EFFECTS OF LED ON THE MICROPROPAGATION AND CALLUS INDUCTION OF *Zingiber officinale* var. *rubrum*

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EFFECTS OF LED ON THE MICROPROPAGATION AND CALLUS INDUCTION OF *Zingiber officinale* **var.** *rubrum*

by

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TO MY RESPECTFUL SUPERVISOR
PROFESSOR SREERAMANAN SUBRAMANIAM

TO MY BELOVED FAMILY

TO ALL THE ASPIRING PhD CANDIDATES

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LIST OF ACRONYMS AND ABBREVIATIONS

MS	Murashige and Skoog
PGRs	Plant growth regulators
BAP	Benzylaminopurine
EDTA	Ethylenediaminetetraacetic acid
TBA	Tertiary-butyl alcohol
TBA	Thiobarbituric acid
FAA	Formaline-acetic acid-alcohol
LED	Light emitting diode
UV	Ultraviolet
DNA	Deoxyribonucleic acid
DAMD	Directed amplification minisatellite DNA
GCMS	Gas chromatography-mass spectrometry
ROS	Reactive oxygen species
H_2O_2	Hydrogen peroxide
OH	Hydroxyl radical
SOD	Superoxide dismutase
CAT	Catalase
APX	Ascorbate Peroxidase
PCR	Polymerase chain reaction
PAR	Photosynthetic active reaction
ANOVA	Analysis of variance
SE	Standard error
SI	Similarity index
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy
M	Molar

mM	Millimolar
mg/L	Milligram per litre
mg/g	Milligram per gram
mg/mL	Miligram per mililitre
A	Absorbance
s^{-1}	per second
min^{-1}	Minute persecond
H	Hour
μL	Microlitre
μm	Micrometre
$U.mg^{-1}$	Unit per milligram
RPM	Rotation per minute
°C	Degree celcius
CLS	Cultivated leaf sheath
IVLS	*In vitro* leaf sheath
C2	Callus 2mg/L Picloram
C4	Callus 4mg/L Picloram
C8	Callus 8mg/L Picloram

KESAN LED TERHADAP MIKROPROPAGASI DAN INDUKSI KALUS

Zingiber officinale var. *rubrum*

ABSTRAK

Mikropropagasi *Zingiber officinale* var. *rubrum* yang berpotensi sebagai ubatan akan menghasilkan klon *in vitro* bebas penyakit yang tidak terhingga untuk tanaman; mencegah penempahan rimpang benih daripada penuaian. Oleh itu, penerapan diod pemancar cahaya (LED) ditentukan untuk mikropropagasi Z. *officinale* var. *rubrum*. Pemanjangan tunas (12.07 cm) dan bilangan akar (13.29) yang ketara dihasilkan di bawah LED merah. Spektrum ungu menghasilkan tumbuhan padat dengan bilangan daun (20) dan pucuk (5.57) yang tertinggi. LED hijau, merah, dan ungu mempersembahkan pembentukan mikrotunas yang paling hebat (5–5.9). Pengawalan sifat antioksidan Z. *officinale* var. *rubrum* yang bergantung pada kualiti cahaya menunjukkan LED biru (0.186 U.mg^{-1}) dan hijau (0.183 U.mg^{-1}) mengakibatkan peningkatan superoksida dismutase. Sebaliknya, LED putih dan merah-jauh meningkatkan aktiviti katalase (0.58 hingga 0.60 U.mg^{-1}) dan askorbat peroksidase (0.017 hingga 0.030 U.mg^{-1}) selain mengumpul karbohidrat dan prolin. Tahap Porphyrin (14.14 µg/g) dan karotenoid (2109.14 µg/g) ditingkatkan oleh LED hijau sementara kepekatan klorofil dipengaruhi oleh LED biru, hijau dan ungu dalam lingkungan 0.516 hingga 0.541 µg/mL. LED merah-jauh, hijau dan merah menyimpang ultrastruktur kloroplas dengan pengumpulan butiran kanji. Tumbuhan yang dijana semula dengan penyinaran LED menghasilkan tunas adventitus dengan meristemoid dan mengesahkan keseragaman genetik 99%. Eksplan tangkai daun dari *Zingiber officinale* var. *rubrum* menunjukkan frekuensi kalogenesis tertinggi (93.75%) dan pengumpulan biomas (3.68g) pada medium yang ditambah dengan 8 mg/L

picloram. Subkultur kalus sangat sesuai dilakukan 45 hari selepas inokulasi sementara LED putih berkesan untuk merebakkan biomas kalus berasal dari 4 mg/L kepada 3.35g berat segar dan 0.162g berat kering. Analisis histologi menunjukkan bahawa kalus primer dan subkultur bersifat bukan embriogenik. Ekstrak metanol tangkai daun dari tumbuhan yang ditanam menunjukkan sebatian antioksidan seperti metil linoleat, asid palmitik, beta-sitosterol, dan phenol, 2,4- bis (1,1-dimetiletil). Selanjutnya, jumlah kandungan fenolik dan flavonoid yang tinggi dengan nilai 191.26 mg GAE/g ekstrak kering dan 4.54mg QE/g ekstrak kering, masing-masing menyumbang kepada aktiviti antiradikal dengan EC_{50} bernilai 0.208 mg/mL. Pencahayaan *in vitro* yang mencukupi mendorong perkembangan daun yang sepadan untuk hidup. Semasa aklimasi, tinggi tunas (32.9 cm), tinggi tangkai daun (11.55 cm), panjang daun (8.48 cm), lebar daun (0.38 mm) dan nilai SPAD (38.80) meningkat terutamanya pada tanaman yang berasal dari sinar LED hijau. Penyinaran LED biru dan putih *in vitro* mempunyai kesan mendalam terhadap ketebalan daun (0.18 mm) dan bilangan pucuk (0.8), masing-masing. Kadar survival semua rawatan kekal pada 100%. Organisasi dedaun yang utuh dengan pengumpulan kanji didapati pada keratan daun yang terdedah kepada rawatan LED. Daun yang bertumbuh di bawah LED hijau menunjukkan peningkatan kepadatan stomata adaksial (8 N/mm2) dan abaksial (40 N/mm2) sementara LED merah meningkatkan indeks stomata pada permukaan adaksial (10.66) dan abaksial (2.020). Ciri-ciri fotomorfogenetik dan fisiologi, dan pertumbuhan *ex vitro* yang memuaskan oleh tumbuhan mikropropagasi menekankan pada kesesuaian LED untuk pencahayaan buatan. Kultur kalus memastikan sumber antioksidan semula jadi yang lestari dan aktiviti antiradikal menyokong penggunaan tradisional *Z. officinale* var. *rubrum* untuk menyembuhkan pelbagai kecelaruan.

EFFECTS OF LED ON THE MICROPROPAGATION AND CALLUS

INDUCTION OF *Zingiber officinale* var. *rubrum*

ABSTRACT

Micropropagation of medicinally important *Zingiber officinale* var. *rubrum* will generate infinite disease-free *in vitro* clones for plantation; preventing reservation of seed rhizome from harvest. Hence, the applicability of light-emitting diodes (LEDs) was determined for the micropropagation of *Zingiber officinale* var. *rubrum.* Significant shoot elongation (12.07 cm) and number of roots (13.29) were produced under red LED. Purple spectrum produced compact plants with highest number of leaves (20) and shoots (5.57). Green, red, and purple LEDs displayed greatest microshoot formation (5–5.9). Light quality-dependent regulation of antioxidant properties of *Z. officinale* var. *rubrum* indicated blue (0.186 U.mg^{-1}) and green (0.183 U.mg^{-1}) LED resulted in increase of superoxide dismutase. By contrast, white and far-red LEDs increased activities of catalase (0.58 to 0.60 U.mg^{-1}) and ascorbate peroxidases (0.017 to 0.030 U.mg^{-1}) besides accumulating carbohydrate and proline. Porphyrin (14.14 μg/g) and carotenoid (2109.14 μg/g) levels were enhanced by green LED while chlorophyll concentration was influenced by the blue, green and purple LEDs in the range of 0.516 to 0.541 μg/mL. Far-red, green and red LEDs distorted chloroplast ultrastructure with accumulation of starch grains. Plants regenerated under LED irradiation produced adventitious shoot buds with meristemoids and confirmed 99% genetic uniformity. Leaf sheath explant of *Zingiber officinale* var. *rubrum* exhibited highest callogenesis frequency (93.75%) and biomass accumulation (3.68g) on medium supplemented with 8 mg/L picloram. Subculture of callus was ideal to be

done 45 days post inoculation while white LED effectively proliferated 4 mg/L picloram grown callus biomass to 3.35 g og fresh weight and 0.162 g of dry weight. Histological analysis revealed the non-embryogenic nature of the primary and subcultured callus. Cultivated leaf sheath methanolic extract presented antioxidant compounds like methyl linoleate, palmitic acid, beta-sitosterol, and phenol, 2,4-bis(1,1-dimethylethyl). Furthermore, high total phenolic and flavonoid contents with values of 191.26 mg GAE/g dry extract and 4.54 mg QE/g dry extract, respectively contributed to the scavenging activity with an EC50 of 0.208 mg/mL. Adequate *in vitro* lighting induced life compatible leaf development. During acclimation, shoot height (32.9 cm), leaf sheath height (11.55 cm), leaf length (8.48 cm), leaf width (0.38 mm) and SPAD value (38.80) increased principally in plantlets originating from green LED irradiance. *In vitro* blue and white LED irradiation had profound impact on leaf thickness (0.18 mm) and number of buds (0.8), respectively. Survival rate of all treatments remained at 100%. Intact foliar organization with starch accumulation was spotted in the cross-section of leaves exposed to the LED treatment. Leaves developed under green LED indicated increased adaxial (8 N/mm2) and abaxial (40 N/ mm2) stomatal density while red LED enhanced stomatal index on the adaxial (10.66) and abaxial (2.02) surfaces. Photomorphogenetic and physiological traits, and promising *ex vitro* growth of the micropropagated plants emphasizes on the suitability of LED for artificial lighting. Callus culture ensures sustainable source of natural antioxidants and the scavenging activity supports the traditional use of *Z. officinale* var. *rubrum* to heal various disorders.

CHAPTER 1

INTRODUCTION

Zingiber officinale var. *rubrum* which is commonly known as red ginger or '*Halia bara*' (Malaysia) has a role in traditional medicine (Levita et al., 2018; Razali et al., 2020) which is contributed by the bioactive components including the essential oil and oleoresin content (Fikri et al., 2016; Suciyati and Adnyana, 2017; Kurniawati et al., 2020). In Indonesia, red ginger is a highly priced agricultural export commodity (Ministry of Trade of The Republic of Indonesia, 2016; Daulay, 2017; Mardiningsih et al., 2021) and the demand for fresh rhizome and derivatives have increased during COVID-19 pandemic (SMART, 2020; Mufti, 2020). However, ginger cultivation in Malaysia is conducted at small scale despite of the medicinal and economical demand (Zuraida et al., 2020). Malaysian Ministry of Agriculture and Food Industry (MAFI) indicated that in the year 2018, ginger cultivation has decreased by 136 hectares while harvest reduced by 794 tonnes in comparison to year 2017, thus, increasing import of ginger to 45261 tonnes (MAFI, 2018). Apart from reducing plantation areas, slower asexual propagation via rhizome seeds, and possibility of disease infestation of the soil or reserved rhizome seeds from the previous harvest constraint ginger cultivation severely (Zuraida et al., 2020). Destructive pathogen of ginger, *Ralstonia pseudosolanacearum* causes lethal wilting disease constraints ginger cultivation (Prameela and Bhai, 2020; Huang et al., 2021; Snigdha and Prasath, 2021). Therefore, development of a micropropagation procedure for *Z. officinale* var. *rubrum* will permit growers to get access to infinite clean planting materials to ensure a good harvest whereas farmers traditionally propagated themselves in the field under uncontrolled growth conditions via vegetative way of propagation. Consequently, a sustainable

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solution will be met to feed the commercial demand for *Z. officinale* var. *rubrum* at the local and international arena.

Growth and development of *in vitro* plants are influenced by spectral quality set by artificial lighting (Bidabadi and Jain, 2020; Kulus and Woźny, 2020; Neto et al., 2020). Since conventional lighting consumes more energy (Yu et al., 2020), light-emitting diodes (LEDs) are used to support plant growth in controlled environments such as plant tissue culture room due to their energy efficiencies and flexibility of controlling the wavelengths (Olle and Viršilė, 2013; Li et al., 2019). Hence, the present study has explored into the interactions of *Z. officinale* var. *rubrum* plants with far-red, blue, green, red and purple LEDs in terms of morphology, biochemical content and genetic stability as growth responses of plants towards LED spectra is species-specific. Shooting (Johnson et al., 2020; Dogan, 2020), rooting (Alallaq et al., 2020; Xu et al., 2020), foliar (Wang et al., 2020) and shooting bud (Jeong and Sivanesan, 2018) development are morphogenic processes occur in response to light quality (Bantis et al., 2020). Hence, in addition to vegetative growth analysis, sensitivity of *Z. officinale* var. *rubrum* plants to various LED spectra will be explored by analyzing the content of antioxidant enzymes (Rehman et al., 2020), pigment concentration (Tran and Jung, 2017), accumulation of metabolites, ultrastructure of leaf (Chen et al., 2020; Dogan, 2020).

Alternatively, inducing callus culture of *Z. officinale* var. *rubrum* will develop a plant cell biomass based substitute system which may act as a reservoir of secondary metabolites. Callus cultures eliminate the effect of interaction of different plant parts, environmental stress and harvesting conditions on the synthesized bioactive compounds (Yang et al., 2018; Applequist et al., 2020). Therefore, callus induction will be explored on various explants of *Z. officinale* var. *rubrum* in the presence of

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exogenous picloram. Apart from the routine procedures such as establishing callus growth curve and histology, preliminary phytochemical analysis including screening for free radical scavenging properties and compound identification from gas chromatography/mass spectrometry (GC-MS) data were conducted on callus extracts. Polyphenols in the plant extracts contribute to the antiradical and antioxidant potential (Lin et al., 2018; Mayouf et al., 2019). Hence, phytochemical analysis of the methanolic *Z. officinale* var. *rubrum* extracts will prove on the importance of the extracts as a sustainable source of antioxidant.

The ultimate goal of *in vitro* mass propagation is achived when plants micropropagated via a new procedure exhibit successful *ex vitro* establishment with normal development (Bag et al., 2019). Modified ROS metabolism and development of incompetent photosynthetic machinery during *in vitro* environment may result in morphological and physiological disorders of the regenerated plants which may impede the *ex vitro* survival efficiency and growth performance (Gupta and Agarwal, 2017[b]). *In vitro* light environment is a critical factor in developing the photosynthetic machinery of the plantlets (Gupta and Agarwal, 2017[b]). Therefore, spectral quality of LEDs were evaluated on growth performance and survival of *in vitro* regenerated *Z. officinale* var. *rubrum* plants upon transfer to *ex vitro* environment. Consequence of spectral quality on growth traits, leaf characteristics, chlorophyll content, survival rate, foliar anatomy and stomatal development of plants were evaluated after transition to the *ex vitro* environment.

1.1   Problem statements

Lack of planting materials due to disease infestation and slower asexual mode of reproduction constrict *Zingiber officinale* var. *rubrum* cultivation. Therefore, development of a micropropagation procedure for *Z. officinale* var. *rubrum* will permit growers to get access to infinite clean planting materials. Given the need to adapt micropropagation for *Z. officinale* var. *rubrum*, this study aimed to evaluate the effect of light quality on *in vitro* vegetative growth stage of red ginger as different wavelengths influence plant metabolism and *in vitro* morphogenesis of the plant. Establishing callus culture of *Z. officinale* var. *rubrum* represent a sustainable low-cost biomass source of easy and scalable production of secondary metabolites as well as reduce the dependence on wild plants. *In vitro* culture condition may produce plantlets with abnormal morphology, anatomy and physiology which impair the survival upon *ex vitro* transfer. Successful *ex vitro* establishment with normal development is the ultimate goal of micropropagation.

1.2   Research objectives

I.   To inspect the impact of light spectra on vegetative growth, biochemical, and genetic stability of *in vitro* plant;

II.   To develop an effective protocol for optimum callus induction;

III.   To determine the chemical constituents and the antioxidant activity of the leaf sheath and callus extracts;

IV.   To study the influence of *in vitro* lighting on the physiological performance and survival of *ex vitro* plants.

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CHAPTER 2

LITERATURE REVIEW

2.1   Zingiberaceae – An overview

Zingiberaceae, commonly known as the ginger family is categorised as vascularised, seeded and flowering monocots (Prince and Kress, 2002). Zingiberaceae is grouped into 52 genera with 1600 species (Christenhusz and Byng, 2016) and exhibits pantropical distribution along Africa, Asia, and America but prevalent with great diversity in Southeast Asia (Tamokou et al., 2017). Zingerberaceae family is cultivated for the economic and medicinal value of the rhizome (Bhunia and Mondal., 2012; Devi et al., 2016).

Zingiberaceae is characterised as aromatic perennial medium-sized herbs with sympodially branched creeping horizontal or tuberous rhizomes (Nair, 2013). Unlike tubers which reproduce via nodes in omnidirection, rhizomes grow horizontally under the ground by sprouting roots and stems along the bottom and top of the horizontal growth, respectively. Morphologically, rhizome varies from other underground storage organs such as taproots (yam and carrot), tubers (potato), tuberous roots (cassava and sweetpotato) (Zierer et al., 2021). Pseudostems are formed by layers of alternating leaf sheaths which are connected to distichous leaves by ligule (Kumar et al., 2013). The inflorescence made of closely grouped overlapping bracts is usually observed on a separate shoot emerging from the rhizome.

Commercially important genus *Zingiber* Mill. of the Zingiberaceae family is well known because of *Zingiber officinale* (L.) Roscoe (Bai et al., 2015). First studied by Roxburgh (1810), *Zingiber* is comprised of 150 perennial species native to Thailand, China, Indian Subcontinent and New Guinea (Anamthawat-Jónsson and

Umpunjun, 2020). *Zingiber* species are characterised by cushion-like pulvinus leaf base which distinguishes it from the other genera (Bai et al., 2015).

2.2 Tissue culture of ginger

In addition to the increased rate of deforestation, reducing plantation areas, insufficient ginger seeds for cultivation as it's the commercial commodity, disease transmittance via bud or rhizome seed, variations and degeneration exhibited by ginger rhizomes under long term vegetative propagation, unsuccessful breeding due to poor flowering and lack of seed set and low propagation rate caused by reproductive sterility have constrained ginger propagation and caused heavy loss on the yield (Taha et al., 2013; Mosie, 2019). Hence, *in vitro* tissue culture techniques would be the viable alternative to apply to increase planting materials for commercial propagation and research studies when conventional propagation proved inadequate. It is imperative to ensure the bioavailability of the plant to exploit the medicinal properties of *Zingiber* species which may reduce in number in the future due to indiscriminate uprooting of rhizomes to prepare of indigenous herbal medicine and essential oils.

Susceptibility of ginger crop to various soil-borne pathogens of viral, bacterial, fungal, and nematode origin constraints ginger propagation and yield severely. For example, *Ralstonia pseudosolanacearum*, the most destructive pathogen of ginger dysfunctions vascular bundle system resulting in lethal wilting disease which constraints ginger cultivation (Prameela and Bhai, 2020; Huang et al., 2021; Snigdha and Prasath, 2021). Ginger is vulnerable to *Serratia marcescens* (Huang et al., 2020) and *Phytium* species that induce ginger rhizomes soft rot disease (Nair, 2019). *Fusarium oxysporum* f. sp. *Zingiberi* and *Phyllosticta zingiberi* specifically infect ginger rhizome which develops *Fusarium* yellows dry rot and leaf spot, respectively. Root-knot nematode impair ginger reproduction by causing galling and stunting on the

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rhizome (Hajihassani and Hampton, 2019). Vulnerability of ginger to soil-borne diseases and transmittance of pathogens from generations to generations is enhanced by asexual propagation since pathogens accumulated on ginger rhizomes (Meenu and Kaushal, 2017). Therefore, nomadic cultivation is practiced for ginger plantation as next growing session on the same land could only be resumed after six years to prevent disease infestation of the ginger rhizome which affects the yield (Suhaimi et al., 2014). Infections are also prevalent in the germplasm collections meant for clonal repositories (Shaik and Rajani Kanth, 2018).

According to Inden et al. (1988), an efficient micropropagation technique using a single shoot tip explant of *Z. officinale* is capable of producing 750 thousands of plantlets in a year. Therefore, clonal multiplication via micropropagation will generate infinite disease-free planting materials for field cultivation, commercial utilisation, and scientific investigations. Moreover, *in vitro* cultures of ginger will also contribute for germplasm collection and crop improvement programs via genetic modification (Shaik and Rajani Kanth, 2018). Therefore, optimisation of explant characteristics, nutrient content, and light quality are investigated to update *in vitro* protocol for rapid propagation of true-to-type ginger (Kasilingam et al., 2018). Tissue culture research studies on *Zingiber* species such as micropropagation (Miri, 2020; Tewelde et al., 2020) as well as induction of callus (Ibrahim et al., 2015), microrhizome (Abbas et al., 2020), microshoot (Al-Taha et al., 2020), and indirect somatic embryogenesis (Musfir Mehaboob et al., 2019) using sterilised rhizome buds (Bhowmik et al., 2016; Zuraida et al., 2016; Chavan et al., 2018), germinating shoot (Cafino et al., 2016) and shoot apical meristems (Macalalad et al., 2016) have been reported in the yesteryears.

2.3   *Zingiber officinale* **var.** *rubrum* **Theilade**

Zingiber officinale is divided into different varieties according to the size and colour of the rhizome. Thus, white ginger is known as *Z. officinale* var. *officinale* while emprit or small ginger is named as *Z. officinale* var. amarum, and red ginger is identified as *Z. officinale* var. *rubrum* (Setyawan et al., 2014; Suciyati and Adnyana, 2017).

Z. officinale var. *rubrum* is cultivated in the South-east Asian countries (Weiss, 2002; Kizhakkayil and Sasikumar, 2011). It is known as Chiang, ganjiang, or kanchiang by the Chinese while in the United Kingdom it is recognised as Canton ginger, whereas Arabians named it zenjabil (Quattrocchi, 2012). However, *Z. officinale* var. *rubrum* is known as '*Jahe Merah*' and '*Halia Bara*' in Indonesia and Malaysia, respectively, owing to the intense scarlet colouration on the surface of the rhizome (Shimoda et al., 2010).

Z. officinale var. *rubrum* is an annual plant that grows upto 50-100 cm (Supu et al., 2018) and distinguished by the characteristic smaller and thicker scarlet coloured pungent rhizome (Suciyati and Adnyana, 2017). Exterior of the rhizome is scarlet coloured due to the accumulation of anthocyanin (Shimoda et al., 2010) but the cross-section appears yellow while layer beneath the skin is pinkish. Lancet-tipped narrow leaves of *Z. officinale* var. *rubrum* may measure to 5-25 cm length and 8-20 mm width. The leaves adjoin to reddish petiole which is extended to leaf sheath with less frequent vertical lines of red colour but intensified scarlet base emerging from the rhizome. Inflorescence stem emerging from the rhizome locates 2 to 2.5 cm long funnel-shaped flower which has distinctive scarlet red lip with creamy-yellow spots (Ross, 2003; Ibrahim et al., 2008).

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Similar to the white ginger, *Z. officinale* var. *rubrum* is used in culinary and perfume industries due to the pungent yet aromatic volatile constituents (Ding et al., 2012). Furthermore, *Zingiber. officinale* var. *rubrum* has been used in folklore medicine by the Indonesians, Chinese, and Malaysians to ease asthma, cough, diabetics, neurological disease, stroke, flatulence, gingivitis, muscle spasms and excessive sweating (Jayanudin et al., 2015). Indonesians specifically uses the rhizome as an ingredient of post-partum medicine and '*Jamu*' which cures stomach discomfort and rheumatism (Ibrahim et al., 2008). It is consumed in the form of paste, dried powder, crystallised candy and tea (Jayanudin and Rochmadi, 2017).

Red ginger is one of the highly priced main agricultural products and spice export commodity in Indonesia with continuos demand from Jordan, Pakistan, Taiwan and Vietnam (Ministry of Trade of The Republic of Indonesia, 2016; Daulay, 2017; Mardiningsih et al., 2021). Particularly, the demand for red ginger has skyrocketed by 138% (SMART, 2020) and herbal supplement made from red ginger extract increased by about 50% during the COVID-19 pandemic (Mufti, 2020). Despite of the medicinal and economical demand, ginger plant cultivation in Malaysia is conducted at small scale as a single crop or as an intercrop grown with perennial crops such as pineapple, rubber and oil palm (Zuraida et al., 2020). In Malaysia, *Z. officinale* var. *rubrum* is cultivated in Pahang (Bentong), Sabah (Keningau and Tambunan), Sarawak, Selangor and Johor as one of the four ginger varieties (Ghasemzadeh et al., 2014; Shuhaimi et al., 2016). According to Ministry of Agriculture and Food Industry (MAFI), Malaysia, ginger cultivation has decreased by 136 hectares in 2018 while ginger production reduced by 794 tonnes in comparison to year 2017 (MAFI, 2018). Furthermore, from 34353 tonnes in 2004, imported ginger rose steeply to 45261 tonnes of ginger in 2018

(MAFI, 2018). Extreme shortage of ginger supply by the local plantation was sorted out by importation of ginger by the Malaysia government.

2.3.1 Phytochemicals of *Zingiber officinale* var. *rubrum*

Primary metabolism of plants involves reactions and pathways necessary for survival, and secondary metabolism encompasses on phytochemicals required for growth and development including the interaction of the plant with the environment (Pott et al., 2019). Ginger produces numerous bioactive compounds including phenols (gingerols, shogaols, and paradols) and terpenes (α-zingiberene, ar-curcumene, β-bisabolene, and β-sesquiphellandrene) (Wohlmuth et al., 2006; Mao et al., 2019). Particularly, *Zingiber officinale* var. *rubrum* contains the highest oleoresin, essential oil, phenol and flavonoid in comparison to *Z. officinale* Roscoe and *Z. officinale* var. amarum (Oboh et al., 2012; Cahyono et al., 2018).

Sivasothy et al. (2011) isolated forty six constituents from the leaf and the major compound was classified as b-caryophyllene (31.7%) among others such as geranial (13.1%), neral (9.8%) and caryophyllene oxide (6.3%) geraniol (4.4%), a-pinene (4.1%) and trans,-trans-a-farnesene (3.2%). Essential oil extracted from *Z. officinale* var. *rubrum* rhizomes yielded 54 identified constituents including monoterpenoids such as camphene (14.5%), geranial (14.3%), and geranyl acetate (13.7%) apart from lower concentrations of neral (7.7%), geraniol (7.3%), and 1,8-cineole (5.0%) (Sivasothy et al., 2011).

Organic solvent extraction of dried *Z. officinale* var. *rubrum* produces oleoresin which is a viscous reddish-brown liquid containing both volatile and nonvolatile pungent components (Jayanudin et al., 2013). Shogaol and zingerone were the principal compounds identified in oleoresin produced by ethanolic extraction of *Z.*

officinale var. *rubrum* rhizome (Jayanudin et al., 2013). Zingerone and shogaol are dehydration products of the gingerols (Nwaoha et al., 2013; Levita et al., 2018).

Neral and geranial contribute to the lemon-like aroma (Wohlmuth et al., 2006), borneol, bornyl acetate, and 1,8-cineole emits camphoraceous odour (Bauer et al., 2001) while gingerol and shogaol are responsible for the pungency (Mulia et al., 2018) of *Z. officinale* var. *rubrum* rhizomes.

An array of pharmaceutically important compounds of *Z. officinale* var. *rubrum* demonstreate bioactivities such as antimicrobial (Sharifi-Rad et al., 2017), antidiabetic (Supu et al., 2018), extracellular melanogenesis inhibitory activity (Yamauchi et al., 2019), antihyperalgesic activity (Fajrin et al., 2019).

2.4   Callus culture: Principals and significance

Plant tissue culture is built on the fundamentals of cellular totipotency, the inherent ability of a somatic cell to divide and differentiate into any types of cells to be organised into an organ or complete organism (Fehér, 2019) upon exposure to a suitable nutrient medium at the optimally controlled environment. Subsequent regeneration phase via environmental adaptation and surviving injuries are mediated by plasticity nature of plants. Hence, totipotency and plasticity characteristics of plants are the pillars of big-scale clonal propagation of plant in a controlled environment (Gaillochet and Lohmann, 2015).

The objective of plant tissue culture is to facilitate rapid clonal propagation of disease-free plant materials for commercialisation and research studies. Manipulations of the plant system via *in vitro* protocols are fundamentally supported by totipotent, dedifferentiation, differentiation, plasticity and competency characteristics of plants. Dedifferentiation is the process of specialised cells physiologically reverts to stem cell-like state with pluripotent ability (Jopling et al., 2011). In plants, dedifferentiation

process allows the pericycle layer to form amorphous proliferative callus mass with pluripotentiality on a callus induction media (Fehér 2019). Hence, callus could be induced on any competent explants such as leaves, stems, seeds, nodes, roots and seedlings or part of it.

Individual or combination of plant growth regulators are supplemented to callus induction media to optimize callus induction (Islam and Alam, 2018). Wound-induced callus formation (Ikeuchi et al., 2013) is controlled by the WOUND INDUCED DEDIFFERENTIATION$_1$ transcription factor and its close homologs WIND2, WIND3, and WIND4 (Iwase et al., 2011) by up-regulating cytokinin biosynthesis leading to cell division, proliferation and callus formation (Ikeuchi et al., 2017). As the thin-walled parenchymatous callus cells begin to differentiate, the mass will be then made of both unorganised and less differentiated parenchymatous tissue (Bisht et al., 2016). Therefore, callus mass may contain pluri- or totipotent cells (Fehér, 2019).

Morphologically callus could be compact or friable, organogenic or non-organogenic, pigmented or creamy whitish, and composed of spherical to elongated cells which are are smaller in size with reduced vacuole and dense cytoplasm (Bhatia, 2015).

Growth index of callus mass is analysed based on fresh weight and dry weight (Kapoor et al., 2018). Callus culture exhibits sigmoid pattern of growth (Coimbra et al., 2019) consist of lag phase, exponential phase, linear phase, deceleration phase and stationary phase during which cells prepare to get divided, divide at the highest rate, slow down on division but increase expansion, both cell division and elongation decrease, and eventually number and size of cells remain constant, respectively (Bhatia, 2015).

Extracts of callus cultures have been proven to demonstrate medicinal properties such as anticancer abilities comparable to the drug (Deshpande et al., 2012). According to Efferth (2019), callus culture has the advantage of producing secondary metabolites to be supplied to pharmaceutical, food and cosmetic industries without sacrificing the mother plant or being intervened by climate, environment, and pathogen attack. Callus cultures produce various bioactive phytochemicals including resveratrol, shikonin, camptothecin, α-tocopherol, quercetin, rosmarinic acid, ginkgolide β, anthocyanins, stilbene, and paclitaxel (Efferth, 2019). However, precaution to minimize and scale-up of the biosynthesis process will enhance the production of the bioactive compound (Yue et al., 2016; Efferth, 2019).

Exogenous antioxidant intake of plant origin received a great deal of attention because of their free radical scavenging abilities associated with disease-fighting potentials (Wiseman et al., 2001; Andreu et al., 2018). Phenolic compounds categorised as flavonoids and non-flavonoid phenolic acids are the largest phytochemical molecules with antioxidant capabilities are synthesized by plants to armour against pathogens and to promote growth under unfavourable conditions. Plant cell culture-synthesized compounds may resort as a renewable source of phytochemicals and demonstrate strong antioxidant activities compared to the wild plants as well as serve as substitutes for synthetic medicine (Yang et al., 2019). A positive correlation between plant-derived antioxidants and free-radical scavenging activity is commonly evaluated by DPPH (2,2-diphenyl-1- picrylhydrazyl) spectrophotometric protocol.

2.5   Significance of spectral quality for plant growth and physiology

Plants are sessile organisms equipped with sophisticated sensory system to integrate exogenous signals such as light and temperature with endogenous

developmental process to support optimal growth, survival, reproduction, dry mass and crop yield (Van Ieperen, 2016). Plants are photoautotrophic and photosynthesis is triggered by light. Light is a significant element besides water and oxygen because photons fuel the photosynthetic machinery. Hence, plants respond to the quality (spectral composition), quantity, duration and direction of light stimuli (Zoratti et al., 2014) to generate reversible or irreversible physiological responses such as stomata opening and seed germination, respectively (Kami et al., 2010).

Pigments selectively transduce wide range of wavebands to trigger a cascade of events (Taiz et al., 2015) to regulate gene expression (Van Ieperen, 2016) which in turn influences photomorphogenesis (Kathare et al., 2021). Specific absorption spectra is harvested by plants via photosynthetic pigments known as chlorophyll A, chlorophyll B, carotenoid, zeaxanthin, lutein, and lycopene (Rehman et al., 2017). The solar radiation spectrum that directly impact plant growth and development is composed of 10% ultraviolet, 40% visible, and 50% infrared rays (Prasanth et al., 2020). Leaf tissues preferentially absorb photosynthetically active radiation wavelengths spectra (400–700 nm) and quantum yield of photosynthesis conducted efficiently by red (600–700 nm) and blue (400–500 nm) photons of the visible light spectrum (Zhen et al., 2021).

2.5.1 Application of light-emitting diodes to control plant behavior

Optimisation of light quality is critical to enhance the photosynthetic performance of leaves on the long term (Van Ieperen, 2016). Effect of light wavelength on plant growth is explained briefly on Table 1.

Highly integrated signal-response networks enable plants to optimally react to modern growth environments and lighting facilities since applied lighting differs from natural intensity, photoperiod and spectral composition besides modified by neighboring plants and weather. *In vitro* plant tissue culture facilities are generally fitted with fluorescent lamp with complete visible light spectrum which is redundant and inadequate to promote plant growth development and pose limitations for photosynthetic performance besides consuming electrical energy and generating heat (Rehman et al., 2017). Hence, economical artificial lighting with high energy-conversion efficiency and most importantly spectral adjustment ability deemed a prerequisite to optimally support plant physiological processes.

Studies on artificial irradiation sources for indoor and greenhouse is an active research niche. LED is increasingly adapted in *in vitro* plant tissue culture facilities as a source of radiation to influence photosynthesis and photomorphogenesis. Earlier trials in the 1980s to study the impact of simple red-only LED arrays as a source of plant lighting for space-based plant-growth systems using lettuce, potato, spinach, and wheat eventually led to the development of LED-based systems for plant physiology experimentation such as seed germination, response of root cuttings and tissue culture studies (Morrow, 2008). Surpassing 20 years of existence as grow light, LED technology has been updated rigorously to be more efficient and economically feasible for large-scale lighting applications.

Innovative LED artificial lighting with controllable spectral composition using narrowband wavelengths and tunable intensity manipulate primary and secondary metabolism, growth and development of plants by influencing their internal light-signal-response system (Ahlman et al., 2018; Hammock et al., 2021). Landi et al., (2020) applauded that LEDs effectively provides the platform to study the plant

performance under increased proportion of a desired wavelength besides investigating the effect of a specific wavelength on secondary plant metabolism or plasticity of the photosynthetic apparatus. In other words, the advancement of LED technology of determining specific wavelength for specific crops will develop effective light recipes to provide required spectral quality for optimal and targeted plant response.

Table 2.1: Effect of light wavelength on plant growth

Wavelength (nm)	Effect on plants
200 – 280 (UV-C radiation)	• Harmful to plants • Absorbed by stratospheric ozone layer before reaching earth
280 – 315 (UV-B radiation)	• Harmful to plants under intense applications (pigment bleaching and degradation of secondary metabolites) • Increase secondary metabolite production
315 – 380 (UV-A radiation)	• Inefficiently harvested by chlorophyll due to the flavonoid shield • Controversial impacts on plant morphology and secondary metabolite production
380–400	• Transition from UV-A to the visible light spectrum • Chlorophyll and carotenoid pigments absorb light within this range.
400 – 520 (Violet, blue, and green light)	• Chlorophyll A and Chlorophyll B pigments obtain peak energy absorption of blue (420–450 nm) at 430 and 453 nm, respectively • Strongly influence photosynthesis, vegetative growth and development
520 – 610 (Green, yellow, and orange light)	• Green (500–600 nm) photons penetrate deeper into leaves • Limited influence on vegetative growth and development • Specific impacts on secondary metabolite production
610 – 720 (Red light)	• Chlorophyll A and Chlorophyll B pigments obtain peak energy absorption of red (600–700 nm) at 665 and 642 nm, respectively • Matches the absorption peaks of various phytochemicals and secondary pigments • Influence vegetative growth, photosynthesis, and reproductive growth
720 – 1000 (Far-red and infrared light)	• Direct impact on germination and flowering
Beyond 1000 (Infrared radiation)	• Primarily converted into heat energy • Not useful for primary or secondary metabolism • Not used for photosynthesis

(Prasanth et al., 2020; Hammock et al., 2021; Zhen et al., 2021)

Technically, unidirectional, direct current usage, long operating hours, ease of handling, designed to be simple with bright irradiance, absence of mercury, integration of digital control systems since LED is a solid-state device and cooler due to almost nonexistent heat emission add to the merits of LEDs (Landi et al., 2020). Excessive heat dissipation from artificial light may raise ambient temperature, thus, affecting photomorphogenesis of *in vitro* culture and increase the expenditure on air conditioning while proven to be fatal to the plant material (Gupta and Agarwal, 2017[a]). However, plant materials can be kept in close proximity to the LED source to allow more photon to be efficiently intercepted by the plant without damaging the foliar. Narrowband uniqueness to provide spectrum matching the absorption peak of plant photoreceptors (Bello-Bello et al., 2017) eliminating electrical energy spending on nonproductive wavelengths yet dynamically controlling the plant morphology make LED lamps the ideal grow light in small- and large-scale operations.

Generally, white (400-700 nm), far-red (720nm), blue (460 nm), green (530 nm), red (660 nm) and purple (400 - 660 nm) LEDs are used either as monochrome or in combination to promote *in vitro* plant growth. LEDs positively influencing the growth and development of plants have been reported including but not limited to potato (Jiang et al., 2019), Strawberry (Kepenek, 2019), Coleus (Cho et al., 2019), and Goji berry (de Oliveira Prudente et al., 2019).

The advent of LED technology is a milestone in controlling the environmental parameters and morphogenetic responses, as mediated by the light quality fixed to the culture shelves. Blue light influences several physiological processes such as stomatal opening (Hayashi et al., 2017), chloroplast maturation (Chen et al., 2020), chlorophyll accumulation (Ma et al., 2021), chlorophyll biosynthesis (Manoh et al., 2021), as well as mediates blue light-dependent photomorphogenic responses such as inhibition of

hypocotyl elongation (Zhong et al., 2021) and essential for the functionality of the photosynthetic apparatus than red light (Izzo et al., 2020). Exposure of red LED stimulates adventitious root formation (Alallaq et al., 2020) via phytochrome (Christiaens et al., 2019) but triggers shade avoidance syndrome to form slender stalk and reduce investment on leaf properties (Chen et al., 2020). Hence, combination of red and blue LEDs is supplied to the *in vitro* cultures to mimic natural light by activating phytochromes, phototrophins, and cryptochromes to promote higher photosynthetic activity while reducing the effect of monochromatic light (Kepenek, 2019). Green LED on the other hand stimulate photosynthesis deep within the leaf contributing to carbon gain and crop yield apart from resulting in developmental adaptation (Smith et al., 2017). Far-red radiation is weakly absorbed (Taiz et al., 2015) and intensifies shade stress by increasing plant height and leaf area besides causing physiological changes in the photosynthetic apparatus (Li et al., 2021) without affecting plant growth (Zou et al., 2019).

2.6 Physiological and biochemical responses of plants to photostress

Lighting conditions strongly influence the growth and development of plants because they are sessile and photo-autotrophic. Changes in light quality is detected by pigments and photoreceptors may impact the anatomical, physiological, morphological, and biochemical parameters of leaves and significantly affect the growth and survival (Saleem et al., 2020). Hence, influence of light quality is comparably more significant than light intensity and photoperiod because specific spectrum stimulates a different response (Wang et al., 2016).

In view of survival of the fittest, plants adapt to the radiation by producing more photosynthetic pigments, modifying photosynthesis rate and altering metabolic

content during low light while resume photoinhibition during high light intensity to prevent photooxidative stress (Idris et al., 2018). Photostress is light associated detrimental effect such as insufficient light reduced photosynthetic activity or excess light energy induced impairment of the photosynthetic apparatus which is injurious to the plant function and development. Although heat emission from LED is relatively low (Gupta and Agarwal, 2017[a]), mild photo-stress is induced by light spectra as well as photoperiod (Sirtautas and Samuolienė, 2013) and plants response to photostress is species specific (Rehman et al., 2020).

Shin et al. (2011) proclaimed that certain LED spectra may induce photooxidative stress. A balanced reactive oxygen species (ROS) generation and elimination will be impaired under detrimental environment and lead to ROS accumulation that induces oxidative stresses such as membrane lipid peroxidation and collapse of membranous system (Saleem et al., 2020). However, accumulation of ROS including superoxide radical, hydrogen peroxide, singlet oxygen and hydroxyl radicals are efficiently scavenged by plants enzymtic defense system composed of superoxidase dismutase, catalase and peroxidase or by enhancing antioxidant compounds such as glutathione, ascorbic acid and carotenoid (Rana et al., 2020). For example, SOD regulates the cellular concentration of O_2^- to decrease OH formation but generates H_2O_2 which inhibits photosynthesis ($O_2^- \rightarrow H_2O_2 + O_2$), however, peroxisomal CATs and APXs in the chloroplasts scavenge on H_2O_2 (Dvorak et al., 2020; Meitha et al., 2020).

Proline accumulation as a result of up regulated synthesis or down regulated catabolism is correspondingly an adaptive approach by plants in response to stressful conditions (Forlani et al., 2019; Rehman et al., 2020). According to Rehman et al. (2021), proline does quench singlet oxygen and superoxide both in *in vitro* systems

and isolated thylakoids relatively at a lower efficiency but physiologically relevant in assisting the antioxidant ROS scavengers of plant cells. Furthermore, Yu et al., (2017) observed reduced lipid peroxidation in exogenous proline-treated rice seedlings which was correlated to a decrease in MDA content.

Light quality affects the biosynthesis and accumulation of carbohydrate (Chen et al., 2018). For example, red LED improved the synthesis of fructose and glucose while combination of blue and red LEDs accumulated total carbohydrate, starch and sucrose significantly in tomato seedlings (Li et al., 2017). As an adaptive responsive, plants accumulate sugars and carbohydrates to maintain plant performance during aberrated environmental conditions (Sami et al., 2016). For example, sugar molecules either function as ROS scavengers or act as secondary messengers that activate other antioxidants to tolerate stressful environment (Gangola and Ramadoss, 2018; Ahmad, 2019). The severity of stress and metabolic state at cellular level or plant on the whole could be interpreted based on the molecular structure of the sugar molecule (Ahmad, 2019). Carbohydrate deposition could also be caused by alteration of starch hydrolytic enzymes activity or suppression of carbon fixation process caused by defunct photosynthetic pigments (Sidhu et al., 2017).

Light energy in the form of a photon is absorbed by the light-harvesting pigments like chlorophyll A (Chl A) or the accessory pigments such as chlorophyll B (Chl B) and carotenoids. Chl A absorbs photons from the orange-red and violet-blue spectrum while Chl B harvest light energy from blue region to be streamed to the electron transport chain (Muharfiza et al., 2017; Martin, 2019). Carotenoid which coexist with chlorophyll is made of xanthophylls and carotenes that absorb photons from blue to green spectra (Aramrueang et al., 2019; Xie et al., 2019). Furthermore, carotenoids play photoprotective function by quench chlorophyll triplets to prevent

their quenching by oxygen which will generate ROS singlet molecular oxygen ($1O_2$) (Khoroshyy et al., 2018) besides dissipate excess of excitation energy of the photosystem in the form of heat (Lin et al., 2020). According to Vahtmäe et al. (2018), health and productivity of vegetation is precisely indicated by the presence and density of photosynthesizing pigments since pigment content, photosynthetic potential, and primary production are interrelated. Spectral quality of lights significantly influence vegetative growth and photosynthetic pigment contents such as chlorophyll and carotenoid (Gupta and Karmakar, 2017; Jiang et al., 2019; Pereira and Otero, 2020). For example, accumulation of carotenoid was far more enhanced by red LED in comparison to blue LED (Ma et al., 2015) or by supplementary wavelengths in the green domain (Thoma et al., 2020). Pigment accumulation and expression of related gene expression were consistently increased indicating on the regulatory mechanism of LED on pigments metabolism (Zhang et al., 2015; Yuan et al., 2017).

Leaf development, and internal anatomy are equally affected by light quality and crucial because leaf characteristics such as chlorophyll content, leaf thickness as well as differentiation of palisade and spongy mesophyll determines the absorption of light energy to drive photosynthesis (Zheng and Van Labeke, 2017[b]). For example, plants exposed to blue light develop thicker leaf blades with increased chlorophyll content to compensate smaller structure caused by limited cell expansion and division which may potentially reduce light harvest (Clavijo-Herrera et al., 2018). Plants undergo intricate network of interactions among the photoreceptors to collect information about the multiple light signals available in the environment. For instance, low red to far-red ratio coupled with cryptochrome inactivation under low blue light triggers shade-avoidance syndrome manifested as ethiolated leaf but increased expansion and elevated angle to accommodate efficient light harvest to drive plant

photosynthesis (Roig-Villanova and Martínez-García, 2016; Fernández-Milmanda and Ballaré, 2021). On the other hand, dichromatic LED developed by combining red and blue lights also gainining momentum to positively influence chloroplast plus leaf mesophyll cell development which accelerates photosynthesis as well as growth of crops (Liu et al., 2018).

Stomatal development and patterning on the epidermis is controlled by coordination between environmental cues and gene expression (Vráblová et al., 2018). Light quality impart obvious effects on stomata formation and stomatal aperture which in turn will influence the gas conductivity (do Nascimento vilela et al., 2015) and subsequent photosynthesis activity. It was further clarified that *in vitro* LED irradiation driven stomata formation could be advantageous to the plantlets to survive acclimatization phase. However, Ferreira et al. (2017) accounted high water loss exhibited by *in vitro* plants irradiated by LED to greater stomatal density on adaxial and abaxial leaf surfaces. Generally, blue light has been documented widely to increase stomatal index and stomatal density (Zheng and Van Labeke, 2017 [a]). Blue light signal transmitted by phototropin and cryptochrome modulate guard cell for stomatal opening to improve stomatal conductance (Clavijo-Herrera et al., 2018).

Light spectrum of various wavelengths induces morphological, ultrastructural, biochemical, and antioxidative responses associated with photosynthetic activity of a plant. However, the impacts of light quality on physiological and photosynthetic responses of plant indicated as changes in transcription to cope with lighting-stress (Yavari et al., 2021). Furthermore, Neves et al. (2017) emphasized that in response to stress events, plants develop stress memory which is associated with physiological and molecular changes. The plant responses towards light quality could be contradictory due to species specificity. Hence, optimisation of light quality provided via artificial

lighting will impart a positive benefit on the growth, development and productivity of the plant.

2.7   Genetic stability assessment of *in vitro* plants by molecular marker

Any plants with superior characteristics could be regenerated and micropropagated at large scale via *in vitro* technique due to massive advancements in the plant tissue culture industry. Clonal propagation either for commercial need or germplasm conservation necessitates genetic uniformity among the regenerated plants. High percentage of genetic homogeneity among regenerated *in vitro* plantlets ensures true-to-type clonal propagation of the regenerates (Bidabadi and Jain, 2020).

Despite of the significance of *in vitro* cell and tissue culture systems, genetic makeup of intact plant organ and tissue is threatened. Transformation phase of the explant to *in vitro* state imposes stress to the plant cells during tissue culture phase which induces genomic shock, hence, restructures the genomes (Ranghoo-Sanmukhiya, 2021). Somaclonal variation is the polymorphic modification of the genome of regenerated plant material occurred due to epigenetic influence of tissue culture condition. It could be caused by plant species or varieties, genotype and ploidy level, explant characteristics such as developmental stage, duration of the callus phase, longer duration of culture, continuous subculturing, nutrient content of the growth medium or combination and concentration of plant growth regulator (Aydin et al., 2016; Bradaï et al., 2016; Cao et al., 2016; Weckx et al., 2019). Somaclonal variation arose from changes in number of chromosome, breakage and rearrangement of chromosome, modifications in DNA amplification, point mutations, activation of transposons, changes in DNA of organelles and changes in DNA methylation (Azizi

et al., 2020; Noormohammadi et al., 2020). Incidence of somaclonal variation is a major drawback apart from contamination issues in the field of plant tissue culture.

Genetic fidelity of regenerated plants could be studied via morpho-physiological, biochemical, cytological, and DNA-based molecular markers techniques (Krishna et al., 2016). Advancement of molecular marker techniques is preferentially used to analyse somaclonal variations over morphological observations such as plaant height and leaf morphology or cytological methods involving chromosomal structural changes (Rastogi et al., 2015). Highly reliable and reproducible molecular confirmation of genetic homogeneity in micropropagated plants is conducted using complimentary primers on the polymerase chain reaction and gel electrophoresis platform (Bidabadi and Jain, 2020). Different molecular markers have been used to study somaclonal variations. From hybridizing radioactive labelled probe technique of Restriction Fragment Length Polymorphism, analysis of genetic polymorphism has been taken over by PCR-based techniques such as Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), and Simple sequence repeat (SSR), Single Nucleotide Polymorphism (SNP), Start codon targeted (SCoT) and Conserved DNA derived polymorphism (CDDP) (Rastogi et al., 2015; Amom and Nongdam, 2017).

Of the available techniques, Directed Amplification of Minisatellite-region DNA (DAMD) technique was invented by Heath et al. (1993) to identify polymorphism in the minisatellites region. Since the construct of DAMD primers is longer than RAPD-PCR primers and constructed from conserved region of gene, thus, enhancing the stringency and reproducibility of the amplification process (Saidi et al., 2017; Saleh, 2019). Moreover, the absence of tedious steps of RFLP such as Southern blot makes DAMD a favourable method to analyse genetic variation. Another added