MICROPROPAGATION OF COMMON FIG (*Ficus carica* L.) CV. VIOLETTE DE SOLLIÈS

LING WAN TING

UNIVERSITI SAINS MALAYSIA

2020

MICROPROPAGATION OF COMMON FIG (*Ficus carica* L.) CV. VIOLETTE DE SOLLIÈS

by

LING WAN TING

Thesis submitted in fulfilment of the requirements
for the degree of
Master of Science

March 2020

ACKNOWLEDGEMENT

I am indebted to my supervisor, Dr. Chew Bee Lynn for her help, valuable suggestions and discussions. I want to express my gratitude for giving me the opportunity to work on this project. I also gratefully acknowledge the help provided by my co-supervisor, Professor Dr. Sreeramanan Subramaniam. Thank you for taking the time to give me those guidance and advices. I am particularly grateful to my seniors and colleagues from Plant Tissue and Cell Culture Laboratory/Plant Biotechnology Laboratory for sharing their experiences and honest feedback about the project. I would like to thank the science officers, assistant science officer, lab assistants and technicians of School of Biological Science, Universiti Sains Malaysia (USM) for taking the time to help me throughout the studies. I truly appreciate their help, without them, those experiments would never be achieved.

I am grateful and blessed to have my parents, brother and sisters in my life. I would like to express my heartfelt thanks for always being there for me and supporting my study. I am so thankful to my friends, Fui Joo, Rui Xuan, Sin Pei and Ya Fen. I just want to say thanks for encouraging and helping me through those difficult times.

I also appreciate Mr. Lim Wei Yong for his ongoing collaboration with our experimental work. Thank you for providing plant materials and other gardening equipments of this study and sharing your experiences and opinion about planting. I also would like to thank Agricultural Crop Trust (ACT) for the financial support provided in my master study.

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LIST OF ABBREVIATIONS

AC	Activated charcoal
ANOVA	Analysis of variance
BAP	6-Benzylaminopurine
CDR	Completely randomized design
DMRT	Duncan's multiple range test
DNA	Deoxyribonucleic acid
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
HCl	Hydrochloric acid
HMDS	Hexamethyldisilazane
Kn	Kinetin
LEDs	Light-emitting diodes
MS	Murashige and Skoog
NAA	1-Naphthaleneacetic acid
NaOH	Sodium hydroxide
OsO_4	Osmium tetroxide
PCR	Polymerase chain reaction
PGRs	Plant growth regulators
RAPD	Randomly Amplified Polymorphic DNA
SCoT	Start Codon Targeted
SEM	Scanning electron microscope
TBE	Tris-Borate-EDTA
VDS	Violette de Solli ès
WPM	Woody Plant Medium
Zn	Zeatin

MIKROPROPAGASI POKOK TIN (*Ficus carica* L.) CV. VIOLETTE DE SOLLIÈS

ABSTRAK

Pokok tin atau secara saintifiknya dikenali sebagai *Ficus carica* merupakan antara tanaman terawal yang ditanam oleh manusia dan tergolong dalam keluarga Moraceae. Pokok tin berasal dari Asia Barat dan diperkenalkan di seluruh negara lembangan Mediterranean bersama dengan penghijrahan manusia. Ia merupakan tanaman makanan yang penting dengan nilai ekonomi, pemakanan dan farmaseutikal yang tinggi. Pokok tin biasanya ditanam melalui pembiakan vegetatif seperti keratan, cantuman dan tut tetapi kadar pembiakan secara konversional ini adalah rendah disebabkan oleh perakaran yang kurang baik dan masalah kontaminasi yang sama dengan tanaman induk. Kultur tisu adalah cara alternatif yang efektif untuk menghasilkan tumbuhan yang bebas penyakit dan virus secara besar-besaran dari stok tumbuhan yang terpilih. Kajian ini bertujuan untuk menubuhkan protokol mikropropagasi untuk pokok tin (*F. carica* L.) cv. 'Violette de Solliès' bagi pengeluaran secara besar-besaran tumbuhan baru yang sama dengan tumbuhan induk melalui kultur tunas apikal. Dalam kajian ini, tunas apikal yang muda dikumpulkan dan permukaan disterilkan dengan kepekatan dan tempoh ejen steril yang berbeza untuk menghasilkan protokol sterilisasi permukaan yang cekap. Eksplan telah dibiakkan dalam medium MS ditambah dengan 4 mg/L BAP untuk penubuhan kultur *in vitro*. Pucuk yang terhasil dikulturkan dalam kepekatan sitokinin (BAP, TDZ, Kn dan Zn) dan arang teraktifkan yang berbeza untuk inisiasi peanggandaan pucuk dan seterusnya sitokinin terpilih digabungkan dengan auksin (IAA) bagi menentukan kesan gabungan terhadap penggandaan pucuk. Pucuk individu dikeluarkan dan dipindahkan ke Woody

Plant Medium (WPM) ditambah dengan kepekatan auksin (NAA, IAA dan IBA) yang berlainan untuk induksi akar. Eksplan pucuk yang berakar diaklimatisasi dengan menggunakan substrat tanah yang berlainan (pelet Jiffy, lumut gambut, perlite, vermiculite dan tanah *bio-char*). Pucuk dari kitaran subkultur yang berbeza diperoleh, DNA diekstrak dan tertakluk kepada analisis RAPD dan SCoT. Mikroskopi elektron pengimbasan digunakan untuk mengkaji stoma daun pada tumbuhan *in vitro*, aklimatisasi dan induk. Dalam kajian ini, media MS ditambah dengan 2.0 mg/L BAP dan 0.5 mg/L IAA didapati paling berkesan untuk penggandaan pucuk dengan jumlah pucuk tertinggi, 17.20 ±2.38. Sementara itu, WPM ditambah dengan 3.0 mg/L IBA memperoleh peratusan pembentukan akar yang tertinggi (93.33%) Tanaman yang diaklimatisasi dalam pelet Jiffy dan tanah *bio-char* memperoleh kadar kemandirian 100%. Keputusan dari analisis mikroskop SEM menunjukkan keadaan stomata yang normal pada daun yang diaklimatisasi Jalur monomorfi dari analisis RAPD and SCoT membuktikan bahawa tumbuhan mikropropagasi yang sama dengan tumbuhan induk telah dihasilkan dalam akhir kajian. Kajian ini telah berjaya menubuhkan protokol mikropropagasi yang cekep bagi pokok tin (*F. carica* L.) cv. Violette de solliès sesuai untuk pengeluaran secara komersial dan penanaman pada tanah tempatan.

MICROPROPAGATION OF COMMON FIG (*Ficus carica* L.) CV. VIOLETTE DE SOLLIÈS

ABSTRACT

Common fig or scientifically known as *Ficus carica* is one of the earliest cultivated crops belonging to the family Moraceae. Fig is native to Western Asia and introduced throughout the Mediterranean basin countries together with the human migration. It is an important food crop known for its high economic, nutritional and pharmaceutical values. The fig plant is commonly cultivated via vegetative propagation such as cutting, grafting and air layering but the multiplication rate of these conventional methods is low due to poor rooting and the presence of same contamination issues as mother plants. Plant tissue culture is an efficient alternative in mass propagation plants from selected plant stocks yielding plants that are disease and virus free. The current study aims to establish a micropropagation protocol for the common fig (*F. carica* L.) cv. 'Violette de Solliès' to mass propagate true-to-type fig plantlets via apical bud culture. Young apical buds were surface sterilized using different concentrations and duration of disinfectants and cultured on MS medium supplemented with 4 mg/L BAP for the establishment of *in vitro* cultures. Induced shoots were subjected to different concentrations of cytokinins (BAP, TDZ, Kn and Zn) and activated charcoal for the initiation of multiple shoots and selected cytokinin was combined with auxin (IAA) to evaluate the combined effects on the shoot multiplication. Individual shoots were transferred to WPM supplemented with different concentrations of auxins (NAA, IAA and IBA) for root induction. Rooted explants were acclimatized using different soil substrates (Jiffy pellet, peat moss, perlite, vermiculite and bio-char soil) and garden soil mixture. Shoots from different subculture cycles were

harvested, DNA extracted and subjected to RAPD and SCoT analysis. Scanning electron microscopy was carried out to study the stomata of leaves on *in vitro*, acclimatized and mother plants. MS media supplemented with 2.0 mg/L BAP and 0.5 mg/L IAA was found to be the most effective treatment for shoot multiplication with the highest number of induced shoots (17.20±2.38). Meanwhile, WPM media supplemented with 3.0 mg/L IBA produced the highest percentage of root formation (93.33%). Plantlets acclimatized on Jiffy pellet and bio-char soil resulted in 100% survival rate. Results from SEM microscopy analysis revealed normal stomata on leaves for acclimatized plants. The monomorphic bands from RAPD and SCoT analysis indicated true-to-type micropropagated plants throughout six subculture cycles. This study has successfully established an efficient micropropagation protocol for common fig (*F. carica* L.) cv. Violette de Solliès suitable for commercialization and cultivation in Malaysia.

CHAPTER 1

INTRODUCTION

Ficus is a genus that belongs to the Moraceae family and is endemic to southwest Asia and the eastern Mediterranean (Chawla *et al*., 2012). *Ficus carica* L. (common fig) is the one of the earliest cultivated crops with the greatest commercial value among 800 species of genus *ficus* (Dhage *et al*., 2012). It is a deciduous tree or shrub with gray and smooth bark, palmate leaves, and fibrous roots. Fig cultivars produce fruits in different skin colour, size, taste and texture. *F. carica* is economically important and propagated abundantly in the Mediterranean countries, especially in Turkey, Egypt, Greece, Iran and Morocco (Flaishman *et al.*, 2007). A report by Food and Agriculture Organization of the United Nations (FAO) (2020) indicated that approximately 1.1 million tonnes of figs were harvested worldwide, of which 306 499 tonnes were produced in Turkey in 2018 (Food and Agriculture Organization of the United Nations (FAO), 2020). Fig is well known as one of the most successful species due to it is widely used either medically or nutritionally for human. Fig fruit is widely known for its high nutritional contents which are mainly fiber, potassium, calcium and iron higher than other fruits, namely banana, orange and apple (Crisosto *et al.*, 2011). Several pharmacological studies demonstrated the fig possess antibacterial, antioxidant, antidiabetic, anti-inflammatory, antipyretic, anticancer and other pharmacological effects. The methanolic leaf and bark extract of *Ficus carica* were found to have antidiabetic effects by reducing blood glucose levels and triglycerides on diabetic rats (Stalin *et al*., 2012; Ahmad *et al.*, 2013)

Ficus carica is not native to the tropical climate and is not commonly grown in Malaysia. As the seed of the fruit does not resemble the quality of the mother plant, current propagation method for fig plants still relies on conventional methods such as cutting, grafting and air layering. These methods might be inefficient to produce high quality plants at a consistent rate, especially for commercial purpose and the establishment of fig farms in Malaysia. The high demand of good quality novel cultivars have caused the price of plant sticks to escalate indirectly hampers the start-up of farms throughout the country. The cultivar 'Violette de Solliès' (VDS) is one of the premium fig cultivars rarely found and sold in Malaysia. It was originated from the Solliès region of France and successfully cultivated in the Malaysia at the Superfruits Valley in Chuping, Perlis, Malaysia. It is recognized as one of the sweetest cultivar with dark purple skin and pinkish red pulp. VDS fruits are large and sweet with hint of the berry and cherry flavors. The demand of high-quality fig plant stock to cater the demand from local supermarkets and juice processing industries have encouraged commercial cultivation of sweet figs on the local grounds. However, the establishment of farms across the local soils has been very slow due to the low availability of plant stocks.

Plant tissue culture is a technique used to propagate plants via cell, tissue or organ culture under sterile and controlled conditions such as proper temperature, humidity, light and nutrients, for their growth and multiplication (Akin-Idowu *et al*., 2009). The culture medium compositions, especially the nitrogen source and the addition of plant growth regulators have significant effects on the growth of the culture. This alternative comes in as a reliable and simple technology in mass propagating plants at a consistent rate producing clones that resembles the good qualities of the mother plants.

A major problem associated with genetic variation arises in many micropropagation protocols. Somaclonal variation is defined as the variation detected in regenerated plants from *in vitro* culture and it is often occurring between sub-clones of one parental line (Larkin and Scowcroft, 1981). Micropropagation possess problems in obtaining true-to-type *in vitro* propagated plants because of the chromosomal rearrangement, gene amplification, gene mutation and retrotransposon activation that can occur through many subculture cycles (Saha *et al*., 2016). The assessment of somaclonal variation within *in vitro* propagated plants plays a pivotal role in the mass production of plant stocks for commercial purposes.

The current study aims to establish an efficient protocol for the micropropagation of 'Violette de Solliès' via apical bud culture and to assess the genetic stability of micropropagated plantlets for mass production of plant stocks fit for commercialization purposes. The objectives of this study are:

1. To induce multiple shoots from shoot and nodal explants using different concentrations and combinations of plant growth regulators.
2. To induce rooting of micropropagated shoots using different basal media and concentrations of auxins.
3. To evaluate the different types of soil substrates suitable for acclimatization.
4. To validate the genetic stability of the micropropagated plantlets using RAPD and SCoT markers.

CHAPTER 2

LITERATURE REVIEW

2.1 *Ficus carica* L.

2.1.1 Plant morphology and development

Common fig, scientifically knows as *Ficus carica* is recognised as *pokok tin* in Malaysia. The Latin name, *carica*, refers to the Caria region in Asia Minor which is the origin of fig (Starr *et al*., 2003) while the Malay name, *tin*, is derived from the Arabic word, *At-Tīn*, mentioned in Holy Qur'an specifically Surah 95. It is a woody plant belonging to the Moraceae family consisting of 40 genera and more than 1100 species. The common fig is a deciduous tree that can grow up to 7 to 10 metres in height with fragrant lobed leaves (Darwesh *et al*., 2014). Fig trees have a diverse branching habit, varied from the open to compact as well as from pendulous to upright to spreading branching along with the cultivars. For example, the cultivar 'Brown Turkey' has a weeping type of compact, down-spreading branching system whilst 'Sierra' and 'Autumn Honey' have a vigorous upright-rising branching system (Flaishman *et al*., 2007). Tree size and density are also different among cultivars, even though the key factors are cultural factors and soil quality. Some of the cultivars are round-topped tree with many spur shoots while others have more apical branches with less spur shoot, creating an open or leggy appearance. Fig tree has an abundant, fibrous and shallow root system (Dom ńguez, 1990). Its roots may grow either laterally or vertically according to the soil condition. Fig roots can sometime cover up to 50 feet in the ground, but it may cover up to 20 feet only in

permeable soil (Chawla *et al*., 2012). Fig trees are relatively drought and salinity tolerant once they are well-established (Golombek & Lüdders, 1990).

Fig wood is soft and condensed with thick branches. The specific gravity of its wood (dried/fresh weight) is 0.43. Fig tree has a pithy trunk and large branches covered with smooth grey bark without fissures (Crisosto *et al*., 2011). The young twigs of fig are generally smooth and brownish-green, and turn into grey once the branches matured. Its lenticels grow to be corky, rough and darker over the time. The internode also has elongated in the direction of the median section of the shoot. Similar to many other higher plant genera, *Ficus* species also produce milky white sap within their vascular system known as latex (Lansky and Paavilainen, 2011) It can cause skin irritation, such as burning or itching due to the proteolytic enzyme ficins.

Fig tree has palmate, large, petiolate leaves with three to seven lobed (Ferguson *et al*., 1990; Morton, 1987). Different fig cultivars have a diverse leaf characters and it was used as an indicator for cultivar identification (Ferguson *et al*., 1990), namely lobes, leaf teeth, leaf venation, colour, presence of hairy leaves. The growing season of fig leaves is starting from the early spring until the temperature drops in autumn. The leaf fall can be influenced by temperature, photoperiod, wind, and rain and other environmental conditions at the end of the growing season (Lansky and Paavilainen, 2011).

Fig has been known as '無花果'; 'Wu Hua Guo' in Chinese which means "no flower fruit" (Flaishman *et al*., 2007). The fruit is borne from syconium inflorescence, which multiple ovaries are hidden within a fleshy hollow receptacle. According to Lisci and Pacini (1994), in fact fig fruit is developed from syconium and considered as a 'false

fruit'. Morton (1987) also stated that the true fruits are drupelets give rise from flowers inside the syconium. Figs are generally separated into four categories based on their sex and types of pollination namely Caprifig ('Pouz Donbali' and 'Shah Anjir' cultivars), common fig ('Brown Turkey' and 'Violette de Solliès cultivars), Smyrna ('Sarilop'and 'Zidi' cultivars), and San Pedro ('Dauphine' and 'King' cultivars) (Crisosto *et al*., 2011). Common fig, Smyrna and San Pedro are edible with the exception of Caprifig. They consist of long-styled female pistillate flowers that produce succulent fruitlets and reproductive function as female plants while Caprifig consists of staminate and short-styled female pistillate flowers and reproductive function as male plants (Hong and Chen, 2003). Common fig requires no caprification (or fig pollination) for fruit growth and maturation. On the other hand, Smyrna, San Pedro and Caprifig require caprification by fig wasp (*Blastophaga psenes*) to develop main fig crop while San Pedro will produce two types of crop, main crop (with caprification) and breba crop (without caprification) (Crisosto *et al*., 2011). Caprifig produces the most crops per year, which is three crops, followed by Common fig and San Pedro with two crops per year and Smyrna only one crop per year (Ferguson *et al.*, 1990).

Fig cultivars have a wide variety of taste, texture and skin colours of fruits, ranged from yellowish-green to copper, bronze, or dark-purple (Danial *et al.*, 2014). 'Blanche d'Argenteuil' has been known as the oldest fig propagated in Argenteuil, Paris (Himelrick, 1999). It produces greenish yellow fruits with white pulp. Its fruits have a light fruity taste with floral aroma. 'Panachée' is a unique French fig cultivar with green and yellow stripes fruits. It is a mutated cultivar or chimera which was first revealed in 1668 (Himelrick, 1999). Its fruits have strawberry pulp with mediocre flavoured and mealy texture.

‘Violette de Bordeaux’ is also one of the French fig cultivars that produce large dark-purple fruits with deep-red pulp. It grows two crops per year, main crop fig and brebas fig. Its fruits have a distinctive but acceptable acid flavour (Himelrick, 1999). The fig cultivar used in this study was ‘Violette de Solliès’ (VDS). The name ‘Solliès’ is derived from its origin, Solliès region of France. Similar to ‘Violette de Bordeaux’, it is also produces dark-purple fruits and pinkish-red pulp. Its fruits are large and flat with berry and cherry flavors. Interestingly, its petioles are red at the base which makes it differs from other cultivars. VDS tree has all the characteristics of the common fig including lobbed leaves, upright branches, grey bark and fibrous roots as illustrated in Figure 2.1 (Trew, 1750).

2.1.2 History

Fig is native to Western Asia and was introduced throughout the Mediterranean basin countries along with the human migration (Flaishman *et al.*, 2007). Before the recorded history, figs have already been introduced into Italy. The introduction of figs to England occurred between the years 1525 to 1548, and then the European figs were transported into other countries, namely China, Japan, India, South Africa, and Australia (Flaishman *et al*., 2007). In 1560, the fig plants cultivated in Mexico were the earliest figs in the New World. Figs were transported to Virginia in 1669, followed by California when the Mission San Diego was built in 1769. Different varieties of fig were later obtained from Europe. The caducous fig was introduced into California in 1881, but it had been commercially cultivated since 1900 due to the presence of the fig wasp (Flaishman *et al*., 2007). The fig was recognized as a popular courtyard plant in the West Indies, as well as

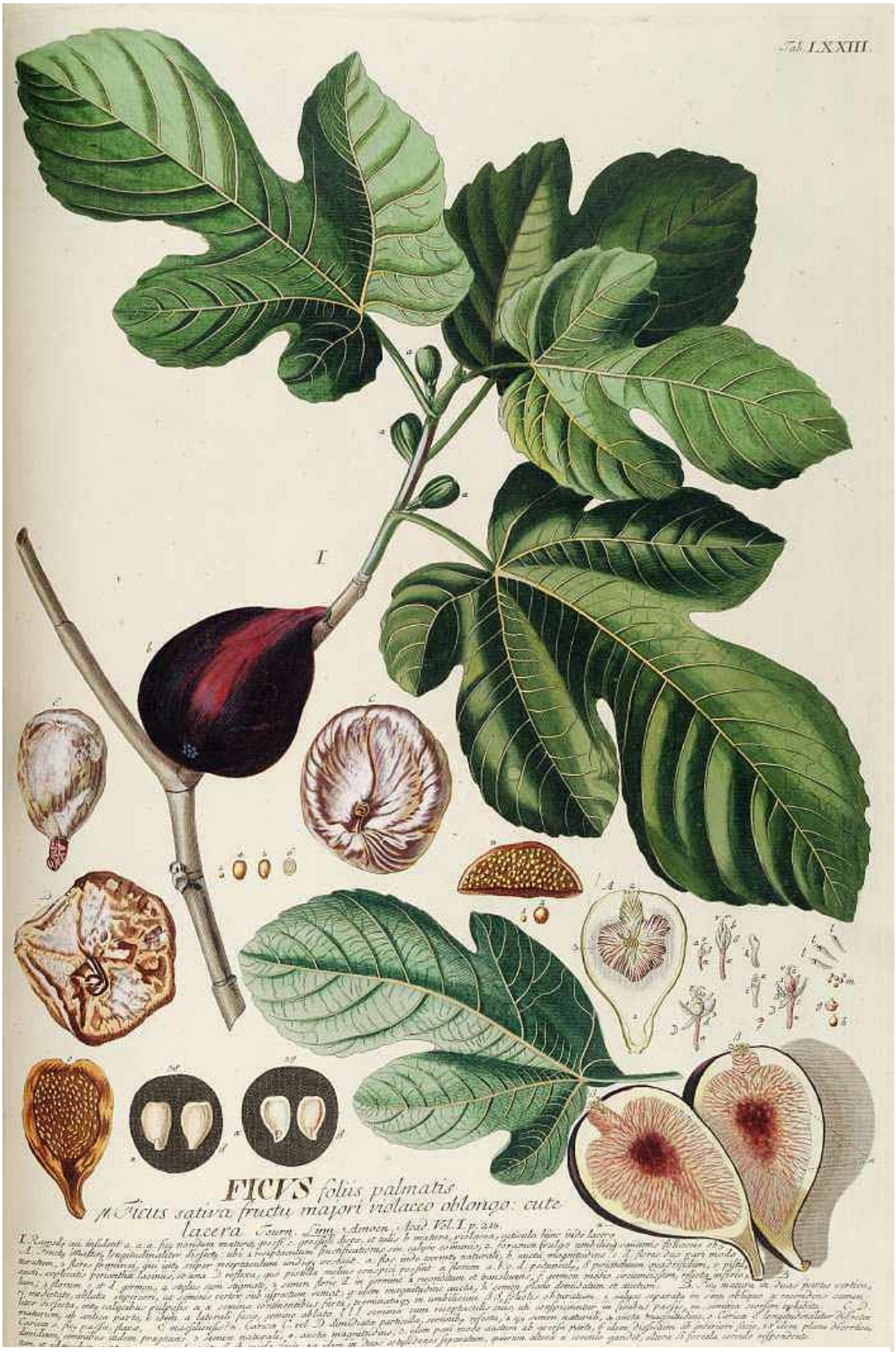

Figure 2.1: Plant morphology of *Ficus carica* L. (Trew, 1750).

Central America and northern South America particularly at medium and low altitudes. Its plantations also had been found on hillsides in Honduras and at low altitude on the part of the Costa Rica area that adjacent to the Pacific Ocean. Only the common fig was cultivated from Florida to northern South America and India. Moreover, the cool tolerant fig varieties were planted in the Chile and Argentina (Flaishman *et al*., 2007).

According to the Inter-communal Tourist Office of Vallée du Gapeau (2019), 'Violette de Solliès' was mentioned by Olivier de Serres, a French author and soil scientist, of the importance of fig trees in the Solliès region in 1620. The local, national and North-West European markets were filled with fig fruits and a total of 18 000 kg of fresh figs were shipped out during the high season in 1907 (Inter-communal Tourist Office of Vallée du Gapeau, 2019). Fig orchards were developed during the interwar period and the production of figs was dramatically increased to 1260 tons in 1932. Figs were dominant the Solliès region and became the representative fruit product in the early 1950's. In 1960's, the Solliès region produced around 1,600 tons fresh figs which about 25% of the total production of figs marketed. Fig production increases to 2500 tons after improving the irrigation system in the mid-1980's (Inter-communal Tourist Office of Vallée du Gapeau, 2019).

2.1.3 Cultures and religious significance

The fig plant is an ancient plant found as early as thirtieth century BC (Ferguson *et al.*, 1990) with religious and cultural significance. The pulchritude of fig leaves and its "sweetness and good fruit" were widely known in the earliest times, where the fig tree is

also often be mentioned in the Bibles and the Koran (Flaishman *et al*., 2007). The fig tree was first introduced in the Holy Bible, Book of Genesis, when Adam and Eve, the first man and woman created by the Lord God, use the fig leaves to cover their nudity after eating the "Forbidden Fruit" from the Tree of Knowledge of Good and Evil in the Garden of Eden (Genesis 3:7). The fig leaf is then extensively used as covering of nudity in painting and sculpture, especially in the Northern Renaissance art, such as the Michelangelo's David (Flaishman *et al*., 2007).

Fig also mentioned in the first Qur'anic verses in Sura al Teen. The Qur'an saith: "I swear by the Fig and the Olive". Fig had been stated as one of the only five plants in the Holy Quran, apart from olives, grapes, pomegranate and dates. As said by Hazrat Abu Darda (Radiallaho Anhu), someone sent fig fruits to the prophet Muhammed and he said, "Eat it as it cures various diseases". Ibn Seereen, a scholar in the science of dreams, proposed that if figs was presented in dreams, it had indicated the wealth and prosperity (Ahmad *et al*., 2009).

In the Greek mythology, the fig was presented to Bacchus (Roman God of Agriculture) and used in religious ceremonies (Flaishman *et al*., 2007). Fig has also made an appearance in the Olympic Games, which winners were crowned with fig headdress and ate the fig Ovid, a Roman poet, mentioned that figs were sent as a gift during the Roman New Year celebration (as cited in Flaishman *et al.*, 2007).

2.1.4 Distribution, habitat and climatic conditions

F. carica is native to the Southwest Asia and the Eastern Mediterranean region, and spread from the Turkey to Spain and Portugal (Chawla *et al*., 2012). It had been introduced either deliberately or accidentally to countries that are out of its native distributional range due to human migration (Flaishman *et al*., 2007). The 'Mission' fig that origin to California was introduced to the New World by the Spanish missionaries in the mid-sixteenth century and it was once again widely propagated in southern Spain for centuries (Condit, 1955). It has been commercially cultivated in the United State and Chile, and small areas of Arabia, Persia, India, China and Japan due to high global market demands. There is also the fig plantation located in India, next to Pune (Maharastra), and Bellary and Anantapur regions (South India). Moreover, the fig tree becomes the well-known courtyard plant in Punjab, Uttar Pradesh and Mysore (Chawla *et al*., 2012).

F. carica was originally growing well in semi-arid subtropical regions with cool winter and dry summers, but it can adapt to the tropical and subtropical region (Stover *et al*., 2007). Fig can be found in the diverse habitats, namely infertile rocky land, woodland, scrubland, and hot dry soil (Lansky and Paavilainen, 2011). It prefers well-drained soil such as sandy-clay loam with pH ranging from 6.0 to 8.0, but it has a high adaptability to a wide range of soil types, such as limestone, light sand, rich loam and heavy clay. Fig tree is moderately salt tolerant which had been proven on *F. carica*, cv. 'Brown Turkey' of which it was able to withstand water with salinity levels of 5.2dSm^{-1} (Alswalmeh *et al*., 2015; Morton, 1987)

2.1.5 Socioeconomic values

With reference to a report by Food and Agriculture Organization of the United Nations (FAO) (2020), the plantation areas for figs were reported to cover about 303 506 hectares worldwide with the production of 1 157 025 tonnes in 2018. The countries lead in fig production are Turkey and Egypt, they produced approximately 50% of the world's production, next are Iran, Algeria, and Morocco. A total of 64% of fig production was achieved by these top five countries. Nevertheless, Turkey is also the world's biggest exporter of fig, followed by the United States, Spain, Syria and Greece. On the other hand, FAO also reported that the leading import countries are Germany, France, Italy and the United States. The United States had exported fig worth more than $15.5 million in 2017/2018 with the total value of both fresh and dried figs imported into the country nearly $52.1 million. In Malaysia, fig fruits are rarely found in the local markets due to the limited commercial farms with constant fruit production. Most of the fig orchards in Malaysia are either producing limited number of fruits or are having issues with the consistent quality and supply of fruits from the commercial cultivars. There is great potential of fig commercialisation in Malaysia due to the high market demand for fresh and processed produce. Due to the limited number of plant stocks, the local prices of fig cutting is approximately RM30 to RM100 per cutting and can go as high as a few hundreds depending on the cultivar (Mohammad Rahimi *et al*., 2019)

Different cultivars can be processed into different products based on their taste and texture The 'Mission' cultivar can be manufactured into dried fruit, paste or concentrated juice, while the cultivars 'Kadota' and 'Adriatic' are mostly used for paste (Crisosto *et al*., 2011). In California, fig production was mainly channelled to the dried fig market (Tous and Ferguson, 1996), and also used in cookie and energy bar companies. Figs can be used

in the preparation of food products such as pastries, snack, cooked dishes and jam. In addition, low quality dried figs can also used to make coffee and juice concentrate (Crisosto *et al*., 2011). Its latex also functioned as a curdling agent in milk production (Lansky and Paavilainen, 2011). Its wood is used for hoops, wreaths, ornaments and others. Fig wood also can be saturated with oil, coated with emery and used as a hone (Marwat *et al.*, 2009). In the past few years, the facial cream containing fig fruit extract was proven to significantly effect on skin melanin, trans-epidermal water loss, hydration and sebum values (Khan *et al.*, 2014). The facial cream supplemented with 4% concentrated fruit extract was found to be able to reduce the skin melanin, epidermal water loss and skin sebum, and improve the skin hydration. It showed the potential of fig facial cream to against hyperpigmentation, acne, freckles and wrinkle (Khan *et al.*, 2014).

2.1.6 Nutritional and pharmacological values

Consumption of fig is known as one of the healthiest diet and is related to longevity (Trichopoulou *et al.*, 2006). The leaves, fruits and latex of *F. carica* can be used to appease one's hunger and enhance the human health (Barolo *et al.*, 2014). In the present day, most commercial fig production is focusing on dried or otherwise processed forms, since the ripe fruit easily damaged and rotten (Flaishman *et al.*, 2007). According to the data of the Dietary Reference Intakes (DRI) and the Nutrient Composition reported by the Food and Nutrition Board of the United States Institute of Medicine and the United States Department of Agriculture (USDA) respectively, dried figs have a higher nutritional score among the other dried fruits namely dried dates, raisins and dried apricot reported (Khatib and Vaya, 2010).

With reference to the nutrient composition of dried figs shown in Table 2.1, it is evident that the fig fruits consists of a high concentration of minerals and vitamins, and is free from fat, sodium and cholesterol that helps build up a strong immune system (Vinson, 1999). Fig contains high levels of calcium and is an essential calcium resource for birds and mammals. The potassium and magnesium contents of fig also help in stabilizing the insulin release. Besides, both fresh and dried figs also provide many fibres and polyphenols that possess the health-promoting potentials (Barolo *et al*., 2014). For instance, soluble fibre plays a significant role in controlling blood glucose and decreasing the blood cholesterol contributing to weight loss when added as a supplement to the diet (Barolo *et al*., 2014). Phenolic compounds are the secondary metabolites commonly found in plant species including fig plants. It acts as natural antioxidants, plays significant roles in decreasing or transferring hydrogen atoms to other compounds, eliminating free radicals, and quenching singlet oxygen (Çalişkan and Aytekin Polat, 2011). The elimination of free radicals such as reactive oxygen species prevents lipid peroxidation and then decreases the pathways of inflammation (Aktumsek *et al*., 2011; Mothana *et al*., 2011). As a result, the phytochemical composition of fig may prevent serious health disorders, namely neurodegenerative disorders, diabetes, arteriosclerosis and cancer.

Table 2.1: Nutrient composition of uncooked dried 'Mission' figs (USDA National Nutrient Database for Standard Reference 1 April 2018)

Nutrient	Unit	1Value per 100 g	1 cup = 149.0g	1 fig = 8.4g
Proximates				
Water	g	30.05	44.77	2.52
Energy	kcal	249	371	21
Protein	g	3.3	4.92	0.28
Total lipid (fat)	g	0.93	1.39	0.08
Carbohydrate, by difference	g	63.87	95.17	5.37
Fiber, total dietary	g	9.8	14.6	0.8
Sugars, total	g	47.92	71.4	4.03
Minerals				
Calcium, Ca	mg	162	241	14
Iron, Fe	mg	2.03	3.02	0.17
Magnesium, Mg	mg	68	101	6
Phosphorus, P	mg	67	100	6
Potassium, K	mg	680	1013	57
Sodium, Na	mg	10	15	1
Zinc, Zn	mg	0.55	0.82	0.05
Vitamins				
Vitamin C, total ascorbic acid	mg	1.2	1.8	0.1
Thiamin	mg	0.085	0.127	0.007
Riboflavin	mg	0.082	0.122	0.007
Niacin	mg	0.619	0.922	0.052
Vitamin B-6	mg	0.106	0.158	0.009
Folate, DFE	µg	9	13	1
Vitamin B-12	µg	0	0	0
Vitamin A, RAE	µg	0	0	0
Vitamin A, IU	IU	10	15	1
Vitamin E (alpha-tocopherol)	mg	0.35	0.52	0.03
Vitamin D (D2 + D3)	µg	0	0	0
Vitamin D	IU	0	0	0
Vitamin K (phylloquinone)	µg	15.6	23.2	1.3
Lipids				
Fatty acids, total saturated	g	0.144	0.215	0.012
Fatty acids, total monounsaturated	g	0.159	0.237	0.013

Fatty acids, total polyunsaturated	g	0.345	0.514	0.029
Cholesterol	mg	0	0	0
Amino Acids				
Tryptophan	g	0.019	0.028	0.002
Leucine	g	0.116	0.173	0.01
Lysine	g	0.081	0.121	0.007
Cystine	g	0.033	0.049	0.003
Aspartic acid	g	0.618	0.921	0.052
Glutamic acid	g	0.217	0.404	0.023
Glycine	g	0.098	0.146	0.008
Proline	g	0.556	0.828	0.047
Other				
Caffeine	mg	0	0	0
Alcohol, ethyl	g	0	0	0

2.1.7 Conventional propagation of fig plants

The fig tree is often cultivated via vegetative propagation namely, cutting and air layering (Brien and Hardy, 2002). For instance, rooted hardwood cuttings (20 to 25 cm in length) with many nodes are widely used for fig propagation (Brien and Hardy, 2002). Hardwood cutting exiced from the basal and median section of branches was treated with root inducing hormone like lndole-3-butyric acid (IBA) prior to cultivated in well-drained soil mix to grow roots (Flaishman *et al*., 2007). Antunes *et al*., (2003) found that 100 $mg.L^{-1}$ IBA was most efficient concentration to improve the sprouting percentage of cuttings of 'Roxo de Valinhos' (100%). Air layering is an alternative propagation method for fig cultivars that results in low rooting ability as observed in the cultivar 'Brunswick', 'Nazareth' and 'Montes' (Bisi *et al*., 2016). This method assist fig propagation especially cultivars with desirable traits like superior fruiting and also used for the selection of planting materials (Flaishman *et al*., 2007)

In Malaysia, fig is still a novel plant as it is new and only limited plant stocks are available for commercialisation. Besides, the conventional propagation method are easy, but its multiplication rates are comparatively low (20-40%) due to the plant materials excised only from upright branches causing difficulties in rooting which is not an efficient propagation method for large scale planting (Darwesh *et al*., 2014). The plant stocks propagated via conventional method is also slow glowing resulting in less fruit production. Thus, microproagation is introduced as an alternative propagation method that can ensure rapid and mass propagation for this plant. It can provide an incessant supply of the fig plantlet stocks for large scale field propagation at a consistent rate without being afftected by the external environment.

2.2 Tissue culture of *Ficus carica*

Plant tissue culture is the techniques applied in plant propagation by culturing living cell, tissues or organ (also known as explants) on the culture medium under aseptic conditions (Yildiz, 2012). An effective tissue culture protocol ensures the rapid and mass propagation of new cultivars, plants with desirable traits and genetically identical plants. Tissue culture is also an alternative propagation method, especially for the plants with nonviable seeds or without seeds. It can produce disease-, pest- and pathogen-free plants for commercial purpose (Yildiz, 2012).

Numerous studies regarding to micropropagation of fig had been published in the past. Researchers used different explants for their studies, including single shoot tips (Demiralay *et al*., 1998; Gella *et al*., 1998; Hepaksoy and Aksoy, 2006; Murithi *et al.*, *1982)*, nodal explants (Fráguas *et al*., 2004), leaves (Dhage *et al*., 2012; Kim *et al*., 2007; Soliman *et al*., 2010) or apical meristems and axillary buds (Al-Khaybari, 2008; Fráguas *et al*., 2004; Soliman *et al*., 2010). The explant is the plant cell, tissue or organ used to start-up a tissue culture experiment. The selection of proper explant influences the results of tissue cultures on their rates of growth and regeneration since different explants have different regeneration potentials. It is significantly affected by the stage of cells in cell cycle, the availability or capability of plant tissue to transport phytohormones and the metabolic abilities of the cells. The meristemic tissue located in shoot tip, axiliary bud tip and root tip often used in tissue culture due to its cells are actively dividing, and containing or producing natural phytohormone, such as auxins and cytokinins (Akin-Idowu *et al*., 2009). These explants have high shoot regeneration ability without the callus formation at the proper concentration and combination of plant growth regulators. Genetic stability of

culture can be maintained via meristem tissue culture and somaclonal variations occurrence can be reduced via preventing indirect organogenesis. Several studies regarding to the use of meristem tissues in the micropropagation of fig have been reported, those explants included single shoot tip (Danial *et al*., 2014; Hepaksoy & Aksoy, 2006; Taha *et al*., 2013), apical buds (Kumar *et al*., 1998), or both apical shoot buds and nodal (Fráguas, 2004; Kim *et al.*, 2007). Apical buds with meristemic tissue was selected in the current study since this approach produces plants with high genetic stability. It does not required the cell dedifferentiation of differentiated cells instead it promote shoot induction from pre-existing meristems (Ngezahayo and Liu, 2014)

The culture media used in micropropagation of fig plants are varied depends on the explant type, cultivar, and propagation stage such as establishment, shoot multiplication and root induction (Pasqual and Ferreira, 2007). Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) and WPM (Woody Plant Medium) (Lloyd and McCown, 1981) are the media that widely used in the micropropagation of fig. MS medium is the most commonly used medium in plant tissue culture studies followed by WPM. It consists of high levels of mineral salt contents that are significantly improving the tissue and cell growth (Pasqual and Ferreira, 2007). Meanwhile, WPM is previously designed for the shoot cultivation of woody plants and has been applied in the propagation of bushes and trees (Pasqual and Ferreira, 2007). Several studies, for instance, Hepaksoy and Aksoy (2006), Kim *et al*. (2007), Mustafa *et al*. (2013), Danial *et al*. (2014) and Darwesh *et al*. (2014), has been reported using MS medium in the micropropagtion of fig. A study showed MS medium, either full or double strength, significantly improved the shoot number, leaf number and shoot length on *in vitro* multiplication of ‘Sultany’ and

‘Aboudi’ fig via shoot tip culture (Mustafa *et al*., 2013). In contract to previous study conducted by Mustafa *et al*. (2013), Brum (2001) compared the effect of five culture media (MS, Knudson, WPM, White and Gamborg (B5) media) and different sucrose concentrations (0, 15, 30 and 45 g L^{-1}) on the micropropagation of ‘Roxo de Valinhos’ and results indicated that WPM medium supplemented with 20g sucrose improved the shoot multiplication and root induction of fig. Both media were chosen and studied on ‘Violette de Solliès’ because they are the most reliable media for most of the plant species.

Plant growth regulators (PRGs) have played significant roles in controlling the plant morphogenesis in the plant tissue culture. PRGs such as auxins, cytokinins, gibberellins, abscisic acid (ABA), and ethylene are widely used in the tissue culture studies. Shoot proliferation is one of the important stages in tissue culture and mainly regulated by cytokinins that trigger the initiation and activity of axillary meristems. Cytokinins used in shoot multiplication of fig consist of BAP (6-benzyloaminopurine), kinetin (N-2-furanylmethyl-1H-purine-6-amine), zeatin (6-4-hydroxy-3-methyl-trans-2-butenylaminopurine) and TDZ (thiazuron-N-phenyl-N-1,2,3 thiadiazol-5ylurea). Cytokinins usually involve in promoting cell division, shoot formation and axillary shoot proliferation, and inhibiting root formation (Saad and Elshahed, 2012). Several studies have been carried out to study the effectiveness of cytokinins on the multiple shoot formation of different fig cultivars. Fráguas *et al*. (2004), Mustafa and Taha (2012) and Shatnawi *et al*. (2019) studied the effect of cytokinins on shoot proliferation of fig cultvars, Roxo de Valinhos’, ‘Sultany’, ‘Aboudi’ ‘White Adcy’ and ‘Salti Kodari’. However, the addition of low concentration of auxins might required despite the auxin-effect is indirect (Ward and Leyser, 2004). The combination of auxins and cytokinins at specific rations

are significant for the shoot and root induction of *F. carica*. The profound effect of combined hormones (auxin-cytokinin) have been observed in the fig studies conducted by Mustafa and Taha (2012) and Shatnawi *et al*. (2019). Both single and combination hormone effects were tested on VDS cultivar to optimise the culture media used for plant growth and development.

Activated charcoal is one of the complex additives that widely used in tissue culture which also part of the experiment in present study. It derived from wood and has many small pores with large internal surface area. The addition of AC may promote or inhibit the cultures depend on the species and tissues, due to its ability to promote darkening of culture media; adsorb unwanted/inhibitory substances; absorb or release of PGRs and other organic compounds (Pan and Staden, 1998). AC plays a momentous role in micropropagation, somatic embryogenesis, synthetic seed production, shoot multiplication, rooting, orchid tissue culture and others (Thomas, 2008). For *F. carica*, the promotive effect of AC has also been confirmed in published reports namely Fráguas *et al*., (2004), Dessoky *et al.* (2016) and Dhage *et al.* (2012).

At rooting stage, auxins (namely NAA, IAA and IBA) are added into culture media to induce adventitious root formation from explants (Dobránszki and Silva, 2010). Auxins that often supplemented in basal media comprise of indole-3-acetic acid (IAA), indole-3-butric acid (IBA), 2, 4-dichlorophenoxy-acetic acid (2, 4-D) and naphthalene-acetic acid (NAA). The effects of auxin on tissue culture, including the callus induction, stimulation of cell growth, shoots and root formation, formation of somatic embryos, and the stimulation of growth for shoot apices and shoot stem culture (Saad and Elshahed, 2012). The addition of auxins also had been reported to improve rooting of many fig cultivars,

namely ‘Conadria’ (Zayed *et al.*, 2018), ‘Sultany’, ‘Gizy’, ‘Aboudi’ (Hepaksoy and Aksoy, 2006), ‘Black Mission’, ‘Brown Turkey’ and ‘Brunswick’ (Metwali *et al*., 2014)

Acclimatization is the process of transferring micropropagated plants from *in vitro* to *ex vitro* environment which is vital for the structural and physiological adaptation of plants (Dobránszki and Silva, 2010). During acclimatization, the key points are slowly decreasing the humidity and increasing the light intensity at the same time to avoid plant loss (Dobránszki and Silva, 2010). Furthermore, soil pathogens and nematodes are one of the significant problems that lead to the loss of plants (Kilinç *et al*., 2007). Therefore the alternative ways established previously was soilless cultivation (Burrage, 1999). Growth substrates, namely Jiffy peat pellet, peat moss, perlite and vermiculite are commonly used during acclimatization. Besides, there is a propagation mix that specially designed for plant nursery such as BioChar soil mix. It is 100% natural soiless and free from contaminants such as bacteria, insect, fungus, heavy metal and animal waste. The water and nutrient retention capacity of these soil substrates were much better than soil and they also provide good aeration and drainage (Sirin *et al*., 2010). Soilless cultivation had also been successfully applied in the acclimatization of fig studies (Sirin *et al*., 2010; Kim *et al*., 2007).

2.3 Scanning electron microscopy (SEM) analysis

In vitro cultures with anatomical and physiological abnormalities often suffer from severe water loss during acclimatization and eventually died (Noi and Bonini, 1996). The excess of water loss might due to abnormal stomatal functioning, insufficient epicuticular

wax, and improper cuticle development (Majada *et al*., 1998). The occurrence of these abnormalities often linked to the high relative humidity of *in vitro* environment. Despite the high humidity improves shoot development of *in vitro* plants yet it also produces abnormal leaves (Kozai *et al*., 1992). To understand the stomata characteristics of *in vitro* leaves, scanning electron microscopy (SEM) is essential to observe and study leaf surfaces at high resolution. Prior to SEM analysis, plant tissues must undergo dehydration in order to withstand water removal during coating system and the microscopes operates under high vacuum (Pathan *et al*., 2009).

2.4 Genetic analysis using Random Amplified Polymorphic DNA (RAPD) and Start Codon Targeted (SCoT) markers

DNA-based molecular markers are the significant tools to assess the true-to-type and uniformity of the tissue culture plants. Somaclonal variation normally happened when callus is induced and results in the production of off-types plantlets. The production of off-types plantlets with undesirable character(s) will be rejected in commercial purpose. Factor inducing somaclonal variation, namely genotype, explant type, subcultures frequency, degree of dedifferentiation and hormone concentration should be considered in the micropropagation studies (Devi *et al*., 2017). The early assessment of genetic stability of micropropagated plants is required, particularly for long living woody plants, in order to avoid any severe deleterious effects in the future that cause the economic impact (Dessoky *et al*., 2016). DNA-based molecular markers namely, Restriction Fragment Length Polymorphisms (RFLPs), Random Amplified Polymorphic DNAs (RAPDs), Amplified Fragment Length Polymorphisms (AFLPs), Simple Sequence Repeats (SSRs), Inter Simple Sequence Repeats (ISSRs), DNA Amplification

Fingerprinting (DAF) and Start Codon Targeted (SCoT), have the ability to detect the any kind of genetic variability at the DNA level.

RAPD (pronounced 'rapid') analysis is widely used in identifying germplasm, screening of off-types, studying genetic diversity and evaluating the genetic stability of conserved germplasm (Devi *et al*., 2017). RAPD markers are decamer DNA fragments amplified from the PCR reaction of random segments of genomic DNA (gDNA) with single primer of arbitrary nucleotide sequence. RAPD will bind randomly to the sequence so no details of the DNA sequence of targeted gene is required, which differs with conventional PCR analysis. RAPD markers extensively applied in the assessment of genetic stability of many plant species such as mulberry (Saha *et al*., 2016), cassava (Osena *et al.*, 2017) and banana (Devi *et al*., 2017).

Most importantly, this marker system was previously used to access the genetic stability of different fig variety such as KSA variety (Dessoky *et al*., 2016) and investigates the genetic diversity of fig varieties including 'Violette de solliès'(Ciarmiello *et al*., 2015; Khadari *et al.*, 1995). Dessoky *et al*. (2016) conducted a study on the establishment of micropropagation protocol of KSA variety of *F. carica* and assessment of the genetic fidelity of micropropagated plants using RAPD and ISSR markers. Selected six RAPD and five ISSR primers produced 109 clear and scorable bands which ranged from 250 to 1550 bp in size. A total of 103 monomorphic bands (94.5%) and 6 polymorphic bands (5.5%) was obtained in this study. These results indicated the occurrence of somaclonal variation, but its effect to the micropropagated plants was small due to the low ratio of polymorphism between mother plants and micropropagated plants.