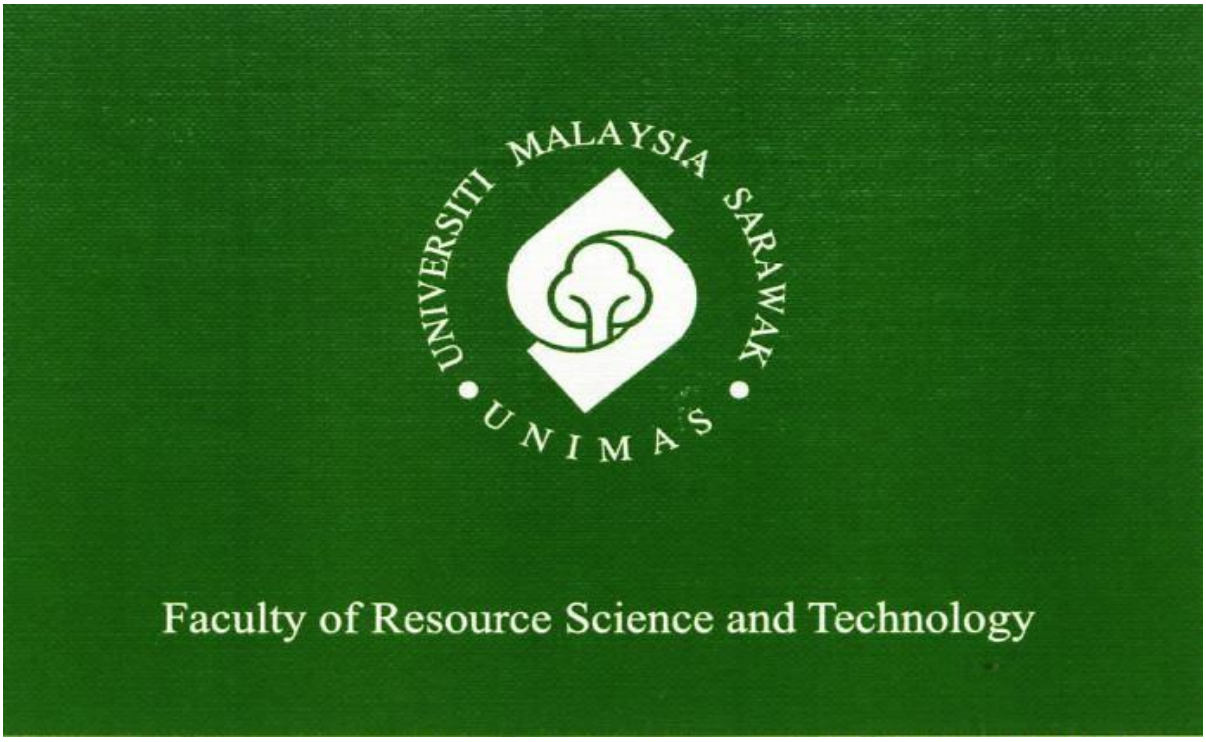

De novo shoot regeneration of *Neolamarckia cadamba* (Roxb.) Bosser

Mohd. Azrin bin Abdullah (70518)

Bachelor of Science with Honours
(Resource Biotechnology)
2022

De novo shoot regeneration of *Neolamarckia cadamba* (Roxb.) Bosser

Mohd. Azrin bin Abdullah

A thesis submitted in partial fulfilment of the Requirement of the Degree
Bachelor of Science with Honours
(Resource Biotechnology)

Supervisor: Associate Professor Dr. Ho Wei Seng

Programme of Resource Biotechnology
Faculty of Resource Science and Technology
UNIVERSITI MALAYSIA SARAWAK
2022

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De novo shoot regeneration of *Neolamarckia cadamba* (Roxb.) Bosser

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ABSTRACT

Neolamarckia cadamba (Roxb.) Bosser, locally known as Kelampayan, is a multipurpose tree species that has the ability to grow faster than many other tropical timber species, which can help in reducing the impact of deforestation, as well as to continuously cater the raw materials for commercial purposes. Despite many studies have been conducted on *de novo* shoot regeneration, the application of this method on *N. cadamba* is limited, and thorough studies on the process is needed to diversify *in vitro* regeneration methods for *N. cadamba*. Also, lack of research has been done to study the effects of different thidiazuron (TDZ) concentrations on *de novo* shoot regeneration for *N. cadamba*. This study focused on generating *N. cadamba* through *de novo* shoot regeneration and determining the optimum TDZ concentration for *N. cadamba* using leaf explants. Three different TDZ concentrations (2.0, 4.0 and 6.0 mgL^{-1}) were tested, focusing on three factors, which are the length of leaf explants, leaf counts and shoot counts. After 5 weeks of culturing, 6.0 mgL^{-1} TDZ exhibited the best results, posting the highest mean length of leaf explants, leaf counts and shoot counts. However, it is not enough to conclude that the concentration stated is the optimum TDZ concentration for *N. cadamba*. Based on the preliminary results, the method can be further improved to fully utilise TDZ as it becomes more common to be used to induce a systematic micropropagation for woody plant species. Thus, by determining the optimum TDZ concentration for *N. cadamba*, it can be used as an indicator to determine the optimum TDZ concentration for other tropical timber species.

Keywords: *Neolamarckia cadamba, de novo,* shoot regeneration, thidiazuron, leaf explants

Penjanaan semula pucuk Neolamarckia cadamba (Roxb.) Bosser secara de novo

ABSTRAK

Neolamarckia cadamba (Roxb.) Bosser, juga dikenali sebagai Kelampayan, ialah spesies pokok serbaguna yang mempunyai keupayaan untuk tumbuh dengan pantas berbanding spesies kayu balak tropika yang lain di mana ia dapat mengurangkan impak penebangan hutan dan dapat membekalkan bahan mentah secara berterusan untuk kegunaan komersial. Walaupun terdapat banyak kajian mengenai penjanaan semula pucuk, pengaplikasian kaedah ini pada N. cadamba adalah terhad, dan kajian secara terperinci tentang kaedah ini diperlukan untuk mempelbagaikan kaedah penjanaan semula untuk N. cadamba secara in vitro. Juga, kajian berkenaan kesan kepekatan thidiazuron (TDZ) yang berbeza terhadap penjanaan semula pucuk N. cadamba adalah terhad. Kajian ini memfokuskan penjanaan N. cadamba melalui penjanaan semula pucuk secara de novo dan menentukan kepekatan TDZ yang optimum menggunakan eksplan daun. Tiga kepekatan TDZ yang berbeza (2.0, 4.0 dan 6.0 mgL^{-1}) diuji, memfokuskan tiga faktor iaitu panjang eksplan daun, kiraan daun dan kiraan pucuk. Selepas 5 minggu pengkulturan, 6.0 mgL^{-1} TDZ menunjukkan hasil yang terbaik. Akan tetapi, ia tidak mencukupi untuk membuat kesimpulan bahawa kepekatan tersebut adalah kepekatan optimum TDZ bagi N. cadamba. Berdasarkan keputusan awal, kaedah ini dapat ditambah baik untuk menggunakan keupayaan TDZ sepenuhnya kerana ia sering digunakan untuk mendorong pembiakan mikro secara sistematik bagi spesies tumbuhan berkayu. Oleh yang demikian, dengan menentukan kepekatan optimum TDZ untuk N. cadamba, ia dapat digunakan sebagai pengukur untuk menentukan kepekatan optimum TDZ bagi tumbuhan berkayu tropika yang lain.

Kata kunci: *N. cadamba, de novo, penjanaan semula pucuk, thidiazuron, eksplan daun*

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LIST OF ABBREVIATIONS

2,4-D	2,4-dichlorophenoxyacetic acid
BAP	6-benzylaminopurine
IAA	Indole-3-acetic acid
MAS	Marker-assisted selection
NAA	1-naphthalenacetic acid
TDZ	Thidiazuron

CHAPTER 1

INTRODUCTION

Deforestation is one of the biggest threats the world is facing at the moment, which has become one of the factors that contribute to the global climate change. Food and Agriculture Organisation (2020) described deforestation as a process by which the forest is converted to other land uses. The resources obtained from deforestation are utilised by humans for economic purposes, such as furniture making, paper production, where the products produced will be exported or traded domestically and internationally (Omran & Schwarz-Herion, 2020). Interestingly, Omran and Schwarz-Herion (2020) also mentioned that deforestation is one of the methods used by developing countries to boost their nation income by exporting the resources obtained from deforestation and products produced, which led to higher rate of deforestation as compared to developed countries.

Neolamarckia cadamba (Roxb.) Bosser, also known as *kadam,* burflower tree and *Kelampayan*, is categorised under the family of Rubiaceae and considered to be one of the tree species that possess the ability to grow faster and multipurpose. Native in countries such as Indonesia, Malaysia, and Thailand in Southeast Asia, and Bangladesh and India in South Asia, *N. cadamba* can be found in low level areas and montane forests up to the level of altitude of 1,000m (Lim et al., 2005). *N. cadamba* is a multipurpose tree species as it can be utilised in many ways, showing its significance in economy sectors. As mentioned by Huang et al. (2020), *N. cadamba* is known to be a “miracle” tree as every part of the tree possesses many benefits in many ways to the growers.

Therefore, to overcome and reduce the effect of deforestation, as well as to meet the wood demand for commercial purposes, many research and methods have been conducted and implemented to restore our forests. One of the methods is direct seeding, where it is

considered to be a practicable method to restore forest in a large scale. However, according to Freitas et al. (2019), little information can be found regarding the success rate of tropical forest restoration through the method of direct seeding, thus requiring in depth research to validate the method. Another method used is developing marker-assisted selection (MAS) to improve the selection and replanting process. Such method is practical for simple characteristics or traits that are controlled by some quantitative trait loci (QTLs), contributing some large parts of the phenotypic variability overall (Lebedev et al., 2020). Nonetheless, due to the quantitative and complexity of inheritance patterns of valuable plant traits such as the rate of growth and the wood properties, MAS cannot be used effectively.

One of the tools used that is considered to be vital in the field of plant biotechnology is plant tissue culture. Raspor et al. (2021) highlighted the significance of plant tissue cultures, mentioning how the techniques are continuously evolving to maximise the procedure efficiency to fully utilise plant phenotype plasticity for research and development, conservation, agricultural and industrial purposes. The method where tissues or organs detached from the plants are cultured in the regeneration media with plant growth regulators (PGRs) needed such as auxin and cytokinin to manipulate the shoot growth from callus is known as *de novo* shoot regeneration (Shinohara et al., 2014). Despite many studies have been conducted on *de novo* shoot regeneration, research on *de novo* shoot regeneration on *N. cadamba* is limited, and a thorough studies on the process is needed to diversify the regeneration methods for *N. cadamba*. It is believed that the method can be implemented to mass generate and produce *N. cadamba* to overcome the impact of deforestation, as well as to cater the wood supplies for commercial purposes.

De novo shoot regeneration is a process where the plant cells or tissues are capable of exhibiting the entire process of postembryonic shoot formation, triggering responses from the explant cells to grow into new plants. Few research has been conducted on *de novo* shoot

regeneration of *N. cadamba*, which led to establishment of *de novo* shoot regeneration protocols for the plant species such through cotyledons, and direct adventitious shoot. A protocol on the regeneration of *N. cadamba* from leaf culture was published by Li et al. (2019), comparing two different cytokinins which were 5 mgL^{-1} thidizuron (TDZ) and 3 and 5 mgL^{-1} 6-benzylaminopurine (BAP), together with 0.1 mgL^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.05 mgL^{-1} 1-naphthaleneacetic acid (NAA). With the establishment of such protocols, it helps in regenerating *N. cadamba* in a large scale, and less time is required to propagate the commercial cultivars. However, BAP is the most common cytokinin used to induce plant regeneration of *N. cadamba*, thus showing lack of research done to study the effect of TDZ on the species in general, as well as the effects of different TDZ concentrations on the species' morphologies when performing *de novo* shoot regeneration on *N. cadamba*.

Objectives:

1. To regenerate *N. cadamba* through the process of *de novo* shoot regeneration.
2. To determine the optimum TDZ concentration for *de novo* shoot regeneration on *N. cadamba*.

CHAPTER 2

LITERATURE REVIEW

2.1 Species studied

2.1.1 *Neolamarckia cadamba* (Roxb.) Bosser

Figure 2.1 *Neolamarckia cadamba* (Roxb.) Bosser, also known as *Kelampayan* in Malaysia. Adapted from *Neolamarckia cadamba* (Roxb.) Bosser, 1984 (p. 1) by A. R. Mojiol, W. Lintangah, M. Maid and K. Julius, 2014, John Wiley & Sons. Copyright 2014 by Wiley-VCH Verlag GmbH & Co.

Neolamarckia cadamba (Roxb.) Bosser (Figure 2.1), commonly known as burflower tree or kadam and locally known as *Kelampayan*, is categorised under the family of Rubiaceae. Historically, the name *Neolamarckia* cadamba was created by Jean Marie Bosser back in 1984 to honour Jean-Baptiste Lamarck for his finding of the Asian genus (Razafimandimbison, 2002). Some other terms or synonyms used to refer *N. cadamba* are *Anthocephalus cadamba* (Roxb.) Miq., *Sarcocephalus cadamba* (Roxb.) Kurz., and *Nauclea cadamba* Roxb., *N. cadamba* is an angiosperm, perennial plant, where its maximum height can reach from the range of 30 to 45 metres (NParks Flora & Fauna Web, 2021). As

mentioned by Lim et al. (2005), this tropical, evergreen tree is a native species in Southeast Asia countries such as Indonesia, Malaysia, and Thailand, as well as in South Asia such as Bangladesh, India, and Sri Lanka, and it can be found growing in the low-level areas and montane forests up to the level of altitude of 1,000 metres. It is worth to note that the plant has been introduced in countries such as Taiwan, Puerto Rico, Costa Rica, Surinam, South Africa, and some other tropical and subtropical nations, as stated by Orwa et al. (2009).

Figure 2.2 The bark of *Neolamarckia cadamba.* Adapted from *Neolamarckia cadamba* (Roxb.) Bosser, 1984 (p. 1) by A. R. Mojiol, W. Lintangah, M. Maid and K. Julius, 2014, John Wiley & Sons. Copyright 2014 by Wiley-VCH Verlag GmbH & Co.

Figure 2.3 The leaves of *N. cadamba.* Adapted from "Traditional Uses, Phytochemistry and Pharmacological Properties of *Neolamarckia cadamba*: A Review", by A. Pandey and P. S. Negi, 2016, *Journal of Ethnopharmacology, 181*, p. 135. Copyright 2016 by Elsevier.

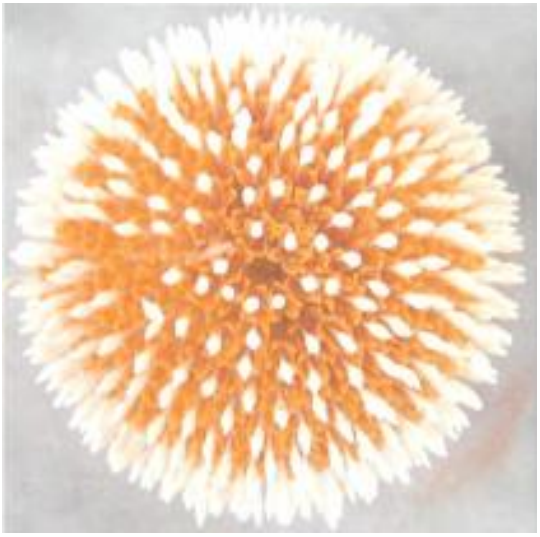

Figure 2.4 The flower of *N. cadamba.* Adapted from "Traditional Uses, Phytochemistry and Pharmacological Properties of *Neolamarckia cadamba*: A Review", by A. Pandey and P. S. Negi, 2016, *Journal of Ethnopharmacology, 181*, p. 135. Copyright 2016 by Elsevier.

In terms of physical appearance, *N. cadamba* can be recognised with the presence of its broad, umbrella-shaped crown, small buttresses and its branches are usually growing up to 25 metres and arranged in tiers (Tan et al., 2007). Its stem diameter can range from 100 to 160 centimetres and its bark (Figure 2.2) can provide some indicators to the conditions of the trees, where usually grey, very light and smooth bark can be found on young *N. cadamba* trees while rough-surfaced, longitudinally cracked, or fissured bark can be observed on the

old ones (Krisnawati et al., 2011). As for the leaves (Figure 2.3), *N. cadamba* has deciduous, shiny green leaves with a wide of 8 to 25 centimetres and 15 to 50 centimetres long. According to Institute of Forest Genetics and Tree Breeding (2019), when the tree enters the flowering phase and starts producing flowers (Figure 2.4), they appear to be hairy, dense, and small orange balls about 5.5 centimetres in diameter, producing sweet fragrant. The fruits (Figure 2.5) are found in capsules that are small in size, and closely packed to one another, forming a fleshy yellow-orange infructescence with about 8,000 seeds. The shapes of the seeds are observed to be irregular or trigonal (Seorianegara & Lemmens, 1993). Once entering the maturation period, the small capsules will be split into 4 parts, releasing its seeds for germination process of new trees to take place.

Figure 2.5 The fruits of *N. cadamba*. Adapted from "Evaluation of the Anti-Oxidative, Erythrocyte Membrane Stabilizing Effect and Nutritional Status of *Neolamarckia cadamba* fruit", by M. M. Sarkar, M. Hasan, S. Bhowmick, J. Hussain, M. H. Bhuiyan, M. A. Khan and S. Hossain, *American Journal of Food and Nutrition, 7*(1), p. 7. Copyright 2019 by Science and Education Publishing.

2.1.2 The uses of *Neolamarckia cadamba* (Roxb.) Bosser

Neolamarckia cadamba is known as a "miracle tree", as stated by Huang et al. (2020) due to its ability to grow faster and every part of the tree can be utilised by the growers. With the density ranges from 290 kg/m^3 to 560 kg/m^3 and 15% moisture content, *N. cadamba* has a smooth to medium surface texture and does not giving out any unpleasant taste or odour (Joker, 2000). Also, to enhance the quality of the *N. cadamba* wood, it can be infused with

synthetic resins to improve its compressive strength and density. Such characteristics possessed by this timber tree species make it an excellent raw material for economic and commercialised purposes, such as paper, pulp, plywood, and furniture productions. Interestingly, as noted by Orwa et al. (2009), the plant has the ability to naturally produce tannin, which can be obtained from its root bark.

Other than that, parts of *N. cadamba* are very well utilised for traditional medicine formulation. According to Umachigi et al. (2007), some parts of *N. cadamba* are used in the making of traditional remedies to treat skin diseases, eye infection, fever, and stomach pain. Also, the leaf extract of the plant is used to treat throat infection by gurgling the extract obtained as it possesses astringent properties (Pandey & Negi, 2016). Additionally, with the presence of flavonoids, glycosides, alkaloids, and steroids as the major phytochemicals found in *N. cadamba* leaves, it is found that the phytochemicals possess anti-microbial, anti-diabetic, anti-oxidant, and anti-inflammatory activities (Khandelwal et al., 2019). Institute of Forest Genetics and Tree Breeding (2019) mentioned that the plant has some of the largest secondary metabolites, thus it enhances biological and pharmacological properties of *N. cadamba* as they can be used as an alternative to a number of synthetic chemical compounds.

As mentioned previously, one of the characteristics possessed by *N. cadamba*, which is fast growth rate, makes it one of the sought-after timber trees in Malaysia, especially in Sarawak. Such characteristic shown by this species is important to overcome the deforestation that is happening in the state. Therefore, the Sarawak State Government has allocated one million hectares for forest plantation development, where plant species such as *N. cadamba* is planted for the purpose of establishing commercial forest plantations (Ho et al., 2012). Also, this plant species is seen to be a suitable alternative to diversify the ecosystem of tree plantation and to improve the productivity, as discussed by Wei and Zhu (2019). It is worth to note that *N. cadamba* helps to improve the chemical and physical properties of the

soil under its canopy, enhancing the capacity of cation exchange, soil organic carbon and nutrient supplies (Orwa et al., 2009). Interestingly, under the Aggressive Industrial Forest Plantation programme headed by Forest Department Sarawak, trees of *N. cadamba* are planted widely at palm estates in Sarawak as to provide alternative timber sources, as well as to fully utilise the lands (Ta Ann Group, n.d.).

2.1.3 Growth conditions of *Neolamarckia cadamba* (Roxb.) Bosser

Countries in Southeast Asia and South Asia regions such as Malaysia, Indonesia, Singapore, Thailand, Vietnam, Myanmar, the Philippines, India, Pakistan, and Bangladesh are the home of *N. cadamba*. This fast-growing timber species can also be found in countries such as Taiwan, South Africa, Puerto Rico, and Suriname as a result of introduction of the species in countries that have similar climates to the tropical regions (Orwa et al., 2009). Under similar climates, the rate of growth of *N. cadamba* is almost the same, or outperforming, some other fast-growing species such as pines, acacias, and eucalypts, according to previous planting and experimental reports published (Zhang et al., 2016).

As a tropical timber tree species, it requires continuous light exposure, making it one of the most important growth conditions for *N. cadamba*. According to Krisnawati et al. (2011), *N. cadamba* strives well in the tropical and subtropical climates, where the maximum temperature ranges from 32˚C to 42˚C, whereas the minimum temperature ranges from 3˚C to 15.5˚C. It is worth to note that the species is easily affected by the cold environment. This species requires alluvial soil with good drainage system, and with the average annual rainfall of 1,500mm to 5,000mm, it can grow well. However, Luna (1996) stated that *N. cadamba* can also grow and survive in an arid environment with low annual rainfall. Geographically, the tree can be found in the low-level areas and montane forests up to the level of altitude of 1,000m (Lim at al., 2005), and it can be up to 1,400m (Martawijaya et al., 1989).

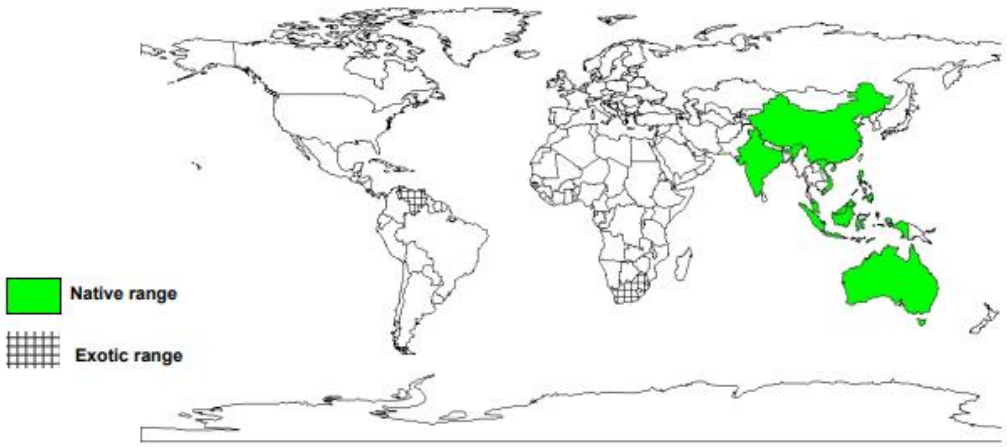

Figure 2.6 The distribution of *N. cadamba* globally. Adapted from Athocephalus cadamba from *World Agroforestry* by C. Orwa, A. Mutua, R. Kindt, R. Jamnadass and A. Simons, 2009, from http://apps.worldagroforestry.org/treedb2/AFTPDFS/Anthocephalus_cadamba.PDF. Copyright 2009 by World Agroforestry.

2.2 *De novo* shoot regeneration

2.2.1 Background of *de novo* shoot regeneration

Due to its inability to move from one place to another, plants have mastered the ability to adapt to the new environment when the basic needs are provided, and most importantly, being able to replace, restore and redevelop broken structures and tissues. As defined by Phillips et al. (1995), the process which is known as regeneration can take place through organogenesis – individual organs are formed through regeneration of shoots or roots, and somatic embryogenesis – a bipolar structure is formed which contains both shoot and root meristems, similarly to zygotic embryos development. Both processes include direct method, where the plant can be regenerated from tissues obtained from non-meristematic sections, referred as an adventitious origin, and indirect method, where the plant can be regenerated from cell cultures and callus, which can also be referred to as *de novo* origin (Phillips et al., 1995; Ikeuchi et al., 2019). These processes that take place in response to the damage or loss

on the body structures, depending on the environmental stressors faced by plants. This plasticity also comprises the capability of the plant to generate *de novo* meristems and organs from tissues that are differentiated, which may lead to the formation of a complete organ, in accordance with the identity switch by the cells (Hnatuszko-Konka et al., 2021).

In general, the term "*de novo*" is an expression derived from Latin which refers to something arises from the beginning or from the start again. Biologically, the term "*de novo*" is used to describe the biological processes that have begun again, as explained by BiologyOnline (2020). Whereas shoot regeneration is explained as a vital process that involves vast fate transition in callus cells and spatial reorganisation of the identities of the cells (Gordon et al., 2007). Therefore, *de novo* shoot organogenesis refers to the capability of the plant cells or tissues to exhibit the entire process of postembryonic shoot formation, where inductive signals are induced during the process of organogenesis and somatic embryogenesis obtained from the mature organs of the plants to trigger responses from the explant cells (Hnatuszko-Konka et al., 2021).

In plant regeneration, there are two steps involved: pluripotency acquisition and *de novo* shoot organogenesis. Chen et al. (2014) brought up the fact that naturally, organs that are detached from the plant can regenerate itself into a whole plant from adventitious roots and shoots, depending on the situations and conditions. *De novo* shoot regeneration can be divided into four stages (Figure 2.7): pluripotency acquisition, shoot pro-meristem formation, shoot progenitor establishment, and shoot outgrowth, as stated by Shin et al. (2020). Table 2.1 shows the main genes or key regulators that are vital in the process of *de novo* shoot regeneration. To begin the process of *de novo* organogenesis, it is important to produce a pluripotent callus, where the tissues or organs extracted from the plant are cultured on the callus-inducing medium which is rich of auxin hormone (Shin et al., 2020). Once pluripotent callus has established its competence for organogenesis to take place, a subgroup of callus

cells will acquire the shoot identity and when incubated in shoot-inducing medium that is rich of cytokinin, it continues to develop into a fertile shoot (Liu et al., 2018).

Table 2.1: List of genes or key regulators that are involved in *de novo* shoot regeneration.

Stage	Genes involved	Reference
Pluripotency Acquisition	• Plethora 1, 2, 3, 5, 7 (PLT1, 2, 3, 5, 7) • WOX11-Lateral organ boundaries domain 16 (LBD16) • Wuschel-related homebox 5 (WOX5) • Scarecrow (SCR)	Sugimoto et al. (2010)
Shoot Pro-meristem	• Cup-shaped cotyledon 1 and 2 (CUC1 & CUC2) • Enhancer of shoot regeneration 1 and 2 (ESR1 & ESR2) • PIN1	Gordon et al. (2007)
Shoot Progenitor	• Wuschel (WUS) • Shoot meristemless (STM) • CLAVATA3 (CLV3)	Negin et al. (2017)
Shoot Outgrowth	• Sawtooth 1 and 2 (SAW1 & SAW2) • TCP family transcription factor 10 (TCP10) • *Arabidopsis thaliana* homeobox (ATH1) • MicroRNA165 and 166 (miRNA165 & miRNA166)	Lee et al. (2019).

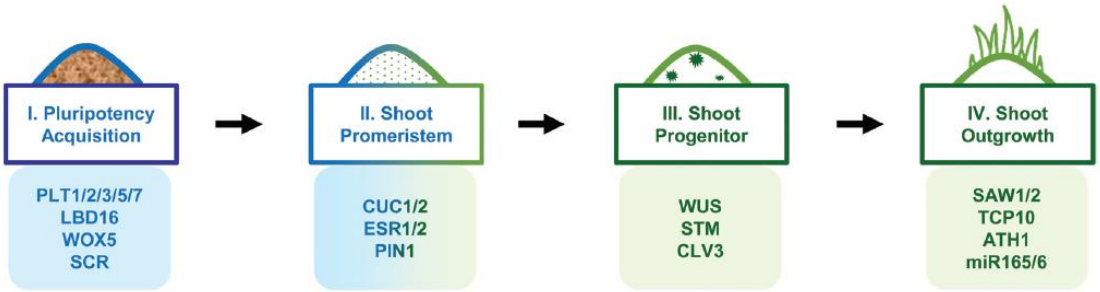

Figure 2.7 Main stages involved in *de novo* shoot regeneration. Adapted from "*De novo* Shoot Organogenesis during Plant Regeneration", by J. W. Shin, S. H. Bae, and P. J. Seo, 2020, *Journal of Experimental Botany, 71*(1), p. 65. Copyright 2020 by Oxford Academic.

2.3 Plant tissue culture

2.3.1 Background of plant tissue culture

In general, plant tissue culture is a method by which the plant seeds, cells, tissues, organs, and protoplasts are cultured on a synthetic, known nutrient media under sterile and controlled environments. This method possesses a number of advantages as compared to the conventional, traditional propagation methods. It has become one of the preeminent tools in plant biotechnology, leading numerous of breakthroughs to understand and apply the fundamental towards gaining more benefits from plants. Historically, Gottlieb Haberlandt, a German scientist, proposed the theory of plant tissue culture back in 1902, thus creating a stepping stone for plant tissue culture to continue developing (Thorpe, 2007). The root, the embryo and the tissue or callus cultures were some of the early studies conducted, and as expressed by Thorpe (2007), the 1940s until 1960s were the periods where new techniques and methods were introduced, as well as some improvements done on the previous ones. Such introductions and improvements have led to the significant applications in fields such as plant modification and improvement, cell behaviour, clonal propagation, and product formation.