In vitro PROPAGATION OF *Carica papaya* L. cv. EKSOTIKA VIA SOMATIC EMBRYOGENESIS

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In vitro PROPAGATION OF *Carica papaya* L. cv. EKSOTIKA VIA SOMATIC EMBRYOGENESIS

ABSTRACT

This thesis summarizes the optimal protocols to achieve *in vitro* micropropagation of *Carica papaya 'Eksotika'* using somatic embryogenesis. Half strength MS medium supplemented with 10 mg L^{-1} Dichlorophenoxyacetic acid (2,4-D) and 18 mg L^{-1} of boric acid produced high yields of somatic embryos. ½ MS medium augmented with 10 mg L^{-1} phloroglucinol was found to be best suited for embryo maturation. The well-formed embryos produced shoots in MS medium supplemented with 0.05% activated charcoal, elongated in MS medium supplemented with 1 mg L^{-1} gibberellic acid (GA_3), 0.5 mg L^{-1} indol-3- acetic acid (IBA), 100 mg L^{-1} *Myo*-insitol and 3.76 mg L^{-1} riboflavin and produced roots in MS medium after being cultured for four days in full strength MS medium supplemented with 2 mg L^{-1} IBA. After the rooting phase, *in vitro* plantlets were acclimatized in peatmos soil. For artificial seeds, the somatic embryos encapsulated in MS medium without calcium demonstrated the highest percentage of shoot formation and the lowest percentage of callus formation at the base end of somatic embryos. Histological analysis demonstrated that somatic embryogenesis could be induced from embryogenic cells that originated in meristematic centers or from clusters of cells. The pro-embryos, globular-shaped embryos, heart-shaped embryos, torpedo-shaped embryos and cotyledonary stages were found to closely resemble the ontogeny of zygotic embryos. Active gene expression of all selected genes, namely *CpLAX2*, *CpLAX3* and *CpPIN4*, were detected in all somatic embryos collected during the maturation and germination phases. All genes (*CpLAX2*, *CpLAX3* and *CpPIN4*) were involved in the development and growth of somatic embryos.

Keywords: Somatic embryogenesis, Artificial seeds, Real time PCR, *Carica papaya* 'Eksotika', Micropropagation.

PROPAGASI *in vitro Carica papaya* L. cv. EKSOTIKA MELALUI EMBRIOGENESIS SOMATIK

ABSTRAK

Tesis ini meringkaskan protokol optimum untuk mencapai mikropropagasi *in vitro Carica papaya* 'Eksotika' menggunakan embriogenesis somatik. Separuh kekuatan MS medium ditambah dengan 10 mg L^{-1} Dichlorophenoxyacetic acid (2,4-D) dan 18 mg L^{-1} asid borik menghasilkan hasil tinggi embrio somatik. Medium ½ MS ditambah dengan 10 mg L^{-1} phloroglucinol didapati paling sesuai untuk pematangan embrio. Embrio yang terbentuk dengan baik menghasilkan pucuk dalam medium MS ditambah dengan arang aktif 0.05%, memanjangkan dalam medium MS ditambah dengan 1 mg L^{-1} asid gibberellic (GA_3), 0.5 mg L^{-1} indol-3 asid asetik (IBA), 100 mg L^{-1} *Myo*-insitol dan 3.76 mg L^{-1} riboflavin dan akar yang dihasilkan dalam medium MS selepas dibiakkan selama empat hari dalam kekuatan penuh MS medium ditambah dengan 2 mg L^{-1} IBA. Selepas fasa rooting, in vitro plantlets telah disesuaikan di tanah peatmos. Untuk biji buatan, embrio somatik yang terkandung dalam medium MS tanpa kalsium menunjukkan peratusan tertinggi pembentukan menembak dan peratusan terendah pembentukan kalus pada akhir asas embrio somatik. Analisis histologi menunjukkan bahawa embriogenesis somatik boleh diinduksi dari sel-sel embriogenik yang berasal dari pusat meristematik atau dari kelompok sel. Pro-embrio, embrio berbentuk globular, embrio berbentuk hati, embrio berbentuk torpedo dan peringkat cotyledonary didapati hampir mirip dengan ontogeny embrio zygotik. Ekspresi gen aktif semua gen terpilih, iaitu *CpLAX2, CpLAX 3* dan *CpPIN4*, dikesan dalam semua embrio somatik yang dikumpulkan semasa fasa pematangan dan percambahan. Semua gen (*CpLAX2*, *CpLAX3* dan *CpPIN4)* terlibat dalam pembangunan dan pertumbuhan embrio somatik.

Kata kunci: embriogenesis somatik, biji Buatan, PCR masa nyata, *Carica papaya* 'Eksotika', Mikropropagasi.

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LIST OF SYMBOLS AND ABBREVIATIONS

%	:	Percentage
½ MS	:	Half strength Murashige and Skoog medium
½WPM	:	Half strength McCown Woody Plant medium
2,4,5-T	:	Trichlorophonexy acetic acid
2,4-D	:	2,4-Dichlorophenoxyacetic acid
AC	:	Activated charcoal
AVG	:	Aminoethoxy Vinyl Glycine
B	:	Boron
BAP	:	6-Benzylaminopurine
Cdna	:	Complementary DNA
Cm	:	Centimeter
CO_2	:	Carbon dioxide
DNA	:	Deoxyribonucleic acid
FAO	:	Food and agriculture organization
G	:	Gram
H	:	Hour
HCl	:	Hydrochloric acid
Hg/Ha	:	Hectogram/ Hectare
IAA	:	Indole-3-acetic acid
IBA	:	Indol-3 butyric acid
IRAP	:	Inter- Retrotransposons amplified polymorphism
IZE	:	Immature zygotic embryo
L	:	Liter
Ma	:	Million years

MARDI	:	Malaysian Agricultural Research & Development Institute
Mg	:	Milligram
min	:	Minute
mL	:	Milliliter
MS	:	Murashige and Skoog medium
MTD	:	Malformed Top Disease
NAA	:	Naphthaleneacetic acid
NaOH	:	Sodium hypochlorite
NH_4^+	:	Ammonium ions
NO_3^-	:	Nitrate ions
pCPA	:	*p*- Chlorophenoxyacetic acid
PCR	:	Polymerase chain reaction
PEG	:	Polyethylene glycol
PG	:	Phloroglucinol
PRSV	:	Papaya Ring Spot Virus
PVS2	:	Plant vitrification solution 2
RAF	:	Randomly amplified DNA fingerprinting
REL	:	Relative expression level
RNA	:	Ribonucleic acid
SA	:	Sodium alginate
SE	:	Somatic embryogenesis
STS	:	Silver thiosulphate
TDZ	:	Thidiazuron
WPM	:	McCown Woody Plant medium

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

The history of *Carica papaya* L. (*C. papaya*) in Malaysia began when Spaniards brought the plant to the Philippines and eventually transferred and grew the *C. papaya* L. in Malaya around 1598. Malaysians and Singaporeans refer to the *C. papaya* L. fruit as "betik" (Chan, 2009). *C. papaya* L. is considered as an economically important fruit tree, especially for tropical and subtropical populations. The *C. papaya* fruit can either be consumed as a fresh fruit or used as a treated product. The ripe *C. papaya* fruits are usually eaten fresh but can also be used as an element in fruit cocktails as well as made into candy, jelly or jam. The unripe fruits contain latex which is the source of papain, a plant proteolytic enzyme (Mendoza *et al.*, 2008). The papain enzyme is able to break down protein and polypeptide. Therefore, it plays a crucial role as drug purposes and pharmaceutical industry. It is also used for clearing beer, tenderizing meat, leather industry, cosmetics industry and candy and chewing gum industry (Nakasone & Paull, 1998; Mamboya, 2012). The *C. papaya* tree is characterized by crops that have early production rates and high yields compared to other perennial plant crops. *C. papaya* trees can begin flowering four months after seed germination and subsequently produce fruits eight months after being planted. Each *C. papaya* tree can produce about 30-40 fruits yearly (Farzana *et al.*, 2008).

C. papaya 'Eksotika' is listed as one of the most important exported fruits in Malaysia. It is mainly cultivated in Johore, Perak, and certain parts of Selangor. The most popular *C. papaya* cultivar grown in Malaysia is 'Eksotika'. This cultivar resulted from a cross breed between two different cultivars, the Subang 6 (old Malaysian cultivar) and the Hawaiian Sunrise Solo (Amarican cultivar). Moreover, the 'Eksotika' was developed by the Malaysian Agricultural Research and Development Institute (MARDI) in 1987 (Chen *et al.*, 1987). This cultivar is better than the 'Sunrise Solo' cultivar in size and texture (Chan & Raveendranathan 1990).

The ‘Eksotika’ is mostly grown from seeds in order to produce hermaphrodite and female trees in a (3:1) ratio respectively and with a 100% rate of fruit-producing trees (Chan & Teo, 1993). The ‘Eksotika’ is very sensitve to many diseases, especially the Papaya Ringspot Virus (PRSV) which infected *C. papaya* crops in Johor in 1991 (Vilasini *et al.*, 2000). The PRSV disease and rapid damage of the fruit were two major challenges faced by the *C. papaya* industry in Malaysia (Chan, 2002; Chan *et al.*, 2015). Also, the ‘Eksotika’ papaya cultivars are most susceptible to the Malformed Top Disease (MTD) caused by the *Cladosporium- thrip* complex. Malformed Top Disease is a destructive disease, especially to small developing plant parts. Trees infected with this fungus show many symptoms in the vegetative parts that lead to the weakening and eventual death of the tree (Chan & Mak, 1993). Chan and Teo (1993), reported that 99–100% of the explants obtained from mature field-grown trees were contaminated with fungus and bacteria.

The majority of *C. papaya* farms depend on seeds for propagation. *C. papaya* propagation through seeds is the preferable method for farmers because it is easy and cheap (Bhattacharya & Khuspe, 2001). However, farmers tend to produce low quality seeds from fruits grown in the farm. This conventionalism caused the spread of dangerous diseases in *C. papaya* farms. In conventional propagation, two seeds or seedlings are usually planted together and when the flower sexes are determined, only the wanted sex is kept (Wu *et al.*, 2012). These practices are very costly for farmers and subsequently for consumers as well (Schmildt *et al.*, 2015).

Although *C. papaya* is a perennial plant, parasitic infestation often prevents farmers from growing *C. papaya* annually. These parasitic infestations reduce the productivity of *C. papaya* trees after 2-3 years (Wu *et al.*, 2012). Apart from that, the absence of complete similarity to the mother plant, high incidence of diseases, high percentage of unwanted

male plants and significant genetic variability are the major problems faced during conventional propagation that limit *C. papaya* propagation in commercial quantities (Farzana *et al.*, 2008; Paull *et al.*, 2008; Kavitha *et al.*, 2010)

In some countries, such as South Africa, vegetative propagation of *C. papaya* through grafting, budding, and root cutting is preferred. However, these methods are not widely used for all *C. papaya* cultivars, since not all *C. papaya* cultivars have the propensity to produce multi-branching. The limited number of plants produced per mother plant is another reason these methods are not widely used (Farzana *et al.*, 2008).

The usage of tissue culture techniques is considered an accessible choice, especially when used for large farms (Magdalita & Mercado, 2003). Generally, the commercial *in vitro* mass propagation of *C. papaya* has not been established due to various difficulties in micro-propagation. For example, microbial contamination caused by endogenous bacteria, especially when mature plants grown in the field are used as explant. Other difficulties include low propagation rates during subculturing and difficulties in establishing normal roots and acclimatization (Wu *et al.*, 2012). According to Litz and Conover (1981), the maintenance of proliferating cultures was missing during the time. Also, apical dominance in shoots were affected with time in multiplication medium (Rajeevan & Pandey, 1986). Many factors have an effect on the development of roots in *in vitro* cultures such as auxin type and concentration, shoot quality, donor age, temperature and substrate (Mohammed & Vidaver, 1988). Plantlet regeneration using shoot tips of hermaphrodite 'Eksotika' papaya was not accomplished because the root quality was very poor (Panjaitan *et al.*, 2007). Also, there was a problem during acclimatization of regenerated plants, whereby over 2/3 of the plants were lost before being moved to the field (Malabadi *et al.*, 2011). Acclimatization is a critical stage in all *in vitro* propagation protocols. In this phase, the relative humidity should start to gradually

decrease to improve the stomata function, enhance cuticle development, and decrease water loss (Pérez *et al.*, 2015).

Somatic embryogenesis (SE) has been employed in *C. papaya* propagation to overcome the difficulties faced during conventional seed propagation (Litz & Conover, 1977; Fitch, 1993; Anandan *et al.*, 2012). Somatic embryogenesis is an appropriate micro-propagation technique in *C. papaya* to overcome the challenges relating to the absence of an actual method for early sex determination (Abreu *et al.*, 2014). Somatic embryogenesis has a superior advantage over other plant tissue culture techniques as it offers an excellent solution for the rooting problem commmonly faced in other micro-propagation techniques. The somatic embryo has bipolar structures containing both shoot and root apices. Also, embryogenic cultures have the ability to produce large numbers of embryos per culture especially when grown in liquid media. Embryos float freely in liquid media after mechanical separation through agitation and therefore do not need manual separation and mechanical handling (Farzana *et al.*, 2008). Unfortunately, the germination of somatic embryos have problems associated with root development due to the accumulation of callus at the base end of somatic embryos which prevents proper root development and weakens the process of being joined to the stem (Fitch & Manshardt, 1990; Sekeli *et al.*, 2012).

In fact, according to the statistics from Food and Agriculture Organization (FAO, 2018), the production and yield of *C. papaya* in Malaysia (Figures 1.1 & 1.2) is low compared to other Asian countries such as the Philippines and Indonesia (Figures 1.3 & 1.4) as well as the world *C. papaya* production (Figures 1.5). Recent years increase in papaya production in Indonesia are mainly the result significant increase in harvested area and fruit yield in Indonesia.

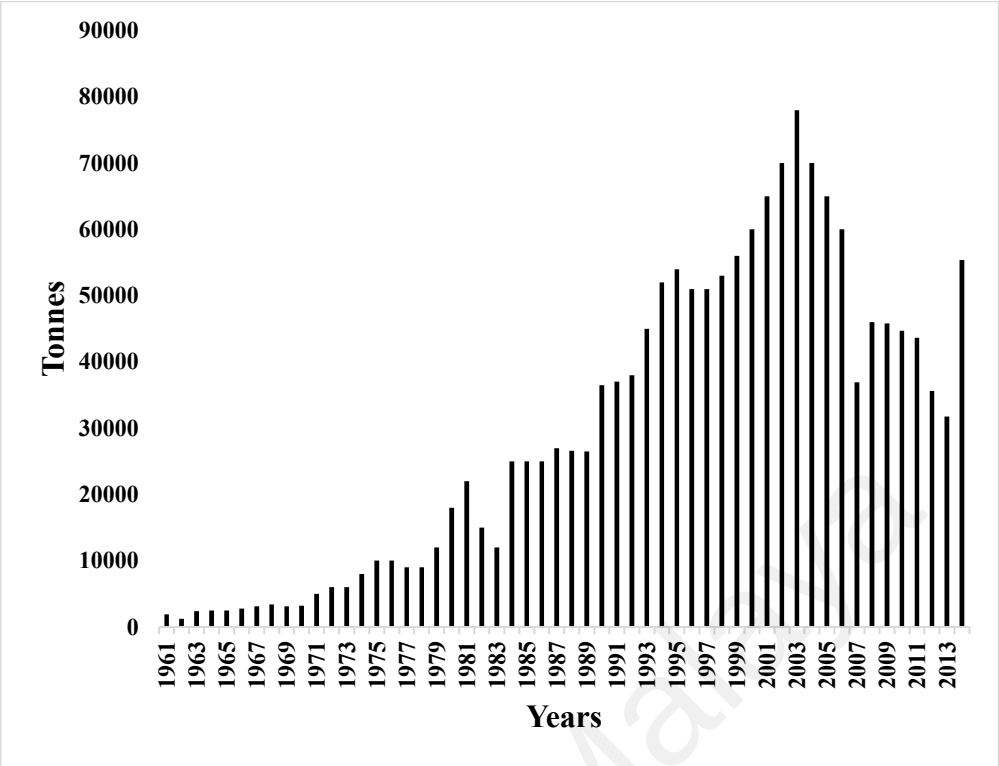

Figure 1.1: Production of *C. papaya* in Malaysia from 1961-2014, (FAO, 2018).

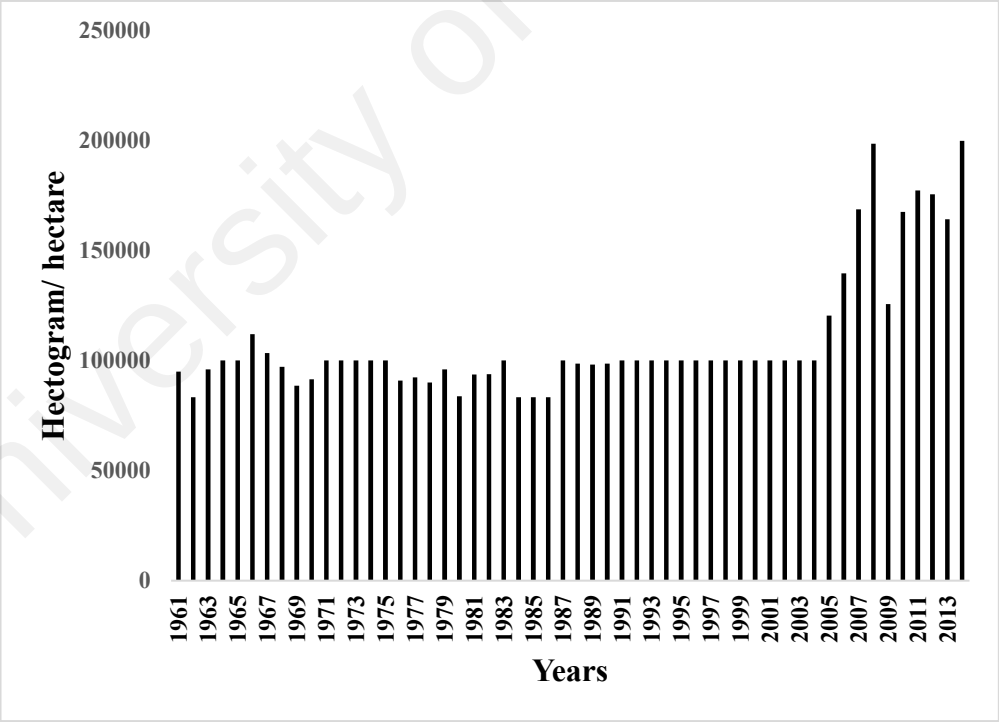

Figure 1.2: Production yield of *C. papaya* in Malaysia from 1961-2014, (FAO, 2018).

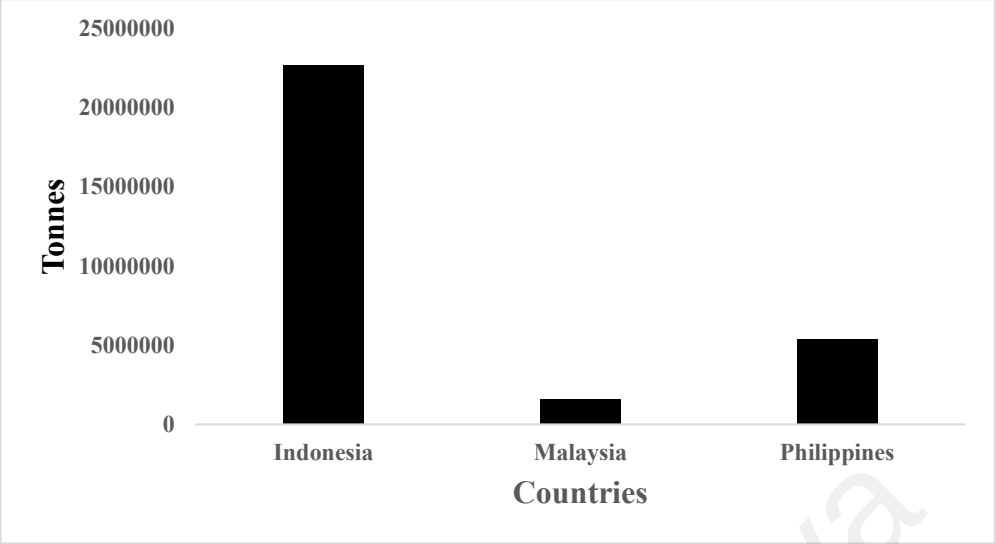

Figure 1.3: Sum of *C. papaya* production in Indonesia, Malaysia and Philippines from 1961-2014, (FAO, 2018).

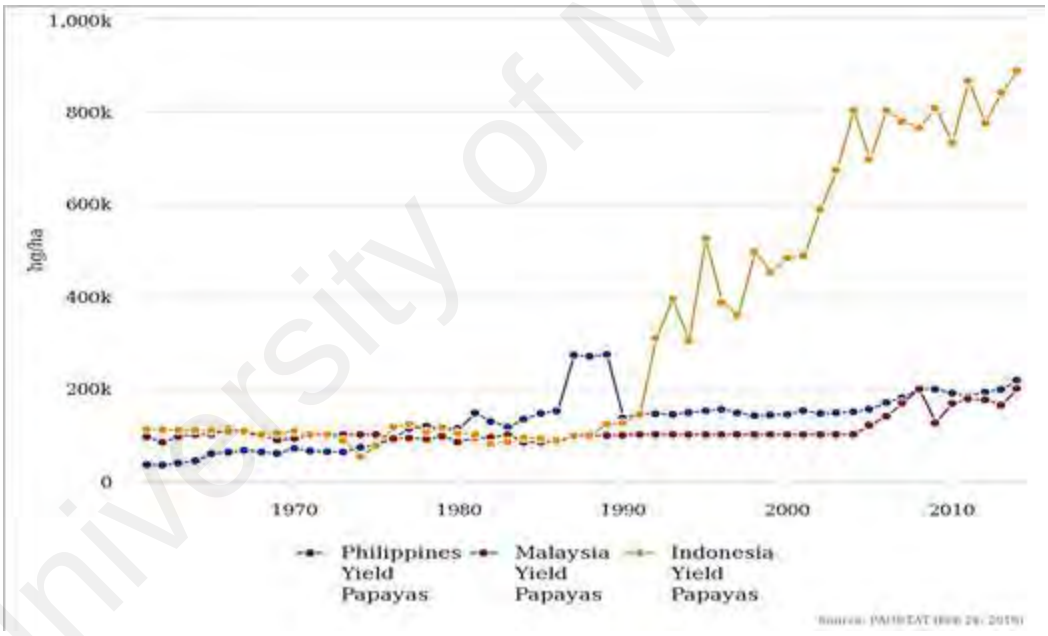

Figure 1.4: Production yield of *C. papaya* in the Philippines, Malaysia and Indonesia from 1961-2014, (FAO, 2018).

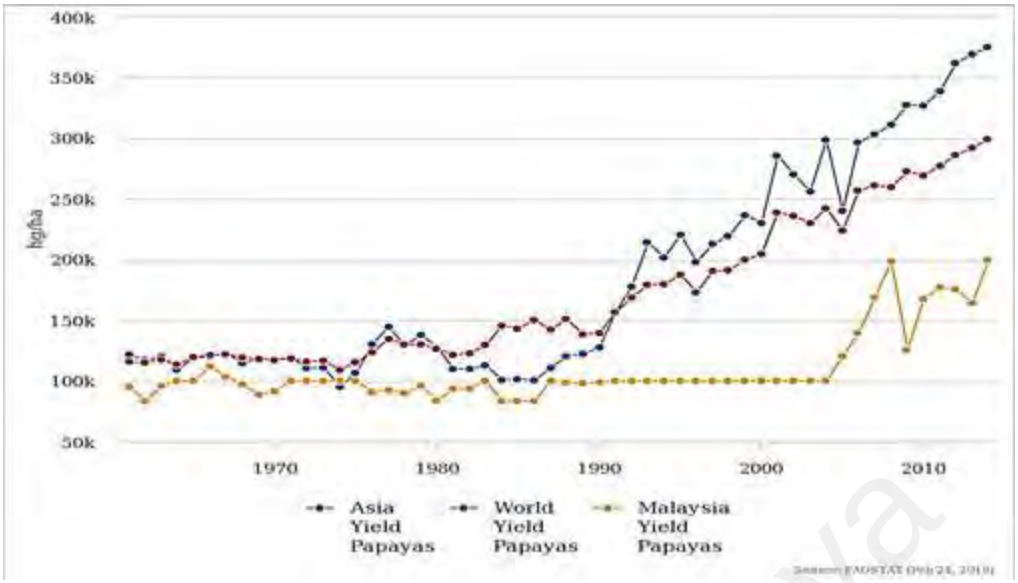

Figure 1.5: Production yield of *C. papaya* in Asia, the world and Malaysia from 1961-2014, (FAO, 2018).

There is clear interest for improving plant cell culture techniques that could help rapid propagation of elite cultivar plants. In Malaysia, there is an urgent need to meet the large requirement of improving *C. papaya* seedlings which are considered an economically important crop (Khalid *et al.*, 2007). To achieve this, we must first solve the two major difficulties faced in developing transgenic Malaysian 'Eksotika'. These difficulties are low efficiency of regenerated roots from shoots and low acclimatization rates of rooted transgenic 'Eksotika' plants in the field. Therefore, rooting efficiency with high-quality root formations is critical in ensuring successful and continuous production of transgenic 'Eksotika' (Sekeli *et al.*, 2014).

1.1 Research Aims

Since 'Eksotika' is an important crop in Malaysia there is a particular interest in genetic improvement programs. The multiplication of 'Eksotika' by seeds is considered as a conventional method. Farmers tend to produce their seeds from fruits grown in the farm. Conventionally, they plant two to three seeds in each hole, wait six to eight months until the sex properties of the plants appear and then remove seedlings of the unwanted sex. So, the farmers have to take care for two to three plants for more than eight months,

fertilizing and spraying them until the flowers appear and then removing plants of the unwanted sex. Essentially, they end up losing 50 to 70% of their crop after eight months of care. Furthermore, the productivity of plants that are propagated by seeds varies in their characteristics.

Thus, the propagation of elite 'Eksotika' plants will increase the economic returns of farmers. One of the methods which can be applied to the propagation of elite 'Eksotika' at a large scale is plant tissue cultures such as SE. However, this cannot happen without standardized steps starting from the sterlization of explants until acclimatization. One defect in any of these steps could adversely affect the final output of the process, either in quality or quantity.

Therefore, this research will examine all the steps in 'Eksotika' production by SE and set the conditions to create a suitable and highly efficient protocol that can be applied at an economic scale. The production of artificial seeds to facilitate the transference and handling of seeds will also be examined. Also, this protocol can be used for both hybridization and transformation experiments to generate strains that are resistant to various diseases. A molecular study was conducted to better understand the fundamental biological factors involved in the maturation of SE of *C. papaya*. Additionally, the process of root formation in SE and the process of callus formation at the base end of somatic embryos was also investigated. So far, no sequence data relating to SE has been available in papaya.

1.2 Research Objectives

To achieve the above above-mentioned the following objectives were considered:

1- To standardize the seed sterilization protocol.

2- To standardize the protocol for SE from immature zygotic embryos (IZE) through the following procedures:

   a- Standardize the induction media and conditions.

   b- Standardize the maturation media and conditions.

   c- Standardize the germination media and conditions.

   d- Standardize the root media and conditions.

3- To establish the synthetic seed production protocol.

4- To study the anatomy of different developmental stages in SE.

5- To detect and follow up the differences in expression levels of *CpLAX2* *CpLAX3* and *CpPIN4* genes during different SE developmental stages.

There is currently no established protocol for 'Eksotika' seeds sterilization. Hence, the first step taken in this research was to adopt a protocol to sterilize the seeds and to ensure sterilization of seeds with a high survival rate. Then, different media with different plant growth regulator concentrations were examined to standardize the different SE steps starting from the induction stage all the way to the rooting stage. Achieving this protocol and using it in the commercial propagation of elite 'Eksotika' and genetic transformation experiments will help to improve the quality and quantity of 'Eksotika' by producing 'Eksotika' that are resistant to environmental changes and diseases.

Standardizing artificial seed production for 'Eksotika' will help in the transportation of elite 'Eksotika' cultivar plants between laboratories. Moreover, it will ease the distribution process of elite plants for farmers.

In addition to the above, a histological study was used to track somatic embryo development and callus formation at the base end of somatic embryos. This will help diagnose SE in early stages and better understand the developmental stages of SE.

A molecular study was conducted to better understand the fundamental biological factors involved in the maturation of SE of *C. papaya*. Additionally, the process of root formation in SE and the process of callus formation at the base end of somatic embryos was also investigated. So far, no sequence data relating to SE has been available in *C. papaya*.

CHAPTER 2: LITERATURE REVIEW

According to FAO (2018) the world produced 12,698,728 tons of *C. papaya* during 2014 with yield reach to 299,239 hg/ha and Asia production was 7,212,748 tones with yield reach to 375,140 hg/ha. Whereas, Malaysia production was 55,358 tons produced from growing 2,771 ha during 2014, with yield reach to 199,776 hg/ha. The production and yield of *C. papaya* in Malaysia are low compared to other Asian countries such as the Philippines and Indonesia as well as the world *C. papaya* production.

2.1 Taxonomy and Origin of Caricaceae

The Caricaceae classification was revised by Badillo to include six genera; *Carica, Jacaratia, Horovitzia, Jarilla, Vasconcella*, and *Cylicomorpha* (Badillo, 2000). Of the 35 species belonging to the Caricaceae family, 32 are dioecious, two are trioecious (*Vasconcella cundinamarcensis* and *Carica papaya*) and one is monoecious (*Vasconcella monoica*) (Badillo, 2000; Ming *et al.*, 2008a). The genus *Carica* consists of only one species (*Carica papaya*). The genus *Cylicomorpha* consists of two species found in tropical Africa, namely the *Cylicomorpha solmsii* found in West Africa and the *Cylicomorpha parviflora* found in East Africa. The genus *Horovitzia* consists of one species (*Horovitzia cnidoscoloides*). The genus *Jarilla* comprises of three species of perennial herbs. The genus *Jacaratia* has 83 species while the *Vasconcellea* genus has 20 species (Badillo, 2000; Carvalho & Renner, 2012).

In the past, scientists commonly supposed that *C. papaya* originated from Central America, specifically from Chile and Argentina to Mexico (Manshardt *et al.*, 1992).

Moringaceae is the sister group of Caricaceae. The DNA sequence in the chloroplast gene *rbc L* props in Caricaceae differs from that in Moringaceae (Olson, 2002). The deepest split in the Caricaceae took place during the Late Eocene around 56.0 million years (Ma) (Carvalho & Renner, 2012). Recently, it was discovered that the *C. papaya* is

related to *Jarilla* and *Horovitzia*, and that all three were spread from South America in the Oligocene around 22–33 Ma ago. The discovery that *C. papaya* is the closest to a herbaceous species has presented a better substitute for plant breeders who have so far only used woody highland papayas (*Vasconcellea*) to cross with *C. papaya* (Carvalho & Renner, 2012).

2.2 General Introduction and Taxonomy of *Carica papaya*

C. papaya L. is the only species in the genus *Carica*. *C. papaya* is dicotyledonous and is considered a polygamous species; the *C. papaya* plant has three types of sex; male, female and hermaphrodite (Bhattacharya & Khuspe, 2001; Liu *et al.*, 2004). Pistillate and hermaphrodite *C. papaya* plants produce fruit all year-round in tropical regions while staminate *C. papaya* plants usually do not produce fruit (Storey, 1953). Consumers prefer *C. papaya* that have flavor and fragrance, flesh color, with standards shape, firmness and size. The taxonomic positions of *C. papaya* L. as below

Kingdom	Plantae
Subkingdom	Viridiplantae
Division	Tracheophyta
Subdivision	Spermatophytina
Class	Magnoliopsida
Order	Brassicales
Family	Caricaceae
Genus	*Carica*
Species	*Papaya*

C. papaya cultivars are differentiated by various vegetative morphological traits such as; leaf shape, central leaf veins, the number of lobes, stomata type, wax structures in leaves, and the petiole color. It generates flowers unceasingly throughout the year and has been conventionally cultivated by seed (Bhattacharya & Khuspe, 2001; Liu *et al.*, 2004). The life cycle of *C. papaya* is short, it only takes between 8 to 12 months from seed to ripe fruit (Storey, 1953).

C. papaya seeds consist of many layers namely, the seed coat that is derived from the multiple outer epidermises of the outer integument of the ovule. The outer region of the seed layer of *C. papaya* is fleshy and becomes a gelatinous sarcotesta at maturity. The mesotesta of *C. papaya* seeds is compact and hard at maturity, consisting of a series of sculptured, soft, and hydroscopic longitudinal folds, derived from sub-epidermal layers of the outer integument. Endosperm consists of superfine walled cells. The embryo is white with ovoid cotyledons (Fisher, 1980).

Morphologically, the *C. papaya* stem is unbranched, hollow and contains leaf scars. It rarely has branching stems without damaging the apical meristem. The wood of the trunk and tap root is soft and succulent. Internodes are hollow (Storey, 1953; Badillo, 2000). The leaves of *C. papaya* are spirally arranged and clustered at the crown. Mature *C. papaya* leaves are palmate with broad pinnatifid lobes. The petioles are green or purple in color, and are lengthy and blank (Storey, 1953; Campostrini & Yamanishi, 2001).

C. papaya has three kinds of flowers: staminate, pistillate and hermaphrodite. Staminate inflorescences are cymose, lateral, long, pendulous and have many branching inflorescences. The staminate flower consists of solid pedicel, and the calyx cup is five toothed. The corolla tubular consists of five light yellow lobes. There are ten stamens arranged in two whorls. The first whorl alternates with petals from the second whorl. The filaments are long, and the anthers have four locules (Fisher, 1980). The pistillate flowers are solitary, the pedicel is solid, and the calyx cup is also five toothed. The corolla in pistillate flowers consist of five petals that are twisted and fused at the base. The ovary is inferior with parietal placentation containing five carpellate that have numerous ovules and a fan-shaped stigma. The style canal is short and lined with stigmatic hairs and mucilage (Fisher, 1980). The hermaphrodite flower is intermediate in size compared to the staminate and pistillate flowers. There are two types of hermaphrodite flowers:

elongated and pentandria. The elongated type consists of short pedicles with a corolla that has united lobes, one-third its length. The ten stamens have the same arrangement as in male flowers. The pistil is functional and elongated. On the other hand, the pentandria type is similar to female flowers but has five stamens with long filaments attached to the base of the ovary. The pistil is functional and not elongated (Fisher, 1980).

C. papaya fruit is a fleshy berry. Its shape varies according to sex; female flowers produce spherical shape fruits, hermaphrodite flowers provide pyriform to cylindrical shape fruits (Fisher, 1980). While the immature fruit is green and rich in white latex, mature fruits are yellow-orange in color and do not have latex. They contain plenty of grey-black ovoid seeds attached by soft, white, fibrous tissue with flesh. The shape of the fruit is a discriminatory characteristic for sex determination. The fruits from pistillate flowers vary from sphere-shaped to ovoid, while the fruits from hermaphrodite flowers are elongated, cylinder-shaped or pyriform. The color of ripe fruits are yellow, orange or red depending on the carotenoids present in the *C. papaya* (Paull *et al.*, 2008; Aikpokpodion, 2012).

2.3 Plant Tissue Culture Media Constituents

Determining the type of media to be used is crucial for successful plant tissue culture systems. The components of media in plant tissue cultures were carefully calculated to grow plant tissues in an artificial environment. Several different media for plant tissue cultures were developed such as MS media (Murashige & Skoog, 1962). The MS media, developed by Murashige and Skoog (1962), is considered the most famous media for *in vitro* plant cultures. The MS media consists of macronutrients, micronutrients, and vitamins.

2.3.1 Macronutrients

Macronutrients consist of the major elements that is, Potassium (K), Nitrogen (N), Calcium (Ca), Phosphate (P), Magnesium (Mg), and Sulphur (S). Plants need them for growth and development. They are usually required in large quantities (George *et al.*, 2008).

2.3.1.1 Potassium (K)

Potassium is one of the fundamental elements in plant media that is needed for plant growth and development. The amount of K needed for plant growth and development differs with plant species. The concentration of K in media is commonly correlated with the nitrate concentration in media. Potassium in the form of potassium dihydrogen phosphate and potassium sulfate is commonly used in media (George *et al.*, 2008).

2.3.1.2 Nitrogen **(N)**

Nitrogen is one of the fundamental elements needed for plant growth and development. The source of N in media can be found in two forms. The first is inorganic N such as ammonium ions (NH_4^+) and nitrate ions (NO_3^-). The total amount of N in media and the ratio between NO_3^- and NH_4^+ are important. The second is organic N such as amino acids rich in N. For example, proline, glutamine, and casein hydrolysate. Organic N is reduced to ensure that the N form is similar to that found in plants in order to facilitate N uptake (George *et al.*, 2008).

2.3.1.3 Calcium (Ca)

Calcium is one of the major elements in plant media. Calcium in the form of $CaCl_2$ or calcium nitrate is usually used in plant media as they act as a co-factor of many enzymes (George *et al.*, 2008).

2.3.1.4 Phosphorous (P)

Phosphorus is one of the major elements in plant media. It is a vital portion in nucleic acids and further cellular structural compounds. Phosphorous in the form of potassium dihydrogen phosphate is usually used in plant media (George *et al.*, 2008).

2.3.1.5 Magnesium (Mg)

Magnesium is one of the major elements in plant media. It is essential for the working of enzymes that are responsible for photosynthesis and chlorophyll formation. Magnesium in the form of magnesium sulfate is usually used in plant media (George *et al.*, 2008).

2.3.1.6 Sulfur (S)

Sulfur is one of the major elements in plant media. It is a vital portion in some amino acids and has an important function in protein folding and structure. Sulfur in the form of magnesium sulfate is usually used in plant media (George *et al.*, 2008).

2.3.2 Micronutrients

Micronutrients consist of minor elements such as Iron (Fe), Manganese (Mn), Zinc (Zn), Boron (B), Copper (Cu), Molybdenum (Mo), Cobalt (Co), and Iodine (I). Plants need them for growth and development. They are usually required in small quantities (i.e., less than 0.5 mM) (George *et al.*, 2008).

2.3.2.1 Iron (Fe)

Iron is one of the minor elements in plant media. Plants need iron for chlorophyll synthesis and functions. The addition of Fe into culture media forms precipitate compounds, especially in alkaline conditions. This enables the binding of Fe with chelating agents, such as ethylenediaminetetraacetic acid (EDTA), that help to stabilize and make Fe available to plant tissues (George *et al.*, 2008).

2.3.2.2 Manganese (Mn)

Manganese is one of the minor elements in plant media. Most enzyme reactions in respiratory and photosynthetic processes heavily depend on Mn. It is usually found in media as Manganese sulfate monohydrate (George *et al.*, 2008).

2.3.2.3 Zinc (Zn)

Zinc is one of the minor elements in plant media. Zinc is necessary for enzyme activities. It is usually found in media as Zinc sulfate tetrahydrate (George *et al.*, 2008).

2.3.2.4 Boron (B)

Boron is one of the minor elements in plant media. Boron is necessary for lignin biosynthesis and metabolism of phenolic acids. It is usually found in media as Boric acid (George *et al.*, 2008).

Boron influnces many essential physiological processes such as maintaining the structural organization of the cell wall (Pandey *et al.*, 2012). The majority of the cell is localized Boron in the cell wall and is associated with pectins within the cell wall. Furthermore, tissue that is deficient in Boron substantially reduced cell wall plasticity and damaged normal cell elongation in growing plant tissues (Hu & Brown, 1994). Recently, scientists have focused on studying the function of Boron in the induction and maturation of embryogenesis (Pandey *et al.*, 2012). Boron is required to maintain normal pore sizes and to stabilize the structure of cell walls during the stationary phase. A deficiency in boric acid causes plant cells to be more enlarged and detached resulting in ruptured plant cells during the stationary phase (Fleischer *et al.*, 1998). Boron affects the endogenous levels of auxin. The absence of B in the media leads to an increase of endogenous auxin and may also swich-on other genetic and cellular trans factors that are necessary for cell reprogramming and inducing embryogenesis (Pandey *et al.*, 2012).

2.3.2.5 Copper (Cu)

Copper is one of the minor elements in plant media. Copper is necessary for enzyme reactions, especially in the cytochrome oxidase system. It is usually found in media as Copper(II) sulfate pentahydrate (George *et al.*, 2008).

2.3.2.6 Molybdenum (Mo)

Molybdenum is one of the minor elements in plant media. Molybdenum helps in the alteration of nitrate to ammonium. It is usually found in media as Sodium molybdate monohydrate (George *et al.*, 2008).

2.3.2.7 Cobalt (Co)

Cobalt is one of the minor elements in plant media. It is usually found in media as Cobalt dichloride hexahydrate (George *et al.*, 2008).

2.3.2.8 Iodine (I)

Iodine is one of the minor elements in plant media. Iodine improves the growth of roots and callus *in vitro*. It is usually found in media as potassium iodide (George *et al.*, 2008).

2.3.3 Organic Compounds

Organic compounds are mostly used as components in or additives to plant culture media that help in the growth and development of plants. Sugar is an organic compound that is important for plant growth and development while organic acids, vitamins and undefined compounds are organic compounds that are not important for plant growth and development but may instead enhance the growth and development of plants (George *et al.*, 2008).

2.3.3.1 Sugars

Plants that grow *in vitro* are considered heterotrophic which means that they cannot fix carbon through the photosynthesis process. Therefore, media augmented with sugar acts as an energy source. There are many types of sugar that can be used as energy sources in plant media such as sorbitol, glucose, fructose and sucrose. Sucrose is the most common type of sugar that is added to plant media (George *et al.*, 2008). Plants in *in vitro* culture conditions need an exogenous carbohydrate source because most of the plants grown *in vitro* tend to shift from autotroph to heterotroph. Selecting the best carbohydrate source and concentration in culture media depends on the plant species and the micropropagation phase. Most *C. papaya* species induced SE in media supplemented with 6% (w/v) sucrose, which promoted a high frequency of embryogenesis. Also, under similar concentrations and mediums, sucrose is better than maltose in inducing embryogenic callus (Vilasini *et al.*, 2000). Sucrose plays an important role and affects the formation of somatic embryogenic callus. In 'Eksotika', 60 g L^{-1} sucrose produced the highest frequency of somatic embryo callus from IZE of hermaphrodite 'Eksotika'(Razak *et al.*, 2015). On the other hand, decreasing or sucrose absent media reduced the risk of contamination, enhanced the photosynthetic capacity of the plant and also, improved acclimatization of plants propagated *in vitro* (Xiao *et al.*, 2011).

2.3.3.2 *Myo*-inositol

Myo-inositol ($C_6H_{12}O_6$) is a sugar alcohol frequently added to plant culture media. *Myo*-inositol can improve *in vitro* responses, mainly in monocots (George *et al.*, 2008).

2.3.3.3 Vitamins

There are many vitamins that can be used in plant tissue cultures such as vitamin E, biotin, ascorbic acid, thiamine, pyridoxine and nicotinic acid. The most widely used vitamins in plant media are thiamine, pyridoxine, and nicotinic acid (George *et al.*, 2008).

2.3.4 Solidifying Agents

Solidifying agents are utilized to produce solid media to support *in vitro* explants and to equally distribute nutrients in the media (George *et al.*, 2008).

2.3.4.1 Agar

Agar is extracted from marine red algae. Agar is a polysaccharide and is the most commonly used gelling agent in plant tissue cultures. Agar consists of two main ingredients, agarose and agaropectin, as well as other impurities such as phenolics and organic compounds. Both agarose and agaropectin polymerize to form gels when mixed with liquid. Agarose and agaropectin have the ability to trap high amounts of H_2O. The gel formed from agarose and agaropectin melts when heated to 100°C and begins solidifying when cooled down to 45°C. Agar is an inert and stable compound which does not react with media components and enzymes released from plant tissues. It does not solidify well when mixed in media augmented with Activated Charcoal (George *et al.*, 2008).

2.3.4.2 Gelrite

Gelrite consists of a polysaccharide and some impurities such as inorganic ions. It is produced by the bacterium *Pseudomonas elodea*. Gelrite produces media that is clearer compared to agar. Therefore, contamination can be distinguished easily. However, Gelrite requires more stirring than agar during media preparation. Additionally, it cannot be reheated and gelled successfully like agar (George *et al.*, 2008).

2.3.4.3 Phytagel

Phytagel consists of a polysaccharide and some impurities**.** It is produced from a bacterial substrate and consists of glucuronic acid, glucose and rhamnose (George *et al.*, 2008).

2.3.5 Media Formulations

Murashige and Skoog (MS) media is the most commonly used media in plant tissue cultures. Murashige and Skoog developed MS media for culturing *Nicotiana tabacum.* The chemical compositions of MS media were designed based on the mineral compounds present in the *Nicotiana tabacum* tissue. Murashige and Skoog media contains a higher salt concentration compared to other media types mainly K and N. Linsmaier-Skoog medium (LS) is a modification of the MS medium using different organic components (Linsmaier & Skoog, 1965). Gamborg's B5 medium was designed with a larger amount of nitrate compared to ammonium ions for soybean callus cultures (Gamborg *et al.*, 1968). Nitsch and Nitsch's (1969) medium was designed with lower salt concentrations than the MS medium (Nitsch & Nitsch, 1969). Lloyd and McCown's Woody Plant Medium (WPM) (Lloyd & McCown, 1980) was designed for tree species. Knudson's medium was designed for orchard cultures (Knudson, 1946).

2.4 Conventional Propagation of *Carica papaya*

The majority of *C. papaya* plantations have been based on seed propagation. The propagation of *C. papaya* by seed is a preferable method for farmers to grow *C. papaya* because it is relatively cheap (Bhattacharya & Khuspe, 2001). Employing tissue culturing techniques are easily accessible, however, they are exhausting and costly especially when used for small farms (Magdalita & Mercado, 2003).

C. papaya is classified as a perennial, but common parasitic infestation often prevents farmers from growing *C. papaya* annually. This results in the reduced productivity of *C. papaya* trees after 2-3 years. Farmers tend to produce low quality seeds from fruits grown in the farm. This conventionalism can spread severe diseases in *C. papaya* farms. In conventional propagation, two seeds or seedlings are usually planted together and when the flower sexes are determined, only the preferred sexes are kept (Wu *et al.*, 2012). These

practices are very costly for farmers and subsequently consumers (Schmildt *et al.*, 2015). Many problems such as high variability in agronomic characteristics resulting from cross-pollination, dioecious nature, and susceptibility to a large number of diseases hamper *C. papaya* cultivation (Paull *et al.*, 2008; Kavitha *et al.*, 2010). The absence of complete similarity to the mother strain, frequent occurence of diseases, high percentages of unwanted male plants, and significant genetic variability are the major limitations of *C. papaya* propagation in commercial quantities (Farzana *et al.*, 2008).

Heterogeneity caused by cross-pollination has many disadvantages. Seeds derived from open pollinated flowers can produce plants with an extensive difference in sex types and variation in fruit quality and type, in addition to the higher possibility of polyploidy, aneuploidy or even chromosomal aberrations (Kim *et al.*, 2002; Clarindo *et al.*, 2008).

In some countries, such as South Africa, vegetative propagation of *C. papaya* is achieved by grafting, budding, and root cutting but these methods are not used widely for *C. papaya* breeding as not all *C. papaya* varieties have the same tendency to produce multi-branches. Also, there is a limited number of plants produced per mother plant (Farzana *et al.*, 2008).

2.5 *In vitro* Propagation of *Carica papaya*

Plant cell cultures can manifest in three different ways: the production of secondary metabolites, micropropagation, and the study of a plant cell in different aspect levels (Zhong, 2001). Cell culture propagation systems are more suitable compared to the conventional spread of whole plants. Since plants propagated in cell cultures are under controlled conditions, they are independent of environmental conditions. Therefore, the cells can proliferate at higher growth rates, and are free of microbes and insects (Vanisree *et al.*, 2004).

In vitro micropropagation seems to be a good alternative technique over seed propagation. It enables the plants to be generated in clean or aseptic conditions, allowing for the plants to grow healthily (Drew, 1988). Litz and Conover (1977) defined tissue culture propagation systems for *C. papaya* in two instances. The first one obtained proliferating plants from tissues isolated from 5-6 cm young seedlings on medium supplemented with kinetin. The other system utilised mature *C. papaya* tissues by rapid callus induction from petiole segments. Subsequently, embryo and shoot formations were induced from this callus by a more complex media.

2.5.1 Propagation of *Carica papaya* by Shoot Tips and Axillary Buds

Vegetative propagation from carefully chosen clones is in high demand. Propagation of preferred genotypes to obtain high similarity plants on a large scale is one of the many applications of plant tissue cultures (Hossain *et al.*, 1993; Panjaitan *et al.*, 2007).

Commercial *in vitro* mass propagation of *C. papaya* has not yet been established. This is due to the various difficulties in micropropagation such as: microbial contamination by endogenous bacteria, especially when mature plants are grown in the field and used as explants, low proliferation rate during subculturing and difficulty in establishing normal roots and acclimatization (Wu *et al.*, 2012). According to Litz and Conover (1981), maintenance of proliferating cultures is inadequate during the time. Also, apical dominance in shoots are affected with time on the multiplication medium (Rajeevan & Pandey, 1986).

In vitro propagation of *C. papaya* is generated using a single node in a modified De Fossard medium supplemented with 0.5 µM of both 6-Benzylaminopurine (BAP) and Naphthaleneacetic acid (NAA) for shoot induction and development, 10 µM Indol-3 butyric acid (IBA) for root induction and is then transferred to a hormone-free Drew-Smith medium for development (Drew, 1992). Dioecious *C. papaya* can be propagated

in vitro by using axillary buds as explants. Axillary buds are cultured in MS basal salt with full strength vitamins supplemented with 0.5 mg L^{-1} benzyl adenine (BA) and 0.1 mg L^{-1} NAA for establishment and proliferation. Then, it is transferred to a MS medium supplemented with 1.0 mg L^{-1} kinetin and 0.05 mg L^{-1} NAA for the elongation stage and finally, half-strength macronutrient of MS medium with full strength of other components is supplemented with 1.0 mg L^{-1} IBA for rooting (Reuveni *et al.*, 1990). Chan and Teo (1994), applied the clonal propagation method to induce the *in vitro* direct establishment of multiple shoots from field grown trees explants. These shoots could then be rooted and grown in the field (Chan & Teo, 1994). Other micropropagation of *C. papaya* studies are summarized in table 1.

2.5.2 Propagation of *Carica papaya* by Organogenesis

There are limited studies on the propagation of *C. papaya* through organogenesis. Anandan *et al.* (2011) developed a protocol for micropropagation of Indian *C. papaya* L. 'Co7'. using epicotyl segments by direct organogenesis from *in vitro C. papaya* seedling plants. Hossain *et al.* (1993) generated a high efficacy protocol for plant regeneration from petiole of *C. papaya* L. 'Rajshahi-red' by using indirect organogenesis. The callus cultures obtained from MS medium augmented with NAA (0.5-10.5 µM) in combination with BA (0.5-5 µM) were the best callus observed at a low ratio of cytokinin and auxin which resulted in hard, green and nodular callus. Then, shoots were regenerated in MS medium supplemented with 100 mg L^{-1} casein hydrolysates 2 µM BA and 0.1 µM NAA. For shoot elongation, the regenerated shoots were transferred to the medium without plant growth regulators. Finally, for root regeneration, the elongated shoots were subcultured in half (½) MS medium supplemented with 3 µM NAA and 0.5 µM Gibberellic acid (GA_3).

Table 2.1: Summary of micropropagation studies of *C. papaya*

Authors	Cultivar and explant	Shoot development, plant growth regulator concentration and culture condition	Root development, plant growth regulator concentration and culture condition
(Litz & Conover, 1977)	NA Shoot apex and small petioles	**Establishment medium:** 50 µM kinetin + 10 µM NAA **Proliferation medium**: 2.0 µM BA + 0.5µM NAA Difco Bacto agar 8 g L^{-1}, 28 ^{o}C with 16 hr. light (3500 lux) and 8 hr. darkness. Then, transferred to media without growth regulators	**Root medium**: Rooting was induced in media supplemented with 0.5 µM NAA
(Reuveni *et al*. 1990)	NA Open pollinated dioecious *Carica papaya*, axillary buds	MS medium supplemented with 1.0 mg L^{-1} Kin and 0.05 mg L^{-1} NAA.	Half-strength macro-elements of MS basal medium supplemented with 1 mg L^{-1} IBA.
(Drew, 1992)	Female *C. papaya* L. 'Hybrid 14' nodal segment	A modified De Fossard *et al.* (1974) medium containing high concentrations of minerals supplemented with 0.5 µM of both BAP and NAA, 20 g L^{-1} sucrose and 8 g L^{-1} Difco Bacto-agar. Culture condition at 25 ± 1^{o}C with cool-white fluorescent tubes	**Rooting medium** A modified De Fossard *et al*. (1974) containing intermediate concentrations of minerals supplemented with 10 µM IBA, 20 g L^{-1} sucrose, 8 g L^{-1} Difco Bacto-agar. Culture condition at 25 ± 1^{o}C with cool-white fluorescent tubes
(Panjaitan *et al*. 2007)	*C. papaya* cv. 'Eksotika'	**Shoot induction medium:** MS medium supplemented with 1.0 mg L^{-1} BAP and 0.05 mg	**Root medium:** MS medium supplemented with 1.0 mg L^{-1} IBA

Table 2.1, Continued.

	Shoot tips of field grown hermaphrodite	L^{-1} NAA, 500 mg L^{-1} casein hydrolysate and 30 g L^{-1} sucrose **Proliferation medium**: MS medium without plant growth regulators for one week	**Acclimatization** MS medium with or without vermiculite for further root development.
(Anandan *et al*. 2011)	*C. papaya* L. var. Co7. Shoot bud from epicotyl segments	**Induction medium** MS medium supplemented with 2.5 µM TDZ and 30 g L^{-1} sucrose **Shoot and multiplication medium:** MS medium supplemented with B5 vitamins, 5.0 µM BAP and 0.05 µM NAA and 30 g L^{-1} sucrose **Shoot elongation medium:** ½ MS basal salts supplemented with B5 vitamins, 400 mg L^{-1} L-glutamine 1.5 µM GA_3 and 30 g L^{-1} sucrose	**Root induction medium:** ½ MS medium supplemented 2.5 µM IBA, and 30 g L^{-1} sucrose **Acclimatization:** 1/4 MS salts without sucrose for 24-48 h and then transferred to pots containing autoclaved soil and soilrite (1:1, w/w) and covered with polybags.
(Wu *et al.*, 2012)	*C. papaya* Hermaphrodites cv. 'Meizhonghong' Hybrid of 'Sunrise' with 'Shuizhonghong' Shoot buds and axillary	**Shoot initiation medium:** MS medium supplemented with 0.5 mg L^{-1} BA and 40 g L^{-1} sucrose. **Proliferation medium:** MS medium supplemented with 0.25 mg L^{-1} BA and 40 g L^{-1} sucrose. **Shoot elongation medium:** MS medium supplemented with 0.25 mg L^{-1} BA 1.0 mg L^{-1} GA_3 and 40 g L^{-1} sucrose.	**Root medium:** The 3/2 MS medium supplemented with 500 mg L^{-1} activated charcoal and 5 g L^{-1} sucrose.

2.5.3 Propagation of *Carica papaya* by Somatic Embryogenesis

The cultivation of *C. papaya* by seeds is hampered by many problems such as considerable variability in a commercial population resulting from open pollination, dioeciously nature, and susceptibility to a large number of diseases (Litz & Conover, 1977; Paull *et al.*, 2008; Kavitha *et al.*, 2010). Somatic embryogenesis has been employed in *C. papaya* crops to solve these problems. Elite plantlets with desirable characteristics and tolerance to distortion ringspot are multiplied and maintained (Litz & Conover, 1977; Fitch, 1993; Anandan *et al.*, 2012). Somatic embryogenesis is an appropriate micropropagation technique in *Carica papaya.* This technique helps to address the difficulties that occur during conventional seed proliferation as well as the absence of an actual method for early sex determination (Abreu *et al.*, 2014).

Tissue culture techniques are considered useful tools for overcoming incompatibility that arises between cross pollination of the *C. cauliflora* and *C. papaya*. Chen *et al.* (1991) induced SE from interspecific hybridization generated from cross breeding between *C. papaya* and *C. cauliflora*. The result indicated that the somatic embryos can proliferate continuously and can induce plant growth by manipulating plant growth regulators in the culture medium (Chen *et al.*, 1991). Plant regeneration through plant tissue culture techniques, especially SE, is necessary for many applications of biotechnology such as artificial seeds (synseeds), transgenic plants, and micropropagation (Bukhori, 2013). Somatic embryogenesis can be applied for producing artificial seeds which can be moved easly like regular seeds, preserved and planted. Similarly, the somatic embryo is suitable for long-range stockpiling methods such as cryopreservation and is an ideal model for genetic transformation research.

A light microscopic study of SE revealed that the daughter embryos produced during SE originated from single cells found on the external surface of parent embryos. Somatic

embryo cultures of cross-pollinated *C. papaya* with *C. cauliflora* are considered an appropriate system for the study of somatic cell genetics due to many reasons. Firstly, the origin of the somatic embryo is a single cell. Next, there is potential for frequent subculturing. Finally, the ability of the plant to regenerate is another reason (Chen *et al.*, 1991). Somatic embryogenesis addresses the problem of rooting caused by micropropagated plantlets and the bipolar structures of embryos containing both shoot and root apices. Also, embryogenic cultures have the ability to produce scores of embryos per culture, especially when grown in liquid mediums. The embryos in somatic embryo callus separated mechanicaly by agitation and float freely in the medium, therefore, manual separation and mechanical handling is not needed in lquid medium (Farzana *et al.*, 2008).

Numerous protocols based on SE have been presented for the propagation of *C. papaya in vitro*. Malabadi *et al.* (2011) induced SE via IZE of thirteen popular cultivars of *C. papaya* grown in India. The results demonstrated that a lower concentration of Thidiazuron (TDZ) (2.27 µM) was optimal for inducing the highest percentage of SE in all the tested *C. papaya* cultivars. Also, the ability to induce SE is varied for different cultivar of *Carica papaya*.

Somatic embryogenesis protocol generated from leaves of hermaphrodite *C. papaya* plants in culture mediums supplemented with 2,4 Dichlorophenoxyacetic acid (2,4-D) demonstrated asynchronicity, with new globular embryos continuously forming from the friable embryonic callus (Koehler *et al.*, 2013). Somatic embryos of *C. papaya* faced complications to develop a reproducible procedure to obtain normal *C. papaya* plants. Complications included low germination, callus production, and unsuccessful acclimatization due to poor root quality (Ascencio-Cabral *et al.*, 2008). Other SE protocols for various purposes are described in table 2.

2.6 Factors Affecting Plant Tissue Culture of *Carica papaya*

2.6.1 *Carica papaya* Cultivars

Different *C. papaya* cultivars respond differently to *in vitro* cultures. Also, the age or degree of maturity of the plant causes different responses. This is due to the fact that the physiological state relating to the endogenous hormonal concentration of the explant at various ages differs significantly. The *C. papaya* 'Taiping' produces 5–6 times more shoots in cultures compared to the *C. papaya* 'Eksotika' even when the medium is controlled for. Also, the continuous usage of papaya cultures to generate shoots in liquid media produced abnormal shoots. The *C. papaya* 'Eksotika' was more susceptible to abnormality than the *C. papaya* 'Taiping' (Chan & Teo, 1993). The callus produced from cross-pollinated *Carica papaya*, and *Carica* wild species *Carica cauliflora* did not have the ability to produce embryogenic calli. Only the *Carica* wild species *Carica goudotiana* was able to produce embryogenic calli. Also, the media constituent and the size of the flower bud had a substantial effect on callus induction in anther culture (Azad *et al.*, 2013). Callus induction for SE is affected by *C. papaya* cultivars. For instance, the *C. papaya* 'Solo' produced more callus than the explants from the *C. papaya* 'Sunrise' in the same type of media (Chen *et al.*, 1987). The different cultivars of *C. papaya* responded differently during embryogenesis that took place in the same concentrations of 2,4-D (Fitch & Manshardt, 1990).

Table 2.2: Summary of protocol used for propagation of *C. papaya* using SE

Authors and years	*C. papaya* Cultivar and Organs	Initiation Media	Induction Media	Maturation Media	Regeneration
(Chen *et al.*, 1987)	*C. papaya* 'Sunrise' and 'Solo' Somatic embryo from the stem, shoot tip, leaf,cotyledon, and root	½ MS medium supplemented with 160 mg L^{-1} adenine sulfate, and 1.0 mg L^{-1} NAA, 0.5 mg L^{-1} kinetin and 1.0 mg L^{-1} GA_3	½ MS with full strength vitamins supplemented with casein hydrolysate, I00 mg L^{-1} *Myo*-inositol, 160 mg L^{-1} adenine sulfate, 30 g L^{-1} sucrose, 1.0 mg L^{-1} NAA, 0.5 mg L^{-1} kinetin and 1.0 mg L^{-1} GA_3 and 8 g L^{-1} agar.		**Germinated medium:** MS medium supplemented with NAA only. 16 h light (2000 lux) and 8 h darkness. **Acclimatization**: mixture of sand: soil (1:1: v/v). One percent IAA was sprayed on the plantlets for two weeks.
(Fitch & Manshardt, 1990)	*C. papaya* hybrid 'Sunrise', 'Sunset', 'Waimanalo' and 'Kapoho' Hermaphroditic, IZE from open pollination and self-90 to 114 days		MS medium supplemented with 20% coconut water and 2 mg L^{-1} 2,4-D. Also, different combinations of plant growth regulators, 0.4 mg L^{-1} BA, 0.02 mg L^{-1} TZD, 1 mg L^{-1} 2,4-D 0.5 mg L^{-1} picloram.)	MS basal salts supplemented with, 5 mg L^{-1} Kinetin, 100 mg L^{-1} *Myo*-inositol, 30 g L^{-1} sucrose and 10 g L^{-1} Difco Bactoagar	**Germination medium:** Same somatic embryo germination medium without Kinetin
(Chen *et al.*, 1991)	*C. papaya* hybrid between Costarica Red *C. papaya* as female with male cauliflora 60 days after pollination.		½ MS medium with full strength vitamin, supplemented with 0.001 mg L^{-1} BA, 1 mg L^{-1} casein hydrolysate, 20 g L^{-1} sucrose and 0.6% agar. 25 ± 2°C with 16 h/8h light dark condition	**Proliferation Medium:** same induction medium but without ABA	**Germination medium:** MS medium without growth regulators.

Table 2.2, Continued.

(Castillo *et al.*, 1998)	Hermaphroditic *C. papaya* 'Solo' Self-pollinated Immature zygotic embryo (65–70 days post-anthesis)	MS basal salts supplemented with 10 µM 2,4-D, 50 mg L^{-1} *Myo*-inositol, 30 g L^{-1} sucrose, and 7 g L^{-1} Difco Bacto-agar. Incubated in the dark at 27°C	Similar to induction medium but with 60 g L^{-1} sucrose and without plant growth regulators. Dark condition **Liquid maturation medium**: Supplemented with 0.5 µM ABA. At 120 rpm under 40 µmol m–2 s–1 constant irradiance.	
(Vilasini *et al.*, 2000)	Hermaphrodite *C. papaya* 'Eksotika' Immature zygotic embryo from 90–100 days old after anthesis.	½ MS medium with full strength vitamins, supplemented with 10 mg L^{-1} 2,4-D, 50 mg L^{-1} *Myo*-inositol, 45 mg L^{-1} adenine sulphate, 100 mg L^{-1}glutamine, 60 g L^{-1} sucrose and 10 g L^{-1} Difco Bacto agar. At 25–27 °C in dark condition	MS medium without plant growth regulators. Light condition.	MS medium supplemented with a 0.1 mg L^{-1} NAA and 0.1 mg L^{-1} 6-BAP, 30 g L^{-1}sucrose. **Root medium** according to Drew *et al*. (1991) in the light **Acclimatization**: soil: sand: vermiculite mixture (1:1:1).
(Fernando *et al.*, 2001)	*C. papaya* 'Sunrise Solo' Mature zygotic embryos	MS medium supplemented with 2 mg L^{-1} 2,4-D, 7 g L^{-1} agar. at 25+2ºC under dark condition		

Table 2.2, Continued.

(Yu *et al.*, 2003)	*C. papaya* L. 'Tainung No. 2' Root segment	MS basal salts supplemented with B vitamins, 4.5 µM 2,4-D, 0.45 µM BA, 30 g L^{-1}sucrose, and 8 g L^{-1} agar. At 28°C under dark condition		**Germination medium**: MS medium supplemented with 0.1 µM NAA and 0.8 µM BA. Under light condition	**Root medium**: MS salts supplemented with B vitamins, 2.5 mM IBA, 30 g L^{-1} sucrose and 8 g L^{-1} agar.
(Clarindo *et al.*, 2008) As recommend by (Castillo *et al.*, 1998)	Hermaphroditic *C. papaya* 'Golden' Immature zygotic embryo 90 to 114 days post-anthesis		½ MS basal salts with full strength vitamins supplemented 9.05 mM 2,4-D, 0.55 mM *Myo*-inositol, 2.75 mM L-glutamine, 60 g L^{-1} sucrose and 2.8 g L^{-1} Phytagel. At 27°C under dark condition.	**Maturation medium**: ½ MS basal salts with full strength vitamins supplemented with 0.5 µM ABA, 0.55 mM *Myo*-inositol, 2.75 mM L-glutamine, and 60 g L^{-1} sucrose. At 27°C in a 16/8 h light/dark condition	**Germination medium**: MS medium supplemented with 0.55 mM *Myo*- inositol, 30 g L^{-1} sucrose and 7 g L^{-1} agar under dark condition. **Multiplication medium:** MS medium supplemented with 0.88 µM BAP, 0.11 µM NAA, 0.55 mM *Myo*-inositol, 30 g L^{-1}sucrose, and 7 g L^{-1} agar. At 27°C under 16/8 h light/dark condition

Table 2.2, Continued.

(Ascêncio *et al*. 2008)	Hermaphrodite *C. papaya* 'Maradol' zygotic embryos	½ MS medium supplemented with 0.02 mg L^{-1} 2,4-D, 0.2 mg L^{-1} kinetin, 0.02 mg L^{-1} ABA, 100 mg L^{-1} L-glutamine, 40 g L^{-1} sucrose and 7.5 g L^{-1} Difco1 Bacto agar. At 27- 28 ^{o}C under light condition.		**Maturation and Germination medium:** ½ MS medium supplemented with Chen vitamins (Chen *et al*., 1987), 100 mg L^{-1} L-glutamine, 68 mg L^{-1} adenine hemisulphate, 16 g L^{-1} sucrose, and 0.35 mg L^{-1} GA_3.	**Root medium:** ½ MS medium without plant growth regulators supplemented with 3.0 g L^{-1} activated charcoal **Acclimatization:** Mixture (70:30:10 of peat moss: vermiculite: pine bark)
(Malabadi *et al.*, 2011)	*C. papaya* 'Coorg Honey dew', 'Honey dew', 'Washington', 'Pusa nanha', 'Pusa delicious', 'Taiwan 785', 'Taiwan 786', 'Sunrise', 'Solo', 'Co-1', 'Co-3', 'Co-7' Immature zygotic embryo.		MS medium supplemented with 4.52 µM 2,4-D, 2.27 µM TDZ, 0.5 mg L^{-1} *Myo*-inositol, 1.0 mg L^{-1} casein hydrolysate, 0.5 mg L^{-1} glutamine, 250 mg L^{-1} peptone, 0.2 mg L^{-1} p-aminobenzoic acid,0.1 mg L^{-1} biotin, 30 g L^{-1} sucrose and 7 g L^{-1} agar. Under dark condition	MS medium supplemented with 30 g L^{-1} sucrose, 5 µM ABA, and 8 g L^{-1} agar. Dark condition	½ MS medium with 7 g L^{-1} agar without plant growth regulators
(Anandan *et al*. 2012)	*C. papaya* 'Co7' Immature zygotic embryo	½ MS medium supplemented with 2 mg L^{-1} 2,4-D, 400 mg L^{-1} glutamine, 1.0% activated charcoal, 60 g L^{-1}		**Maturation liquid phase**: liquid MS medium supplemented with 10.0 mg L^{-1} ABA and 10 g L^{-1} sucrose. At 25 ±	MS medium supplemented with 0.4 mg L^{-1} BAP, 0.02 NAA, 100 mg L^{-1} casein hydrolysate, 100 mg L^{-1} malt extract, 30 g L^{-1}

Table 2.2, Continued.

		sucrose and 4% phytagel. At 25ºC under dark condition		2ºC Agitated at 110 rpm under a 16 h light condition. **Maturation solid phase**: containing same initiation media with 4% phytagel and 10 g L^{-1} sucrose	sucrose and 4 % phytagel. at 22-25 ºC
(Azad *et al.*, 2012)	hybrid between *C. papaya* 'Shahi' and *C. cauliflora* Immature hybrid embryos	½ MS medium supplemented with 5 mg L^{-1} 2,4-D, 100 mg L^{1} casein, 100 mg L^{-1} glutamine, hydrolysate and 60 g L^{-1} sucrose	MS medium without plant growth regulator	½ MS medium supplemented with 0.5 mg L^{-1} BAP, 0.2 mg L^{-1} NAA, and 60 g L^{-1} sucrose. At 24 ± 2 °C under 16 h photoperiod	MS medium without plant growth regulator **Acclimatization:** amixture of autoclaved cocopeat, sand and garden soil (1:1:1).
(Bukhori, 2013)	Hermaphrodite *C. papaya* 'Eksotika' 90-100 days old Immature zygotic embryo		½ MS with full strength vitamins supplemented with 10 mg L^{-1} 2,4-D, 50 mg L^{-1} *Myo*-insitol, 45 mg L^{-1} adenine sulphate, 250 mg L^{-1} carbenicillin, 100 mg L^{-1} L-glutamine 60 g L^{-1} sucrose, and 0.195% Phytagel. At ± 25ºC under dark condition. **Liquid multiplication medium** same induction medium, but decreasing 2,4-D	**Germination medium** MS basal salts supplemented with 0.2 mg L^{-1} of both BAP and NAA, 108 mg L^{-1} *Myo*-inositol, 30 g L^{-1} sucrose and 0.195% phytagel. Under light condition	**Regeneration medium:** MS medium supplemented with 1 mg L^{-1} GA_3, 0.5 mg L^{-1} IBA, and 3.76 mg L^{-1} riboflavin **Rooting medium:** MS medium supplemented with 2.0 mg L^{-1} IBA. At ± 25ºC, under a 16 photoperiod

Table 2.2, Continued.

(Abreu *et al.*, 2014)	Hermaphroditic *Carica. papaya* 'Golden' Immature zygotic embryo 90-114 day	½ MS with full strength vitamin supplemented with 9.05 µM 2,4-D, 0.1 g L^{-1} *Myo*-insitol, 0.4g L^{-1} glutamine, 0.04 g L^{-1} Cysteine, 30 g L^{-1} sucrose and 3 % phytagel	Liquid media ½ MS with full strength vitamin supplemented with 9.05µM 2,4-D, 2.25 µM BAP,0.1 g L^{-1} *Myo*-insitol, 0.4 g L^{-1} L-glutamine, 0.04 g L^{-1} Cysteine, 0.1 g L^{-1}Malt extract, and 3% sucrose. At 100 rpm and maintained at 27°C under a 16/8 h light/dark condition	½ MS with full strength vitamin supplemented with 0.5 µM ABA, 0.1 g L^{-1}*Myo*-insitol, 0.4g L^{-1} L-glutamine, 0.04 g L^{-1} L-Cysteine, 0.1 g L^{-1} Malt extract, and 30 g L^{-1} sucrose. At 100 rpm and maintained at 27°C under a 16/8 h light/dark condition	MS medium supplemented with 0.5 µM GA_3, 0.1 g L^{-1} *Myo*-insitol and 30 g L^{-1} sucrose. At 27°C under dark condition **Acclimatization:** Vermiculite: peat moss (2: 1).
(Razak *et al.*, 2015)	*C. papaya* L. 'Eksotika' Immature zygotic embryo		½ MS medium with full strength vitamins supplemented with 45.2 µM 2,4-D, 50 mg L^{-1} *Myo*-inositol, 0.14 mg L^{-1} adenine hemisulphate, 400 mg L^{-1} glutamine, 250 mg L^{-1} carbenicillin, 60 g L^{-1} sucrose and 3.2 mg L^{-1} Gelrite. At 25 ± °C under light condition **Maturation medium** De Fossard (De Fossard, 1974) without growth regulator	De Fossard medium supplemented with 0.89 µM 6- BA 1.1 µM NAA and 150 mL coconut water under a light photoperiod at 26 ± 2°C	

Table 2.2, Continued.

(Heringer *et al.*, 2013)	hybrid *C. papaya* UENF/CALIMAN 01 Immature zygotic embryo 120 - 130 day	**Induction medium**: MS medium supplemented with 20 µM 2,4-D 30 g L^{-1} sucrose, and 2 % Phytagel. At 25± 2°C under dark condition. **Maturation medium**: MS medium supplemented with 0.05 g L^{-1} *Myo*-inositol, 6 % PEG (MW 3350) 30 g L^{-1} sucrose and 2% Phytagel. At 25± 1°C under dark condition for the first week then light condition	
(Koehler *et al.*, 2013)	Hermaphrodite *C. papaya* Young leaves	**Induction media**: ½ MS full strength vitamins supplemented with 9.05 µM 2, 4- D, 0.55 mM *Myo*-insitol, 2.75 mM glutamine, 87.6 mM sucrose and 0.28% (w/v) Phytagel. At 27°C under dark condition. **Maturation medium:** ½ MS with full strength vitamins supplemented with 5 µM abscisic acid, 100 mg L^{-1} malt extract, 5% polyethylene glycol 2000, 0.2% activated charcoal, and 0.55 mM *Myo*-insitol, 2.75 mM L-glutamine, and 87.6 mM sucrose.	**Germination medium**: MS basal medium, supplemented with 0.55 mM *Myo*-inositol, 58.4 mM sucrose and 0.65% (w/v) agar. at 27°C under a 16/8h light/dark photoperiod,

2.6.2 Explant Source

The source of explants is vital for the successful establishment of *C. papaya* in cultures (Reuveni *et al.*, 1990). During *in vitro* micropropagation, the age of explants played a powerful role in determining successful propagation. This is due to the fact that immature or less differentiated plants are easier to sterilize and initiate cultures. They also have the ability to multiply and form roots better than explants from mature plants (Anandan *et al.*, 2012). According to Wu *et al.* (2012), the induction of shoot buds and proliferation varied with the age of explants. Chen *et al.* (1987) studied the ability of different *C. papaya* organs (i.e. stem, leaf, root, shoot tip and cotyledon explants) and different *C. papaya* cultivars ('Solo' and 'Sunrise') to generate SE. It was found that the shoot tips and stems of these two *C. papaya* cultivars were most appropriate for forming a callus, whereas cotyledons, leaves, and roots were difficult to induce callus. Zygotic embryos of *C. papaya* were high in embryogenic potential and responded quickly to tissue culture conditions (Anandan *et al.*, 2012). A large number of embryogenic calluses can be generated from an IZE (110 to 120 days old) in MS medium augmented with 2,4-D (2.0 mg L^{-1}) after 6–8 weeks of culturing (Anandan *et al.*, 2012).

2.6.3 Plant Growth Regulators

Plant growth regulators were one of the essential factors for callus induction from petiole explants (Hossain *et al.*, 1993), whereas, auxin was the key to the initiation and continuous growth of callus. Conversely, kinetin and GA_3 were not essential for the induction of callus (Arora & Singh, 1978). Somatic embryos initiated in culture mediums supplemented with picloram, BA, TDZ, and coconut water, showed inhibitory effects to SE. On the other hand, zygotic embryos remained inactive or terminated in media without growth regulators (Fitch & Manshardt, 1990).

The 2,4-D hormone was appropriate for prompting SE in *C. papaya* (Clarindo *et al.*, 2008). Low concentration level of the 2,4-D was substantial for the initiation of somatic embryos in *Carica papaya*, especially from IZE. Higher concentration levels tended to induce genetic changes and caused variations between the *in vitro* propagated plants (Anandan *et al.*, 2012).

Abscisic acid (ABA) played a substantial role in regulating the *in vitro* SE in *Carica papaya.* ABA allowed direct SE without callus formation. Abscisic acid could initiate growth of the early embryo and set the accumulation of storage proteins. Induced somatic embryos from IZE of *C. papaya* x *C. cauliflora* in the absence of ABA generated a small number of abnormal embryos (Chen *et al.*, 1991; Anandan *et al.*, 2012). The presence of BAP and NAA in the medium enhanced the number of shoots produced from shoot tip explants. The BAP helped to prevent inertness and to begin shoot initiation, while NAA increased elongation of meristem cells (Panjaitan *et al.*, 2007).

2.6.3.1 General Introduction of Auxin

Auxin was the first type of plant growth regulator discovered. The term "auxin" arises from the Greek word "auxein" which means "to grow" (Moore, 1979). Auxin consists of indole rings linked with functional groups that are responsible for creating many chemical signals in vascular plants. These chemical signals regulate many mechanisms in plants during growth and development at both the cellular level (e.g., cell division and elongation) and the whole plant level (i.e., organ development) (Robert & Friml, 2009). Auxin plays a large role in regulating roots during growth and development. It interacts in a complicated system with other plant growth regulators, either synergistically or antagonistically, during the various stages of root development (Overvoorde *et al.*, 2010; Saini *et al.*, 2013). It also regulates plant responses to the environment such as phototropism, gravitropism and thigmotropism (Zažímalová *et al.*, 2010).

2.6.3.2 Auxin and Plant Tissue Cultures

Conventional experiments in plant tissue cultures have demonstrated that root formation is promoted when grown in media supplemented with a high auxin to cytokinin ratio whereas shoot regeneration is promoted when grown in media supplemented with a low auxin to cytokinin ratio (Kakani *et al.*, 2009). Auxin induces SE in the majority of plant species. It generates signals that play an important role in controlling molecular mechanism during dedifferentiation and re-differentiation of somatic cells during SE (Wójcikowska & Gaj, 2017). Auxin transport, biosynthesis and response have been detected in most developmental stages during SE, especially during the initial or induction phase (Möller & Weijers, 2009). Both the transport and distribution of auxin during SE control many developmental processes. The distribution of auxin can be monitored through different ways such as marking the embryogenic cells that contain the *GUS* reporter gene, as well as the embryonic cells that contain the promoters of these genes, namely DR5 and LEC2 (Kurczyńska *et al.*, 2007).

Kurczyńska *et al.* (2007) used the *GUS* gene method to monitor the transport of auxins during the SE of IZE from transgenic *Arabidopsis*. Results demonstrated that during the beginning of the culture, auxins accumulated in all explant tissues and then relocated to accumulate in the margin of explants. During the globular stage, auxins were detected in all cells whereas during the heart stage, auxins were detected in the shoot, root apex and cotyledon primordia.

Roots are a vital organ system in plants which contribute to water and nutrient absorption, anchorage of plants, storage functions, secondary metabolite synthesis and its accumulation can also be used for plant propagation (Saini *et al.*, 2013).

The interaction of phytohormone during root development and growth is not fully understood (Saini *et al.*, 2013). Root development in plants goes through three stages. In

the first stage, auxin is created through biosynthesis. In the second stage, auxin transport maintains the auxin gradient. Finally, in the last stage, auxin signaling affects the development of roots. (Saini *et al.*, 2013) Auxin is considered an important hormone in developing diverse plant tissues. The biosynthesis of auxin and direction flow within tissues are regulated by auxin efflux transporters that are polarly localized in cells. This dynamic pathway of auxin affects developmental and environmental responses (Leyser, 2005). Overvoorde *et al*. (2010) observed that active auxin efflux has a significant effect in transporting auxin out of the cell and distributing auxin into the root to maintain the optimum cellular auxin concentration that is needed for root growth and development (Overvoorde *et al.*, 2010). Generally, there are two ways in which auxin is transported. The first is the long distance movement of auxin whereby auxin is transported from the site of auxin synthesis to the roots via the mature phloem. The second is the short distance movement of auxin whereby auxin moves from cell to cell through specific auxin influx and efflux carriers (Friml *et al.*, 2003).

Friml *et al.* (2002a) focused their efforts to understand the molecular mechanisms responsible for auxin biosynthesis. They found that the cellular levels of auxin can be regulated through different processes such as conjugation, deconjugation, production, degradation, and directional transport. The interesting finding was the long distance movement of auxin, from the site of auxin synthesis towards the root, through mature phloem (Friml *et al.*, 2002a). Conversely, Leyser (2005) described the short distance pathway of auxin transfer between and inside cells. Here, auxin was considered a weak acid that significantly protonated outside cells where the extracellular environment had relatively low pH. Auxin then entered the cells through diffusion which was facilllitated by carriers called auxin influx carriers. Once inside the cells, auxin was ionized and trapped due to the low acidity inside the cells or cytoplasm where the pH was higher. Therefore, the movement of auxin outside the cells occurred through active transport or

carrier-dependent processes which were dependent on the PIN protein. The actual mechanism for transporting auxin using this protein is not yet fully understood (Leyser, 2005).

The main source of auxin is from the shoot. Auxin is then transported to the root tip via both long distance pathways using the phloem and short distance pathways using the polar transport stream (Petrášek & Friml, 2009). The pattern of auxin distribution in the root tip is dynamic and preserved regardless of the source of auxin. Also, auxin can be transferred from the auxin concentrated area in the root to the root cap and then back up to the root and away from the tip in the epidermal tissues though active transport mediated by PIN proteins (Friml *et al.*, 2003). This dynamic movement or redistribution of auxin is crucial to regulate the elongation of cells and to enable the meristematic cells in the meristematic zone to enter the elongation zone (Petrášek & Friml, 2009). Thus, regardless of the source and sink of auxin, the distribution of auxin between them is actively maintained by the PIN protein network. Consistent with this, auxin that is added exogenously can be rapidly redistributed to maintain the auxin gradient pattern (Friml *et al.*, 2002a; Vieten *et al.*, 2005).

The development of the root apical meristem occurs in response to the accumulation of auxin at the basal end of the embryo (Feher *et al.*, 2003). Due to the asymmetric cell division of zygotes, auxin-transporting pathways and auxin-responsive daughter cells are generated (Friml *et al.*, 2003). The polar distribution of auxin regulates organ development, cell elongation and cell division in the root system and is also important for root branching. Auxin is distributed in the primary root tip via polar transport using auxin transporters, such as PIN proteins (Friml *et al.*, 2003; Petrášek & Friml, 2009; Robert & Friml, 2009).

According to Jürgens (2001), root formation in natural zygotic embryos is initiated from the uppermost suspensor cell (Hypophysis or lens-shaped cell) during the late globular stage. This cell works as an organizing center for the embryonic root (Jürgens, 2001). Hypophysis specialization occurs when the pre-embryo consists of approximately 30 cells and when notes a maximum of auxin in hypophysis (Friml et al., 2003). Weijers *et al*. (2006) suggest that hypophysis specialization in root formation is not the direct result of auxin response but instead depends on cell-to-cell signaling that is generated by auxin in adjacent cells (Weijers *et al.*, 2006).

2.6.3.3 Auxin Genes

The *C. papaya* genome is relatively small (372 Mbp) and consists of 8 chromosomes with primitive sex chromosomes (Ming *et al.*, 2008b). Epigenetic regulation plays a crucial role in regulating many plant developmental processes by affecting the expression of many genes in response to exogenous or endogenous signals (Nguyen *et al.*, 2013). Chromatin modification is an example of the epigenetic regulation mechanism. It occurs under two types of regulation, DNA methylation and histone modification, and can either positively or negatively affect transcriptional states (Luo *et al.*, 2012). DNA methylation can inhibit transcription of genes by modifying target sites of transcriptional factors in order to prevent them from binding to these sites. The second type of epigenetic mechanism is changes of histone, such as acetylation and deacetylation (Nguyen *et al.*, 2013). The histone deacetylase inhibitors regulate root development in *Arabidopsis* via the regulation of *PIN1* degradation (Nguyen *et al.*, 2013).

It is well known that not all plant cells have the capability of synthesizing auxin. Auxin transportation between cells depends on two mechanisms, namely, auxin influx transporters (AIT) and auxin efflux transporters (AET) (Robert *et al.*, 2015) PIN proteins are a plant transmembrane protein belonging to AET which facilitates the transport of

auxin from cells. These proteins are found inside the cells and direct intercellular auxin flow based on their polarity (Křeček *et al.*, 2009). The AUX1/LAX proteins are another type of transmembrane proteins that belong to AIT and facilitates the transport of auxin across cell membranes. Both AUX1/LAX and PIN proteins play a role in plant embryogenesis and development by directing intercellular auxin flow (Estrella-Maldonado *et al.*, 2016).

There are three major classes of auxin transporters in *Arabidopsis thaliana*, namely, PIN proteins, AUX1 proteins, and p-glycoproteins (PGP). While PIN proteins are considered auxin efflux carriers and LAX proteins are considered auxin influx carriers, PGPs influence both the efflux and influx of auxins (Leyser, 2005).

Auxin/Indoleacetic acid (*AUX/IAA*) genes participate in the auxin signaling pathway and play an important role in plant growth and development such as embryogenesis, root growth and root development. In addition, *Aux/IAA* genes also play an important role during drought tolerance (Liu *et al.*, 2011; Haitao & Jing, 2017). The *AUX1/LAX* genes family is considered as AIT and plays a crucial role in the development of embryonic root apexes in *Arabidopsis thaliana*. *AUX1/LAX* mutations were shown to have a major effect on cell proliferation and growth of radical tip compared to wild type *aux1/lax* mutants (Ugartechea-Chirino *et al.*, 2009).

In *Arabidopsis*, PIN1 protein influences vascular tissue differentiation and organogenesis (Gälweiler *et al.*, 1998; Benková *et al.*, 2003), PIN2 protein influences root gravitropic growth (Müller *et al.*, 1998), PIN3 influences shoot differential growth (Friml *et al.*, 2002b), PIN4 influences developing and mature root meristems (Friml *et al.*, 2002a), and PIN7 influences early embryo development (Friml *et al.*, 2003).

In *Medicago truncatula*, there are ten auxin efflux carriers (MtPINs) and five auxin influx carriers (MtLAXs). The genomic sequence of each of these genes was determined and their expression was examined in the shoot and root tissue of *Medicago truncatula*. The expression of *MtPIN2* was limited to the roots, while all other genes were detected in both shoots and roots. Both the *PIN* and *LAX* gene families contained more members in *M. truncatula* than in *Arabidopsis* (Schnabel & Frugoli, 2004). In strawberry fruits, two of the early auxin-responsive *AUX/IAA* genes, termed *FaAUX/IAA1* and *FaAUX/IAA2* respectively, were isolated and characterized. Both genes were involved in early fruit development. The enhancement of *FaAUXIAAs* transcripts may induce fruit growth and delay fruit ripening (Liu *et al.*, 2011).

2.6.4 Solidifying Agent

Gelling agent had considerable effects compared to light and phloridzin. Solidified media bacto Agar achieved a higher germination percentage with lesser genetic variation. On the other hand, phytagel and agar produced genetic variations in the callus and plantlet (Ascencio-Cabral *et al.*, 2008).

2.6.5 Light

Light is one of the physical factors that affects many physiological processes of *in vitro* plantlets, for instance, photomorphogenesis and photosynthesis. The light quality, quantity, and exposure duration can affect plant growth and development (Gupta & Jatothu, 2013). Ascencio-Cabral *et al*. (2008) studied the effects of light quality on the germination of *C. papaya* somatic embryos to promote efficient and reproducible protocol for propagation. They found that light quality had significant effects on the development of SE and plant growth. The plant exposed to gro-lux lamps showed higher percentages of survival without hyperhydricity.

2.6.6 Gaseous Atmosphere

The growth and development of *in vitro* plants depend on the constituents of the culture medium and the constituents of the gaseous atmosphere. The ventilation of culture flasks has an important effect on the oxygen and ethylene concentration inside the culture flasks, which subsequently affects the number of shoots and leaves as well as leaf expansion. Moreover, plants grown in non-aerated flask media for an extended duration demonstrated growth retardation (Lai *et al.*, 2000). The type of culture flask is an important factor for *in vitro* culturing. The ethylene and carbon dioxide (CO_2) concentration increases when light is absent in smaller-sized airtight culture flask. It was proposed that incubating nodal cultures of *C. papaya* with different ethylene levels caused poor performance (Magdalita *et al.*, 1997). On the other hand, the addition of some chemicals to reduce ethylene such as aminoethoxy vinyl glycine (AVG), and silver thiosulphate (STS), significantly improved nodal culture growth and leaf area production as well as reduced leaf deterioration (Magdalita *et al.*, 1997).

2.6.7 Sucrose

Plants in *in vitro* culture conditions need an exogenous carbohydrate source because most of the plants grown *in vitro* tend to shift from autotroph to heterotroph. Selecting the best carbohydrate source and concentration in culture media depends on the plant species and the micropropagation phase. Most *C. papaya* cultivars induced SE in media supplemented with 6% (w/v) sucrose, which promoted a high frequency of embryogenesis. Also, under similar concentrations and mediums, sucrose is better than maltose in inducing embryogenic callus (Vilasini *et al.*, 2000). Sucrose plays an important role and affects the formation of somatic embryogenic callus. In 'Eksotika', 60 g L^{-1} sucrose produced the highest frequency of somatic embryo callus from IZE of hermaphrodite *C. papaya* (Razak *et al.*, 2015). On the other hand, decreasing or sucrose absent media reduced the risk of contamination, enhanced the photosynthetic capacity of

the plant and also, improved acclimatization of plants propagated *in vitro* (Xiao *et al.*, 2011).

2.6.8 Other Culture Media Components

Induction of embryogenic callus is affected by the nutrient media and plant growth regulators used (Jordan, 1986). Adenine hemisulfate is an example of supplements that can improve the capability of *C. papaya* shoots to regenerate from calluses (Drew, 1987). MS medium containing 160 mg L^{-1} adenine sulfate was found to be optimal for the propagation rate of *C. papaya* (Reuveni *et al.*, 1990). The addition of coconut water to the media does not give significant results when compared with media without coconut water (Fitch & Manshardt, 1990).

In tissue culture media, combinations of vitamins with other media constituents, directly and indirectly affects all phases of SE from induction to rooting. Adding components of cytokinin with thiamine positively affect the induction of callus growth and rooting. Also, a combination of biotin and riboflavin play a role in the development of callus. Furthermore, riboflavin can positively affect the plant rooting of *Carica papaya*. On the other hand, vitamin D improves cell elongation and meristematic cell division, which enhances shoot and rooting growth (Abrahamian & Kantharajah, 2011).

The culture media constituent has a significant effect on callus induction. The ½ MS medium with full strength Na-Fe-EDTA supplemented with NAA (2.0 mg L^{-1}), BAP (1.0 mg L^{-1}) produced the highest percentage of callus in three *Carica* species: *C. papaya, C. cauliflora*, and *C. goudotiana* (Azad *et al.*, 2013).

2.6.8.1 Polyethylene Glycol (PEG)

There are two types of osmotica, namely plasmolyzing, such as D-mannitol and D-sorbitol, and non-plasmolyzing such as PEG (Walker & Parrott, 2001). Polyethylene

glycol is a large molecule that cannot pass through cell walls, reduces water uptake and reduces turgor pressure (Langhansova *et al.*, 2004). Gradually increasing the PEG concentration causes a gradual decrease in the water content inside cells and increases the endogenous free proline inside cells (Al-Khayri & Al-Bahrany, 2004).

Many researchers studed the effect of PEG during different stages of SE (Heringer *et al.*, 2013). Rai *et al.* (2009) evaluated the effect of two amino acids (L-proline and L-glutamine) and PEG during the maturation and germination of somatic embryos in *Psidium guajava* L. The results demonstrated that only L-proline amino acid and PEG had a significant effect in enhancing the maturation frequency of somatic embryos (Rai *et al.*, 2009). Heringer *et al.* (2013) found that the addition of PEG to MS media led to an increased number of somatic embryos that were matured and converted at the end of the process (Heringer *et al.*, 2013).

Polyethylene glycol was used to induce water deficit for *in vitro* selection of *Helianthus annus* L callus, for water stress tolerance (Hassan *et al.*, 2004) and to enhance the development of somatic proembryos of conifers (Santos *et al.*, 2002). Polyethylene glycol had a significant effect on the quality of somatic embryos by lowering the osmotic potential in the maturation medium. The effect of carbon sources and PEG on the maturation of Maritime pine somatic embryos varied widely between varieties (Ramarosandratana *et al.*, 2001). Increasing the levels of PEG (0 - 2.0 %) created water pressure and reduced the ability of callus induction and plant regeneration in *Oryza sativa* L (Wani *et al.*, 2010). The addition of ABA and PEG, was necessary for the functional development of somatic embryos in coniferous species (Stasolla *et al.*, 2002). Polyethylene glycol helped to improve the roots in *Panax ginseng* during SE by enhancing the root meristem organization during the torpedo-stage of somatic embryos (Langhansova *et al.*, 2004).

The nature of stress produced by PEG is crucial to somatic embryos during development in some plant species as this stress stimulates DNA methylation that leads to an increase in the accumulation of storage compounds that are essential for the development of somatic embryos during the maturation phase of SE (Leustek & Kirby, 1988; Smulders & De Klerk, 2011).

2.6.8.2 Phloroglucinol (PG)

Phenol compounds affect the induction, development and germination of somatic embryos during SE of *Feijoa*. They also affect the isolation of somatic embryos from the mother tissues by forming zones consisting of cells that have high contents of phenolic compound (Reis *et al.*, 2008). The efficiency of phenolic compounds depends on the number and position of hydroxy groups (OH) in the aromatic rings (Bandurski *et al.*, 1995). Phenol compounds that contain two or more OH protect endogenous IAA and reduce the wound response that may inhibit regeneration processes. Conversely, monophenol, such as salicylic acid, stimulates IAA decarboxylation and the wound response resulting in a negative effect on rooting (De Klerk *et al.*, 2011).

Phloroglucinol is not familiar to many researchers in plant biotechnology labs and is mostly used as a complement in combination with other plant growth regulators as its actual effect is hidden by commonly used plant growth regulators (da Silva *et al.*, 2013). Phloroglucinol (1,3,5-trihydroxybenzene) has growth-promoting properties. Phloroglucinol gave a positive effect and enhanced the germination of *Feijoa sellowiana* that was regenerated through SE when used in induction media (Reis *et al.*, 2008). The addition of PG to rooting media supplemented with IBA stimulated rooting (Pérez *et al.*, 2016). Phloroglucinol had a positive effect on the rooting of *C. papaya* cv. 'Maradol Roja' *in vitro* (Pérez *et al.*, 2016). It also played a critical role in controlling hyperhydricity throughout the lignification process (da Silva *et al.*, 2013).

The addition of PG in culture media helped oxidize the phenolic substances released from explants. These phenolic substances caused browning of the callus (Kim *et al.*, 2007). Phloroglucinol is an antioxidant that protects the tissue from oxidative stress and IAA from decarboxylation (catabolism). Indole-3-acetic acid is metabolized in media through oxidation and conjugation (De Klerk *et al.*, 2011). The presence of PG in the culture media enhanced the production of various metabolites and enzymes in *Aristolochia tagala* callus that originated from leaves, such as catalase and peroxidase which is an oxidative enzyme. It also increased the production of protein and carbohydrate (metabolites). In addition, it decreased the amount of polyphenol oxidase (Remya *et al.*, 2013).

Many researchers reported the significant effect of PG in stimulating shoot development when added to culture media (Sarkar & Naik, 2000; Steephen *et al.*, 2010), enhancing root induction in *Bacopa monnieri* (Ceasar *et al.*, 2010), enhancing rooting induction in *Rosa damascena* (Noodezh *et al.*, 2012), enhancing rooting frequency in *Asparagus racemosus* (Bopana & Saxena, 2008), enhancing micro-shoot rooting and plant survival of walnut trees during acclimatization (Licea-Moreno *et al.*, 2015), and enhancing bud induction responses and formation in *Capsicum annuum* on the inverted hypocotyls (Kumar *et al.*, 2005). The presence of PG in culture medium of nodal segments from *Stevia rebaudiana* reduced the time needed for rooting and acclimatization. It also improved the photosynthetic activity in new leaves grown *ex vitro* and the survival rate of plants (Piqueras, 2014).

2.6.8.3 Activated Charcoal (AC)

The growth and development of a plant *in vitro* is affected by many factors such as the type of media and compositions, plant variety, culture condition and some organic substances (George *et al.*, 2008). Morphological and physiological studies have

demonstrated that the SE process consists of at least two phases: firstly, the induction of embryogenic competence in cells with high concentrations of auxins; secondly, the development of embryogenic masses into embryos in the absence of or decreased concentrations of auxins. There are some anomalies associated with somatic embryos such as, lateral root development, multiple cotyledons, multiple embryos attached as a single unit, and secondary embryos associated with cotyledons and hypocotyls (Lazzeri *et al.*, 1987; Chengalrayan *et al.*, 1997; De-la-Peña *et al.*, 2015).

Callus formation on shoots that were transferred to media containing auxin is a major limitation for the *in vitro* rooting development of *Jatropha curcas*. Most callus formed on the shoot due to the type of auxins and cytokinin's used in the culture media. The induction of roots in media containing IBA reduced the callus formation more than that containing NAA and IAA (Daud *et al.*, 2013).

Activated charcoal originates from wood, is characteized by a large surface area and consists of small pores with large inner surface areas (Thomas, 2008). It absorbs colloidal solids, gases and vapors such as aromatic (phenolics compound, cytokinin and auxin) and polar molecules such as glucose, sorbitol, mannitol and inositol (George *et al.*, 2008).

Activated charcoal is widely used in plant tissue cultures to improve growth and development of various plant organs (Pan & Van Staden, 1998; Thomas, 2008). The absorptive capacity of charcoal varies when augmented to the medium. It plays a vital role in micro-propagation, SE, artificial seed production and rooting. The positive effects of AC on morphogenesis may be due to many reasons. The first reason is the adsorption of inhibitory compounds that are found in the culture medium such as toxic metabolites and phenolic compounds. The second reason is the release of some ingredients that are naturally present in AC which stimulates growth. The third reason is providing the dark environment for culture media (Pan & Van Staden, 1998; Thomas, 2008). The adsorptive

capacity of AC is generally dependent on a variety of factors such as density, purity and pH values (Halhouli *et al.*, 1995). Activated charcoal is able to adsorb substances presumed to be deleterious and/or inhibitory to *in vitro* cultures. However, the adsorption of growth regulators applied to the tissue by AC could also occur at the same time and non-selective adsorption may result in negative effects on cultured explants (Pan *et al.*, 2002).

Activated charcoal prevented the occurrence of hyperhydricity in *Platycerium bifurcatum* and enhanced regeneration efficiency (Teng, 1997). MS medium supplemented with different auxins (2,4-D, IAA and NAA) could not produce roots in *Daucus carota* without AC as AC was important for root formation and development (Pan *et al.*, 2002). The lack of accurate information on the exact concentrations of plant growth regulators in culture media when mixed with AC was a major obstacle to determining the exact concentration of growth regulators that must be added to culture media (Ebert *et al.*, 1993).

Recently, many reports have confirmed the positive role of AC in plant tissue cultures, especially in promoting the growth and development of plant tissues. Activated charcoal helped improve the different stages of SE (Thomas, 2008). The addition of AC was observed to significantly improve embryo germination in *Panax quinquefolium* L (Tirajoh *et al.*, 1998). In *Pinus thunbergii,* media augmented with AC increased the maturation and germination frequencies of somatic embryos (Maruyama *et al.*, 2005). Germination medium augmented with AC (0.25 and 0.5 g L^{-1}) enhanced the germination of somatic embryos in *Phoenix dactylifera* (Zouine *et al.*, 2005). Activated charcoal enhanced shoot regeneration of *Lilium. longiflorum* (Nhut *et al.*, 2001). It also had a beneficial effect on the regeneration of somatic embryos into plantlets in *Paspalum scrobiculatum* L (Rashid, 2001). Activated charcoal had a significant effect on the

induction and maturation of SE in *Daucus carota* whereby the presence of AC increased the number of SE and enhanced the regeneration of normal plantlets (Pan & Van Staden, 2001).

2.7 Root Formation of *Carica papaya*

The possibility of large-scale propagation via *in vitro* protocols depends on the capability to produce high numbers of plants with low prices. Also, the ability of propagated plants to adapt to the *ex vitro* conditions to ensure high quality and survival rates is another factor to be considered (Hazarika, 2006). Riboflavin and IBA promoted root initiation. Riboflavin and IBA concentrations decreased rapidly in media exposed to light. So, dark conditions are recommended when IBA and riboflavin are added in the media. On the other hand, increasing concentrations of riboflavin in the media quickened the reduction of IBA (Drew *et al.*, 1991). The use of riboflavin injections instead of transferring to media without growth regulators saved time and cost of subculturing (Drew *et al.*, 1991; Drew *et al.*, 1993). Indole-3-butyric acid (IBA) is better than other plant growth regulators, such as indole-3-acetic acid (IAA), NAA or *p*-Chlorophenoxyacetic acid (pCPA) for root initiation of *Carica papaya*. Moreover, the exposure of shoots to a medium containing 10 µM of IBA for two days after being transfered to free media with 31 µM riboflavin under dark condition produced the best result for root initiation (Drew *et al.*, 1993). Apart from incurring a lower cost, it is also an easy and economical protocol to acclimatize root systems of *C. papaya* L which promotes large-scale micropropagation. This protocol depended on root induction in medium supplemented with low concentrations of IBA followed by root development in ½ MS medium supported with vermiculite under ventilated conditions (Yu *et al.*, 2000).

2.7.1 Problems Associated with Root Development and Acclimatization

A plant that is grown *in vitro* differs from those produced *in vivo*. Plants grown *in vitro* are heterotrophic, whereas plants grown *in vivo* are photoautotrophic. The gaseous, light, and nutrition varies between *in vitro* and *in vivo* (Kadleček *et al.*, 2001). Plant growth *in vitro* is classified into three types according to carbon and energy source. The first type, photoautotrophic growth, occurs when the plant is dependent on photosynthesis in *in vitro* culture. The second type, heterotrophic growth, occurs when the plant is dependent on sugar found in the culture medium. The third type, photo mixotrophic growth, occurs when the plant depends on photosynthesis and sugar in common cultures (Kozai *et al.*, 2005). Micropropagation of the plant in culture mediums without a carbon source is called photoautotrophic micropropagation (Zobayed *et al.*, 2001; Kozai *et al.*, 2005). Photoautotrophic can be stimulated *in vitro* by eliminating carbohydrates from the culture medium and increasing gas exchange in the culture flask (Xiao *et al.*, 2011).

Many factors influence the development of roots in *in vitro* cultures such as auxin type and concentration, shoot quality, donor age, and temperature (Mohammed & Vidaver, 1988). The germination of somatic embryos suffered problems associated with root development due to the accumulation of callus at the base end of somatic embryos which prevented the proper development of roots and weakened the joining of roots to the stem (Fitch & Manshardt, 1990; Sekeli *et al.*, 2012).

Induction of roots *in vitro* from young *C. papaya* leaves was possible after being exposed to several auxins such as a 2,4,5-trichlorophenoxyacetic acid (2,4,5-T); IAA; IBA and α- NAA. Recently, Pérez *et al.* (2015) studied the effects of phloroglucinol (PG) in rooting. PG stimulated construction of new roots and enhanced root elongation, especially when added to ½ MS medium without sucrose, fortified with IBA (9.8 μM).

This resulted in excellent rooting development with 100% rooting and a larger number of roots for each plant.

One of the biggest problems associated with SE of *C. papaya* is the acclimatization of regenerated plants, whereby over $^2/_3$ of the plants are lost before being moved to the field (Malabadi *et al.*, 2011) due to the inability to adapt to the new environment. Reducing the relative humidity and increasing the ventilation inside the culture containers seems to have a larger influence on the adaptation and increasing plant survival of *C. papaya* under *ex vitro* acclimatization (Pérez *et al.*, 2015). Acclimatization is a critical stage in all *in vitro* propagation protocols. In this phase, the relative humidity should be gradually decreased to improve stomata function, enhance cuticle development, and decrease water loss. Acclimatization of *C. papaya* using ½ MS medium supplemented with IBA without sucrose and with zeolite as supporting material can also increase ventilation, improve root quality and increase the survival rate of plants (Pérez *et al.*, 2015). Poor photosynthetic rate of *in vitro* plants is associated with CO_2 concentration. So, improving CO_2 concentration and light intensity inside the culture flask and decreasing relative humidity will improve the photosynthetic rate (Kozai *et al.*, 2005).

2.8 Somaclonal Variation

Plant tissue culture technique causes genetic variation. This variation can prevent and block some stages of *in vitro* cultures and generate undesired characteristics especially during genetic transformation. However, it can also cause useful genetic variability for crop improvement (Clarindo *et al.*, 2008). There are many problems associated with somatic embryos of *C. papaya* in achieving a reproducible and secure protocol to produce healthy plants without genetic changes. Examples include decreased germination rate, accumulation of callus that prevents root germination, the occurrence of abnormal plantlets and unsuccessful acclimatization (Ascencio-Cabral *et al.*, 2008).

The occurrence of polyploidy is one of the factors that generates abnormal plants during SE, especially when used for propagation. In the past, most scientists used conventional chromosome counting to detect ploidy level whereas now, flow cytometry is usually used. For instance, Clarindo *et al.* (2008) used flow cytometry to detect polyploidy of SE of *C. papaya* derived from IZE. There were no changes in ploidy level recorded in all *C. papaya* plantlets in the multiplication medium (Clarindo *et al.*, 2008). The flow cytometry level and cytogenetic analyses are necessary for assessing the DNA amount (ploidy level), investigating chromosomal stability and providing a rapid, reliable and final assessment of the genetic constancy of *Carica. papaya* plantlets restored by SE (Abreu *et al.*, 2014).

2.9 Cryopreservation

Cryopreservation can be used for the conservation of germplasm as well as the storage of embryogenic cultures and somatic embryos for micropropagation, genetic transformation, and selection studies (Fitch, 2005). Cryopreservation can affect incubation media, time of incubation, vitrification, exposure time, concentration of vitrification, temperature of vitrification condition and explant type for instance. Plant vitrification solutions 2 (PVS2) contain large concentrations of highly toxic material that harms plant tissue. Therefore, minimum exposure to the solution is recommended to obtain sufficient dehydration and to prevent toxic effects (Azimi *et al.*, 2005). Desiccated *C. papaya* seeds differing in moisture content (i.e. ranging from 5% to 40%) and frozen in liquid nitrogen demonstrated a significant increase in germination rate (48%) at 10% moisture content. On the other hand, the germination rate decreased for cryopreserved seeds when the moisture content was less than 10% (Azimi *et al.*, 2005). According to Perveen *et al.* (2007), temperature and humidity were the main important factors in pollen viability. Decreasing the temperature and humidity helped to increase the period of

viability. Pollen stored at –60ºC demonstrated the best result with 60% viability after being preserved for 48 weeks.

Azimi *et al.* (2005) utilized two procedures for storing *C. papaya* germplasm namely, desiccation and cryopreservation of seeds and vitrification-based cryopreservation of shoot tips. Results demonstrated that shoot tips were successfully cryopreserved by incubation for 1 to 4 days before vitrification. 100% PVS2 for 20 minutes of exposure to vitrification at 0 ºC gave 70% recovery from the shoot tips.

Cryopreservation technique based on vitrification was used to store 12 genotypes of *Carica papaya*. Where the shoot tips were used as explant, after that the genetic stability was examined for screened the genetic and epigenetic changes. Results indicated that the regenerated plantlets were abnormal with numerous genetic variations. In addition, the exposure of shoot tips to cryoprotectants reduced recovery growth rates and induced genetic variation by inducing methylation and DNA changes (Kaity *et al.*, 2008).

Randomly amplified DNA fingerprinting (RAF) is a technique used for screening changes in genomic DNA. This technique is useful and easy since information regarding DNA sequence is not necessary. Additionally, it does not require a restriction template but instead only requires one primer and amplification reaction. Therefore, it is possible to obtain a rapid and reproducible amplification result and to determine the markers from any organism. Furthermore, this technique has the capacity to generate identical profiles regardless of the concentrations, as well as the ability to create profiles using small samples (Kaity *et al.*, 2008).

2.9.1 Artificial Seeds

The production and encapsulation of somatic embryos allow mass propagation of genetically engineered *C. papaya* (Castillo *et al.*, 1998). The occurrence of regeneration from encapsulated embryos of *Carica papaya.* L. is affected by many factors, such as the

presence or absence of nutrient salts in the capsule, the concentration of sodium alginate (SA), and finally, the duration of exposure to calcium chloride ($CaCl_2$). Castillo *et al.* (1998), presented a protocol for the alginate encapsulation of leaves or leaf-derived callus (soft or hard) from two *C. papaya* cultivars, 'Rainbow' and 'Sunrise Solo'. The purpose was to preserve valuable germplasm using cryopreservation. The ability to store these encapsulated alginate beads at low temperatures and to cryopreserve them upon their successful regeneration provides a useful and innovative mid- to long-term method of preserving *C. papaya* germplasm.

Castillo *et al.* (1998) also achieved an optimized protocol for encapsulation that ensured proper regeneration of *C. papaya* using a SA (2.5%) concentration in a ½ MS with short exposure time to $CaCl_2$ (less than 10 min).

2.9.1.1 Advantage of Artificial Seeds

Artificial seeds have many advantages and solve many problems in plant biotechnology, especially when used as tissue culture for commercial use. Some of their advantages include the fact that artificial seeds are easy to control and carry, can be stored for a long period of time and can be used to propagate large quantities of plants. There is also the possibility to computerize the whole production process (Singh *et al.*, 2007). Artificial seeds used for propagation can be preserved for various time periods, allowing them to be used as and when needed (Ray & Bhattacharya, 2008). This allows the conservation and management of important plant species, especially endangered plants. *In vitro* conservation or preservation using artificial seeds offers a cheap, effective and simplified option for germplasm preservation (Danso & Ford-Lloyd, 2003).

The transportation of *in vitro* plantlets by conventional methods such as using test tubes containing the plantlets often fail because the test tubes are fragile and tend to break during transfers. Also, packaging samples in test tubes may damage the gelled substrate

and expose the plantlet to shock, which may subsequently affect regeneration or acclimatization (Danso & Ford-Lloyd, 2003). Also, the alginate matrix acts as an artificial endosperm when supported with nutrients such as carbon sources and plant growth regulators that are necessary for both the storage and conversion competences of the encapsulated propagules (Naik & Chand, 2006).

Utomo *et al.* (2008) described artificial seeds as a fast transport system of somatic embryos. It is suggested that artificial seeds merge the benefits of clonal propagation, seed propagation and storage (Ara *et al.*, 1999). Artificial seeds can be suitable for the preservation of clonal germplasm from elite cultivars and endangered plants species by optimizing the protocol for storage of these explants (Maruyama *et al.*, 1997). Kamińska *et al.* (2017) developed an efficient protocol for using artificial seeds to store the shoot tips of endangered *Taraxacum pieninicum* (Kamińska *et al.*, 2017).

The artificial seed technique has been broadly studied and successfully completed for different plant species including, cereals, vegetables (Oceania *et al.*, 2015; Ghanbarali *et al.*, 2016), fruits, ornamentals, medicinal plants (Prasad *et al.*, 2014; Gantait *et al.*, 2015) and forest trees (Pintos *et al.*, 2008).

2.9.1.2 Factors Affecting on Artificial Seeds

There are many factors that affect the production of artificial seeds. The best characteristic of artificial seeds have a high conversion ability and forms a compact and firm shape (Nieves *et al.*, 1995). The most important factor that controls artificial seed production is the shape, texture, and size of beads. To get uniform, compact and firm bead shapes with specific sizes, various concentrations of SA and $CaCl_2$ must be examined. Using various combinations of SA and $CaCl_2$ produces beads with different textures, shapes, diameters and transparencies (Ahmed *et al.*, 2015).

Sodium alginate (SA) (gel matrix) has been widely used for encapsulating plant materials during artificial seed production. It is used for various purposes such as enhancing nodal segment propagation (West *et al.*, 2006), shoot tip propagation (Singh *et al.*, 2006) bud propagation (Pattnaik & Chand, 2000), and storage under low temperatures (Lisek & Orlikowska, 2004). Softer gel matrixes offer a more suitable condition for somatic embryo growth than harder gel matrixes. Harder gel matrixes tend to reduce regeneration rates. Encapsulation with 1% SA produces the most suitable condition for somatic embryo growth and produces high regeneration rate. However, it is too soft for handling and transportation purposes. Also, lower concentrations (1-2%) of SA cannot polymerize, especially when exposed to high temperatures during autoclaving (Larkin *et al.*, 1988).

Many reports demonstrate the direct relationship between germination rates from encapsulated somatic embryos and exposure time to $CaCl_2$ (Castillo *et al.*, 1998). Both high concentrations of $CaCl_2$ and longtime exposure of somatic embryos to $CaCl_2$ led to more absorption and penetration of $CaCl_2$ to the embryo, which affected plant regeneration (Redenbaugh *et al.*, 1986; Malabadi & Van Staden, 2005). Recently, Cheruvathur *et al.* (2013) studied the relationship between the duration of exposure of SA (3% w/v) to $CaCl_2.2H_2O$ (100 µM) and the germination frequency of somatic embryos in *Rhinacanthus nasutus*. The results demonstrated a negative relationship between the duration of exposure of SA to $CaCl_2$ and the germination frequency of somatic embryos in *Rhinacanthus nasutus*. Exposing SA to $CaCl_2.2H_2O$ for thirty min resulted in the highest rate of survival and conversion of plantlets (Cheruvathur *et al.*, 2013).

Many researchers studied the effect of encapsulation mixture compositions on survival, regeneration and beads formation. The effect of the encapsulation matrix constitution on the regeneration of encapsulated micro-cuttings of hybrid aspen (*Populus*

tremula L. × *P. tremuloides* Mincx.) was studied. The results demonstrated that the ingredients of the matrix such as nutrient medium salts, sugars and growth regulators significantly affected the initial development of the micro-cuttings, especially the sucrose (Tsvetkov *et al.*, 2006). Ganapathi *et al*. (2001) reported that the constitution of the encapsulation mixture influenced the survival of the somatic embryos of banana (Ganapathi *et al.*, 2001).

Past research has investigated the best concentrations of SA and $CaCl_2$ as well as the optimum exposure of SA to $CaCl_2$ in order to form suitable beads for handling and regeneration. However, their findings were not constant for all species as the researchers used different solvents when forming the gel mixture. Also, the conditions of the experiments such as the brand of gelling agents and complexing agents ($CaCl_2$) were not constant. Generally, lower concentrations of SA (1-2 %) or $CaCl_2.2H_2O$ (less than 50 mM) delayed the ion exchange between sodium and calcium and produced fragile capsules. Conversely, higher concentrations of SA (above 4%) produced very hard capsules that hampered embryo growth (Kumar & Thomas, 2012). Naik and Chand (2006) used 3% SA with MS liquid medium supplemented with 4.44 mM BA, 0.54mM NAA and 100 mM $CaCl_2$ to form ideal artificial seeds (Naik & Chand, 2006). Danso and Ford-Lloyd (2003) used calcium-free MS medium supplemented with various concentrations of sucrose and growth regulators as solvents to dissolve 3% of SA, which was then exposed to 100 mM $CaCl_2$ to form ideal beads (Danso & Ford-Lloyd, 2003). Kamińska *et al.* (2017) used 3% SA prepared in liquid MS medium to capsulate shoot tips and was then exposed to sterile 100mM $CaCl_2.2H_2O$ solution for 25 min (Kamińska *et al.*, 2017). Preece and West (2006) used 2.75% SA which was then placed into sterile 50 µM $CaCl_2$ solution for 30 min (Preece & West, 2006). Rihan *et al.* (2011) used 2% SA which was then dropped into an autoclaved solution of $CaCl_2$ (15 g L^{-1}) for 30 min for full complexion (Rihan *et al.*, 2011). Poor viability of stored artificial seeds may be

related to both oxygen deficiency in the gel bead and rapid drying (Redenbaugh *et al.*, 1991).

2.9.1.3 Artificial Seeds Using Somatic Embryos

The artificial seed technique has been broadly studied and successfully completed in different plant species using somatic embryos. The somatic embryos of *Quercus suber* were coated with SA for the commercial production of artificial seeds (Pintos *et al.*, 2008). The somatic embryos of *Chamaecyparis pisifera* (Maruyama *et al.*, 2003) and *Paulownia elongata* (Ipekci & Gozukirmizi, 2003) have also been used.

3% of SA dissolved in liquid MS medium and exposed to 50 mM $CaCl_2$ for 30 min provided uniform encapsulation of somatic embryos from *Paulownia elongate* (Ipekci & Gozukirmizi, 2003). Somatic embryos of *Rhinacanthus nasutus* were collected during the torpedo stage and were suspended in a matrix of 3% SA dissolved in MS medium and exposed to 100mM $CaCl_2.2H_2O$ for 30 min for the production of artificial seeds (Cheruvathur *et al.*, 2013). Somatic embryos from IZE of *Pinus radiata* were mixed in 3% of SA that was dissolved in LP media without calcium and exposed to 100 mM $CaCl_2$ for 30 min in order to produce large quantities of artificial seeds (Aquea *et al.*, 2008). Somatic embryos were used to develop artificial seeds for *Mondia whitei* which is considered an endangered medicinal plant. A concentration of 3% SA that was exposed to 100mM $CaCl_2.2H_2O$ for 10 min resulted in the highest survival (95.7%) and germination (73%) frequencies of artificial seeds (Baskaran *et al.*, 2015). 4% SA with ½ MS medium supplemented with BA were used to encapsulate the somatic embryos of *Quercus serrata* L, but only root elongation was observed upon conversion (Ishii *et al.*, 1999)

2.9.1.4 Artificial Seeds Using Shoot Tip

The artificial seeds technique has been used to encapsulate nodal segments (Danso & Ford-Lloyd, 2003), micro-shoots (Rihan *et al.*, 2011) and shoot tips (Ray & Bhattacharya, 2008). Shoot tips of *Rauvolfia serpentina* were preserved in three different temperatures (20 °C, 12 °C and 4 °C) by encapsulating shoot buds (Ray & Bhattacharya, 2008). Also, nodal cuttings or shoot tips of cassava were encapsulated in 3% SA for storage and germplasm exchange purposes (Danso & Ford-Lloyd, 2003). Nodal segments of *Hibiscus moscheutos* were coated with 2.75% SA and placed into sterile 50 μM $CaCl_2$ solution for 30 min to produce artificial seeds (Preece & West, 2006). Micro-shoots from cauliflower (*Brassica oleracea*) were coated in 2% (w/v) SA and dropped into an autoclaved solution of $CaCl_2$ (15g L^{-1}) for 30 min to produce artificial seeds (Rihan *et al.*, 2011).

2.10 *Carica papaya* of Malaysia

The history of *C. papaya* in Malaysia started when Spaniards brought the plant to the Philippines in 1598 and then transferred and grown in Malaya (Chan *et al.*, 2015). *C. papaya* is listed as one of the most important fruits export in Malaysia. It is mainly cultivated in Johore, Perak, and parts of Selangor. The most popular grown cultivar of *C. papaya* in Malaysia is 'Eksotika'. This cultivar resulted from a cross breed between 'Subang 6' and the Hawaiian 'Sunrise Solo' (Chen *et al.*, 1987; Chan *et al.*, 2015). The 'Eksotika' fruit is superior to 'Sunrise Solo' fruit in size, weights and firm texture (Chan & Raveendranathan 1990). The 'Eksotika' cultivar, grown from seeds, tends to produce hermaphrodite and female populations in a (3:1) proportion respectively, with a 100% fruit-producing population (Chan & Teo, 1993). The 'Eksotika' is very sensitive to many diseases especially the PRSVwhich infected *C. papaya* crops in Johor in 1991 (Vilasini *et al.*, 2000). The PRSV disease and rapid damage to the fruit are two major restriction challenges for the *C. papaya* industry in Malaysia (Chan, 2002; Chan *et al.*, 2015). The MTD caused by *Cladosporium*- thrip complex is another challenge. The *C. papaya*

'Eksotika' and 'Solo' cultivars are considered the most susceptible to this disease. It is very destructive specially to developing vegetation. Trees infected with this fungus shows many symptoms such as; the leaves display typical shot-hole symptom and trees will be weakened (Chan & Mak, 1993). Chan and Teo (1993), reported that 99–100% of the explants obtained from mature field-grown trees were contaminated with fungus and bacteria.

There is a keen rising interest in plant cell cultures that can help rapid propagation of selected plants. In Malaysia, there is an urgent need for sufficient supply to meet the significant demand for seedlings, which are an important plantation and cash crop (Khalid *et al.*, 2007). Plantlet regeneration using shoot tips of hermaphrodite 'Eksotika' was achieved by culturing in MS medium supported with combinations of BAP (1.0 mg L^{-1}) and NAA (0.05 mg L^{-1}). The result demonstrated that the maximum rooting percentage was obtained when shoots explants were cultured in media augmented with IBA (1.0 mg L^{-1}) (Panjaitan *et al.*, 2007). Unfortunately, the roots generated were abnormal, when ½ MS medium, with full-strength vitamins and 2,4-D (10 mg L^{-1}) were used for induction of SE from IZE 'Eksotika' (Vilasini *et al.*, 2000). Bukhori (2013), generated an efficient *in vitro* protocol for the production of 'Eksotika', using IZE grown in different culture media. Induction media cultures consisted of ½ MS medium supplemented with carbenicillin (250 mg L^{-l}) and 2,4-D (10 mg L^{-l}). Germination media consisted of MS supplemented with BAP and NAA (0.2 mg L^{-l}), whereas rooting media consisted of MS fortified with IBA (0.5 mg L^{-l}).

'Eksotika' resistant to PRSV were generated via microprojectile bombardment of coat protein gene isolated from the local virus. The same cultivar was also engineered for fruits with improved shelf life (Vilasini *et al.*, 1998). The two major difficulties faced in developing transgenic Malaysian 'Eksotika' plants are low efficiency of regenerated roots

from shoots and low acclimatization rate of rooted transgenic *C. papaya* plants in the field. Therefore, rooting efficiency with high-quality roots formation are critical in ensuring successful and continuous production of transgenic 'Eksotika' (Sekeli *et al.*, 2014). It is also possible to develop new *C. papaya* varieties with enhanced performance and disease resistance using irradiation. Irradiation-induced mutation is progressively being used as a complementary tool in plant breeding. It is most suitable for developing one or two easily identifiable traits and well-accepted breeding lines. There is an excellent response regarding selecting dwarf trees, lower fruit bearing stature, higher total soluble solids in fruits and for resistance to MTD. Unfortunately, irradiation did not seem to be favorable for developing PRSV resistance in 'Eksotika' (Chan *et al.*, 2007).

Inter-Retrotransposons Amplified Polymorphism (IRAP) molecular markers are used to simplify the chosen *C. papaya* that have resistance to PRSV in breeding programs. The result showed significant polymorphism in DNA banding patterns to determine parental-progeny relations and categorized progenies based on their resistance to PRSV. The best primers regarding inheritance pattern based on the PRSV resistance trait was a primer combination between LTR 6150 and Nikita primers (Rashid *et al.*, 2014). Razak *et al.* (2015) developed marker-free positive selection of transgenic *C. papaya* plants using phospho- mannose isomerase (pmi) genes. Only transformed cells can employ mannose as a carbon source to grow. They also evaluated the effect of mannose on the growth and development of embryogenic 'Eksotika' callus. Mannose at 30 g L^{-1} was found to be effective for screening transformed embryogenic calli.

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CHAPTER 3: METHODOLOGY

3.1 Seed Sterilization experiment

3.1.1 Seed Source

C. papaya 'Eksotika' seeds were provided by MARDI, Selangor, Malaysia. All seeds were stored for two months and preserved in a closed nylon bag at 20-30^{o}C. Seeds of each accession were sterilized and germinated *in vitro*. The seedlings were scored for contamination every two days.

3.1.2 Seed Sterilization

Ten different sterilization methods were tested. The first three methods were based on 80% ethanol for 1 min and (1, 5 or 10 min) of 0. 1% mercuric chloride ($HgCl_2$) for seeds sterilization. The following three methods were based on 70% ethanol for one min, (10, 20 or 30%) Clorox for 5 min and 0.1% $HgCl_2$ for 5 min. The last four methods were based on 70% ethanol for 2 min, (40 or 50%) Clorox for 5 min and 0.1% $HgCl_2$ for 5 and 10 min.

For the first three treatments, 120 seeds were used. Seed sterilization took place in a 50ml Erlenmeyer flask as follows:

1- The seeds were washed under running tap water for one hour.
2- The seeds were transferred to laminar flow and immersed in 80% ethanol for one min.
3- The seeds were then washed three times with autoclaved distilled water (dH_2O) and then rinsed with 0.1% $HgCl_2$ for either 1, 5, or 10 min.
4- The seeds were washed three times with dH_2O and then rinsed in 80% ethanol for 1 min.
5- Then, the seeds were washed three times with dH_2O.

6- Finally, the seeds were rinsed in dH_2O for 30-60 min under laminar flow until used for further experiments.

Sterilized seeds were inoculated in 10 plates and each plate contained 4 seeds.

For the following three treatments, 100 seeds were used. Seed sterilization took place in a 50 ml Erlenmeyer flask as follows:

1- The seeds were soaked for 30 min under running tap water with two to four drops of soap.
2- The seeds were transferred to laminar flow and immersed in 70% ethanol for one min.
3- The seeds were washed three times with dH_2O.
4- Then, the seeds were washed with different concentrations (10, 20 or 30%) of Clorox for five min each.
5- The seeds were once again washed three times with dH_2O.
6- The seeds were rinsed in 0.1% $HgCl_2$ for five min.
7- The seeds were washed three times with dH_2O.
8- The seeds were washed with 70% ethanol for one min to remove the remaining $HgCl_2$.
9- After that, the seeds were washed three times with dH_2O.
10- Finally, the seeds were rinsed for 30- 60 min under laminar flow until used for further experiments.

Six plates, each containing three seeds, were used for every sterilization method.

The last four treatments underwent a similar protocol as above, with some modifications in timing and concentrations.

1- The seeds were soaked for 30 min under running tap water with two to four drops of soap.
2- Then, the seeds were transferred to laminar flow and immersed in 70% ethanol for two min.
3- After that, the seeds were washed three times with dH_2O.
4- The seeds were washed with different concentrations (40 or 50%) of Clorox for five min each.
5- Then, they were washed three times with dH_2O.
6- After that, the seeds were rinsed in 0.1% $HgCl_2$ for either five or ten min.
7- The seeds were washed three times with dH_2O.
8- The seeds were washed with 70% ethanol for two min to remove the remaining mercuric chloride.
9- Finaly, the seeds were washed three times with dH_2O and rinsed for 2 hours under laminar flow until used for further experiments.

3.1.3 Seeds Sterilization Medium

Murashige and Skoog medium (1962) without growth regulators was used as the basic media for seed sterilization experiment. To prepare one liter of MS basic media, a 1000 ml conical flask was used and filled up with 800 ml of distilled water. Then, 4.4g L^{-1} MS powder and 3.3 g L^{-1} Gelrite were added into the conical flask. The media solution was stirred on a hot plate until all components were dissolved. After that, distilled water was added to reach the final volume of one liter. The pH of the media solution was adjusted to 5.8 using 1.0 N hydrochloric acid (HCl) and 1.0 M sodium hydroxide (NaOH). The media was autoclaved for 20 min at 104 kPa pressure and at 121ºC. After the media was autoclaved and cooled (50ºC), it was dispensed into sterile petri dishes under laminar flow.

3.2 Induction of Somatic Embryogenesis From Mature Zygotic Embryos Experiment

3.2.1 Seeds Sterilization

The seeds were sterilized as following:

1- The 'Eksotika'seeds were soaked for 30 min under running tap water with two to four drops of soap.
2- Then, the seeds were transferred to laminar flow and immersed in 70% ethanol for two min.
3- After that, the seeds were washed three times with dH_2O.
4- The seeds were washed with 40% of Clorox for five min.
5- Then, they were washed three times with dH_2O.
6- After that, the seeds were rinsed in 0.1% $HgCl_2$ for ten min.
7- The seeds were washed three times with dH_2O.
8- The seeds were washed with 70% ethanol for two min to remove the remaining mercuric chloride.
9- Finaly, the seeds were washed three times with dH_2O and rinsed for 2 hours under laminar flow until used.

3.2.2 Media Preparation

3.2.2.1 Preparation of Stock Solutions for McCown Woody Plant Medium

McCown Woody Plant medium (WPM) basal salts were prepared as stock solutions (20X concentration for macronutrients and 200X for micronutrients). The vitamin for the WPM media were prepared as stock solutions (200X concentrations). All stocks were stored in a refrigerator at $4^{o}C$ until used.

(a) ***Macronutrients Stock Solutions***

The macronutrients stock solution (20X) for WPM was prepared as follows: all macronutrients found in table 3.1 were weighed and dissolved separately in an Erlenmeyer flask filled with the proper amount of distilled water (Table 3.1).

Table 3.1: The compositions of McCown Woody Plant medium (WPM) as described by Lloyd & McCown **(**Lloyd & McCown, 1980**).**

Macronutrients in WPM medium	**Concentration (mg L^{-1}) in medium**	**Amount in (g) to prepared 500 ml stock (20X)**
1. NH_4NO_3	400.00	4.00
2. K_2SO_4	990.00	9.90
3. $CaCl_2.2H_2O$	96.00	0.96
4. $MgSO_4.7H_2O$	370.00	3.70
5. KH_2PO_4	170.00	1.70
6. $(Ca(NO_3)_2.4H_2O)$	556	5.56
Micronutrients in WPM medium	**Concentration (mg L^{-1}) in medium**	**Amount in (g) to prepared 500 ml stock (200X)**
1. H_3BO_3	6.20	0.620
2. $MnSO_4.H_2O$	22.30	2.230
3. $ZnSO_4.4H_2O$	8.60	0.860
4. $Na_2MoO_4.2H_2O$	0.25	0.025
5. $CuSO_4.5H_2O$	0.25	0.025
Vitamins in WPM medium	**Concentration mg L^{-1} in medium**	**Amount in (g) to prepared 500 ml stock (200X)**
1. Thiamine (HCl)	1.0	0. 10
2. Nicotinic acid	0.5	0.05
3. Glycine	2.0	0.20
4.Pyrodoxine (HCl)	0.5	0.05
5. *Myo* – Inositol	100	
Iron in WPM medium, 500 ml Stock (200X)		
37.3 mg of Na_2EDTA (Ethylenediamine tetra acetic acid, disodium salt) was dissolved in 250 ml dH_2O by using Erlenmeyer flask (500 ml). 27.80 mg of $FeSO_4.7H_2O$ was dissolved in 250ml dH_2O by using Erlenmeyer flask (500 ml). Na_2EDTA solution was boiled and $FeSO_4$ solution was added gently to it, with stirring.		

Then they were slowly mixed in a 500 ml Erlenmeyer flask containing the proper amount of distilled water. They were stirred to avoid precipitation. A 500 ml graduated

cylinder was used to measure the solution and distilled water was added to reach the final volume of 500 ml. The macronutrients stock solution was then stored in a refrigerator.

(b) ***Micronutrients Stock Solutions***

The micronutrients stock solution (200X) for WPM was prepared as follows: all micronutrients found in table 3.1 were weighed and dissolved separately in an Erlenmeyer flask filled with the proper amount of distilled water (Table 3.1). Then, they were slowly mixed in a 500 ml Erlenmeyer flask containing the proper amount of distilled water. They were stirred to avoid precipitation. A 500 ml graduated cylinder was used to measure the solution and distilled water was added to reach the final volume of 500 ml. The micronutrients stock solution was then stored in a refrigerator.

(c) ***Iron-EDTA Stock Solutions***

The iron-EDTA stock solution (200X) for WPM was prepared as follows: all iron components found in table 3.1 were weighed and dissolved separately in an Erlenmeyer flask filled with the proper amount of distilled water (Table 3.1). Then, the Na_2EDTA solution was boiled and the $FeSO_4$ solution was gently added to it. It was stirred to avoid precipitation. A 500 ml graduated cylinder was used to measure the solution and distilled water was added to reach the final volume of 500 ml. The iron-EDTA stock solution was then stored in a refrigerator.

(d) ***Vitamins Stock Solutions***

The vitamins stock solution (200X) for WPM was prepared as follows: all vitamins found in table 3.1 were weighed and dissolved separately in an Erlenmeyer flask filled with the proper amount of distilled water (Table 3.1). Then they were slowly mixed in a 500 ml Erlenmeyer flask containing the proper amount of distilled water. They were stirred to avoid precipitation. A 500 ml graduated cylinder was used to measure the

solution and distilled water was then added to reach the final volume of 500 ml. The vitamins stock solution was then stored in a refrigerator.

(c) ***Preparation of I Liter of WPM Medium (1000 ml)***

To prepare 1000 ml of full-strength WPM medium, 60 g of sucrose was dissolved (by stirring) in 500 ml of dH_2O in a 1000 ml Erlenmeyer flask. After that, 50 ml of the WPM macronutrients stock solution (20X), 5ml of micronutrients (200X), 5 ml of iron stock solution (200X) and 5 ml of vitamins stock solution (200X) were added to the sucrose solution and stirred. 3.3 g Gelrite and other vitamins or amino acids were added to the previous solution. Distilled water was added to reach the final volume of 1000 ml.

To prepare 1000 ml of half strength WPM medium with full strength vitamins, 60 g of sucrose was dissolved (by stirring) in 500 ml of dH_2O in a 1000 ml Erlenmeyer flask. After that, 25 ml of the WPM macronutrients stock solution (20X), 2.5 ml of micronutrients (200X), 2.5 ml of iron stock solution (200X) and 5 ml of vitamins stock solution (200X) were added to the sucrose solution and stirred. 3.3 g Gelrite and other vitamins or amino acids were added to the previous solution. Distilled water was added to reach the final volume of 1000 ml.

To prepare 1000 ml of $^3/_2$ WPM medium with full strength vitamins, 60 g of sucrose was dissolved (by stirring) in 500 ml of dH_2O in a 1000 ml Erlenmeyer flask. After that, 75 ml of the WPM macronutrients stock solution (20X), 7.5 ml of micronutrients (200X), 7.5 ml of iron stock solution (200X) and 5 ml of vitamins stock solution (200X) were added to the sucrose solution and stirred. 3.3 g Gelrite and other vitamins or amino acids were added to the previous solution. Distilled water was added to reach the final volume of 1000 ml.

3.2.2.2 Preparation of Stock Solutions for MS Medium

MS basal salts were prepared as stock solutions (20X concentration for macronutrients and 200X for micronutrients). The vitamins for the MS media were prepared as stock solutions (200X concentrations). All stock solutions were stored in a refrigerator at 4^{o}C until used.

(a) ***Macronutrients Stock Solutions***

The macronutrients stock solution (20X) for MS medium was prepared as follows: all macronutrients found in table 3.2 were weighed and dissolved separately in an Erlenmeyer flask filled with the proper amount of distilled water (Table 3.2). Then they were slowly mixed in a 500 ml Erlenmeyer flask containing the proper amount of distilled water. They were stirred to avoid precipitation. A 500 ml graduated cylinder was used to measure the solution and distilled water was then added to reach the final volume of 500 ml. The macronutrients stock solution was then stored in a refrigerator.

(b) ***Micronutrients Stock Solutions***

The micronutrients stock solution (200X) for MS medium was prepared as follows: all micronutrients found in table 3.2 were weighed and dissolved separately in an Erlenmeyer flask filled with the proper amount of distilled water (Table 3.2). Then they were slowly mixed in a 500 ml Erlenmeyer flask containing the proper amount of distilled water. They were stirred to avoid precipitation. A 500 ml graduated cylinder was used to measure the solution and distilled water was then added to reach the final volume of 500 ml. The micronutrients stock solution was then stored in a refrigerator.

(c) ***Iron-EDTA Stock Solutions***

The iron-EDTA stock solution (200X) for MS was prepared as follows: all components of iron found in table 3.2 were weighed and dissolved separately in an Erlenmeyer flask filled with the proper amount of distilled water (Table 3.2). The Na_2EDTA solution was

Table 3.2: The composition of MS medium as described by Murashige and Skoog (Murashige & Skoog, 1962).

Macronutrients in MS medium	**Concentration (mg L^{-1}) in medium**	**Amount in (g) to prepared 500 ml stock (20X)**
1. NH_4NO_3	1650	16.5
2. KNO_3	1900	19.0
3. $CaCl_2.2H_2O$	440	4.4
4. $MgSO_4.7H_2O$	370	3.7
5. KH_2PO_4	170	1.7
Micronutrients in MS medium	**Concentration (mg L^{-1}) in medium**	**Amount in (g) to prepared 500 ml stock (200X)**
1. H_3BO_3	6.200	0.6200
2. $MnSO_4.H_2O$	16.900	1.6900
3. $ZnSO_4.4H_2O$	8.600	0.8600
4. KI	0.830	0.0830
5. $Na_2MoO_4.2H_2O$	0.250	0.0250
6. $CoCl_2.6H_2O$	0.025	0.0025
7. $CuSO_4.5H_2O$	0.025	0.0025
Vitamins in WPM medium	**Concentration (mg L^{-1}) in medium**	**Amount in (g) to prepared 500 ml stock (200X**
1. Thiamine (HCl)	0.1	0.01
2. Nicotinic acid	0.5	0.05
3. Glycine	2.0	0.20
4.Pyrodoxine (HCl)	0.5	0.05
5. *Myo* – Inositol	100 mg	
Iron in MS medium, 500 ml Stock (200X)		
37.3 mg of Na_2EDTA (Ethylenediaminetetra acetic acid, disodium salt) was dissolved in 250 ml dH_2O by using Erlenmeyer flask (500 ml). 27.80 mg of $FeSO_4.7H_2O$ was dissolved in 250 ml dH_2O by using Erlenmeyer flask (500 ml).Na_2EDTA solution was boiled and $FeSO_4$ solution was added gently to it, with stirring.		

then boiled and the $FeSO_4$ solution was gently added to it. It was stirred to avoid precipitation. A 500 ml graduated cylinder was used to measure the solution and distilled

water was then added to reach the final volume of 500 ml. The iron-EDTA stock solution was then stored in a refrigerator.

(d) ***Vitamins Stock Solutions***

The vitamins stock solution (200X) for MS medium was prepared as follows: all vitamins found in table 3.2 were weighed and dissolved separately in an Erlenmeyer flask filled with the proper amount of distilled water (Table 3.2). Then they were slowly mixed in a 500 ml Erlenmeyer flask containing the proper amount of distilled water. They were stirred to avoid precipitation. A 500 ml graduated cylinder was used to measure the solution and distilled water was then added to reach the final volume of 500 ml. The vitamins stock solution was then stored in a refrigerator.

(e) ***Preparation of I Liter of MS Medium (1000 ml)***

To prepare 1000 ml of full-strength MS medium, 60 g of sucrose was dissolved (by stirring) in 500 ml of dH_2O in a 1000 ml Erlenmeyer flask. After that, 50 ml of the MS macronutrients stock solution (20X), 5 ml of micronutrients stock solution (200X), 5 ml of iron stock solution (200X) and 5 ml of vitamins stock solution (200X) were added to the sucrose solution and stirred. 3.3 g Gelrite and other vitamins or amino acid were added to the previous solution and water was added to reach the final volume of 1000 ml.

To prepare 100 ml of half strength MS medium with full strength vitamins, 60 g of sucrose was dissolved (by stirring) in 500 ml of dH_2O in a 1000 ml Erlenmeyer flask. After that, 25 ml of the MS macronutrients stock solution (20X), 2.5 ml of micronutrients stock solution (200X), 2.5 ml of iron stock solution (200X) and 5 ml of vitamins stock solution (200X) were added to the sucrose solution and stirred. 3.3 g Gelrite and other vitamins or amino acid were added to the previous solution and water was added to reach the final volume of 1000 ml.

(f) ***Preparation of Basic Media***

MS medium (1962) and WPM were used as the basic media for inducing callus. Specifically, MS and WPM media powder from Duchefa were used. To prepare one liter of full MS media, a 1000 ml conical flask was used and filled up with 800 ml of distilled water. Then, 60 g L^{-1} sucrose, 4.4 g L^{-1} MS powder and 3.3 g L^{-1} Gelrite were added into the conical flask. The media solution was stirred on a hot plate until all components were dissolved. After that, distilled water was added to reach the final volume of one liter. The pH of the media solution was adjusted to 5.8 using 1.0 N hydrochloric acid (HCl) and 1.0 M sodium hydroxide (NaOH). The media was autoclaved for 20 min at 104 kPa pressure and at 121ºC. After the media was autoclaved and cooled (50ºC), it was dispensed into sterile petri dishes under laminar flow. The same steps were used to prepare the WPM media apart from replacing the 4.4 g L^{-1} MS with 2.3 g L^{-1} WPM media.

The same steps were used to prepare ½ MS medium with decreasing the amount of medium 2.2 g L^{-1} instead of 4.4 g L^{-1}.

(g) ***Preparation of Media with Hormones***

Culture media supplemented with hormones were prepared as basic media but the hormones were added into the media before adjusting the pH of the media. For heat sensitive hormones and amino acids such as GA_3 and glutamine, the hormones and amino acids were added into the media solution after being autoclaved and cooled down until 50ºC. The hormones and amino acids that were added into the media after the autoclave process needed to be sterilized by using syringe filter 0.22 µm.

3.2.3 First Treatment: Induction of Somatic Embryos on Various Media at Different Concentrations of 2,4-D from Mature Zygotic Embryos

All the media were used in this treatment was prepared as mentioned in sections (3. 2. 4. 2) page 74 and 77. Two types of media with different salt concentrations were used: MS (half and full strength) with full strength vitamins and WPM (half, full and $^3/_2$ basal

salt strength) with full strength vitamins (Table 3.3). Various concentrations of 2,4- D (i.e., 0, 2, 10, and 15 mg L^{-1}) were examined for every media mentioned. Induction media were supplemented with 50 mg L^{-1} *Myo*-inositol, 45 mg L^{-1} adenine hemisulfate, 100 mg L^{-1} L-glutamine, 3.3 mg L^{-1} Gelrite and 6% sucrose. The medium pH was adjusted to 5.8 before autoclaving. Seven petri dishes were used for each concentration with four embryos in each plate. The subculture was done every two weeks for eight weeks. The final subculture took place in glass jars with the same media composition for 20 weeks without subculturing. The percentage of callus formed, callus diameter, and callus fresh weight were recorded for each subculture.

3.2.4 Second Treatment: Induction of Somatic Embryos on ½ MS Medium Supplemented with Different Concentrations of Boric Acid

The medium was used in this treatment was prepared as mentioned in sections (3. 2. 4. 2) page 78. All the media were used in this treatment was prepared as mentioned in sections (3. 2. 4. 2) b & d page 74 and 77. One type of medium was used for callus induction. Induction medium: ½ MS with full strength vitamins, 50 mg L^{-1} *Myo*-inositol, 45 mg L^{-1} adenine sulphate, 100 mg L^{-1}, L-glutamine, 10 mg L^{-1} 2,4-D, 3.3 g L^{-1} Gelrite, 6% sucrose, with different concentrations of boric acid H_3BO_3 (M= 61.84 g/mol) (0, 6, 18, 36, 54, and 72 mg L^{-1}). The cultures were maintained in darkness at 27± 1 ºC for eight weeks and subcultured every two weeks.

3.2.4.1 Media Condition

The induction media used in these experiments were adjusted to pH 5.8 before adding 3.3% Gelrite (Duchefa, Netherland). The media was then sterilized by autoclaving at 104 kPa (121ºC) for 20 min. Induction of callus was performed by culturing explants in petri dishes (20 ml medium). They were sub-cultured at 2-week intervals for eight weeks and were maintained at 27 ± 1ºC under dark condition.

Table 3.3: Comparison The compositions of MS medium as described by Murashige and Skoog (1962) and WPM medium as described by Lloyd and McCown (1980).

Macronutrients	**Concentration (mg L^{-1}) in WPM medium**	**Concentration (mg L^{-1}) in MS medium**
1. NH_4NO_3	400.00	1650
2. K_2SO_4	990	------
3. $CaCl_2.2H_2O$	96	440
4. $MgSO_4.7H_2O$	370	370
5. KH_2PO_4	170	170
6. $(Ca(NO_3)_2.4H_2O)$	556	------
KNO_3	------	1900
Micronutrients	**Concentration (mg L^{-1}) in WPM medium**	**Concentration (mg L^{-1}) in MS medium**
1. H_3BO_3	6.20	6.200
2. $MnSO_4.H_2O$	22.30	16.900
3. $ZnSO_4.4H_2O$	8.60	8.600
4. KI	------	0.830
5. $Na_2MoO_4.2H_2O$	0.25	0.250
6. $CoCl_2.6H_2O$	------	0.025
7. $CuSO_4.5H_2O$	0.25	0.025
Vitamins	**Concentration (mg L^{-1}) in WPM medium**	**Concentration (mg L^{-1}) in MS medium**
1. Thiamine (HCl)	1.0	0.1
2. Nicotinic acid	0.5	0.5
3. Glycine	2.0	2.0
4. Pyrodoxine (HCl)	0.5	0.5
5. *Myo* – Inositol	100	100
Iron, 500 ml Stock (200X)		
37.3 mg of Na_2EDTA (Ethylenediaminetetra acetic acid, disodium salt) was dissolved in 250 ml dH_2O by using Erlenmeyer flask (500 ml). 27.80 mg of $FeSO_4.7H_2O$ was dissolved in 250ml dH_2O by using Erlenmeyer flask (500 ml). Na_2EDTA solution was boiled and $FeSO_4$ solution was added gently to it, with stirring.		

3.2.4.2 Experimental Design

For culture initiation, four explants were cultured per petri dish with seven replicates for each treatment. The cultures were covered with aluminum foil after being randomly arranged on the shelves in the culture room. For statistical analysis, the normality distribution of all quantitative data expressed as percentages were first examined. All experiments were set up in a completely randomized design. Percentages of explants forming callus, callus weight and callus diameter were determined after each subculture

in callus induction media. The difference between means was scored with Duncan's multiplication range test.

3.3 Induction of SE from Immature Zygotic Embryo Experiment

3.3.1 Fruit Collection and Sterilization

'Eksotika' fruits were collected from organic farms near Desaru Bandar Penawar, Johor Bahru, which is located 391 km southwest of Kuala Lumpur. The 'Eksotika' fruits were 95-100 days old when harvested. They were sprayed with 70% ethanol and kept in an ice box until used. The fruits were once again sprayed with 70% ethanol, then washed under running tap water with soap for 15 minutes to remove dust. The fruits were then sprayed again with 70% ethanol in the laminar flow. They were cut open with a sterile knife and the seeds were removed using a sterile spoon and was then kept in sterile vessels at 4^{o}C until used.

3.3.2 First Treatment: Induction of Somatic Embryos on Various Media with Different Concentrations of 2,4-D

All the media were used in this treatment were prepared as mentioned in sections (3. 2. 2) page 73 and 76. Two types of media with different salt concentrations were used : ½ MS basal salt strength with full strength vitamins and WPM (half, and full salt strength) with full strength vitamins. Various concentrations of 2,4-D (0, 2.5, 5, 7.5 and 10 mg L^{-1}) were added for every media mentioned. Induction media was supplemented with 50 mg L^{-1} *Myo*-inositol, 45 mg L^{-1} adenine sulphate, 100 mg L^{-1} L-glutamine, 3.3 g L^{-1} Gelrite and 6% sucrose. The filter sterilized L-glutamine was added to autoclaved media after the media was cooled down. The media pH was adjusted to 5.8 before autoclaving. Ten petri dishes were used for each concentration with nine embryos in each plate. The subculture was done every two weeks for eight weeks. Data was recorded every 2-weeks interval.

3.3.3 Second Treatment: Induction of Somatic Embryos on ½ MS Medium Supplemented with Different Concentrations of Boric Acid

The medium was used in this treatment was prepared as mentioned in sections (3. 2. 4. 2) page 78. Induction medium, ½ MS with full strength vitamins supplemented with 10 mg L^{-1} 2,4-D, was used with different concentrations of boric acid (0, 6, 18, 36, 52 and 72 mg L^{-1}). Induction medium was supplemented with 50 mg L^{-1} *Myo*-inositol, 45 mg L^{-1} adenine sulphate, 100 mg L^{-1} L-glutamine, 3.3 g L^{-1} Gelrite and 6% sucrose. The medium pH was adjusted to 5.8 before autoclaving. Ten petri dishes were used for each concentration with nine embryos in each plate.

3.3.4 Media Condition

Media used in experiments were adjusted to pH 5.8 before adding 3.3% Gelrite (Duchefa, Netherland), and were then sterilized by autoclaving at 104 kPa (121^{o}C) for 20 min. Induction of callus was performed in a petri dish (20 ml medium) and subcultured at a 2-week interval for eight weeks. Cultures were maintained at 27 ± 1^{o}C under dark condition. The frequency of embryogenesis was calculated as the percentage of cultures showing at least one somatic embryo. All media and chemicals were prepared fresh and kept three days before culturing. Callus diameter was recorded for every subculture. Callus fresh weight was recorded under laminar flow condition.

3.3.5 Experimental Design

For culture initiation, eight explants were cultured per petri dish with ten replicates for each treatment. The cultures were covered with aluminum foil after being randomly arranged on the shelves in the culture room. For statistical analysis, the normality distribution of all quantitative data expressed as percentages were first examined. All experiments were set up in a completely randomized design. Percentages of explants forming callus, callus weight and callus diameter were determined after each subculture

in callus induction media. The difference between means was scored with Duncan's multiplication range test.

3.3.6 Identification of Embryogenic Callus

The callus formed after induction can be divided into two types, embryogenic callus that has the ability to form new plants and non-embryogenic callus that consists of undifferentiated cells and generates undifferentiated cells. The double staining technique (acetocarmine and Evan's blue dye) was used to distinguish the embryogenic callus from the non-embryogenic callus (Gupta & Durzan, 1987).

3.3.6.1 Double Staining Technique:

The preparation of 100 ml of 2% acetocarmine under fume hood

1. 2 g of acetocarmine was added to 55 ml of distilled water and poured into a 200 ml beaker containing a magnetic stirrer.

2. 45 ml of glacial acetic acid was added to the beaker containing distilled water.

3. The solution was stirred and boiled on a hot plate for 5 min, then cooled down and filtered in a funnel using Whatmant filter paper.

The preparation of 100 ml of 0.5% Evan's Blue

1. 0.5 g of Evan's Blue was added to the 100 ml distilled water in the flask (250 ml).

Acetocarmine and Evan's blue staining protocol. The callus was stained as described in Gupta and Durzan (1987):

1- Small portions of callus (3-5 mm) were placed on glass slides.

2- Two to four drops of acetocarmine were added until the callus was submerged.

3- Forceps were used to gently divide the callus on the slide containing the acetocarmine solution.

4- The slide was gently heated without boiling it.

5- The slide was washed two to three times with distilled water to remove any excess acetocarmine stains.

6- Two drops of 0.5% Evan's Blue were added to the slide.

7- The slide was washed two to three times with water to remove any excess Evan's Blue stains.

8- One drop of glycerol was added after which the sample was covered with a cover slip.

9- The slides were observed under a light microscope to identify the embryogenic and non-embryogenic callus.

3.4 Maturation Phase

3.4.1 Media Preparation

The maturation media were prepared as described in chapter three section (3. 2. 2) page 77.

3.4.2 Maturation Experiments

The embryogenic callus produced from induction media was transferred to different maturation media as listed below:

1- Embryogenic callus from IM1 medium was cultured in M1 medium: which consisted of ½ MS medium supplemented with different concentrations of PG (0, 1, 5 and 10 mg L^{-1}).
2- Embryogenic callus from IM2 medium was cultured in M2 medium: which consisted of ½ MS medium supplemented with different concentrations of PEG (0, 2, 4, 8 and 12%).
3- Embryogenic callus from IM3 medium was cultured in M3 medium: which consisted of ½ MS medium supplemented with different concentrations of PG (0, 1, 5 and 10 mg L^{-1}).
4- Embryogenic callus from IM4 medium was cultured in M4 medium: which consisted of ½ WPM medium supplemented with 5 mg L^{-1} for first subculture then the 2,4-D was decreased to 2.5 and 0 for each subculture.

The somatic embryos were maintained for 6 weeks in the maturation medium to study the effect of PG, PEG and media composition. Five petri dishes were used for each concentration, and 0.4 g of embryogenic callus was transferred to each petri dish. Subculturing was done biweekly for six weeks. The concentration of 2,4-D was decreased to half in the first two subcultures and then the third subculture was done in ½ MS without 2,4-D. The number of somatic embryos at different developmental stages were recorded after finished maturation phase.

3.4.3 Media Conditions

Maturation media used in the experiments were adjusted to pH 5.8 before adding 3.8% Gelrite (Duchefa, Netherland). All maturation media were supplemented with 100 mg L^{-1} *Myo*-inositol, 68 mg L^{-1} adenine sulphate, 100 mg L^{-1} L-glutamine, 3.8 g L^{-1} Gelrite and 3% sucrose. They were then sterilized by autoclaving at 104 kPa (121°C) for 20 min. Somatic embryos were cultured in 20 ml medium in Petri dishes which were eventually

subcultured biweekly interval for six weeks. All the cultures were maintained at 27 ± 1ºC under a 16/8 light- dark condition.

3.5 Somatic Embryos Development (Shoot Formation) Phase

3.5.1 Media Preparation

The shoot formation media were prepared as described in in chapter three section (3. 2. 2) page 77, with 0.8% agar as solidifying agent instead Gelrite.

3.5.2 Somatic Embryos Development Experiments

The two types of media used for shoot formation were as follows; SF1 medium: which consisted of MS medium without growth regulators and, SF2 medium: which consisted of MS medium supplemented with 0.05% AC. All shooting media were supplemented with 3% sucrose, and 8 g L^{-1} agar. The media were adjusted to pH 5.8 before autoclaving. Five glass jars (30 ml medium for each) were used for each treatment with 25 cotyledonary stage embryos (size ≥ 3mm) cultured in each jar. The cultures were maintained in the culture room at 27 ± 1ºC under a 16/8 light- dark condition. The percentage of shoot formation was recorded after 6 weeks of culturing.

3.5.3 Media Conditions

Shoot formation media used in the experiments were adjusted to pH 5.8 before adding 0.8% agar. All shoot formation media were supplemented with 3% sucrose. They were then sterilized by autoclaving at 104 kPa (121ºC) for 20 min. Somatic embryos were cultured in 30 ml medium in glass flasks which were eventually subcultured biweekly interval for six weeks. All the cultures were maintained at 27 ± 1ºC under a 16/8 light-dark condition.

3.6 Elongation Phase

3.6.1 Media Preparation

The elongation media were prepared as described in chapter three section (3. 2. 2) page 77.

3.6.2 Elongation Experiments

One type of medium was used for elongation; EL medium: which consisted of MS medium supplemented with 1 mg L^{-1} GA_3, 0.5 mg L^{-1} IBA, 100 mg L^{-1} *Myo*-insitol and 3.76 mg L^{-1} riboflavin. The pH was adjusted to 5.8 before autoclaving. Five glass jars (50 ml medium for each) were used for each treatment. The cultures were maintained in the culture room at 27 ± 1ºC under a 16/8 light- dark condition. The percentage of survival rate was recorded after 6 weeks of culturing.

3.6.3 Media Conditions

Elongation medium used in the experiments were adjusted to pH 5.8 before adding 3.8% Gelrite. All shoot formation media were supplemented with 3% sucrose. They were then sterilized by autoclaving at 104 kPa (121ºC) for 20 min. Somatic embryos were cultured in 30 ml medium in glass flasks which were eventually subcultured biweekly interval for six weeks. All the cultures were maintained at 27 ± 1ºC under a 16/8 light-dark condition.

3.7 Rooting Phase

3.7.1 Media Preparation

The rooting media were prepared as described in chapter three section (3. 2. 2) page 77. For liquid media vermiculate was used for supporting media instead Gelrite.

3.7.2 Rooting Experiments

For rooting, two types of explants were used EX1: explants from M2+SF1 medium were used to examine the effect of riboflavin on rooting and EX2: explants from M3+SF2 were used to examine the effect of PG on rooting. Both explants EX1 and EX2 were cultured for four days in full strength MS medium supplemented with 2 mg L^{-1} IBA. After 4 days in the MS medium containing IBA, the shoots were transferred to four types of media supported by vermiculite; R1 medium: which consisted of MS medium supplemented with 3.76 mg L^{-1} riboflavin and, R2 medium: which consisted of MS medium without riboflavin, R3 medium: which consisted of MS medium supplemented with 7.9 mg L^{-1} PG and R4 medium: which consisted of MS medium without PG. To study the effect of IBA on rooting, EX1 explants were cultured directly in R5 medium: which consisted of MS medium supplemented with 7.9 mg L^{-1} PG without IBA and supported with vermiculate. The cultures were maintained at 27 $\pm$ 2°C for 6 weeks under a 16-hour photoperiod. The number and length of roots formed, as well as the quality of the roots were recorded after 6 weeks of culturing.

3.7.3 Media Conditions

All rooting media used in the experiments were adjusted to pH 5.8 before adding 3.8% Gelrite. All shoot formation media were supplemented with 3% sucrose. They were then sterilized by autoclaving at 104 kPa (121°C) for 20 min. Somatic embryos were cultured in 50 ml medium in glass flasks. All the cultures were maintained at 27 ± 1°C under a 16/8 light- dark condition.

3.8 Acclimatization

After rooting phase, *in vitro* plantlets were transferred to pots (80 x 60 mm) filled with peatmos soil. The agar medium and vermiculate was carefully washed off the roots with distilled water before planted in the pot. The potted plantlets were first kept in the culture

room at 25 ± 1 °C under 16 hours light and 8 hours dark for 3 weeks. The plantlets were watered every 3 days.

3.9 Artificial Seeds Production from Somatic Embryos of *Carica papaya* L. cv. Eksotika

An encapsulation matrix was prepared for the production of artificial seeds. Three different concentrations (2.5, 3.5, and 4.5%) of SA (R & M Chemicals, $C_6H_7O_6Na$) were dissolved in 5 different solvents (distilled water, MS medium, MS medium without calcium component, ½ MS medium and ½ MS medium without calcium component). Additionally, three different concentrations (50, 75 and 100 mM) of $CaCl_2$ (SYSTERM, ChemAR, $CaCl_2.2H_2O$, Mw= 147.02 g $mole^{-1}$) were prepared. Subsequently, the encapsulation matrix and $CaCl_2$ solution were autoclaved for 20 min under 104kPa pressure at 120°C. The pH of the encapsulation matrix was adjusted to 5.8 before autoclaving. The beads produced from the different concentrations of SA were soaked in different concentrations of $CaCl_2$ for different durations (10, 20 and 30 min) to examine the artificial seed formation and shape. Thus, the best concentration of SA and $CaCl_2$ were determined by the formation of firm, clear and rounded beads.

3.9.1 Explant Source

The somatic embryos at cotyledonary stage were used in this experiment. The IZE of *C. papaya* were induced and matured as described in chapter four sections (3. 3 & 3. 4) page 80 &83, IM2 media for induction phase and M2 (8% PEG) for maturation phase.

3.9.2 Artificial Seeds Experiment

During the cotyledonary stage, somatic embryos were excised (2 mm< size) from the embryogenic callus and subsequently encapsulated with the encapsulation matrix. The encapsulated somatic embryos were individually dropped into the $CaCl_2$ solution using wide blue tips (4 mm diameter). Following encapsulation, the beads were immersed and

washed thrice with sterilized distilled water. Then, they were immersed in liquid MS medium before inoculation in the germination media. Two germination media were used for the artificial seed experiment; SY1 medium: which consisted of MS medium without growth regulators and, SY2 medium: which consisted of MS medium supplemented with 0.2 mg L^{-1} NAA+ 0.2 mg L^{-1} BAP. Twenty-four replicates were used for each treatment with mostly one bead for each plate. The percentage of germination was calculated after 8 weeks of culturing.

3.9.2.1 Media Preparation

The media were prepared as mentioned in chapter three sections (3. 4. 2) page 76 for liquid media with excluded the solidified agent (Gelrite) and calcium ($CaCl_2.H_2O$) in macronutrients. Germination media were prepared as mentioned in chapter three sections (3. 4. 2) page 77.

3.10 Histological Analysis of The Developmental Stages of Somatic Embryogenesis Induced from Immature Zygotic Embryos of *Carica papaya* L. cv. Eksotika

3.10.1 Explant Source

C. papaya 'Eksotika' fruits were collected and prepared as mentioned in chapter three section (3. 3. 1) page 80.

3.10.2 Induction and Maturation Media

The IZE of *C. papaya* 'Eksotika' were excised and incubated in the induction medium as mentioned in Vilasini *et al.* (2000). The induction medium consisted of ½ MS media with full strength vitamins supplemented with 10 mg L^{-1} 2,4 D, 45 mg L^{-1} adenine hemisulfate, 50 mg L^{-1} *Myo*-insitol and 100 mg L^{-1} L-Glutamine. The induced somatic embryos were then transferred to the maturation medium which consisted of ½ MS medium supplemented with 100 mg L^{-1} L-glutamine, 68 mg L^{-1} adenine hemisulfate, 100

mg L^{-1} *Myo*-insitol, with half the amount of 2,4-D (5 mg L^{-1} and 2.5 mg L^{-1}) for two subcultures. The last subculture occurred in ½ MS medium without growth regulators.

3.10.3 Histological Samples Preparation

For histological examination, samples of somatic callus were collected and fixed randomly after (25, 27, 28 30, 32, 34, 38, 40, 42, 44, 46, 48, 50, 52, 55, 57, 59, 60, 70 and 80) days of culturing. At least 3 explants (callus) were randomly collected each time and processed for histological examination.

3.10.3.1 Fixation

Samples (0.5 cm) were fixed in a cool formaldehyde- acetic acid- ethanol (FAA) solution in a ratio of 5: 5: 90 (v/v/v). The ratio between sample and fixative was (1:10) respectively. After that, the specimens with fixatives were transferred to a refrigerator at 4^{o}C for 48 h (Huang & Yeung, 2015).

3.10.3.2 Dehydration

The specimens were dehydrated through nine graded series of tert-butyl alcohol (TBA) (Table 6.1 steps (1-8)). The samples were treated sequentially for 2 h per step. Two drops of eosin were added to the first 100% TBA to dye the transparent embryo in the wax in order to facilitate trimming and sectioning.

3.10.3.3 Infiltration and Embedding

The samples were embedded in paraffin wax. The somatic embryos were redirected in the molds to get longitudinal sectioning. Then, the molds were kept in a refrigerator to speed up hardening and to prevent bubbles formation (Table 3.4 steps (9-13)).

3.10.3.4 Sectioning

Serial 10 µm sections were cut using Leica, RM2125 rotary microtome (Leica Microsystems, Wetzlar, Germany). After that, the wax ribbon was floated on the surface

of a water bath (40- 45ºC) to stretch and smooth the wax sections and to affix them on frosted slides. Then, they were dried overnight in an incubator at approximately 35ºC and were arranged in slide racks.

3.10.3.5 Staining

The samples slides were exposed to a series of staining solutions Toluidine Blue O and Safranin- O Fast green (Table 3.5 & 3.6). Sections were stained according to Johansen (1940) with 0.05% (w/v) toluidine blue O (TBO) for common histology.

3.10.3.6 Observation

Prepared slides were observed through OLYMPUS CX22 (Tokyo Japan) supplemented with Dino-Lite USB microscope cameras, including *DinoCapture 2.0* software and Zeiss Axioscope microscope supplemented with a Zeiss Axiocam MRe digital camera with AxioVision software.

Table 3.4: The dehydration and imbedding protocol for prepared microscopic slides.

Step	Treatment	Duration of process (h)
1	TBA: 95% ethanol: water (1: 4: 5)	1
2	TBA: 95% ethanol: water (2: 5: 3)	1
3	TBA: 95% ethanol: water (3.5: 5: 1.5)	1
4	TBA: 95% ethanol: water (5.5: 4.5: 0)	1
5	TBA: 95% ethanol: water (7.5: 2.5: 0)	1
6	Pure TBA+ 2 drops of Eosin	1
7	Pure TBA	1
8	Pure TBA (oven at 60 ºC)	1
9	TBA+ Wax (2: 1) (oven at 60 ºC)	12
10	TBA+ Wax (1: 1) (oven at 60 ºC)	12
11	TBA+ Wax (1: 2) (oven at 60 ºC)	12
12	Pure Wax (oven at 60 ºC)	12
13	Pure Wax (oven at 60 ºC)	12

Table 3.5: The de-waxing and TBO staining protocol for prepared microscopic slides.

Step	Treatment	Duration of process
1	Xylene 2 times	5 min for each
2	100% ethanol 2 times	2 min for each
3	95 % ethanol	1 min
4	85 % ethanol	1 min
5	70 % ethanol	2 min
6	50 % ethanol	2 min
7	H_2O	2 min
8	TBO	20 sec
9	H_2O 3 times	10 dips for each
10	95% ethanol	10 dips
11	100% ethanol 2 times	10 dips for each
12	Xylene 2 times	5 min for each

Table 3.6: The de-waxing and "Safranin and fast green" staining protocol for prepared microscopic slides.

Step	Treatment	Duration of process
1	Xylene 2 times	5 min for each
2	100% ethanol 2 times	5 min for each
3	95 % ethanol	2 min
4	85 % ethanol	2 min
5	70 % ethanol	2 min
6	Safranin O	3 h
7	H_2O 2 times	5 min for each
8	95 % ethanol	10 sec
9	95% ethanol	10 sec
10	100% ethanol	10 sec
11	Fast green	15 sec
12	Diluted clearing solution	One Rinse
13	Clearing solution	10 sec
14	Xylene 2 times	5 min for each

3.11 The Expression of *CpLAX2*, *CpLAX3* and *CpPIN4* Genes During Maturation and Germination Phases of Somatic Embryogenesis in *Carica papaya* cv. Eksotika

3.11.1 Explant Source and Sterilization

C. papaya 'Eksotika' fruits were collected and prepared as mentioned in chapter three section (3. 3. 1) page 80.

3.11.2 Induction Phase

The explants, IZE, were first incubated for 8 weeks in an induction medium which consisted of ½ MS medium supplemented with 10 mg L^{-1} 2,4-D, 50 mg L^{-1} *Myo*-inositol, 45 mg L^{-1} adenine sulphate, 100 mg L^{-1} L-glutamine, 3.8 g L^{-1} Gelrite and 6% sucrose. The induction medium was adjusted to pH 5.8 before 3.8% Gelrite was added (Duchefa, Netherland). The explants were then sterilized by autoclaving at 104 kPa (121^{o}C) for 20 minutes. The IZE were cultured in petri dishes containing 20 ml medium and were subcultured at a biweekly interval for eight weeks. All cultures were maintained at 27 ± 1^{o}C under dark condition.

3.11.3 Maturation Phase

The embryogenic callus produced from the induction medium was then transferred to the maturation medium, which consisted of ½ MS medium supplemented with 5mg L^{-1} of 2,4-D, 100 mg L^{-1} *Myo*-inositol, 68 mg L^{-1} adenine sulphate, 100 mg L^{-1} L-glutamine, 3.8 g L^{-1} Gelrite and 3% sucrose, for 6 weeks. The 2,4-D concentration was decreased to half (2.5 mg L^{-1}) in the second subculture while the third subculture took place in ½ MS without 2,4-D. They were then sterilized by autoclaving at 104 kPa (121^{o}C) for 20 minutes. The somatic embryos were cultured in petri dishes containing 20ml medium and were eventually subcultured at a biweekly interval for six weeks. All cultures were maintained at 27 ± 1^{o}C under a 16/8 light- dark condition.

3.11.4 Total RNA Isolation

For molecular analyses, samples were collected from the maturation and germination medium after (59, 61, 63, 70, 90, 100, 105, 115, 130, 135, 143 and 158) days of culturing in the induction medium. Total RNA was extracted in at least three independent replications. Frozen SE callus (0.35 g) were kept at -80^{o}C for subsequent RNA extraction. All frozen tissues were ground in liquid nitrogen for RNA isolation. Total RNA was

extracted from the fine powder of SE callus using RNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's instructions as follows:

1- 300 mg of callus were weighed and crushed in liquid nitrogen using a mortar and pestle.
2- The tissue powder and liquid nitrogen were poured into sterile vessels and left until the liquid nitrogen evaporated without allowing the tissue to thaw.
3- 450 µL RLC buffer was added to 100 mg of tissue powder in a sterile microcentrifuge tube and was vortexed vigorously.
4- The lysate produced was transferred to the GIA shredder spin column and centrifuged for 2 min at 13500 rpm.
5- The supernatant was carefully transferred to a new sterile microcentrifuge tube without disrupting the pellet.
6- ½ volume of absolute ethanol was added to the supernatant and mixed by pipetting before being transferred to the RNeasy mini spin column. The lid was closed and centrifuged for 15 sec at 10000 rpm.
7- Precipitate fluid was removed.
8- 700 µL RW1 buffer was added to the RNeasy mini spin column. The lid was closed and centrifuged for 15 sec at 10000 rpm.
9- Precipitate fluid was removed.
10- 500 µL RPE1 buffer was added to the RNeasy mini spin column. The lid was closed and centrifuged for 15 sec at 10000 rpm.
11- Precipitate fluid was removed.
12- 500 µL RPE1 buffer was added to the RNeasy mini spin column. The lid was closed and centrifuged for 2 min at 10000 rpm.
13- Precipitate fluid was removed.

14- RNeasy mini spin column was added in a new 1.5 ml sterile microcentrifuge tube and 40 µL RNase free water was added to the RNeasy mini spin column. The lid was closed and centrifuged for 1 min at 10000 rpm to elute the RNA.

The total RNA was resuspended in 40 µLof RNase free water. The RNA concentration and purity were evaluated by measuring absorbance at 230, 260 and 280nm respectively, using Nanodrop 2000 (Eppendorf, Germany). Only RNA samples with a 260/280 ratio between 1.8 and 2.1 and 260/230 ratio higher than 1.5 were used for further experiments.

3.11.5 RNA Integrity

The integrity of the extracted RNA sample was examined using a gel electrophoresis system. The gel was prepared by dissolving 1% of agarose gel with 1 X Tris-acetate-EDTA (TAE) buffer, followed by boiling the mixture until the agorose was completely dissolved and then leaving it to cool down. Red safe stain (1µL/ 10 ml of gel mixture) was then added to the mixture before pouring it into a cast of electrophoresis and leaving it to cool down. The comb was then removed and the cast was set inside the electrophoresis tank before pouring Tris-Borate-EDTA (TBE) buffer. After that, the comb was removed and the quality of the wells were checked. The first well was filled with 4µL of ladder (100 bp) (Thermo Fisher Scientific, USA) and the sample was loaded one by one using a micropipite. The electrophoresis system was operated at 100-Voltage, 200 Amp for 30 min.

3.11.6 Genomic DNA Removal

Before initiating cDNA synthesis, the genomic DNA was removed using a QuantiNova Rev. Transcription Kit (Qiagen, Germany) according to the manufacturer's intstructions. The master mix was prepared by mixing 2 µL of gDNA removal mix with 10 µL of RNA sample, 1µL of internal control RNA and 2 µL of RNase free water for

each reaction. The reaction components were Incubated for 2 minutes at 45°C, after which it was immediately placed on ice.

3.11.7 cDNA Synthesis

The cleaned total RNA extracted were used as templates for the cDNA synthesis. 0.2 microgram of total RNA was reverse-transcribed using the QuantiNova Rev. Transcription Kit (Qiagen, Germany) according to the manufacturer's instructions. The following thermal cycle conditions were used: 3 min at 25°C for the annealing step, followed by 10 min at 45°C for the reverse transcription step, and 5 min at 85°C for the inactivation of reaction. The final cDNA products were diluted 10-fold prior to being used in RT-qPCR.

3.11.8 Primer Design

A search on the *LAX2*, *LAX3* and *PIN4* genes of *Arabidopsis thaliana* was initially conducted through the "bioinformatics" website followed by a search on the orthologs using the integrative orthology viewer. The best ortholog for the *LAX2* gene, *LAX3* gene, and *PIN4* gene were (Cpa.g.sc37.169), (Cpa.g.sc36.16), and (Cpa.g.sc115.56) respectively. Then, the sequence of *Arabidopsis* genes was blasted without the intron portion and with genomic *C. papaya* using the (https://blast.ncbi.nlm.nih.gov/Blast.cgi) website. Next, the ortholog sequence was used to go to GenPept and find REFSEQ in order to pick primers. The primers were chosen according to temperature (i.e., forward and reversed primers had to have similar temperatures), GC content (i.e. ensuring that it was 50-80%). The predicted protein sequence for LAX2, LAX3 and PIN4 in *C. papaya* were (gene id: Cpa.g.sc37.169, Cpa.g.sc36.16 and Cpa.g.sc92.84) respectively (Appendix 1). These primers were synthesized through integrated DNA technologies (Malaysia). The lyophilized primers were dissolved and diluted using Nuclease free water (Table 3.7).

Table 3.7: List of Primers with length, Tm and GC content.

Gene name	Sequence (5'->3')	Template strand	Length	Tm	GC %
LAX2	Forward	TGTGCTCACACTGACACTCC	20	59.90	55.0
	Reverse	CAGCCATGTCTCGGAAAGGT	20	60.04	55.0
LAX3	Forward	GGCCAGCCAAAATGGTTCTG	20	60.04	55.0
	Reverse	TCCACTGTCACAGCATGTCC	20	59.96	55.0
PIN4	Forward	CCAACTCCGGCCTTTCTCTT	20	59.96	55.0
	Reverse	TAGAGCATCTTGGCGTTGGG	20	60.11	55.0
EIF	Forward	CGCGGAAGACAAACCCAATC	20	59.83	55.0
	Reverse	CTGGAGCAAAACGGCTGATG	20	59.83	55.0

3.11.9 PCR (Polymerase chain reaction) Primers and Reaction Conditions

Gene-specific PCR primer pairs were tested to optimize the annealing temperature when amplifying products from cDNA. A normal PCR kit was used. The primers were tested for gene specificity using *EIF*, *LAX2*, *LAX3* and *PIN4* genes as well as cDNA clones as templates. The PCRs (10 µL) consisted of a master mix that included 2 µL of buffer, 0.2 µL of DNTP, 0.1 Taq polymerase, 0.6 µL $MgCl_2$, 6.22 µL DNase free water plus 0.2 µL of both forward and reverse primers and 200 ng of cDNA as a template. Thermocycling conditions were 95 ^oC for 5 min, followed by 35 cycles of 95 ^oC for 45 sec, 62 ^oC for 45 seconds, and 72^oC for 30 sec and final elongation of 72 ^oC for 5 min before finally maintaining the temperature at 10 ^oC. Products were analyzed on 2 % (w/v) agarose gels in 1X TBE buffer and were visualized by staining with red safe stain.

3.11.10 Real Time-Quantitative PCR Conditions

The primers were synthesized using an Agilent technologies stratagene Mx 3005P (USA) PCR machine using QuantiNova SYBR Green PCR Kit (Qiagen, Germany) according to the manufacturer's recommendations. Each sample was run in triplicate in a

final volume of 10 µL containing 2.6 uL of the cDNA (26ng), 0.7 uM of each specific primer, 1µL of RNase free water and 5 µL 2X SYBR green PCR Master Mix solution, according to the manufacturer's instructions. The cycling conditions were as follows: 2 min at 95 ^{o}C for initial heat of polymerase activation, and 40 cycles at 95 ^{o}C for 5 sec and 62 ^{o}C for 10 sec. Each assay was conducted in triplicate and included a no-template control (NTC). The threshold cycle (Ct) of the primary amplification curve was used for calculations. The *EIF* gene was selected as a reference gene. The gene expression level was calculated according to the following formula:

$$\Delta\Delta Ct = (Ct_{target} - Ct_{EIF})_{\text{time x}} - (Ct_{target} - Ct_{EIF})_{\text{time 0}}$$

$$\text{Relative gene expression} = 2^{-\Delta\Delta Ct}$$

Data were analyzed according to the relative quantification methods of (Livak & Schmittgen, 2001).

CHAPTER 4: RESULTS

4.1 Seed Sterilization Experiment

A successful micropropagation procedure heavily depends on keeping explants free from contamination. A higher survival percentage and a lower contamination rate were observed when explants were treated with 70% ethanol for 2 min, then soaked in 40% and 50% Clorox for 5 min, followed by soaking in 0.1 $HgCl_2$ for 10 min and finally soaked in 70% ethanol for 2 min (Table 4.1 and Figure 4.1). Conversely using 80% ethanol for 1 min, followed by various exposure times (1, 3 and 5 min) of 0.1 % $HgCl_2$, then soaking in 80% of ethanol for 1 min, showed 100% contamination and most of the explants died. Also, using 70% ethanol for 1 min, then soaking in different concentrations of Clorox (10, 20 and 30%) followed by soaking in 0.1% $HgCl_2$ for 5 min and then washing in 70% ethanol for 1 min resulted in 100% contamination.

Table 4.1: Types, time of exposure and concentrations of sterilizing agents used for sterilizing seeds of *C. papaya* **'**Eksotika'.

Treatment	Chemical	Time (min)	Concentration (%)
Treatment 7	Ethanol	2	70
	Clorox	5	40
	Mercuric chloride	5	0. 1
	Ethanol	2	70
Treatment 8	Ethanol	2	70
	Clorox	5	40
	Mercuric chloride	10	0. 1
	Ethanol	2	70
Treatment 9	Ethanol	2	70
	Clorox	5	50
	Mercuric chloride	5	0. 1
	Ethanol	2	70
Treatment 10	Ethanol	2	70
	Clorox	5	50
	Mercuric chloride	10	0. 1
	Ethanol	2	70

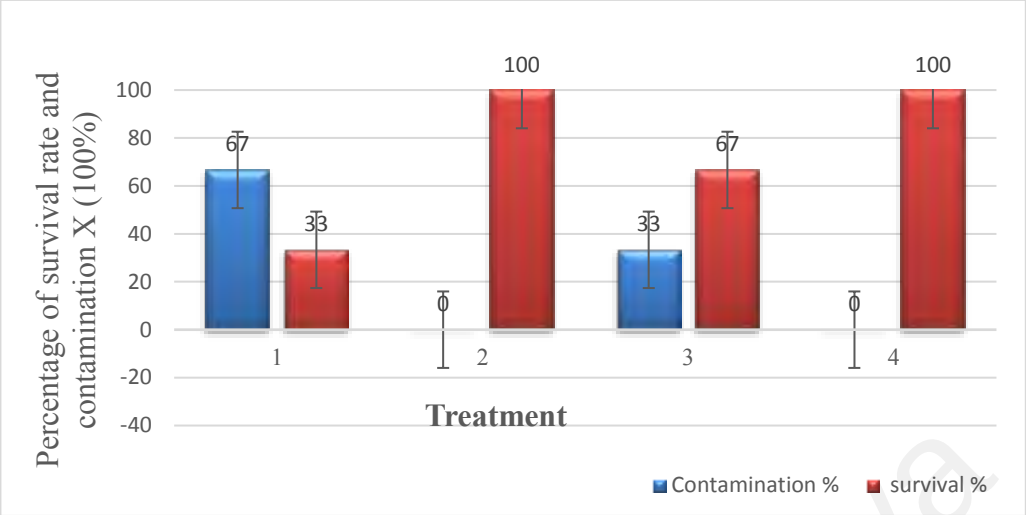

Figure 4.1: The effect of different treatment in seed surface sterilization on the percentage of survival rate and contamination of C. papaya 'Eksotika' seeds inoculated in MS medium.

4.2 First Experiment: Somatic Embryogenesis Induction from Mature Zygotic Embryos

4.2.1 First Treatment: Induction of Somatic Embryos on Various Media at Different Concentrations of 2,4-D from Mature Zygotic Embryos

After a few days in culture, mature zygotic embryo explants developed calli after ten days of inoculation in all media and 2,4-D concentrations tested. There was no obvious indication of callus proliferation in media without growth regulators. In the media without growth regulators, the zygotic embryos produced shoots and roots.

The average callus weight and average callus diameter were measured after 6, 8 and 20 weeks respectively. After 6 weeks, the highest average callus weight (0.04 g) was recorded in ½ MS medium supplemented with 2 mg L^{-1} 2,4-D. After 8 weeks, ½ MS medium supplemented with 2 mg L^{-1} 2,4-D recorded the highest average callus weight (0.05 g). Finally, after 20 weeks, the ½ MS medium supplemented with 10 mg L^{-1} 2,4-D and ½ WPM with 2 mg L^{-1} recorded the highest average callus weight (0.78 g) (Table 4.2).

Table 4.2: Means comparison of the average callus weight (g) ± Standard Error of different types of media supplemented with different 2,4-D concentrations after 6, 8 and 20 weeks of culture.

Time (weeks)	2, 4- D Concentration (mg L^{-1})	½ MS	½ WPM	WPM	$^3/_2$WPM
6	2	0.04±0.01^{a}	0.01±0.01^{d}	0.02±0.00^{bcd}	0.02±0.01^{d}
	10	0.04±0.02^{ab}	0.04±0.01^{ab}	0.02±0.01^{d}	0.02±0.01^{d}
	15	0.02±0.01^{cd}	0.02±0.01^{bcd}	0.02±0.01^{d}	0.04±0.00^{abc}
8	2	0.05±0.02^{a}	0.03±0.00^{bc}	0.02±0.01^{d}	0.01±0.01^{d}
	10	0.04±0.01^{ab}	0.02±0.01^{d}	0.02±0.0^{cd}	0.02±0.01^{d}
	15	0.02±0.00^{d}	0.01±0.01^{d}	0.02±0.01^{d}	0.03±0.00^{bc}
20	2	0.10±0.01^{a}	0.78±0.04^{a}	0.04±0.02^{b}	0.02±0.00^{b}
	10	0.78±0.02^{a}	0.03±0.01^{b}	0.03±0.01^{b}	0.02±0.01^{b}
	15	0.02±0.00^{b}	0.02±0.01^{b}	0.02±0.00^{b}	0.02±0.01^{b}

Same letters are not significantly different at p= 0.05 level

The highest callus diameter (0.53 cm) after 2 weeks of culturing was observed in ½ MS medium supplemented with 10 mg L^{-1} 2,4-D. Conversely, the lowest callus diameter (0.29 cm) was recorded in $^3/_2$ WPM medium supplemented with 15 mg L^{-1} 2,4-D (Table 4.3). The highest callus diameter after 4, 6 and 8 weeks were 0.87, 0.89 and 1.05 cm, respectively in ½ MS media supplemented with 2 mg L^{-1} 2,4-D, whereas, the lowest callus diameter was recorded in $^3/_2$ WPM medium supplemented with 15 mg L^{-1} 2,4-D.

Table 4.3: Means comparison of the average callus diameter (cm) ± Standard Error of different types of media supplemented with different 2,4-D concentrations after 2, 4, 6 and 8 weeks of culture.

Subculture Time (weeks)	Concentration of 2,4-D (mg L^{-1})	½ MS	½ WPM	WPM	$^3/_2$ WPM
2	2	0.51±0.03^{a}	0.52±0.00^{a}	0.42±0.03^{ab}	0.31±0.02^{b}
	10	0.53±0.04^{a}	0.34±0.05^{b}	0.31±0.02^{b}	0.34±0.01^{b}
	15	0.29±0.03^{a}	0.47±0.03^{ab}	0.41±0.03^{ab}	0.29±0.03^{b}
4	2	0.87±0.04^{a}	0.51±0.04^{cd}	0.56±0.03^{bcd}	0.41±0.04^{cd}
	10	0.73±0.04^{ab}	0.50±0.04^{cd}	0.63±0.01^{bc}	0.48±0.03^{cd}
	15	0.38±0.01^{d}	0.44±0.05^{cd}	0.48±0.02^{cd}	0.40±0.02^{d}
6	2	0.89±0.07^{a}	0.80±0.03^{abc}	0.62±0.04^{cd}	0.39±0.03^{de}
	10	0.86±0.05^{ab}	0.61±0.04^{cd}	0.64±0.02^{bcd}	0.47±0.03^{de}
	15	0.57±0.02^{cde}	0.53±0.04^{de}	0.51±0.04^{de}	0.35±0.03^{e}
8	2	1.05±0.07^{a}	0.75±0.01^{bc}	0.64±0.05^{bc}	0.48±0.03^{c}
	10	0.96±0.03^{a}	0.64±0.01^{bc}	0.68±0.02^{bc}	0.55±0.02^{bc}
	15	0.59±0.02^{bc}	0.60±0.04^{bc}	0.58±0.05^{bc}	0.47±0.02^{c}

Same letters are not significantly different at p= 0.05 level

4.2.2 Second Treatment: Induction of Somatic Embryos on Half (½) MS Medium Supplemented with Different Concentrations of Boric Acid

After four weeks, the highest percentage of callus induction (86.10%) and the highest average callus weight (0.04 g) were recorded in the medium supplemented with 72 mg L^{-1} boric acid. The medium supplemented with 6 mg L^{-1} boric acid recorded the highest average callus diameter (0.70 cm) while the medium supplemented with 18 mg L^{-1} of boric acid recorded the highest percentage of somatic embryos (81.90%) (Table 4.4).

Table 4.4: Means comparison of callus induction percentage (%) ± Standard Error, average callus diameter (cm) ± Standard Error, average callus weight (g) ± Standard Error and percentage of somatic embryos (%) ± Standard Error of different boric acid concentrations after 4 weeks of culture.

Boric acid concentration (mg L^{-1})	Callus induction percentage (%)	Average callus diameter (cm)	Average callus weight (g)	Percentage of somatic embryo (%)
6	51.20±0.05^{b}	0.70±0.01^{a}	0.02±0.01^{bc}	72.10±3.29^{a}
18	34.90±0.02^{b}	0.67±0.02^{ab}	0.02±0.01^{bc}	81.90±3.55^{a}
36	51.30±0.04^{b}	0.55±0.01^{c}	0.02±0.01^{c}	68.40±3.76^{a}
54	46.90±0.03^{b}	0.67±0.01^{ab}	0.03±0.01^{ab}	70.10±2.29^{a}
72	86.10±0.03^{a}	0.58±0.01^{c}	0.04±0.01^{a}	65.90±3.59^{a}

Same letters in a column are not significantly different at p=0.05 level

After 8 weeks, the medium supplemented with 6 mg L^{-1} of boric acid recorded the highest callus weight (0.16 g) (Table 4.5). Conversely, the lowest callus weight (0.08 g) was observed in the medium supplemented with 36 mg L^{-1} of boric acid (Table 4.5). The medium supplemented with 18 mg L^{-1} of boric acid recorded the highest somatic embryos percentage (89.3%) (Table 4.5).

Table 4.5: Means comparison of average callus weight (g) ± Standard Error and percentage of the somatic embryo (%) ± Standard Error of different boric acid concentrations after 8 weeks of culture.

Boric acid concentration (mg L^{-1})	Callus weight (g) ± SE	Percentage of somatic embryos (%) ± SE
0	0.15±0.05^{a}	70±5.90^{b}
6	0.16±0.01^{a}	83±3.18^{ab}
18	0.1±0.02^{b}	89.3±2.53^{a}
36	0.08±0.02^{b}	79±1.81^{ab}
54	0.12±0.05^{a}	85.7±1.49^{ab}
72	0.15±0.02^{a}	81.8±2.14^{ab}

Same letters in a column are not significantly different at p= 0.05 level

4.3 Second Experiment: Induction of SE from Immature Zygotic Embryo

4.3.1 First Treatment: Induction of Somatic Embryos on Various Media with Different Concentrations of 2,4-D

The IZE were obtained from immature *C. papaya* 'Eksotika' fruits (95-100 days after flowering) (Figure 4.2). After two weeks of culturing, most explants produced callus in the different media with various 2,4-D concentrations, except in the control medium (i.e., medium without plant growth regulators), where roots and shoots developed. ½ MS medium supplemented with 2.5 mg L^{-1} 2,4-D produced the highest average callus weight after 2 weeks (Figure 4.3). Conversely, the WPM medium supplemented with 2.5 mg L^{-1} 2,4-D produced the lowest average callus weight (Figure 4.3).

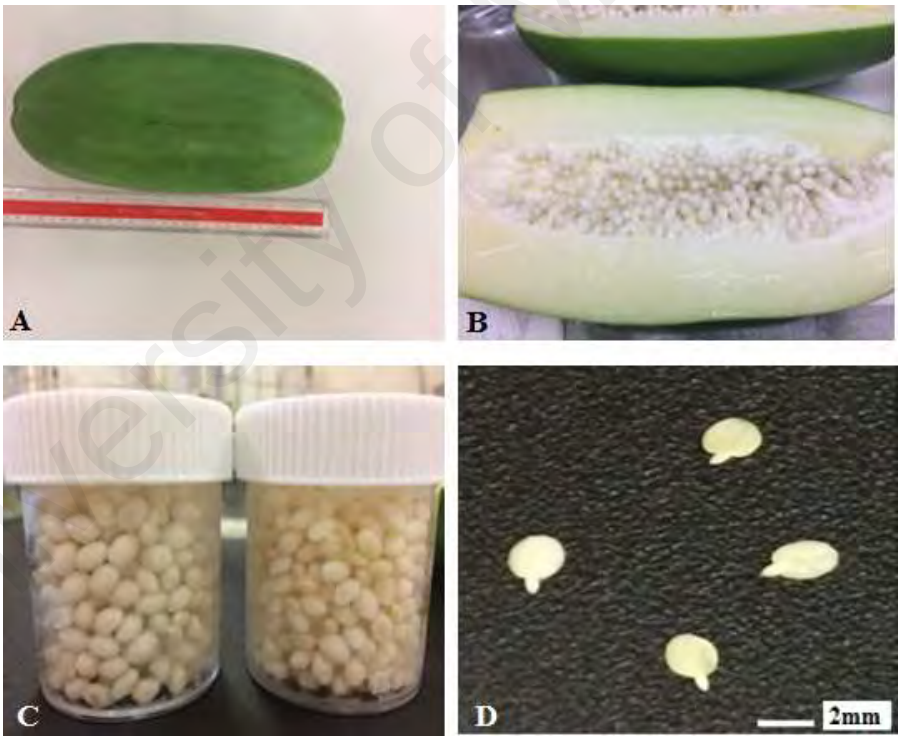

Figure 4.2: A & B- Immature *C. papaya* 'Eksotika' fruit, C- Immature *C. papaya* 'Eksotika' seeds, D- Immature *C. papaya* 'Eksotika' embryos.

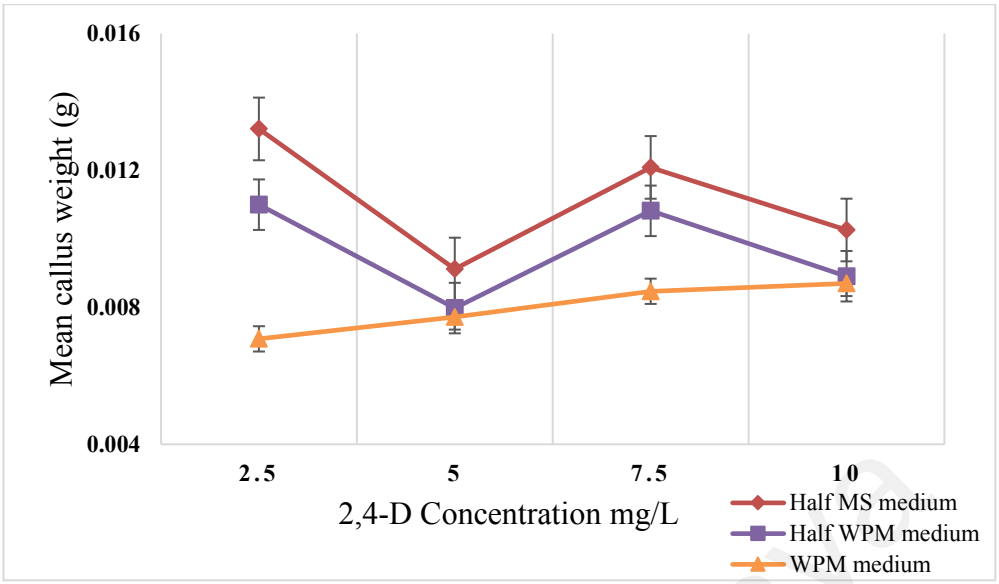

Figure 4.3: The marginal means of average callus weight (g) in different media with different concentrations of 2,4-D after 2 weeks of culture.

The highest average callus diameter (0.04 cm) was obtained in ½ MS medium supplemented with 2.5 mg L^{-1} 2,4-D while the lowest callus diameter (0.02 cm) was obtained in ½ WPM medium supplemented with 5 mg L^{-1} 2,4-D after 2 weeks (Figure 4.4 and Table 4.6).

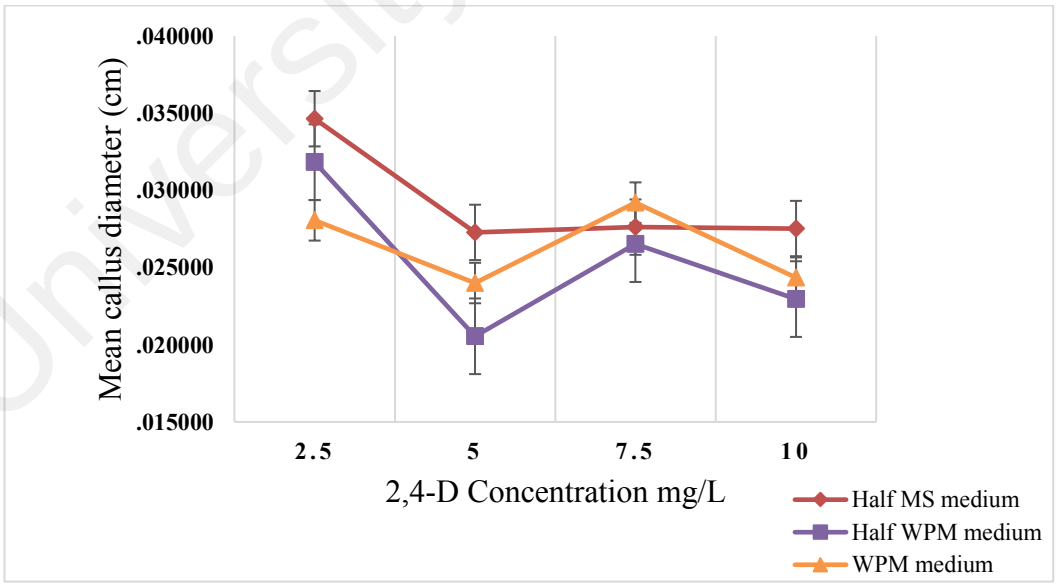

Figure 4.4: The marginal means of average callus diameter (cm) in different media with different concentrations of 2,4-D after 2 weeks of culture.

Table 4.6: Means comparison of the average callus diameter (cm) ± Standard Error of different media and 2,4-D concentrations after 2 weeks of culture.

2,4-D concentration (mg L^{-1})	½ MS	½ WPM	WPM
2.5	0.04 ± 0.00^{a}	0.03 ± 0.00^{ab}	0.03 ± 0.00^{bc}
5.0	0.03 ± 0.00^{bc}	0.02 ± 0.00^{d}	0.02 ± 0.00^{cd}
7.5	0.03 ± 0.00^{bc}	0.03 ± 0.00^{bcd}	0.03 ± 0.00^{abc}
10	0.03 ± 0.00^{bc}	0.02 ± 0.00^{cd}	0.02 ± 0.00^{cd}

Same letters in a column are not significantly different at p= 0.05 level

After 6 weeks of culturing, majority of the petri dishes showed friable yellow callus with somatic embryo formation. The highest average callus weight was obtained in ½ MS medium supplemented with 7.5 mg L^{-1} 2,4-D while the lowest average callus weight was obtained in ½ WPM medium supplemented with 5 mg L^{-1} 2,4-D (Figure 4.5).

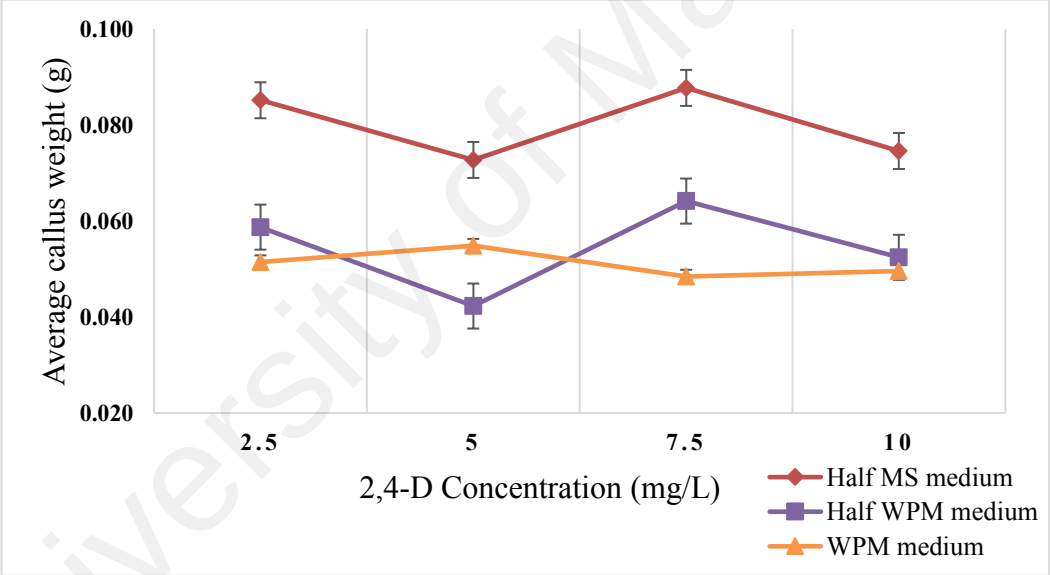

Figure 4.5: The marginal means of average callus weight (g) in different media with different 2,4-D concentrations after 6 weeks of culture.

However, the highest percentage of somatic embryos after 6 weeks (66.10%) was observed in WPM medium supplemented with 2.5 mg L^{-1} 2,4-D while the lowest percentage of somatic embryos (34.3%) was observed in ½ WPM medium supplemented with 2.5 mg L^{-1} 2,4-D (Table 4.7).

Table 4.7: Means comparison of the somatic embryos percentage (% ± Standard Error) of different media supplemented with different 2,4-D concentrations after 6 weeks of subculture.

2,4-D Concentration (mg L^{-1})	½ MS	½ WPM	WPM
2.5	52.00±2.94^{abc}	34.30±2.06^{c}	66.10±2.33^{a}
5	57.10±2.06^{abc}	34.60±2.63^{c}	52.40±1.25^{abc}
7.5	62.70±2.64^{ab}	34.90±1.92^{c}	39.90±1.58^{bc}
10	53.60±2.73^{abc}	48.00±1.81^{abc}	50.60±3.42^{abc}

Same letters are not significantly different at p=0.05 level

The highest average callus weight after 6 weeks (0.09 g) was observed in ½ MS medium supplemented with 2.5 and 7.5 mg L^{-1} 2,4-D whereas the lowest average callus weight (0.04 g) was observed in ½ WPM medium supplemented with 5 mg L^{-1} 2,4-D (Table 4.8).

Table 4.8: Means comparison of the average callus weight (g) ± Standard Error of different media supplemented with different 2,4-D concentrations after 6 weeks of culture.

2,4-D Concentration (mg L^{-1})	½ MS	½ WPM	WPM
2.5	0.085±0.028^{a}	0.059±0.019bcd	0.051±0.018^{cd}
5	0.073±0.026ab	0.042±0.014d	0.055±0.017^{cd}
7.5	0.088±0.018^{a}	0.064±0.019bc	0.048±0.008^{cd}
10	0.075±0.026ab	0.052±0.012cd	0.050±0.018^{cd}

Same letters are not significantly different at p=0.05 level

The highest average callus diameter after 6 weeks (0.79 cm) was observed in ½ MS medium supplemented with 7.5 mg L^{-1} 2,4-D while the lowest average callus diameter (0.56 cm) was observed in ½ WPM medium supplemented with 5 mg L^{-1} 2,4-D (Table 4.9).

Table 4.9: Means comparison of the callus diameter (cm) ± Standard Error, of different media supplemented with different 2,4-D concentrations after 6 weeks of culture.

2,4-D concentration (mg L^{-1})	½ MS	½ WPM	WPM
2.5	0.76±0.01^{ab}	0.66±0.01^{bcd}	0.60±0.01^{de}
5	0.73±0.02^{abc}	0.56±0.01^{e}	0.66±0.01^{cd}
7.5	0.79±0.01^{a}	0.68±0.01^{bcd}	0.60±0.01^{de}
10	0.75±0.01^{abc}	0.62±0.01^{de}	0.62±0.01^{de}

Same letters are not significantly different at p=0.05 level

4.3.2 Second Treatment: Induction of Somatic Embryos on ½ MS Medium Supplemented with Different Concentrations of Boric Acid

After 2 weeks of culturing, the highest average callus diameter (0.229 cm) was observed in medium supplemented with 18 mg L^{-1} of boric acid. The highest average callus weight after 2 weeks (0.009 g) was produced in the control medium (i.e., medium without boric acid) while the lowest average callus weight (0.005 g) was produced in the medium supplemented with 36 mg L^{-1} of boric acid (Table 4.10).

Table 4.10: Means comparison of the callus diameter (cm) ± Standard Error and callus weight (g) ± Standard Error after 2 weeks of culture.

Boric acid concentration (mg L^{-1})	Average callus diameter (cm)	Average callus weight (g)
Control	0.206±0.018ab	0.009±0.002a
6 mg L^{-1}	0.210±0.023ab	0.007±0.002ab
18 mg L^{-1}	0.229±0.029a	0.008±0.002a
36 mg L^{-1}	0.193±0.031ab	0.005±0.002b
54 mg L^{-1}	0.218±0.038ab	0.008±0.002a
72 mg L^{-1}	0.211±0.030ab	0.008±0.002a

After 6 weeks, the highest average callus diameter (0.70 cm) was observed in the medium supplemented with 18 mg L^{-1} of boric acid, while the lowest average callus diameter (0.62 cm) was observed in the medium supplemented with 36 mg L^{-1} boric acid (Table 4.11). The highest average callus weight (0.06 g) was observed in two media: the control media (i.e., media without addition of boric acid) and the medium supplemented with 18 mg L^{-1} of boric acid (Table 4.11). The highest percentage of somatic embryos (68.70%) was formed in the medium supplemented with 54 mg L^{-1} of boric acid while the lowest percentage of somatic embryos (44.40%) was obtained from the control medium (Table 4.11).

Table 4.11: Means comparison of the average callus diameter (cm) ± Standard Error, callus weight (g) ± Standard Error and percentage of somatic embryos (%) ± Standard Error after 6 weeks of culture.

Boric acid concentration (mg/L)	Average callus diameter (cm)	Average callus weight (g)	Percentage of the somatic embryo (%)
Control	0.674±0.050ab	0.063±0.011a	44.4±0.119b
6 mg/L	0.678±0.071ab	0.058±0.005ab	64.6±0.145a
18 mg/L	0.700±0.071a	0.063±0.007a	60.3±0.114ab
36 mg/L	0.618±0.072b	0.051±0.005b	60.2±0.208ab
54 mg/L	0.670±0.057ab	0.061±0.007a	68.7±0.170a
72 mg/L	0.658±0.051ab	0.057±0.009ab	45.9±0.188b

Same letters in column are not significantly different at p=0.05 level

4.4 Maturation Phase

Subsequently, the somatic embryos were transferred to a maturation medium where the globular developed into heart-shaped, torpedo and cotyledonary stages (Figure 4.7). The potential for somatic embryo development in response to the various maturation treatments was analyzed through growth analysis (i.e. embryogenic callus weight) and the number of different developmental somatic embryos stages (globular, heart, torpedo, cup-shape and cotyledonary). Growth analysis under different treatments was measured based on the increase of fresh weight.

Figure 4.6: Different somatic embryos stages developed from immature zygotic embryo of *C. papaya* 'Eksotika' a- Heart-shaped embryos, b, c, and d: Cup-shaped embryos and, e, f, g and h: Cotyledonary stage embryos (bar: 2 mm).

The embryogenic callus induced in IM1 medium was used to examine the effect of PG on the maturation of somatic embryos. Maturation medium supplemented with 5 mg L^{-1} PG produced the highest number of large embryos (32.80 ± 1.31), small embryos (41.20 ± 3.56), globular-shaped (26.20 ± 1.67) and cotyledonary stage (13.20 ± 1.21). Conversely, maturation medium supplemented with 1 mg L^{-1} PG produced the lowest (3.00 ± 0.70) callus formation at the base end of somatic embryos and the highest number of cup-shaped (11.60 ± 0.3) embryos. Meanwhile, the maturation medium supplemented with 10 mg L^{-1} of PG produced the highest root formation (12.20 ± 3.05) embryos. The highest number of callus formation at the base end of somatic embryos (6.20 ± 1.40) was observed in the control media (Table 4.12).

Table 4.12: Means comparison of different embryo size (≤ 3 mm and > 3 mm), developmental stages of somatic embryos (globular, heart, torpedo, cup-shape and cotyledon), callus and, root formation ± Standard Error in different concentrations of PG after 8 weeks of subculture.

PG (mg L^{-1})	> 3 mm large embryos	≤ 3 mm small embryos	Globular	Heart	Torpedo	Cup-shape	Cotyledon	Callus	Root
0	27.00±1.01a	25.20±1.68a	13.60±0.81b	10.00±0.67a	14.80±0.34a	10.60±1.82a	7.20±0.67b	6.20±1.40a	5.60±0.87a
1	30.00±0.34a	26.60±0.64a	10.80±1.69b	10.60±0.88a	19.80±0.60a	11.60±0.33a	8.00±1.30b	3.00±0.70a	9.60±1.62a
5	32.80±1.31a	41.20±3.56a	26.20±1.67a	10.20±1.32a	15.00±2.26a	9.20±0.28a	13.20±1.21a	3.80±1.11a	9.80±1.09a
10	31.80±1.38a	27.60±2.10a	15.80±0.78b	5.60±1.02b	16.60±1.44a	11.20±2.84a	10.00±0.50ab	4.20±0.72a	12.20±3.05a

Same letters in a column are not significantly different at p=0.05 level.

Table 4.13: Means comparison of different embryo size (≤ 3 mm and > 3 mm), developmental stages of somatic embryos (globular, heart, torpedo, cup-shape and cotyledon), callus and, root formation ± Standard Error in different concentrations of PEG (% w/v) after 8 weeks of subculture.

PEG %	> 3 mm large embryos	≤3 mm small embryos	Globular	Heart	Torpedo	Cup-shape	Cotyledon	Callus	Root
0	20.80±0.52c	27.20±1.78a	15.20±1.15a	5.20±1.56b	18.20±1.22a	7.60±0.91b	10.80±1.69a	2.60±0.71a	6.40±0.91b
2	30.40±0.28b	19.60±1.68a	11.00±0.30a	5.00±1.14b	18.80±0.87a	4.80±0.59b	13.60±1.96a	1.00±0.00bc	12.20±1.59ab
4	31.00±0.96b	17.80±2.03a	11.00±2.32a	7.00±1.36ab	13.60±1.61a	4.20±1.26b	16.40±1.38a	1.00±1.22bc	13.60±1.56ab
8	37.00±0.37ab	26.00±0.93a	9.60±1.22a	8.80±0.65ab	17.60±0.40a	13.80±1.98a	17.60±1.91a	1.20±0.77b	10.20±0.8ab
12	41.80±1.68a	21.80±0.70a	14.00±0.65a	10.00±0.39a	20.00±1.78a	14.80±1.39a	13.40±1.70a	0±0c	18.60±2.44a

Same letters in a column are not significantly different at p=0.05 level.

The embryogenic callus induced in IM2 medium was used to examine the effect of PEG on the maturation of somatic embryos. Maturation medium supplemented with 12% of PEG produced the highest number of large embryos (41.80 ± 1.68), heart-shaped (10.00 ± 0.39), cup-shaped (14.80 ± 1.39) and torpedo-shaped (20.00 ± 1.78). However, no callus formation was observed at 12% of PEG (Table 4.13). Meanwhile, the highest number of cotyledonary stage (17.60 ± 1.91) was produced in the maturation medium supplemented with 8% of PEG (Table 4.13). Conversely, the control media produced the highest number of small embryos (26.00 ± 0.93) and callus formation (2.60 ± 0.71) which were observed at the base end of the somatic embryos.

The embryogenic callus induced in IM3 medium (Figure 4.8) was used to examine the effect of PG on the maturation of somatic embryos. Maturation medium supplemented with 10 mg L^{-1} PG produced the greatest number of large embryos (36.50 ± 0.87), torpedo-shaped embryos (22.75 ± 0.69), cup-shaped embryos (13.00 ± 1.20) and root formation embryos (11.50 ± 1.31). In contrast, maturation medium supplemented with 10 mg L^{-1} PG produced the lowest callus formation (2.75 ± 0.76) at the base end of somatic embryos (Table 4.14) while the maturation medium supplemented with 5 mg L^{-1} of PG produced the greatest number of globular-shaped (16.25 ± 3.11) and cotyledonary stage (11.75 ± 0.91). The control media produced the greatest amount of callus formation (8.75 ± 1.55) observed at the base end of somatic embryos (Table 4.14).

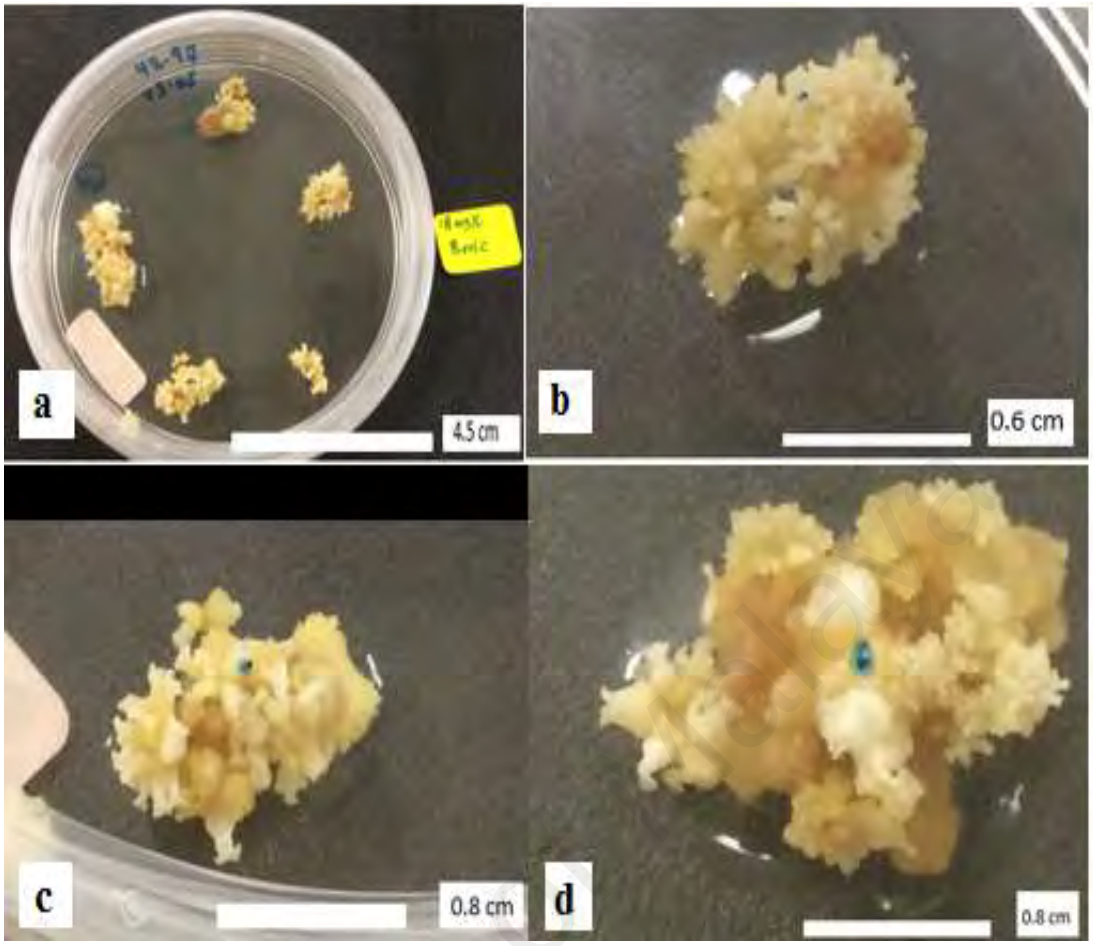

Figure 4.7: Somatic embryogenic callus culture in ½ MS media supplemented with 10 mg L^{-1} 2,4-D and 18 mg L^{-1} boric acid concentration, after 12 weeks of subculture.

Table 4.14: Means comparison of different embryo size (≤ 3 mm and > 3 mm), developmental stages of somatic embryos (globular, heart, torpedo, cup-shape and cotyledon), callus and, root formation ± Standard Error in different concentrations of PG after 8 weeks of subculture.

PG (mg L^{-1})	> 3 mm large embryos	≤ 3 mm small embryos	Globular	Heart	Torpedo	Cup-shape	Cotyledon	Callus	Root
0	23.75±1.52bc	43.25±2.79a	15.50±1.97a	12.25±1.29a	21.75±2.63a	9.00±1.56a	10.75±0.91a	8.75±1.55a	6.75±0.79ab
1	18.25±1.32c	31.75±1.7a	10.50±1.77a	10.75±1.48a	13.25±0.61a	7.50±1.41a	8.75±1.36a	4.75±1.77ab	6.00±1.29b
5	31.25±1.05ab	38.00±3.00a	16.25±3.11a	9.75±2.00a	20.50±1.46a	12.25±2.46a	11.75±1.15a	3.25±0.83b	5.50±1.01b
10	36.50±0.87a	24.25±2.44a	12.25±1.27a	6.75±0.91a	22.75±0.69a	13.00±1.20a	10.00±1.95a	2.75±0.76b	11.50±1.31a

Same letters in a column are not significantly different at p=0.05 level.

The growth analysis (weight (g)) of somatic embryos after maturation phase was recorded (Table 4.15). The somatic embryos grown in M1 medium supplemented with 5 mg L^{-1} PG resulted in the highest growth rate (3.98 ± 0.65 g) and those grown in M2 medium supplemented with 12% PEG resulted in the lowest growth rate (0.77 ± 0.13 g).

Table 4.15: Means comparison of the somatic embryos weight (g) ± Standard Error in different concentrations of PG and PEG after 6 weeks of culture on maturation media.

Media	Concentration	Weight (g)
M1	0 mg/L	3.49±0.54abc
M1	7.9 mg/L	2.82±0.29abcd
M1	39 mg/L	3.98±0.65a
M1	79 mg/L	3.47±0.60abc
M2	0 mg/L	3.80±0.25ab
M2	2%	2.79±0.19bcd
M2	4%	2.62±0.19bcd
M2	8%	1.46±0.22ef
M2	12%	0.77±0.13f
M3	0 mg/L	2.63±0.51bcd
M3	7.9 mg/L	3.37±0.20abc
M3	39 mg/L	3.13±0.39abc
M3	79 mg/L	2.75±0.24bcd
M4	0 mg/L	2.42±0.19cde

Same letters are not significantly different at p= 0.05 level

4.5 Somatic Embryos Development (Shoot Formation) Phase

The effect of AC during germination was studied. The somatic embryos that were grown in the best concentration of each maturation media (M1: 5 mg L^{-1} PG, M2: 12% PEG and M3: 10 mg L^{-1} PG) were cultured in two types of shoot formation media; SF1 medium: which consisted of MS medium without growth regulators and SF2 medium: which consisted of MS medium supplemented with 0.05 % AC. The percentage of germination was recorded after 6 weeks of culturing. The highest percentage of germination (62 ± 1.19%) was recorded for mature somatic embryos grown in medium supplemented with 12% PEG that were transferred to SF1 medium. The lowest percentage of germination (32.8 ± 1.06%) was recorded for mature somatic embryos grown in control media and then transferred to SF1 medium (Table 4.16). The SF1

medium showed higher or similar percentages of germination compared to SF2 medium for all explants.

Table 4.16: Means comparison of the germination (%) ± Standard Error of somatic embryos after 6 weeks of culture in germination media.

Media	Germination %
M1+ SF1	57.6±1.06ab
M1+ SF2	51±1.19ab
M2+ SF1	62±1.19a
M2+ SF2	55±1.19ab
M3+ SF1	40±1.06bc
M3+ SF2	41.6±1.06bc
M4+ SF1	58±1.19ab
M4+ SF2	49.6±1.06abc
MS+ SF1	32.8±1.06c

Same letters are not significantly different at p= 0.05 level

4.6 Elongation Phase

Elongation medium (EL) was used for elongation of somatic embryos. The percentage of somatic embryos germination was recorded 6 weeks after being cultured in the EL medium. The highest germination percentage (77.18 ± 9.49%) after elongation was observed for somatic embryos grown in M4 medium and transferred to SF2 medium (Table 4.17) whereas the lowest germination percentage (44 ± 3.10%) was observed in M1 medium and transferred to SF2 medium (Table 4.17).

Table 4.17: Means comparison of the germination (%) ± Standard Error of somatic embryos after 6 weeks culture in elongation media.

Media	Germination %
M1+ SF1+ E1	59.80±7.84abc
M1+ SF2+ E1	44.00±3.10c
M2+ SF1+ E1	60.24±9.77abc
M2+ SF2+ E1	56.16±12.06abc
M3+ SF2+ E1	64.33±5.28abc
M3+ SF1+ E1	51.49±6.51bc
M4+ SF1+ E1	71.89±2.75ab
M4 +SF2+ E1	77.18±9.49a

Same letters are not significantly different at p= 0.05 level

4.7 Root Phase

Five types of media were used in rooting experiments to study the effect of riboflavin, PG and IBA on root formation. The explants treated in R1 and R3 media showed no callus

formation at the base end of stem explants (Figures 4.9 & 4.10). The highest number of roots (3.75 ± 0.52) was observed in R3 medium (Figure 4.5). the longest root length (10.2 ± 1.37 cm), shoot length (8.00 ± 1.26 cm) and highest number of leaves (4.50 ± 0.68) were observed in R4 medium (Figure 4.11). The highest germination percentage (100 ± 7.31 %) was observed in R5 medium (Table 4.18 & Figure 4.12).

Table 4.18: Means comparison of the germination (%) ± Standard Error, root number ± Standard Error, root length (cm) ± Standard Error, shoot length (cm) ± Standard Error, leaves number ± Standard Error, and callus percentage (%) ± Standard Error of somatic embryos after 6 weeks culture in different root media.

Media Types	Germination percentage (%)	Root number	Root length (cm)	Shoot length (cm)	Leaves number	Callus percentage (%)
R1	52.75±7.31b	2.25±0.52a	6.25±1.37ab	7.00±1.26a	4.25±0.68a	0.00±11.85b
R2	93.75±7.31a	2.50±0.52a	3.50±1.37b	4.75±1.26a	3.00±0.68ab	14.50±11.85b
R3	88.75±7.31a	3.75±0.52a	4.50±1.37b	7.00±1.26a	4.00±0.68a	0.00±11.85b
R4	83.5±7.31a	3.00±0.52a	10.2±1.37a	8.00±1.26a	4.50±0.68a	29.00±11.85b
R5	100±7.31a	2.25±0.52a	5.00±1.37b	7.75±1.26a	3.75±0.68a	29.00±11.85b

Same letters in a column are not significantly different at p=0.05 level

Figure 4.8: *C. papaya* 'Eksotika' explants rooted in R1 medium (A) Showing 2 main roots with root hair, (B) Explant showing more than 10 root hairs.

Figure 4.9: *C. papaya* **'**Eksotika' explants rooted in R2 medium, explant showing callus at the base end of stem (*Black arrow*).

Figure 4.10: *C. papaya* 'Eksotika' explants rooted in R3 medium (A& B) Callus formation at the base end of stem (black & white arrows).

Figure 4.11: *C. papaya* **'**Eksotika' explants rooted in R4 medium (A) Explant showing many root hairs with thick stem (B) Explant showing the stem evaginated to give 2 stems.

Figure 4.12: *C. papaya* 'Eksotika' explants rooted in R5 medium (A) Explant showing the stem evaginated to give 2 stems (B) Explant showing more than 30 root hairs.

4.8 Acclimatization phase

The explants grown in different rooting media were transferred for acclimatization process. One type of media was used for acclimatization i.e. peatmos soil (Figures 14.14, 14.15, 4.16 &4.17). The percentage of germination was recorded after one month. The highest germination percentage (100 ± 24.73%) was observed on explants grown in R4 medium (Table 4.8 & Figure 4.17).

Table 4.19: Means comparison of the germination (%) ± Standard Error of explants after 6 weeks of grown in peatmos soil.

Explant source	Parentage of germination
R1 medium	41.67±17.49ab
R2 medium	66.67±20.19ab
R3 medium	85.71±13.22a
R4 medium	100±24.73a
R5 medium	16.67±17.49b

Same letters in a column are not significantly different at p=0.05 level.

Figure 4.13: (A & B) *C. papaya* 'Eksotika' explants regenerated from R1 medium after one month.

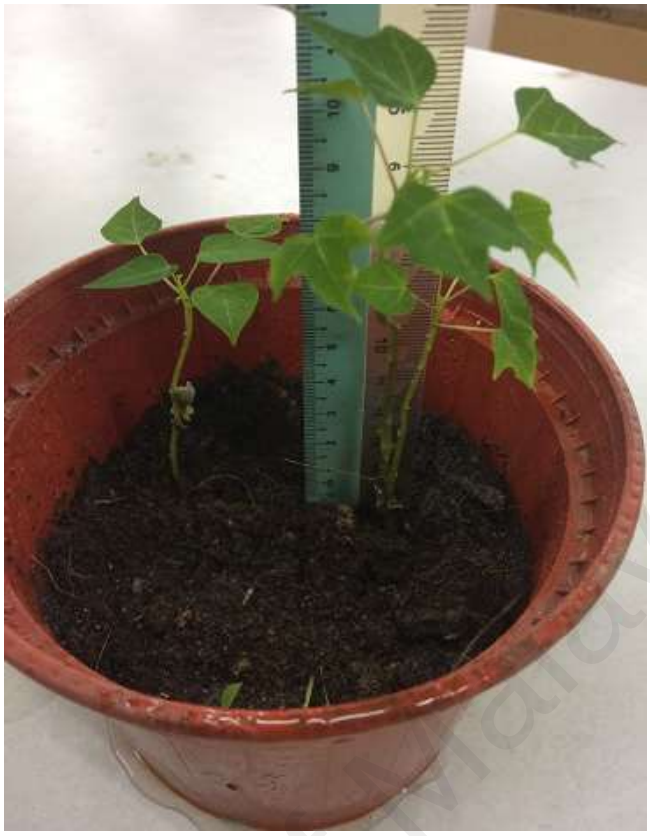

Figure 4.14: *C. papaya* 'Eksotika' explants regenerated from R2 medium after one month.

Figure 4.15: *C. papaya* 'Eksotika' explants regenerated from R3 medium after one month.

Figure 4.16: *C. papaya* 'Eksotika' explants regenerated from R4 medium after one month.

4.9 Artificial Seeds Production from Somatic Embryos of *Carica papaya* L. cv. Eksotika

4.9.1 Standardized Artificial Seed Conditions

For the artificial seeds study, the optimum concentration for the formation of firm, clear and rounded beads for each encapsulation matrix (water, MS medium without calcium component, MS medium, ½ MS medium without calcium component, and ½ MS medium) was 4.5% SA that was immersed in 50 mM $CaCl_2$ for 10 min (Appendix A). Thus, a subsequent experiment was carried out by encapsulating somatic embryos with the optimum concentration of encapsulation matrix.

4.9.2 Artificial Seeds Experiment

The artificial seeds produced were cultured in two different media (SY1 medium: which consisted of MS media without growth regulators and SY2 medium: which consisted of MS media with 0.2 mg L^{-1} NAA+ 0.2 mg L^{-1} BAP). The germination percentage and morphological characteristics were observed after 8 weeks of culturing (Figure 4.18). The somatic embryos encapsulated in MS medium without a calcium component produced the highest percentage (39%) of shoot formation, the highest percentage (56%) of root formation and the lowest percentage (11%) of callus formation at the base end of somatic embryos. The highest percentage of normal leaves (100%) was obtained when somatic embryos encapsulated in MS medium were cultured in SY2 medium (Figure 4.19 & 4.20).

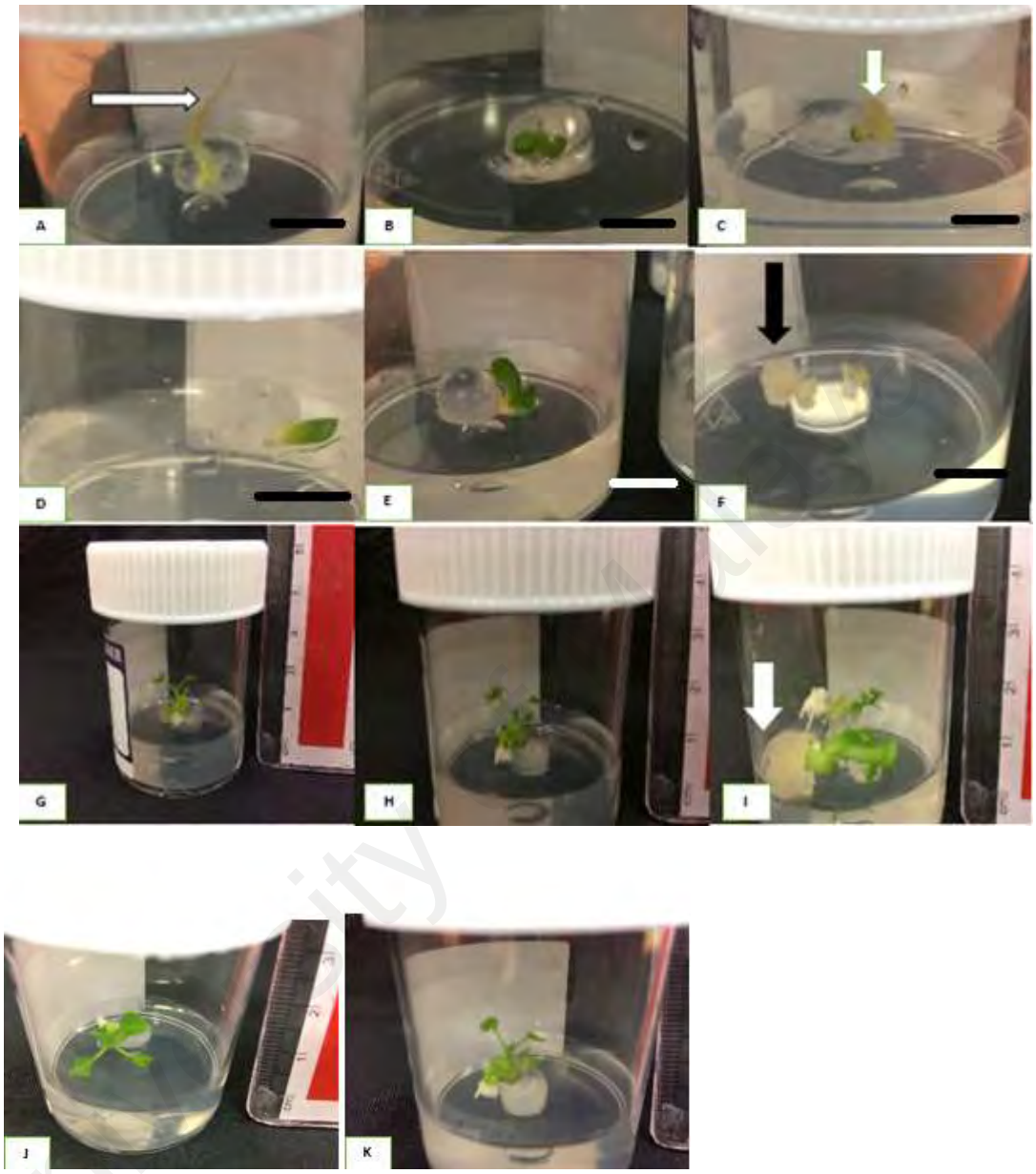

Figure 4.17: Somatic embryos of *C. papaya* 'Eksotika' encapsulated with different encapsulation matrix and grown in different germination media. A: Somatic embryos capsulated with water and germinated in SY1 medium, B: Somatic embryos capsulated with water, C &D: Somatic embryos capsulated with MS without Ca component and germinated in SY1medium and germinated in SY2 medium, E: Somatic embryos capsulated with MS-Ca and germinated in SY2 medium, F: Somatic embryos capsulated with MS and germinated in SY2 medium G, H and I: Somatic embryos capsulated with MS and germinated in SY2 medium, J & K: Somatic embryos capsulated with MS-Ca and germinated in SY2 medium. (Bar = 1 cm)

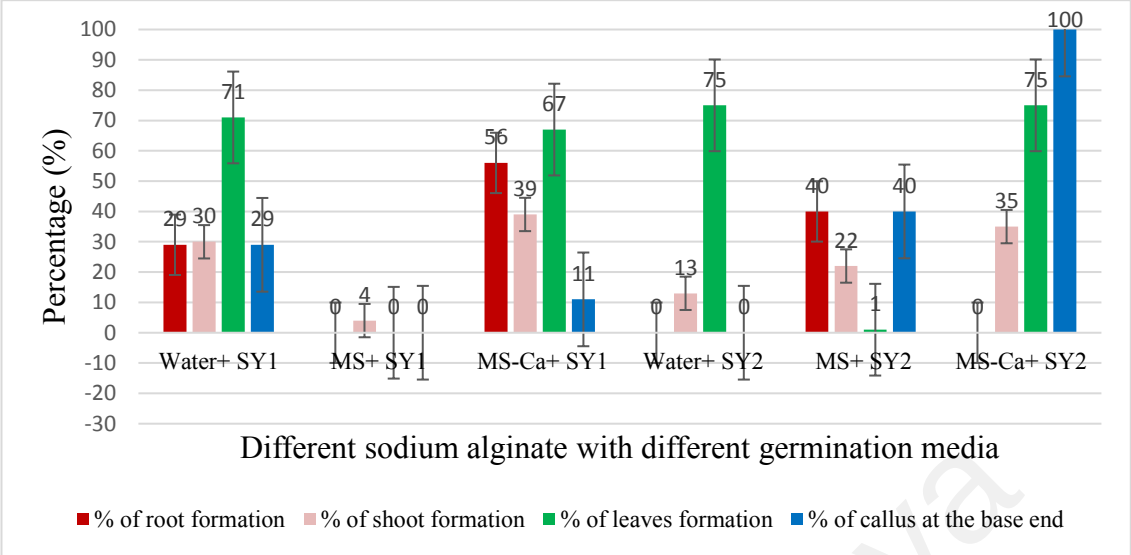

Figure 4.18: The effect of different encapsulation matrix with different germination media on the percentage of root formation, shoot formation, leaves formation and callus formation at the base end of somatic embryos. SY1: MS medium without growth regulator and SY2: MS media supplemented with 0.2 mg L^{-1} NAA+ 0.2 mg L^{-1} BAP.

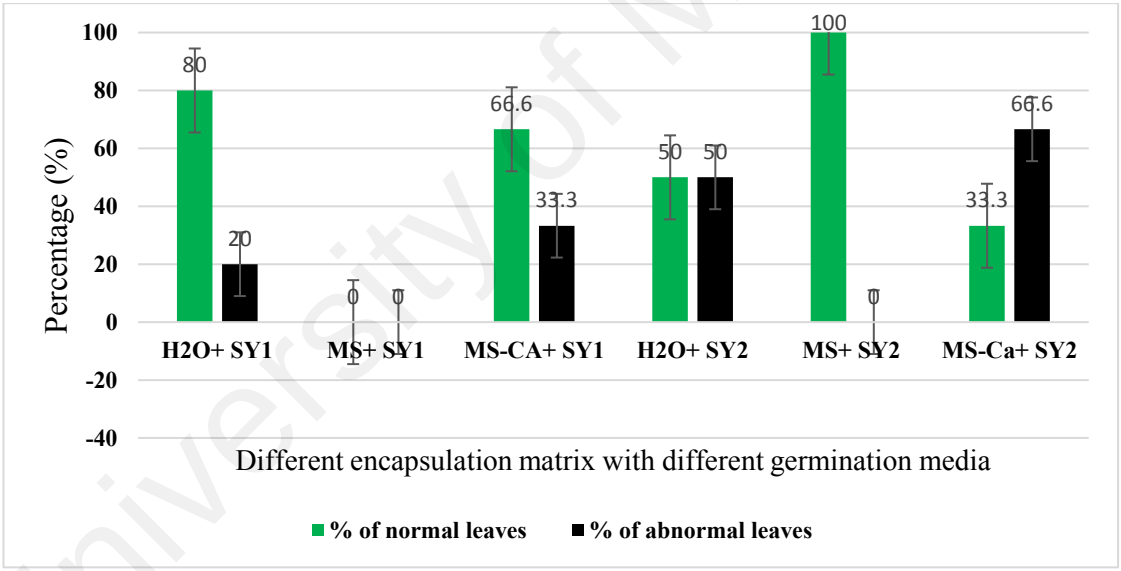

Figure 4.19: The effect of encapsulation matrix with different germination media on the percentage of normal leaves and abnormal leaves of somatic embryos. SY1: MS medium without growth regulator and SY2: MS medium supplemented with 0.2 mg L^{-1} NAA+ 0.2 mg L^{-1} BAP.

4.10 Histological Analysis of The Developmental Stages of Somatic Embryogenesis Induced from Immature Zygotic Embryos of *Carica papaya* L. cv. Eksotika

The anatomy during different developmental stages such as induction, maturation and development of somatic embryos were studied via histological sections. Histological analysis using acetocarmine and Evan blue revealed originated of somatic embryos after 25 days (Figure 4.21). A small number of somatic embryos were identified on the exterior surface of the embryonic calli in the medium. Observations were made using the naked eye as well as through a dissecting microscope. Histological analysis using safranin and fast green stain revealed the groups of cells called pro-embryonal complex, which categorized the origin of somatic embryo. The embryogenic calli consisted of small and compact cells with small vacuoles and condensed nuclei (Figures 4.22 A & B). These somatic embryos were loosely attached to the surface of the explant and consisted of bipolar axes (Figure 4.22C). The internal explant cells consisted of parenchymal cells. The parenchyma cells were large in size, more vacuolated, varied in cell shape and were bigger than marginal cells with lengthy and widened intercellular spaces (Figure 4.22A). Also, a meristematic center that formed from concentrated cell divisions was observed. The cells that formed the meristematic center were characterized by small size, uniform shape, thick cell wall, central nucleus, dense cytoplasm and the absence of intracellular space (Figure 6.4.23).

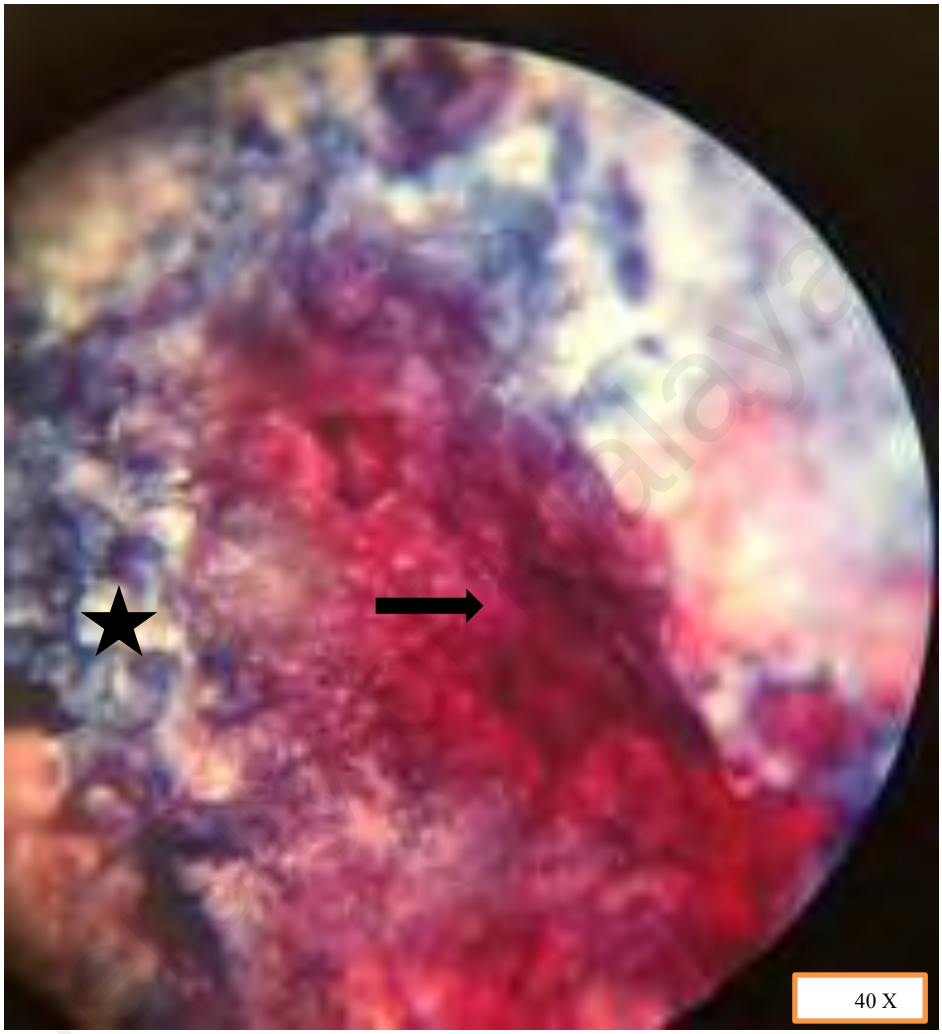

Figure 4.20: Embryogenic callus stained in red color using acetocarmine stain (arrow), and non-embryonic callus stained in blue color using Evan blue stain (asterisk) (40 X).

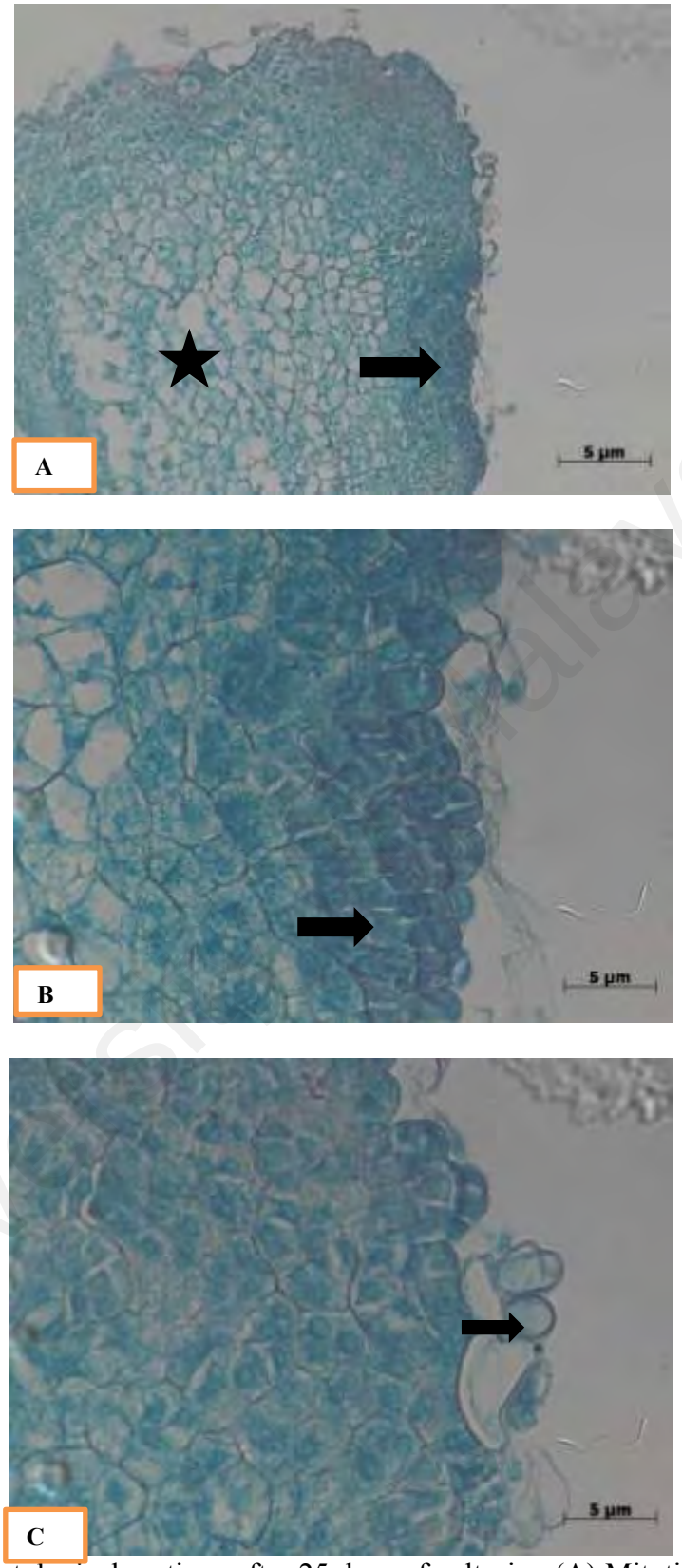

Figure 4.21: Histological sections after 25 days of culturing (A) Mitotic divisions were induced on the surface of the cotyledon. Embryonic cells (arrow) appeared at the surface of the callus and consisted of small and dense cells with small vacuoles and loose intracellular space. The inner cells consisted of parenchyma cells that were large, irregular cells with large vacuoles (asterisk). (B) Somatic embryos were loosely attached to the surface of the explant and consisted of clear bipolar axes (arrow). (C) Magnified marginal part of callus demonstrates pro-embryonic cells in early stages (arrow).

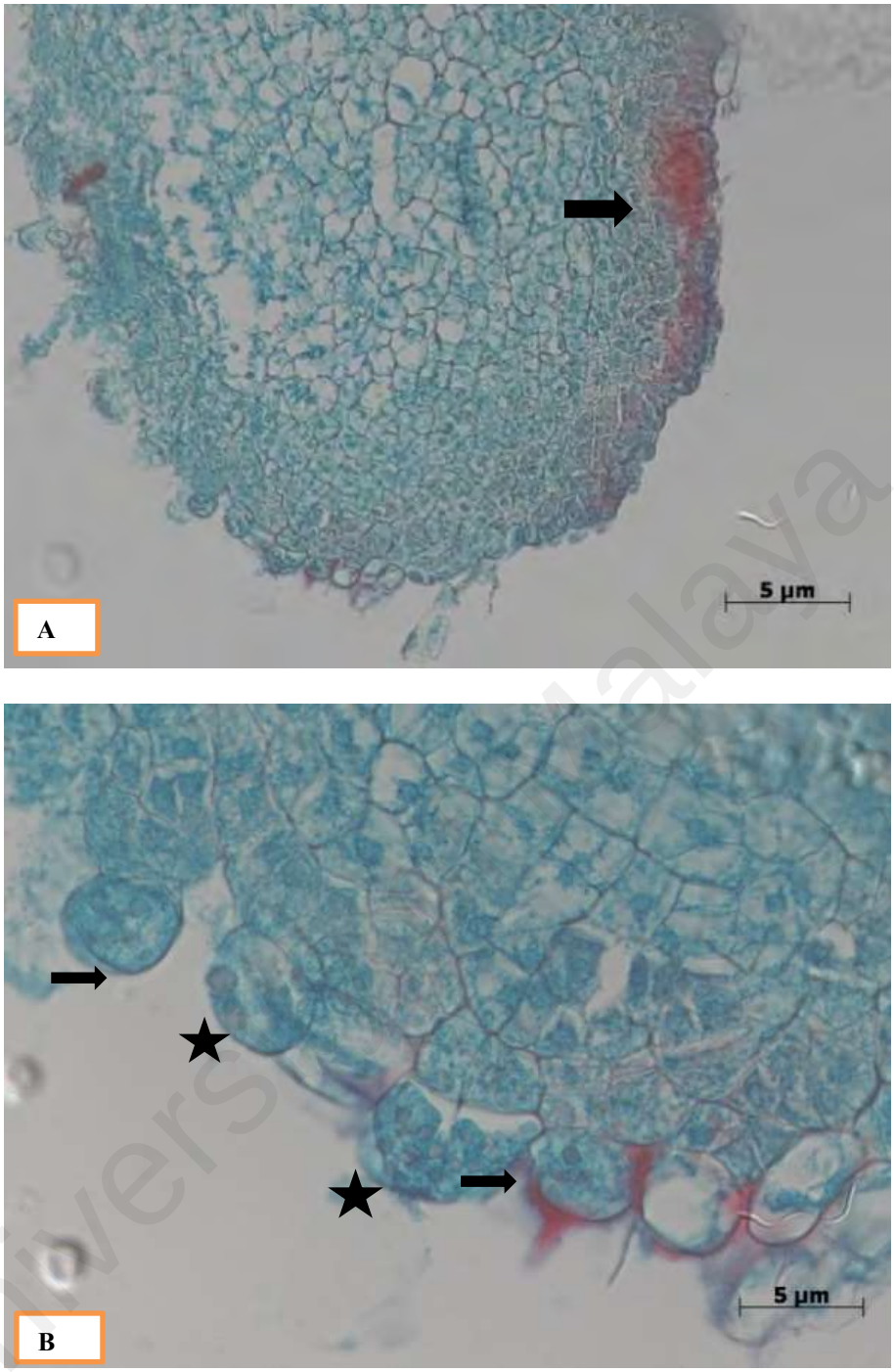

Figure 4.22: Histological sections after 25 days of culturing. (A) The epidermis of the callus was irregular in shape and internal cells proliferated and developed. (B) Some pro-embryonic cells began organizing and developing and could be distinguished from other cells based on the thickness of cell walls (*arrow*). Starch granules (asterisk) were detected in callus cells.

The somatic embryo was initiated by the periclinal division (i.e. the division of cells parallel to an adjacent layer of cells) of one epidermal cell of the explant. This formed two cells; the internal basal cell and the external apical cell. Then, the internal basal cell divided anticlinally (i.e., perpendicular to the adjacent layer of cells), forming two cells that subsequently divided periclinally, where the apical cell was sustained as primary cell (Figure 4.24). After that, the apical cell began dividing and forming a mass of undifferentiated cells in a globular shape (Figure 4.25). The protoderm on globular shape was observed in the late stages of globular shape formation without the observation of any differentiation in the inner cells (Figure 4.26).

Subsequently, evagination appeared in the globular which represented the heart-shaped stage (Figure 4.27). During the heart-shaped stage, the root pole of the embryo could be distinguished whereby it consisted of a mass of dense cytoplasmic cells without observation of any vascular connection that linked the mother explant to the somatic embryos (Figure 4.27). The procambial appeared in the late stages of heart-shaped embryos. Then, the evagination grew continuously and formed a torpedo-shape, representing the cotyledonary stage or mature somatic embryo (Figure 4.28). Bilateral symmetry was observed in most mature somatic embryos whereby the apical shoot meristems and the apical root meristem was clearly observed and showed intense staining (Figure 4.28a). In the longitudinal section, a concave shape of the shoot meristem could be observed in the mature somatic embryo and consisted of more than one layer (Figure 4.28b).

Some abnormalities in the developing somatic embryos were observed, such as differing amounts of cotyledons and the presence of fused somatic embryos (Figure 4.29). The formation of somatic callus, especially in the root pole, during the cotyledonary stage of somatic embryos was also observed.

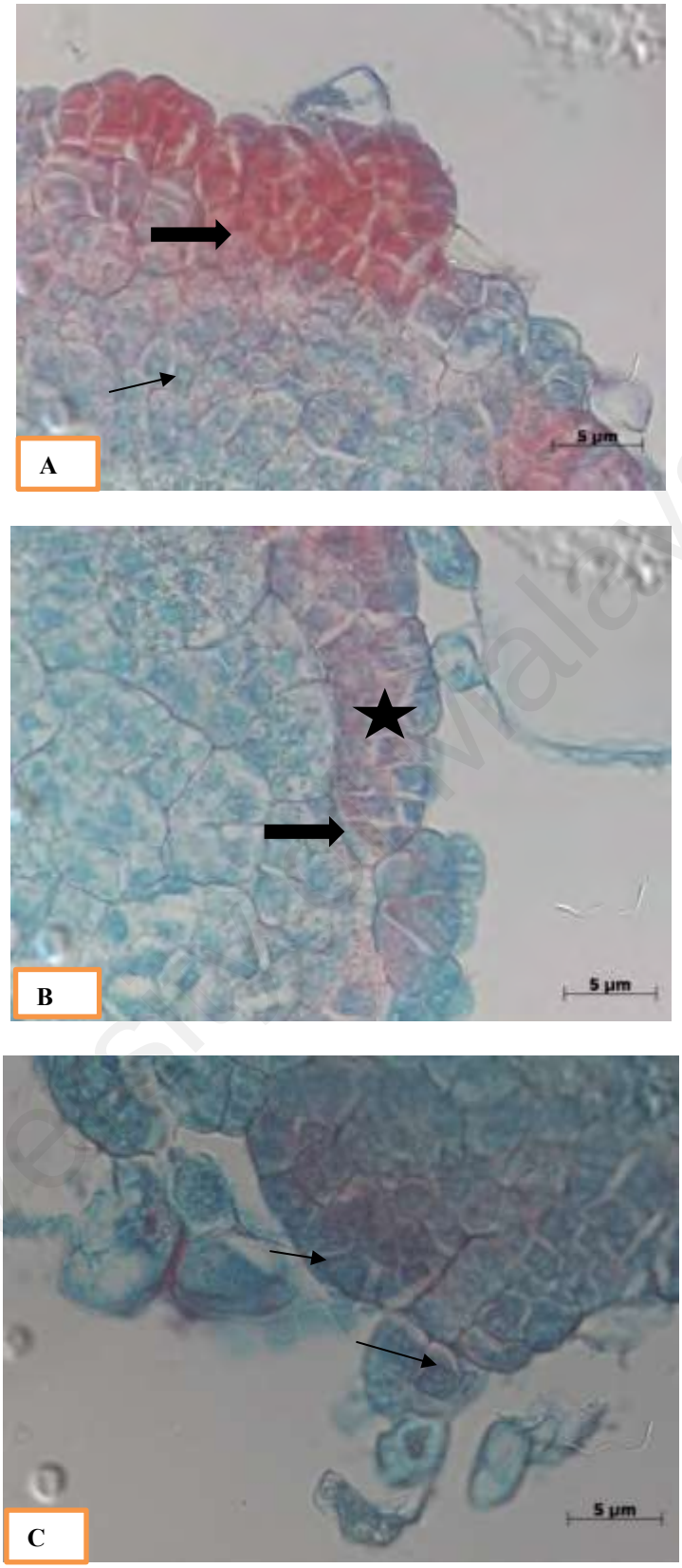

Figure 4.23: Histological sections, after 25 days of culturing, illustrating mitotic divisions that were induced on the surface of the callus. (A) The cells divided continuously and meristematic zones were formed (wide black arrow). Cluster of epidermal and subepidermal cells (asterisk). (B) Periclinal division (narrow arrow) and anticlinal division of the basal cell (wide arrow). (C) The early stage of globular embryos.

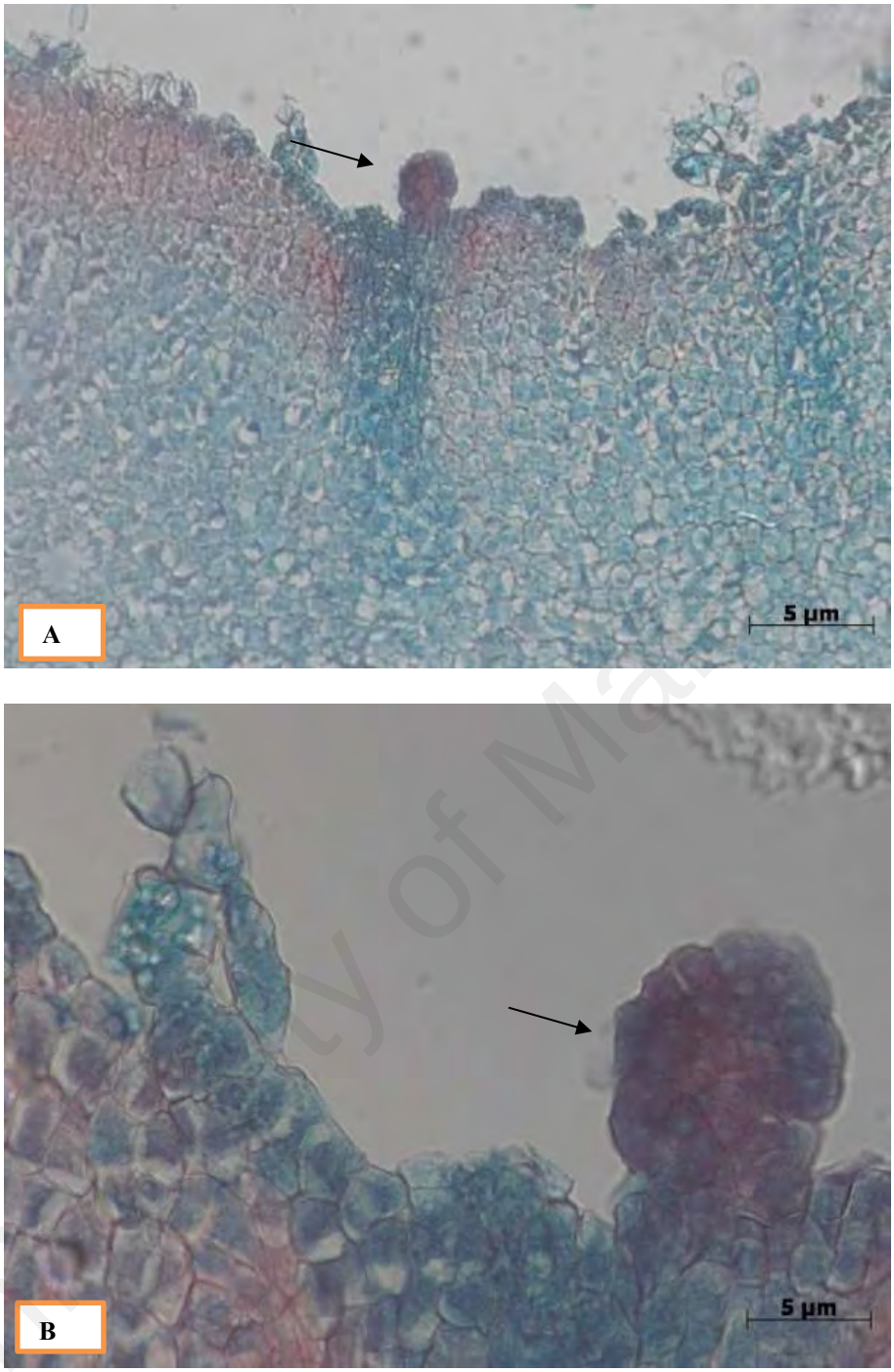

Figure 4.24: Histological longitudinal section after 28 days of culturing. (A) Illustration of the globular embryo during the early stage. (B) Highly magnified illustration of the globular embryo during the early stage.

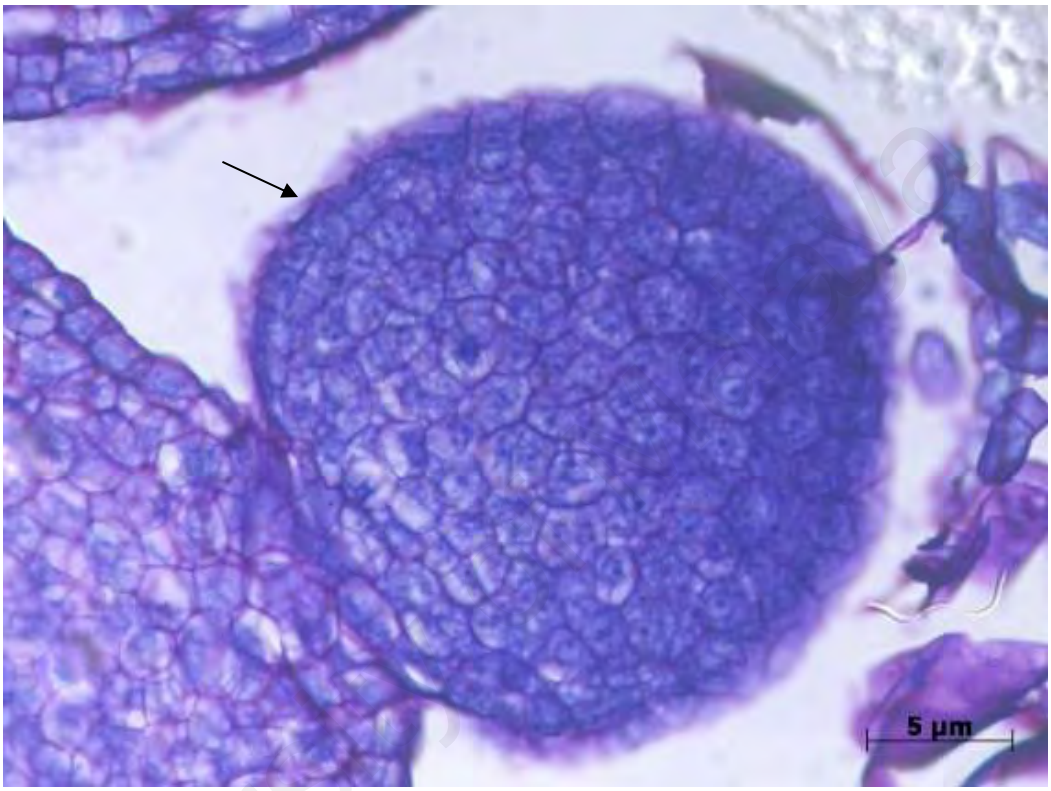

Figure 4.25: Histological section after 30 days of culturing. (A) The globular embryo at late stage of development which consisted of small, highly cytoplasmic cells. The protoderm was identified (arrow) without observation of any organization in inner cell during globular stage.

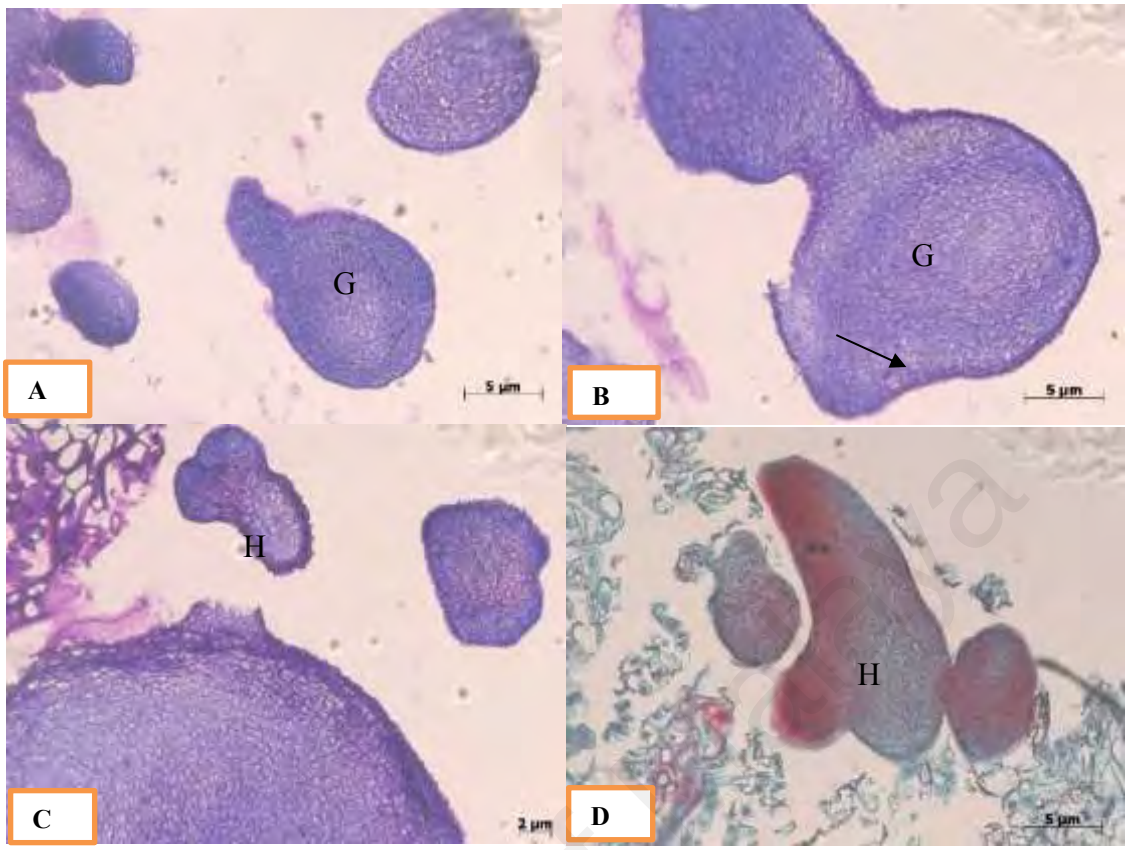

Figure 4.26: Histological sections after 52 or 56 days of culturing. (A & B) The globular embryo (G) started evagination (black arrow) to start forming a heart shape (H). (C & D) Heart shape embryos without observation of any vascular tissue in the shoot and root part.

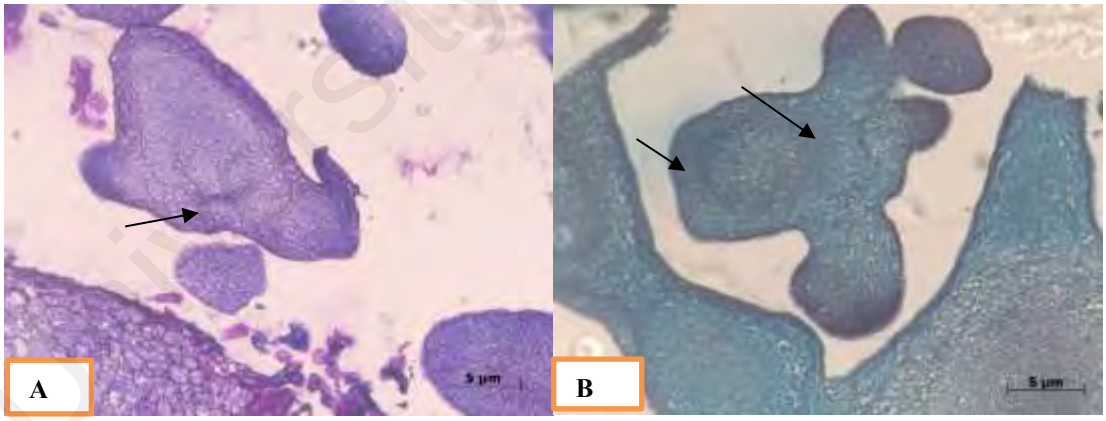

Figure 4.27: Histological sections after 52 days of culturing. (A) The late heart shape or early torpedo shape whereby evagination is larger and procambial tissue (arrow) started appearing. (B) The mature cotyledon embryo with root and shoot poles as well as clearly distinguished procambial in the shoot and root pole (arrow).

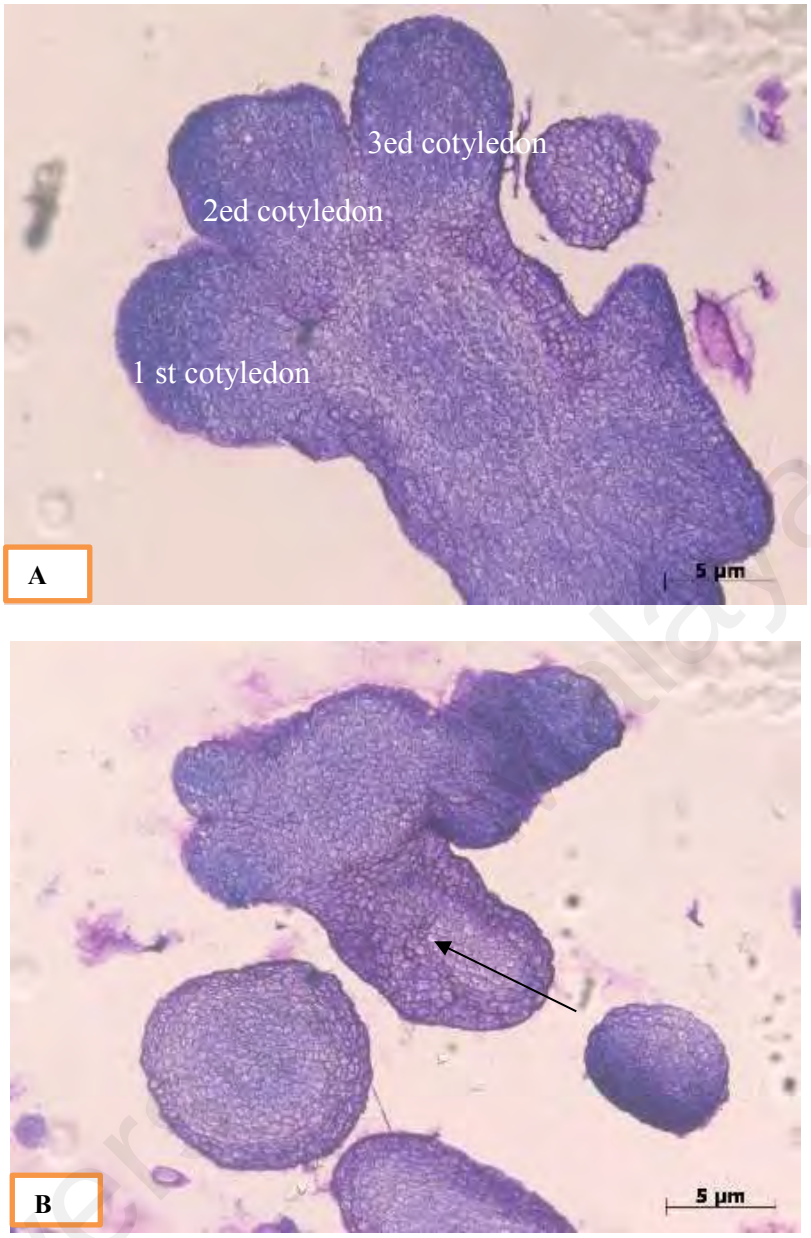

Figure 4.28: Histological sections of a somatic embryo after 52 days of culturing illustrating some abnormalities that were found during SE. (A) Altered number of cotyledons. (B) Fused somatic embryos (black arrow).

The formation of this callus prevented the formation of functional roots and thus resulted in an incomplete plant. Histological examination revealed the formation of somatic callus at the root base end of somatic embryos (Figure 4.30). Most somatic callus were concentrated near the root cap of cotyledonary embryos. The somatic embryos started developing from these calli and were observed after the 52nd day of culturing (Figures 4.31 & 4.32). Many new embryos with different developmental stages were

observed. New embryos originated from the marginal region of primary somatic embryos, especially during the cotyledonary stage.

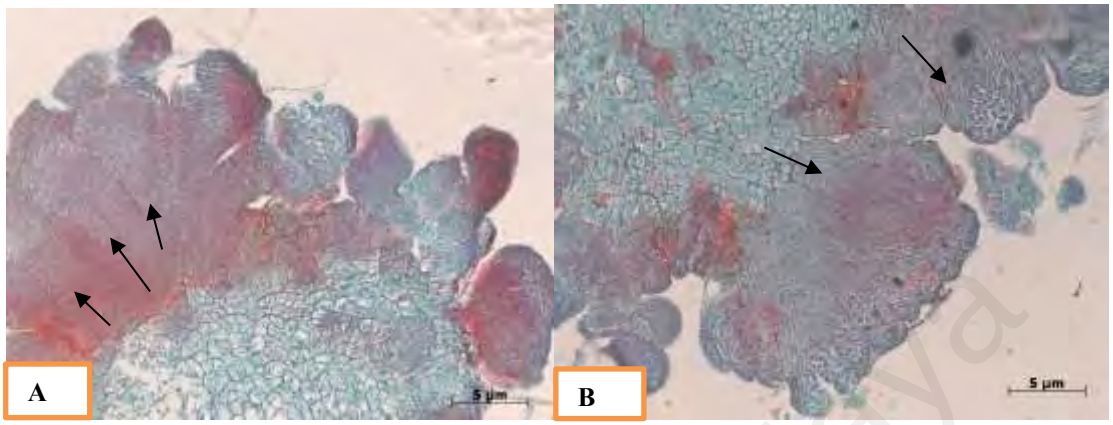

Figure 4.29: Histological sections after 56 days of culturing illustrating the embedded cellular protrusions of the deformed embryo that varied in width, length and shape especially in the root region (black arrows).

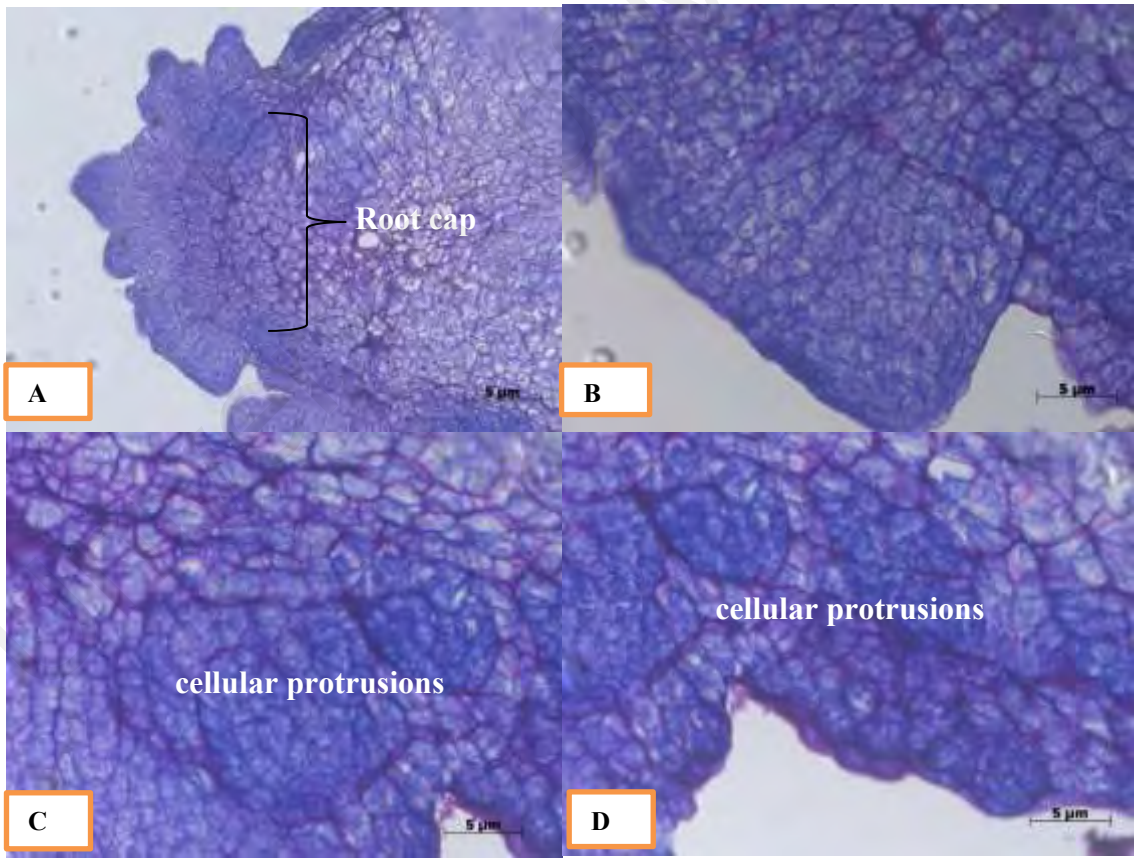

Figure 4.30: Histological sections after 70 days of culturing illustrating the embedded cellular protrusions of the malformed embryo that varied in width, length and shape and covered the root cap (RC)in the root region.

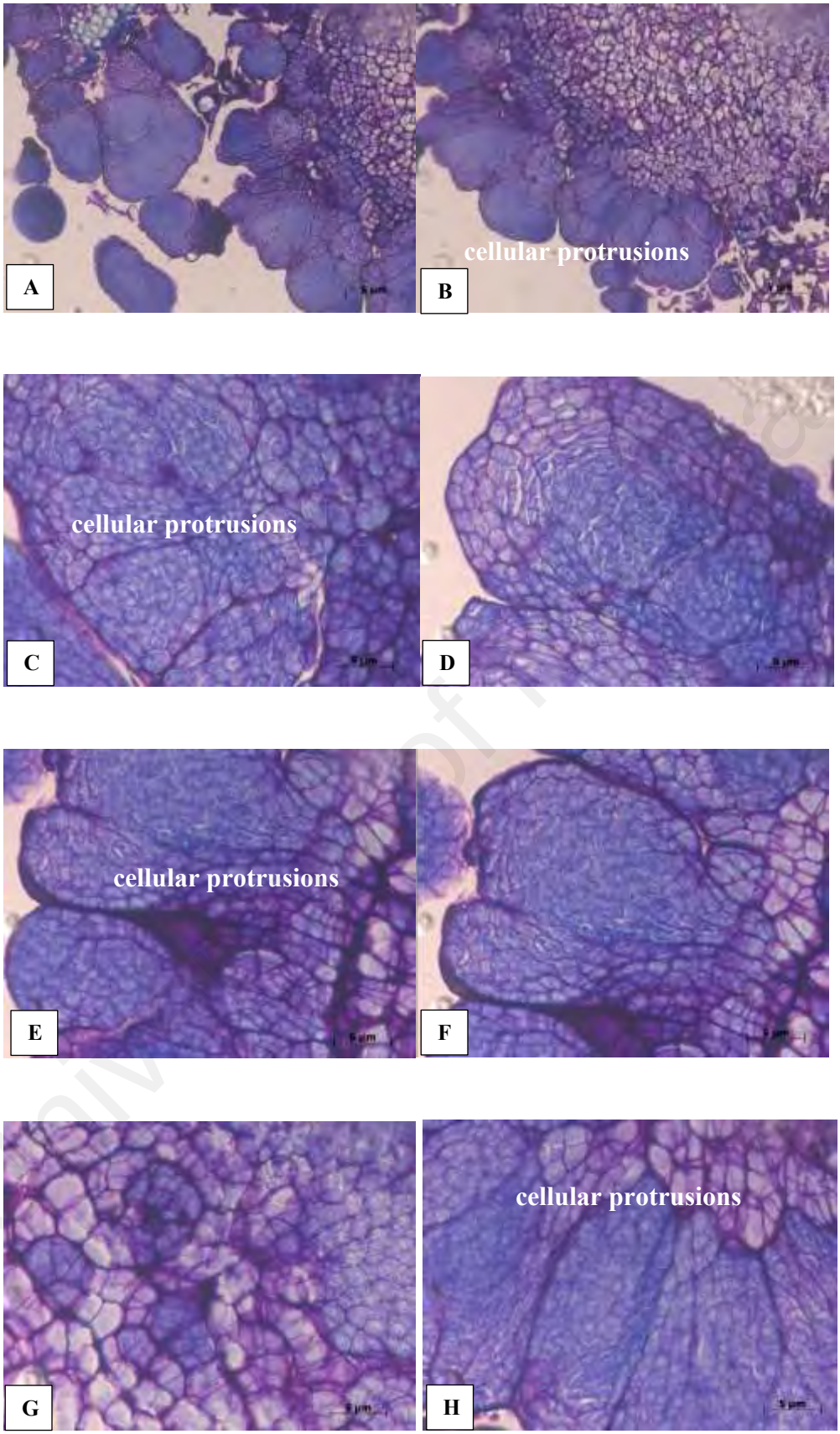

Figure 4.31: Histological sections after 70 days of culturing illustrating the embedded cellular protrusions of the malformed embryo that varied in width, length and shape.

4.11 The Expression of *CpLAX2*, *CpLAX3* and *CpPIN4* Genes During Maturation and Germination Phases of Somatic Embryogenesis in *Carica papaya* cv. Eksotika

This study investigated the relative expression of the *CpLAX2, CpLAX3* and *CpPIN4* genes compared to the *EIF* gene (housekeeping gene) during the different developmental stages of SE induced from IZE. The specificity of PCR results was checked through melting-curve analyses, and only primer sets producing a single sequence-specific peak were conserved. Quantitative RT-PCR was employed to assess the transcript levels of *CpLAX2*, *CpLAX3* and *CpPIN4* genes during the different periods of somatic embryo development. The relative transcript levels were estimated comparatively to the *CpEIF* gene. Transcripts of all selected genes were detected in all analyzed samples (Figure 4.33).

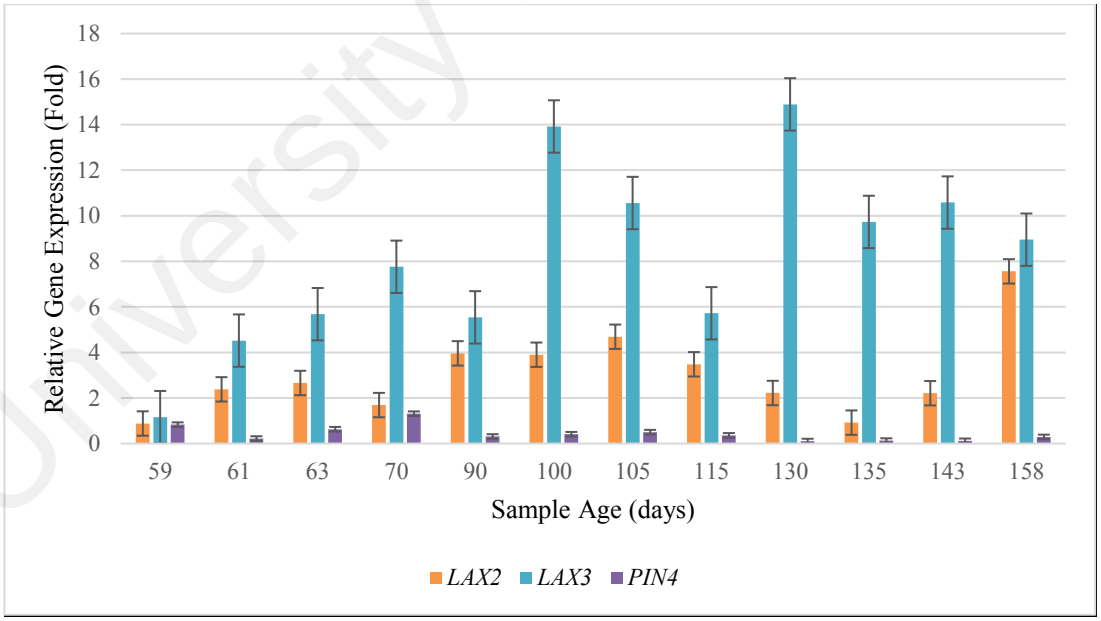

Figure 4.32: Relative expression of *CpLAX2*, *CpLAX3* and *CpPIN4* genes at different developmental stages of SE.

Gene names, accession numbers, the primer sequences and amplicon characteristics including Tm, length, and amplification efficiency are listed in table 1. Primer pairs performed well in the melting curve obtained after 40 cycles of amplification. Only those

showing single product and no product in the no-template control (NTC) were selected for this experiment. The specificity of the amplifications was also confirmed through agarose gel electrophoresis, which revealed that the majority of primer pairs selected by melting curve analysis amplified a specific PCR product with the expected size (Figures 4.34 & 4.35). The CT values of reference genes ranged from 22.64 to 31.62 in all tested samples. The average CT value of all reference genes within the datasets was approximately 25.85 cycles. The individual reference gene had different expression ranges across all studied sample sets.

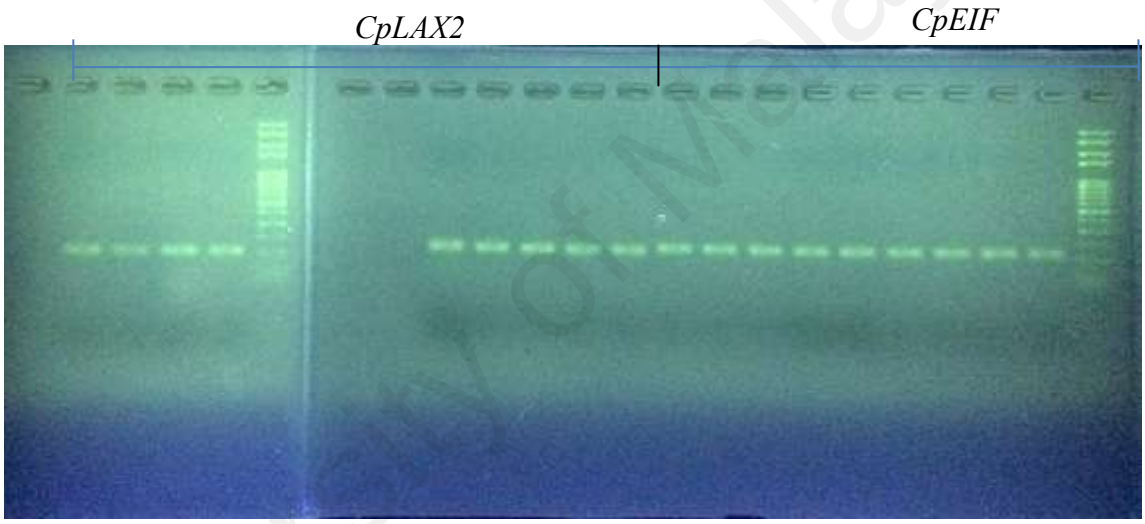

Figure 4.33: Showing the quality of primers for *EIF* and *LAX2* genes with different temperature (50.2, 52.0, 53.4, 54.9, 56.5, 58.1, 59.5, 60.7, and 62.0 ^{o}C).

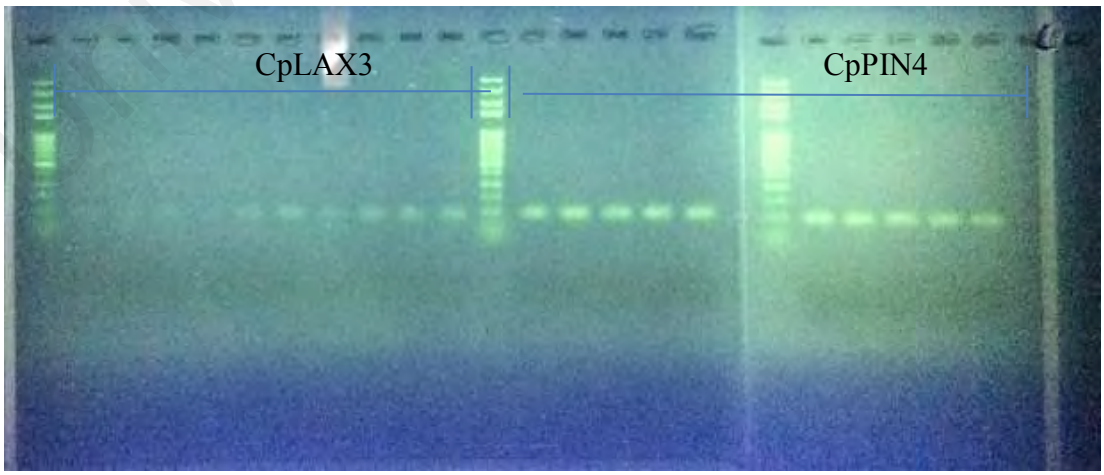

Figure 4.34: Showing the quality of primers for *LAX3* and *PIN4* genes with different temperature (50.2, 50.9, 52.0, 53.4, 54.9, 56.5, 58.1, 59.5, 60.7, and 62.0 ^{o}C)

Regarding the *CpLAX2* gene, as shown in figure 4. 36, the highest level of gene expression was recorded in the sample collected after 158 days of culturing in the germination medium while the lowest level of gene expression was recorded in the sample collected after 59 days of culturing in the maturation medium (first day of culturing in maturation medium). The level of expression showed 3 cycles of gene expression. The first cycle began after 59 days of culturing in the induction medium where the gene expression of *CpLAX2* began to increase until day 63. Then, the gene expression started to decline until day 70. The second cycle began when the gene expression of *CpLAX2* began to increase again on day 70 until day 105 where it began to decline again until day 135. The third cycle began when the gene expression of *CpLAX2* once again increased on day 135 and reached the maximum level of gene expression on day 158.

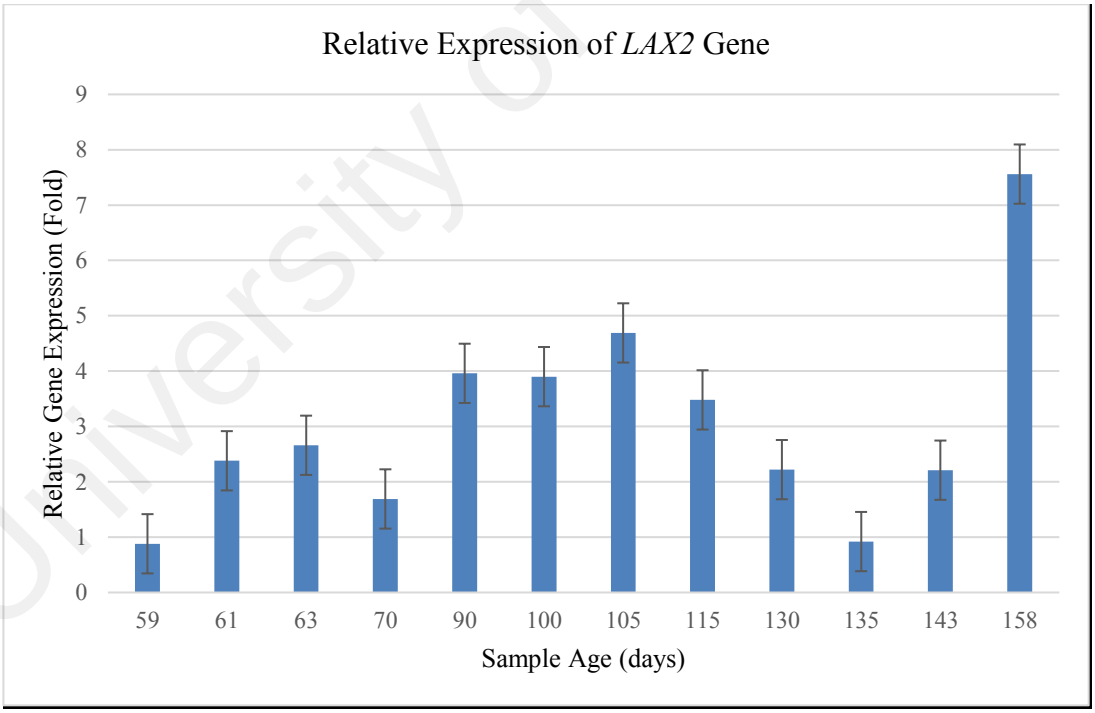

Figure 4.35: Relative expression of *CpLAX2* at different developmental stages of SE.

Regarding the *CpLAX3* gene, as shown in figure.4.37, the highest level of gene expression was recorded in the sample collected after 130 days of culturing in the germination medium while the lowest level of gene expression was recorded after 59 days of culturing in the induction medium (first day of culturing in maturation medium). The

level of gene expression showed 3 cycles. The first cycle began when the gene expression of *CpLAX3* increased from day 59 until day 70 then started to decline until day 90. The second cycle started when the gene expression of *CpLAX3* increased on day 90 until day 100. Then, the gene expression started to decline again until day 115. The third cycle started when the gene expression of *CpLAX3* started to increase on day 115 until it reached the maximum level of gene expression on day 130. Then, the gene expression started to decline again until day 158.

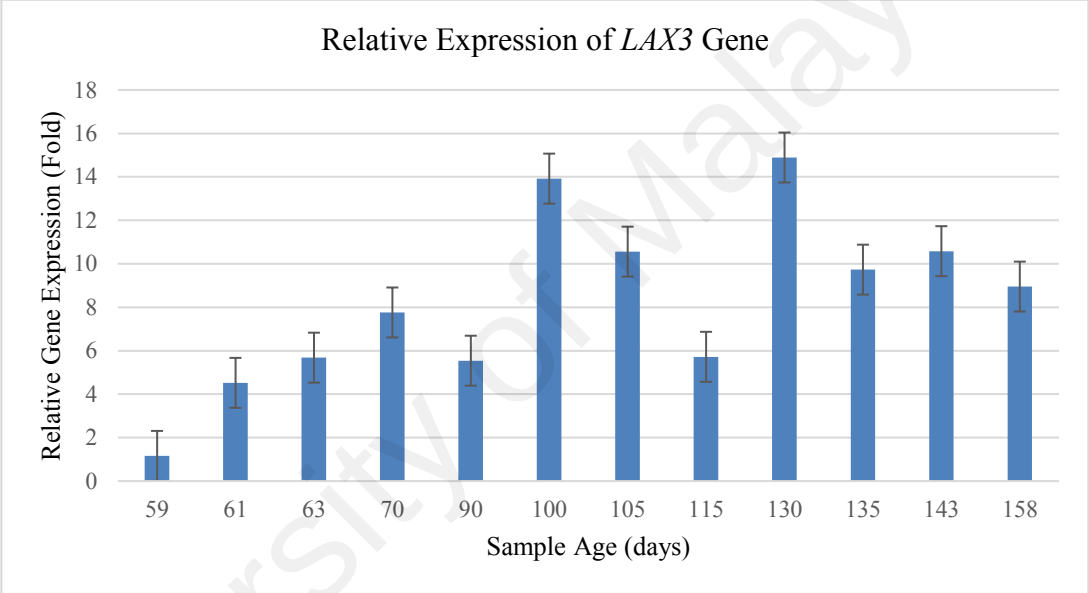

Figure 4.36: Relative expression of *CpLAX3* at different developmental stages of SE.

Regarding the *CpPIN4* gene, as shown in figure. 4.38, the highest level of gene expression was recorded in the sample collected after 70 days of culturing in the maturation medium while the lowest level of gene expression was recorded after 130 days of culturing in the germination medium. The level of gene expression showed fluctuations in the expression of *CpPIN4* gene. The gene expression started to decline from day 59 until day 61. After that, the gene expression started to increase until day 70. Then, the gene expression started to decline again on day 90. After that, the gene expression started to increase again until day 105 before declining again until day 130. After that, the gene expression increased slightly until day 158.

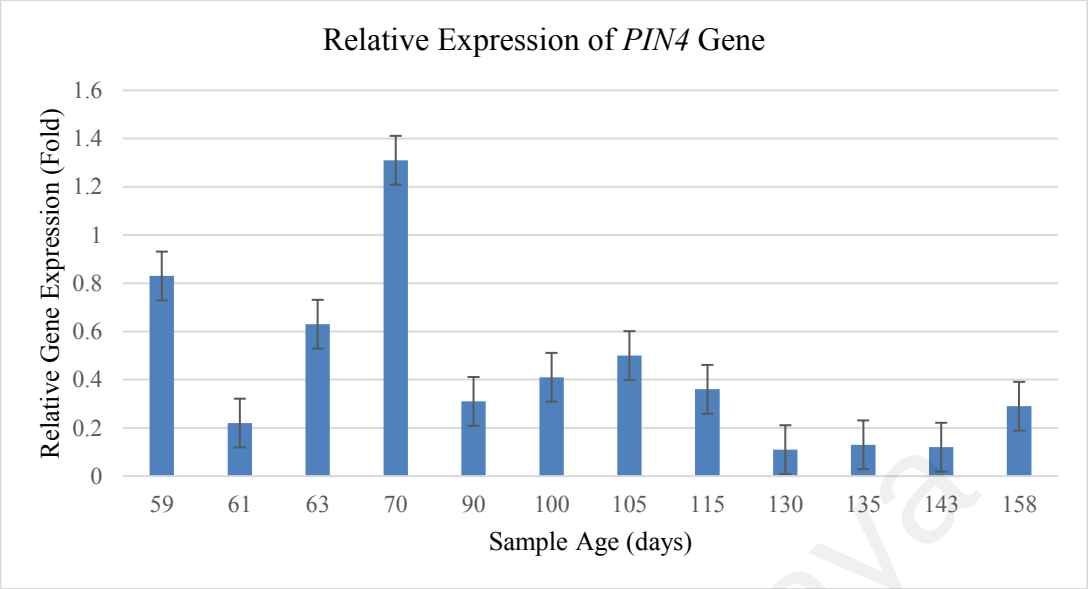

Figure 4.37: Relative expression of *CpPIN4* at different developmental stages of SE.

4.12 Summary of Results

- The best sterilization method involved treating *C. papaya* 'Eksotika' seeds with 70% ethanol for 2 min, then soaking in either 40% or 50% Clorox for 5 min, followed by soaking in 0.1 $HgCl_2$ for 10 min and finally soaking in 70% ethanol for 2 min.
- The best media for the preservation and induction of somatic embryos from mature zygotic embryos in *C. papaya* 'Eksotika' was ½ MS supplemented with 10 mg L^{-1} 2,4-D whereby the callus grew continuously without any browning for more than 20 weeks of subculturing.
- The addition of 18 mg L^{-1} of boric acid to ½ MS media augmented with 10 mg L^{-1} of 2,4-D enhanced the percentage of somatic embryos form mature zygotic embryos in *C. papaya* 'Eksotika' after 6 weeks of subculturing.
- Half strength MS media supplemented with 7.5 mg L^{-1} 2,4-D was the best medium for the induction of SE from IZE in *C. papaya* 'Eksotika'. This media produced the highest average callus weight, average callus diameter and percentage of somatic embryos

- The addition of 54 mg L^{-1} of boric acid to ½ MS media augmented with 10 mg L^{-1} of 2,4-D enhanced the average callus weight, average callus diameter and percentage of somatic embryos from IZE in *C. papaya* 'Eksotika' after 6 weeks of subculturing.
- Maturation medium supplemented with 12% PEG has beneficial effect in increasing the number and quality of large embryos.
- Activated charcoal has inhibitory effect during germination phase.
- M1 medium supplemented with 5 mg L^{-1} PG produced the highest number of large embryos (32.80 ± 1.31), small embryos (41.20 ± 3.56), globular-shaped embryos (26.20 ± 1.67) and cotyledonary stage embryos (13.20 ± 1.21).
- M2 medium supplemented with 12 % of PEG produced the highest number of large embryos (41.80 ± 1.68), heart-shaped embryos (10.00 ± 0.39), cup-shaped embryos (14.80 ± 1.39) and torpedo-shaped embryos (20.00 ± 1.78). However, no callus formation was observed on the base end of somatic embryos at concentration of 12% of polyethylene glycol.
- M3 medium supplemented with 10 mg L^{-1} PG produced the greatest number of large embryos (36.50 ± 0.89), torpedo-shaped embryos (22.75 ± 0.69), cup-shaped embryos (13.00 ± 1.20) and root formation embryos (11.50 ± 1.31).
- The largest percentage of shoot formation (62 ± 1.19%) was recorded for mature somatic embryos grown in M2 medium supplemented with 12% PEG after SF1 medium.
- The highest germination percentage (77.18 ± 9.49%) after elongation was observed for somatic embryos grown in M2 medium and transferred to SF1 medium.
- The highest number of root (3.75 ± 0.52) was observed in R3 medium without callus at the base end of the stem explants.

- On the other hand, the highest length of root (10.2 ± 1.37 cm), shoot (8.00 ± 1.26 cm) and number of leaves (4.50 ± 0.68) were observed in R4 medium.
- 4.5% SA with 50 mM $CaCl_2$ that was soaked for 10 min was found to be the best combination for the formation of ideal, uniform, and good quality beads.
- The somatic embryos encapsulated in MS medium without calcium showed the highest percentage (39%) of shoot formation and lowest percentage (11%) of callus formation at the base end of somatic embryos.
- MS media supplemented with NAA and BAP increased the percentage of callus formation at the base end of somatic embryos.
- The ontogeny and anatomy of somatic embryo structures during the induction phase of SE was examined using light microscopy.
- Histological study, under light microscopy, of the continuous embryogenic cultures demonstrated two ways in which somatic embryos developed. The first was that somatic embryos developed from single epidermal cells and the second was that somatic embryos developed from groups of cells.
- ½ MS media supplemented with 10 mg L^{-1} 2,4-D was enhanced callus formation at the base end of somatic embryos and not suitable for the induction and maturation of SE from IZE of *C. papaya* 'Eksotika'.
- Active gene expression of all selected genes (i.e., *CpLAX2*, *CpLAX3* and *CpPIN4*) were detected in all somatic embryos collected during maturation and germination phases.
- *CpLAX3* showed the highest level of expression compared to the rest of the genes used in the experiment while *CpPIN4* showed the lowest level of expression.
- Gene expression of *CpLAX2* showed the highest level of gene expression in the sample collected after 158 days of culturing in the germination medium while

the lowest level of gene expression was recorded after 59 days of culturing in the induction medium (first day of culturing in the maturation medium).

- Gene expression of *CpLAX3* showed the highest level of gene expression in the sample collected after 130 days of culturing in the germination medium while the lowest level of gene expression was recorded after 59 days of culturing in the induction medium (first day of culturing in the maturation medium).
- Gene expression of *CpPIN4* showed the highest level of gene expression in the sample collected after 70 days of culturing in the maturation medium while the lowest level of gene expression was recorded after 130 days of culturing in the germination medium.
- The level of gene expression showed fluctuations in the expression of *CpPIN4* gene.
- All genes (*CpLAX2*, *CpLAX3* and *CpPIN4*) were involved in the development and growth of somatic embryos.

CHAPTER 5: DISCUSSION

A higher survival percentage and a lower contamination rate were observed when explants were treated with 70% ethanol (2 min), followed by either 40% or 50% Clorox (5 min), then soaked in 0.1 $HgCl_2$ (10 min) and finally immersed in 70% ethanol for 2 min.

Somatic embryogenesis is one of the pathways for *in vitro* plant morphogenesis. The relationship between the culture medium composition and explant types lead to SE formation. Unfortunately, this relationship is complex and remains poorly understood. Most researchers use classical approaches by manipulating ratios between plant growth regulators to optimize the quality and number of embryos. However, many species and varieties do not respond according to this classical approach and need additional optimization through the manipulation of other chemicals or physical factors (Ramage & Williams, 2002).

Numerous investigations in *C. papaya* have concentrated on the effects of genotypes (Chan & Teo, 1993; Malabadi *et al.*, 2011), explant age (Chen *et al.*, 1987), plant growth regulators and cultural conditions on the induction of SE in several *C. papaya* species (Bhattacharya *et al.*, 2002; Ascencio-Cabral *et al.*, 2008). Different cultivars of *C. papaya* produce different responses to *in vitro* conditions. Also, the age or degree of maturity of the plants resulted in differential responses. This is because the physiological state related to the endogenous hormonal concentration of the explants at various ages differs significantly. The *C. papaya* 'Taiping' reduced 5–6 times more shoots in the culture than the *C. papaya* 'Eksotika' when in the same media. The 'Eksotika' cultivar was more susceptible to abnormality than the 'Taiping' cultivar when maintained in the *C. papaya* culture to generate shoots in liquid media (Chan & Teo, 1993). A complete understanding of the factors that regulate SE induction, maturation and germination will increase the

efficiency of protocols. Generally, SE development *in vitro* depends on the interactions between internal factors (e.g., genotype, type of explants, and age) and external factors (e.g., growth regulators, culture medium composition, and light) (Bukhori, 2013). In general, little attention has been given to the constituent of media in the *C. papaya* cultivars. Somatic embryogenesis processes have been examined just by using MS medium (Fitch & Manshardt, 1990), and ½ MS (Malabadi *et al.*, 2011; Anandan *et al.*, 2012; Razak *et al.*, 2015).

The embryogenic potential of *C. papaya* explants varied with genotype and the type of explants used. However, better results were usually obtained from regeneration protocols that utilized explants from IZE (Fitch & Manshardt, 1990; Vilasini *et al.*, 2000; Malabadi *et al.*, 2011). Callus induction for SE is affected by *C. papaya* cultivars. For instance, the 'Solo' cultivar produced more callus than the explants from 'Sunrise' in the same type of media (Chen *et al.*, 1987). The different cultivars of *C. papaya* responded differently during SE even when in the same concentrations of 2,4-D (Fitch & Manshardt, 1990). Also, induction of embryogenic callus was affected by nutrients in the media and the plant growth regulators used (Jordan, 1986).

In the present study, mature zygotic embryos (size ≥ 3mm) were cultured in different media supplemented with different concentrations of 2,4-D for somatic embryo induction. The somatic embryos were induced in all media with different concentrations of 2,4-D after 3 weeks of culturing. This result is in agreement with the findings of Fitch and Manshardt (1990) when they similarly observed SE occurring after only three weeks in the culture. The best growth of embryogenic calli (i.e., highest average callus weight and highest average callus diameter) was observed in ½ MS medium supplemented with 2 mg L^{-1} 2,4-D after 6, 8 and 20 weeks, whereas after 6, 8, 20 weeks the lowest growth of callus was recorded in $^3/_2$ WPM media supplemented with 15, 2, 15 mg L^{-1} 2,4-D respectively.

Our results also are in agreement with previous studies by Fernando *et al.* (2001), whereby they found that the induction of SE from mature zygotic embryos of *C. papaya* 'Sunrise Solo' in MS medium (Murashige & Skoog, 1962) supplemented with 2 mg L^{-1} of 2,4-D produced the best result. Most of the media with a lower concentration of 2,4-D stimulated higher callus formation. This result is also similar to the findings of Fitch and Manshardt (1990) when they found higher 2,4-D concentrations were inhibitory to the SE process.

The types of media used differed in their total ionic strength, nitrogen level, ammonium concentration, and the ammonium-to-nitrate ratio. It may be inferred that one of these nutritional parameters could be responsible for the differences, though further study would be necessary to establish the critical factor determining the efficiency of the callus induction and somatic embryo production in *Carica papaya*. Pěnčík *et al.* (2015) found that NH_4^+ induced embryogenic callus through the increased accumulation of endogenous IAA and perhaps this macronutrient is responsible for the differences.

For the boric acid experiment, after 4 weeks of culturing, the maximum callus weight and callus induction percentage were recorded in the medium supplemented with 72 mg L^{-1} of boric acid, whereas after 8 weeks of culturing, explants cultured in the medium supplemented with 6 mg L^{-1} of boric acid produced the highest average callus weight. However, there were no significant differences in the average callus weight in the medium supplemented with 6 and 72 mg L^{-1} of boric acid. Conversely, the lowest callus weight was observed in the medium supplemented with 36 mg L^{-1} of boric acid after 4 and 8 weeks of subculturing.

Regarding the formation of somatic embryos, the highest somatic embryo percentage was obtained in the medium supplemented with 18 mg L^{-1} boric acid after 6 and 8 weeks of subculturing. This result contradicted Renukdas *et al.* (2003) that stated 62 mg L^{-1} of

boric acid was the best concentration for SE in *C. papaya* 'Honey Dew'. This contradiction may be due to the different *C. papaya* cultivar or boric acid concentration used.

In this study, the IZE (mostly size ≥3mm) were also used as starting materials for callus induction and subsequent SE. In the Caricaceae family, many patterns have been proposed for the development of SE since the first study in 1981 by Litz and Conover (Litz & Conover, 1981). The occurrence of SE in *C. papaya* plants has been reported from different explants for various genotypes such as hypocotyl (Fitch, 1993), root (Chen *et al.*, 1987), adventitious root (Yu *et al.*, 2001), stem (Chen *et al.*, 1987; Fitch, 1995), petiole (Hossain *et al.*, 1993), leaves (Koehler *et al.*, 2013) and IZE (Fitch & Manshardt, 1990; Chen *et al.*, 1991; Vilasini *et al.*, 2000; Bhattacharya *et al.*, 2002; Ascencio-Cabral *et al.*, 2008; Malabadi *et al.*, 2011). Callus induction in *C. papaya* is frequently accomplished by supplementing 2,4-D in MS or ½ MS medium (Fitch & Manshardt, 1990; Chen *et al.*, 1991; Vilasini *et al.*, 2000; Bhattacharya *et al.*, 2002; Ascencio-Cabral *et al.*, 2008). In the current study, three different types of media were tested in combination with different concentrations of 2,4-D with 60 g L^{-1} sucrose to induce callus and to form subsequent embryogenic callus.

In the present study, the highest percentage of somatic embryos (66.1%) was achieved in WPM medium supplemented with 2.5 mg L^{-1} 2,4-D. So far, no research has reported using WPM media for the induction of somatic embryos from *Carica papaya*. The explants cultured in WPM medium supplemented with 2.5 mg L^{-1} 2,4-D produced compact and embryogenic calli. Various studies have shown induction of embryogenic calli using WPM media in other species such as *Fraxinus pennsylvanica* (Li *et al.*, 2014) and *Ocotea catharinensis* (Catarina *et al.*, 2004). Unfortunately, the average callus weight was low, and the texture of callus was compact with a small friable portion.

Our results were different from the previous study by Fitch and Manshardt (1990) who found that the largest number of embryogenic zygotic embryos were produced by each of the four genotypes tested and cultured in ½ MS medium supplemented with 5 mg L^{-1} 2,4-D. Again, this contradiction may be due to the differences in *C. papaya* varieties and/or media composition.

Malabadi *et al.* (2011) induced embryogenic callus from IZE after 4-6 weeks of culturing in full-strength MS medium supplemented with 4.52 µM 2,4-D and 2.27 µM TDZ but after three weeks of culturing, the IZE turned brown and died. Our results showed no browning of callus and can possibly be explained by the differences in *C. papaya* variety. Also, Bhattacharya and Khuspe (2000) found that using IZE as explants in *C. papaya* 'Honey Dew' and 'CO2' produced the highest SE frequency. Our results also agreed with Fitch and Manshardt (1990) that 2,4-D is considered the best auxin for the initiation of somatic embryos from IZE in *Carica papaya*.

Also, the addition of boric acid to ½ MS medium supplemented with 10 mg L^{-1} 2,4-D increased the percentage of somatic embryo formation. The highest percentage of somatic embryos was observed in 54 mg L^{-1} of boric acid while the lowest percentage was found in the control medium (i.e., medium without addition of boric acid). The effect of boric acid on SE has been previously reported (Behrendt & Zoglauer, 1996; Sahasrabudhe *et al.*, 1999; Renukdas *et al.*, 2003; Mashayekhi & Neumann, 2006). Boric acid is essential mainly during the early phases of organogenesis for stimulating the mechanisms that lead to cell differentiation (Redondo-Nieto *et al.*, 2008).

In the boric acid experiment, the highest callus weight was recorded in control medium whereas the lowest callus weight was observed at 36 mg L^{-1} of boric acid after eight weeks. Other researchers studied the induction of SE from IZE of *C. papaya* 'Honey Dew' using MS basal salt with B5 vitamin supplemented with 2 mg L^{-1} 2,4- D, in addition

to different concentrations of boric acid (30-500 mg L^{-1}). The result showed that the highest somatic embryo induction was observed in MS medium supplemented with 2 mg L^{-1} 2,4- D added with 62 mg L^{-1} boric acid. Somatic embryogenesis was completely inhibited when the concentration of boric acid was more than 100 mg L^{-1} (Renukdas *et al.*, 2003). Boron promoted the destruction of natural auxin and increased its translocation. Endogenous IAA levels increased in the absence of B and translocation was reduced (Goldbach & Amberger, 1986). Boron deficiency may affect plant water balance by reducing water uptake due to a reduced root tip number and root length (Mottonen *et al.*, 2001). Root growth was enhanced under nitrogen and phosphorus deficiencies, but not under deficiencies of nutrients of low mobility in the phloem, such as calcium and B. Enhanced root growth under nutrient deficiency relies on the importation of both photosynthetic and mineral nutrients (Marschner *et al.*, 1996).

Boron may also affect the ratio between endogenous levels of IAA and some cytokinin, which causes morphogenesis, whereby lower B concentrations promote root development with simultaneous retardation of shoot development and higher B concentrations promote shoot development with decreased root system development. Furthermore, increased B concentrations in the medium reduced the concentration of endogenous abscisic acid (Mashayekhi & Neumann, 2006).

Little studies refer to the effect of Phloroglucinol (PG) on the induction (Reis *et al.*, 2008) maturation and germination (Murali *et al.*, 1996) of somatic embryos. So far, there have not been any published research on the effect of PG on the maturation phase of somatic embryos of *C. papaya* 'Eksotika'.

Maturation medium 1 (M1) medium supplemented with 5 mg L^{-1} PG produced the highest number of large embryos (32.80 ± 1.31), small embryos (41.20 ± 3.56), globular-shaped embryos (26.20 ± 1.67) and cotyledonary stage embryos (13.20 ± 1.21).

Nevertheless, maturation medium supplemented with 1 mg L^{-1} PG produced the lowest number of (3.00 ± 0.70) callus formation at the base end of somatic embryos and the highest number of cup-shaped (11.60 ± 0.3) (Table 4.12). The results of the present study are similar to Murali *et al.* (1996) where PG had a positive effect and enhanced the germination of somatic embryos (Murali *et al.*, 1996). The positive effect maybe back to the increased the amount of phenolic compounds in the explants cultured in the media supplemented with PG (Reis *et al.*, 2008). Other researchers have reported that the addition of PG to *in vitro* culture medium acts as synergists auxin for that stimulated shoot development (Sarkar & Naik, 2000; Steephen *et al.*, 2010), and root development (Pérez *et al.*, 2016)

Numerous research have proven the positive effect of PEG on the maturation of somatic embryos (Li *et al.*, 1998; Walker & Parrott, 2001; Yadollahi *et al.*, 2011) and the germination of somatic embryos (Walker & Parrott, 2001; Langhansova *et al.*, 2004). The quality of somatic embryos can be significantly influenced by a low osmotic potential in the maturation medium by using PEG (Körbes & Droste, 2005). The PEG 8000 helps to improve the development of somatic embryos in conifers (Santos *et al.*, 2002). Additionally, it enhances the maturation of somatic embryos in *Psidium guajava* by increasing the maturation frequency (Rai *et al.*, 2009).

In the present study, maturation medium supplemented with 12% of PEG produced the highest number of large embryos (41.80 ± 1.68), heart-shaped embryos (10.00 ± 0.39), cup-shaped embryos (14.80 ± 1.39) and torpedo shaped embryos (20.00 ± 1.78). Besides, no callus formation was observed at the base end of somatic embryos (Table 4.13). Many researchers used PEG in plant physiology and plant tissue cultures to simulate the effects of water stress and salinity (Castroluna *et al.*, 2014; Shaygan *et al.*, 2017). Polyethylene glycol cannot penetrate into the plant cells, it limits water uptake by creating water stress

under *in vitro* conditions and simulates drought stress during somatic embryo development (Langhansova *et al.*, 2004; Wani *et al.*, 2010). Water stress changes the DNA methylation pattern and causes changes in gene expression of proteins, which is crucial for somatic embryo development (Smulders & De Klerk, 2011). Al-Khayri and Al-Bahrany (2004) found that PEG concentration in media has a negative correlation with water content inside plant cells and a positive correlation with the level of endogenous free proline (Al-Khayri & Al-Bahrany, 2004). The presence of L-proline in the culture medium increases the nitrogen and carbon source, and finally enhances maturation of somatic embryos (Moghaddam *et al.*, 2000).

The results of the present study demonstrate that no callus is formed at the base end of somatic embryos in M2 medium supplemented with 12% PEG which may contribute to root enhancement. Many researchers proved that callus formation at the base end of somatic embryos is the main cause of poor root formation and low percentage of acclimatization in *C. papaya* (Michalczuk *et al.*, 1992; Drew *et al.*, 1993). The results of the present study are similar to those obtained by Langhansova and colleagues (2004) whereby PEG enhanced root meristem organization of torpedo-stage during SE which has also been observed in *Panax ginseng*. It could be that PEG inhibited callus formation at the base end of somatic embryos due to changes in DNA methylation pattern that either caused changes in the gene expression of proteins or increases in the level of endogenous free L- proline.

Manipulation of a micronutrient in the induction media (i.e. boric acid) may affect the result in the maturation phase. Comparison between the results in the maturation phase showed that the optimum concentration of PG in M1 medium was 5 mg L^{-1} (Table 4.12) whereas in M3 was 10 mg L^{-1} (Table 4.14). This clarifies that the composition of the

induction and maturation media played important roles in the development of SE of *Carica papaya*.

Many research have demonstrated the positive role of activated charcoal (AC) in plant tissue cultures, especially in supporting the growth and development of plant tissues (Paek & Hahn, 2000; Thomas, 2008). Activated charcoal helps in the induction (Manchanda & Gosal, 2012) and maturation (Krajňáková *et al.*, 2009) of somatic embryos. Activated charcoal plays a critical role in SE (Sáenz *et al.*, 2010), anther culture (Abdollahi *et al.*, 2017), synthetic seed production (Pehwal *et al.*, 2013), rooting (Nhut *et al.*, 2001), and plant regeneration (Mittal *et al.*, 2016). The addition of AC to culture media may either encourage or inhibit growth, depending on the species and tissues used (Pan & Van Staden, 1998).

Somatic embryos grown in the best maturation medium concentration (i.e. M1 supplemented with 5 mg L^{-1} PG, M2 supplemented with 12% PEG and M3 supplemented with 10 mg L^{-1} PG) were transferred to two types of shoot formation media; SF1 and SF2. The highest germination percentage (62 ± 1.19%) was recorded for mature somatic embryos cultured in M2 medium supplemented with 12% PEG and then transferred to SF1 medium. Conversely, the lowest germination percentage (32.8 ± 1.06%) was recorded for mature somatic embryos cultured in control media and transferred to SF1 medium (Table 4.16). Activated charcoal is characterized by a high adsorptive capacity for gases, vapors and colloidal solids (Halhouli *et al.*, 1995). Activated charcoal is able to adsorb substances such as plant growth regulators, suggesting that AC is a non-selective adsorber (Pan *et al.*, 2002). This raises concerns regarding the optimum concentration of growth regulators in tissue culture media that contain Activated Charcoal (Ebert *et al.*, 1993).

Activated charcoal may release substances that promote *in vitro* growth (Pan & Van Staden, 1998). The present study demonstrated the negative effects of AC during somatic embryo germination. This contradicts past researches that found AC significantly enhanced the germination of embryos in *Panax quinquefolium* L. (Tirajoh *et al.*, 1998) and *Phoenix dactylifera* (Zouine *et al.*, 2005) as well as increased maturation frequencies of somatic embryos in *Pinus thunbergii* (Maruyama *et al.*, 2005) and *Daucus carota* (Pan & Van Staden, 2001). Also, AC had beneficial effects on the regeneration of somatic embryos into plantlets in *Paspalum scrobiculatum* L. (Rashid, 2001) *Lilium longiflorum* (Nhut *et al.*, 2001), and *Platycerium bifurcatum* (Teng, 1997).

The somatic embryos were elongated in the same media. Most of the somatic embryos germinated in SF1 medium and transferred to EL showed higher germination percentages than somatic embryos germinated in SF2 medium and transferred to EL medium. However, there were no significant differences in germination percentages in both media for explants matured in M3 medium.

The major problems faced developing transgenic *C. papaya* plants are low rooting efficiency of regenerated shoots and low acclimatization rate of rooted transgenic *C. papaya* plants in the field (Malabadi *et al.*, 2011). These problems could probably due to poor quality roots such as thickened, callused, and absent lateral roots and root hair formation, which affect the uptake of nutrients by the plants after being transferred to soil (Fitch & Manshardt, 1990; Sekeli *et al.*, 2012). Therefore, efficiency and quality of root are critical in ensuring successful and continuous production of transgenic *C. papaya* 'Eksotika'. IBA had been reported by many researchers as the best auxin for root induction in *C. papaya* (Drew *et al.*, 1993; Sekeli *et al.*, 2012; Pérez *et al.*, 2016). IBA is known to have a greater ability to promote rooting with less callus formation compare with other types of auxin. Drew and coworker (1993) reported that rooting in agar

medium produced thick, short, and stumpy roots. Many authors reported that vermiculite was able to improve rooting efficiency and produced good quality roots that were finer with more abundant lateral branches and root hairs (Kaity *et al.*, 2009; Wu *et al.*, 2012; Pérez *et al.*, 2015). Langhansov et al., (2004) found that plants obtained from somatic embryos treated with PEG displayed better root formation, facilitating subsequent acclimatization.

Five types of media were used in rooting experiments to study the effect of riboflavin, PG and IBA on root formation. The highest germination percentage (100 ± 7.31%) was observed in R5 medium. The highest number of roots (3.75 ± 0.52) was observed in R3 medium. The explants treated in R1 and R3 media showed no callus at the base end of the stem explant. Conversely, the longest root length (10.2 ± 1.37 cm), shoot length (8.00 ± 1.26 cm) and highest number of leaves (4.50 ± 0.68) were observed in R4 medium (Table 4.18). This demonstrated a positive significant effect of PG in the number of roots formed, whereby adding this component to the root media eliminated callus formation and enhanced the number of roots until IBA was removed from the media. Also, riboflavin prevented callus formation. Conversely, using IBA without riboflavin and PG, enhanced callus formation at the base end of explants. Our result similar to previous finding where, many research have demonstrated the positive role of PG for the enhancement of root frequency (Bopana & Saxena, 2008), root induction (Ceasar *et al.*, 2010; Petri & Scorza, 2010) in different plant species, as well as rooting and plant survival of walnut trees during acclimatization (Licea-Moreno *et al.*, 2015).

Nowadays, artificial seeds are introduced as a new technology for future plant production and germplasm preservation during offseason periods (Ahmad & Anis, 2010). The establishment of an efficient SE system is a major prerequisite for a successful program on synthetic seeds. In the present study, 4.5% SA with 50 mM $CaCl_2$ that was

soaked for 10 min was found to be the best combination for the formation of ideal, uniform, and good quality beads. Both SA and $CaCl_2$ played an important role in capsule quality (Singh *et al.*, 2010). Our results were similar to Saha *et al.* (2004) study that used the same concentration of SA (4.5%) for the production of synthetic seeds in *C. papaya* (Saha *et al.*, 2004).

Sodium alginate mixed with liquid media probably increased shoot emergence due to the existence of nutrients in the gel matrix that acted as artificial endosperm around the embryo and facilitated growth and survival, thus allowing them to germinate (Antonietta *et al.*, 1998; Ara *et al.*, 1999). In the present study, artificial seeds that consisted of SA with MS media without calcium component enhanced the percentage of root and shoot formation as well as decreased the percentage of callus formation at the base end of somatic embryos. This is the first study on the effects of calcium in the artificial seed production of *C. papaya* 'Eksotika'. There are many past research that show the direct relationship between germination rate from encapsulated somatic embryos and exposure time to $CaCl_2$ (Castillo *et al.*, 1998). Both high concentrations of $CaCl_2$ and longtime exposure of somatic embryos to $CaCl_2$ led to more absorption and penetration of $CaCl_2$ in the embryo, which affected the plants response to regeneration (Redenbaugh *et al.*, 1986; Malabadi & Van Staden, 2005). Also, many researchers have studied the effect of encapsulation mixture compositions on the survival, regeneration and formation of beads. Ganapathi *et al.* (2001) reported that the composition of the encapsulation mixture influenced the survival of somatic embryos of bananas (Ganapathi *et al.*, 2001). The effect of encapsulation matrix composition on the regeneration of encapsulated micro-cuttings of hybrid aspen (*Populus tremula* L. × *P. tremuloides* Mincx.) has also been studied. The results demonstrated that the ingredients of the matrix such as nutrient medium salts, sugars (especially sucrose) and growth regulators significantly affected the initial development of the micro-cuttings (Tsvetkov *et al.*, 2006). The conversion rate of

artificial seeds was higher in capsules augmented with mineral nutrients than capsules without nutrients (Pintos *et al.*, 2008).

MS media supplemented with NAA and BAP increased the percentage of callus formation at the base end of somatic embryos. Most researchers used a combination of NAA and BAP in the germination of somatic embryos of *Carica papaya*, and obtained unsatisfactory results for root formation due to the formation of callus at the base end of somatic embryos (Vilasini *et al.*, 1998; Clarindo *et al.*, 2008; Bukhori, 2013).

To a certain extent, SE is similar to zygotic embryogenesis, whereby a single cell or a group of somatic cells are the origin for the formation of a somatic embryo (Von Arnold *et al.*, 1996). The type of callus during SE is usually recognized using either visual observation or a dissecting microscope and is confirmed by cytological examination using acetocarmine-Evan blue stain (Gupta & Durzan, 1987). The identification of embryogenic calli during early stages is crucial for plant transformation and propagation. This helps to improve callus development and to increase the production of appropriate calli for plant transformation and propagation (Bevitori *et al.*, 2014).

Embryogenic competence is limited to particular tissues whereby each genotype possesses specific tissue and has a high likelihood of becoming embryogenic competent. Mostly, embryogenic competence is highly found in tissues that have embryonic origin such as IZE (Neumann, 2000). Somatic embryogenesis from IZE of *C. papaya* 'Eksotika' was induced in ½ MS medium augmented with 10 mg L^{-1} 2,4-D, 50 mg L^{-1} *Myo*-insitol, 100 mg L^{-1} glutamine and 45 mg L^{-1} adenine hemisulfate. Induction of SE began after 3 weeks of culturing. The results of the present study was comparable to Kurczyńska *et al.* (2007) finding that direct SE resulted from IZE of *Arabidopsis* when cultured in media augmented with auxin. The highly active somatic cells that originated directly from somatic embryos (competent cells) were detected in the protodermis and sometimes

subprotodermis of the upper surface of cotyledons (adaxial side) (Kurczyńska *et al.*, 2007).

Embryogenic callus was formed from rounded cells with large nucleus and dense cytoplasm. Conversely, elongated or irregular cells with small nucleus and light cytoplasm caused non-embryogenic callus to form. These results were similar to Silveira *et al.* (2013) they found that some cells on the surface of somatic embryo had dense cytoplasm with a large nucleus and was considered the origin of daughter embryos. Also, Sharp *et al.* (1980) studied the histological sections of somatic embryos of *Tilia cordata* and found that some epidermal cells were characterized by dense cytoplasm with large nuclei and these characteristics distinguished embryogenic cells from other cells (Sharp *et al.*, 1980).

The present results demonstrated that throughout the globular embryo stage, most, if not all, embryonic cells had similar meristematic characteristics such as large nucleus and dense cytoplasm. However, during the early heart stage, the cells began to lose these characteristics. Similar histological characteristics of embryogenic cells have been described by Ma *et al.* (2012). According to Feher *et al.* (2003) the development of meristematic centers and organs were associated with the cell's capability to react to specific hormonal or environmental signals (Feher *et al.*, 2003).

The current histological study, under light microscopy, of the continuous embryogenic cultures demonstrated two ways in which somatic embryos developed. The first was that somatic embryos developed from single epidermal cells and the second was that somatic embryos developed from groups of cells. These findings were in agreement with observations from (Canhoto *et al.*, 1996). According to El Dawayati *et al.* (2012) there were two types of meristematic cells in embryogenic tissue. The first type of meristematic cells was found in surface layers which generally have intensive mitotic activity. The

second type of meristematic cells appeared as several meristematic multicellular centers, which occurred in the peripheral and/or the entire embryonic tissue, and had definite fragmentation lines produced by thickened cell walls (El Dawayati *et al.*, 2012).

Many researchers have investigated and garnered mixed results regarding the origin of somatic embryos during the early stages of SE. Some found that somatic embryos either originated or developed from unicellular or multicellular (Maximova *et al.*, 2002) while some maintain that they originated from both (Kurczyńska *et al.*, 2007). The multicellular origin of SE generally resulted in somatic embryos that were directly connected to parental tissue, whereas the unicellular origin generated somatic embryos that were connected to the explant by a suspensor (Santos *et al.*, 2006). Jayasankar *et al.* (2003) proposed that the presence of a suspensor controlled the development and improved the rate of germination and regeneration (Jayasankar *et al.*, 2003). The absence of suspensors and delays in the formation of inner organizations were the main differences between somatic embryos obtained from IZE and those obtained from zygotic embryos (Santos *et al.*, 2006).

Nonohay *et al.* (1999) distinguished between the unicellular and multicellular origin of somatic embryos based on their place of generation. Somatic embryos that had unicellular origins generated from a superficial callus cell while those that had multicellular origins generated from epidermal and subepidermal callus cells (Nonohay *et al.*, 1999). Pescador *et al.* (2008) found many sources for the generation of embryos during SE. Specifically, somatic embryos could be generated from a single protoderm cell, a cluster of protoderm cells or a cluster of ground meristematic cells near the protoderm (Pescador *et al.*, 2008).These types of cells either originated from meristematic tissues or could be formed from elongated, vacuolized cells under specific conditions, such as treatment with 2,4-D in specific concentrations (Fehér, 2005).

In the present study, daughter embryos of various sizes and stages were detected on the surface of mother somatic embryos. These observations suggested that the surface cells in somatic embryos that have dense cytoplasm and large nuclei could be divided to form daughter embryos. The daughter embryos were connected to the parent tissue without suspensors and vascular connections akin to the results in Chen *et al.* (1991) study. Bharathy and Agrawal (2008) found that daughter embryos developed on the hypocotyls in the root–shoot zone, cotyledon and sometimes form the entire embryo in grapevine SE (Bharathy & Agrawal, 2008). These observations could be explained by Fehér (2005) study that found explants could maintain the embryogenic potential of meristematic cells when cultured in medium supplemented with 2,4-D, whereby embryogenic callus were formed in response to auxin. The embryogenic callus then generated somatic embryos when transferred into media without growth regulators (Fehér, 2005). These somatic embryos then generated daughter embryos, possibly because the 2,4-D enabled the somatic embryos to maintain their embryogenic potential. Also, 2,4-D is considered a synthetic auxin that works as a natural auxin, but produces more varied effects (Fehér, 2005). As a synthetic auxin, 2,4-D affects more classes of genes and these effects can be observed in signal transduction, cellular transport, protein movement and subcellular localization (Fehér, 2005). Kurczyńska *et al.* (2007) studied auxin distribution during the different developmental stages of somatic embryos to prove the embryogenic nature of protodermal and subprotodermal. The results showed concentrated auxin in protodermis during the globular stage of somatic embryos which then transferred and concentrated in the shoot, root pole and cotyledon primordia during the heart and cotyledonary stages (Kurczyńska *et al.*, 2007).

According to histological examination in the present study, the root began developing in the early heart shape stage. This finding was similar to Jürgens (2001) who found that the root began forming during the late globular stage when an asymmetric division

occurred in the upper suspensor cell and produced small lens-shaped cells that formed the center for embryonic root development (Jürgens, 2001).

Somaclonal variation is a random change in any region of the genome that causes changes in genotypes and phenotypes (Krishna *et al.*, 2016). These variations can be detected and characterized through a variety of ways such as by analyzing the morphology of the plant (Pérez *et al.*, 2011), analyzing the structure and number of chromosomes using cytological studies (Currais *et al.*, 2013), analyzing the DNA content of the cells using molecular studies (Jin *et al.*, 2008) or analyzing the profile of protein using biochemical studies (Yaacob *et al.*, 2013). There are many factors that cause somaclonal variation, such as physical and chemical factors. Physical factors include light intensity, temperature and photoperiod whereas the chemical factors include plant growth regulators and some osmoprotectants. Additionally, the species genotype, explant source and period of *in vitro* culture also highly influence somaclonal variation (Bordallo *et al.*, 2004).

The present study observed numerous abnormalities throughout somatic embryo development, such as differing number of cotyledons and the presence of fused somatic embryos. The formation of somatic callus, especially on the root pole, during the cotyledonary stage of somatic embryos was also observed. The formation of this callus prevented the formation of functional roots and thus resulted in incomplete plants. The anomalous or morphological abnormalities of somatic embryos have frequently occurred especially in plant species that were cultivated in medium augmented with 2,4-D (Pescador *et al.*, 2008). These abnormalities can be observed based on their morphology and failure to develop into plants (Kong *et al.*, 2012). Histological analyses on *Acca sellowiana* showed that the abnormal embryos were formed either directly from explants or indirectly from callus formed on the explant. This suggested that the formation of

abnormal somatic embryos reflected the disturbances in either physiology, genetics or both due to the presence of 2,4-D in the medium (Pescador *et al.*, 2008).

The present study is in agreement with past studies that found somatic embryos of most species developed numerous morphological abnormalities such as variation in shape, size, and cotyledon number (Jayasankar *et al.*, 2003). Halperin and Wetherell (1964) found that carrot embryos treated with high concentrations of 2,4-D demonstrated abnormal development in the apical meristems which subsequently inhibited embryo growth (Halperin & Wetherell, 1964). Recently, Araújo et al. (2005) grew explants in medium augmented with 2,4-D and found that 2,4-D strongly affected the expression of different transposable elements (Araujo *et al.*, 2005). 2,4-D concentrations had the potential to alter the gene expression of PIN genes and affect PIN proteins since PIN proteins are a plant transmembrane protein belonging to auxin efflux transporters which participate in the transportation of auxin from cells. These proteins are found inside the cells and direct intercellular auxin flow according to their polarity (Friml *et al.*, 2003).

Based on the aforementioned, the formation of anomalous somatic embryos that causes low conversion rates in *C. papaya* is possibly due to the effect of 2,4-D in the medium which changes normal genetic and physiological processes. Therefore, appropriate endogenous auxin levels in explants play a critical role in ensuring normal somatic embryos are produced (Fehér, 2005).

Auxin plays a critical role in regulating plant growth and development. Auxin is transported directly from cell to cell through polar auxin transport (PAT) by the action of auxin influx (AUX1/LAXs) and efflux (PINs) transporter proteins. The biological function of numerous auxin transporter genes in *Arabidopsis thaliana* has been well characterized (Yu *et al.*, 2017). The role of 2,4-D as inducer of SE in *C. papaya* from IZE has been previously confirmed (Vilasini *et al.*, 2000). Furthermore, the greatest

embryogenetic response was detected in 10 mgL^{-1} of 2,4-D. Therefore, 10 mgL^{-1} of 2,4-D was chosen for this study. The ability of embryos to germinate and convert into plantlets is dependent on the quality of the shoot apical meristem and root apical meristem produced during development (Kong & Yeung, 1992). In *Carica papaya*, the capacity of embryogenic callus to develop somatic embryos during the proliferation phase is considered an important step for determining the quality of cotyledonary somatic embryos. Well-developed bipolar somatic embryos are able to develop cotyledonary embryos of *C. papaya* (Chen *et al.,* 1991). *C. papaya* plantlets generated *in vitro* from SE usually show low rooting capacity (Chen *et al.*, 1987; Fitch, 2005; Farzana *et al.*, 2008). It has been suggested in other species that the auxin concentration at root tissues is the result of auxin influx transporters (AIT; AUX1/LAX) and auxin efflux transporters (AET; PIN) that regulate the mechanism of the initiation and development of lateral roots (Friml *et al.*, 2002a; Friml *et al.*, 2003; Ugartechea-Chirino *et al.*, 2009).

In *Carica papaya*, there are four *AUX1/LAX* genes (*CpAUX1*, *CpLAX1*, *CpLAX2* and *CpLAX3*) and six *PIN* genes (*CpPIN1*, *CpPIN2*, *CpPIN3*, *CpPIN4*, *CpPIN5* and *CpPIN6*) (Estrella-Maldonado *et al.*, 2018). Defects in *AUX1/ LAXs* and *PINs* can reduce various stages of SE and also reduce the formation of the apical pole or radicle (inhibition of lateral roots) which is associated with a reduction of auxin movement (Marchant *et al.*, 2002). Therefore, in the present study, experiments were designed to compare the expression patterns of the *CpLAX2*, *LAX3* and *CpPIN4* genes during different developmental stages of SE. Molecular analysis was conducted to gain a better understanding of the fundamental events that occur during SE induced from IZE of *C. papaya*.

In *Arabidopsis thaliana*, PAT plays an important role in the formation of apical and radicular meristems during SE (Péret *et al.*, 2012). However, little is known regarding the

expression patterns of AIT and AET genes, and their possible role in the initiation and development of the SE process in tropical species such as *Carica papaya*.

Based on genome sequences available from *C. papaya*, as well as *LAX2*, *LAX3* and *PIN4* genes functionally characterized in *A. thaliana*, specific primer pairs were designed. For each gene family, the homologs existing in the *C. papaya* genome were demonstrated. Nucleic acid alignments showed that *LAX2*, *LAX3* and *PIN4* sequences shared high similarity with *Arabidopsis thaliana* sequences (Appendix A).

Real-time PCR is a preferred method for rapid and accurate methods for study gene expression analysis. Gene expression analysis is an important step to understand the roles of genes in developmental and cellular processes, such as the signaling and metabolic pathways (Hu *et al.*, 2009). Nevertheless, to accurately quantify gene expression, several experimental variations, such as quality and amount of starting material, presence of inhibitors in different sample materials, primer design, RNA extraction and retro-transcription efficiencies, should be taken into account (Ginzinger, 2002). Appropriate application of RT-qPCR requires accurate normalization through the use of reference genes (Zhu *et al.*, 2012). An ideal reference gene should be expressed at a constant level across various conditions and its expression is assumed to be unaffected by experimental parameters (Thellin *et al.*, 1999; Schmittgen & Zakrajsek, 2000). Moreover, the reference gene and the target gene should have similar ranges of expression in the samples to be analyzed (Cappelli *et al.*, 2008). Zhu *et al.* (2012) selected 21 candidate reference genes and evaluated their expression stability in 246 *C. papaya* fruit samples using three algorithms, geNorm, NormFinder and RefFinder. In general, the internal reference genes EIF (Eukaryotic initiation factor 4A), TBP1 (TATA binding protein 1) and TBP2 (TATA binding protein 2) demonstrated good performance under most experimental conditions, whereas the most widely used reference genes, *ACTIN* (Actin 2), 18S *rRNA* (18S

ribosomal RNA) and *GAPDH* (Glyceraldehyde-3-phosphate dehydrogenase) were not suitable in many experimental conditions (Zhu *et al.*, 2012). Therefore, the *CpEIF* gene was used as an internal reference gene in the present study.

In this study, the transcripts of all analyzed genes were detected in all samples of somatic embryos. The expression pattern of the selected auxin transporter genes during SE of *C. papaya* was evaluated using qRT-PCR. The variance expression level of most of the *CpLAX2*, *LAX3* and *CpPIN4* genes during the different developmental stages of somatic embryos suggested that they might be involved in the regulation of growth and development of somatic embryos of *Carica papaya*. Studies of gene expression in *Arabidopsis* showed that the *AtLAX2* gene has functions in vascular development and cell division in the Quiescent Center (Friml *et al.*, 2003). Conversely, *AtLAX3* regulates apical hook development and lateral root development (Swarup *et al.*, 2008). Also, *PIN* family genes have been previously elucidated to participate in growth and development in a variety of plant species (Friml *et al.*, 2002a; Schnabel & Frugoli, 2004).

In this study, the highest level of *LAX2* expression was registered in somatic embryos at day 158 of culturing (germination phase). The somatic embryos were germinated in media without auxin (i.e. without growth regulators) and most somatic embryos were at cotyledonary stage. During the maturation period, the reduction of *LAX2* transcript levels could be associated with the activation of the cellular division process which leads to subsequent callus formation observed from the 70 days of culture. Similarly, Chen *et al.* (2001) found that the position of maximum cell divisions correlated with lower *LAX2* levels in tobacco leaf tissues. During the maturation phase, the fluctuation of transcript levels were in agreement with previous studies where most of these genes were involved in cell division control and had a probable involvement in developmental regulation (Ugartechea-Chirino *et al.*, 2009). The expression profile of *LAX2* showed two main

phases followed by a final decline. During the maturation phase, the relatively low and declining levels observed seem to be associated with the cellular dedifferentiation phase confirmed by a histological study (previous chapter). The second phase, characterized by an important increase to peak at day 90-105, seems to correspond to the activation of cell proliferation and callus formation processes observed in histological slide (previous chapter). This result is in good agreement with the observation that *LAX2* is mainly expressed in dividing tissues. The final decline (observed after day 105) might correlate with the progressive reduction of cell divisions. The variety of expression patterns indicate *LAX2* plays different roles during the development of somatic embryos of *Carica papaya*. *AtLAX2* functions in vascular development and cell division in the QC (Péret *et al.*, 2012). Therefore, the expression was the highest after 158 days of culturing.

In *Arabidopsis*, *AtLAX1* and *AtLAX3* were highly induced by 2,4-D in the roots (Péret *et al.*, 2012). Both *AtLAX3* and *AtAUX1* coordinated the regulation of lateral root development by regulating the emergence and initiation of lateral root primordia. AtAUX1 and AtLAX3 were found to be high-affinity auxin transporters by auxin uptake experiments in heterologous expression systems (Yang *et al.*, 2006; Swarup *et al.*, 2008; Péret *et al.*, 2012). The *AUX /LAX* gene family affects phyllotactic patterning and is needed to establish the embryonic root cell organization and plant embryogenesis in *Arabidopsis* (Bainbridge *et al.*, 2008; Ugartechea-Chirino *et al.*, 2009). The *ClLAX* genes might play similar or different roles during watermelon development because of their variety of expression patterns (Robert *et al.*, 2013). Our results showed that the auxin influx transporter (AIT) genes (*CpLAX 2, 3*) appeared to be more related to the development of somatic embryos at the torpedo and cotyledonary stages. The results of this experiment agreed with the previous study where the tissue-specific expression analysis of *ClLAX*, and *ClPIN* genes indicated that the transcriptional level of these auxin transporter genes expressed in the roots, cotyledons, leaves, shoots and flowers varied

greatly. *LAX* and *PIN* genes have been found to be involved in plant growth and development (Kaneda *et al.*, 2011; Péret *et al.*, 2012).

The PIN family genes play crucial roles in various aspects of developmental processes, including root meristem patterning, root hair growth, lateral root development, vascular bundle differentiation, phototropism and embryo development (Ganguly *et al.*, 2010; Robert *et al.*, 2013). Twenty-three members of the *PIN* gene family were identified in the soybean genome through homology searches (Wang *et al*., 2015). Five of the *Arabidopsis PINs (PIN1-4* and *PIN7*) are located in the plasma membrane and they play a prominent role in the directional, cell-to-cell auxin transport (Adamowski & Friml, 2015). The results in this research agreed with the previous research where the spatiotemporal expression patterns and the auxin-dependent cross regulation of their expressions made them functionally redundant and complementary in a variety of plant developmental processes, including embryogenesis and tissue differentiation (Michniewicz *et al.*, 2007). At*PIN4* expression is up-regulated in response to auxin signaling, most likely through the direct action of auxin response factors (Vieten *et al.*, 2005). *PIN4* expression is confined to the center of root meristem and is detectable during the globular stage of embryogenesis (Bassuner *et al.*, 2007). *AtPIN4* is essential for the establishment of an auxin gradient as it regulates both auxin homeostasis and patterning through sink-mediated auxin distribution in root tips (Friml *et al.*, 2002a). The results of this experiment demonstrated that the gene expression of *CpPIN4* during SE was not highly affected by the exogenous auxin (2,4-D) inserted to the medium and the effect of expression was mostly related to developmental stages rather than the auxin concentration. Also, there may be a possibility that the formation of callus at the base end of the explants affected the expression level of this gene. Furthermore, this result contradicted with the previous study where the gene expression of *CpPIN4* showed a

gradual increase in expression during the maturation process of embryos (Estrella-Maldonado *et al.*, 2018).

CHAPTER 6: GENERAL CONCLUSION

The best sterilization method involved treating *C. papaya* seeds with 70% ethanol for 2 min, then soaking in either 40% or 50% Clorox for 5 min, followed by soaking in 0.1 $HgCl_2$ for 10 min and finally soaking in 70% ethanol for 2 min.

The best media for the preservation and induction of somatic embryos from mature zygotic embryos in *C. papaya* was ½ MS supplemented with 10 mg L^{-1} 2,4-D whereby the callus grew continuously without any browning for more than 20 weeks. The addition of 18 mg L^{-1} of boric acid to ½ MS media augmented with 10 mg L^{-1} of 2,4-D enhanced the percentage of somatic embryos form mature zygotic embryos in *C. papaya* after 6 weeks of culturing.

Half strength MS media supplemented with 7.5 mg L^{-1} 2,4-D was the best medium for the induction of SE from IZE in *Carica papaya*. This media produced the highest average callus weight, average callus diameter and percentage of somatic embryos. The addition of 18 mg L^{-1} of boric acid to ½ MS media augmented with 10 mg L^{-1} of 2,4-D enhanced the average callus weight, average callus diameter and percentage of somatic embryos in *C. papaya* after 6 weeks of culturing.

M1 medium supplemented with 5 mg L^{-1} PG produced the highest number of large embryos (32.80 ± 1.31), small embryos (41.20 ± 3.56), globular-shaped embryos (26.20 ± 1.67) and cotyledonary stage embryos (13.20 ± 1.21). Maturation medium supplemented with 12% PEG has beneficial effect in the number and quality of large embryos. M2 medium supplemented with 12 % of PEG produced the highest number of large embryos (41.80 ± 1.68), heart-shaped embryos (10.00 ± 0.39), cup-shaped embryos (14.80 ± 1.39) and torpedo-shaped embryos (20.00 ± 1.78). However, no callus formation was observed at 12% of polyethylene glycol. M3 medium supplemented with 10 mg L^{-1}

PG produced the greatest number of large embryos (36.50 ± 0.89), torpedo-shaped embryos (22.75 ± 0.69), cup-shaped embryos (13.00 ± 1.20) and root formation embryos (11.50 ± 1.31).

Activated charcoal has inhibitory effect during germination phase. The largest percentage of shoot formation (62 ± 1.19%) was recorded for mature somatic embryos grown in M2 medium supplemented with 12% PEG after SF1 medium. The highest germination percentage (77.18 ± 9.49%) after elongation was observed for somatic embryos grown in M2 medium and transferred to SF1 medium.

The highest number of root (3.75 ± 0.52) was observed in R3 medium without callus at the base end of the stem explants. On the other hand, the highest length of root (10.2 ± 1.37 cm), shoot (8.00 ± 1.26 cm) and number of leaves (4.50 ± 0.68) were observed in R4 medium.

The best protocols to achieve *in vitro* micropropagation of *Carica papaya* 'Eksotika' using somatic embryogenesis. Half strength MS media supplemented with 10 mg L^{-1} 2,4-D and 18 mg L^{-1} of boric acid yielded somatic embryos with high efficiency. ½ MS medium augmented with 10 mg L^{-1} phloroglucinol was found suitable for embryo maturation. The well-formed embryos produced shoot formation on MS medium supplemented with 0.05% AC. The well-formed embryos elongated on MS medium supplemented with 1 mg L^{-1} GA_3, 0.5 mg L^{-1} IBA, 100 mg L^{-1} *Myo*-insitol and 3.76 mg L^{-1} riboflavin. The well-formed embryos produced root in MS medium after cultured for four days in full strength MS medium supplemented with 2 mg L^{-1} IBA. After rooting phase, *in vitro* plantlets were acclimatized in peatmos soil

4.5% SA with 50mM $CaCl_2$ that was soaked for 10 min was found to be the best combination for the formation of ideal, uniform, and good quality beads. The somatic embryos encapsulated in MS medium without calcium showed the highest percentage (39%) of shoot formation and lowest percentage (11%) of callus formation at the base end of somatic embryos. MS media supplemented with NAA and BAP increased the percentage of callus formation at the base end of somatic embryos during regeneration of artificial seeds.

The ontogeny and anatomy of somatic embryo structures during the induction phase of SE was examined using light microscopy. Histological study, under light microscopy, of the continuous embryogenic cultures demonstrated two ways in which somatic embryos developed. The first was that somatic embryos developed from single epidermal cells and the second was that somatic embryos developed from groups of cells. ½ MS media supplemented with 10 mg L^{-1} 2,4-D was enhanced callus formation at the base end of somatic embryos and not suitable for the induction and maturation of SE from IZE of *C. papaya.*

Active gene expression of all selected genes, namely *CpLAX2*, *CpLAX3* and *CpPIN4*, were detected in all somatic embryos collected during the maturation and germination phases. The expression level of *CpLAX3* demonstrated the highest level of expression compared to the rest of the genes used in the experiment while *CpPIN4* demonstrated the lowest level of expression. All genes (*CpLAX2*, *CpLAX3* and *CpPIN4*) were involved in the development and growth of somatic embryos. Gene expression of *CpLAX2* showed the highest level of gene expression in the sample collected after 158 days of culturing in the germination medium while the lowest level of gene expression was recorded after 59 days of culturing in the induction medium (first day of culturing in the maturation medium). Gene expression of *CpLAX3* showed the highest level of gene expression in the

sample collected after 130 days of culturing in the induction medium while the lowest level of gene expression was recorded after 59 days of culturing in the induction medium (first day of culturing in the maturation medium). Gene expression of *CpPIN4* showed the highest level of gene expression in the sample collected after 70 days of culturing in the maturation medium while the lowest level of gene expression was recorded after 130 days of culturing in the induction medium. The level of gene expression showed fluctuations in the expression of *CpPIN4* gene.

REFERENCES

Abdollahi, M. R., Eshaghi, Z. C., & Majdi, M. (2017). Improvement in androgenic response of borage (*Borago officinalis* L.) cultured anthers using antibrowning agents and picloram. *Turkish Journal of Biology, 41*(2), 354-363.

Abrahamian, P., & Kantharajah, A. (2011). Effect of vitamins on *in vitro* organogenesis of plant. *American Journal of Plant Sciences*, 2(05), 669-674.

Abreu, I. S., Carvalho, C. R., & Clarindo, W. R. (2014). Massal induction of *Carica papaya* L.'Golden'somatic embryos and somaclone screening by flow cytometry and cytogenetic analysis. *Cytologia (Tokyo), 79*(4), 475-484.

Adamowski, M., & Friml, J. (2015). PIN-dependent auxin transport: Action, regulation, and evolution. *The Plant Cell, 27*(1), 20-32.

Ahmad, N., & Anis, M. (2010). Direct plant regeneration from encapsulated nodal segments of *Vitex negundo*. *Biologia Plantarum, 54*(4), 748-752.

Ahmed, M. R., Anis, M., & Al-Etta, H. A. (2015). Encapsulation technology for short-term storage and germplasm exchange of *Vitex trifolia* L. *Rendiconti Lincei, 26*(2), 133-139.

Aikpokpodion, P. O. (2012). Assessment of genetic diversity in horticultural and morphological traits among papaya (*Carica papaya*) accessions in Nigeria. *Fruits, 67*(3), 173-187.

Al-Khayri, J., & Al-Bahrany, A. (2004). Growth, water content, and proline accumulation in drought-stressed callus of date palm. *Biologia Plantarum, 48*(1), 105-108.

Anandan, R., Sudhakar, D., Balasubramanian, P., & Gutieirrez-Mora, A. (2012). *In vitro* somatic embryogenesis from suspension cultures of *Carica papaya* L. *Scientia Horticulturae, 136*, 43-49.

Anandan, R., Thirugnanakumar, S., Sudhakar, D., & Balasubramanian, P. (2011). *In vitro* organogenesis and plantlet regeneration of (*Carica papaya* L.). *Journal of Agricultural Technology, 7*(5), 1339-1348.

Antonietta, G. M., Emanuele, P., & Alvaro, S. (1998). Effects of encapsulation on *Citrus reticulata* Blanco somatic embryo conversion. *Plant Cell Tissue and Organ Culture, 55*(3), 235-238.

Aquea, F., Poupin, M. J., Matus, J. T., Gebauer, M., Medina, C., & Arce-Johnson, P. (2008). Synthetic seed production from somatic embryos of *Pinus radiata*. *Biotechnology Letters, 30*(10), 1847-1852.

Ara, H., Jaiswal, U., & Jaiswal, V. (1999). Germination and plantlet regeneration from encapsulated somatic embryos of mango (*Mangifera indica* L.). *Plant Cell Report, 19*(2), 166-170.

Araujo, P. G., Rossi, M., Jesus, E. M., Saccaro, N. L., Kajihara, D., Massa, R., . . . Chabregas, S. M. (2005). Transcriptionally active transposable elements in recent hybrid sugarcane. *The Plant Journal, 44*(5), 707-717.

Arora, I. K., & Singh, R. N. (1978). Growth hormones and *in vitro* callus formation of papaya. *Scientia Horticulturae, 8*(4), 357-361.

Ascencio-Cabral, A., Gutierrez-Pulido, H., Rodriguez-Garay, B., & Gutierrez-Mora, A. (2008). Plant regeneration of *Carica papaya* L. Through somatic embryogenesis in response to light quality, gelling agent and phloridzin. *Scientia Horticulturae, 118*(2), 155-160.

Azad, M. A., Rabbani, M. G., & Amin, L. (2012). Plant regeneration and somatic embryogenesis from immature embryos derived through interspecific hybridization among different *Carica* species. *International Journal of Molecular Sciences, 13*(12), 17065-17076.

Azad, M. A. K., Rabbani, M. G., & Amin, L. (2013). Effects of different culture media and flower bud size on haploid plant production through anther culture of three *Carica* species. *Journal of Food Agriculture & Environment, 11*(2), 296-300.

Azimi, M., O'Brien, C., Ashmore, S., & Drew, R. (2005). Cryopreservation of papaya germplasm. In W. C. Chang & R. Drew (Eds.), *Proceedings of the second international symposium on biotechnology of tropical and subtropical species* (pp. 43-50). Leuven 1: International Society Horticultural Science.

Badillo, V. (2000). *Carica* L. vs. *Vasconcella* St. Hil.(Caricaceae) con la rehabilitación de este último. *Ernstia, 10*(2), 74-79.

Bainbridge, K., Guyomarc'h, S., Bayer, E., Swarup, R., Bennett, M., Mandel, T., & Kuhlemeier, C. (2008). Auxin influx carriers stabilize phyllotactic patterning. *Genes & Development, 22*(6), 810-823.

Bandurski, R. S., Cohen, J. D., Slovin, J. P., & Reinecke, D. M. (1995). Auxin biosynthesis and metabolism. In *Plant Hormones* (pp.39-65): Springer, Dordrecht.

Baskaran, P., Kumari, A., & Van Staden, J. (2015). Embryogenesis and synthetic seed production in *Mondia whitei*. *Plant Cell Tissue and Organ Culture, 121*(1), 205-214.

Bassuner, B. M., Lam, R., Lukowitz, W., & Yeung, E. C. (2007). Auxin and root initiation in somatic embryos of *Arabidopsis*. *Plant Cell Reports, 26*(1), 1-11.

Behrendt, U., & Zoglauer, K. (1996). Boron controls suspensor development in embryogenic cultures of *Larix decidua*. *Physiologia Plantarum, 97*(2), 321-326.

Benková, E., Michniewicz, M., Sauer, M., Teichmann, T., Seifertová, D., Jürgens, G., & Friml, J. (2003). Local, efflux-dependent auxin gradients as a common module for plant organ formation. *Cell, 115*(5), 591-602.

Bevitori, R., Popielarska-Konieczna, M., Dos Santos, E., Grossi-de-Sá, M. F., & Petrofeza, S. (2014). Morpho-anatomical characterization of mature embryo-derived callus of rice (*Oryza sativa* L.) suitable for transformation. *Protoplasma, 251*(3), 545-554.

Bharathy, P., & Agrawal, D. C. (2008). High frequency occurrence of single cotyledonary embryo morphotype and repetitive somatic embryogenesis in 'Thompson seedless' crossed with seven grapevine male parents. *Vitis, 47*(3), 169-174.

Bhattacharya, J., & Khuspe, S. (2000). 2, 4, 5-T induced somatic embryogenesis in papaya (*Carica papaya* L.). *Journal of Applied Horticulture, 2*(2), 84-87.

Bhattacharya, J., Khuspe, S., Renukdas, N., & Rawal, S. (2002). Somatic embryogenesis and plant regeneration from immature embryo explant of papaya (*Carica papaya* L. cv. Washington and honey dew). *Indian Journal of Experimental Biology, 40*(5), 624-627.

Bhattacharya, J., & Khuspe, S. S. (2001). *In vitro* and *in vivo* germination of papaya (*Carica papaya* L.) seeds. *Scientia Horticulturae, 91*(1-2), 39-49.

Bopana, N., & Saxena, S. (2008). *In vitro* propagation of a high value medicinal plant: *Asparagus racemosus* Willd. *In Vitro Cellular & Developmental Biology-Plant, 44*(6), 525-532.

Bordallo, P. N., Silva, D. H., Maria, J., Cruz, C. D., & Fontes, E. P. (2004). Somaclonal variation on *in vitro* callus culture potato cultivars. *Horticultura Brasileira, 22*(2), 300-304.

Bukhori, M. F. M. (2013). Improved protocol for high frequency plant regeneration through somatic embryogenesis in *Carica papaya*. *Research in Biotechnology, 4*(5), 9-19.

Campostrini, E., & Yamanishi, O. K. (2001). Estimation of papaya leaf area using the central vein length. *Scientia Agricola, 58*(1), 39-42.

Canhoto, J., Mesquita, J., & Cruz, G. (1996). Ultrastructural changes in cotyledons of Pineappple guava (Myrtaceae) during somatic embryogenesis. *Annals of Botany*, *78*(4), 513-521.

Cappelli, K., Felicetti, M., Capomaccio, S., Spinsanti, G., Silvestrelli, M., & Supplizi, A. V. (2008). Exercise induced stress in horses: Selection of the most stable reference genes for quantitative RT-PCR normalization. *BMC Molecular Biology, 9*(1), 49.

Carvalho, F. A., & Renner, S. S. (2012). A dated phylogeny of the papaya family (Caricaceae) reveals the crop's closest relatives and the family's biogeographic history. *Molecular Phylogenetics and Evolution, 65*(1), 46-53.

Castillo, B., Smith, M., & Yadava, U. (1998). Plant regeneration from encapsulated somatic embryos of *Carica papaya* L. *Plant Cell Report, 17*(3), 172-176.

Castroluna, A., Ruiz, O., Quiroga, A., & Pedranzani, H. (2014). Effects of salinity and drought stress on germination, biomass and growth in three varieties of *Medicago sativa* L. *Avances en Investigación Agropecuaria, 18*(1), 39‑50.

Catarina, C. S., dos Santos Olmedo, A., de Andrade Meyer, G., Macedo, J., De Amorim, W., & Viana, A. M. (2004). Repetitive somatic embryogenesis of *Ocotea catharinensis* Mez.(Lauraceae): Effect of somatic embryo developmental stage and dehydration. *Plant Cell Tissue and Organ Culture, 78*(1), 55-62.

Ceasar, S. A., Maxwell, S. L., Prasad, K. B., Karthigan, M., & Ignacimuthu, S. (2010). Highly efficient shoot regeneration of *Bacopa monnieri* L. using a two-stage culture procedure and assessment of genetic integrity of micropropagated plants by RAPD. *Acta Physiologiae Plantarum, 32*(3), 443-452.

Chan, L., & Teo, C. (1993). *In vitro* production of multiple shoots in papaya as affected by plant tissue maturity and genotype. *MARDI Research Journal, 21*, 105-111.

Chan, L. K., & Teo, C. K. (1994). Culture of papaya explant in solid-liquid media sequence as a rapid method for producing multiple shoots. *Pertanika Journal of Tropical Agricultural Science, 17*(2), 103-106.

Chan, Y. K. (2009). Breeding papaya (*Carica papaya* L.). In *Breeding plantation tree crops: Tropical species* (pp. 121-159), New York, NY, Springer.

Chan, Y. and Raveendranathan (1990). Proceedings of the MARDI-MAPPS Seminar on Eksotika Papaya Problems and Prospects. In *MARDI-MAPPS Seminar on Eksotika Papaya: Problems and Prospects*. Johor Baharu, Malaysia.

Chan, Y. K. (2002). Breeding papaya for resistance to ringspot virus disease in Malaysia. In *XXVI International Horticultural Congress: Citrus and Other Subtropical and Tropical Fruit Crops: Issues, Advances and 632* (57-64).

Chan, Y., Lee, H., & Rusna, I. (2007). Irradiation-induced variations in M2 populations of Eksotika papaya. *Journal of Tropical Agriculture and Food Science, 35*(1), 49.

Chan, Y., & Mak, C. (1993). Resistance of six papaya varieties and their hybrids to Malformed top disease. *MARDI Research Journal, 21*, 7-13.

Chan, Y. K., Lee, H. K., & Isa, R. (2015). Irradiation-induced variations in M2 populations of Eksotika papaya (variasi iradiasi-aruhan dalam populasi M2 betik Eksotika). *Journal of Tropical Agriculture and Food Science, 35*, 49-57.

Chen, J. G., Shimomura, S., Sitbon, F., Sandberg, G., & Jones, A. M. (2001). The role of auxin-binding protein 1 in the expansion of tobacco leaf cells. *The Plant Journal, 28*(6), 607-617.

Chen, M., Chen, C., Wang, D., & Chen, F. (1991). Somatic embryogenesis and plant regeneration from immature embryos of *Carica papaya*× *Carica cauliflora* cultured *in vitro*. *Canadian Journal of Botany, 69*(9), 1913-1918.

Chen, M., Wang, P., & Maeda, E. (1987). Somatic embryogenesis and plant regeneration in *Carica papaya* L. Tissue culture derived from root explants. *Plant Cell Reports, 6*(5), 348-351.

Chengalrayan, K., Mhaske, V., & Hazra, S. (1997). High-frequency conversion of abnormal peanut somatic embryos. *Plant Cell Reports, 16*(11), 783-786.

Cheruvathur, M. K., Kumar, G. K., & Thomas, T. D. (2013). Somatic embryogenesis and synthetic seed production in *Rhinacanthus nasutus* (L.) Kurz. *Plant Cell Tissue and Organ Culture, 113*(1), 63-71.

Clarindo, W. R., de Carvalho, C. R., Araujo, F. S., de Abreu, I. S., & Otoni, W. C. (2008). Recovering polyploid papaya *in vitro* regenerants as screened by flow cytometry. *Plant Cell Tissue and Organ Culture, 92*(2), 207-214.

Currais, L., Loureiro, J., Santos, C., & Canhoto, J. M. (2013). Ploidy stability in embryogenic cultures and regenerated plantlets of Tamarillo. *Plant Cell Tissue and Organ Culture, 114*(2), 149-159.

da Silva, J. A. T., Dobránszki, J., & Ross, S. (2013). Phloroglucinol in plant tissue culture. *In Vitro Cellular & Developmental Biology-Plant, 49*(1), 1-16.

Danso, K., & Ford-Lloyd, B. (2003). Encapsulation of nodal cuttings and shoot tips for storage and exchange of *Cassava* germplasm. *Plant Cell Reports, 21*(8), 718-725.

Daud, N., Faizal, A., & Geelen, D. (2013). Adventitious rooting of *Jatropha curcas* L. Is stimulated by phloroglucinol and by red led light. *In Vitro Cellular & Developmental Biology-Plant, 49*(2), 183-190.

De-la-Peña, C., Nic-Can, G. I., Galaz-Ávalos, R. M., Avilez-Montalvo, R., & Loyola-Vargas, V. M. (2015). The role of chromatin modifications in somatic embryogenesis in plants. *Frontiers in Plant Science, 6*, 635.

De Fossard, R., Myint, A., & LEE, E. C. (1974). A broad spectrum tissue culture experiment with tobacco (*Nicotiana tabacum)* pith tissue callus. *Physiologia Plantarum, 31*(2), 125-130.

De Klerk, G. J., Guan, H., Huisman, P., & Marinova, S. (2011). Effects of phenolic compounds on adventitious root formation and oxidative decarboxylation of applied indoleacetic acid in Malus 'Jork 9'. *Plant Growth Regulation, 63*(2), 175-185.

Drew, R. (1987). The effects of medium composition and cultural conditions on *in vitro* root initiation and growth of papaya (*Carica papaya* L.). *Journal of Horticultural Science, 62*(4), 551-556.

Drew, R. A. (1988). Rapid clonal propagation of papaya *in vitro* from mature field-grown trees. *Journal of Horticultural Science, 23*(3), 609-611.

Drew, R. A. (1992). Improved techniques for *in vitro*-propagation and germplasm storage of papaya. *Journal of Horticultural Science, 27*(10), 1122-1124.

Drew, R. A., McComb, J. A., & Considine, J. A. (1993). Rhizogenesis and root growth of *Carica papaya* L. *In vitro* in relation to auxin sensitive phases and use of riboflavin. *Plant Cell Tissue and Organ Culture, 33*(1), 1-7.

Drew, R. A., Simpson, B. W., & Osborne, W. J. (1991). Degradation of exogenous Indole-3-butyric acid and riboflavin and their influence on rooting response of papaya *in vitro*. *Plant Cell Tissue and Organ Culture, 26*(1), 29-34.

Ebert, A., Taylor, F., & Blake, J. (1993). Changes of 6-Benzylaminopurine and 2,4-Dichlorophenoxyacetic acid concentrations in plant tissue culture media in the presence of activated charcoal. *Plant Cell Tissue and Organ Culture, 33*(2), 157-162.

El Dawayati, M. M., Bar, O. H. A. E., Zaid, Z. E., & El Din, A. F. Z. (2012). *In vitro* morpho-histological studies of newly developed embryos from abnormal malformed embryos of date palm cv. Gundila under desiccation effect of polyethelyne glycol treatments. *Annals of Agricultural Sciences, 57*(2), 117-128.

Estrella-Maldonado, H., Ortíz, G. F., León, A. C. C., Zapata, L. C. R., May, C. T., y Gil, F. E., . . . Santamaría, J. M. (2016). The papaya *CpAUX1/LAX* and *CpPIN* genes: Structure, phylogeny and expression analysis related to root formation on *in vitro* plantlets. *Plant Cell Tissue and Organ Culture, 126*(2), 187-204.

Estrella-Maldonado, H., Posada-Pérez, L., Talavera, M. C., Barredo, P. F., Gómez-Kosky, R., & Santamaría, J. M. (2018). The expression of *CpAUX1/LAXs* and most of the long-distance *CpPIN*s genes increases as the somatic embryogenesis process develops in *C. papaya* cv. Red maradol. *Journal of Plant Growth Regulation*, *37*(2), 502-516.

Farzana, A., Palkadapala, P., Meddegoda, K., Samarajeewa, P., & Eeswara, J. (2008). Somatic embryogenesis in papaya (*Carica papaya* L. cv. Rathna). *Journal of the National Science Foundation of Sri Lanka, 36*(1), 41-50.

Fehér, A. (2005). Why somatic plant cells start to form embryos?. In *Somatic embryogenesis* (pp. 85-101). Springer, Berlin, Heidelberg.

Feher, A., Pasternak, T. P., & Dudits, D. (2003). Transition of somatic plant cells to an embryogenic state. *Plant Cell Tissue and Organ Culture, 74*(3), 201-228.

Fernando, J. A., Melo, M., Soares, M. K., & Appezzato-da-Glória, B. (2001). Anatomy of somatic embryogenesis in *Carica papaya* L. *Brazilian Archives of Biology and Technology, 44*(3), 247-255.

Fisher, J. B. (1980). The vegetative and reproductive structure of papaya (*Carica papaya*). *Lyonia, 1*(4), 191-208.

Fitch, M. (1995). Somatic embryogenesis in papaya (*Carica papaya* L.) In *Somatic embryogenesis and synthetic seed I* (pp. 260-279). Springer, Berlin, Heidelberg.

Fitch, M. M. (1993). High frequency somatic embryogenesis and plant regeneration from papaya hypocotyl callus. *Plant Cell Tissue and Organ Culture, 32*(2), 205-212.

Fitch MMM (2005) 6.1 *Carica papaya* Papaya. In: Biotechnology of fruit and nut crops Litz RE (ed), (pp174–207). CABI, Cambridge, London, United Kingdom.

Fitch, M. M., & Manshardt, R. M. (1990). Somatic embryogenesis and plant regeneration from immature zygotic embryos of papaya (*Carica papaya* L.). *Plant Cell Reports, 9*(6), 320-324.

Fleischer, A., Titel, C., & Ehwald, R. (1998). The boron requirement and cell wall properties of growing and stationary suspension-culture *Dchenopodium album* L. Cells. *Plant Physiology, 117*(4), 1401-1410.

Food and Agriculture Organization of the United Nations. (2018). F*AOSTAT statistics database.* Retrived on February 22, 2018 at*:* http://www.fao.org/faostat/en/#compare.

Friml, J., Benková, E., Blilou, I., Wisniewska, J., Hamann, T., Ljung, K., . . . Jürgens, G. (2002a). *AtPIN4* mediates sink-driven auxin gradients and root patterning in *Arabidopsis*. *Cell, 108*(5), 661-673.

Friml, J., Vieten, A., Sauer, M., Weijers, D., Schwarz, H., Hamann, T., . . . Jürgens, G. (2003). Efflux-dependent auxin gradients establish the apical–basal axis of *Arabidopsis*. *Nature, 426*(6963), 147-153.

Friml, J., Wiśniewska, J., Benková, E., Mendgen, K., & Palme, K. (2002b). Lateral relocation of auxin efflux regulator *PIN3* mediates tropism in *Arabidopsis*. *Nature, 415*(6873), 806-809.

Gälweiler, L., Guan, C., Müller, A., Wisman, E., Mendgen, K., Yephremov, A., & Palme, K. (1998). Regulation of polar auxin transport by *AtPIN1* in *Arabidopsis* vascular tissue. *Science, 282*(5397), 2226-2230.

Gamborg, O. L. c., Miller, R. A., & Ojima, K. (1968). Nutrient requirements of suspension cultures of soybean root cells. *Experimental Cell Research, 50*(1), 151-158.

Ganapathi, T., Srinivas, L., Suprasanna, P., & Bapat, V. (2001). Regeneration of plants from alginate-encapsulated somatic embryos of banana cv. Rasthali (Musa spp. AAB group). *In vitro Cellular & Developmental Biology-Plant, 37*(2), 178-181.

Ganguly, A., Lee, S. H., Cho, M., Lee, O. R., Yoo, H., & Cho, H.-T. (2010). Differential auxin-transporting activities of PIN-FORMED proteins in *Arabidopsis* root hair cells. *Plant Physiology, 153*(3), 1046-1061.

Gantait, S., Kundu, S., Ali, N., & Sahu, N. C. (2015). Synthetic seed production of medicinal plants: A review on influence of explants, encapsulation agent and matrix. *Acta Physiologiae Plantarum, 37*(5), 98.

George, E. F., Hall, M. A., & De Klerk, G.-J. (2008). The components of plant tissue culture media i: Macro-and micro-nutrients. In *Plant propagation by tissue culture* (pp. 65-113): Springer, Netherlands.

Ghanbarali, S., Abdollahi, M. R., Zolnorian, H., Moosavi, S. S., & Seguí-Simarro, J. M. (2016). Optimization of the conditions for production of synthetic seeds by encapsulation of axillary buds derived from minituber sprouts in potato (*Solanum tuberosum*). *Plant Cell Tissue and Organ Culture, 126*(3), 449-458.

Ginzinger, D. G. (2002). Gene quantification using real-time quantitative PCR: An emerging technology hits the mainstream. *Experimental Hematology, 30*(6), 503-512.

Goldbach, H., & Amberger, A. (1986). Influence of boron deficiency on 3H indole-3yl-acetic acid uptake and efflux in cell cultures of *Daucus carota* L. *Plant Growth Regulation, 4*(1), 81-86.

Gupta, P. K., & Durzan, D. J. (1987). Biotechnology of somatic polyembryogenesis and plantlet regeneration in Loblolly pine. *Nature Biotechnology, 5*(2), 147.

Gupta, S. D., & Jatothu, B. (2013). Fundamentals and applications of light-emitting diodes (LEDs) in *in vitro* plant growth and morphogenesis. *Plant Biotechnology Reports, 7*(3), 211-220.

Haitao, L., & Jing, W. (2017). Genome-wide analysis of the auxin/Indoleacetic acid (*AUX/IAA*) gene family in allotetraploid rapeseed (*Brassica napus* L.). *BMC Plant Biology*, *17*(1), 204.

Halhouli, K. A., Darwish, N. A., & Al-Dhoon, N. M. (1995). Effects of pH and inorganic salts on the adsorption of phenol from aqueous systems on activated decolorizing charcoal. *Separation Science and Technology, 30*(17), 3313-3324.

Halperin, W., & Wetherell, D. (1964). Adventive embryony in tissue cultures of the wild carrot, *Daucus carota*. *American Journal of Botany*, 274-283.

Hassan, N. S., Shaaban, L. D., Hashem, E., & Seleem, E. E. (2004). *In vitro* selection for water stress tolerant callus line of *Helianthus annus* L. cv Myak. *International Journal of Agriculture and Biology*, *6*(1), 13-18.

Hazarika, B. (2006). Morpho-physiological disorders in *in vitro* culture of plants. *Scientia Horticulturae, 108*(2), 105-120.

Heringer, A. S., Vale, E. M., Barroso, T., Santa-Catarina, C., & Silveira, V. (2013). Polyethylene glycol effects on somatic embryogenesis of papaya hybrid UENF/CALIMAN 01 seeds. *Theoretical and Experimental Plant Physiology, 25*(2), 116-124.

Hossain, M., Rahman, S. M., Islam, R., & Joarder, O. I. (1993). High-efficiency plant-regeneration from petiole explants of *Carica papaya* L. through organogenesis. *Plant Cell Reports, 13*(2), 99-102.

Hu, H., & Brown, P. H. (1994). Localization of boron in cell walls of squash and tobacco and its association with pectin (evidence for a structural role of boron in the cell wall). *Plant Physiology, 105*(2), 681-689.

Hu, R., Fan, C., Li, H., Zhang, Q., & Fu, Y. F. (2009). Evaluation of putative reference genes for gene expression normalization in soybean by quantitative real-time RT-PCR. *BMC Molecular Biology, 10*(1), 93.

Huang, B. Q., & Yeung, E. C. (2015). Chemical and physical fixation of cells and tissues: An overview. In *Plant microtechniques and protocols* (pp. 23-43): Springer Cham.

Ipekci, Z., & Gozukirmizi, N. (2003). Direct somatic embryogenesis and synthetic seed production from *Paulownia elongata*. *Plant Cell Reports, 22*(1), 16-24.

Ishii, K., Thakur, R., & Jain, S. M. (1999). Somatic embryogenesis and evaluation of variability in somatic seedlings of *Quercus serrata* by RAPD markers. In *Somatic embryogenesis in woody plants* (pp. 403-414): Springer, Dordrecht.

Jayasankar, S., Bondada, B. R., Li, Z., & Gray, D. (2003). Comparative anatomy and morphology of *Vitis vinifera* (Vitaceae) somatic embryos from solid and liquid culture derived proembryogenic masses. *American Journal of Botany, 90*(7), 973-979.

Jin, S., Mushke, R., Zhu, H., Tu, L., Lin, Z., Zhang, Y., & Zhang, X. (2008). Detection of somaclonal variation of cotton (*Gossypium hirsutum*) using cytogenetics, flow cytometry and molecular markers. *Plant Cell Reports, 27*(8), 1303-1316.

Johansen, D. A. (1940). *Plant microtechnique*. McGraw-Hill Book Company, London, United Kingdom.

Jordan, M. (1986). Somatic embryogenesis from cell suspension cultures in *Carica candamarcensis*. *Plant Cell Tissue and Organ Culture, 7*(3), 257-261.

Jürgens, G. (2001). Apical–basal pattern formation in *Arabidopsis* embryogenesis. *The EMBO Journal, 20*(14), 3609-3616.

Kadleček, P., Tichá, I., Haisel, D., Čapková, V., & Schäfer, C. (2001). Importance of *in vitro* pretreatment for *ex vitro* acclimatization and growth. *Plant Science, 161*(4), 695-701.

Kaity, A., Ashmore, S., Drew, R. A., & Dulloo, M. (2008). Assessment of genetic and epigenetic changes following cryopreservation in papaya. *Plant Cell Reports, 27*(9), 1529-1539.

Kaity, A., Parisi, A., Ashmore, S., & Drew, R. (2009). Root initiation and acclimatization of papaya plants. *Acta Horticulturae,* 812, 387-394.

Kakani, A., Li, G., & Peng, Z. (2009). Role of *AUX1* in the control of organ identity during *in vitro* organogenesis and in mediating tissue specific auxin and cytokinin interaction in *Arabidopsis*. *Planta, 229*(3), 645-657.

Kamińska, M., Gołębiewski, M., Tretyn, A., & Trejgell, A. (2017). Efficient long-term conservation of taraxacum pieninicum synthetic seeds in slow growth conditions. *Plant Cell Tissue and Organ Culture*, *132*(3), 469-478.

Kaneda, M., Schuetz, M., Lin, B. S., Chanis, C., Hamberger, B., Western, T., . . . Samuels, A. (2011). ABC transporters coordinately expressed during lignification of *Arabidopsis* stems include a set of ABCBs associated with auxin transport. *Journal of Experimental Botany, 62*(6), 2063-2077.

Kavitha, P. S., Chezhiyan, N., & Kumar, N. (2010). Regeneration of papaya embryos under *in vitro* condition. *Acta Horticulturae, 851,* 213-218.

Khalid, N., Chee, W. W., Bokhari, F., Kiat, T. S., Idris, F. D., Mohamad, M., . . . Rahman, N. A. (2007). Banana, gingers and papaya cell cultures for high throughput agriculture. *Asia-Pacific Journal of Molecular Biology and Biotechnology*, *18*(1), 73-74.

Kim, K. M., Kim, M. Y., Yun, P. Y., Chandrasekhar, T., Lee, H. Y., & Song, P. S. (2007). Production of multiple shoots and plant regeneration from leaf segments of fig tree (*Ficus carica* L.). *Journal of Plant Biology, 50*(4), 440-446.

Kim, M., Moore, P., Zee, F., Fitch, M. M., Steiger, D., Manshardt, R., . . . Ming, R. (2002). Genetic diversity of *Carica papaya* as revealed by AFLP markers. *Genome, 45*(3), 503-512.

Knudson, L. (1946). A new nutrient solution for the germination of orchid seed. *American Orchid Society Bulletin, 15*, 214-217.

Koehler, A. D., Carvalho, C. R., Abreu, I. S., & Clarindo, W. R. (2013). Somatic embryogenesis from leaf explants of hermaphrodite *Carica papaya*: A new approach for clonal propagation. *African Journal of Biotechnology, 12*(18), 2386.

Kong, D., Shen, H., & Li, N. (2012). Influence of $AgNO_3$ on somatic embryo induction and development in Manchurian ash (*Fraxinus mandshurica* Rupr.). *African Journal of Biotechnology, 11*(1), 120-125.

Kong, L., & Yeung, E. C. (1992). Development of white spruce somatic embryos: Ii. Continual shoot meristem development during germination. *In Vitro Cellular & Developmental Biology - Plant, 28*(3), 125-131.

Körbes, A. P., & Droste, A. (2005). Carbon sources and polyethylene glycol on soybean somatic embryo conversion. *Pesquisa Agropecuária Brasileira, 40*(3), 211-216.

Kozai, T., Xiao, Y., Nguyen, Q. T., Afreen, F., & Zobayed, S. M. (2005). Photoautotrophic (sugar-free medium) micropropagation systems for large-scale commercialization. *Propagation of Ornamental Plants, 5*(1), 23-34.

Krajňáková, J., Häggman, H., & Gömöry, D. (2009). Effect of sucrose concentration, polyethylene glycol and activated charcoal on maturation and regeneration of *Abies cephalonica* somatic embryos. *Plant Cell Tissue and Organ Culture, 96*(3), 251.

Křeček, P., Skůpa, P., Libus, J., Naramoto, S., Tejos, R., Friml, J., & Zažímalová, E. (2009). The PIN-formed (PIN) protein family of auxin transporters. *Genome Biology, 10*(12), 249.

Krishna, H., Alizadeh, M., Singh, D., Singh, U., Chauhan, N., Eftekhari, M., & Sadh, R. K. (2016). Somaclonal variations and their applications in horticultural crops improvement. *3 Biotech, 6*(1), 54.

Kumar, G. K., & Thomas, T. D. (2012). High frequency somatic embryogenesis and synthetic seed production in *Clitoria ternatea* Linn. *Plant Cell Tissue and Organ Culture, 110*(1), 141-151.

Kumar, V., Gururaj, H., Prasad, B. N., Giridhar, P., & Ravishankar, G. (2005). Direct shoot organogenesis on shoot apex from seedling explants of *Capsicum annuum* L. *Scientia Horticulturae, 106*(2), 237-246.

Kurczyńska, E. U., Gaj, M. D., Ujczak, A., & Mazur, E. (2007). Histological analysis of direct somatic embryogenesis in *Arabidopsis thaliana* (L.) Heynh. *Planta, 226*(3), 619-628.

Lai, C. C., Yeh, S. D., & Yang, J. S. (2000). Enhancement of papaya axillary shoot proliferation *in vitro* by controlling the available ethylene. *Botanical Bulletin of Academia Sinica, 41*(3), 203-212.

Langhansova, L., Konradova, H., & Vaněk, T. (2004). Polyethylene glycol and abscisic acid improve maturation and regeneration of *Panax ginseng* somatic embryos. *Plant Cell Reports., 22*(10), 725-730.

Larkin, P. J., Davies, P. A., & Tanner, G. J. (1988). Nurse culture of low numbers of *Medicago* and *Nicotiana* protoplasts using calcium alginate beads. *Plant Science, 58*(2), 203-210.

Lazzeri, P. A., Hildebrand, D. F., & Collins, G. B. (1987). Soybean somatic embryogenesis: Effects of hormones and culture manipulations. *Plant Cell, Tissue Organ and Culture, 10*(3), 197-208.

Leustek, T., & Kirby, E. G. (1988). The influence of glutamine on growth and viability of cell suspension cultures of Douglas-fir after exposure to polyethylene glycol. *Tree Physiology, 4*(4), 371-380.

Leyser, O. (2005). Auxin distribution and plant pattern formation: How many angels can dance on the point of PIN?. *Cell, 121*(6), 819-822.

Li, D., Zhang, J., & Merkle, S. A. (2014). Induction of green ash embryogenic cultures with potential for scalable somatic embryo production using suspension culture. *Trees, 28*(1), 253-262.

Li, X. Y., Huang, F. H., Murphy, J. B., & Gbur, E. E. (1998). Polyethylene glycol and maltose enhance somatic embryo maturation in Loblolly pine (*Pinus taeda* L.). *In vitro Cellular & Developmental Biology-Plant, 34*(1), 22-26.

Licea-Moreno, R. J., Contreras, A., Morales, A. V., Urban, I., Daquinta, M., & Gomez, L. (2015). Improved walnut mass micropropagation through the combined use of phloroglucinol and feeddha. *Plant Cell Tissue and Organ Culture, 123*(1), 143-154.

Linsmaier, E. M., & Skoog, F. (1965). Organic growth factor requirements of tobacco tissue cultures. *Physiologia Plantarum, 18*(1), 100-127.

Lisek, A., & Orlikowska, T. (2004). *In vitro* storage of strawberry and raspberry in calcium-alginate beads at 4°C. *Plant Cell Tissue and Organ Culture, 78*(2), 167-172.

Litz, R. E., & Conover, R. A. (1977). Tissue culture propagation of papaya. In *Proceedings of the Florida State Horticultural Society*, 90, 245-246.

Litz, R. E., & Conover, R. A. (1981). Effect of sex type, season, and other factors on *in vitro* establishment and culture of *Carica papaya* L explants. *Journal of the American Society for Horticultural Science, 106*(6), 792-794.

Liu, D. J., Chen, J. Y., & Lu, W. J. (2011). Expression and regulation of the early auxin-responsive *AUX/IAA* genes during strawberry fruit development. *Molecular Biology Reports, 38*(2), 1187-1193.

Liu, Z., Moore, P. H., Ma, H., Ackerman, C. M., Ragiba, M., Yu, Q., . . . Stiles, J. I. (2004). A primitive Y chromosome in papaya marks incipient sex chromosome evolution. *Nature, 427*(6972), 348-352.

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta ct}$ method. *Methods, 25*(4), 402-408.

Lloyd, G., & McCown, B. (1980). Commercially-feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot-tip culture. Combined Proceedings, International Plant Propagators' Society*, 30*, 421-427.

Luo, M., Liu, X., Singh, P., Cui, Y., Zimmerli, L., & Wu, K. (2012). Chromatin modifications and remodeling in plant abiotic stress responses. *Biochimica et Biophysica Acta (BBA)-Gene Regulatory Mechanisms, 1819*(2), 129-136.

Ma, L., Xie, L., Lin, G., Jiang, S., Chen, H., Li, H., . . . Xu, C. (2012). Histological changes and differences in activities of some antioxidant enzymes and hydrogen peroxide content during somatic embryogenesis of *Musa* AAA cv. Yueyoukang 1. *Scientia Horticulturae, 144*, 87-92.

Magdalita, P. M., Godwin, I. D., Drew, R. A., & Adkins, S. W. (1997). Effect of ethylene and culture environment on development of papaya nodal cultures. *Plant Cell Tissue and Organ Culture, 49*(2), 93-100.

Magdalita, P. M., & Mercado, C. P. (2003). Determination the sex of papaya for improved produced. *Bulletin Food and Fertilizer Technology Center Philippines*, 1-12.

Malabadi, R. B., Kumar, S. V., Mulgund, G. S., & Nataraja, K. (2011). Induction of somatic embryogenesis in papaya (*Carica papaya*). *Research in Biotechnology, 2*(5) 40-55.

Malabadi, R. B., & Van Staden, J. (2005). Storability and germination of sodium alginate encapsulated somatic embryos derived from the vegetative shoot apices of mature *Pinus patula* trees. *Plant Cell Tissue & Organ Culture, 82*(3), 259-265.

Mamboya, E. A. F. (2012). Papain, a plant enzyme of biological importance: A review. *American Journal of Biochemistry and Biotechnology, 8*(2), 99-104.

Manchanda, P., & Gosal, S. (2012). Effect of activated charcoal, carbon sources and gelling agents on direct somatic embryogenesis and regeneration in sugarcane via leaf roll segments. *Sugar Tech, 14*(2), 168-173.

Manshardt, R., Hammerschlag, F., & Litz, R. (1992). Papaya. *Biotechnology of Perennial Fruit Crops.*489-511.

Marchant, A., Bhalerao, R., Casimiro, I., Eklöf, J., Casero, P. J., Bennett, M., & Sandberg, G. (2002). *AUX1* promotes lateral root formation by facilitating indole-3-acetic acid distribution between sink and source tissues in the *Arabidopsis* seedling. *The Plant Cell, 14*(3), 589-597.

Marschner, H., Kirkby, E., & Cakmak, I. (1996). Effect of mineral nutritional status on shoot-root partitioning of photoassimilates and cycling of mineral nutrients. *Journal of Experimental Botany, 47*(Special Issue), 1255-1263.

Maruyama, E., Hosoi, Y., & Ishii, K. (2003). Somatic embryo culture for propagation, artificial seed production, and conservation of sawara cypress (*Chamaecyparis pisifera* Sieb. et Zucc.). *Journal of Forest Research, 8*(1), 1-8.

Maruyama, E., Hosoi, Y., & Ishii, K. (2005). Somatic embryo production and plant regeneration of Japanese Black Pine (*Pinus thunbergii*). *Journal of Forest Research, 10*(5), 403-407.

Maruyama, E., Kinoshita, I., Ishii, K., Ohba, K., & Saito, A. (1997). Germplasm conservation of the tropical forest trees, *Cedrela odorata* L., *Guazuma crinita* mart., and *Jacaranda mimosaefolia* D. Don., by shoot tip encapsulation in calcium-alginate and storage at 12–25°C. *Plant Cell Reports, 16*(6), 393-396.

Mashayekhi, K., & Neumann, K. (2006). Effects of boron on somatic embryogenesis of *Daucus carota*. *Plant Cell, Tissue Organ Culture, 84*(3), 279-283.

Maximova, S. N., Alemanno, L., Young, A., Ferriere, N., Traore, A., & Guiltinan, M. J. (2002). Efficiency, genotypic variability, and cellular origin of primary and secondary somatic embryogenesis of *Theobroma cacao* L. *In vitro Cellular & Developmental Biology-Plant, 38*(3), 252-259.

Mendoza, E. M. T., Laurena, A. C., & Botella, J. R. (2008). Recent advances in the development of transgenic papaya technology. *Biotechnology Annual Review, 14*, 423-462.

Michalczuk, L., Cooke, T. J., & Cohen, J. D. (1992). Auxin levels at different stages of carrot somatic embryogenesis. *Phytochemistry, 31*(4), 1097-1103.

Michniewicz, M., Brewer, P. B., & Friml, J. (2007). Polar auxin transport and asymmetric auxin distribution. In *The Arabidopsis Book*, *5*, e0108.

Ming, R., Hou, S., Feng, Y., Yu, Q., Dionne-Laporte, A., Saw, J. H., . . . Lewis, K. L. (2008a). The draft genome of the transgenic tropical fruit tree papaya (*Carica papaya* Linnaeus). *Nature, 452*(7190), 991-996.

Ming, R., Yu, Q., Blas, A., Chen, C., Na, J. K., & Moore, P. H. (2008b). Genomics of papaya a common source of vitamins in the tropics. *Genomics of Tropical Crop Plants*, 405-420.

Mittal, P., Devi, R., & Gosal, S. (2016). Effect of genotypes and activated charcoal on high frequency *in vitro* plant regeneration in sugarcane. *Indian Journal of Biotehnology*, *15*(2) 261-265.

Moghaddam, B. E., Mesbah, M., & Yavari, N. (2000). The effect of in planta tiba and proline treatment on somatic embryogenesis of sugar beet (*Beta vulgaris* L.). *Euphytica, 112*(2), 151-156.

Mohammed, G. H., & Vidaver, W. E. (1988). Root production and plantlet development in tissue-cultured conifers. *Plant Cell Tissue & Organ Culture, 14*(3), 137-160.

Möller, B., & Weijers, D. (2009). Auxin control of embryo patterning. *Cold Spring Harbor Perspectives in Biology, 1*(5), a001545.

Moore, T. C. (1979). Auxins. In *Biochemistry and physiology of plant hormones* (pp. 32-89): Springer, New York, United States.

Mottonen, M., Aphalo, P. J., & Lehto, T. (2001). Role of boron in drought resistance in Norway spruce (*Picea abies*) seedlings. *Tree Physiology, 21*(10), 673-682.

Müller, A., Guan, C., Gälweiler, L., Tänzler, P., Huijser, P., Marchant, A., . . . Palme, K. (1998). *AtPIN2* defines a locus of *Arabidopsis* for root gravitropism control. *The EMBO Journal, 17*(23), 6903-6911.

Murali, S., Sreedhar, D., & Lokeswari, T. (1996). Regeneration through somatic embryogenesis from petal-derived calli of *Rosa hybrida* L. cv Arizona (hybrid tea). *Euphytica, 91*(3), 271-275.

Murashige, T., & Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum, 15*(3), 473-497.

Naik, S. K., & Chand, P. K. (2006). Nutrient-alginate encapsulation of *in vitro* nodal segments of Pomegranate (*Punica granatum* L.) for germplasm distribution and exchange. *Scientia Horticulturae, 108*(3), 247-252.

Nakasone, H. Y., & Paull, R. E. (1998). The production of papain an agri-cultural industry for tropical America. *Tropical Fruits. CAB International, Wallingford, UK*, 62-79.

Neumann, K. H. (2000). Some studies on somatic embryogenesis. A tool in plant biotechnology. Pflanzliche Zell-Gewebekulturen. *Ulmer Verlag, Stuttgart*, *304*, 1-27.

Nguyen, H. N., Kim, J. H., Jeong, C. Y., Hong, S.-W., & Lee, H. (2013). Inhibition of histone deacetylation alters *Arabidopsis* root growth in response to auxin via *PIN1* degradation. *Plant Cell Report, 32*(10), 1625-1636.

Nhut, D. T., Van Le, B., Fukai, S., Tanaka, M., & Van, K. T. T. (2001). Effects of activated charcoal, explant size, explant position and sucrose concentration on plant and shoot regeneration of L*ilium longiflorum* via young stem culture. *Plant Growth Regulation, 33*(1), 59-65.

Nieves, N., Lorenzo, J., Blanco, M., Gonzáles, J., Borroto, E., Concepción, O., . . . Borroto, C. (1995). Artificial endosperm assays for embryos of Cleopatra mandarin (*Citrus Reshni* Hort ex Tan). *Turrialba, 45*(1/2), 64-69.

Nitsch, J. P., & Nitsch, C. (1969). Haploid plants from pollen grains. *Science, 163*(3862), 85-87.

Nonohay, J., Mariath, J., & Winge, H. (1999). Histological analysis of somatic embryogenesis in Brazilian cultivars of barley, *Hordeum vulgare vulgare*, Poaceae. *Plant Cell Report, 18*(11), 929.

Noodezh, H. M., Moieni, A., & Baghizadeh, A. (2012). *In vitro* propagation of the Damask rose (*Rosa damascena* Mill.). *In Vitro Cellular & Developmental Biology-Plant, 48*(5), 530-538.

Oceania, C., Doni, T., Tikendra, L., & Nongdam, P. (2015). Establishment of efficient *in vitro* culture and plantlet generation of tomato (*Lycopersicon esculentum* Mill.) and development of synthetic seeds. *Journal of Plant Sciences, 10*(1), 15-24.

Olson, M. E. (2002). Intergeneric relationships within the Caricaceae-Moringaceae clade (Brassicales) and potential morphological synapomorphies of the clade and its families. *International Journal of Plant Sciences, 163*(1), 51-65.

Overvoorde, P., Fukaki, H., & Beeckman, T. (2010). Auxin control of root development. *Cold Spring Harbor Perspectives in Biology, 2*(6), a001537.

Paek, K. Y., & Hahn, E. j. (2000). Cytokinins, auxins and activated charcoal affect organogenesis and anatomical characteristics of shoot-tip cultures of lisianthus [*Eustoma grandiflorum* (RAF.) Shinn]. *In Vitro Cellular & Developmental Biology-Plant, 36*(2), 128-132.

Pan, M., & Van Staden, J. (1998). The use of charcoal in *in vitro* culture–a review. *Plant Growth Regulation, 26*(3), 155-163.

Pan, M., & Van Staden, J. (2001). The effect of activated charcoal on the production and development of somatic embryos in cultures of carrot *Daucus carota*. *South African Journal of Botany, 67*(4), 629-635.

Pan, M., Van Staden, J., & Debergh, P. (2002). The effect of activated charcoal and auxins on root formation by hypocotyl segments of *Daucus carota*. *South African Journal of Botany, 68*(3), 349-356.

Pandey, D. K., Singh, A. K., & Chaudhary, B. (2012). Boron-mediated plant somatic embryogenesis: A provocative model. *Journal of Botany,* 1-9.

Panjaitan, S., Aziz, M., Rashid, A., & Saleh, N. (2007). *In vitro* plantlet regeneration from shoot tip of field-grown hermaphrodite papaya (*Carica papaya* L. cv. Eksotika). *International Journal of Agriculture & Biology, 9*(6), 827-832.

Pattnaik, S., & Chand, P. K. (2000). Morphogenic response of the alginate-encapsulated axillary buds from *in vitro* shoot cultures of six Mulberries. *Plant Cell Tissue & Organ Culture, 60*(3), 177-185.

Paull, R. E., Irikura, B., Wu, P., Turano, H., Chen, N. J., Blas, A., . . . Yu, Q. (2008). Fruit development, ripening and quality related genes in the papaya genome. *Tropical Plant Biology, 1*(3-4), 246-277.

Pehwal, A., Vij, S. P., Pathak, P., & Attri, L. K. (2013). Augmented shelf-life and regeneration competence of activated charcoal (AC) supplemented synthetic seeds in *Cymbidium pendulum* (Roxb.) Sw. *Current Botany, 3*(5) 30-34.

Pěnčík, A., Turečková, V., Paulišić, S., Rolčík, J., Strnad, M., & Mihaljević, S. (2015). Ammonium regulates embryogenic potential in *Cucurbita pepo* through pH-mediated changes in endogenous auxin and abscisic acid. *Plant Cell Tissue and Organ Culture, 122*(1), 89-100.

Péret, B., Swarup, K., Ferguson, A., Seth, M., Yang, Y., Dhondt, S., . . . Syed, A. (2012). *AUX/LAX* genes encode a family of auxin influx transporters that perform distinct functions during *Arabidopsis* development. *The Plant Cell, 24*(7), 2874-2885.

Pérez, G., Mbogholi, A., Sagarra, F., Aragón, C., González, J., Isidrón, M., & Lorenzo, J. C. (2011). Morphological and physiological characterization of two new pineapple somaclones derived from *in vitro* culture. *In Vitro Cellular & Developmental Biology-Plant, 47*(3), 428-433.

Pérez, L. P., Montesinos, Y. P., Olmedo, J. G., Rodriguez, R. B., Sánchez, R. R., Montenegro, O. N., . . . Gómez-Kosky, R. (2016). Effect of phloroglucinol on rooting and *in vitro* acclimatization of papaya (*Carica papaya* L. var. Maradol roja). *In Vitro Cellular & Developmental Biology-Plant, 52*(2), 196-203.

Pérez, L. P., Montesinos, Y. P., Olmedo, J. G., Sánchez, R. R., Montenegro, O. N., Rodriguez, R. B., . . . Gómez-Kosky, R. (2015). Effects of different culture conditions (photoautotrophic, photomixotrophic) and the auxin indole-butyric acid on the *in vitro* acclimatization of papaya (*Carica papaya* L. var. Red maradol) plants using zeolite as support. *African Journal of Biotechnology, 14*(35), 2622-2635.

Perveen, A., Alikhan, R. A., & Abid, R. (2007). Maintenance of pollen germination capacity of *Carica papaya* L.(Caricaceae). *Pakistan Journal of Botany, 39*(5), 1403-1406.

Pescador, R., Kerbauy, G. B., Viviani, D., & Kraus, J. E. (2008). Anomalous somatic embryos in *Acca sellowiana* (O. Berg) Burret (Myrtaceae). *Brazilian Journal of Botany, 31*(1), 155-164.

Petrášek, J., & Friml, J. (2009). Auxin transport routes in plant development. *Development, 136*(16), 2675-2688.

Petri, C., & Scorza, R. (2010). Factors affecting adventitious regeneration from *in vitro* leaf explants of 'Improved french' plum, the most important dried plum cultivar in the USA. *Annals of Applied Biology, 156*(1), 79-89.

Pintos, B., Bueno, M., Cuenca, B., & Manzanera, J. (2008). Synthetic seed production from encapsulated somatic embryos of cork oak (*Quercus suber* L.) and automated growth monitoring. *Plant Cell Tissue & Organ Culture, 95*(2), 217.

Piqueras, A. (2014). Metatopolin and phloroglucinol improve shoot production and plant quality in micropropagated *Stevia rebaudiana* by the activation of lignin metabolism. *In Vitro Cellular & Developmental Biology-Animal*, 50, 65-65.

Prasad, A., Singh, M., Yadav, N. P., Mathur, A. K., & Mathur, A. (2014). Molecular, chemical and biological stability of plants derived from artificial seeds of *Centella asiatica* (L.) Urban an industrially important medicinal herb. *Industrial Crops and Products, 60*, 205-211.

Preece, J. E., & West, T. P. (2006). Greenhouse growth and acclimatization of encapsulated *Hibiscus moscheutos* nodal segments. *Plant Cell Tissue & Organ Culture, 87*(2), 127-138.

Rai, M. K., Jaiswal, V., & Jaiswal, U. (2009). Effect of selected amino acids and polyethylene glycol on maturation and germination of somatic embryos of guava (*Psidium guajava* L.). *Scientia Horticulturae, 121*(2), 233-236.

Rajeevan, M. S., & Pandey, R. M. (1986). Lateral bud culture of papaya (*Carica papaya* L.) for clonal propagation. *Plant Cell Tissue and Organ Culture, 6*(2), 181-188.

Ramage, C. M., & Williams, R. R. (2002). Mineral nutrition and plant morphogenesis. *In Vitro Cellular & Developmental Biology-Plant, 38*(2), 116-124.

Ramarosandratana, A., Harvengt, L., Bouvet, A., Calvayrac, R., & Pâques, M. (2001). Effects of carbohydrate source, polyethylene glycol and gellan gum concentration on embryonal-suspensor mass (ESM) proliferation and maturation of Maritime pine somatic embryos. *Vitro Cellular & Developmental Biology-Plant, 37*(1), 29-34.

Rashid, A. (2001). Direct as well as indirect somatic embryogenesis from immature (unemerged) inflorescence of a minor millet *Paspalum scrobiculatum* L. *Euphytica, 120*(2), 167-172.

Rashid, K., Othman, R. Y., Ali, B. S. B. K. S., Yusof, Y. M., & Nezhadahmadi, A. (2014). The application of IRAP markers in the breeding of papaya (*Carica papaya* L.). *Indian Journal of Science and Technology, 7*(11), 1720-1728.

Ray, A., & Bhattacharya, S. (2008). Storage and plant regeneration from encapsulated shoot tips of *Rauvolfia serpentina* an effective way of conservation and mass propagation. *South African Journal of Botany, 74*(4), 776-779.

Razak, R. A., Sekeli, R., Shaharuddin, N. A., & Abdullah, J. O. (2015). Using mannose as a positive selection of transformed *Carica papaya* L. var 'Eksotika'. *Jurnal Teknologi, 77*(31) 13-18.

Redenbaugh, K., Fujii, J., Slade, D., Viss, P., & Kossler, M. (1991). Artificial seed encapsulated somatic embryos. In *High-Tech and Micropropagation I* (pp. 395-416). Springer, Berlin, Heidelberg.

Redenbaugh, K., Paasch, B. D., Nichol, J. W., Kossler, M. E., Viss, P. R., & Walker, K. A. (1986). Somatic seeds: Encapsulation of asexual plant embryos. *Nature Biotechnology, 4*(9), 797-801.

Redondo-Nieto, M., Reguera, M., Bonilla, I., & Bolaños, L. (2008). Boron dependent membrane glycoproteins in symbiosome development and nodule organogenesis: A model for a common role of boron in organogenesis. *Plant Signaling & Behavior, 3*(5), 298-300.

Reis, E., Batista, M., & Canhoto, J. (2008). Effect and analysis of phenolic compounds during somatic embryogenesis induction in *Feijoa sellowiana* Berg. *Protoplasma, 232*(3), 193-202.

Remya, M., Bai, V. N., & Mutharaian, V. (2013). *In vitro* regeneration of *Aristolochia tagala* and production of artificial seeds. *Biol. Plant., 57*(2), 210-218.

Renukdas, N., Mohan, M., Khuspe, S., & Rawal, S. (2003). Influence of boron on somatic embryogenesis in papaya. *Biologia Plantarum, 47*(1), 129-132.

Reuveni, O., Shlesinger, D. R., & Lavi, U. (1990). *In vitro* clonal propagation of dioecious *Carica papaya. Plant Cell Tissue and Organ Culture, 20*(1), 41-46.

Rihan, H. Z., Al-Issawi, M., Burchett, S., & Fuller, M. P. (2011). Encapsulation of cauliflower (*Brassica oleracea* var. Botrytis) microshoots as artificial seeds and their conversion and growth in commercial substrates. *Plant Cell Tissue and Organ Culture, 107*(2), 243-250.

Robert, H. S., & Friml, J. (2009). Auxin and other signals on the move in plants. *Nature Chemical Biology, 5*(5), 325-332.

Robert, H. S., Grones, P., Stepanova, A. N., Robles, L. M., Lokerse, A. S., Alonso, J. M., . . . Friml, J. (2013). Local auxin sources orient the apical-basal axis in *Arabidopsis* embryos. *Current Biology, 23*(24), 2506-2512.

Robert, H. S., Grunewald, W., Sauer, M., Cannoot, B., Soriano, M., Swarup, R., . . . Friml, J. (2015). Plant embryogenesis requires AUX/LAX-mediated auxin influx. *Development, 142*(4), 702-711.

Sáenz, L., Herrera-Herrera, G., Uicab-Ballote, F., Chan, J. L., & Oropeza, C. (2010). Influence of form of activated charcoal on embryogenic callus formation in coconut (*Cocos nucifera*). *Plant Cell Tissue and Organ Culture, 100*(3), 301-308.

Saha, M., Phatak, A., & Chandra, N. (2004). Generation of synthetic seeds from different varieties of *Carica papaya* L. *Journal of Tissue Research, 4*, 207-209.

Sahasrabudhe, N., Nandi, M., & Bahulikar, R. (1999). Influence of boric acid on somatic embryogenesis of a cytosterile line of indica rice. *Plant Cell Tissue & Organ Culture, 58*(1), 73-75.

Saini, S., Sharma, I., Kaur, N., & Pati, P. K. (2013). Auxin: A master regulator in plant root development. *Plant Cell Report, 32*(6), 741-757.

Santos, A. L. W. D., Silveira, V., Steiner, N., Vidor, M., & Guerra, M. P. (2002). Somatic embryogenesis in parana pine (*Araucaria angustifolia* (Bert.) O. Kuntze). *Brazilian Archives of Biology and Technology, 45*(1), 97-106.

Santos, K. G. B. D., Mariath, J. E. D. A., Moço, M. C. C., & Bodanese-Zanettini, M. H. (2006). Somatic embryogenesis from immature cotyledons of soybean (*Glycine max* (L.) Merr.): Ontogeny of somatic embryos. *Brazilian Archives of Biology and Technology, 49*(1), 49-55.

Sarkar, D., & Naik, P. S. (2000). Phloroglucinol enhances growth and rate of axillary shoot proliferation in potato shoot tip cultures *in vitro*. *Plant Cell Tissue & Organ Culture, 60*(2), 139-149.

Schmildt, O., Netto, A. T., Schmildt, E. R., Carvalho, V. S., Otoni, W. C., & Campostrini, E. (2015). Photosynthetic capacity, growth and water relations in 'Golden' papaya cultivated *in vitro* with modifications in light quality, sucrose concentration and ventilation. *Theoretical and Experimental Plant Physiology, 27*(1), 7-18.

Schmittgen, T. D., & Zakrajsek, B. A. (2000). Effect of experimental treatment on housekeeping gene expression: Validation by real-time, quantitative RT-PCR. *Journal of Biochemical and Biophysical Methods, 46*(1-2), 69-81.

Schnabel, E., & Frugoli, J. (2004). The pin and lax families of auxin transport genes in *Medicago truncatula*. *Molecular Genetics and Genomics, 272*(4), 420-432.

Sekeli, R., Abdullah, J. O., Namasivayam, P., Muda, P., & Abu Bakar, U. K. (2012). Better rooting procedure to enhance survival rate of field grown Malaysian Eksotika papaya transformed with 1-Aminocyclopropane-1-carboxylic acid oxidase gene. *ISRN Biotechnolo*gy, 13, 1–10.

Sekeli, R., Abdullah, J. O., Namasivayam, P., Muda, P., Abu Bakar, U. K., Yeong, W. C., & Pillai, V. (2014). RNA interference of 1-Aminocyclopropane-1-carboxylic acid oxidase (ACO1 and ACO2) genes expression prolongs the shelf life of Eksotika (*Carica papaya* L.) papaya fruit. *Molecules, 19*(6), 8350-8362.

Sharp, W., Sondahl, M., Caldas, L., & Maraffa, S. (1980). The physiology of *in vitro* asexual embryogenesis. *Horticultural Reviews, 2*, 268-310.

Shaygan, M., Baumgartl, T., & Arnold, S. (2017). Germination of *Atriplex halimus* seeds under salinity and water stress. *Ecological Engineering, 102*, 636-640.

Silveira, V., de Vita, A. M., Macedo, A. F., Dias, M. F. R., Floh, E. I. S., & Santa-Catarina, C. (2013). Morphological and polyamine content changes in embryogenic and non-embryogenic callus of sugarcane. *Plant Cell Tissue and Organ Culture, 114*(3), 351-364.

Singh, A. K., Varshney, R., Sharma, M., Agarwal, S. S., & Bansal, K. C. (2006). Regeneration of plants from alginate-encapsulated shoot tips of *Withania somnifera* (L.) dunal, a medicinally important plant species. *Journal of Plant Physiology, 163*(2), 220-223.

Singh, B., Sharma, S., Rani, G., Virk, G., Zaidi, A., & Nagpal, A. (2007). *In vitro* response of encapsulated and non-encapsulated somatic embryos of Kinnow mandarin (*Citrus nobilis* Lour × *Citrus Deliciosa* Tenora). *Plant Biotechnology Reports, 1*(2), 101-107.

Singh, S. K., Rai, M. K., Asthana, P., & Sahoo, L. (2010). Alginate-encapsulation of nodal segments for propagation, short-term conservation and germplasm exchange and distribution of *Eclipta alba* (L.). *Acta Physiologiae Plantarum, 32*(3), 607-610.

Smulders, M., & De Klerk, G. (2011). Epigenetics in plant tissue culture. *Plant Growth Regulation, 63*(2), 137-146.

Stasolla, C., Kong, L., Yeung, E. C., & Thorpe, T. A. (2002). Maturation of somatic embryos in conifers: Morphogenesis, physiology, biochemistry, and molecular biology. *In Vitro Cellular & Developmental Biology -Plant, 38*(2), 93-105.

Steephen, M., Nagarajan, S., & Ganesh, D. (2010). Phloroglucinol and silver nitrate enhances axillary shoot proliferation in nodal explants of *Vitex negundo* L. an aromatic medicinal plant. *Iranian Journal of Biotechnology, 8*(2), 82-89.

Storey, W. B. (1953). Genetics of the papaya. *Journal of Heredity, 44*(2), 70-78.

Swarup, K., Benková, E., Swarup, R., Casimiro, I., Péret, B., Yang, Y., . . . Vanneste, S. (2008). The auxin influx carrier *LAX3* promotes lateral root emergence. *Nature cell biology, 10*(8), 946.

Teng, W. L. (1997). Activated charcoal affects morphogenesis and enhances sporophyte regeneration during leaf cell suspension culture of *Platycerium bifurcatum*. *Plant Cell Report, 17*(2), 77-83.

Thellin, O., Zorzi, W., Lakaye, B., De Borman, B., Coumans, B., Hennen, G., . . . Heinen, E. (1999). Housekeeping genes as internal standards: Use and limits. *Journal of Biotechnology, 75*(2-3), 291-295.

Thomas, T. D. (2008). The role of activated charcoal in plant tissue culture. *Biotechnology Advances, 26*(6), 618-631.

Tirajoh, A., Kyung, T., & Punja, Z. (1998). Somatic embryogenesis and plantlet regeneration in American ginseng (*Panax quinquefolium* L.). *In Vitro Cellular & Developmental Biology-Plant, 34*(3), 203-211.

Tsvetkov, I., Jouve, L., & Hausman, J.-F. (2006). Effect of alginate matrix composition on regrowth of *in vitro* derived encapsulated apical microcuttings of hybrid Aspen. *Biologia Plantarum, 50*(4), 722-724.

Ugartechea-Chirino, Y., Swarup, R., Swarup, K., Péret, B., Whitworth, M., Bennett, M., & Bougourd, S. (2009). The *AUX1 LAX* family of auxin influx carriers is required for the establishment of embryonic root cell organization in *Arabidopsis thaliana. Annals of Botany, 105*(2), 277-289.

Utomo, H., Wenefrida, I., Meche, M., & Nash, J. (2008). Synthetic seed as a potential direct delivery system of mass produced somatic embryos in the coastal marsh plant smooth cordgrass (*Spartina alterniflora*). *Plant Cell Tissue & Organ Culture, 92*(3), 281-291.

Vanisree, M., Lee, C. Y., Lo, S. F., Nalawade, S. M., Lin, C. Y., & Tsay, H. S. (2004). Studies on the production of some important secondary metabolites from medicinal plants by plant tissue cultures. *Botanical Bulletin Academia Sinica, 45*(1), 22.

Vieten, A., Vanneste, S., Wiśniewska, J., Benková, E., Benjamins, R., Beeckman, T., . . . Friml, J. (2005). Functional redundancy of pin proteins is accompanied by auxin-dependent cross-regulation of *PIN* expression. *Development, 132*(20), 4521-4531.

Vilasini, P., Chan, Y. K., Hassan, M. D., Lam, P. F., Ong, C. A., Tan, C. S., & Umi Kalsom, A. B. (1998). Development of improved variety of Eksotika papaya using conventional and non-conventional methods. In *Proceedings of the 4th Asia-Pacific Conference on Agricultural Biotechnology. Fyshwick, Canberra, Australia: CPN Publications* (359-61).

Vilasini, P., Latipah, Z., & Salasiah, A. (2000). Induction of somatic embryogenesis and plant regeneration from immature embryos of Eksotika papaya (*Carica papaya*). *Journal of Tropical Agriculture and Food Science, 28*, 121-126.

Von Arnold, S., Clapham, D., Egertsdotter, U., & Mo, L. (1996). Somatic embryogenesis in conifers a case study of induction and development of somatic embryos in *Picea abies*. *Plant Growth Regulation, 20*(1), 3-9.

Walker, D. R., & Parrott, W. A. (2001). Effect of polyethylene glycol and sugar alcohols on soybean somatic embryo germination and conversion. *Plant Cell Tissue & Organ Culture., 64*(1), 55-62.

Wang, Y., Chai, C., Valliyodan, B., Maupin, C., Annen, B., & Nguyen, H. T. (2015). Genome wide analysis and expression profiling of the *PIN* auxin transporter gene family in soybean (*Glycine max)*. *BMC Genomics, 16*(1), 951.

Wani, S. H., Sofi, P. A., Gosal, S. S., & Singh, N. B. (2010). *In vitro* screening of rice (*Oryza sativa* L) callus for drought tolerance. *Commun Biometry Crop Science, 5*, 108-115.

Weijers, D., Schlereth, A., Ehrismann, J. S., Schwank, G., Kientz, M., & Jürgens, G. (2006). Auxin triggers transient local signaling for cell specification in *Arabidopsis* embryogenesis. *Developmental Cell, 10*(2), 265-270.

West, T. P., Ravindra, M., & Preece, J. E. (2006). Encapsulation, cold storage, and growth of *Hibiscus moscheutos* nodal segments. *Plant Cell Tissue & Organ Culture, 87*(3), 223-231.

Wójcikowska, B., & Gaj, M. D. (2017). Expression profiling of auxin response factor genes during somatic embryogenesis induction in *Arabidopsis*. *Plant Cell Report, 36*(6), 843-858.

Wu, K. L., Zeng, S. J., Chen, Z. L., & Duan, J. (2012). *In vitro* mass propagation of hermaphroditic *Carica papaya* cv. Meizhonghong. *Pakistan Journal of Botany, 44*(5), 1669-1676.

Xiao, Y., Niu, G., & Kozai, T. (2011). Development and application of photoautotrophic micropropagation plant system. *Plant Cell Tissue and Organ Culture, 105*(2), 149-158.

Yaacob, J. S., Loh, H. S., & Mat Taha, R. (2013). Protein profiling and histone deacetylation activities in somaclonal variants of oil palm (*Elaeis guineensis* Jacq.). *The Scientific World Journal, 13,* 1-8.

Yadollahi, A., Abdollahi, M., Moieni, A., & Danaee, M. (2011). Effects of carbon source, polyethylene glycol and abscisic acid on secondary embryo induction and maturation in rapeseed (*Brassica napus* L.) microspore-derived embryos. *Acta Physiologiae Plantarum, 33*(5), 1905-1912.

Yang, Y., Hammes, U. Z., Taylor, C. G., Schachtman, D. P., & Nielsen, E. (2006). High-affinity auxin transport by the AUX1 influx carrier protein. *Current Biology, 16*(11), 1123-1127.

Yu, C., Dong, W., Zhan, Y., Huang, Z.-a., Li, Z., Kim, I. S., & Zhang, C. (2017). Genome-wide identification and expression analysis of *clLAX, clPIN* and *clABCB* genes families in *Citrullus lanatus* under various abiotic stresses and grafting. *BMC Genetics, 18*(1), 33.

Yu, T. A., Yeh, S. D., & Yang, J. S. (2001). Effects of carbenicillin and cefotaxime on callus growth and somatic embryogenesis from adventitious roots of papaya. *Botanical Bulletin of Academia Sinica, 42*:281-286.

Yu, T. A., Yeh, S. D., Cheng, Y. H., & Yang, J. S. (2000). Efficient rooting for establishment of papaya plantlets by micropropagation. *Plant Cell Tissue & Organ Culture, 61*(1), 29-35.

Yu, T. A., Yeh, S. D., & Yang, J. S. (2003). Comparison of the effects of kanamycin and geneticin on regeneration of papaya from root tissue. *Plant Cell Tissue & Organ Culture., 74*(2), 169-178.

Zažímalová, E., Murphy, A. S., Yang, H., Hoyerová, K., & Hošek, P. (2010). Auxin transporters why so many? *Cold Spring Harbor Perspectives in Biology, 2*(3), a001552.

Zhong, J. J. (2001). Biochemical engineering of the production of plant-specific secondary metabolites by cell suspension cultures. In *Plant cells* (pp. 1-26). Springer Berlin Heidelberg.

Zhu, X., Li, X., Chen, W., Chen, J., Lu, W., Chen, L., & Fu, D. (2012). Evaluation of new reference genes in papaya for accurate transcript normalization under different experimental conditions. *PLoS ONE, 7*(8), e44405.

Zobayed, S., Afreen, F., & Kozai, T. (2001). Physiology of eucalyptus plantlets grown photoautotrophically in a scaled-up vessel. *In Vitro Cellular & Developmental Biology-Plant, 37*(6), 807-813.

Zouine, J., El Bellaj, M., Meddich, A., Verdeil, J. L., & El Hadrami, I. (2005). Proliferation and germination of somatic embryos from embryogenic suspension cultures in *Phoenix dactylifera*. *Plant Cell Tissue & Organ Culture, 82*(1), 83-92.

LIST OF PUBLICATIONS AND PAPERS PRESENTED

(A) PUBLICATIONS

1. **Al-Shara, B.**, Taha, R. M., & Rashid, K. (2018). Biotechnological methods and limitations of micropropagation in papaya (*C. papaya* L.) production: a review. *The Journal of Animal and Plant Sciences*, 28*(5),* 1208-1226.

(B) PAPERS PRESENTED

1. *Efficient in vitro surface sterilization protocols and callus formation from seed explants of Carica papaya. L (Eksotika I).* International Postgraduate Research Awarded Seminar 2016, Oral presentation, University of Malaya, 6-7 April 2016.