ESTABLISHMENT OF TISSUE CULTURE PLANTING MATERIALS OF WHITE STRAWBERRY [*Fragaria* × *ananassa* var. Pearl White (Iroha-001)] FOR INDOOR FRUIT FARMING WITH LED SYSTEM

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by

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LIST OF ACRONYMS AND ABBREVIATIONS

%	Percentage
µ mol/s	Micromole per second
µg/mL	Microgram per microlitre
µL	Microlitre
µM	Micromolar
^{o}C	Degree Celsius
µ mol/s	Micromole per second
2,4-D	2,4-Dichlorophenoxyacetic acid
2-iP	6-(γ,γ-Dimethylallylamino)purine
A	Absorbance
AFLP	Amplified fragment length polymorphism
ANOVA	Analysis of variance
APX	Ascorbate peroxidase
ASC	Ascorbate
BAC	Biologically active compounds
BAP	6-Benzylaminopurine
bp	Base pair
BR	Blue and red
BSA	Bovine serum albumin
CAT	Catalase
CO_2	Carbon dioxide
cm	Centimetre
DAMD	Directed amplification of minisatellite DNA
DMHDA	Dimethylhexadecylamine
DNA	Deoxyribonucleic acid
ddH_2O	Double distilled water
EC	Electrical conductivity
EM	Electromagnetic
EDTA	Ethylenediaminetetraacetic acid

FAA	Formalin: Acetic acid: 95% alcohol
FR	Far-red
g	Gram
g/L	Gram per litre
GSH	Glutathione
h	hour
HCl	Hydrochloric acid
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
ISSRs	Inter simple sequence repeats
LECA	Lightweight expanded clay aggregate
LEDs	Light-emitting diodes
MDA	Malondialdehyde
MEL	Melatonin
mg/L	Milligram per litre
mL	Millilitre
mm	Millimeter
MS medium	Murashige and Skoog's (1962) medium
NAA	1-Naphthaleneacetic acid
NaOH	Sodium hydroxide
nm	Nanometer
PCIB	p-chlorophenoxyisobutyric acid
PAR	photosynthetically active region
PCR	Polymerase chain reaction
PGR	Plant growth regulator
POX	Peroxidase
PPFD	Photosynthetic Photon Flux Density
pH	Potential hydrogen
PSI	Photosystem I
PSII	Photosystem II

R^2	R-squared, coefficient of determination in regression
RAPD	Random amplified polymorphic DNA
RGB	Red: green: blue
ROS	Reactive oxygen species
rpm	Revolutions per minute
SEM	Scanning electron microscope
SOD	Superoxide dismutase
SPAR	Single primer amplification reaction
SSR	Simple sequence repeats
T_a	Annealing temperature
TBA	Tert-butyl alcohol
TBE	Tris-Borate-EDTA
TDZ	Thidizuron
TEM	Transmission electron microscopy
TIBA	2,3,5-triiodobenzoic acid
UV	Ultraviolet
v/v	Volume per volume
w/v	Weight per volume
UV	Ultraviolet

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PENUBUHAN BAHAN PENANAMAN KULTUR TISU STRAWBERI PUTIH [*Fragaria* × *ananassa* var. Pearl White (Iroha-001)] UNTUK PERTANIAN BUAH DALAM RUANGAN DENGAN SISTEM LED

ABSTRAK

Penyelidikan ini telah dijalankan untuk mengkaji kepelbagaian kesan spektrum cahaya yang berbeza dan kesan penambahan melatonin terhadap penumbuhan, pembangunan, dan kestabilan genetik bahan kultur tisu daripada pokok strawberi Jepun Pearl White [*Fragaria* × *ananassa* var. Pearl White (Iroha-001)]. Strawberi Pearl White adalah suatu buah yang jarang dan terpilih kerana penampilannya yang menonjol dan mempunyai keunikan rasa tersendiri. Warnanya putih yang lazat dan rasa manis, menjadikan buah ini hidangan istimewa yang digemari oleh peminat dan pakar buah. Strawberi putih telah terpilih sebagai tumbuhan model kerana ia merupakan sejenis tumbuhan yang bernilai tinggi, tetapi sukar untuk ditanam. Disebabkan penghasilan buah yang kurang, keperluan sinaran cahaya yang tepat dan pemeliharaan yang memerlukan tenaga buruh yang tinggi, ia mendapat permintaan yang tinggi dan justeru sesuai untuk penyelidikan buah yang terbaik ini. Kajian ini menggunakan pendekatan menyeluruh, dengan menggabungkan analisis fisiologi, histologi, dan molekul untuk memperjelas interaksi yang rumit antara faktor tersebut serta implikasi terhadap pembiakan berasaskan teknik kultur tisu. Pensterilan permukaan dengan larutan Clorox 30% (Kaedah 1) telah dikenal pasti sebagai protokol yang paling berkesan, memastikan sumber bahan yang bersih dan bebas daripada sebarang pencemaran untuk eksperimen. Penyelidikan selanjutnya mendedahkan bahawa medium optima untuk menggalakkan pertumbuhan

tunas gandaan dan pembiakan melibatkan penambahan 2 µM TDZ dalam medium Murashige dan Skoog (MS), tanpa keperluan auxin semasa pertumbuhan akar. Pokok yang telah berakar berjaya menyesuaikan diri dengan keadaan *ex vitro*, menghasilkan buah yang sihat dalam tempoh tiga bulan. Pencahayaan memainkan peranan penting dalam penggalakan tunas, dengan set yang melibatkan RGB + FR LED pada intensiti cahaya 41.70 µmol/s menunjukkan keberkesanan yang lebih tinggi, menghasilkan jumlah tunas yang tertinggi. Sebaliknya, tunas individu yang berakar adakah paling berjaya dengan medium tanpa hormon di bawah pencahayaan LED putih pada intensiti cahaya 33.00 µmol/s. Analisis mikroskopi pengimbas elektron (SEM) mengesahkan bahawa LED yang digunakan tidak mempunyai kesan buruk terhadap morfologi daun dan susunan sel. Selain itu, kajian ini menekankan peranan penting melatonin dalam mengawal organogenesis akar dan tunas, memelihara tisu tumbuhan, dan melambatkan penuaan daun dalam pokok kultur tisu strawberi Pearl White Jepun. Kepekatan melatonin yang optima sebanyak 15 µM telah dkenalpasti untuk menggalakkan perkembangan tunas dan akar. Penilaian kestabilan genetik yang dijalankan dengan menggunakan penanda molekul ISSR, DAMD, dan SCoT menunjukkan keseragaman genetik pokok kultur tisu Pearl White Jepun yang disemai dalam radas. Walau bagaimanapun, kajian ini mendedahkan bahawa peningkatan dalam kitaran subkultur menyebabkan variasi somaklonal, menekankan keperluan pengurusan teliti dalam proses subkultur untuk mengekalkan konsistensi genetik. Kesimpulannya, penyelidikan ini menyediakan informasi penting dalam mengoptimakan pertumbuhan dan memastikan kelestarian teknik propagasi berasaskan kultur tisu untuk pokok strawberi Pearl White Jepun [*Fragaria* × *ananassa* var. Pearl White (Iroha-001)].

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ABSTRACT

This research investigates the multiple effects of variations in light spectrum and melatonin supplementation on the growth, development and genetic stability of *in vitro*-cultured Japanese Pearl White strawberry plants [*Fragaria* × *ananassa* var. Pearl White (Iroha-001)]. The Pearl White strawberry is a rare and exquisite fruit known for its striking appearance and unique flavour profile. Its luscious white colour and sweet, floral taste make it a coveted delicacy among fruit enthusiasts and connoisseurs. The white strawberry was chosen as a model plant because it is a high-quality, difficult-to-grow plant. Due to their low fruit yield, precise light treatment and labour-intensive cultivation, they are in high demand and therefore an ideal fruit for research. The study uses a comprehensive approach combining physiological, histological, and molecular analyses to elucidate the intricate interactions between these factors and their impact on tissue culture-based propagation. Surface sterilisation with a 30% Clorox solution (protocol 1) proved to be the most effective protocol, ensuring a clean and contamination-free starting point for the experiment. Subsequent studies revealed that the optimal medium for inducing multiple shoots and promoting proliferation involved the use of a 2 µM TDZ supplement in the Murashige and Skoog (MS) medium and without the need of auxins during root induction. The rooted plants successfully acclimatised to the *ex vitro* conditions and bore healthy fruit within a period of three months. Light conditions played a pivotal role in shoot

induction, with the RGB + FR LED treatment with a light intensity of 41.70 µmol/s showing superior efficacy and producing the highest number of shoots. In contrast, rooting of individual shoots in a hormone-free medium under white LED illumination with a light intensity of 33.00 µmol/s was the most successful. Scanning electron microscopy (SEM) analyses confirmed that the LEDs used had no detrimental effects on leaf morphology and cellular arrangement. Furthermore, the study emphasises the important role of melatonin in regulating root and shoot organogenesis, maintaining plant tissue and delaying leaf senescence in Japanese Pearl White strawberry. An optimal concentration of 15 µM melatonin was found to promote shoot and root development. Evaluation of genetic stability using ISSR, DAMD and SCoT molecular markers emphasised the genetic uniformity of *in vitro* cultivated Japanese Pearl White strawberry plants. However, the study showed that an increase in subculturing cycles led to the occurrence of somaclonal variations, emphasising the need for careful management of the subculturing process to maintain genetic consistency. In conclusion, this research provides valuable insights into optimising growing conditions and ensuring the sustainability of tissue culture-based propagation techniques for Japanese Pearl White strawberry [*Fragaria* × *ananassa* var. Pearl White (Iroha-001)].

CHAPTER 1

INTRODUCTION

White strawberries, which botanically belong to the genus *Fragaria*, are an extremely rare fruit speciality from the Rosaceae family. White strawberries are grown commercially worldwide in different varieties, and each variety differs in appearance, flavour, and texture. White strawberries are not a new variety. Alpine, Beach, and Pineberry varieties have occurred spontaneously in the wild for generations as mutations of red strawberries. More than 50 varieties of white strawberries have been developed for the modern market, and the fruit has been sold as a delicacy since its introduction to the market. Among the most popular strawberry varieties are Pearl White, White Rabbit, and White Jewel (Specialty Produce, 2023).

The presence of one of the critical ripening proteins called 'Fra a1' found in strawberries is the main explanation for this colour variation. At the beginning of a strawberry's life cycle, the flower turns into a pea-sized fruit. This protein is insufficient or not present at all in most white strawberries. This is why they remain white even when ripe and do not turn red. In summary, white strawberries are white because they lack the potential to turn red (Muñoz et al., 2010; Strawberry, 2022). Apart from their colour, white strawberries are bigger, softer, and sweeter than red strawberries (Alexandra, 2022). Due to their novelty and scarcity, they can fetch exceptionally high prices of around $10 (RM40) per fruit.

White strawberries were chosen as a model crop because they are high-value plants that are notoriously difficult to grow. Their plants have limited fruit production and require

precise light treatment and a labour-intensive growing method. Furthermore, importing mother plants is not economically feasible, and sourcing healthy stock for propagation using conventional methods is not feasible. In addition, white strawberries are in high demand, but due to limited global availability, the cost of them is exorbitantly high (up to $10 per fruit). This makes them a perfect candidate for research and development. The white strawberry variety selected in this study is the Japanese Pearl White strawberry.

In plant tissue culture, plants are propagated in a sterile laboratory environment using small pieces of plant tissue, such as buds, leaves or stem cells. The tissue is placed on a nutrient-rich growth medium, and divides and differentiates under controlled conditions of light, temperature, and humidity to form a new plant (Singh, 2015; Phillip & Garda, 2019). This approach is used for various applications, including plant breeding, genetic engineering, and the commercial production of disease-free plants (Arencibia, 2000). Since the entire process is carried out in a controlled sterile environment, external environmental factors such as seasons and weather are no longer a problem, so that fresh plantlets can be produced at any time (Ahloowalia et al., 2002).

The tissue culture of plants is strongly dependent on light. White fluorescent light is generally used to illuminate *in vitro* plants in the tissue culture room. However, fluorescent light consumes more energy (Yu et al., 2020). Therefore, light-emitting diodes (LEDs) are nowadays used to support plant growth in plant tissue culture laboratories. LEDs are practical lighting devices that convert electrical energy into electromagnetic (EM) radiation. Their main features are low heat generation, high efficiency in converting electrical energy, and variable specific wavelengths of LEDs (Mickens et al., 2019). Sunlight, which emits light in all wavelengths of the visible, ultraviolet, and infrared

spectrum, has generally been planted as the primary light source (Kumar et al., 2018). However, due to the absorption of chlorophyll, plants generally require more red and blue light, making them the most important light wavelength ranges for photosynthetic assimilation of CO_2 by plants (Batista et al., 2018). Since plant growth responses to LED spectra are species-specific, the current study investigated the interactions of white strawberry plants with far-red, blue, green, red and violet LEDs in terms of morphology, biochemical content, and genetic stability.

Indoleamine N-acetyl-5-methoxytryptamine, also known as melatonin (MEL), is a non-toxic biological molecule with various functions in plants (Sharid et al., 2018; Erland & Saxena et al., 2018). The physiological functions of MEL in plants include functions in rhythmic and cyclic processes such as chronoregulation, seasonal and senescence processes, as well as modulation of reproductive development, seed germination, control of root and shoot organogenesis, maintenance of plant tissues and defence against biotic and/or abiotic stress (Erland & Saxena, 2018; Arnao & Hernández-Ruiz., 2019). Melatonin is also considered a master plant regulator due to its multiple functions for plants (Arnao & Hernández-Ruiz, 2019). MEL was used in this study to determine the effects on *in vitro* cultures of Pearl White strawberries by reviewing relevant studies.

The ultimate success of *in vitro* mass propagation is demonstrated by micropropagation of plants with a new approach when they exhibit successful *ex vitro* establishment and typical development (Bag et al., 2019). Thus, the quality of the regenerated plantlets was evaluated by anatomical, genetic and biochemical means. These evaluations clearly show how effective the clonal propagation techniques are.

Biochemical, histological and molecular DNA analyses were performed on the induced white strawberry plants under different LED illuminations and MEL concentration. The internal structure of the plant cell changes was evaluated using histological techniques. Biochemical analyses such as the determination of total carotenoid and chlorophyll (Su et al., 2014) and the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay (Shen et al., 2010) were performed on regenerated white strawberry plants to understand the relationships between growth and numerous applied treatments. At the same time, molecular analysis was performed to detect any polymorphisms or molecular abnormalities.

The aim of this study is to develop an efficient micropropagation protocol for the Pearl White strawberry and then to analyse the variations in the DNA and the structural, and biochemical characteristics of the regenerated plant in order to produce a clone of white strawberries that is disease-free and healthy so that it can later be used for indoor cultivar farming.

1.1 Research objectives:

I. To establish contamination-free *in vitro* Pearl White strawberry explants via surface sterilisation of *ex vitro* plant materials,

II. To establish an efficient tissue culture system of Pearl White strawberry to produce high-quality runners,

III. To investigate the effects of LEDs on the improvement of growth of Pearl White strawberry plantlets,

IV. To determine the effects of different melatonin concentrations on the growth of Pearl White strawberry plantlets,

V. To determine biochemical, SPAR-DNA molecular markers, and histology on plant growth characteristics of Pearl White strawberry.

CHAPTER 2

LITERATURE REVIEW

2.1 Rosaceae-An overview

Rosaceae is the rose family (order Rosales), which is medium-sized and comprises over 2,500 species in more than 90 genera (Moustafa& Cross, 2019). The family is mainly native to the northern temperate zone, where it occurs in various environments. Apples, almonds, cherries, pears, raspberries, and strawberries are economically important food plants; others, such as the rose, are cultivated as ornamental plants (Li et al., 2021). Members of the Rosaceae are typically woody plants, mainly shrubs or small to medium-sized trees that have thorns, spines, or prickles to deter herbivores (Zhao et al., 2021). Strawberry (*Fragaria*), cinquefoil (*Potentilla*), columbine (*Geum*), and goatsbeard (*Aruncus*) are examples of herbaceous perennials. Most members of the family have alternate leaves, and stipules, and small leaf-like structures at the base of the petioles are common (Sytsma, 2016).

2.1.1 Strawberry

The strawberry (genus *Fragaria*) belongs to the Rosaceae plant family. Strawberries are native to the temperate zones of the northern hemisphere, and cultivated varieties are widely grown worldwide (Britannica, 2022). Strawberries have a high content of phenols, known as flavonoids, and micronutrients such as folate, vitamin C, and minerals, that contribute to health (Giampiere et al., 2012; Giampiere et al., 2014). The fruit is highly valued for its characteristic flavour, bright red colour, juicy texture, and

sweetness (Zhang et al., 2008). It is consumed in large quantities, fresh or in prepared foods such as jam, juice, cakes, biscuits, milkshakes, and chocolates.

Strawberry plants are herbaceous plants with a fibrous root structure and a crown from which basal leaves emerge. The leaves are complex, with three leaflets that are often saw-toothed and hairy. The mostly white, but also scarlet-red flowers sit in tiny clusters on slender stems that emerge from the leaf axils, similar to the creeping stems above ground. The root system of an older plant becomes woody, and the “mother” crown sends out runners (e.g. stolons) that touch the ground and take root, thus expanding the plant vegetatively. Botanists classify the strawberry fruit as a “secondary fruit” and not as an actual berry. The pulp consists of the clearly expanded inflorescence and many natural fruits or achenes, commonly referred to as seeds (Britannica, 2022).

2.1.2 Economic importance of strawberry

Strawberries are highly valued commercially as berry fruits due to their nutritional value and delightful flavour. They belong to the rose family and are cultivated worldwide, which is particularly significant in Malaysia's horticultural sector. According to Simpson (2018), strawberries are grown commercially in 76 countries, with major producers including the United States, South Korea, Poland, Mexico, Egypt, Turkey, and Spain (Arief et al., 2023). Global production in 2012 totalled 4,516,810 tons, with significant cultivation in Estonia (Mirmajlessi et al., 2015). In the United States, strawberries rank as the fifth most popular fruit after bananas, apples, oranges, and grapes (Padmanabhan et al., 2016).

Strawberries are the most commercially produced and consumed berries due to their aroma, taste, and biochemical properties, leading to increased global production and

consumption (Oğuz et al., 2023). The strawberry production industry was valued at €2.05 billion (USD 2.2 billion) in 2015 (Horth & Campbell, 2017). Strawberry cultivation is expanding into subtropical conditions, reflecting a growing trend (Singh et al., 2023). Quebec, Canada, is recognized as North America's third-largest strawberry producer, following Florida and California (Gendron et al., 2018). Research focuses on various factors affecting strawberry quality, including farming systems, postharvest storage, and cultivation techniques (Barbieri et al., 2015; Hamilton et al., 2021). Pollinators, such as native mason bees, play a critical role in enhancing strawberry yield (Horth & Campbell, 2017). Diverse pollinator communities in strawberry fields have been linked to improved pollination and marketability, emphasizing the importance of biodiversity in agriculture (Sciligo et al., 2022; Ahrenfeldt et al., 2019).

Strawberries' cultivation in 76 countries underscores their global popularity and economic importance. Despite advancements in shelf-life extension and marketing, fresh berries remain perishable, necessitating local production to avoid high transport costs. Unlike tree fruits that can be stored longer for international distribution, strawberries require proximity to markets, influencing production locations and labour dynamics. Hand-harvesting of dessert strawberries creates local jobs and benefits the economy in growing areas. In regions like North America and Western Europe, harvesting relies heavily on seasonal and migrant labour, often accommodated on fruit farms during the season (Simpson, 2018).

2.1.3 White strawberry

The red colour is inseparable from strawberries. Most people do not realise that there are also many white strawberries. Some *Fragaria* species can be completely white

in colour. White strawberries come in various sizes, from tiny to large, and have an oval shape with curved, occasionally broad shoulders that taper to a rounded tip (Specialty Produce, 2023). The fruits of this extremely rare strawberry subspecies can grow very large, around 50 g, with the price per piece being USD 10 (RM 46.69). This high price is due to years of breeding, low yield, labour-intensive cultivation, the difficulty of growing some varieties and space (Laura, 2016). White strawberries have a unique flavour. It tastes like pineapple and has a sweet, candy-like aftertaste.

The most striking difference between white strawberries and red strawberries is the colour. The presence of one of the most important ripening proteins, 'Fra a1', in strawberries is the most important explanation for these colour differences. In the life cycle of a strawberry, the flower normally first turns into a pea-sized fruit. When strawberries containing this protein reach full maturity, they turn a red colour, which means they are ready to eat. In most white strawberries, this protein is either insufficient or not present at all. As a result, even when fully ripe, they remain white instead of turning red. So the reason why white strawberries are white is because they cannot turn red (Erik, 2017).

White strawberries are more distinctive and softer than red strawberries (Figueroa et al., 2008) and sweeter than their conventional counterparts (red strawberries). More than 50 cultivars of white strawberries are grown worldwide, each with its own flavour (Specialty Produce, 2023). The white subspecies of *Fragaria vesca* belong to the white strawberry cultivars, Pineberry, *Fragaria chiloensis*, White Jewel Strawberry and several more. Coastal strawberries, wild strawberries, Chilean strawberries (*Frutilla Chilena*) and South American strawberries are all names for white beach strawberries. *Fragaria*

chiloensis is the generic name for all these white strawberries. This strawberry species was decisive for the crossbreeding that led to today's large, red, plump strawberry varieties.

2.1.4 Pearl White

When it comes to white strawberries: The "fragrance of first love" has gained popularity, and several new varieties have been developed, including "Light Snow". However, this Pearl White variety is characterized by its exceptional whiteness and striking appearance. The shape is a slightly vertically elongated oval, the colour of the epidermis is partly light pink on a white background, and Pearl White feels much whiter in comparison to "Light Snow'. The achene is also red in colour and the red drops on a white background are beautiful (Figure 2.1).

Pearl White is a white strawberry variety bred by crossing by Mr Mitsuki Maeda, a biotechnology researcher and strawberry producer in Nara Prefecture, Japan. The registration application was published in 2013 (Heisei, 2013), and the variety was registered in 2015 (Heisei, 2015, p. 27). The official variety name is "Iroha-001", and Pearl White is a nickname or the product name in distribution. As the name "Pearl White" implies, it is known as a strawberry that stands out from the various white strawberries, and only the "achene" (crushed part) that can be seen on the surface of the strawberry is red（Kudamononavi，2018 "July to see first harvest of potential new brand of white 'Snow strawberry' 2021"）.

2.1.5 Characteristics of Pearl White

The new strawberry variety, Pearl White strawberry (Figure 2.1), is said to have a less acidic flavour and a more refined sweetness and fragrance. The shape of the fruit is a vertically elongated oval, and the size is medium. When ripe, the pericarp is white to pinkish white in colour and the bruised part turns red. As the pericarp is firm, it is easy to transport and is very popular as an order or gift. The shipping season is from December to April. It is mainly grown in the prefectures of Nara and Saga (Kudamononavi, 2018).

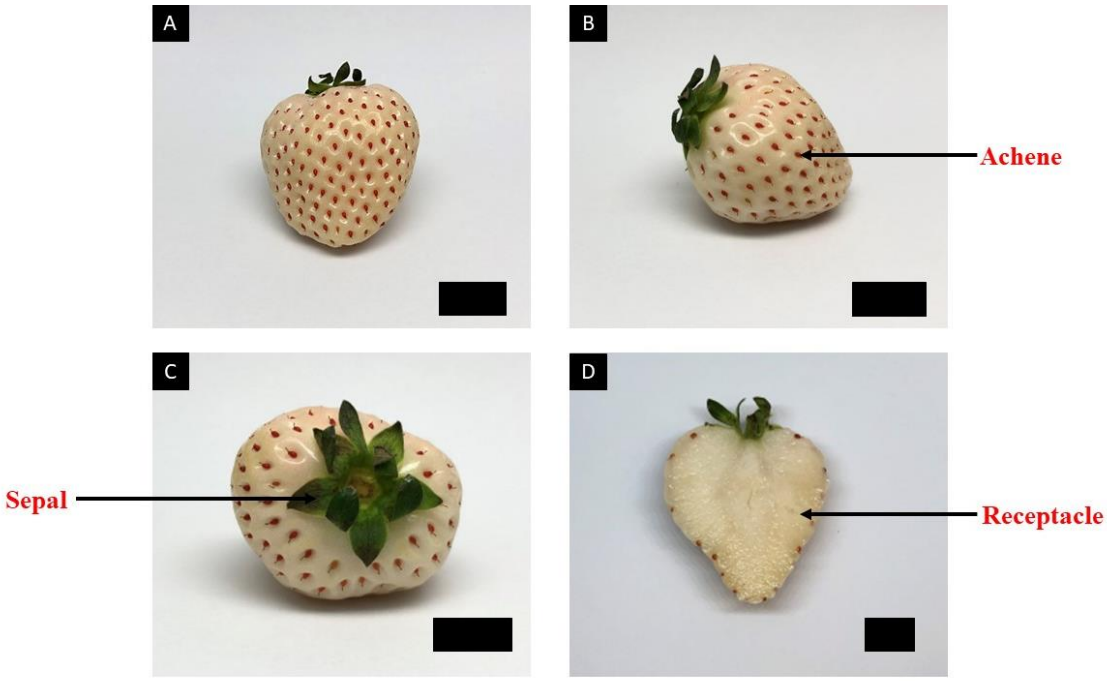

Figure 2.1: **The morphology and anatomy of a white strawberry fruit. (A) Pearl White strawberry, (B) Achene, (C) Sepal, and (D) The cross section is also pure white as shown in the photo, and it is also characterized by its low acidity and strong scent (Receptacle).** Scale bar: 1 column represents 1cm.

2.1.6 Strawberry diseases

Several soil infections damage the strawberry roots so that they eventually lose vigour and die. According to Hancock et al. (2008), there are two main problems that are common worldwide, namely red stele or red centre, caused by *Phytophthora fragariae* Hickman, and Verticillium wilt, caused by *Verticillium albo-atrum* and *V. dahlia*. Black root rot, caused by various organisms including *Pythium*, *Rhizoctonia* and the root lesion nematode (*Pratylenchus penetrans*), is also widespread.

Fusarium wilt or Fusarium yellows (*Fusarium oxysporum*) is a major problem in Japan, Korea, and Australia. Although fumigation with methyl bromide will soon be banned, the widespread use of fumigation to control soil diseases has increased interest in the development of resistant varieties. These varieties generally produce 50% less fruit without fumigation (Hancock et al., 2008). Three leaf diseases, including leaf spot (*Phomopsis obscurans*), Ramularia leaf spot (*Mycosphaerella fragariae*), and leaf blight (*Diplocarpon earliana*), are widespread and can cause significant damage. In Europe, New Zealand, and Korea, Alternaria leaf spot disease, often referred to as black leaf spot [*Alternaria alternata* (FR.) Keissler], also causes considerable damage.

Although it rarely causes financial damage, powdery mildew (*Sphaerotheca macularis*) is also present in most strawberry-growing regions. Throughout the world, strawberries are increasingly affected by angular leaf spot (*Xanthomonas fragariae*) (Hancock et al., 2008). Another widespread problem in strawberries is anthracnose, which leads to various symptoms such as fruit rot, crown rot and lesions on the runners, petioles and leaves. *Colletotrichum fragariae* and *Colletotrichum acutatum* are responsible for this strawberry anthracnose infection. Therefore, *in vitro* propagation of strawberries offers

significant prospects for commercial cultivation and overcoming the problem of strawberry diseases.

2.2 Tissue culture of strawberry

The tissue culture of plants, also known as micropropagation, is a process in which cells, tissue or the mother plant are cultivated in an aseptic environment. It is mainly used in forestry, agriculture, and horticulture (Oseni et al., 2018). Plant tissue culture is a powerful method for breeding the desired plant, as it enables the rapid and efficient production of many true-to-type clones of the selected plant.

Many living organisms can regenerate, and each has a different ability to regrow organs and tissues (Pulianmackal et al., 2014). This type of plant regenerative capacity is known as totipotency. It is a unique form of regeneration in which a single cell can regenerate an entire plant (Ikeuchi et al., 2016), and the ability of plant cells to become totipotent is an important factor in the success of plant tissue culture (Fehér, 2019). The plant material used in this method is called an explant. Explants are cells or tissues that are separated from a mother plant or a donor plant (Phillips & Garda, 2019). Apical buds, leaves, stem segments, and seeds are some types of explants that are used.

Strawberries were first propagated using micropropagation in 1974 due to two key factors: soil fungi, which severely damaged strawberry fields, and plants produced *in vitro* produced plants that, which appeared to generate rapidly produce more runners per plant. Due to genetic variation and particular the specific requirements for seed germination needs, it is challenging to grow strawberry plants from seeds. In addition, the percentage of cuttings that take root is low (Mohan et al., 2005; Palei et al., 2015). Strawberry plants grown using tissue culture are thought to be more expensive than plants derived grown

commercially from traditional propagation on a commercial basis. However, micropropagation of strawberries has several benefits advantages, including the ability to quickly increase rapidly propagate a virus-free stock and the enhanced improved ability of these plants to produce runners for sowing in the field (Lopez-Aranda et al., 1994; Palei et al., 2015).

In addition, tissue-engineered propagules require less storage space compared to conventional stoloniferous plants and *in vitro* storage can begin at any point in the production cycle (Swartz et al., 1981; Palei et al., 2015). Past experience with strawberry micropropagation shows that fruit yield increases by 24%, plants are more uniform, produce more runners and survive the field better than traditionally propagated plants (Kikas et al., 2006). Assuming that the source plant is disease free, another advantage of micropropagation is that stress from pests and pathogens is removed during the production cycle (Palei et al., 2015) to determine if the tissue culture approach could be utilised on a larger scale.

2.2.1 Surface sterilisation

To create aseptic cultures, the explants are separated from the mother or donor plant. They are then washed using the aseptic technique in a laminar flow bonnet, using various detergents or surfactants. This process is known as surface sterilisation (Cassells & Doyle, 2006; Phillips & Garda, 2019). There is an abundance of contaminants in the natural environment; spores and dust are two well-known examples. This also applies to plant parts, which have a variety of contaminants adhering to their surfaces. Before the desired plant tissue is inoculated onto a culture medium, the outer surface of the tissue must be sterilised in a series of procedures using various chemicals and surfactants such

as 70% ethanol and Clorox® to prevent contamination (Bhojwani & Razdan, 1986; Bhojwani, 2012).

However, surface sterilisation is only used on the surface of the explants. Trichomes are various epidermal appendages that can grow on any part of a plant to adapt to its environment (Evert, 2006). Trichomes on the explants can trap fungi and bacteria so that they are not washed away by chemical cleaners. Therefore, Tween 20® or 80® are usually used to increase the effectiveness of the surfactants. Even after surface sterilisation, endogenous, disease-infected plants are still contaminated. After surface sterilisation, endophytic bacteria and fungi that have invaded the vascular system remain inside the explant. Mercuric chloride has been used in various concentrations as a surfactant because it effectively sterilises the explants containing endophytic bacteria (Iliev et al., 2010).

Surface sterilisation must be optimised based on several criteria that should be taken into account. Before the explants are introduced into the *in vitro* environment, their sterility against contaminants can be influenced by several factors, including the type of plant material used, the species, the surface structure and the cleanliness of the environment. Until the desired axenic explants are achieved, optimisations can be made by changing the duration and concentration of sterilising agents (Hermayani et al., 2017).

2.2.2 Seed culture

The embryo, the endosperm, the perisperm, and the testa form a typical angiosperm seed. The imbibition process, during which the seeds absorb water, usually marks the beginning of seed germination. Germination is complete when the radicle emerges from the seed (Bewley & Black, 1994).

In general, seeds are used to establish a sterile culture for various reasons. Since this method is practical when there are few mother plants, it can be used to save the genetics of endangered ornamental plants such as orchids (Kauth et al., 2006). Furthermore, this technique avoids the problems associated with the sterilisation of *ex vitro* plant material. According to Loyola-Vargas and Ochoa-Alejo (2018), another advantage of using seeds as starting culture material is the possibility of maintaining and preserving the desired genetic diversity of a species. This is because crops can be planted with plants that have been bred from germ-free seeds that are easy to sow.

The strawberry is an aggregate fruit with a series of achenes (the actual fruit containing the seed) systematically arranged and attached to the epidermis of the receptor (the floral axis to which the various floral parts are attached). The huge, fleshy receptor, which comprises a pith, a rind and a vascular system, is the juicy, edible part of the fruit. After fertilisation, it grows in response to hormone production by the ovules (Smith et al., 2003). An effective way to produce many actively growing aseptic plants could be to optimise and further develop the process of *in vitro* seed culture. In the present study, the achenes were used as initial explants for the establishment of *in vitro* cultures, and the seedlings produced are then used in the course of the study.

2.2.3 Multiple shoot induction

Stem cuttings and seed germination are two time-consuming and financially costly traditional cultivation methods that utilise traditional cultivation methods. The large-scale and economic yield of crops grown using traditional farming methods needs to be questioned more (Gopi et al., 2006; Gana, 2011). The ability to produce multiple disease-free clonal plants from a single explant using the micropropagation technique is its main

advantage over traditional methods. Explants such as shoot tips or axillary buds are usually used for the mass production of new shoots (Sharma et al., 2015; Handique, 2016; Rohela et al., 2019). The explants are grown on solid media that also contain optimal plant growth regulators (PGRs) for shoot formation. Most of the multiple shoots formed per explant on the growth medium are usually obtained with cytokinins such as (6-Benzylaminopurine BAP), kinetin and thidiazuron (TDZ) (Shen et al., 2010; Abbas et al., 2018; Arti et al., 2018).

The induction of multiple shoots aims to increase the number of shoots from a single explant in order to increase propagation. In addition to promoting the growth of multiple shoots, cytokinin is often used to produce numerous shoots (Gaba, 2005). The desired effect only occurs if the cytokinin concentration is within the ideal range. However, if it is outside this range, growth is slowed or prevented (Huetteman & Preece, 1993). In practise, various cytokinins and additives have been used in different amounts and mixtures. Research results showed that the most popular technique was the induction of multiple shoots using plant parts from seedlings produced from seeds (Sangeetha & Venkatachalam, 2011; Padmanabhan et al., 2017; Tan et al., 2018).

The desired starting material was then selected and excised from the plantlets after the seeds had developed into plantlets of a certain predetermined age and size or after the desired plant component had emerged. Shoot tips, nodal segments and leaves are examples of these components. Most successful attempts to produce numerous shoots appear to involve BAP (Hiregoudar et al., 2003; Usman et al., 2005; Sangeetha & Venkatachalam, 2011; Padmanabhan et al., 2017; Tan et al., 2018). *In vitro* plants were used as the source of plant material, as this method ensures that the plant material is free of contaminants and

has not been affected by previous external environmental influences. Overall, the results were encouraging. Genetic analysis can be used to determine whether there are deviations when the stability of the genetics is questioned (Venkatachalam et al., 2018).

The induction of multiple shoots is interesting because it often produces multiple plantlets when detached and subcultured with shoot-inducing plant growth regulators (Sangeetha & Venkatachalam, 2011; Rency et al., 2018). Even if the group of numerous shoots is not separated to develop separately, the eventual plantlet contains many developmental sites that essentially function as a single shoot plant with a significant number of branches.

2.2.4 Root induction

Plants have different parts that are visible in their natural environment, including shoots, leaves, stems and roots. These organs were necessary for survival under *ex vitro* conditions and were therefore present. Under *in vitro* conditions, explants often take up nutrients through their cut surfaces, with larger surface areas facilitating the uptake of nutrients and growth regulators (Agustina et al., 2020). However, when not in an *in vitro* environment, the water and nutrient uptake system relies mainly on the roots, making them the only organ that regenerates after shoot induction. Many research results show that rooting can be successfully established. As the example of *Eurycoma longifolia* shows, root induction is often performed when an object of interest is made available by creating a root or a specific root trait (Hussein et al., 2012). Alternatively, *Agrobacterium tumefaciens* introduces the hairy root gene, followed by the ability of the tissue culture to regrow the desired region (Cardarelli et al., 1987).

Rooting is achieved by the addition of auxins in regeneration protocols aimed at producing complete plantlets. Indole-3-acetic acid (IAA), Indole-3-butyric acid (IBA) and naphthyl acetic acid (NAA) are three commonly used auxins that affect plants in different ways (Usman et al, 2005; Sangeetha & Venkatachalam, 2011; Rency et al, 2018; Tan et al, 2018). The initiation of roots generally does not follow the formation of shoots. When cytokinin is supplied, roots can occasionally form simultaneously with the development of shoots, a phenomenon known as auxin autonomy (George et al., 2008). In addition, in some bulb or perennial plants, the root can grow before the shoots if the root tissue is not completely removed from the explant (Panayotova, 2015). When cytokinin and auxin are combined, their different developmental patterns — which are both necessary but opposite — generally lead to cancelling effects, resulting in callus instead of the desired growth (Punja et al., 1990). It is therefore necessary to first promote either the shoot or the root before focussing on the other.

In an optimisation phase, different auxins can be tested to ensure the best rooting for Pearl White strawberries. IAA, IBA and NAA are some of them that are commonly available and often give convincing results (Adelberg, 1998; Lin et al., 2011). Once the optimal concentrations of each auxin have been determined, the concentrations can be modified by possible combination treatments.

2.2.5 Acclimatisation

Plants that have been micropropagated *in vitro* have characteristics that distinguish them from plants grown outdoors. Due to the perfect sterility of the *in vitro* environment, the plants cannot be invaded by bacteria, fungi or pests. The rapid transition from the *in vitro* environment to the outdoor environment would result in reduced survivability of the

plantlets and the loss of critical traits necessary for survival in the outdoor environment in the plantlets (Afreen, 2005). Any micropropagation study must end with the rooted plantlets being successfully acclimatised *ex vitro*. When the *in vitro* plants undergo the process of hardening, this is crucial for their survival. Therefore, a suitable procedure must be developed so that the plantlets survive the environmental changes.

During acclimatisation, *in vitro* plants experience biotic and abiotic stress. Abiotic factors such as light intensity, humidity and biochemical concentration are altered during acclimatisation. Biotic factors such as biotisation (soil microorganisms) can also affect the growth of *ex vitro* acclimatised plants (Chandra et al., 2010). Therefore, axenic root cultures are planted in tiny pots filled with sterile soil before the cultures are transferred to *ex vitro* settings. This demonstrates that the *in vitro* plants can successfully adapt to the soil conditions (Hazarika et al., 2006).

Despite all the successful efforts that have been reported and documented, they have hardly ever been explained in detail, as there seem to be significant information gaps when it comes to ensuring the high survival rate of acclimatised plants. Therefore, a clearly defined process is required for the correct acclimatisation of Pearl White strawberry seedlings.

2.3 Application of light emitting diodes (LEDs) in plant tissue culture system

Rapid technological progress is expected in plant tissue culture. A semiconductor diode known as a light-emitting diode (LED) emits multiple colours depending on the wavelength of the light (Olle &Viršile, 2013). Many different types of illumination lamps are currently available on the market. For *in vitro* plant tissue culture, conventional high-pressure sodium vapour lamps, fluorescent tubes, incandescent bulbs and metal halide

lamps are the general illumination sources available (Kim et al., 2004). However, these lamps have a broad spectrum of wavelengths that appear superfluous and are of low quality for promoting plant growth. They consume a lot of electricity but generate heat in an enclosed space without adequate ventilation (Dutta Gupta & Jatothu, 2013).

The quantity, quality and duration of light are the three main aspects of lighting that generally have a significant influence on the development or yield of plants. The amount of light available to plants influences how effectively they can photosynthesise. This quantity of light is also known as intensity. The quality of light, often referred to as the light spectrum, is used as an energy source to control the photomorphology of plants, including growth aspects such as plant height. The initiation of flowering is highly influenced by the duration of light, also known as the photoperiod. In a tissue culture room, the photoperiod is normally sixteen hours of light and eight hours of darkness. Nevertheless, different plants require different amounts of photoperiod for optimal growth depending on the species and variety (Siddique & Islam, 2015).

Fluctuations in light wavelengths can severely affect the growth and development of plants, as light is necessary for photosynthesis in plants. Due to their energy efficiency, low heat generation and tunable light wavelength specificity, LEDs have the potential to be a useful light source for growing plants (Mickens et al., 2019). Compared to conventional lighting, LEDs can provide additional illumination with higher efficiency (Van level, 2017). With the help of LED lights, the spectral composition is controllable by humans and allows the selection of the ideal light spectrum that influences photosynthesis and photomorphogenesis (Samuolienė et al., 2017).

LEDs have low energy requirements and produce special light spectra and radiation that have proven to be very effective in promoting plant development (Gupta & Jatothu, 2013). According to Gupta and Jatothu (2013), LEDs are referred to as cool light because they produce less heat than fluorescent tubes. LEDs have been shown to be a suitable substitute in plant tissue culture to promote plant growth and morphogenesis. They can replace fluorescent white light in the room where plant tissue cultures are incubated. In addition to blue:red in a variable ratio, red, green and blue (RGB) LEDs are often produced to replace fluorescent white light. The experimental setup proved to be effective and best suited to investigate the relationship between the brightness of the LEDs in different spectra and the complement of the composition of the medium.

2.4 Melatonin

Melatonin (5-methoxy-N-acetyltryptamine) is a very popular chemical active ingredient in both plants and animals. More than 100 plant species contain the melatonin hormone, which was first isolated in edible plants in 1995 (Dubbels et al., 1995; Paredes et al., 2009). Melatonin is a low molecular weight chemical found in all living organisms. It has a simple structure and a wide range of biological functions in all living organisms, from bacteria to mammals (Hardeland et al., 2011). It is associated with essential substances such as serotonin, tryptophan and indole-3-acetic acid (IAA). As a biological modulator of sleep, mood, retinal physiology, sexual behaviour, seasonal reproductive physiology, circadian rhythms, and immunological enhancement, melatonin is now the subject of numerous studies in humans (Arnao & Hernández, 2006).

A sizable and continuously expanding body of literature has examined the function of melatonin in plants ever since it was found in plants. Melatonin has generally been

found to be engaged in specific physiological processes in plants, acting as a cytoprotectant, circadian regulator, and growth promoter (Arnao & Hernández, 2006). Moreover, research has shown that it is crucial for biotic stress (Arnao & Hernández, 2015) and stimulates gene expression that aids plants in coping with biotic and abiotic challenges (Zhang et al., 2015).

As melatonin is an indoleamine, it might have typical auxin-like properties, such as controlling morphogenesis and promoting the development of new roots (Murch et a., 2001). According to a study by Sarropoulou et al. (2012), the explants of the sweet cherry rootstocks CAB-6P, Gisela 6, and M X M 60 benefit from melatonin's promotion of rooting. Besides, when combined with IAA, melatonin influences the regeneration of the lateral root system and adventitious roots in *Lupinus albus* seedlings and encourages rooting (Arnao & Hernández, 2007).

In addition, melatonin has strong scavenging abilities for reactive oxygen and nitrogen species (Galano et al., 2011). Melatonin has several beneficial properties, including its ability to scavenge free radicals, defend plants from oxidative stress or any other stressful situation at the cellular level, and minimise harm to macromolecules (Tan et al., 2002; Tan et al., 2007).

2.5 Histological analyses

The microscopic identification and examination of tissue is a crucial component of tissue culture research. It deals with the inspection of crucial internal tissue structures. Histology is the sectioning, staining and microscopic examination of animal and plant tissues to examine their anatomical structures. In histological studies, the plant or animal tissues to be examined are fixed, prepared by dehydration, embedded, and blocked in wax,