ESTABLISHMENT OF TISSUE CULTURE PLANTING MATERIALS OF MANGO (*Mangifera indica* L.) cv. HARUMANIS

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ESTABLISHMENT OF TISSUE CULTURE PLANTING MATERIALS OF MANGO (*Mangifera indica* L.) cv. HARUMANIS

by

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LIST OF ABBREVIATIONS AND SYMBOLS

%	Percent
μmol	Micromole
μM	Micromolar
m	Meter
cm	Centimeter
nm	Nanometer
g/L	Gram per liter
mg/L	Microgram per liter
mL/L	Milliliter per liter
hr	Hour
min	Minute
ATP	Adenosine triphosphate
WPM	Woody plant medium
MS	Murashige and Skoog
TCL	Thin cell layer
tTCL	Transverse thin cell layer
lTCL	Longitudinal thin cell layer
NaOCl	Sodium hypochlorite
NaCl	Sodium chloride
HCl	Hydrochloric acid
$HOCl^-$	Hypochlorous acid
OCl^-	Hypochlorite ions
$HgCl_2$	Mercury (II) chloride
PPM™	Plant preservative mixture™
v/v	Volume over volume
dH_2O	Distilled water
sdH_2O	Sterile distilled water
PGR	Plant growth regulator
2iP	6-(γ,γ-dimethylallylamino)purine
TDZ	Thidiazuron
CPPU	N-(2-Chloro-4-pyridyl)-N′-phenylurea

BAP	Benzylaminopurine
LED	Light emitting diode
SEM	Scanning electron microscope
TEM	Transmission electron microscope
RSM	Response surface methodology
ANOVA	Analysis of variance
R^2	R-squared
Adj. R^2	Adjusted R-squared
Pred. R^2	Predicted R-squared
SE	Standard error
SPD	Spectral power distribution
USM	Universiti Sains Malaysia

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PENUBUHAN BAHAN PENANAMAN KULTUR TISU MANGGA

(*Mangifera indica* L.) cv. HARUMANIS

ABSTRAK

Permintaan mangga Harumanis yang tinggi di Malaysia disebabkan oleh rasa manis yang luar biasa dan aroma yang menyenangkan. Mangga ini dibiakkan di negeri bahagian utara Malaysia, iaitu Perlis. Namun, pelbagai halangan propagasinya menyebabkan produksi mangga ini tidak dapat memenuhi permintaan pasaran. Kelemahan aktiviti cantuman dan jumlah benih yang terhad (hanya satu musim menuai dalam setahun) untuk membiak Harumanis masing-masing secara vegetatif dan bukan vegetatif memerlukan masa dan tenaga yang banyak. Untuk mengatasi masalah ini, mikropropagasi dengan teknik kultur tisu tumbuhan merupakan pengganti yang amat handal dan berkesan untuk menghasilkan klon tumbuhan induk secara cepat. Namun, beberapa kajian sebelumnya yang menggunakan segmen nodus sebagai bahan permulaan telah melaporkan kadar percambahan yang rendah disebabkan oleh endofit dan sifat tanaman yang rekalsitran. Oleh kerana itu, tujuan dari kajian ini adalah untuk menubuhkan protokol sterilasi dan propagasi tunas bagi *Mangifera indica* cv. Harumanis. Kadar kesterilan sebanyak 87.5% telah dicapai dengan merawat tumbuhan asal dengan 0.5 mL/L azoxystrobin dua hari sebelum eksperimen dan membersihkan segmen nodus pada peringkat daun hijau tidak matang dengan 40% Clorox® selama 20 minit, 0.5 g/L benomyl selama 1 jam dan 300 mg/L cefotaxime selama 5 minit. Teknik sel lapisan nipis melintang batang (tTCL) kemudian digunakan pada segmen nodus dan kultur diinokulasikan pada WPM dengan 0.5 g/L benomyl dan 300 mg/L cefotaxime. Penambahan arang aktif sebanyak 1 g/L ke dalam WPM membantu mengurangi kalus dan memperpanjang umur biakan. WPM yang dibekalkan dengan 4 dan 6 µM BAP serta

2 µM kinetin, merangsang pertumbuhan primordia daun dari kultur yang dirawat tTCL. Selain itu, cahaya LED RGB meningkatkan pertumbuhan kultur. Analisis histologi menunjukkan bahawa bahagian atas dan tengah batang memiliki tunas aksilar yang lebih layak dibandingkan dengan bahagian bawah. Analisis SEM mendedahkan adanya bakteria dan fungi endofit di dalam tunas aksilar. Kajian histologi dan SEM menunjukkan bahawa pembuluh xilem segmen nodus di kedua-dua bahagian atas dan tengah batang mempunyai bilangan bakteria endofitik yang lebih rendah. Berdasarkan hasil ini, dicadangkan untuk menggunakan segmen nodal dari bahagian atas dan tengah batang sebagai bahan permulaan untuk eksperimen selanjutnya. Hasil kajian ini menerangkan kepentingan pra-rawatan dan penyelenggaraan kultur dalam mengurangi kontaminasi endofit, dan menunjukkan potensi kultur Harumanis yang dirawat dengan tTCL untuk propagasi tunas dan pengurangan kontaminasi.

ESTABLISHMENT OF TISSUE CULTURE PLANTING MATERIALS OF MANGO (*MANGIFERA INDICA* L.) CV. HARUMANIS

ABSTRACT

Harumanis mango is one of the highest demand mango cultivars in Malaysia due to its exceptional sweetness and fabulous fragrance. It is grown in a northern state of Malaysia, Perlis. However, the production of this mango cannot meet the market demand as there are several limitations in its propagation. The limited grafting activity and the limited number of seeds (only one harvest season per year) make vegetative and non-vegetative propagation of Harumanis time and labour intensive. Micropropagation using tissue culture techniques is a reliable and effective alternative for mass *in vitro* propagation of Harumanis at a consistent and faster rate, producing clones of the mother plants. Few studies have used nodal segments as starting material and the reported success rate is low. This is due to the two main problems, namely deep-seated contaminants and the recalcitrant nature of the plant. The objective of the present study is to develop an effective disinfection and shoot proliferation protocol for *Mangifera indica* cv. Harumanis. A sterility rate of 87.5% was achieved by pretreating mother plants with 0.5 mL/L azoxystrobin two days before the experiment and surface sterilising nodal segments at the immature green leaf stage with 40% Clorox® for 20 minutes, 0.5 g/L benomyl for 1 hour and 300 mg/L cefotaxime for 5 minutes. The transverse thin layer (tTCL) technique was applied to the nodal segments and cultures were maintained at WPM with 0.5 g/L benomyl and 300 mg/L cefotaxime. The addition of 1 g/L activated charcoal to WPM effectively reduced browning and extended the life of the cultures. WPM, supplemented with 4 and 6 μM BAP and 2 μM kinetin, promoted the growth of leaf primordia from the tTCL-treated cultures. In addition, RGB LED

light promoted the growth of the cultures. Histological analysis showed that axillary buds at the upper and middle ends of the stem were more viable than those at the lower end. Endophytic bacteria and fungi were observed in the axillary buds at SEM. Both histological and SEM analysis showed that the xylem vessels of the nodal segments at these two ends of the stem tended to have lower numbers of endophytic bacteria. This suggests that the nodal segments at the upper and middle ends of the stem are the best starting material for future experiments. The present results show the importance of pre-treatment and culture maintenance in reducing endophytic contamination. Furthermore, the results showed the potential of tTCL-treated Harumanis cultures in shoot proliferation and minimising the contamination rate.

CHAPTER 1

INTRODUCTION

Mangifera indica, commonly known as mango, is a delicious tropical fruit revered for its juicy sweetness and distinctive flavour. The plant originates from northeast India, the border region between Indo-Myanmar and Bangladesh. It has a rich history dating back thousands of years and is one of the most widely cultivated fruits in the world (Singh, 2016; Yadav and Singh, 2017). There is even a mango-like leaf fossil from the Palaeocene found near Damalgiri in India (Mehrotra et al., 1998). According to Dinesh and colleagues (2011), there are 73 genera and 1000 named species of mango in the genus *Mangifera*, but only 27 species of this genus produce edible fruits.

Almost every part of the *Mangifera* plants is useful and has been used as folk medicine for thousands of years. The leaves, root and bark of *M. indica* cv. Sewe and Bouka have been found to have high levels of antiglycation and antioxidant activities, which may play a potential role in combating ageing, diabetic complications and diseases associated with oxidative stress (Ndoye et al., 2018). According to The Food and Agriculture Organisation of the United Nations (FAO), mango is one of the three most traded tropical fruits in terms of export volumes in 2021 (FAO, 2022). The mango can be marketed in many forms. It can be sold as fresh fruit, fruit peels or pickled fruit, or as primary products such as mango puree, pulp and concentrate. The mango pulp can also be used to enrich or flavour secondary products such as ice cream, yoghurt, beverages, and soft drinks (Owino and Ambuko, 2021).

Reproduction of this species can be non-vegetative and vegetative. The seeds of this plant used for non-vegetative propagation pose some challenges to growers. *M. indica* is a recalcitrant species in which the viability of the seeds rapidly decreases weeks after fruiting maturity. In addition, the number of progeny is limited by only one

seed per fruit. Therefore, growers usually propagate this plant vegetatively by grafting. However, this technique requires skilled labour and is very labour intensive. There is another technique by which a large number of aseptic progeny can be produced by non-vegetative or vegetative means.

Micropropagation is a plant tissue culture technique that aims to produce large numbers of offspring free of viruses and diseases in a controlled sterile environment. The first report on plant tissue culture was published by Gottlieb Haberlandt in the early 20th century (Thorpe, 2007). To produce aseptic cultures, it is important to sterilise the explants before cultivating them on the medium. The reason for this is that the fungus or bacteria inside or outside the explant will overgrow the cultures and then cause microbial contamination and eventually death of the cultures (necrosis). Therefore, surface sterilisers, fungicides, antibiotics and biocides are used in the protocol to disinfect the explants during micropropagation (Tilahun et al., 2013; Ahmadpoor et al., 2022; Ho et al., 2022; Anjum et al., 2023; Verma et al., 2023). It is also important to maintain a balance between culture mortality and sterility when selecting a suitable steriliser (Wegayehu et al., 2015).

Once the aseptic explants have been established, the next step in micropropagation should be shoot regeneration. Cytokinin, a plant-specific chemical messenger (hormone) that can initiate *de novo* shoot organogenesis, is always used in micropropagation for shoot regeneration. Although cytokinin can stimulate shoot multiplication, an increase in cytokinin concentration does not lead to a higher growth rate of the cultures. It was found that 0.5 mg/L kinetin induced the longest tetraploid shoot, but 1.5 mg/L kinetin inhibited the growth of the shoot of *Plectranthus amboinicus* (Lour.) Spreng (Sari et al., 2021).

Another factor that can improve the growth of the cultures is the source of light. Light is not only a source of energy, but also an important signaller for the plant. Although *in vitro* cultures can extract sucrose (carbon) from the medium as an energy source, the presence of light facilitates the use of this source and the metabolism of other biochemicals relevant to growth (Amoozgar et al., 2017). Light-emitting diodes (LED) have emerged as the better choice over fluorescent lighting in recent years. This is because LED can produce light with high energy in a narrow range of a specific light spectrum, whereas fluorescent tubes cannot. These properties of LED allow researchers to increase the yield of secondary metabolites (Yeow et al., 2020; Jung et al., 2021) and improve the growth of cultures (Kim et al., 2014; Yu et al., 2020). Amoozgar and colleagues (2017) even irradiated lettuce with two monochromatic LED lights in a ratio of 7 red: 3 blue to improve crop yield and quality.

The aim of the present study was to establish an effective disinfection protocol to solve one of the major problems in micropropagation of *M. indica* cv. Harumanis, namely deep-seated contaminants. If the amount of endophytes in the explant cannot be minimised, then the number of explants that can be used for shoot regeneration will be very small. Therefore, the current study focused not only on optimising the surface sterilisation of the explants, but also on the pre-treatment of the mother plants and the post-treatment of the cultures. In addition, studies were conducted on the effect of various cytokinins and LED light to overcome the plant's recalcitrance. Histological and SEM analyses were also carried out to better understand the viability of the explants in the different parts of the strain and the infection mechanism of the deep-seated contaminants.

1.1 Objectives

The objectives of the present study are:

i. To establish a disinfection protocol for *Mangifera indica* cv. Harumanis,

ii. To evaluate the combinational effect between various spectra of LED light and various growth hormones on the growth rate of axillary bud,

iii. To evaluate maturity and sterility of the nodal segments obtained from the field plants.

CHAPTER 2

LITERATURE REVIEW

2.1 Mango

Mango (*Mangifera indica*) is one of the best-known angiosperm species in the Anacardiaceae family, as are cashew and marula. It belongs to the genus *Mangifera*, which comprises 73 genera and 1000 named species of mango, of which about 27 species produce edible fruits (Dinesh et al., 2011). Species such as *M. gedebe*, *M. minor* and *M. mucronulata*, which occur in the Solomon Islands and *M. minor* in Micronesia, either do not bear fruit or the fruit is inedible (Bally, 2006).

There are about hundreds of cultivars of *M. indica* around the world and over 1000 varieties in Asia, especially in India (Singh, 2016). The word mango most probably originated from the Portuguese word “manga”, which is probably derived from Malayalam “māṅṅa”. Mango trees play a sacred role in India. In Hindu culture, hanging fresh mango leaves outside the front door during Ponggol (Hindu New Year) and Deepawali is considered a blessing for the house (Yadav & Singh, 2017).

Bally (2006) explains that *Mangifera* originates from tropical Asia. Borneo, the Malay Peninsula, Sumatra and Java have the most species of this genus. The author also mentions that the most cultivated Mangifera species, *M. indica* (mango), originates from Indo-Burma. However, Yadav and Singh (2017) have confirmed that the Malaysia region is also the origin of *M. indica*, not just Indo-Burma, as most related species grow in Malaysia.

Since the mango tree can grow in an area with temperatures ranging from 6°C to 48°C, it tolerates a wide range of climates. Therefore, the mango tree is grown in many places, from the tropics to temperate climates. The minimum temperature to

which a mango tree can adapt is 6°C. But mango trees grown in places where the temperature is close to the minimum or maximum temperature to which they can adapt do not thrive well. Sukhvibul and his colleagues (1999) found that the inflorescence emergence and elongation of the 4 tested mango tree cultivars (`Nam Dok Mai', `Kensington', `Irwin' and `Sensation') were negatively affected when they were kept in an environment with a day/night temperature of 15/5°C. They found that inflorescence development in all cultivars only occurred at warmer temperatures (20/10°, 25/15°, 30/20°C). Another interesting phenomenon observed by the author was that the inflorescences of all varieties developed differently even at the same temperature.

2.2 Mango trade market and significance

Mango plays an important role in the economy of many countries in tropical regions. According to Gao and her colleagues (2022), the world's largest mango producer is India (24.7 million tonnes), followed by Indonesia (3.6 million tonnes) and China (3.4 million tonnes) in 2020. The Ministry of Commerce and Industry, Government of India, indicated that the country exported 2.7 million tonnes of fresh mangoes worth USD 44.05 million to the world in 2021-2022 (APEDA, 2023a). Instead of exporting the bulk of fresh mangoes to the world, India exports only about 10% of its fresh mangoes. Most of the fresh mangoes remain in the domestic market. Unlike India, Thailand is the world's largest exporter of fresh mangoes. The country shipped USD 734.01 million in 2020 (Mzingaye, 2023). The main importer of Thailand’s exported fresh mangoes is China, with an import value of USD 707.3 million in 2021 (Tridge, 2022). According to Tridge (2022), China is the world's largest importer of fresh mangoes.

Processed products made from fresh mangoes are also in high demand in the market. Fresh mangoes can be processed in 2 stages of ripeness. Unripe mangoes are

processed into foods such as chutney, pickles, curries and dried products, while ripe mangoes can be processed into puree, canned and frozen slices, juices and other similar products.

Instead of processing fresh mangoes according to their degree of ripeness, these mangoes can also be processed into primary and secondary products. In the case of primary products, a distinction is made between mango powder, mango puree, mango pulp and mango concentrate. The secondary product type, on the other hand, is divided into jam, pickles, juice, sweets, preserves, frozen slices and others. According to the Ministry of Commerce and Industry, Government of India, India is the world's largest exporter of mango pulp. The author stated that the country exported 12.3 million tonnes of mango pulp worth USD 124.11 million to the world during 2011-2022 (APEDA, 2023b).

In Malaysia, the 2 main mango varieties sold in the market are Chok Anan and Harumanis (Unit Perangkaan Pertanian et al., 2022). According to the report, 204.146 tonnes of Chok Anan and 45.417 tonnes of Harumanis were produced in 2021 (Unit Perangkaan Pertanian et al., 2022). Although the production of Chok Anan is about 4 to 5 times that of Harumanis, the report states that the net profit from the sale of Harumanis is about 1.7 times higher than that of Chok Anan, RM436,188.83 and RM252,214.88 respectively. These data indicate that an increase in the production of Harumanis will bring Malaysia a large income.

2.3 Plant tissue culture

Plant tissue culture is a collection of techniques for maintaining or cultivating cells, tissues, organs and their components under defined physical and chemical conditions *in vitro*. Henri-Louis Duhamel du Monceau’s experiment in year 1756 on

wound healing in plants is considered to have pioneered the science of plant tissue culture (Thorpe, 2013; Tiku et al., 2021).

Plant tissue culture is widely used in basic research in cell biology, genetics and biochemistry and also in solving problems in agriculture and horticulture. Thorpe (1990) stated that the application of plant tissue culture can be divided into 5 broad areas, namely cell behaviour, plant modification and improvement, pathogen-free plants and germplasm storage, product formation and clonal propagation. Among these 5 areas of application, clonal propagation technique to produce vegetative plants is the most commonly used technique in plant tissue culture.

The clonal propagation technique in plant tissue culture is also known as micropropagation. There are generally 3 approaches to micropropagation, namely promoting axillary bud burst (Yu et al., 2023), adventitious bud production directly or indirectly via callus (Yang et al., 2023) and somatic embryogenesis directly or indirectly on explants (Yang et al., 2023), and somatic embryogenesis directly or indirectly on explants (Chan and Stasolla, 2023). In this technique, genetically identical copies of individual plants are propagated in closed vessels under controlled aseptic conditions. A layer of a nutrient medium is added inside the vessels to support or promote the growth of the explants. The nutrients of the medium consist of macronutrients, micronutrients, vitamins and carbohydrate sources. Other additives such as plant regulators, antimicrobials, antioxidants, gelling agents and hexitols are specifically added to the medium depending on the research objective.

2.4 Micropropagation of *M. indica*

Studies on the micropropagation of *M. indica* have been conducted since the beginning of the 20th century. Most of these studies have used somatic embryogenesis

instead of direct organogenesis (Litz and Schaffer, 1987; Ara et al., 2000; Ermayanti and Rantau, 2009; Mishra et al., 2010).

In somatic embryogenesis, the nucellar tissue in the seed is often used as the explant, as this tissue is genetically identical to the mother plant (Simsek et al., 2019). Nucellus The nucellar are extracted obtained from the immature seed (fruit with a length of 3 – 4 cm long) of *M. indica* (Rivera-Domínguez et al., 2004). Although direct somatic embryogenesis of *M. indica* is well established, there are still some limitations to this technique such as genetic variation in the induced callus and a limited number of seeds. The second problem is indeed a critical one for mango varieties that have only one harvest season per year, such as Harumanis.

To avoid the two shortcomings of the technique, direct organogenesis was used with the shoot tip and nodal segment of this plant. Direct organogenesis is the most important technique in micropropagation to regenerate a whole plant from totipotent somatic cells (Duclercq et al., 2011). The apical bud of the shoot apex and the axillary bud of the nodal segment have meristem cells that fulfil the conditions as starting material for this technique. Although it is known that the sterility of the apical bud is better than that of the axillary bud, the number of axillary buds per branch is greater than that of the apical bud (one apical bud per branch). Therefore, selecting the axillary bud for direct organogenesis of *M. indica* can improve the yield of the progeny. It also increases the accuracy of the results, as a large number of samples allows more replicates in the experiments.

However, deep-seated contaminants in the nodal segment of *M. indica* limit the number of germ-free progeny (Tetsumura et al., 2016). In the same report, growth of the remaining aseptic shoots was found to be slow and inefficient even when gibberellin and plant peptide hormone were added to the growth medium. This finding is further

evidence of the recalcitrance of *M. indica*. To minimise endophytes in the explant, Conde and colleagues (2023) compared the contamination rate between *ex vitro* and *in vitro* germinated seeds. They found that explants obtained from *in vitro* germinated seed had a significantly lower contamination rate. This shows the positive influence of the growth environment of the mother plant on the contamination rate. Although browning of explants and excretion of phenolics into the medium contribute to the recalcitrance of *in vitro* cultures in this plant, this can be overcome by using appropriate plant growth regulators (PGRs), antioxidants and adsorbents or by subculturing the cultures regularly (Tetsumura et al., 2016; Conde et al., 2023; Permadi et al., 2023). Transferring cultures to a new medium once or twice a week was found to minimise the deleterious effects of phenolic by-products in the medium excreted by the banana explant (Permadi et al., 2023).

2.4.1 Disinfection protocols

As mentioned earlier, deep-seated contaminant is a limiting factor in the production of aseptic progeny of *M. indica*. To address this problem, an effective disinfection protocol that balances both culture survival and sterility is required. The most common technique for disinfecting the explant during micropropagation is surface sterilisation. This is an important technique to remove the microbes that are on the surface of the explants (Bhojwani and Razdan, 1986). However, it can also be used the other way around. Sahu and colleagues (2022) optimised the protocol for surface sterilisation of explants to remove the epiphytes and isolate the endophytes without damaging the endophytes. It is very important to optimise the duration and concentration of the surface sterilising agents, as they can inhibit growth or cause necrosis in the cultures if the parameters exceed the limit that the explants can tolerate (Zahid et al., 2021).

However, surface sterilisation alone is sometimes not sufficient to maintain sterility of cultures. Maintaining the health of the mother plants (pre-treatment) is also a crucial part of the disinfection protocol. Jiménez and colleagues (2006), working on micropropagation of giant bamboo, reported spraying a fungicide on the field crop weekly in the rainy season and monthly in the dry season. Krishna and colleagues (2008) sprayed mother plants with 2 g/L imidazole three times at three-day intervals to reduce *in vitro* contamination. Sharma and colleagues (1999) even sprayed antibiotics (50 mg/L gentamycin + 50mg/L streptomycin + 100 mg/L rifampicin) four times on alternate days on the shoot tip of papaya to control both tested Gram positive and negative bacteria.

With a well-established pre-treatment and surface sterilisation protocol, post-treatment of cultures is the final attempt to eliminate the deep-seated contaminants. Post-treatment involves the addition of antimicrobials such as fungicides, antibiotics and/or biocides to the growth medium. It was reported that the addition of 3 mL/L Plant Preservative Mixture (PPM™) and 1 mL/L Carbendazim® to the medium effectively reduced microbial contamination without inhibiting the germination rate of bamboo nodal segments (Jiménez et al., 2006). As with surface sterilisers, the optimal concentration of antimicrobials in the medium should be investigated, as these agents can be harmful to the cultures if the concentration is too high.

2.4.1(a) Surface sterilant

Mercury (II) chloride ($HgCl_2$) and sodium hypochlorite (NaOCl) are regularly used to sterilise the surface of the explants (Anjum et al., 2023; Pourhassan et al., 2023). The mercury ions (Hg^+) of $HgCl_2$ were found to inhibit the archaeal transcriptional activities that stopped cell division, stimulating a cytostatic response at submicromolar concentrations and a cytocidal response at micromolar concentrations (Dixit et al.,

2004). Chlorine is a strong antioxidant that can inhibit bacterial enzymes, leading to irreversible oxidation of the SH groups (sulphydryl group) of essential bacterial enzymes (Estrela et al., 2002).

$HgCl_2$ has long been used for surface sterilisation. Nevertheless, the disposal of $HgCl_2$ into the environment poses dangers to all living things. Algal cells, which are of great benefit to aquatic animals, were found to be severely damaged when treated with 0.9 mg/L $HgCl_2$ (Ge et al., 2022). In addition, improper handling of this chemical can damage the nervous system of the researchers (Li et al., 2022). Therefore, many studies have been conducted to find a substitute for $HgCl_2$ and NaOCl has been found to be a safer disinfectant that also gives promising results in surface sterilisation (Sharifkhani et al., 2011; Tilahun et al., 2013).

NaOCl is an oxidising agent. It is used as a disinfectant and bleaching agent in households, in the food industry, in health care and for the treatment of drinking water. Clorox® with 5.25% NaOCl is a household disinfectant commonly used in Malaysia. Although the chlorine element is found in both $HgCl_2$ and NaOCl, the mode of action of NaOCl is slightly different. Hypochlorous acid ($HOCl^-$) and hypochlorite ion (OCl^-) released by NaOCl when it comes into contact with organic tissue can act as a solvent and release chlorine, which combines with the amino group of the protein and eventually causes the degradation and hydrolysis of the amino acid of the microbes (Estrela et al., 2002). Since the essential enzyme sites of the bacteria were irreversibly inactivated by the hydroxyl ions and chloramination, NaOCl was considered to have antimicrobial activity (Estrela et al., 2002). NaOCl has been widely used for surface sterilisation compared to $HgCl_2$ due to its lower phytotoxicity to plants (Kaya et al., 2023; Pérez-Pazos et al., 2023; Shende et al., 2023).

If microbial contamination is still present after surface sterilisation, one can either assume that the concentration and duration of the protocol are not optimal or that there are endophytes inside the explants that the surface sterilising agents cannot reach. If the second assumption is true, the application of a systemic fungicide, antibiotic and biocide is required..

2.4.1(b) Fungicide

Benomyl is a systemic fungicide used to protect trees and fruits of *M. indica* from infestation by anthracnose. Anthracnose is a term that non-specifically describes a group of fungal diseases that cause dark lesions on the leaves and sunken lesions and cankers on the stems when severe (Downer et al., 2020). This disease also has a great impact on the yield of mango and means a great loss to the growers (Arauz, 2000). Fortunately, benomyl and azoxystrobin have been found to be effective in controlling anthracnose (Muirhead, 1976; Sundravadana et al., 2007; Bharathi et al., 2019; Khaskheli, 2020).

Benomyl is a systemic fungicide that can inhibit a broad spectrum of fungi (Hauptmann et al., 1985). The active ingredient of benomyl, namely benzimidazole, eliminates the fungus by inhibiting the polymerisation of tubulin within the fungal cells (Zhou et al., 2016). Without the polymerisation process, the microtubules, which act as a cytoskeleton to maintain the shape of the cells and serve as a platform for the intercellular transport of proteins, cannot form. This leads to the death of the fungus. Azoxystrobin is also a systemic fungicide that has been used to protect crops in agriculture. It has a quinol oxidation inhibitor (QoI) that can inhibit mitochondrial respiration in fungi and lead to the death of the fungus. In contrast to benomyl, azoxystrobin is only used to a very limited extent in micropropagation. It is mainly used

to protect mother plants from fungi by foliar spray (Sundravadana et al., 2007; Vawdrey et al., 2010).

Benomyl has been widely used for surface sterilisation of explants due to its low phytotoxicity (Filiz et al., 2010; Regalado et al., 2015; Ebrahimi et al., 2023). In addition, it has been added to the growth medium to eliminate endophytic fungi present in the cultures (Ramanayake and Yakandawala, 1997; Santana et al., 2003; Ismail et al., 2016). However, the endophytic fungus is not the only deep-seated contaminant found in *M. indica.* Bacteria that thrive in the plant also contaminate the cultures. It has been reported that surface sterilisation of *M. indica* explants with 0.5 g/L benomyl was effective against fungal contamination but not against bacterial contamination (Andrade et al., 2004). Therefore, an antibiotic that is effective against a wide range of bacteria and has low phytotoxicity to the explants is also required to keep the cultures sterile.

2.4.1(c) Antibiotic

Antibiotics are also used in micropropagation. Cefotaxime is one of the recommended agents for plant tissue culture because it is effective against a wide range of bacteria and has low phytotoxicity (Hamilton-Miller et al., 1978; Pollock et al., 1983). Cefotaxime is a beta-lactam antibiotic belonging to the third-generation cephalosporins. The cephalosporin antibiotic inhibits the synthesis of the bacterial cell wall and eventually leads to cell lysis of the bacteria (Murray and Moellering, 1981). In addition, cefotaxime has a high stability towards β-lactamase (enzyme that hydrolyses beta-lactam antibiotics) produced by various bacterial species (Mitsuhashi et al., 1980). It was reported to be effective against *Agrobacterium tumefaciens* (Ferdous et al., 2021) and *Xanthomonas axonopodis* (Doy, 2021) which infect mango trees and cause crown gall and bacterial black spot diseases respectively (Wang et al., 2010; Almashhadany, 2019)

Prakasha and colleagues (2018) reported that soaking banana suckers in 400 mg/L cefotaxime solution for 15 minutes resulted in significantly lower contamination. In addition, surface sterilisation of shoot tip of sugarcane with 500 mg/L cefotaxime for 30 minutes was reported to produce 70– 90% sterile cultures (Khan et al., 2007).

Instead of using cefotaxime in surface sterilisation, most studies have added this antibiotic to the growth medium (Magdum, 2013; Haddadi et al., 2015; Verma and Modgil, 2023) . It has been reported that cefotaxime can promote growth of cultures at optimum concentration, but it also significantly inhibits growth at high concentration (Manchanda et al., 2011; Asif et al., 2013; Lakshmi et al., 2021). Verma and colleagues (2023) minimised the inhibitory effect of cefotaxime on culture growth by gradually reducing the concentration of cefotaxime in the medium to 200 mg/L over a period of 7 weeks. The same phenomenon was also observed in the medium supplemented with a high concentration of benomyl (Filiz et al., 2010; El-Sharabasy and Zayed, 2018).

2.4.1(d) Plant Preservative Mixture (PPM™)

Besides fungicides and antibiotics, there is also a biocide that can inhibit the growth of bacteria and fungi. This is Plant Preservative Mixture (PPM™) (Fuller and Pizzey, 2001). PPM™ contains a mixture of isothiazolones (methylisothiazolinone and chloromethylisothiazolinone) that are effective against a broad spectrum of bacteria and fungi (Niedz, 1998). These isothiazolones interfere with metabolic pathways involving dehydrogenase enzymes and inhibit growth, respiration (oxygen consumption) and synthesis of adenosine triphosphate (ATP) in microbes (Williams, 2006). This series of actions eventually leads to cell death.

Niedz (1998) first proposed the use of PPM™ in plant tissue culture to eliminate microbial contamination. He also mentioned that this product is phytotoxic when its concentration is higher than the plant materials can tolerate. George and Tripepi (2001)

reported that although PPM™ is effective in inhibiting microbial growth, 2 mL/L PPM™ in the medium completely stopped the growth of chrysanthemum leaf explants and reduced the growth of birch leaf explants when the concentration of PPM™ in the medium increased from 0–4 mL/L. PPM™ can also be used for surface sterilisation. Kushnarenko and colleagues (2022) reported that surface sterilisation of shoot tip explants with 5% (v/v) PPM™ for 10 min prior to culturing in medium supplemented with 0.2% (v/v) PPM™ effectively controlled microbial contamination. However, it was reported that *in vitro* cultures of apples treated with 0.2% (v/v) PPM™ were contaminated when transferred to medium without PPM™ (Romadanova et al., 2022). Fong and colleagues (2012) faced the same phenomenon and suspected that PPM™ in the medium suppressed the growth of microbes instead of eliminating them..

2.4.2 Explant browning and phenolic excretion

The browning of explants is a major obstacle to micropropagation as it inhibits the growth of cultures and causes necrosis. This is a phenomenon in which the surface of the plant material turns brown. This phenomenon usually occurs in most woody plant materials such as apple, walnut and mango (Krishna et al., 2008; Martini et al., 2013; Zhao et al., 2021). This also occurs with some herbaceous plant materials such as banana (Permadi et al., 2023). In daily life, browning of the surface of fruits and vegetables can also be observed when the surface is damaged or attacked by microbes.

In plant tissue culture, an explant is cut out of the mother plant for an experiment. This creates wounds on the surface of the explant. The damaged cells of the explant release phenols, which are then enzymatically oxidised by polyphenol oxidase (PPO) and peroxidase (POD) to quinones (JunHui et al., 2000). These highly reactive quinones polymerise proteins and other cellular components into the amorphous dark pigments known as melanin (Stevens and Davelaar, 1996). Melanin is

the biochemical responsible for the brown colour of the explant and the dark brown appearance of the phenolic exudates that are exuded into the medium.

In addition, the highly reactive quinones bind with cell proteins or polymerise in tissues through the tissue by dehydration or polymerisation, cause leading to disruption of tissue metabolism, growth inhibition of growth, and, eventually, necrosis of the explants (Zhao et al., 2021). He Yang and colleagues (2009) hypothesised that exposure of explants to quinones may lead to accumulation of hydrogen peroxide (H_2O_2) in the explants. This hypothesis proved to be valid as H_2O_2 was reported to be spontaneously formed when chlorogenic acid quinones were reduced in acidic solution (Murata et al., 2002). H_2O_2 is also a strong oxidant that can cause necrosis in explants. Since the accumulation of quinones in the medium can further stimulate the production of H_2O_2, it is always recommended to subculture the explants in fresh medium from time to time to reduce the oxidative stress of the cultures (Leng et al., 2009; Abohatem et al., 2011; Permadi et al., 2023).

To reduce the high oxidative activity of quinone and H_2O_2, antioxidants that can protect plant tissue from these free radicals are often used in tissue culture of woody plants. Antioxidants such as ascorbic acid and citric acid have been used in micropropagation of *M. indica* to reduce enzymatic browning (Thomas and Ravindra, 1997; Samaan et al., 2007; Conde et al., 2023). Ascorbic acid has been reported to reduce quinones to their original, less reactive substrate (catechol) at high concentrations and to inhibit PPO by acting as a competitive inhibitor at low concentrations. Ali and colleagues (2014) also reported that citric acid acts as a non-competitive inhibitor of PPO at low concentration by completely inhibiting the formation of quinone when added to the reagent prior to the addition of PPO.

Adsorbents such as polyvinylpyrrolidone (PVP) and activated charcoal have also been used in micropropagation of *M. indica* (Thomas and Ravindra, 1997; Shamsul, 2021). Activated charcoal and PVP have been shown to be strong adsorbents, adsorbing phenols excreted by explants (Zhou et al., 2010; Bantte and Feyissa, 2015). Without the accumulation of phenols in the medium, PPO cannot convert phenols to quinones and thus reduce oxidative stress in the cultures. However, it has been reported that these adsorbents can also adsorb nutrients in the growth medium and then inhibit the growth of the cultures or induce necrosis (Bantte and Feyissa, 2015; Das and Srivastav, 2015; Verma and Jakhar, 2022). Therefore, the optimal concentration of these adsorbents in the medium should be determined.

In addition, the nutrient concentration in the medium should also be optimised as this may lead to browning of the explants due to osmotic stress on the cultures. Yang and colleagues (2019) reported that osmotic stress induced by sucrose and plant growth regulators in the medium leads to the accumulation of H_2O_2 in the explants and causes more browning of the explants.

2.4.3 Plant growth regulator

Plant growth regulators (PGRs) are a group of chemicals that play a regulatory rather than a nutritional role in plant growth and development. These compounds are generally active at low concentrations and are referred to as plant hormones (George et al., 2008). There are generally five groups of PGRs, namely auxin, cytokinin, gibberellic acid, abscisic acid and ethylene (Roberts and Hooley, 1988; George et al., 2008).

Of these PGRs, auxin and cytokinin are most commonly used in micropropagation. Cytokinin regulates the growth and development of plant tissues by stimulating the proliferation of plant cells. It initiates the signalling pathway by binding

to membrane-associated histidine kinase receptors and passing through a phosphorelay system (Yang et al., 2021). The first cytokinin was discovered in 1955. It was named kinetin by Miller and colleagues (1955) and was extracted from yeast extract. There are two main classes of cytokinin, namely adenine-based cytokinin and phenylurea-based cytokinin. Kinetin, 6-benzylaminopurine (BAP), zeatin, 6-(γ,γ-dimethylallylamino)purine (2iP) and metatopoline are adenine-based cytokinins, while thidiazuron (TDZ) and N-(2-Chlorochloro-4-pyridyl)-N′-phenylurea (CPPU) are phenylurea-based cytokinins.

It was once reported that phenylurea-based cytokinin has higher activity and promotes culture growth more than adenine-based cytokinin (Mok et al., 1987). However, recent studies have shown that the growth response of cultures is more dependent on cytokinin type and dose. Ravanfar and colleagues (2014) reported that cabbage hypocotyl explants treated with 9.84 µM 2iP induced the highest percentage of shoot growth (90%), which was not significantly different from the percentage of shoot formation in cultures treated with 0.23 and 2.27 µM TDZ (80%).

While it was known that PGRs promote shoot growth, apical dominance is different. Apical dominance is a condition where vertical growth exceeds lateral growth in plants. This means that plants prefer to grow longer and taller rather than allowing more branches to grow laterally through axillary bud break. One of the reasons for this is competition for light with other competitors in the field (Irwin and Aarssen, 1996). Auxin produced by the shoot apex is commonly known as the main suppressor of axillary bud break by suppressing the biosynthesis of cytokinin within the nodal stems (Tanaka et al., 2006). Therefore, massive branching can always be observed on the stems when the plants were decapitated. However, cytokinin has been found to be the only factor that can reduce the inhibitory effect of auxin. Mason and colleagues (2014)

found that the lack of sugar in axillary buds causes the buds to remain dormant. The authors mentioned that most of the sucrose was transported to the shoot tip through apical dominance. When the shoot tip was removed, rapid accumulation of sucrose in the axillary buds and subsequent sprouting was observed. Therefore, the concentration and type of cytokinin and the concentration of sucrose should be considered when inducing shoots from the nodal explants of *M. indica*.

2.4.4 Thin cell layer

Thin cell layer (TCL) is a plant tissue culture technique used to induce organogenesis or embryogenesis in cultures from very thin explants. This technique was discovered by Tran Thanh Van (1973) who reported that small explants (0.4 x 1 cm) consisting of 3 – 6 layers of epidermal and subepidermal cells from flowering branches of *Nicotiana tabacum* were capable of producing de novo flower buds without forming callus when cultured on growth medium enriched with cytokinin. After 3 years, he wrote a handbook on TCL and indicated that TCL can be further divided into two groups, namely longitudinal TCL (lTCL) and transverse TCL (tTCL) (Tran Thanh Van and Gendy, 1996). He mentioned that the size of lTCL explants is about 3 – 6 cortical layers in thickness, 1 mm in width and 4 – 8 cm in length. For tTCL, the thickness of the explants should be about 200 µm – 2 mm.

It is widely accepted that TCL promotes higher productivity and less time spent on crop multiplication (Ben Ghnaya et al., 2007; Bravo-Ruiz et al., 2022). One of the reasons for this is that the nutrients or PGRs can more easily reach the target cells (Nhut et al., 2003a). It has been reported that TCL-processed explants responded faster (within 14 – 19 days) than standard explants (within 40 – 48 days) (Aghion-Prat, 1965). Since TCL only uses tiny parts of certain tissues for experiments, the growth responses of plant material from different parts of the plants are different. Monja-Mio and Robert

(2013) pointed out that stem tissue treated with TCL was more sensitive than leaf tissue treated with TCL. In addition, stem tissue from the apical and middle part of the stem reacted more strongly than the lower part. Furthermore, the author mentioned that there is a significant difference between the different genotypes of the Hennequen plant. Based on this report, it can be said that TCL can also be used to explore the potential of different parts of *M. indica* and overcome the recalcitrance of this plant.

2.4.5 Light-emitting diode (LED)

Sunlight, an electromagnetic radiation emanating from the sun, is the main source of energy for plants in nature. The chlorophylls of plants absorb this light energy and use it to convert carbon dioxide into glucose. This process, in which the light energy is stored in the molecular binding of organic molecules (e.g. carbohydrates), is known as photosynthesis. However, carbohydrate (sucrose) was added to the growth medium in the plant tissue culture. Therefore, light is no longer the main source of energy under these conditions.

However, light has another important function for plants, namely the regulation of plant growth and development. Light activates photoreceptors in the plant, thereby stimulating downstream signalling pathways that lead to extensive changes in gene expression responsible for physiological, morphological and developmental responses (Yadav et al., 2020). For example, plants have been found to respond differently to different light spectra (Yeow et al., 2020; Lai et al., 2022; Pang et al., 2023). LED is a better choice than the widely used cool white fluorescent light because it can emit specific light in a narrow spectrum, which fluorescent light cannot. Wu and Toit (2012) reported that *Protea cynaroides* explants treated with white fluorescent light showed more browning than those treated with red and blue LED light. In addition, the authors reported that the leaves of explants treated with blue LED light were the greenest.

However, Kwon and Park (2019) found that explants of *Phalaenopsis* 'Spring Dancer' protocorm-like body (PLB) released less phenolics to the medium when treated with red LED light and a combination of red, blue and green LED light than when treated with fluorescent light and 1 red : 1 blue LED light.

In addition to the light spectrum of LED, light intensity is also a factor that influences the response of plants. It has been reported that high intensity of natural light and additional LED light (red : far red) induces a higher percentage of sprouting axillary buds in roses (Wubs et al., 2013, 2014). Kepenek (2019) reported that 75 $\mu molm^{-2}s^{-1}$ of LED light induced the best growth response in strawberry plantlets compared to those treated with 45 $\mu molm^{-2}s^{-1}$ cool white fluorescent light. The author also reported that strawberry plantlets irradiated with 75 $\mu molm^{-2}s^{-1}$ from LED performed better than those irradiated with 100 $\mu molm^{-2}s^{-1}$ from LED. This indicates that plants grow better when irradiated with optimal rather than high light intensity.

Since LED light has the potential to reduce the browning and phenolic excretion of the explant and break the dormancy of axillary buds, different colours of LED light can be tested on *M. indica* cv. Harumanis to improve the growth response of its axillary buds.

2.5 Histological analysis

The steps for plant histology are as follows: Obtaining plant tissue, fixation, embedding, sectioning, staining and finally observation under the light microscope (Spence, 2001). Since the observation area under the light microscope is small, the size of the plant tissue should be reduced by removing the superfluous tissue that is not of interest for the experiment. Next, the plant tissue was treated with a series of reagents to fix it. This step stops the degeneration process (autolysis) of the plant tissue so that the intact structure of the tissue can be observed. Then the plant tissue is sent for

embedding. The tissues are flooded and coagulated together with paraffin wax for preservation. In addition, the handler can place the tissues in the wax in the desired position, e.g. lengthwise or crosswise, before it coagulates. Solidified paraffin wax provides a good support structure for the section.

The cutting of plant tissues is done with a microtome, a cutting instrument with special precision. The thickness of the tissue is usually 2 – 15 µm, depending on its condition. The sections are then placed on glass slides for staining. There are many types of staining agents that target different cell compartments. For example, Safranin stains lignified and cutinised walls red, Fast Green stains cellulose walls and cytoplasm green and toluidine blue stains cellulose walls, cytoplasm and nucleus blue, nucleolus purple and lignified cell walls green or blue (Spence, 2001). Staining plant tissue with safranin and fast green (counterstain) has also proven useful in nuclear and embryological studies (Moreno-Sanz et al., 2020). Under certain circumstances, plant tissue is stained with a microbial targeting stain to identify the presence of endophytes in plants. Under the light microscope, endophytic bacteria were reported to have infected the secondary xylem of clove, which was stained red with safranin (Trianom et al., 2019).

Since staining the nuclei with safranin and fast green can reveal the viability of meristematic tissue in plants, this technique could be used in *M. indica* cv. Harumanis. In addition, staining the nodal explants with safranin can reveal which part of the tissue is infected with deep-seated contaminants and determine the need for adding antimicrobials to the growth medium.

2.6 Scanning electron microscope analysis

In contrast to the light microscope, the scanning electron microscope (SEM) directs a beam of electrons instead of photons onto the surface of the samples.

Bombarding the specimens with these electrons generates signals (e.g. secondary electrons, X-rays and photons). These signals are received by cathode ray tubes in SEM and converted into an image showing the three-dimensional structure of the sample (Leamy, 1982).

Images taken with SEM are commonly used in plant pathology as these images show the interactions between the endophytes and the infected areas of the plant. Caldwell and Iyer-Pascuzzi (2019) reported that the images taken with SEM clearly show how the bacteria infect the proto- and meta-xylem vessels of *Zea mays*. The authors also took an image under the light microscope, but this image only showed some highly stained substances sticking to the wall of the xylem vessels. They were also able to use SEM to identify the hyphae of the endophytic fungus that infects the xylem vessels of tomato stems. This proves the usefulness of SEM in studying the interaction between microbes and plants. On the other hand, bacterial clumps clogging the xylem vessels of the internodes and petiole of grapes were identified (Fritschi et al., 2008). These occlusions are the main cause of Pierce's disease, a xylem vessels blockage disease caused by *Xylella fastidiosa* in grapevines.

SEM is a very useful tool for identifying deep-seated contaminants in nodal explants of *M. indica*. It can also be used to locate endophytes in the explants and thus provide clear direction for devising an effective disinfection protocol.