MICROPROPAGATION AND THE INCORPORATION OF CYANOBACTERIAL EXTRACTS ON THE REGENERATION OF *Ficus carica* CV. GOLDEN ORPHAN

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by

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LIST OF ABBREVIATIONS

%	Percentage
$\bar{x}$	Mean
µM	Micromolar
2-iP	6-(γ,γ-Dimethylallylamino) purine
ABA	Abscisic acid
ATP	Adenosine triphosphate
ANOVA	Analysis of variance
BAP	6-Benzylaminopurine
BRs	Brassinosteroid
℃	Degree Celcius
DNA	Deoxyribonucleic acid
et al.	Et alia
g	Gram
GAs	Gibberellic acid
HCl	Hydrochloric acid
H_2O_2	Hydrogen peroxide
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
JA	Jasmonic acid
K	Kelvin
kPa	Kilopascal
lm	Lumen
M	Metre

Mg	Milligram
mg/L	Milligram per litre
mL	Millilitre
Mm	Millimetre
MS	Murashige and Skoog
NAA	1-Naphthaleneacetic acid
NaOCl	Sodium hypochlorite
NaOH	Sodium Hydroxide
PCR	Polymerase Chain Reaction
PGR	Plant Growth Regulators
s. e	Standard error
TDZ	Thidiazuron
W	Watt

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MICROPERAMBATAN DAN PENGGABUNGAN EKSTRAK SIANOBAKTERIA DALAM REGENERASI *Ficus carica* CV. GOLDEN ORPHAN

ABSTRAK

Ficus carica L. (Moraceae) merupakan buah yang dipenuhi dengan serat dan vitamin semulajadi. Buah tin kuning dari kultivar 'Golden Orphan' adalah manis dan pejal, sesuai dikomersialkan. Namun, penanaman buah tin secara konvensional di Malaysia kurang berkesan justeru menghalang penubuhan ladang komersial. Penggabungan ekstrak sianobakteria dalam kultur tisu pokok tin masih belum dikaji, apatah lagi dengan strain bukan toksik genus *Nostoc* dan *Anabaena*. Penyelidikan ini bertujuan meneroka aspek propagasi *in vitro* kultivar Golden Orphan menggunakan pengawalatur pertumbuhan tumbuhan dan gabungan ekstrak sianobakteria yang berbeza. Eksplan nodul tunas *in vitro* diinokulasi dalam media MS ditambah dengan 1 mg/L BAP dan pucuk yang terhasil dikultur dalam media MS ditambah dengan sitokinin (BAP, Kinetin, Thidiazuron dan Zeatin) dan auksin (NAA, IAA dan IBA) bagi menentukan kesan gabungan terhadap penggandaan pucuk. Pucuk yang terhasil juga dikaji dengan tiga strain sianobakteria, iaitu *Nostoc* sp. Penang Hill (strain 1), *Nostoc* Antarctic (strain 2) dan *Anabaena* sp. Penang Hill (strain 3) bagi menentukan kesan tindak balas morfogenik eksplan. Pucuk yang terhasil dipindahkan ke media MS ditambah dengan kepekatan auksin (NAA, IAA dan IBA) yang berlainan untuk induksi akar dan diaklimatisasi. DNA daun dari kitaran subkultur yang berbeza (S_1-S_6) dan pokok induk diekstrak dan di analisis menggunakan penanda SCoT dan DAMD. Bilangan pucuk tertinggi (4.15±0.43) diperhatikan dalam rawatan 0.8 mg/L BAP, manakala ketinggian pucuk tertinggi (2.05±0.20 cm) diperhatikan dalam 0.2 mg/L

Zeatin. Penambahan 0.8 mg/L BAP dan 0.5 mg/L IAA dalam media MS telah menghasilkan ketinggian pucuk maksimum (2.23±0.09 cm), berbanding dengan kombinasi 0.8 mg/L BAP dan 2.5 mg/L IBA dalam media MS tidak sesuai kerana morfologi eksplan yang terhasil adalah pertumbuhan kalus dan nekrosis hujung pucuk yang tinggi. Ekstrak ekstrasel *Nostoc* sp. Penang Hill menghasilkan bilangan akar maksimum (6.02±0.50). Manakala, ekstrak intrasel strain 2 pada 0.5% menghasilkan jumlah akar tertinggi (7.93±0.99), 6.0% ekstrak menghasilkan ketinggian pucuk maksimum (3.28±0.64 cm), dan pemanjangan akar maksimum (3.51±0.45 cm) apabila 2.0% ekstrak digunakan. Kemampuan biosintesis fitohormon dalam strain sianobakteria ini ternyata berbanding dengan fitohormon kimia yang boleh menyebabkan kesan toksik pada sel tumbuhan. Manakala, penghasilan akar optima (83.33%) diperhatikan dalam penambahan 0.4 mg/L IBA dalam media MS dan aklimatisasi dalam media sabut kelapa menghasilkan kadar kemandirian maksima. Jalur monomorfik hasil analisis molekular Start Codon Targeted (SCoT) dan Directed Amplification of Minisatellite-region DNA (DAMD) menunjukkan anak pokok hasil mikroperambatan menyerupai pokok induk sepanjang enam kitaran subkultur. Kajian ini membuktikan bahawa propagasi *Ficus carica* cv. Golden Orphan secara *in vitro* berjaya dicapai bagi penghasilan stok tumbuhan berkualiti tinggi yang sesuai untuk pengkomersialan dan penanaman di Malaysia.

MICROPROPAGATION AND THE INCORPORATION OF CYANOBACTERIAL EXTRACTS ON THE REGENERATION OF *Ficus carica* CV. GOLDEN ORPHAN

ABSTRACT

Ficus carica L. (Moraceae) is a nutritious fruit rich in natural fibre and vitamins. The yellow fruited Golden Orphan cultivar is sweet and firm, suitable for commercialisation in Malaysia. However, conventional methods for plant propagation are less efficient, impeding commercial farming in Malaysia. The incorporation of cyanobacteria extracts in fig micropropagation has not been explored, yet alone with the nontoxic strains belonging from the genus *Nostoc* and *Anabaena*. This research aims to establish methods for the micropropagation of Golden Orphan using different plant growth regulators and cyanobacteria extracts. *In vitro* nodal bud explants were established in MS media with 1 mg/L BAP and induced shoots were treated with different concentrations and combinations of cytokinin (BAP, Kinetin, Thidiazuron and Zeatin) and auxins (NAA, IAA and IBA) for shoot multiplication. The induced shoots were also tested with different cyanobacteria strains namely *Nostoc* sp. Penang Hill (strain 1), *Nostoc* Antarctic (strain 2) and *Anabaena* sp. Penang Hill (strain 3) to evaluate explant morphogenic responses. Regenerated shoots were rooted in MS medium supplemented with auxins (NAA, IAA and IBA) and were acclimatised. Leaf DNA extraction from different subculture cycles (S_1-S_6) were subjected to Start Codon Targeted (SCoT) and Directed Amplification of Minisatellite-region DNA (DAMD) marker analysis. The maximum number of shoots (4.15±0.43 shoots) was observed in 0.8 mg/L BAP, and optimum shoot height (2.05±0.20 cm) was observed in 0.2 mg/L Zeatin. MS media supplemented with 0.8 mg/L BAP and 0.5 mg/L IAA produced

maximum shoot height (2.23±0.09 cm) whereas, auxins (NAA, IAA and IBA) combined with 0.8mg/L BAP resulted in morphological deformation such as callus induction and shoot tip necrosis. Moreover, the extracellular extract of *Nostoc* sp. Penang Hill generated 6.02±0.50 roots. Whereas, intracellular extract of strain 2 at 0.5% produced maximum root number (7.93±0.99), 6.0% extract produced maximum shoot height (3.28±0.64 cm), and 2.0% extract produced optimal root elongation at 3.51±0.45 cm. In this study, the incorporation of cyanobacteria exemplifies the possible phytohormone biosynthesis ability of these cyanobacterial strains when compared to traditional phytohormones which are hazardous to plant cells. Optimal rooting (83.33%) was achieved in MS medium with 0.4 mg/L IBA, while maximum survival was attained in cocopeat substrate medium. The monomorphic bands from SCoT and DAMD molecular analysis reveals true-to-type micropropagated plants throughout six subculture cycles. This research posits that rapid *in vitro* regeneration of *Ficus carica* cv. Golden Orphan can successfully provide high-quality plant stocks suitable for commercialization and cultivation in Malaysia.

CHAPTER 1
INTRODUCTION

1.1 INTRODUCTION

Ficus carica L. (Fig) is a vital deciduous plant of the genus *Ficus* which belongs to the Moraceae family. With grey and smooth bark, palmate leaves, and fibrous roots, it is a deciduous tree or shrub with a distinctive appearance. Fig cultivars yield fruits with a variety of characteristics, notably due to its diversity in terms of skin colour, size, flavour, and texture. Figs are geographically diverse as the plant favours suitability in catering to varying climacteric and geographical conditions (Hiwale, 2015). Figs were reportedly cultivated in the eastern Mediterranean area between 2000 and 3000 BC (Marpudi *et al*., 2013). Figs are a significant international crop for its dry and fresh consumption criteria either in dried, canned or varied preservation methods. The fig is a powerhouse of nutrients, amino acids, and antioxidants and it is a wholesome fruit high in fibre, potassium, calcium, and iron in comparison to apples, grapes, and strawberries (Lakshmi *et al*., 2018). Different parts of the fig plant comprise of a myriad of bioactive constituents that can be extracted from different portions of the plant, including phenolic compounds, organic acids, phytosterols, anthocyanins, triterpenoids, coumarins, and volatile compounds, such as hydrocarbons and aliphatic alcohols (Oliveira *et al*., 2009). The leaves and latex of *F. carica* have been utilised as an alternative medicinal treatment approach in a variety of health circumstances, including calluses, warts, gum wounds, cataract therapy, bee stings, and wound healing (Hashemi *et al*., 2011). Ficin, a protein hydrolysing enzyme, is found in the secret milky white latex of *F. carica*, which grows 15–20 feet tall with numerous branches (Badgujar *et al*., 2014). Emollient, laxative, aphrodisiac, cough

suppressor, haemorrhoid suppressant, antiulcer, and hypercholesterolemia constitute some of the traditional uses of *F. carica* (Lalitha *et al*., 2021). In Indian medicine, the fruits of *F. carica* have been employed as a mild laxative, expectorant, and diuretic whereas fresh figs are used to treat skin abnormalities in both contemporary and traditional medicine (Solomon *et al*., 2006).

Countries in the Mediterranean region contribute the vast majority of the world's fig production, dominated by Turkey, Egypt, Algeria, and Morocco accounting for more than 65% of total output. Of these countries, Turkey is the world's biggest exporter of both fresh and dried figs, accounting for 51% of total global fig fruit exports (Allegra *et al*., 2018). Fresh figs are currently imported at a premium cost and are not cultivated commercially in Malaysia for domestic purposes. The use of seedlings is not a feasible method due to the distinctive variations diverging from mother plants, hence propagation techniques such as cuttings, grafting, and air layering are implemented (Ling *et al*., 2018). Apart from that, the success of propagation via stem cuttings or air layering is dependent on factors such as the age and diameter of cuttings, growth media used, application of rooting hormones, and environmental factors (Swarts *et al*., 2018). Hence, these conventional methods do not provide constant growth and development of plants, which results in variable fruit output, particularly at the commercial level for a bigger size market. Moreover, the current fig propagation in Malaysia still relies in conventional methods that are less efficient and is therefore not suitable for the production of plant stocks catered for commercial planting (Isa *et al*., 2020). Plant tissue culture technique allows an incessant, sustainable, affordable, and viable generation of plant stocks that are identical to the mother plant, irrespective of climatic or geographical conditions. Minute pieces of plant tissue (explants) from the mother plant can be employed in a continuous process to generate hundreds and thousands of

plantlets in a short period of time (Chun *et al*., 2020). In plant tissue culture, the manipulation of selected plant tissues in the induction of organogenesis can be attributed to the supplementation of plant growth regulators which primarily consist of various auxins and cytokinins. Although previous literature has garnered sufficient cultivar-based optimisation of shooting and rooting parameters desirable for large-scale *in vitro* propagation, the unsuitability of these optimisations in micropropagation of the Golden Orphan cultivar should be noted as the response towards exogenous phytohormones are cultivar specific. Thus, incorporating plant growth regulators in the culture medium will improve the shoot and root growth of *in vitro F. carica* cv. Golden Orphan explants.

Cyanobacteria, the first group of photosynthetic gram-negative prokaryotes to evolve oxygen, are unique in the microbial world and is well-known for its symbiosis with taxonomically diverse host in terrestrial, freshwater and marine environments (Kumar *et al*., 2019). Cyanobacteria influence their hosts' biology and evolution by offering a variety of advantages namely including carbon, nitrogen fixation, UV protection, and defensive toxins (Usher *et al*., 2007; Sánchez-Baracaldo *et al*., 2022). Hence, studies related to cyanobacteria and their applicability in the fields of biotechnology, medicine, sustainable management and agriculture are well established (Zahra *et al.*, 2020). Specifically, cyanobacterial incorporation into various facets of agriculture such as biofertilizers in rice cultivation and as soil remediators in deteriorated areas have been acknowledged, analysed and reviewed (Zahra *et al.*, 2020). Additionally, the incorporation of cyanobacteria in amended composts has been reported to have facilitated crop growth, yield and quality as well as an increase in soil organic carbon, nitrogen fixation, phosphorus and zinc content (Prasanna *et al*., 2013). However, despite the elaborated incorporation of the group into various fields as

outlined previously, only a few reports have been published with regards to the applicability of cyanobacterial extracts on the *in vitro* morphogenic responses of plants (Molnár and Ördög, 2005; Hussain and Hasnain, 2012; Gurusaravanan *et al*., 2013; Ibrahim *et al*., 2018) despite the conformation on the presence of compounds rendering plant growth regulating properties such as auxin- and cytokinin-like substances (Seyed Hashtroudi *et al*., 2011; Žižková *et al*., 2017). Their auxin-like properties which resulted in *in vitro* rooting have been observed by previous literatures (Shanab *et al*., 2003; Banerjee and Modi, 2010) but studies on the utilization of cyanobacterial extracts in promoting *in vitro* shoot activities have been limited. Therefore, the addition of cyanobacterial extracts in the culture medium as compared to culture medium only is more likely to improve shoot and root growth of *in vitro F. carica* cv. Golden Orphan explants.

Hence, the current study aims to evaluate the efficiency of various plant growth regulators at varying concentrations and the effects of cyanobacterial extracts in the regeneration and micropropagation of *Ficus carica* cv. Golden Orphan. The optimisation of selected parameters such as the number of shoots, shoot height, number of roots and root length which are crucial factors in the determination of healthy plantlets have been demonstrated. The genetic fidelity of the propagated plantlets was assessed via molecular markers for the identification of potential polymorphism occurrence in order to ensure that the *in vitro* regenerated plantlets are true-to-type and uniform at various subculture intervals. Furthermore, since the supplementation of cyanobacterial extracts in the cultivation of *F. carica* has not yet been reported in literature, this study also aims to evaluate the potential organogenesis-promoting properties of three nontoxic cyanobacterial strains. These strains were utilised as an organic source of phytohormone supplementation in the *in vitro*

regeneration of *F. carica* cv. Golden Orphan to achieve a production of healthy plantlets suitable for commercialisation purposes.

1.2 RESEARCH OBJECTIVES

The purpose of this study is to develop an effective protocol for the *in vitro* micropropagation of Golden Orphan figs using nodal explants, as well as to examine the genetic fidelity of the micropropagated plantlets for large-scale production of commercially viable plant stocks. The objectives of this study are:

a) To induce multiple shoots from *in vitro* explants of *F. carica* cv. Golden Orphan by using nodal segments of the stem, with a series of combination of plant growth regulators
b) To assess the role of cyanobacterial extracts in improving *in vitro* quantitative and qualitative growth of *F. carica* cv. Golden Orphan
c) To evaluate and identify polymorphism occurrences of the *in vitro* regenerative shoots via molecular markers
d) To induce *in vitro* rooting and to acclimatize the *F. carica* cv. Golden Orphan plantlets for field adaptation

CHAPTER 2
LITERATURE REVIEW

2.1 GENERAL INFORMATION ON *FICUS CARICA*

2.1.1 Description, Origin and Distribution

Ficus carica L. is a substantial deciduous member of the genus *Ficus*, and is commonly referred to as "fig". Specifically, figs which belong to the Moraceae family are distributed all over the world due to its suitability in adapting to varying climacteric conditions (Lim, 2012). Hailing from a generic diversity of more than 800 angiosperm species of trees, climbers, creepers and hemiepiphytes (Frodin, 2004), *F. carica* is native to southwest Asia and the eastern Mediterranean region, owning the title of being a pioneer among other fruiting crops that were cultivated by humans. The fig is a worldwide essential harvest for its dry and fresh consumption in raw, canned or in its varying forms of preservation. Due to their distinctive flavour, figs are savoured as dried fruit (Hussain *et al*., 2021). For a long time, *F. carica* has been grown worldwide for its edible fleshy, receptacle and hollow fruit, whereby the main producers of edible figs are Turkey, Egypt, Morocco, Spain, Greece, California, Brazil and elsewhere, usually with moderate winters and dry hot summers. The common fig, which accounts for the majority of consumable figs, is still growing wild in the Mediterranean Basin. The Jordan Valley's early Neolithic site lauds the discovery of carbonised figs about 11400-11200 years ago, which indicated the presence of fig domestication prior to the domestication of cereal (Kislev *et al.*, 2006). Hence, the present genetic diversity of figs is attributed to its early migration and domestication history, in addition to human selection and introgression with landraces and pre-existing local wild figs (Aradhya *et al*., 2010).

2.1.2 Morphology and Characteristics

Fig species consists of small trees or shrubs and they are deciduous, with greyish brown and slightly roughened barks. Generally, *F. carica* is identified through its large and deeply lobed fragrant leaves, complex and hidden inflorescence, multiple fruits, and latex (Mamoucha *et al*., 2016). The large leaves of *F. carica* comprise of lesser mechanical tissue as compared to other Mediterranean leaves. They are relatively coarse in texture as *F. carica* defies the general leaf adaptation of Mediterranean plants through the reduction of surface to volume ratio. In order to evade the extremities of heat, the leaves are positioned perpendicularly such that sunlight fall sideways on the leaves (Mamoucha *et al*., 2016). The fig buds comprising of the fruit bud, vegetative bud and mixed bud develop on the same shoot wherein the basal portion harbours the main crop and the distal portion of the shoot bears breba crops (Sedaghat *et al*., 2021). Figs are categorised as dioecious as they are present in two tree morphs namely capri fig and common fig (Mawa *et al*., 2013). The fig fruit namely syconium belongs to the composite fruit type consisting of a receptacle tissue that enfolds numerous individual pedicellate pistillate flowers which develops into drupelets. Tiny seeds are formed as a resultant of fertilised drupelets, apart from the empty drupelets in the case of parthenocarpic fruits (Marcotuli *et al*., 2020). The amount of seeds per fruit may range from 30 to 1600, depending on their size (Shahrajabian *et al*., 2021). The chemotype, caprification and ripening stages significantly contribute to the amount and size of seeds, thereby influencing flavour and taste in fresh and dried fig fruits (Hssaini *et al*., 2021). The fruits are solitarily borne at an axillary position on normal leafy shoots with varying colouration upon maturity (Wu *et al*., 2003). The skin colour of figs ranges from yellow to black, enabling them to be divided into two categories based on their skin colour wherein

light skin fig types are fruits with a yellow, yellow-green or green hue, and dark skin fig types comprise of fruits with a red, purple, black or brown skin colour (Bey and Louaileche, 2015).

2.1.3 Growing Conditions

The high adaptivity in fig species towards varying climatic and soil conditions without the rendition of toxicity or deficiency problems has resulted in its widespread geographically. Figs are highly tolerant of semi-arid tropical and subtropical regions, with exposure to partial or full sunlight in both inland and coastal areas located in lowlands and highlands alike. Hence, fig species can be grown sufficiently under unfavourable conditions despite its optimal growing requirements such as a warm climate with mild winters as low as 10 °C and hot summers between 15-36 °C at an altitude of 1525 metres above sea level. However, figs do not tolerate temperatures beyond 39 °C as it deteriorates fruiting quality thereby resulting in hastened ripening (Hiwale, 2015). They do not tolerate rainy conditions which would cause developing and ripening fruits to split, thereby substantially affecting fruit quality (Isa *et al*., 2020). Moreover, fig fruits expand to their final size during maturation, and they take less than three days for the crop to ripen rapidly.

Figs growing in open fields and in greenhouse conditions are rapidly increasing, as it is a species which responds favourably to growing conditions involving agronomic management and is therefore an excellent alternative to production. Figs planted in the open field also face certain challenges such as high incidences of pests and diseases, fruit rotting during the rainy season and poor plant management. These detrimental conditions are having a significant impact on crop growth and fruit production. Therefore, in the case of unfavourable circumstances,

cultivation of fig under a controlled growing condition tackles the aforementioned impediments. The most intensive crop type with a yield per unit area up to 10 times greater than a field crop is greenhouse cultivation (Shamin-shazwan *et al*., 2019). Additionally, agronomic management intervention would permit the optimisation of crop performance whereby a reduced period of production, early flowering and quality improvement can be achieved in such controlled growing conditions.

2.1.4 Nutritional Value

Figs are one of the most nutrient-packed fruits consumable by humans, as confirmed by various studies. Dried fruits of *F. carica* contains crucial source of minerals, carbohydrates, rich soluble dietary fibres formed by pectin (Trad *et al*., 2014), vitamins, sugars and organic acids (Slatnar *et al*., 2011). Moreover, bioactive compounds namely phenolic compounds, phytosterols, anthocyanin composition, triterpenoids, coumarins and volatile compounds are present in figs (Abdel-Rahman *et al*., 2021). High amounts of amino acids such as aspartic acid and glutamine, and polyphenols such as proanthocyanidins is found in both fresh and dried figs (Vinson *et al*., 2005). Whereas, figs are devoid of sodium, fat and cholesterol content (Rønsted *et al*., 2008). Nutrients essential to human diet such as copper, magnesium, manganese, calcium, potassium, and vitamins E and K are present in adequate amounts, in fig fruits (Khatib and Vaya, 2010). The Food and Nutrition Board of the U.S. Institute of Medicine revealed the nutritional content of dried figs of which 100 g of serving renders 30 % iron, 15.8 % calcium, 14 % potassium, 7.1 % thiamin (B1) 7.1% and 6.2 % riboflavin (B2) (Silva *et al*., 2009).

2.1.5 Traditional Uses and Medicinal Values

In traditional medicine, different parts of the fig plant such as its root, leaves and fruit have been widely used to treat respiratory, gastrointestinal and cardiovascular disorders, besides serving as an anti-inflammatory and antispasmodic remedy (Mawa *et al*., 2013). The decoction of boiled fig, neem, mango and peepal barks were traditionally used in India to alleviate burn-induced ulcers (Joseph and Raj, 2011). The fig fruit possesses antipyretic, purgative, and aphrodisiac characteristics that have been used to cure inflammation and paralysis (Abdel-Rahman *et al*., 2021). The natural antioxidants present possess the ability to inhibit free radical formation through the reduction or donation of hydrogen to other compounds (Arvaniti *et al*., 2019). *Ficus* plants are also used in the treatment of hypertension and other ailments as herbal concoctions and remedies in Sub-Saharan Africa and other parts of the world, according to folklore (Ajeigbe *et al*., 2021).

Fig leaf extracts and constituents possess stronger antioxidant activity in comparison to its fruits, wood and bark, whereby anticancer activity on colon, prostate, breast, cervical and liver cancers have been observed (Li *et al*., 2021a). There are a total of 121 volatile compounds in the leaves and 108 volatile constituents in the fruits, with 18 volatile compounds found in both leaves and fruits (Li *et al*., 2012). *Ficus carica* polysaccharides (FCPS) has been utilised as an efficacious immunostimulant in immunotherapy in animals and humans (Zou *et al*., 2020). A flavonoid namely rutin is a glycoside that has been reported to have inhibit prostate cancer (LNCaP) cells (Romero *et al*., 2002; Li *et al*., 2021b). The ethanolic leaf extract of figs at 1mg/mL have significantly exhibited antioxidant activity on hepatocellular carcinoma (HepG2) and human laryngeal carcinoma (Hep-2) cell lines at 75.7% DPPH scavenging ability (Abdel-Rahman *et al*., 2021). Furthermore, fig fruits has shown to exhibit antidiabetic

activity in streptozotocin-induced albino Wistar rats (Sheikh *et al*., 2015). Different type of extracts and compounds obtained from various *Ficus* species efficaciously alleviated diabetes caused by the induction of streptozotocin and alloxan through the regulation of blood glucose level in animal models through diverse mechanisms of action (Deepa *et al*., 2018).

Mopuri *et al*. (2018) reported the use of *F. carica* in the treatment of skin diseases, ulcers, paralysis, diabetes, anaemia, liver diseases, and propounded its clinical utilisation. Latex released from plucked fig fruits has been observed to treat warts and skin cancer (Elsayed *et al*., 2018). Regarding Alzheimer's disease, the acetone extract of fig fruit resulted in the enhancement of behavioural and cognitive deficits of transgenic mice (Subash *et al*., 2016). Whereas, fig latex demonstrated significant cytotoxic activity towards hepatocellular carcinoma HepG2, breast cancer MCF-7, colorectal HCT-116 and fibroblastic CCD-45 SK cell lines (Yahiaoui *et al*., 2022). In addition, fig latex successfully impeded cell growth in FaDu human hypopharynx squamous carcinoma cells as well as in causing programmed cell death via the caspase 3-9 and Bcl-2 family of proteins (Shin *et al*., 2017).

2.1.6 Economic Value

More than one million tonnes of fig fruit are produced annually, with 82 percent of the production industry dominated by the Mediterranean countries (FAOSTAT, 2015). Countries such as Turkey, Egypt, Morocco, Algeria, Iran, and the Syrian Arab Republic are among the 10 largest global fig growers. Spain is the only European country on the list, and it also includes the United States of America and Brazil (Solana and Romano, 2019). Turkey accounts for about 27 % of the world's new figs, 53 % of dried figs, and 51 % of the world's exports of fig fruit. Output and export volumes of

dry figs are 299 and 278 thousand metric tonnes, respectively, according to recent data published by the Turkish Statistical Institute (Yilmaz *et al*., 2017). In recent years, however, both fresh and dried figs have seen a growing trend in the world market. The fig shape, weight and its maturity index are crucial determinants of trade preferability, wherein the globose-shaped fruit varieties are preferred due to packaging and transportation aptness (Benettayeb *et al*., 2017). Fig cultivation has seen an upward trend in some countries. In Mexico, the area cultivated in 2017 was 1440 hectares, with an average yield of 5.6 tonnes per hectare. However, yields greater than 100 tonnes per hectare have been recorded under greenhouse conditions (Garza-Alonso *et al*., 2019). Fig crop production in Malaysia is still in its infancy, with 16,000 fig trees planted on a 10-hectare plot at the Indonesia, Malaysia and Thailand Growth Triangle (IMT-GT) project site in Chuping, Perlis (Kamarubahrin *et al*., 2019).

2.1.7 Reproduction and Classification of *Ficus carica* Varieties and Cultivars

The genus *Ficus* bears a unique inflorescence (syconium) as a result of synapormorphy, resembling an urn-shaped receptable and is internally borne with unisexual flowers, with a single opening namely ostiole (Teixeira *et al*., 2018). An unusual pollination-based obligate mutualism emerging more than 80 million years ago has resulted between *Ficus* plants and agaonid wasps belonging to the family Agaonidae and Chalcidoidea (Cruaud *et al*., 2012). Fig flowers can be divided into male, female and gall flowers, wherein monoecious species produces pollen, seeds and wasps. Whereas, gynodioecious species differ from their monoecious counterparts as female trees bear only seeds and male trees bear only wasps in their respective syconia (Borges, 2021).

Figs can be categorised into four groups with reference to their sex and pollination namely Caprifig (*F. carica* var. sylvestris Shinn.), Common fig (*F. carica* var. hortensis Shinn.), Smyrna (*F. carica* var. smyrnica Shinn.) and San Pedro (*F. carica* var. intermedia Shinn.). Of these, Caprifigs are nonedible (Crisosto *et al*., 2011), although some types are edible and consist of a more succulent fruitlet when compared to typical Caprifigs. The sexual reproduction of the Caprifig group consists of staminate and short-styled female flowers which acts as male in order to pollinate the female figs (Yahia, 2011). The process of fig pollination (caprification) occurs symbiotically with the fig wasp *Blastophaga psenes*, wherein female wasps exit from the ostiole of the Caprifig with pollen obtained from the male flowers and pollinate female flowers of the Smyrna and San Pedro fig (Crisosto *et al*., 2011). The resultant eggs develop into wingless male and female wasps that mate and then the female wasps exit the syconium to pollinate receptive syconia (Cook and Rasplus, 2003). The remaining three groups, although edible, are further categorised based on its ability to produce fruit with or without fertilisation (parthenocarpy). The usage of the term parthenocarpy, however, is refrained and the term 'persistent' is used instead as figs are not true fruits (Crisosto *et al*., 2011). Common figs are completely parthenocarpic, whereas Smyrna and San Pedro consist of non-parthenocarpic flowers.

In the Mediterranean region, the Brown Turkey figs are known to be widely cultivated due to their yield efficacy and in proving maximum values of fruit weight and width (Pereira *et al*., 2017). Brown Turkey is also undoubtedly a favoured variety in Europe and The USA due to its hardiness and productivity hence the preference by cultivators (Condit, 1955). The Brown Turkey fig fruit is of violet-brown peel with its pulp bearing a combination of white, red and pink colour ranges. Whereas, the Black Jack is a fig variety that is increasingly savoured by Malaysians due to its large, juicy

and sweet fruits, as well as an enticing purplish-brown peel colouration with a pink flesh (Parab *et al*., 2021). According to Moniruzzaman *et al*. (2020), notable cultivars suitable to be commercialised in Malaysia based on parameters such as fruit characteristics, productivity and pest susceptibility are Longue d'Aout, Alma, Dauphine, A132, Masui Dauphine, Wuhan, B110, Orphan, A134 and Fen Chan Huang.

Due to selection and favourability towards desired cultivars, significant habitual fig cultivation areas have decreased; the remaining cultivars are propagated conventionally and maintained through cuttings to achieve commercial or farming scale (Mars, 2001). For variation differences and germplasm classification, self-devised descriptors are efficaciously utilised which includes various pomological, agronomic and technical characteristics as well as molecular markers. Strong genetic variation within cultivated fig germplasm has been observed by morphometric and molecular analysis (Mars, 2001). The pattern in the receptable for most of the genes is unique thereby contributing to the fig drupelets produced within the syconium being implied to operate as parthenocarpy true fruit, which functions to control the ripening processes for the whole accessory fruit (Freiman *et al*., 2015).

2.1.8 The Golden Orphan, A Yellow Fig Cultivar

Yellow figs are light-coloured fig cultivars whereby the peel primarily bears a yellowish colouration with notes of green and is often favoured as dry figs (Trad *et al*., 2014). Light-coloured figs generally contain colossal amounts of glucose, fructose and possess high free radical scavenging activity (Hssaini *et al*., 2020). Besides, total flavonoids and proanthocyanidins were reported to have increased in yellow figs upon drying (Kamiloglu and Capanoglu, 2015). Carotenoids namely β-carotene, zeaxanthin,

β-cryptoxanthin and lutein represent carotenoids that accumulate in yellow fig cultivars (Wang *et al*., 2017).

The Golden Orphan cultivar which is also known as “Jin Ao Fen” has light green to a yellowish colouration upon ripening (Figure 2.1). This cultivar originates from California, USA and its cultivation was subsequently distributed to other countries namely China and Malaysia (Aradhya *et al*., 2010). The flesh of the ripened fruits is of pinkish-yellow in colouration with crunchy pink seeds (Figure 2.2) containing a sweet and milky flavour when it is consumed fresh (Sriskanda *et al*., 2021). According to Chen (2011), the fruits of this cultivar are relatively large when compared to other notable cultivars and has the longest fruit development period of 15 weeks.

Figure 2.1: Fruit (Syconium) of *F. carica* cv. Golden Orphan. Scale bar represents 2 cm.

Figure 1.2: Cross-section of *F. carica* cv. Golden Orphan fruit. Scale bar represents 2 cm.

2.1.9 The Different Propagation Techniques of *Ficus carica*

Conventionally, figs are cultivated through various methods particularly through vegetative propagation. Figs are generally cultivated through cuttings, whereby cuttings from one- or two-year-old shoots are planted in soil to establish new trees (Dolgun and Tekintas, 2008). Hardwood cuttings are propagated by dipping the basal part of the stem into rooting hormone to induce rooting prior to planting. In a study conducted by (Aljane and Nahdi, 2014), best rooting and cumulative plant growth can be achieved through the usage of shoots aged two years old with the length of 40 cm and at a diameter less than 1.5 cm. In another stem cutting technique conducted by Zerhoune (2003), vertically planted stem cuttings were suitable in the induction of vigorous shoots and roots. Plunging and cleft grafting are grafting techniques which has been successfully employed on the Smyrna, Troyana or Palestino, and Roxo de Valinhos fig cultivars with a 100% survival rate of cuttings (A. Boliani *et al*., 2019).

Air layering is a propagation technique used on mature tree branches that are able to blossom and bear fruit. The ring of bark is removed to facilitate the accumulation of photosynthates (Tchoundjeu *et al*., 2010). Once the cambium has been exposed, rooting powder comprising of auxin is applied to encourage rooting. A wet rooting media, such as compost or peat, is then encircled on the exposure and wrapped with a black polythene bag to stimulate roots within the next few weeks or months (Tchoundjeu *et al*., 2010). This method is also used in fig orchards as this technique efficaciously speed up the rooting process due to the providence of water and nutrients via the xylem of the mother plant to the cutting (Dolgun and Tekintas, 2008). Bisi *et al*. (2016) mentioned that the “Roxo de Valinhos” and the “Troiano” cultivar has rendered a colossal success in callus formation beyond 70 % when the air

layering technique was employed, despite the occurrences of branch breaking (Daneluz *et al*., 2009) due to excessive weight especially if air layering is conducted at the apical section (as cited in Dolgun and Tekintas, 2008). Despite, the air layering method gives a low yield, hence is not a favourable option to mass propagate figs.

Another technique involves the mass production of clones genetically identical to the mother plant, namely plant tissue culture or micropropagation which requires a sterile and controlled (*in vitro*) environment dependent on factors namely luminosity, photoperiod, temperature and nutrition supplementation (Gutiérrez *et al*., 2011). The applicability and efficacy of plant tissue culture technology with regards to the rapid propagation of *F. carica* have been widely reported in literature (Dhage *et al*., 2015; Volo *et al*., 2017; Guranna and Huchesh, 2018; Ling *et al*., 2018; Elhomosany and Sayed, 2019), hence evident to be endorsed as a propagation alternative for fig plant stocks.

2.2 PLANT TISSUE CULTURE

2.2.1 Plant Tissue Culture Technique

Plant tissue culture, also known as *in vitro* culture is an aseptic method for plant cells, tissue and organ cultivation. Contrary to traditional cultivation, *in vitro* culture stipulates stringent, suitable physical and chemical requirements, based on the cell's capacity to grow in an autonomous manner, so that a well-developed plant can be cultivated and differentiated into desired outcomes. A technique known as micropropagation in plant tissue culture uses small sections of tissues known as explants, which are grown in a sterile medium to rapidly replicate into shoots and roots (Yancheva and Kondakova, 2018). The explant turned plantlets can then be split into

multiple plantlets during their shooting stage and grown under the designated environmental conditions. By encouraging axillary bud breaking, generating adventitious buds via callus, and somatic embryogenesis on the *in vitro* explant, micropropagation can be achieved (Thorpe, 2006).

Plant production using tissue culture is a rapid process that allows a crop species to be mass propagated, which would otherwise be prolonged and it renders economically inadequate circumstances to growers if traditional vegetative propagation methods are chosen. In comparison to the propagule multiplication rate by traditional propagation, micropropagation enables high multiplication rates of propagules. In order to develop enough clones for a year's supply, small quantities of plant tissue can be utilised via this technique. Micropropagation greatly reduces the time taken to produce plantlets of a new variety by 50 % (Bhatia *et al*., 2015). This approach also aids in the conservation of space by allowing large numbers of plants to be maintained in small places during the *in vitro* propagation phase. In contrast to seed propagation, the clones are free of bacterial, fungal, and viral infections since the propagules generated are genetically comparable and true-to-type to the selected mother plant. Since *in vitro* propagules may be multiplied at any time and in any season, the dependence on plants to reproduce during certain seasons for the acquisition of new plants can be decreased. Under the regulated sterile conditions provided by tissue culture, plants bearing seeds with germination suboptimality may germinate and thrive at an early stage (Bhatia *et al*., 2015).

2.2.2 Tissue Culture and Micropropagation of *Ficus carica*

Various methods in plant tissue culture have been employed by previous studies on figs, with reference to the materials, explant types and micropropagation techniques. Different explant types such as nodal segments, internodal segments,

axillary buds and leaf explants were used to induce organogenesis in *F. carica*. Qrunfleh *et al*. (2013) utilised various concentrations of carbon sources, gelling agent as well as salinity in order to evaluate on their efficacy in parameters such as number of shoots, shoot length, and fresh and dry weight. The rendered results revealed that despite the indifferences in the application of various carbon sources, increased salinity concentrations had decreased shooting frequency and length. Besides, liquid media produced more shoots when compared to the employment of solidifying agents.

Axillary or meristem culture has rendered a high regeneration capacity in inducing microshoots through direct organogenesis, according to Mitrofanova *et al*. (2017), with the supplementation of a combination of cytokinins and auxins in MS medium. The author also established the induction of morphogenic callus, somatic embryos and seedlings development, thereby fortifying the employment of somatic embryogenesis in the micropropagation of figs. Due to its effectiveness in rendering virus-free stock cultures by eradicating the possibility of Fig Mosaic Disease (FMD) on leaves and fruits, shoot-tip culture on explant sizes of 0.5, 1 and 1.5 mm was tested by Bayoudh *et al*. (2015), which significantly increased the potentialities of various fig varieties in direct organogenesis with specificity towards leaf, shoot and root regeneration rate.

Moreover, Abdolinejad *et al*. (2020) utilised the thin cell layer (TCL) technique to develop an indirect organogenesis protocol for *F. carica* by using matured stem segments of 0.5-0.8 mm thickness and 10 mm in diameter. The rendered results were the induction of morphogenic calli which were further divided into small segments and transferred into shoot proliferation medium comprising of plant growth regulators. In a study conducted by Elhomosany and Sayed (2019), the ability of *F. carica* to be cryopreserved was tested. The utilisation of the vitrification technique

through the treatment of shoot tips with Plant Vitrification Solution 2 (PVS2) at 0 °C for 30, 40 and 50 minutes and a subsequent plunging into Liquid Nitrogen (LN) had rendered survivability and regrowth which caters a long-term *in vitro* preservation for this species and its genetic diversity to be safeguarded in gene banks.

Shoot regeneration was obtained optimally through the supplementation of additives such as myo-inositol and thiamine HCL alongside plant growth regulators and carbon source, when the explants were inoculated with the adaxial surface up indicating the importance of dorsoventral orientation of explants in *in vitro* propagation (Yancheva *et al*., 2005). Additionally, the study of Yancheva *et al*. (2005) involved the transformation technique which incorporated the utilisation of *Agrobacterium* to introduce foreign genes into transgenic fig cultivars in order to improve figs in the context of its cultivation, disease resistance and shelf life. The induction and elicitation of hairy roots by plant growth regulators such as Methyl Jasmonate and through the inoculation of fig explants with various strains of *Agrobacterium rhizogenes* has been explored by Amani *et al*. (2020). The study revealed that the hairy root cells produced high amounts of phenolic compounds as well as an increase in the antioxidant capacity upon elicitation which enabled rapid high-yield production of secondary metabolites suitable in an industrial scale through *in vitro* techniques.

2.2.3 Plant Growth Regulators (PGRs)

Plant growth regulators (PGRs) are defined as chemicals utilised in both *in vivo* and *in vitro* conditions in the alteration of plant growth and development via its regulation over physiological and morphogenic functioning that contributes to shoot and root growth, callus induction, defence against abiotic stresses, senescence,

flowering and fruiting (Fishel, 2006; Ahmed *et al*., 2021). Although termed as PGRs or plant hormones, these chemicals are ubiquitous as they are prevalent in higher and lower plants, fungi and bacteria (Yamaguchi *et al*., 2010). Plant shape and size are controlled by a combination of external and endogenous signals that interact with the inherent genetic composition of the plant to direct development and growth (Kazan, 2013). This mechanism acts with many growth regulators such as auxin, cytokinin, gibberellins (GAs), abscisic acid (ABA), ethylene, brassinosteroids (BRs), and jasmonic acid (JA) (Jiang and Asami, 2018). Interactions between auxin, cytokinin and auxin-cytokinin are vital in the regulation of a variety of developmental processes, namely the formation and retention of meristems crucial for the creation encompassing the entire plant body wherein the shoot meristems differentiate into parts of the above-ground plant while the root meristems differentiate into parts of the below-ground plant (Schaller *et al*., 2015).

In addition to supporting cell wall acidification, onset of cell division, organisation of meristems to produce callus or established organs, especially roots, and promoting vascular differentiation, auxins primarily act to promote cell growth expansion (Costa *et al*., 2013). Further, by retaining apical dominance, impacting abscission, encouraging root development, slowing leaf senescence, and fruit maturation, auxins play a role in organised tissues (Iqbal *et al*., 2017). Cytokinin, another widely used plant growth regulator group commonly used in tissue culture, enhances and controls cell division and differentiation primarily as it interacts with auxin, facilitates lateral bud dormancy, growth and leaf proliferation, promotes and improves the synthesis and production of chlorophyll, retards leaf senescence, and induces adventitious bud formation in cuttings and culture (George *et al*., 2007). Auxins control DNA replication throughout the cell cycle, while cytokinins engage in

events leading to mitosis and cytokinesis. Zeatin, 2-iP, di-hydro-zeatin and zeatin riboside are naturally occurring cytokinins. Cytokinin-like growth regulators include substituted purines such as kinetin and 6-benzylaminopurine (BAP). Exogenously applied BAP partakes in the delay of senescence as demonstrated by Chang *et al*. (2003), whereby isopentenyl transferase, a crucial gene in cytokinin biosynthesis present in transgenic petunia flowers, resulted in lengthened flower longevity therefore antagonistic to the action of ethylene. Gibberellins (GAs) usually encourage seed germination, leaf growth, stem elongation, flowering and fruiting induction (Gupta and Chakrabarty, 2013). The supply of endogenous auxins found in plant tissues is impaired by GAs. The addition of GAs in meristem and shoot cultures can enhance the growth of shoots. Abscisic acid (ABA) acts as an enzyme that slows down the elongation of cells and prevents the loosening of auxin-promoted cell wall acidification (Lorrai *et al*., 2018). ABA influences callus growth and organogenesis in tissue culture, and is important in somatic embryo formation. ABA may alter the synthesis or operation of cytokinin, as well as promote IAA oxidation (Lorrai *et al*., 2018).

Ethylene, a gaseous phytohormone, facilitates fruit maturation, senescence and abscission of the stems (Iqbal *et al*., 2017). In plant tissue culture, the effect of ethylene is specific, but its function is difficult to understand because the effects vary with the stage of development; low ethylene concentrations will show counteracting results if the concentration is high. In the production of ethylene, auxin-cytokinin activity is observed, whereby auxin typically stimulates the production of ethylene, whereas cytokinins and growth regulators such as silver thiosulfate (STS) (Hyde *et al*., 2020) can be antagonistic to the action of ethylene. Brassinosteroids are a new category of PGRs that play a role in regulation, in which this phytohormone acts to