MICROPROPAGATION OF AUSTRALIAN PINK FINGER LIME (*CITRUS AUSTRALASICA* CV. MIA ROSE)

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by

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LIST OF SYMBOLS AND ABBREVIATIONS

%	Percent
°C	Degree Celsius
±	Plus-minus
AC	Activated charcoal
$AgNO_3$	Silver nitrate
ANOVA	Analysis of variance
BAP	6-benzylaminopurine
DAMD	Directed Amplification of Minisatellite DNA
DMRT	Duncan's multiple range test
g/L	Gram per litre
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
Kn	Kinetin
mg/L	Milligram per litre
MS	Murashige and Skoog
mT	Meta-topolin
NAA	1-napthaleneacetic acid
S. E.	Standard error
SCoT	Start Codon Targeted
STS	Silver thiosulphate
v/v	Volume per volume
VT	Vermicompost tea
w/v	Weight per volume
WPM	Woody Plant Medium
µM	Micromolar

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MIKROPROPAGASI LIMAU JARI MERAH JAMBU AUSTRALIA (*Citrus australasica* cv. Mia Rose)

ABSTRAK

Citrus australasica, yang lebih dikenali sebagai limau jari atau "kaviar sitrus", terkenal dengan penampilan dan rasanya yang unik, serta kepelbagaian penggunaannya dalam masakan. Ia menawarkan manfaat kesihatan yang signifikan, termasuk kandungan tinggi antioksidan, vitamin C dan mineral. Namun, kaedah pembiakan konvensional, seperti cantuman dan keratan batang tidak efisien dan memakan masa, menyebabkan kesukaran untuk memenuhi permintaan yang semakin meningkat. Pembiakan melalui biji pula perlahan dan menghasilkan anak pokok yang mempunyai variasi genetik disebabkan sifat monoembrionik. Mikropropagasi menawarkan alternatif yang cekap untuk penghasilan pantas tumbuhan bebas penyakit di bawah keadaan terkawal. Kajian ini bertujuan untuk menghasilkan protokol mikropropagasi bagi limau jari merah jambu Australia (*C. australasica* cv. Mia Rose). Eksplan nodal telah dikulturkan pada media Murashige dan Skoog (MS) separuh kekuatan yang ditambah dengan sitokinin seperti 6-benzylaminopurine (BAP), kinetin (Kn), dan meta-topolin (mT), serta BAP dengan indole-3-acetic acid (IAA) untuk regenerasi pucuk. Eksplan juga dikulturkan dalam 2.0 mg/L BAP bersama perak nitrat ($AgNO_3$) atau perak tiosulfat (STS) untuk menilai kesannya terhadap pengguguran daun yang disebabkan oleh etilena. Induksi akar dijalankan pada Woody Plant Medium (WPM) yang ditambah dengan 1.0 g/L arang aktif (AC) secara bersendirian, dan digabungkan dengan auksin seperti IAA, indole-3-butyric acid (IBA), dan 1-naphthaleneacetic acid (NAA). Teh vermikompos (VT) diuji dengan rawatan terpilih (1.0 g/L AC, 2.0 mg/L IAA, dan 1.0 mg/L NAA) untuk meningkatkan keberkesanan

pengakaran. Anak pokok berakar baik diaklimatisasi dalam pelet Jiffy atau campuran benih, diikuti peralihan ke substrat yang mengandungi tanah hitam, perlit, dan gambut (3:1:1). Polimorfisme tumbuhan yang dijana semula dinilai menggunakan penanda molekul SCoT dan DAMD. Regenerasi pucuk yang optimum diperoleh pada media MS separuh kekuatan yang mengandungi 2.0 mg/L BAP sahaja atau digabungkan dengan 1.5 mg/L IAA, menghasilkan 2.39 ± 0.18 dan 2.67 ± 0.18 pucuk baharu, serta panjang pucuk masing-masing 0.23 ± 0.02 cm dan 0.41 ± 0.07 cm. Penambahan 4.0 mg/L $AgNO_3$ atau 10.0 µM STS berjaya mengatasi pengguguran daun. Induksi akar paling berkesan pada WPM dengan 1.0 mg/L NAA, mencapai kadar pengakaran 50.00 ± 28.87%, namun dengan pembentukan kalus sebanyak 100%. Walaupun rawatan VT tidak menunjukkan peningkatan yang ketara dalam parameter pengakaran, gabungan 1.0 g/L AC dan 1.0 mg/L NAA menggalakkan pengakaran tanpa pembentukan kalus, menghasilkan 38.89 ± 13.89% penghasilan akar, 1.67 ± 0.33 akar baharu dengan panjang 2.40 ± 0.31 cm. Anak pokok yang diaklimatisasi menunjukkan kadar kelangsungan hidup yang lebih tinggi (76.19 ± 17.17%) dalam campuran benih. Analisis penanda molekul mengesahkan kestabilan genetik tumbuhan yang dijana secara *in vitro* sepanjang lima kitaran subkultur. Kajian ini telah berjaya melaporkan protokol mikropropagasi terperinci untuk *C. australasica* cv. Mia Rose yang menyumbang kepada potensi penanaman komersial dan pemuliharaan genetiknya.

MICROPROPAGATION OF AUSTRALIAN PINK FINGER LIME (*Citrus australasica* cv. Mia Rose)

ABSTRACT

Citrus australasica, commonly known as Australian finger lime or "citrus caviar", is renowned for its distinctive appearance, flavour and culinary versatility. It offers significant health benefits including high levels of antioxidants, vitamin C and minerals. However, conventional propagation methods, such as grafting and cuttings, are inefficient and time-consuming, often difficult to meet growing demand. Seed propagation is slow and produce genetically variable offsprings due to monoembryonic nature. Micropropagation offers a viable alternative for the rapid multiplication of disease-free plants under controlled conditions, suitable for establishing farm-ready plant stocks. This study aims to develop a complete micropropagation protocol for the Australian pink finger lime (*C. australasica* cv. Mia Rose). Nodal explants were cultured on half-strength Murashige and Skoog (MS) media supplemented with cytokinins, including 6-benzylaminopurine (BAP), kinetin (Kn) and meta-topolin (mT), as well as combination of BAP with indole-3-acetic acid (IAA) for shoot regeneration. Explants were also cultured in 2.0 mg/L BAP with silver nitrate ($AgNO_3$) or silver thiosulphate (STS) to assess their effects on ethylene-induced leaf abscission. Root induction was performed on Woody Plant Medium (WPM) supplemented with 1.0 g/L activated charcoal (AC) alone, and in combination with IAA, indole-3-butyric acid (IBA) and 1-naphthaleneacetic acid (NAA). Vermicompost tea (VT) was also tested with selected treatments (1.0 g/L AC, 2.0 mg/L IAA and 1.0 mg/L NAA) to improve rooting. Well-rooted plantlets were acclimatized in Jiffy pellets or seedling mix, followed by a transition to a substrate of black soil, perlite and

peat moss (3:1:1). Polymorphism of regenerated plants was assessed using SCoT and DAMD molecular markers. Optimal shoot regeneration was achieved on half-strength MS media containing 2.0 mg/L BAP alone or in combination with 1.5 mg/L IAA, yielding 2.39 ± 0.18 and 2.67 ± 0.18 new shoots, and shoot lengths of 0.23 ± 0.02 cm and 0.41 ± 0.07 cm, respectively. The addition of 4.0 mg/L $AgNO_3$ or 10.0 μM STS effectively mitigated leaf abscission. Root induction was most effective on WPM with 1.0 mg/L NAA, resulting in 50.00 ± 28.87% rooting rate coupled with 100% callus formation. Although VT treatments did not significantly enhance rooting parameters, the combination of 1.0 g/L AC and 1.0 mg/L NAA promoted rooting without callus formation, yielding 38.89 ± 13.89% of rooting, 1.67 ± 0.33 new roots, with a length of 2.40 ± 0.31 cm. Acclimatized plantlets showed higher survival rates (76.19 ± 17.17%) in seedling mix. Molecular marker analysis revealed uniform banding patterns across five subculture cycles, confirming the genetic stability of *in vitro* regenerated plants. This study has successfully documented a complete micropropagation protocol for *C. australasica* cv. Mia Rose which contributes to its potential commercial cultivation and genetic conservation.

CHAPTER 1

INTRODUCTION

Citrus fruits, belonging to the genus *Citrus* in the family Rutaceae, are among the most widely cultivated and demanded fruit crops globally. The citrus industry has expanded significantly over recent decades, driven by increased production, exports, utilization, and rising market prices (Food and Agriculture Organisation of the United Nations, 2021). *Citrus australasica*, commonly known as finger lime, is a rare and distinct citrus variety native to the subtropical rainforests of Australia (Lim, 2012). Renowned for its caviar-like vesicles, multicoloured peel and pulp, ranging from clear, yellow, green, pink and black (Ramadugu et al., 2016). Among the cultivars, the pink finger lime cultivar 'Mia Rose' is especially valued for its appealing pink pulp and its flavour profile, which is tangy, sweet and floral, with a mild bitterness and a grapefruit aftertaste. The 'Mia Rose' cultivar is believed to have originated from the 'Pink Ice' cultivar, offering improvements in size, colour and uniformity ("Mia Rose Finger Limes", n.d.; "Varieties", n.d.).

In Australia, the demand for finger limes demonstrates strong growth potential, driven by increasing interest from chefs, who use them as garnishes in high-end restaurants, as well as by value-adders developing products for the food and beverage, botanical, nutraceutical, pharmaceutical and cosmetic industries (Glover et al., 2021). This has further enhanced their market value, with the export market currently exceeding local demand and offering significant opportunities for market expansion and economic benefits for local farmers and producers (Glover et al., 2021). Beyond its culinary and commercial applications, the fruit is recognized for its medicinal properties, including high levels of vitamin C and antioxidants, which supports immunity and skin care (Delort & Yuan, 2018). Finger lime is rich in dietary fibre,

healthy fats, and essential minerals making it a promising ingredient for pharmaceutical and nutraceutical applications (Richmond et al., 2019).

Finger lime is also known for its tolerance to citrus greening disease, or Huanglongbing (HLB), which has devastated citrus populations worldwide. This trait offers potential for developing HLB-tolerant citrus cultivars through conventional breeding and biological methods (Weber et al., 2022). However, conventional propagation methods, such as seed germination, require up to 15 years to bear fruits. Seed progeny also exhibits high genetic variation in fruit shape and colour, with uncertain inheritance of specific traits. Vegetative cuttings are often insufficient due to low success rates and poor rooting percentages (Hardy et al., 2010). Currently, the primary method for vegetative propagation of finger limes is grafting onto *Citrus trifoliata* rootstock, which accelerates growth and enhances vigour and disease resistance (Harvest Savvy, 2024). However, grafting is often less efficient, requiring skilled techniques, and careful maintenance. It also poses risks such as the potential spread of graft-transmissible disease, susceptibility to pests, and vulnerability to mechanical damage from strong winds (Sanderson et al., 2007). These limitations hinder the rapid multiplication of finger lime, making it challenging to meet the growing market demand.

Plant tissue culture, particularly micropropagation, offers a promising alternative to overcome propagation challenges by enabling the rapid production of genetically uniform, disease-free plants under controlled conditions (George et al., 2008). Factors such as genotype, explant type, age, position, and media composition significantly influence *in vitro* regeneration outcomes (Sharma et al., 2011). Micropropagation studies in citrus have explored various explants for shoot organogenesis, including directly using nodal (Tallón et al., 2013; Navarro-García et

al., 2016) and shoot tip explants (Haradzi et al., 2021), as well as indirectly through seeds (Hasan et al., 2019a), nucellus tissues (Hussain et al., 2018) and leaf segments (Tao et al., 2002). The selection of culture media, growth conditions and plant growth regulators played a fundamental role in optimizing micropropagation protocols (Carimi & De Pasquale, 2003). Studies have reported the use of various basal media, such as MS, WPM, MT and DKW in citrus micropropagation (Navarro-García et al., 2016; De Oliveira et al., 2016; Prusty et al., 2023). Cytokinins, either alone or in combination with auxins, have been effective for shoot proliferation, while auxins enhance root formation in many citrus species including *C. limon* (Goswami et al., 2013), *C. sinensis* (Prusty et al., 2023), *C. paradisi* (Bhavishya et al., 2024) and *C. aurantifolia* (Al-Khayri & Al-Bahrany, 2001).

Besides, silver-containing compounds, such as silver nitrate and silver thiosulphate are often employed as potent ethylene antagonists. They effectively prevent abscission and improve overall regeneration efficiency in tissue culture studies (Kumar et al., 2009). Additionally, vermicompost tea, which is an aqueous extract of solid vermicompost, contains active beneficial microbial communities, humic substances and plant growth regulators produced by microorganisms, alongside a chelating effect that enhances micronutrient availability, which nourishes the plant directly (Kiyasudeen et al. 2016). Activated charcoal, known for its adsorption properties, is frequently incorporated into culture media to eliminate inhibitory substance, such as toxic metabolites and phenolic exudates. Its role in improving different stages of micropropagation is well-documented (Thomas, 2008).

To date, the application of *in vitro* techniques for finger limes remains limited, with only two reports documenting the tissue culture work on this species. Supreetha et al. (2023) achieve 100% shoot induction from nodal explants through optimized

surface sterilization, while Supreetha et al. (2024) and Sriskanda et al. (2021) demonstrated successful shoot regeneration in finger limes using different cytokinin treatments. Notably, Mahmoud et al. (2020) and Xie et al. (2023) established effective micropropagation protocols, achieving high shoot regeneration, rooting rates and survival of acclimatized plantlets through the use of silver-containing compounds and plant growth regulators. This gap poses a significant challenge to the commercial establishment of finger limes in Malaysia, where they are currently available only through two physical nurseries located in Perak (Nurseri Kota Lama) and Perlis (Superfruits Valley), and one online nursery, TZF Garden (https://shopee.com.my/tzf_06). This study aims to develop a comprehensive micropropagation protocol for *C. australasica* cv. Mia Rose, focusing on shoot multiplication, root induction and plantlet acclimatization. Polymorphism analysis using SCoT and DAMD markers provides a reliable method for assessing clonal fidelity, which ensures the genetic uniformity of micropropagated plants for commercial cultivation. The findings are anticipated to contribute to the sustainable cultivation and commercialization of this economically important cultivar.

1.1 Objectives

1. To optimize shoot multiplication from nodal explants of the Australian pink finger lime (*Citrus australasica* cv. Mia Rose) using different concentrations and combination treatments of plant growth regulators.
2. To evaluate the effects of silver nitrate and silver thiosulphate on shoot regeneration and leaf abscission in *in vitro* nodal explants of *C. australasica* cv. Mia Rose.
3. To assess the rooting efficiency of regenerated shoots using treatments with auxin, vermicompost tea, and activated charcoal, and to acclimatize in vitro-grown plantlets using different soil substrates.
4. To evaluate and identify polymorphisms between cultivated and regenerated *C. australasica* cv. Mia Rose plants using SCoT and DAMD molecular markers.

CHAPTER 2

LITERATURE REVIEW

2.1 Overview of *Citrus australasica*

The Australian finger lime (*Citrus australasica*) is a member of the Rutaceae family. It is native to the rainforests of southeast Queensland and northern New South Wales (NSW) (Delort & Yuan, 2018). Historically, finger limes grew naturally in the wild, but European colonization led to deforestation for settlements and farming, endangering the species. However, some varieties survived on farmland and private property, eventually becoming the source for the commercial production seen today (Low, 1991).

Finger limes have long been an important bush food for Indigenous Australians, valued for their nutritional content, unique flavour, and use as a natural food colouring. The fruits are rich in antioxidants, vitamins and minerals, contain carotenoids like lutein, which contribute to their orange-red pigmentation. Traditionally, the juice was used as an antiseptic for infected wounds and boils (Richmond et al., 2019). The caviar-like pulp is widely used to garnish seafood and to prepare sauces, jams, jellies and marmalade (Ramadugu et al., 2017). Additionally, Indigenous communities utilized the strong wood of finger lime trees for crafting tools, including hammers, axe handles and walking sticks (Rennie, 2017). In recent decades, global demand for finger limes has grown, with export-grade fruit being sold fresh to markets in the European Union, Asia, the United Kingdom and Canada. This growth is driven by their distinctive appearance, status as a gourmet bushfood, versatile culinary applications, and numerous health benefits (Lim, 2012).

The finger lime plant is a spiny, evergreen subshrub that can grow up to 6 meters in height. The leaves are dark purple when young, transitioning to green as they

mature. They are small, smooth, glossy, and ovate, measuring approximately 1 – 5 cm in length and 0.3 – 2.5 cm in width, with numerous oil glands visible on the surface. The tree produces small, white flowers with pinkish buds that are about 1 cm in diameter, adding to its ornamental value. The most distinctive feature of finger lime is its elongated fruit, which can reach up to 10 cm in length (Lim, 2012; Osborne, 2015). The fruit pulp consists of juice vesicles resembling caviar, earning it the name "citrus caviar". The fruit skin exhibits a wide range of colours, from green, yellow, red to black. The seeds are typically whitish to cream-coloured, embedded within the juice vesicles. The seed number varies depending on cultivar, ranging from seedless to a few or many seeds (Delort & Yuan, 2018). The finger lime peel has a distinctive fresh, floral, citrus aroma, while the pulp gives a strong, sour, tangy taste with occasional hints of bitterness, resinous and citronella-like (Delort et al., 2015). In field, the root system of finger limes tends to be more fibrous, which may contribute to the plant's adaptability in diverse soil conditions (Hardy et al., 2010).

2.1.1 *Citrus australasica* cv. Mia Rose

The Australian pink finger lime (*C. australasica* cv. Mia Rose) is a distinctive variety of finger lime, known for its larger fruit size compared to other varieties. The fruit typically ranges from 5 – 12 cm in length and weights between 10 – 20 grams. It is believed to have originated from the 'Pink Ice' cultivar, which was developed in the late 20th and early 21st centuries for its improved size, colour and uniformity. The 'Mia Rose' shrub is thorny, evergreen, and can reach a height of 1 – 3 meters. Each shrub takes approximately five months from flowering to fruit maturity. The fruit is slender and cylindrical with blunt to slightly pointed ends, within a thin, pebbled, semi-rough skin dotted with oil glands. The peel colour varies from green to brown, red or pink, which is influenced by environmental factors, in which cooler climate enhances the intensity of the colour. The pulp is divided into 5 – 7 locules, each filled with vibrant, caviar-like juice vesicles that range from pink to red. The flavour profile of the 'Mia Rose' is tangy, sweet, and floral, with a mild bitterness and a grapefruit aftertaste ("Mia Rose Finger Limes", n.d.; "Varieties", n.d.).

Figure 2.1: *C. australasica* cv. Mia Rose shrub.

Figure 2.2: *C. australasica* cv. Mia Rose flower.

Figure 2.3: *C. australasica* fruit form.

Figure 2.4: Caviar-like pulps of *C. australasica* cv. Mia Rose.

2.2 Growing conditions

Finger lime trees thrive in both tropical and subtropical regions, adapting well to diverse soil conditions and prefer a well-drained soil. The trees grow best in loamy soils rich in organic matter and in slightly acidic soils with a pH range of 5 – 6.5. Adequate watering is necessary to keep the soil moist, and regular fertilization is essential for optimal growth (Hardy et al., 2010, Grant, 2021). A commonly used fertilizer is a low phosphorus NPK fertilizer with a 15:4:11 ratio. Fertilization is typically avoided from the flowering stage until the fruits reach 1 cm in length to prevent fruit abortion (Hardy et al., 2010). Adequate irrigation is essential throughout the growing season, particularly during flowering, fruit set and fruit growth stages to ensure optimal tree health and fruiting (Hardy et al., 2010). Vashisth (2022) emphasized the importance of nitrogen content in the fertilizer used for finger limes as it is essential for vigorous growth. However, excessive nitrogen application can negatively impact fruit quality. In addition, potassium foliar spray has been shown to improve fruit size, while the application of potassium and calcium has been linked to improved peel integrity, which is crucial for maintaining fruit appearance and marketability (Vashisth, 2022).

Finger lime trees flourish in warm climates with year-round temperatures between 16 – 32°C, but are also tolerant of cooler climates and can withstand mild frost (Hardy et al., 2010; Grant, 2021). Seedling-grown finger lime trees exhibit slow growth, requiring 8 to 12 years to reach maturity and up to 15 years to bear fruit (Hardy et al., 2010). However, growing finger limes from seeds does not result in true-to-type plants, leading to variation in fruit shape and colour. Therefore, vegetative propagation methods such as grafting, are widely used in commercial propagation to ensure genetic

uniformity and to replicate the desirable characteristics of the mother plant (Osborne, 2015).

In commercial propagation, finger limes are often grafted onto *Citrus trifoliata* and Troyer citrange rootstocks for their compatibility (Grant, 2021). In contrast, propagation by cuttings generally have a low success rate, often below 50% (Rennie, 2017). The selection of an appropriate rootstock is crucial, as it significantly affects tree size, fruit quality and yield. Additionally, suitable rootstocks can confer tolerance to various soil types, disease, pests and climatic conditions, thereby enhancing the overall success and resilience of finger lime cultivation (Hardy et al., 2010). Fruiting in finger lime trees typically begins 2 – 3 years after planting, with peak production occurring in the 5^{th} – 6^{th} years. The fruits require approximately five months to mature after flowering and must remain on the tree until fully ripe. By the age of 5 – 6 years, a single tree can produce up to 20 kg of fruits annually, although yields vary depending on the cultivar, weather conditions and water availability (Rennie, 2017).

2.3 Economic importance of *Citrus australasica*

Citrus fruits, including oranges, lemons, limes, grapefruits and mandarins, are widely grown across tropical and subtropical regions, accounting for a significant portion of global fruit production. In 2021, over 158 million tons of citrus were produced globally, with China, Brazil, India and Mexico ranking as the top producers (McGill, 2022).

The cultivation of finger limes spans across multiple countries worldwide beyond its native range in Australia, with notable production occurring in the USA and Guatemala (Glover et al., 2021). In Australia, finger lime production has grown steadily, with annual output reaching approximately 100 tons. Major growers account for 75% of this production, while smaller growers contribute the remaining 25%. Demand for finger limes in Australia is particularly high in the restaurant trade and export markets, compared to the domestic consumption. This demand is expected to rise over the years, with an anticipated 8% increase in production by 2025 (Glover et al, 2021). Finger limes are commonly exported as a high value fruit to countries such as Italy, Singapore and Japan. To meet the demands of distant markets, finger limes are often packed in the form of frozen fruits or frozen pearls, allowing them to maintain their quality and reach a wider range of countries (Australian Tree Crop, 2023).

Finger limes are highly valued both domestically in Australia and in international markets, with demand growing steadily over the past decades, mainly due to their recognition as a bush food (Foreign Agricultural Service, 2024). Their distinctive appearance, texture and flavour profile have made them highly sought after in the gourmet food industry. They command premium prices and are increasingly available in supermarkets and fresh produce retailers (Australian Tree Crop, 2023). In 2010, export-grade finger limes were priced between $40 – $60 per kilogram, while

domestic fresh fruit prices ranged from $25 – $40 per kilogram (Hardy et al., 2010). Notably, there has been an increase in the value of finger limes over the years. By 2020, wholesale prices averaged at $59.17 per kilogram, and retail prices stood at $84.54 per kilogram (Glover et al., 2021).

The economic value of finger lime production has also seen significant growth. The farmgate value rose from $600,000 in 2012 to $3.1 million in 2020, reflecting increased demand and premium market status of finger limes (Glover et al, 2021). This increase highlights the economic importance of finger limes as a niche but highly profitable crop, which has the potential to be cultivated in Malaysia, given by our tropical climate required for their optimal growth, in turn contributing to commercialization of this niche crop for both domestic consumption and export markets.

2.4 Medicinal properties of *Citrus australasica*

Finger limes have gained attention as a healthful addition to the daily diet due to their low sugar, fat and calorie content (Richmond et al., 2019). Adhikari et al. (2021) highlighted that finger limes are rich in antioxidants, primarily attributed to their phenolic compounds. Notably, the peel contains two to three times higher levels of phenolic compounds and antioxidant capacity compared to the pulp. The major phenolic compounds include flavonoids such as naringin and didymin, phenolic acids like caffeic and coumaric acid, as well as limonoids, and citric acid being the most abundant. Additionally, the presence of pyrogallol further enhances the fruit's antioxidant profile (Aznar et al., 2022).

The essential oils in the peel and pulp of finger limes are dominated by monoterpenes and sesquiterpenes, respectively (Cioni et al., 2022). These compounds, which constitute the primary components of plant essential oils, exhibit potent antifungal activities (Mehdizadeh & Moghaddam, 2018). The volatile constituents of citrus peel are a complex mixture of monoterpene and sesquiterpene hydrocarbons along with oxygenated derivatives, such as aldehydes, ketones, acids, alcohols, and esters (Jing et al., 2014). In finger lime peel, limonene is the major compound, with sabinene as the second most abundant (Cozzolino et al., 2022). Delort et al. (2015) identified three unique chemotypes in finger limes, which are limonene/sabinene, limonene/citronellal/isomenthone and limonene/citronellal/citronellol which have not been reported in other citrus species. Citrus essential oils, including those derived from finger limes, are widely used in the food industry for postharvest disease control, food preservation and edible coatings (Jing et al., 2014). Additionally, these oils have pharmaceutical applications, serving as anti-inflammatory, anti-tumour, anti-

ulcerogenic and anti-depressant agents, as well as in aromatherapy treatments (Palazzolo et al., 2013).

Additionally, the fruit's extracts are valued for their antioxidant, anti-aging and photoprotective properties. For instance, Cote Rojas (2024) investigated the peel extract of green and pink varieties of finger lime for their ability to inhibit enzymes associated with aging, such as hyaluronidase, tyrosinase and elastase. Result showed that extracts from both peel colours exhibited strong antioxidant effects and effectively inhibited hyaluronidase and tyrosinase , suggesting potential applications in anti-aging skincare. Indeed, finger lime is already utilized in commercial skincare products, including face, lip and body serums, by brands such as Biologi Skincare (Australia), Lyudè Cosmetics (Italy) and Averr Aglow (Georgia).

In addition, finger lime is recognized for its high ascorbic acid content, the biologically active form of vitamin C. Unlike other vitamins such as vitamin D or B, humans cannot synthesize vitamin C due to the deficiency of a key enzyme in the ascorbic acid biosynthetic pathway (Nishikimi et al., 1994). This essential nutrient plays a crucial role in various bodily functions, particularly in maintaining skin health and supporting immune defense. Vitamin C also promotes collagen synthesis, which helps maintain skin elasticity and integrity, while also acting as a potent antioxidant that reduces oxidative damage by neutralizing free radicals, thereby preventing premature aging (Pullar et al., 2017). Vitamin C further enhances the immune function by maintaining the integrity of the skin barrier, serving as a primary defense against pathogens. Deficiency in this vitamin can lead to scurvy, a condition characterized by rough, scaly skin, swollen and bleeding gums, brittle hair, and impaired wound healing (Carr & Maggini, 2017).

Among the different varieties of finger lime, the pink finger lime contains the highest vitamin C levels, with 91 ± 2 mg/100g, significantly surpassing the red (41 ± 20 mg/100g), green (26 ± 1 mg/100g) and yellow (59 ± 4 mg/100g) varieties. When compared to other common citrus species, the vitamin C content of pink finger lime is approximately 1.56-fold higher than oranges, 1.85-fold higher than grapefruits, and twice as high as lemons. Furthermore, other finger lime varieties also exhibit higher vitamin C levels than key lime and kaffir lime (Konczak et al., 2010; Netzel et al., 2007; Najwa & Azrina, 2017).

Folate, also known as vitamin B_9, is essential for cell division and supports the growth of organs and bone marrow. Its consistent intake during pregnancy, is vital in preventing neural tube defects (NTDs), which can lead to malformations of the brain, skull and spinal cord of the baby (Barnes-Jewish St. Peters Hospital, 2025). Recognizing its importance, the U.S. Food and Drug Administration advises women of childbearing age to consume approximately 400 mcg of folic acid daily to obtain adequate amounts of folate, thereby reducing the risk of NTDs and related congenital anomalies (U.S. Food and Drug Administration, 2016). The pulp of both green and pink finger lime varieties are particularly rich in folate, containing 89.88 μg and 80.84 μg per 100g of fresh weight, respectively. In contrast, the commonly consumed folate-rich citrus fruits, such as oranges, which provide only 30 μg per 100g. This represents a 2.67-fold higher folate content in finger limes compared to oranges (Konczak, 2015; U.S. Department of Agriculture, 2019).

In addition, finger lime is also notable for its high mineral content compared to other native Australian fruits, particularly in calcium, magnesium, phosphorus and potassium (Richmond et al., 2019). Consuming fruits rich in these minerals provides multiple health benefits, including the development of strong and healthy bones and

teeth, supporting muscle and nerve function, and maintaining proper fluid balance (Soetan et al., 2010). Thus, finger lime is not only a versatile and flavourful fruit but also a nutrient-dense superfood with significant health-promoting benefits. Its rich nutritional profile supports potential applications in the pharmaceutical and nutraceutical industries, as well as the development of functional foods.

2.5 The different cultivars of *Citrus australasica*

In Australia, more than 75 cultivars of finger limes have been named and selected (Karp, 2022), with new hybrid cultivars continually being bred and released (Bowman et al., 2019; Dutt, 2024). Currently, only seven finger lime cultivars are officially registered with the Australian Cultivar Registration Authority (ACRA), an organization responsible for ensuring the correct nomenclature of cultivars in commercial trade. These registered cultivars include ‘Alstonville’, ‘Blubonia Pink Crystal’, ‘Byron Sunrise’, ‘Durhams Emerald’, ‘Jali Red’, ‘Judy’s Everbearing’ and ‘Pink Ice’ (Australian Cultivar Registration Authority, n.d.). These varieties have been granted Australian Plant Breeders’ Rights (PBR), thereby promoting the standardized classification and commercialization of Australian plant cultivars.

These cultivars are generally cylindrical in shape, measuring 4 – 8 cm in length and 15 – 25 mm in diameter. Despite their similar shapes, they exhibit significant variation in fruit characteristics, including peel and pulp colouration as well as flavour profiles, as summarized in Table 2.1 (Hardy et al., 2010; “Varieties”, n.d.).

Table 2.1: List of registered varieties of *C. australasica* under ACRA.

No.	Cultivar	Year of Registration	Origin	Description
1	'Alstonville'	February 1, 2007	Alstonville, NSW	A moderately dense, tall shrub or small tree reaching up to 4 meters in height. The fruit has dark green to black skin with translucent, pale green pulps. Flavour is described as tart and slightly sweet.
2	'Blunobia Pink Crystal'	February 1, 2007	Mount Burrell, NSW	A dense, medium-sized shrub growing up to 2 meters. The fruit has green skin with a brown overtone and deep pink pulps. Flavour is described as mild, sweet and slightly acidic.
3	'Byron Sunrise'	June 30, 2010	Alstonville, NSW	A slender, upright tall shrub or small tree, with a maximum height of 2 meters. The fruit is characterized by its green to black skin when fully riped, with bright red pulps. Flavour has a mild lemon taste with low acidity.
4	'Durhams Emerald'	February 1, 2007	Queensland	A moderately dense shrub growing up to 2 meters in height. The fruit is long and slender, with black skin, and emerald green pulp. Flavour is strong as tangy.
5	'Jali Red'	June 30, 2010	Whian Whian, NSW	A tall shrub or small tree with an open canopy structure. The fruit turns dark brown to black when fully ripe, with pulp ranging from light to dark red. Flavour is mild, and sweeter like other dark red varieties.
6	'Judy's Everbearing'	February 1, 2007	Bangalow, NSW	A tall tree, reaching up to 4 meters tall, with a well-developed canopy. The fruit has dark green to maroon skin when fully ripe and clear to red pulps. Flavour is mild with balanced tartness.
7	'Pink Ice'	January 31, 2007	Alstonville, NSW	A moderately dense tall shrub or small tree growing up to 3 meters. The fruit has dark green to maroon skin when completely ripe, and pulp in shades from clear to different shades of pink. The taste is mild, slightly tart, with a grapefruit finish.

2.6 Plant tissue culture and micropropagation

Totipotency, the ability of a single plant cell to regenerate into a whole plant, forms the basis of plant tissue culture technique (Fehér, 2019). This approach involves growing plant cells, tissues or organs under sterile condition on nutrient-enriched media (George et al., 2008). A standard culture medium consists of macro- and micro-nutrients, vitamins, amino acids, carbohydrates, gelling agents and plant growth regulators (Chimdessa, 2020). Macronutrients, such as nitrogen, phosphorus and potassium support cell division, protein synthesis, and cell wall formation. Micronutrients such as iron and manganese are vital for enzymatic activities and metabolic processes (Bhatia, 2015). Carbohydrates, particularly sucrose, serve as the main energy source in plant tissue culture due to their affordability and availability, and function to maintain osmotic balance (Yaseen et al., 2013). Organic supplements, including amino acids mixtures like casein hydrolysate, glycine and glutamine, as well as organic additives like coconut water or banana homogenate, provide additional nitrogen and growth-promoting factors (Saad & Elshahed, 2012; Hamdeni et al., 2022).

Different types of plant tissue culture media have been developed to meet specific nutrient requirements (Bhatia, 2015). Murashige and Skoog (MS) medium, characterized by its high nitrogen content in both nitrate and ammonium forms, is the most widely used across various plant species. In contrast, Gamborg (B5) medium contains lower ammonium levels and is often modified for species-specific needs. Woody Plant Medium (WPM) and Driver and Kuniyuki (DKW) medium are optimized for woody species, with lower total nitrogen and increased sulfate levels compared to MS medium (Phillips & Garda, 2019). Additionally, Vacin and Went (VW) was developed for orchids culture, while Nitsch and Nitsch (NN) medium was optimized for anther and protoplast culture, as well as embryogenesis (Bhatia, 2015).

Plant growth regulators (PGRs), particularly auxins and cytokinins, are pivotal in regulating morphogenesis and cell division. Their balanced interplay determines the developmental pathway, such as shoot or root induction or callus induction (Ahmad et al., 2012; Bhatia, 2015). Auxins such as indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), 2,4-dichlorophenoxyacetic acid (2,4-D) and 1-naphthaleneacetic acid (NAA) are widely used in tissue culture studies to induce rooting (Rathore et al., 2007), callus formation (Al-Hamidi et al., 2023) and somatic embryogenesis (Ho & Vasil, 1983). Callus induction is particularly effective with 2,4-D and NAA across a variety of explants, including cotyledons, nodal sections, and leaf blades (Al-Hamidi et al., 2023). Cytokinins, such as 6-benzylaminopurine (BAP) and meta-topolin (mT), promote shoot formation (Bairu et al., 2007; Singh & Rajam, 2010), and when combined with auxins, they induce and maintain callus cultures (Martinez et al., 2021). Thidiazuron (TDZ), although not a true cytokinin, exhibits strong cytokinin-like activity, and effectively induces somatic embryogenesis and adventitious shoots (Liang et al., 2020).

Other PGRs such as gibberellic acid (GA_3) is widely used for *in vitro* seed germination and zygotic embryo development (Aké et al., 2007; Al-Hawezy, 2013), as well as improving fruit storage quality and stress resistance postharvest (Zhang et al., 2023). Abscisic acid (ABA), a plant growth inhibitor, regulates bud and seed dormancy, stomatal closure and water and ion uptake by roots, as well as flower senescence and abscission (George at al., 2008). ABA often interacts with ethylene to regulate water stress response (Salazar et al., 2015), and works synergistically during fruit ripening (Gupta et al., 2022). Ethylene, a gaseous plant hormone, influences fruit ripening, flower and leaf senescence (Iqbal et al., 2017). In *in vitro* cultures, the effect of ethylene can be species-specific (Biddington, 1992). Ethylene-induced leaf

abscission can inhibit shoot regeneration (Mahmoud et al., 2021), but it has also been reported to promote callus induction, shoot elongation and adventitious root formation (Neves et al., 2023). Recently, additional PGRs such as salicylic acid, jasmonic acid and brassinosteroids have also been discovered (Agudelo-Morales et al., 2021).

Micropropagation, one of the primary applications of tissue culture, facilitates the rapid production of true-to-type, disease-free plantlets from a single plant material (Loberant & Altman, 2010). It involves five sequential stages, including explant selection, culture establishment, multiplication, rooting and acclimatization (Purohit et al., 2011). Micropropagation methods include organogenesis and somatic embryogenesis. Organogenesis, which may follow a direct or indirect pathway, involves shoot and root formation from explants. Direct organogenesis bypasses the intermediate callus phase, ensuring genetic uniformity and efficiency (Halmagyi et al., 2023). Indirect organogenesis involves callus formation from explants (Sharma et al., 2021), is often utilized for genetic modifications and transgenic plant production (Dutt et al., 2011). Somatic embryogenesis is characterized by bipolar regeneration, in which somatic cells differentiate to form both shoot and root meristems simultaneously, leading to the development of a complete plant (Loberant & Altman, 2010). Somatic embryogenesis is particularly advantageous for synthetic seed production, germplasm conservation and cryopreservation (Guan et al., 2016), despite challenges like genotype dependency and poor embryo germination rates persist (Guan et al., 2016; Pais, 2019).

In Malaysia, micropropagation has been successfully applied to the propagation of economically important fruit crops such as bananas (Hui et al., 2012), pineapples (Hamid et al., 2013), and papayas (Al-Shara et al., 2020), vegetable crops such as ‘Bentong ginger’ (Zahid et al., 2021), or ornamental orchids (Chew et al.,

2018). However, challenges such as contamination, somaclonal variation and high operating costs can limit its broader adoption (Loberant & Altman, 2010). Therefore, advancements in plant tissue culture and micropropagation will be crucial for meeting the increasing demand for high-quality, disease-free plants across various industries and applications.