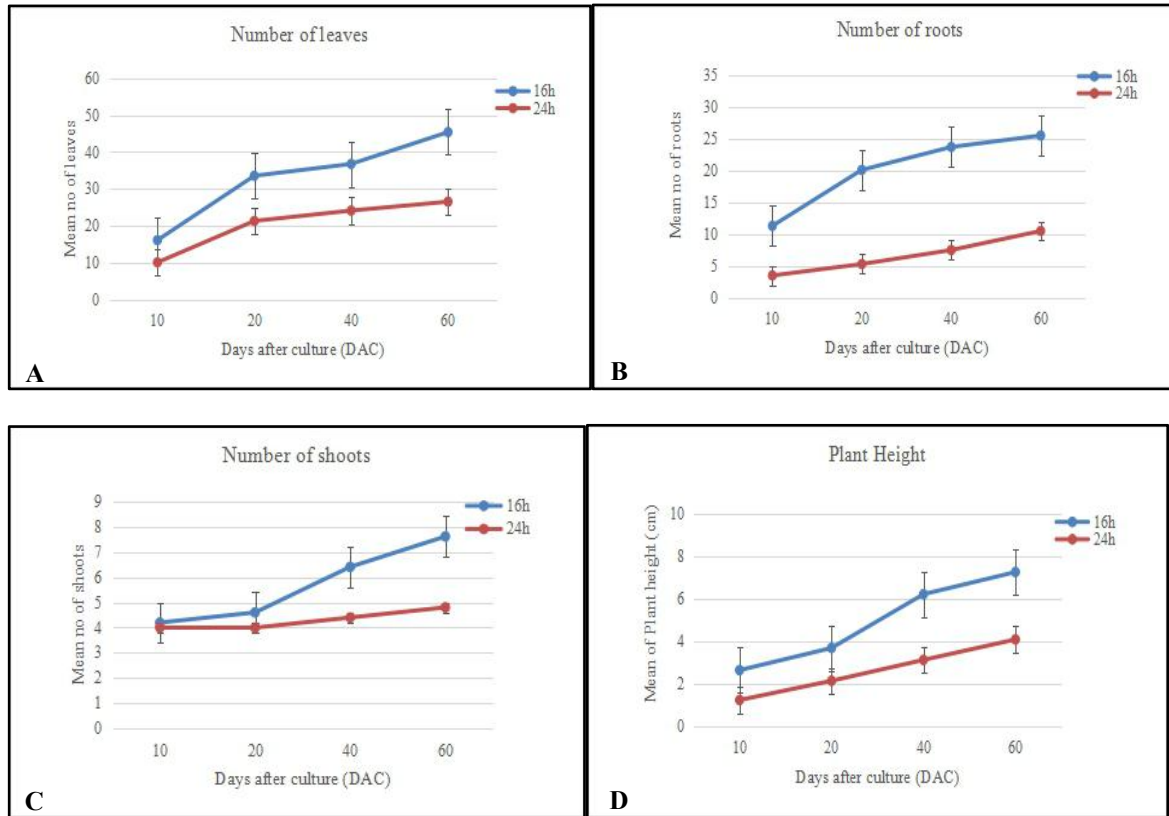
Effect of types basal media on seedling growth and development: Graft (A) Number of leaves; (B) Number of roots; (C) Number of shoots; and (D) Plant height on *in vitro* seedling *Neolamarckia cadamba* according to the day after culture (DAC).

Appendix D



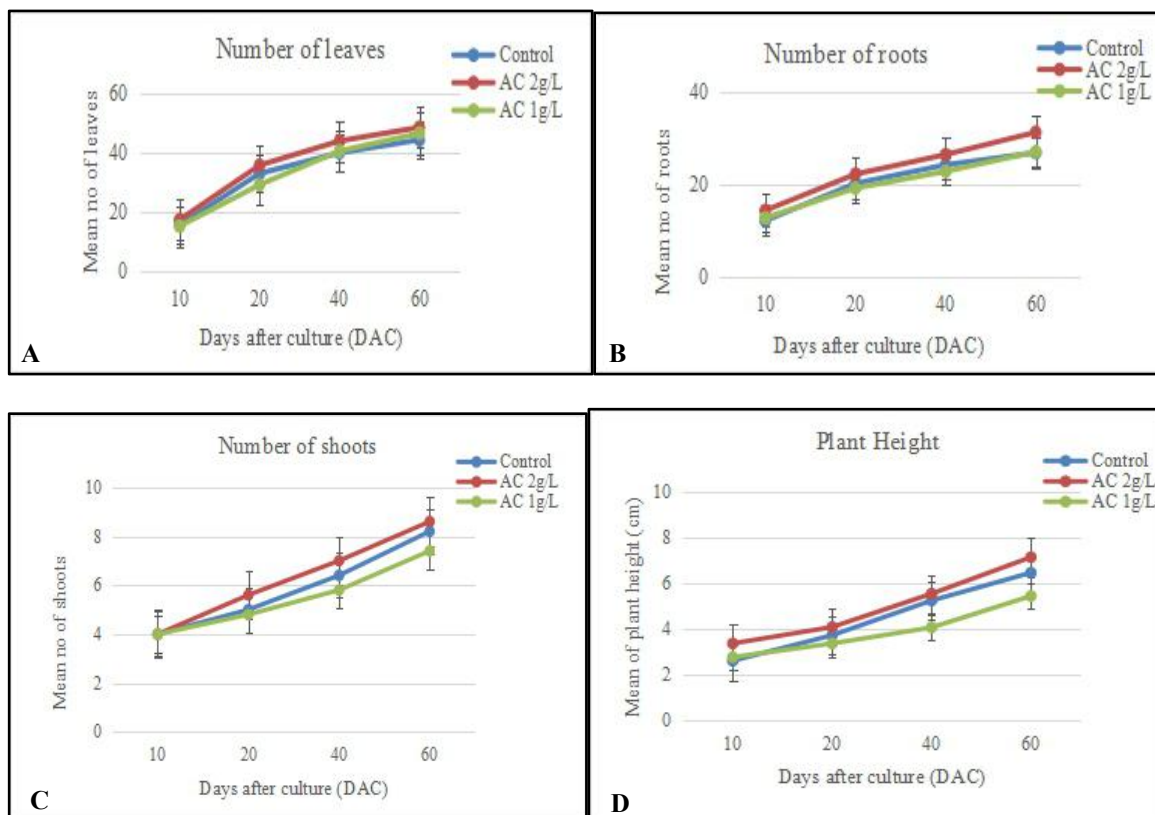
Effect of different concentrations of complex additives on seedling growth and development: Graft (A) Coconut water; (B) Banana homogenate; and (C) Potato homogenate on plant height of *Neolamarckia cadamba* by days after culture (DAC)

Appendix E



Effects of photoperiod on seedling growth and development: Graft (A) leaves, (B) roots, (C) shoots; and (D) Plant height (cm) on seedling growth and development of *Neolamarckia cadamba* according to day after culture.

Appendix F



Effect of activated charcoal on seedling growth and development of *Neolamarckia cadamba* according to days after culture.