IN VITRO MULTIPLICATION AND PLANT REGENERATION OF KIWANO (*Cucumis metuliferus* E. Mey. ex Naudin), A POTENTIAL SUPER NUTRITIOUS FRUIT

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LIST OF ABBREVIATIONS AND SYMBOLS

%	Percent
μM	Micromolar (mikromolaritas)
μm	Micrometre
nm	Nanometre
m	Metre
μmol	Micromole
BAP	6-benzylaminopurine
w/v	Weight/volume
&	and
v/v	Volume/volume
cm	Centimetre
KIN	Kinetin
mT	Meta-topolin
AdS	Adenine sulphate
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
NAA	Naphthaleneacetic acid
DPX	Distrene, plasticiser, xylene
et al.	Et alia
MS	Murashige and Skoog
g	Gram
g/L	Gram per litre
ANOVA	Analysis of variance
Fe-EDTA	Ferric ethylenediaminetetraacetic acid
USM	Universiti Sains Malaysia
mL	Millilitres
TBA	Tertiary-butyl alcohol
mg/L	Milligram/litre

AgNPs	Silver nanoparticles
LED	Light emitting diode
bp	Base pairs
kb	Kilobase pairs
PCR	Polymerase chain reaction
S.E.	Standard error
Tm	Melting temperature
HCl	Hydrochloric acid
NaOH	Sodium hydroxide
ISSR	Inter simple sequence repeats
DAMD	Directed amplification of minisatellite DNA
SI	Similarity index

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ABSTRAK

Cucumis metuliferus merupakan sejenis tumbuhan dari genus yang sama seperti timun dengan buah berduri yang berwarna kuning ke jingga. Tumbuhan ini berasal dari Afrika tropika dan biasanya digunakan oleh masyarakat asli sebagai makanan atau ubatan. Kajian terkini ke atas tumbuhan ini menunjukkan pelbagai kesan farmakologi yang positif selain menjadi tumbuhan yang tahan terhadap jangkitan nematod. Buah berkhasiat tinggi ini sukar didapati walaupun mempunyai nilai pasaran yang tinggi. Bagi mengatasi cabaran dari segi kebolehdapatan, produksi skala besar, dan bahan penanaman yang berkualiti, protokol penggandaan dan regenerasi *in vitro* yang kukuh telah ditubuhkan. Kultur biji telah berjaya melalui kaedah pensterilan permukaan biji yang dibuang kulit biji dan dipusingkan dalam larutan Klorox 5% selama 5 minit. Kadar percambahan dihasilkan adalah sejumlah 76.6%. Regenerasi pucuk dengan pelbagai jenis sitokinin menunjukkan bahawa 5µM AdS menggalakkan pertumbuhan pucuk yang tertinggi dengan 5.56 ± 0.77sm setiap pucuk. Rawatan gabungan 2.5µM mT + 5µM AdS menghasilkan pucuk yang lebih tinggi pada 5.74 ± 0.65sm setiap pucuk. Rawatan gabungan tersebut digunakan atas 6 jenis rawatan diod pemancar cahaya (DPC). Lampu putih mint menghasilkan pucuk tertinggi pada 4.06 ± 0.58sm, manakala lampu putih, biru, merah, dan merah:biru (1:1) menghasilkan keputusan yang sama. Regenerasi akar adalah terbaik apabila auksin IAA, IBA, dan NAA tidak ditambah. Nanopartikel perak (AgNPs) tidak membantu pertumbuhan pucuk dan juga tidak merencat pertumbuhan akar. Analisis histologi anak pokok yang

dirawat dengan 6 jenis DPC menunjukkan anatomi yang sama. Analisis penanda molekul ISSR dan DAMD menunjukkan bahawa rawatan DPC tidak menyebabkan polimorfisme yang signifikan. Kadar polimorfisme yang paling rendah dicatatkan oleh DPC merah untuk ISSR dan DPC merah:biru untuk DAMD. Kandungan klorofil dan karotenoid adalah yang tertinggi dalam anak pokok yang dirawat dengan cahaya merah:biru (1:1) dengan kandungan klorofil sebanyak 0.677 ± 0.007 mg/g dan karotenoid sebanyak 0.158 ± 0.001 mg/g. Aktiviti antioksidan adalah tertinggi dengan rawatan cahaya merah, manakala hasil daripada lampu putih, biru, merah, dan merah:biru (1:1) tidak mempunyai perbezaan yang signifikan antara satu sama lain. DPC merah:biru (1:1) disimpulkan sebagai lampu yang optima untuk pertumbuhan anak pokok *Cucumis metuliferus* kerana pertumbuhan pucuk yang baik, persamaan morfologi yang tinggi dengan pokok induk, kadar mutasi yang rendah dengan kandungan klorofil dan karotenoid yang tinggi. Protokol regenerasi *in vitro* yang ditubuhkan membolehkan propagasi skala besar tumbuhan kiwano untuk keperluan pasaran tempatan dan antarabangsa, serta menyediakan asas kepada kajian berkaitan dengan kultur tisu kiwano pada masa yang sama.

IN VITRO MULTIPLICATION AND PLANT REGENERATION OF KIWANO (*Cucumis metuliferus* E. Mey. ex Naudin), A POTENTIAL SUPER NUTRITIOUS FRUIT

ABSTRACT

Cucumis metuliferus is a plant of the same genus as cucumbers with spike-bearing fruits that are yellow to orange in colour. This plant originated from tropical Africa and is commonly used by native communities as food or medication. Recent studies conducted on this plant showed various positive pharmacological effects other than being a plant resistant to nematode infection. This super nutritious fruit is hardly available even though it possesses high market value. To overcome problems with availability, mass production, and quality planting materials, a reliable *in vitro* multiplication and regeneration protocol was established. Seed culture was successful with surface sterilization protocol of removing seed coat and swirling in 5% Clorox solution for 5 minutes. The germination rate was obtained at 76.6%. Shoot regeneration with various cytokinins showed that 5µM AdS promoted the tallest shoot growth at 5.56 ± 0.77cm per shoot. Combination treatment of 2.5µM mT + 5µM AdS produced taller shoots at 5.74 ± 0.65cm per shoot. This combination treatment was then applied to 6 types of LED treatments. Mint white light produced the tallest shoots at 4.06 ± 0.58cm, while white, blue, red, and red:blue (1:1) lights displayed similar results. Root regeneration was the best when auxins, IAA, IBA, and NAA were not added. Silver nanoparticles (AgNPs) did not improve the growth of shoots and also did not inhibit root growth. Histological analysis of plantlets treated with 6 types of LEDs showed similar anatomy. ISSR and DAMD molecular marker analyses showed

that LED treatments did not cause significant polymorphism. The lowest polymorphism was recorded by red LEDs and red:blue LEDs for ISSR and DAMD respectively. Chlorophyll and carotenoid contents were the highest in red:blue (1:1) light treated plantlets at 0.677 ± 0.007 mg/g and 0.158 ± 0.001 mg/g, respectively. Antioxidant activity was highest with red light treatment, while results from white, blue, red, and red:blue (1:1) lights were not significantly different from each other. Red:blue (1:1) LED was concluded to be the optimum for *Cucumis metuliferus* plantlet growth due to good shoot growth promotion, high morphological resemblance to the mother plant, low rate of mutation, with high chlorophyll and carotenoid contents. The established *in vitro* regeneration protocol enables mass propagation of kiwano plants for local and international market demands, while providing foundation for kiwano tissue culture related research at the same time.

CHAPTER 1

INTRODUCTION

Cucumis metuliferus, commonly known as African horned melon, African cucumber, African horned cucumber, jelly melon, and kiwano, is a member of the Cucurbitaceae family of plants and originated from tropical Africa (Usman *et al*., 2015). The plant has a distinct-looking fruit that is yellow to orange in colour when ripe, possesses protruding spike-like structures, and multiple rows of juicy pulps each containing a seed within. *C. metuliferus* is a close relative of cucumbers and melons in which they may have diverged from the original ancestor species (Helm & Hemleben, 1997; Weng, 2010). These plants are natural climbers that will wrap around structures for support and grow upwards for better access to sunlight (Ferrara, 2018).

The plant is naturally used by native communities as food or medication (Usman *et al*., 2015). Together with multiple studies on various aspects including medicinal, nutrition, and effect on well-being, *C. metuliferus* has shown the potential to become a fruit of high impact and importance. Among the benefits *C. metuliferus*, many characteristics can be associated with extracts and certain aspects derived from the plant such as antitrypanosomal (Abubakar *et al*., 2011), antiviral (Anyanwu *et al*., 2017), antimicrobial (Usman *et al.*, 2014), anti-ulcer (Omale *et al*., 2011), antihyperglycemic (Jimam *et al*., 2010), and antioxidative (Sadou *et al*., 2007). Currently, the plant is part of the export commodities of a few countries (Lim, 2012).

Plant tissue culture is a reliable alternative for mass propagation of plants *in vitro* at a consistent and faster rate. Difficulties in producing mass amounts of planting material due to low germination rates can be overcome by plant tissue culture techniques. The term micropropagation is described as an application of *in vitro* methods to induce fast multiplication of a selected plant (Singh, 2015). An optimised

protocol for micropropagation would allow the production of high-quality, genetically uniform, and disease-free plant material in a relatively shorter time. As the entire process is carried out under a controlled sterile environment, external factors such as seasons and weather will no longer be factors of concern, allowing the production of new plantlets to be done at any time (Ahloowalia *et al*., 2002).

A mass propagation method allowed the establishment of new plantations and the supply of more plant material for research purposes. A small amount of plant material even if not seeds will suffice for the requirement to start a cycle of micropropagation that will eventually provide a large number of plants. This eliminated the limitation of having a new batch of plants grow to flowering, fruiting, and fruit ripening stages for a new batch of seeds to be obtained for further propagation. Under *in vitro* conditions, various factors enhancing growth can be applied and studied. Treatments can be planned and applied to manipulate conditions regarding their nutrition, lighting, and humidity, producing plantlets or plant secondary metabolites of desired quality (Dias *et al.*, 2016). Subsequently, a proper acclimatization protocol plays an important part to bring out developed plantlets to the natural environment, which requires optimisation to achieve a high rate of surviving plants (Hazarika, 2003). Plants require the natural setting as they will outgrow their containment and growing plants to large sizes *in vitro* can be inconvenient and cost-ineffective.

Light-emitting diodes (LEDs) are efficient light supply devices that convert electrical energy into electromagnetic radiation. The main benefits of LEDs usage is the low heat, high-efficiency electrical energy conversion, and adjustable specific wavelengths (Mickens *et al.*, 2019). Light plays a pivotal role in plant photosynthesis and differences in light spectra supplied can potentially bring significant impacts on plant growth. Sunlight has always been the source of light for plant survival, which

provides all light wavelengths in the visible spectrum, ultraviolet spectrum, and infrared spectrum (Kumar *et al.*, 2018). However, plants commonly require red and blue lights the most due to the absorption of chlorophylls, making red and blue lights the most important light wavelength bands for photosynthetic assimilation of CO2 in plants (Batista *et al.*, 2018). Specific red and blue wavelengths had been used to improve the yield from micropropagation plants (Hung *et al.*, 2016; Kong *et al.*, 2018). In the endeavour of improving *C. metuliferus* regeneration protocol, LEDs have been included in treatments.

Silver nanoparticles (AgNPs) are silver atoms clusters in the size range of 1 to 100 nm. Nanoparticles had seen widespread use due to their defined chemical, optical and mechanical properties (Rai *et al.*, 2009). Bionanotechnology is a new field emerging from the application of nanotechnology in biotechnological fields of study. The rise of bionanotechnology enabled the development of biosynthetic and environmentally friendly technology for the synthesis of nanomaterials (Rai *et al.*, 2009). Silver nanoparticles can be produced using a wide variety of methods, including a few examples such as substances of plant origin (Sökmen *et al.*, 2017; Tai *et al.*, 2021), laser ablation of solids in open-air conditions (LASOA) (Boutinguiza *et al.*, 2015), and recycling used batteries (Norouzi *et al.*, 2020). When applied in plant tissue culture, silver nanoparticles acted as growth promoters of plant micropropagation by increasing multiple shoots, bioactive compound production, and reducing defoliation (Mahendran *et al.*, 2019). The addition of AgNPs into MS media was shown to be able to significantly reduce internal bacterial contamination (Priya *et al.*, 2014). Through the evaluation of relevant studies, AgNPs were selected to be used in experiments to determine the effects on *C. metuliferus in vitro* cultures.

The quality of regenerated plantlets was assessed through anatomical, genetic, and biochemical means. Similarity of internal structure was assessed with histological techniques. For the determination of clonal characteristics, plant genetic stability and morphological similarity were assessed based on biochemical methods and microscopy. Such analyses provide a determinate answer to the effectiveness of clonal propagation methods used (Martins *et al*., 2004; Chandrika *et al*., 2008; Zafar *et al*., 2019). Biochemical analyses were performed on chlorophylls, carotenoids (Su *et al.*, 2014), and antioxidant activity (Shen *et al.*, 2010) of kiwano regenerated plantlets to understand the relationships between growth and various treatments applied.

Kiwano has gained attention due to its pest resistance, nutritional, pharmacological, and biotechnological uses which increased the demand for more plants. There is a potential market in Malaysia for this fruit, but efficiency in conventional farming is limited. Multiplication of this plant normally occurs through seed dispersion, but it is difficult to obtain seeds and fruits in Malaysia. *In vitro* regeneration of *C. metuliferus* is also barely studied although it can produce plants in mass quantities. Therefore, this project aims to establish a regeneration protocol for kiwano and subsequently examine differences in DNA, anatomical, and biochemical aspects of regenerated plants. Establishing an *in vitro* tissue culture protocol for kiwano plants will be able to promote future mass production and healthy consumption of this highly nutritious fruit in Malaysia. This will not only create a market for this relatively less known fruit in the country but also provide research opportunities and benefit the community of growers.

1.1 Objectives

The objectives of the present study are:

i. To establish a micropropagation protocol for *Cucumis metuliferus* E. Mey. ex Naudin using various auxins (IAA, IBA, NAA) and cytokinins (AdS, BAP, KIN, mT),

ii. To investigate the effectiveness of silver nanoparticles and LEDs for micropropagation of *Cucumis metuliferus* E. Mey. ex Naudin,

iii. To examine histological, biochemical, and genetic similarity aspects of *in vitro* regenerated plants irradiated under LEDs.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction of Cucurbitaceae family

The Cucurbitaceae family consists of tropical and subtropical species with about 1000 species in 96 genera (Chomicki *et al.*, 2019). Cucurbitaceae originated from Asia during the Late Cretaceous period. This family of plants was proposed to have went through five of its deepest evolutionary divergences in the Late Cretaceous which dates back to 70 to 80 million years ago (Schaefer and Renner, 2016). The oldest discovered fossils of the Cucurbitaceae were seeds that date back to Uppermost Paleocene and Lower Eocene London Clay (65 Ma) (Renner and Schaefer, 2016).

Plants in this family share certain similarities in sexual system traits such as inferior ovaries and parietal placentation with Begoniaceae, Datiscaceae, and Tetramelaceae (Zhang et al. 2006). When compared with other families in the order Cucurbitales, the sexual systems traced suggested that Cucurbitaceae had an ancestry of the dioecious trait (Zhang *et al.*, 2006). Certain genera of Cucurbitaceae reversed back to monoecy, illustrating sexual system flexibility in Cucurbitaceae (Schaefer & Renner, 2010a). About 50 % of their species are monoecious and 50 % dioecious (Renner & Schaefer, 2016). Flower pollen of Cucurbitaceae plants commonly has a thick layer of oily pollenkitt in yellow to orange colour covering them (Schaefer and Renner, 2010b). Pollination of Cucurbitaceae plants mainly occurs with the help of pollen-foraging bees (Schaefer and Renner, 2010b).

Cucurbitaceae was a family established by Linnaeus in 1792 (Guo *et al.*, 2020) based on floral characteristics while considering tendrils as a synapomorphic character and homologous to shoots (Guo *et al.*, 2020). Within the classification of angiosperms

families that has members producing tendrils, the Cucurbitaceae is the second largest family of the order Cucurbitales in the rosid group (Guo *et al.*, 2020). There are nearly 1000 species within Cucurbitaceae that are mainly endemic to tropical and subtropical regions, while a relatively small number of species are found in temperate areas (Guo *et al.*, 2020).

Members of the Cucurbitaceae are usually climbers covered with hair, grow lateral tendrils, and have whitish or yellow flowers (Renner & Schaefer, 2016). Annual and perennial herbs make up most of the Cucurbitaceae family and typically have relatively thin shoots and roots. In many of the perennial species of this family, tuberous roots or pachypodia are present, and their shoots are herbaceous and have an annual cycle of death and regrowth (Schaefer & Renner, 2010b). The pepo type of fruit is a distinctive character of this family, which is described as a fleshy fruit with a relatively hard shell exemplified by cucumber, pumpkin, and watermelon. Simultaneously, other types of fruits such as berry, capsule, and samara are also found among cucurbits (Guo *et al.*, 2020). Tendrils of cucurbits can be distinguished into branchless and branched forms, which either coil below the branch point or not. This trait is included in cucurbit taxonomy when classifying species (Guo *et al.*, 2020).

Out of all the species within Cucurbitaceae, 10 species were cultivated globally and 23 species were cultivated within their native range. The 10 species cultivated globally are considered major crops due to their worldwide economic importance, while the other 23 species are seen as minor crops for their local commercial needs (Chomicki *et al.*, 2019). Major crops cultivated around the world are *Cucumis sativus*, *Cucumis melo*, *Citrullus lanatus*, *Benincasa hispida*, *Cucurbita pepo*, *Lagenaria siceraria*, *Momordica charantia*, *Cucurbita argyrosperma*, *Cucurbita maxima*, and *Cucurbita moschata*. Minor crops include *Benincasa fistulosa*,

Citrullus amarus, *Citrullus colocynthis*, *Citrullus mucosospermus*, *Coccinia grandis*, *Cucumis anguria*, *Cucumis melo* subsp. *meloides*, *Cucumis metuliferus*, *Cyclanthera pedate*, *Hodgsonia macrocarpa*, *Luffa acutangular*, *Luffa aegyptiaca*, *Melothria mannii* (syn. *Cucumeropsis mannii*), *Melothria scabra*, *Momordica dioica*, *Momordica balsamina*, *Momordica cochinchinensis*, *Sicana odorifera*, *Sicyos edulis*, *Siraitia grosvenorii*, *Telfairia occidentalis*, *Telfairia pedate*, and *Tricosanthes cucumerina* (Chomicki *et al.*, 2019).

2.1.1 *Cucumis metuliferus*

Cucumis metuliferus E. Mey. ex Naudin is a plant of the family Cucurbitaceae, native to tropical Africa which includes south Sahara, Senegal, Namibia, Nigeria, South Africa, and Swaziland (Usman *et al*., 2015). Its common name includes African horned melon, African cucumber, African horned cucumber, jelly melon, and kiwano (Yagi *et al*., 2014). In Latin, the word *Cucumis* is equivalent to the common English term of cucumber and is also the name of the genus for plants related to cucumbers. The species name *metuliferus* is made up of two words from Latin, metula, meaning a small pyramid, and ferus, meaning bearing (Usman *et al*., 2015). Therefore, *Cucumis metuliferus* meant small pyramid-like protrusion bearing cucumbers if translated directly, which fits the description of the fruit's appearance. Within the genus *Cucumis*, there are two major crops: *Cucumis sativus* (cucumbers) and *Cucumis melo* (melons), and two minor crops: *Cucumis anguria* (West Indian gherkin) and *Cucumis metuliferus* (kiwano) (Usman *et al*., 2015). *Cucumis metuliferus* is grown in South Africa, Chile, California, Australia, and New Zealand. *Cucumis metuliferus* plants are annual climbing vines with a life cycle that completes in about 156 days (Liang et al., 2015).

2.1.1(a) Morphology

Morphologically, they are generally green with all plant parts being green except the flowers and fruits. The leaves are sub-pentagonal to cordate, linked to the node by a petiole, and possess short and densely packed hair-like structures. Axils of the plant, located at the nodes give rise to leaves, tendrils, and axillary buds. Flowers are yellow for both male and female flowers with their perianth also having similar morphology. Both types of flowers occur on the same plant, making *C. metuliferus* a monoecious plant (Figure 2.1). Male and female reproductive organs occur on separate flowers, differing in the morphology of the part below the flower whereby the male has a lobed receptacle while the female flower has an ellipsoid ovary covered in conical-shaped fleshy spines (Lim, 2012). Fertilization occurs mainly by selfing with the help of insects. The unripe fruit is green in colour, bright orange when ripe, and covered in sharp spikes, with a bright green, gelatinous flesh. The taste was compared to a mix of banana and cucumber. Often, it is eaten raw similar to being a snack, and may also be used in cooking (Usman *et al*., 2015).

2.1.1(b) Fruits

Cucumis metuliferus propagates naturally through seed dispersion and germination. Seed germination was found to be improved when aged for several months and was most optimal at 95-100% when the soil temperature is between 20-35°C. Germination at 12°C was delayed and completely inhibited at an even lower temperature of 8°C. When the temperature rises above 35°C, germination is greatly inhibited (Benzioni *et al*., 1991). Salinity also affects the germination rate whereby the relationship between the two factors is a longer time taken to maximum germination

of seeds when the salinity increases. It is also found that kiwano plants grow well in warmer climates as compared to colder ones (Benzioni *et al*., 1991).

Fruits take 33 days to achieve maximum fruit weight after pollination. The period of fruit ripening and changes in fruit constituent and color took place between 37 to 51 days. On day 33, the fruits were green in colour and is considered unripe (Mendlinger *et al.*, 1992). Ethylene treatment causes the unripe fruits to turn yellow which is the ripe fruits' color in 3 days. Fruits not treated with ethylene took nearly 30 more days to look nearly as ripe as those treated with ethylene (Mendlinger *et al.*, 1992). Such treatment was shown to not have any significant effect on fruit constituents studied, namely fresh fruit weight, pH, electrical conductivity, acidity, total soluble solids, reducing sugar content, and water loss after specific storage times post-harvest (Mendlinger et al., 1992). For better market value in ornamental and consumer markets, kiwano fruits need to be improved in terms of taste (Benzioni *et al*., 1995).

Cucumis metuliferus exists naturally in two forms that differ by taste primarily in the wild: the bitter and non-bitter forms. Non-bitter form of *C. metuliferus* was found to be less toxic compared to the bitter form and is widely cultivated (Usman *et al*., 2015). The fruit is occasionally eaten when food is scarce, cooked, or consumed raw. Leaves of *C. metuliferus* are cooked as spinach or as a mixture with maize meal (Usman *et al*., 2015). It is reported that the fruits and seeds of *C. metuliferus* are eaten raw as supplements by local populations of Africa (Wannang, 2011). It contains comparatively high levels of minerals especially calcium and magnesium according to a study conducted by Odhav *et al*., 2007. Game animals in the Kalahari area of South Africa eat the bitter fruits, and when food is scarce, the fruits are fed to cattle and eaten by bushmen (Burkill, 1985). Kiwano contains saponins, oily glycosides that turn

foamy when shaken in a mixture with water (Burkill, 1985). The Shona tribe in Zimbabwe utilises the root to produce a decoction that relieves pain after childbirth. Additionally, it is claimed that the boiled root is highly beneficial for the treatment of gonorrhoea (Usman *et al*., 2015).

2.1.1(c) Susceptibility and resistances

Kiwano plant is resistant to a few common pests and diseases, while susceptible to several others. Fusarium wilt is one of the common diseases occurring in nature but is resisted by kiwano (Tamilselvi *et al.*, 2016a) when tested with *Fusarium oxysporum* races 1 and 2 (Nisini *et al*., 2002; Matsumoto *et al*., 2011). In terms of viruses, Kiwano is resistant to squash mosaic virus (Provvidenti & Robinson, 1974), papaya ringspot virus, and watermelon mosaic virus 1 (Provvidenti & Gonsalves, 1982). Kiwano is also resistant to *Aphis gossypii*, a type of aphid (MacCarter & Habeck, 1974). The most well-researched resistance is the resistance to the root-knot nematode *Meloidogyne incognita.* The high resistance to this nematode sparked multiple research projects and related articles were abundant (Tamilselvi *et al.*, 2016b; Ling *et al.*, 2017; Ye *et al*., 2017; Expósito *et al*., 2018; Expósito *et al*., 2019). Due to this remarkable trait, studies to use kiwano rootstock for grafting and hybridization were also done by many over the years (Sigüenza *et al*., 2005; El-Eslamboly and Deabes, 2014; Expósito *et al*., 2018).

However, *C. metuliferus* also has several susceptibilities. Kiwano was described as one of the hosts for *Pseudoperonospora cubensis,* a type of downy mildew (Lebeda, 1992). Lebeda (1984) also mentioned susceptibility to powdery mildew (*Erysiphe cichoracearum and Sphaerotheca fuliginea*) infections on *C. metuliferus*. These two fungal diseases were common in the *Cucumis* genus according

to the studies mentioned. *Tetranychus urticae* or commonly called red spider mite appears to infest *Cucumis melo* as a finding by MacCarter and Habeck (1974). This phenomenon may be co-related with cucumber beetles feeding on cucurbitacin (Chambliss and Jones, 1966). The resistance to one type of insect may mean that a plant will be susceptibility to other types of pests (Da Costa and Jones, 1971). *Cucumis metuliferus* did not face cucumber beetle infestation when maintained in Universiti Sains Malaysia but were susceptible to spider mites.

Through analysis using simple sequence repeats to evaluate 36 *C. metuliferus* accessions, genetic variation between various accessions was concluded to be low (Weng, 2010). Exhibition and expression of genes may have shown a high degree of diversity such as in the resistance to fusarium wilt (Liu *et al*., 2015), resistance to papaya ringspot virus, and resistance to root-knot nematodes. Genetic variation may be low, but the minor variations still conferred varying levels of resistance and susceptibilities to pests and diseases (Weng, 2010). Therefore, the source of *C. metuliferus* planting materials plays an important role in negating the detrimental effects of pathogens in the effort to maximize yield.

2.1.1(d) Phylogenetic relationships

Genetic studies are important in providing a deeper understanding of phenomena and traits of organisms which allows the determination of phylogenetic relationships between species. They provide approximate values for the estimation of cellular functions, defense mechanisms, and various other aspects regarding an organism of the same category. These relationships allow estimation of similarity between species which is pivotal for researchers when experimenting on a new species

by referring to neighboring species. A good way to determine the phylogenetic relationship between species is through the identification of satellite DNA.

Satellite DNA is a type of highly repetitive non-coding DNA which is widely used in various genetic analyses. The satellite DNA of *C. metuliferus* constituted about 4.96% of its total nuclear DNA with 48.06% of guanine and cytosine content (Ramachandran and Narayan, 1990). Satellite DNA contributes to a high degree in phylogenetic relationship discovery, whereby *C. metuliferus* was shown to be close to other species in the *Cucumis* genus but is distant enough to be unique proposing an ancestral species that gave rise to the members of *Cucumis* genus. *Cucumis metuliferus* is said to be a separate species in the *Cucumis* genus as the *C. metuliferus* knob satellite DNA was found to be absent in nuclear genomes of *Cucumis melo*, *Cucumis anguria* and *Cucumis sativus* (Ramachandran and Narayan, 1990). Staub *et al*. (1997) reported a distant genetic relationship in *C. metuliferus* from cucumber and *Cucumis melo* through analysis with isozyme and random amplified polymorphic DNA (RAPD) lending support to explaining the dismal success rates of hybridizing *C. metuliferus* and other members of the Cucumis genus (Deakin *et al.,* 1971; Kho *et al.,* 1980; Beharav and Cohen, 1994b). The genetic distance between *C. metuliferus* and *Cucumis melo* however, is smaller than between *C. metuliferus* and cucumber (Weng, 2010). Although genetically distant, there exists a high degree of DNA sequence homology between *C. metuliferus* and cucumber (Weng, 2010).

However, the satellite pMetSat shares short stretches of similarity with other cultivated species in *Cucumis* genus, in particular types of cucumber and *Cucumis melo*. It suggested that a pattern of satellite distribution resembles the Cucumis taxonomic classification, and a set of different but related repeats should have existed in an ancestral species prior to speciation and cultivation (Helm and Hemleben, 1997).

Repetitive DNA is mainly positioned in the subtelomeric regions in cucumber, *Cucumis hystrix* and *C. metuliferus*. At the same time, for *Cucumis melo* and *Cucumis anguria* most of them were placed in the pericentromeric heterochromatin regions. Therefore, it is a safe estimate that the main reason for the divergence of *Cucumis* species from a common ancestor was the evolution of such repetitive sequences in their DNA (Zhang *et al*., 2015). The *Cucumis* genus likely has a common ancestor but branched out at a point in time with varying extents and paths of evolution (Levi *et al*., 2005).

2.2 Pharmacological and economic significance of *Cucumis metuliferus*

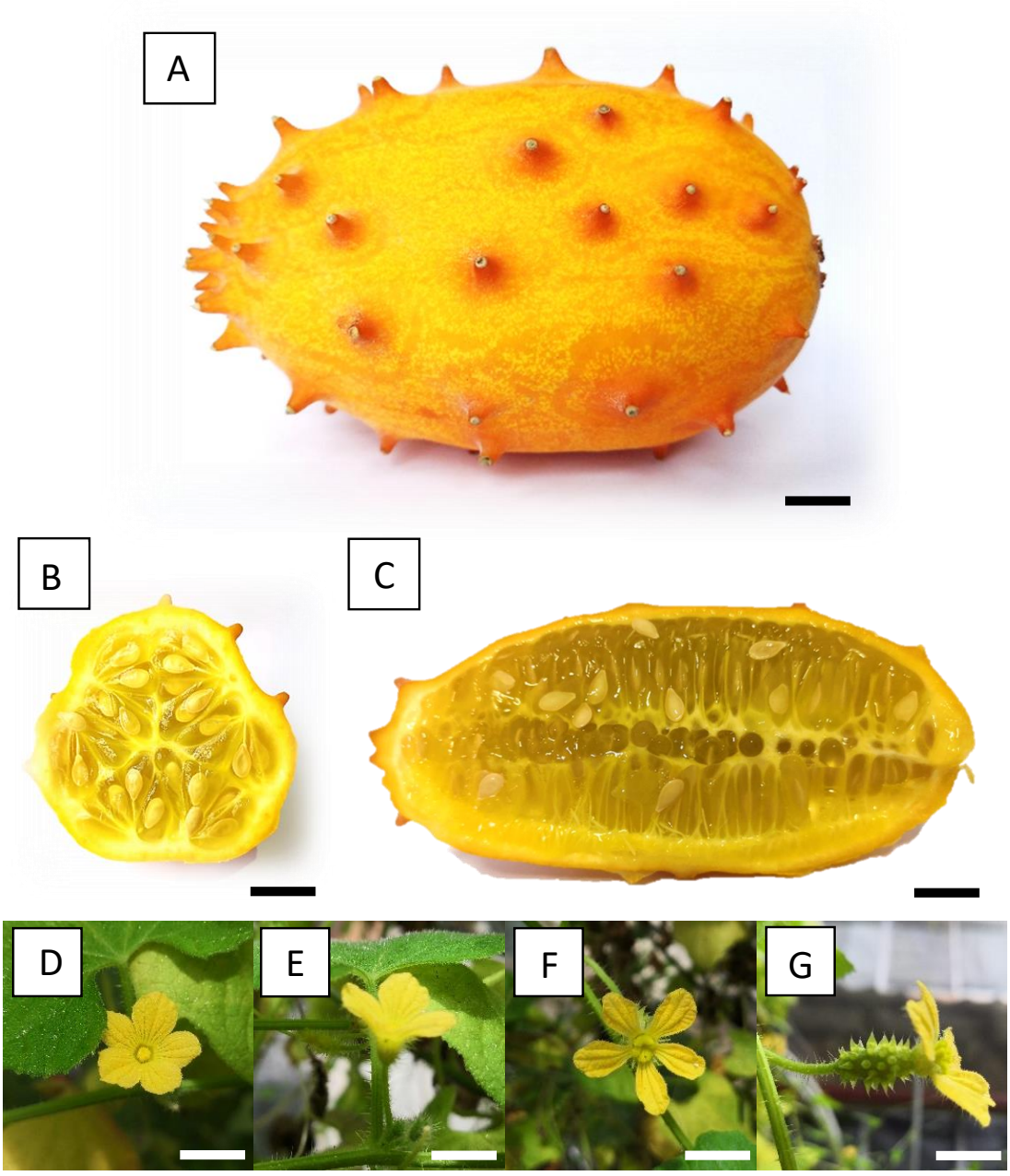

Figure 2.1: **The morphology and anatomy of a kiwano fruit, and flower morphology of *Cucumis metuliferus* plant flowers. (A)** Ripe appearance of fruit **(B)** Latitudinal cross-section of fruit **(C)** Longitudinal cross-section of fruit **(D)** Top view of male flower **(E)** Side view of male flower **(F)** Top view of female flower **(G)** Side view of female flower. (Scale bar = 1cm).

Cucumis metuliferus has a wide range of uses and useful properties. Its fruit pulp extract can be used in haematinics, capable of increasing the production of haemoglobin and red blood cell counts (Usman *et al*., 2018). Through investigation of antitrypanosomal efficacy of fruit pulp extract by using rabbits as test subjects, it was concluded that *C. metuliferus* has antitrypanosomal properties when administered at 500 to 1000 mg/kg body weight (Abubakar *et al*., 2011). At the same time, it promotes weight gain, reduces anaemia, and controls hepatomegaly and splenomegaly in *T. brucei brucei* infection (Abubakar *et al*., 2011). Antiviral effects of *C. metuliferus* on specific viruses were also proven whereby alkaloids extracted from the fruit pulp successfully inhibited replication of Infectious Bursal Disease Virus (IBDV) (Anyanwu *et al*., 2017), and may have certain activities against hepatitis B virus (HBV) (Anyanwu *et al*., 2015). A study by Wannang *et al*. (2010) showed that an alkaloid extract of 600 mg/kg from kiwano reversed haemorrhagic lesions brought about by Newcastle disease virus. On a related note, alkaloids of *C. metuliferus* isolated from the fruit pulp were tested on adult albino rats and results showed suppressed carbon tetrachloride, gentamicin-induced hepatic and nephrotic injury (Anyanwu *et al*., 2014).

According to a study experimenting on antimicrobial effects of *C. metuliferus* fruit extract, it was shown to possess antibacterial activity against *Salmonella gallinarum* possibly due to the various phytochemicals present in the methanolic extract (Usman *et al.*, 2014). Organic leaf extracts of *C. metuliferus* of dosage 1500 mg/kg had a chemo suppression of 98.55%, which was considered high for organic extracts as it is equivalent to 100% chemo suppression of chloroquine. As such, the results suggest a possibility that antimalarial phytochemicals exist in the leaves of the plants, which virtually explains the role of an antimalarial herbal remedy in the Ugweno community (Mzena *et al*., 2018)**.** Anti-ulcer properties of *C. metuliferus* were

also explored whereby mice gastric lesions were induced with ethanol and treated with alkaloids isolated from the pulp of the fruit. Results yielded have shown a significant decrease in haemorrhages and ulcer symptoms of the gastric mucous membrane layer when 500 and 1000 mg/kg of *C. metuliferus* alkaloids were administered to the mice. Hence, said fruit possesses anti-ulcer properties and protective properties to the gastric mucous membrane (Omale *et al*., 2011). Anti-diabetic effects were also reported as glycosides extracted from *C. metuliferus* fruit pulp exhibited antihyperglycemic activities at different doses against alloxan-induced diabetes mellitus in albino rats (Jimam *et al*., 2010; Gotep, 2011; Sharma & Arya, 2011).

Cucumis metuliferus shows positive effects on male reproductive abilities. Continuous oral administration of fruit extract at 500 mg/kg and 1000 mg/kg on albino rats showed an absence of damage on the sertolli/leydig cells after 28 days. However, as 500 mg/kg of the fruit extract produced an increase, 1000 mg/kg decreased both the viable and total sperm counts. This shows dosage of administration is vital for desired positive effects (Wannang *et al*., 2008). *Cucumis metuliferus* fruits have good levels of vitamin C, iron, and potassium, and some amounts of phosphorus, magnesium, zinc, calcium, copper, and sodium. The fruit pulp contains beta carotene and vitamin A (Usman *et al*., 2015). Beta carotene is important to strengthen the immune system, while vitamin A is beneficial for the eyes and required for proper night vision and healthy skin. Diets with sufficient beta carotene, lutein, and lycopene are alleged to aid in slow aging and may also protect and repair DNA (Usman *et al*., 2015). Seeds of *C. metuliferus* contain oleic and linoleic acids (Sadou *et al*., 2007). Oleic acid lowers blood lipids mainly cholesterol, LDL-cholesterol, and triglycerides (Lopez-Huertas, 2010). Linoleic acid is an essential omega-fatty acid for humans (Usman *et al*., 2015). Also, two antioxidants were found in the seeds at higher levels: γ-tocopherol and α-

tocopherol (Sadou *et al*., 2007). Both types of tocopherols are organic types of vitamin E and are antioxidants, which have many health benefits to the body (Packer & Landvik, 1989). Vitamin E functions by neutralizing damage from free radicals which can cause cancer and cardiovascular disease (Rimm *et al*., 1993; Stampfer *et al*., 1993). It has been suggested that Vitamin E may help reduce the risk of Parkinson's (Etminan *et al*., 2005).

In terms of planting in the field, *C. metuliferus* has considerably stronger resistance to nematodes compared to other members of the Cucurbitaceae family. Ling *et al*. (2017) conducted tests and obtained results indicating lesser growth of *Meloidogyne incognita* in *C. metuliferus* than in *Cucumis sativus* (cucumber) of line 9930 and concluded that various genes were differentially expressed in response to infection, and cytoskeleton-related genes are key regulators of said resistance**.** In another related study, it was claimed that the expression of genes results in a series of hardening and repellent substance production processes. This in turn reduced nematode penetration, hindered their growth, and prevented the plant part's hypersensitive necrosis (Ye *et al*., 2017).

2.3 Tissue culture of *Cucumis metuliferus*

Regeneration is an ability of many living organisms with varying levels of organ and tissue regrowth capacity for every organism (Pulianmackal *et al*., 2014). Plants, in particular, have a high capacity for such regrowth, which made it possible for humans to perform cutting, grafting, and clonal propagation (Melnyk and Meyerowitz, 2015). Such regeneration capability of plants is named totipotency, a unique regeneration capability plants possess that can regrow an entire plant from as little material as a single cell (Ikeuchi *et al*., 2016).

Tissue culture was a concept proposed with attempts to regenerate entire plants with tissue fragments or even single cells *in vitro* by Haberlandt (1902). A breakthrough was achieved by Skoog (1957) with the discovery that exogenously applied auxin and cytokinin ratio can determine the regeneration of root or shoot. A higher auxin to cytokinin ratio promotes root regeneration and a higher cytokinin to auxin ratio promotes shoot growth (Ikeuchi *et al.*, 2016).

The capacity of plants to regenerate entire plants or totipotency was demonstrated by Steward et al. (1958) with single cells from carrot vascular phloem cells, highlighting the fact that plant somatic cells retain high regenerative potential. Both in nature and under *in vitro* conditions, plants commonly regenerate through *de novo* organogenesis where cuttings and explants alike form apical meristems in abnormal places followed by the development of shoots and roots. Meristems are actively dividing groups of cells that provide the potential to differentiate into different cell types required for every part of the plant (Ikeuchi *et al.*, 2016).

There are a few types of different methods to regenerate plants. Organogenesis, somatic embryogenesis, and synthetic seeds were among the available methods, with the first two being far more efficient. Organogenesis can stem from various plant parts under *in vitro* conditions. Direct organogenesis is favored, as an unstable multiplication of cells which results in callus gives rise to the possibility of genetic variation. Therefore, structuring a micropropagation or regeneration process should try to avoid indirect organogenesis (Gaba, 2005).

Meristem culture is a type of culture method that uses only the apical meristem with one to two leaf primordia. Shoot tips contain the said meristem and are generally used at 1cm in length after excision to eliminate viral contamination. Cultures of plant material containing small apical meristems provide two pivotal values highly sought

after by tissue culturists, which are viral exclusion and genetic stability (Bhatia *et al*., 2015). Viral exclusion is made possible due to the nature of actively dividing cells in the meristem which outcompetes the virus in using materials for replication (Taşkın *et al*., 2013). Other available research state RNA dependent RNA polymerases delay and prevent viral systemic spread in meristem tissues (Schwach *et al*., 2005; Di Serio *et al*., 2010).

The growing points of plants can be used as culture material which often means apical and axillary buds. These can be cultured in a nutrient medium and growth continues as if they were allowed to normally grow on the donor plant. Shoot initials will grow from buds and develop into plantlets, which can be rooted under the right conditions. Node culture is an adaptation of shoot culture, and they essentially run on the same principles as apical and axillary bud cultures, with the same method of culture establishment. Both plant parts when cultured can give rise to uniform plant appearances and development is relatively free from chromosomal changes and irregularities (Bhatia *et al*., 2015). Nodal segments are more abundant compared to shoot tips on donor plants and would be a better material for tests.

Past regeneration research done on *C. metuliferus* has been using seeds as a starting material, with eventual callus formed or plantlets being excised and tested with various treatments (Punja *et al*., 1990; Raharjo & Punja, 1993; Beharav & Cohen, 1994a; Adelberg, 1998; Lin *et al*., 2011). Transformation of *C. metuliferus* was also attempted with much success (Lin *et al*., 2011). However, regeneration was not tested with different plant growth regulators to achieve a more optimized regeneration rate. The common plant growth regulator used was BAP for shoot regeneration (Raharjo & Punja, 1993; Adelberg, 1998; Lin *et al*., 2011).

2.3.1 Surface sterilisation

The introduction of outside plant material into *in vitro* conditions requires certain steps to be taken. Contaminants are everywhere in the natural surroundings, with obvious examples being spores and dust. Plant parts are no exception to this, carrying a wide range of contaminants on their surfaces. To avoid contamination, the desired plant tissue has to undergo sterilization of its outer surface in a series of steps before inoculation on a nutrient medium (Bhojwani & Razdan, 1986). Generally, the plant material will first be cleaned with running tap water to remove dirt and larger contaminants (Ahloowalia *et al*., 2002). Subsequently, surface sterilization takes place in a laminar flow hood with the plant material being treated with ethyl and isopropyl alcohol, followed by the required amount of sodium hypochlorite solutions (Bhojwani & Razdan, 1986) or mercuric chloride with other substances if needed (Daud *et al*., 2012). After surface sterilization, rinsing of plant material is done a few times to clear any residual sterilizing agent and dried before excision of desired plant material length for culture on plant growth medium (Bhojwani & Razdan, 1986).

Surface sterilization is a step that requires optimization due to several factors that are of concern. Plant material type, species, surface structure, and environmental cleanliness are among the different possible factors that can affect the sterility from contaminants of explants before introduction into *in vitro* environments. Optimizations can be done by altering the duration and concentration of sterilizing agents until desirable axenic explants are obtained (Hermayani *et al*., 2017).

Regarding surface sterilization on *C. metuliferus*, the only reported surface sterilization protocols involve the surface sterilization of seeds. A common trend in all the protocols was the usage of sodium hypochlorite as a sterilizing agent whether alone or with prior treatment of ethanol for a brief period (Punja *et al*., 1990; Raharjo &

Punja, 1993; Beharav & Cohen, 1994a; Lin *et al*., 2011). In comparison to surface sterilization of other plant parts such as nodal and apical buds, surface sterilization of seeds appeared to require less prior cleaning with tap water (Lin *et al*., 2011). Adelberg (1998) used a different method of surface sterilizing by sterilizing the entire *C. metuliferus* fruit before opening the fruit for its seeds under sterile conditions.

2.3.2 Plant culture medium

Explants require a suitable medium after culture for growth to occur. Various plant culture mediums were available with examples such as MS (Murashige and Skoog, 1962), WPM (Lloyd & McCown, 1980), DPD (Durand *et al*., 1973), and B5 (Gamborg *et al*., 1968). Among them, MS medium is arguably the most commonly used formulation, which was originally derived by Toshio Murashige and Folke Skoog in 1962. The medium contains inorganic and organic components in a generally balanced manner for plants to obtain macronutrients, micronutrients, and vitamins.

Their findings showed that nitrogen, phosphorus, potassium, calcium, magnesium, and sulphur affect the growth of plants significantly when the concentrations of each nutrient were altered individually and were defined as macronutrients. Boron, manganese, iron, zinc, copper, cobalt, molybdenum, iodine, and sodium were micronutrients with less effect on plant growth but essential (Murashige and Skoog, 1962). Vitamins are supplied to the medium in relatively minute amounts. Other than that, sucrose is added at 3% (w/v) normally and the entire mixture of macronutrients, micronutrients, vitamins, sucrose, and water were solidified in an agar-like state with a gelling agent. Gelrite has been demonstrated to have better gel strength and clarity compared to agar powder as a gelling agent (Harris, 1985). The pH value is usually maintained between 5.7-5.8 before autoclaving. It

should also be of important note that the basic Murashige and Skoog medium can be altered to suit the specific needs of a plant. Modifications to concentrations of any substance can be made in the process of finding the optimum medium condition for the culture of the desired plant material (Punja *et al*., 1990; Beharav & Cohen, 1994a). Other additions that are commonly added to plant growth medium to influence plant growth *in vitro* are plant growth regulators.

Out of all possible plant growth medium formulations, MS medium was used on *C. metuliferus* explants. All past research on this plant used MS as the base medium with various modifications (Punja *et al*., 1990; Raharjo & Punja, 1993; Beharav & Cohen, 1994a; Lin *et al*., 2011). Any component of the formulation can be altered, with one such study changing the entire vitamin content from regular MS vitamin dosage into B5 vitamin dosage (Lin *et al*., 2011)

2.3.3 Plant growth regulators

Plant growth regulators as their name suggests influence growth and development in plants, affecting stages from germination to reproduction (Gaba, 2005). Also known commonly as a plant hormone, they function by producing chemical signals that trigger transportation to target parts and subsequently induce their intended effects on cells and tissues with specific binding sites that they target (Ferguson & Grafton-Cardwell, 2014). They generally regulate plant growth and development by bringing changes to cell division, cell expansion, and cell differentiation (Coggins & Lovatt, 2014). At minute concentrations, the hormone can still produce effects and is already sufficient in most cases (Ferguson & Grafton-Cardwell, 2014). For certain hormones, the dose of supplemented hormones has to be regulated as they cause different effects based on the amount supplied. The responses to plant growth regulator

concentrations usually depict a hyperbola when displayed on a graph, with the best response being at the top of the curve (Gaba, 2005). Plants respond to plant growth regulators based on several factors, which include a relative concentration of plant growth regulator added, health condition of a plant, nutritional status, water status, and also climate provided to the plant. Similarly, every plant growth regulator produces effects depending on the concentration applied, the type of cells targeted in tissues, and the phase of development the tissue is undergoing where the plant growth regulator is applied (Coggins & Lovatt, 2014).

According to some studies, plant growth regulators are also synthetic replacements of naturally available plant hormones that are being applied exogenously to simulate hormonal effects (Jiménez, 2005; Coggins & Lovatt, 2014). This differentiation was done to separate terms for hormones between naturally occurring and synthetic ones, which made sure they are known as plant hormone and plant growth regulators respectively. Nonetheless, they produce similar effects on plant growth and development.

Plant growth regulators bring about effects individually but can also be combined with other plant growth regulators to bring about compound effects that enhance the intended effect (Huetteman & Preece, 1993; Jiménez, 2005). As an example, a combination of cytokinins, thidiazuron (TDZ) and 6-Benzylaminopurine (BAP) can produce additive effects boosting plant growth significantly (Ramakrishnan, 2014).

Plants react differently towards plant growth regulators depending on species, whereby one plant growth regulator that is known to work well with a specific species may bring about effects that differ drastically when applied to another species. Specificity of hormonal action can occur where contrary to common trends, kinetin,

benzylaminopurine, and zeatin were not effective in inducing proliferation of quality shoots in *Browallia viscosa* but 6-(γ,γ-Dimethylallylamino)purine was effective. Likewise, in *Gerbera* kinetin was able to induce quality shoots, but faster propagation was achieved by BAP (Gaba, 2005).

Cytokinins are known to promote the growth of shoots and at the same time inhibit the formation of roots, meaning that the following step after shoot induction will be root induction (Gaba, 2005). Auxins in general are used for root formation, and auxin alone is usually sufficient to induce roots in explants. The concentration and type of auxin can produce effects that are different based on the plant they are used on. It is to be taken note, that the concentration used should not be at a level that induces callus formation instead of root formation and this will take optimization tests to find out. Sometimes tissue and organs can grow without auxins and this phenomenon is called auxin autonomy (George *et al*., 2008). Auxins used in plant tissue culture are usually synthetically produced with indole-3-acetic acid (IAA) and indole-3-butyric acid being naturally occurring hormones with synthetic options, and 2,4-dichlorophenoxyacetic acid (2,4-D), 1-naphthaleneacetic acid (NAA), dicamba (3,6-dichloro-*o*-anisic acid), picloram (4-amino-3,5,6-trichloropyridine-2-carboxylic acid) being synthetic auxins. 2,4-D is often used in callus induction, NAA is applied when inducing organogenesis, dicamba and picloram are effective in embryogenesis (Gaspar *et al*., 1996). Regeneration by using BAP for shoot proliferation with subsequent rooting by adding IAA was able to give rise to viable plantlets (Kumar *et al*., 2014b; Kumari & Kumar, 2016; Sereda *et al*., 2017).

Among the cytokinins available, BAP is widely used in experiments involving *C. metuliferus*, and the results of its application appeared to be effective (Adelberg, 1998; Lin *et al*., 2011). Plant growth regulators were added in pairs most of the time,