5.1 Choice of Explants

In this study, immature zygotic embryos of *Carica papaya* L. variety Eksotika were used as explants to induce somatic embryos. The choice of explants will influence the type of callus produced.

In this study, immature zygotic embryos were chosen based on reports. According to Bhattacharya *et al*., 2003, the reproductive tissues are usually used for somatic embryogenesis studies. Immature zygotic embryos were used to induce callus and somatic embryos in other plants such as rice which proved the efficacy of this explant as a good target tissue for embryogenic material (Vilasini *et al*., 2000; Renukdas *et al*., 2003 and Bhattacharya *et al*., 2003).

5.2 Choice of Media

Media formulations are the most important aspect for suitable initiation and maintenance of callus cultures from tissue explants of most plant species (Drew *et al*., 1993 and Renukdas *et al*., 2003). It is a known fact that successful mass propagation of papaya by using tissue culture technique is greatly influenced by the medium used. Murashige and Skoog's salt formulation or modifications are commonly used for somatic embryogenesis (Castillo *et al*., 1998). Based on this information, a solidified MS-based media was used in this study.

MS-based medium supplemented with 10 mg/L 2,4-D was used to induce and propagate embryogenic callus and subsequently somatic embryos. In this media, 100 % of the

selected explants excised developed into callus. Similar observation was made by Litz (1992), while other reports used abscisic acid (ABA), adenine sulfate, anandamide (ANA), 6-benzyladenine (BA), 6-benzylaminopurine (BAP), gibberellic acid (GA_3), glutamine, indole-3-acetic acid (IAA), indole butyric acid (IBA), naphthaleneacetic acid (NAA), kinetin, picloram, thidiazuron (TDZ) and zeatin for embryogenic callus initiation. The carbohydrate source in this study was similar to that of Litz and Conover (1983) where 6 % sucrose was used. Although sucrose is the most commonly used carbon source, glucose was reported to be superior for embryogenic cell induction (Stasolla and Yeung, 2003).

Hormone-free medium was used for the germination of somatic embryos of papaya variety Highland (Monmarson *et al*., 1995). BAP and NAA were used for the germination of somatic embryos of papaya. However, Rajeevan and Pandey (1986) and also Yu and Yang (2001) used BAP and NAA supplemented media for somatic embryo germination and this was inconsistence with Eksotika used in this study. The analysis on the effects of cytokinin and auxin at this level suggests a kind of antagonism between multiplication rate of cell development and growth of shoot. It also determined the subsequent shoot growth concomitant with rooting (de Winnaar, 1988).

5.3 Formations of Embryogenic Callus

Optimal papaya callus formation (93.3 % ±11.8) from immature zygotic embryos was obtained on the culture medium with 250 mg/L carbenicillin placed in the dark at ±25 ºC.

A lower percentage of callus formation (65.6 % ±8.6) was obtained from explants cultured on a medium without carbenicillin.

Similar media composition was used by other reports (Fitch and Manshardt, 1990 and Vilasini *et al*., 2000) but without carbenicillin. Comparatively, the induction percentage for embryogenic callus from the same source of explants was more efficient (100 %) than previous reports, where 62 % and 78 % were reported by Vilasini *et al*., 2000 and Fitch and Manshardt, 1990 respectively. Similar observation was made by Yu *et al*., 2000 on callus growth using callus induction media supplemented with 250 to 500 mg/L carbenicillin.

This indicated the dual functions of carbenicillin capabilities, first as an antibiotic with bacteriostatic property, Yu and Yang, 2001 and secondly as an enhancer to the growth of embryogenic callus when bacteria-suppressing effects of carbenicillin occured. It has been reported that endogenous bacteria contaminated cultures without the use of carbenicillin (Yu and Yang, 2001).

5.4 Verification of Embryogenic Cell

Embryogenic cells showed small size, dense cytoplasmic nucleoli, and large nuclei with prominent enlarge nucleoli, small vacuoles and a profusion of starch grains and total proteins. The vacuolated cells also have a large central nucleus containing one or two prominent nucleoli of the friable sector of the callus.

Embryogenic cells were distinguished by double staining because less viable, vacuolated cells with small nuclei (non-embryogenic cells) permitted Evan's blue dye to enter and stain the cells blue, whereas the acetocarmine was unable to stain the cells red.

The red stained cells occurred because the small cells with nuclei were associated with the formation of suspensors derived from embryonal cells. The exclusion of Evan's blue determines of the embryogenic cells.

5.5 Formations of Somatic Embryo

Exogenous supplied growth regulators are essential to the process of somatic embryogenesis. Different concentrations and different types of exogenous growth regulators are required by each plant species (Dixon and Gonzales, 1994). Therefore, somatic embryos need hormones during the initiation phase and the process of induction.

The potencial of 2,4-D to induce somatic embryogenesis on immature zygotic embryos of papaya has been exhibited in other varieties of papaya (Fitch and Manshardt, 1990). de Almeida *et al.* (2000) also reported that many plants produce somatic embryos when their immature zygotic embryos are subjected to medium containing 2,4-D substantiated media.

In contrast, high frequency of somatic embryogenesis has been achieved previously in papaya tissue culture derived from immature zygotic embryos and seedling explants on BAP and NAA containing MS medium (Chen *et al.*, 1987 and Litz and Conover, 1983).

In this study, high frequency of somatic embryos 93.3 % ±11.8 were observed from callus on MS with 10 mg/L 2,4-D supplemented with 250 mg/L carbenicillin in the dark

regime at ±25 ºC, meanwhile the lowest frequency of somatic embryos, 65.6 % ±8.6 were observed from callus on MS medium containing 10 mg/L 2,4-D without 250 mg/L carbenicillin.

Embryogenesis is the production of embryo-like structures from somatic cells. The somatic embryo is an independent bipolar structure and is not physically attached to the tissue of origin (Fernando *et al*., 2001)

5.6 Somatic Embryogenesis in Suspension Culture

5.6.1 Effects of Liquid Multiplication Medium

In this study, the liquid medium used for raising fast growing somatic embryos was generally proven suitable for initiating suspension cultures. The medium used was Murashige and Skoog (MS) basal medium. Most suspension cultures were obtained by transferring friable callus lumps into agitated liquid medium of the same composition as that used for callus growth (Fitch, 1993). The same principles were used in this study accordingly.

In this study, to multiply cell suspension, low initial inoculums was used. Upon sub culturing, partial conditioned medium was added to fresh medium. Although large initial inoculums are advantageous, because this will ensure that sufficient single cell and/or small clumps are released into the medium to provide a suitably high cell density for subsequent growth. Conditioned medium (medium in which tissues has been grown for

some time) has been previously used to reduce the lag phase of growth cycle. Such a medium presumably contains metabolites, essential for growth.

According to the results obtained, the pro-embryos increased in number rapidly, primarily as a result of profuse budding from the globular pro-embryos and yielded suspensions of highly uniform globular somatic embryos. By regularly sub culturing globular somatic embryos in fresh medium with 2 mg/L 2,4-D, it has been possible to produce continuous proliferation without apparent loss of regenerative potential (Litz and Conover, 1982).

5.6.2 Growth and Multiplications of Cells

In this study, somatic embryos cells grow and multiply positively in the MS media with 2 mg/L 2,4-D supplemented with 250 mg/L carbenicillin in the light regime at ±25 ºC compared to somatic embryos cells in MS medium containing 2 mg/L 2,4-D without 250 mg/L carbenicillin. Data were derived using measurements of cell volume after sedimentation (CVS) parameter.

The maintenance of embryogenic suspension cell cultures proceeded as a series of growth cycles. Each cycle is characterized by its sigmoid nature, comprising of lag phase, cell-division phase to a stationary phase (Gupta and Ibaraki, 2006).

According to the results obtained, only cells from ***Plate 4*** in ***Figure 4.2*** showed a typical growth curve of suspension cultures, compared to other cells in various plates (***Plates 1***, ***2***, ***3*** and ***5***) in ***Figure 4.2***. This shows that only cells in plate four were stable cultures.

In light condition and liquid MS medium with 2 mg/L 2,4-D supplemented with 250 mg/L carbenicillin enhanced the production of pro-globular embryos. During lag phase, cells cultured in fresh medium, initiated a series of metabolic processes, which prepare the cells for mitosis. During this stage, half volume of the total growth medium will be replaced with fresh medium in order to promote active cell division, but sudden drop of the cell growth relatively happened due to the conditioning process of the cells towards the culture medium substitution. Cell growth is strongly influenced by the media composition and also auxin type and concentration.

Once cell division has been induced, it will proceed until one or more of the nutrients such as sucrose and casein hydrolysate becomes limited. The increase in cell volume usually precedes the increase in fresh weight, due to the time delay for cell expansion. This situation happened in the exponential phase.

Continuous proliferation was due to profuse budding from globular pro-embryo. The regenerative potential of the suspensions could be further maintained for 2 to 3 months when cultured in liquid media without 2,4-D. However, the single embryos became mature if prolonged culture on this media for a subsequent 5 weeks period.

The enhanced growth of the somatic embryos by the synchronous effect of carbenicillin could be due to the existence of adventive embryogenesis on single cells as in a globular embryo clumps. The growth of cells increased rapidly and more cells became embryogenic after one month of culture.

5.7 Plant Regeneration from Cell Suspension

The use of a single culture medium with varying levels and types of growth regulator often determines the route of morphogenesis *in vitro*. Generally, medium containing high auxin levels will induce callus formation. Lowering the auxin concentration and increasing the cytokinin concentration is traditionally performed to induce shoot organogenesis from callus.

In this study, embryos further develop and germinated into plantlets through the events that correspond with the zygotic occurrences. High percentage of normal plant regenerated from somatic embryos (88 %) was obtained on medium containing 0.2 mg/L BAP and NAA in light regime. However, a low percentage of abnormal plant (12 %) was obtained from somatic embryos on the same medium constitution and cultural conditions.

Generally, in somatic embryogenesis, transfer of callus to medium devoid of growth regulators is usually sufficient to stimulate the later stages of embryo development and subsequent germination (Litz and Conover, 1983; Chen and Chen, 1992; Fitch, 1993; Castillo *et. al*., 1998 and Renukdas *et. al*., 2003).

Besides, according to de Winnaar (1988), auxin was required during the initial phase of adventitious root formation and somatic embryogenesis and cytokinins is required for callus formation and growth in papaya. These results are in accordance to the results obtained in this study.

This might be due to plant genotypic dependent and the endogenous hormone present in the developed somatic embryos.

5.8 Characterization of Alkaloid Carpaine

Other researches have isolated the alkaloid carpaine from the leaves of the *Carica papaya* L. var. Uzbekistan, Nigerian and Solo (Coke and Rice, 1968; Burdick, 1971; Ogan, 1970; Tang, 1978; Khuzhaev and Aripova, 2000 and Govindachari, 2002). However, the amount of carpaine they extracted from the Uzbekistan, Nigerian and Solo variety was quite low, ranging from 0.025 to 0.4 % and rather impure since it contains other compounds such as dehydrocarpaine I and dehydrocarpaine II.

In our work, the major alkaloid isolated from the leaves of *Carica papaya* L. is carpaine (Govindachari *et al*., 1954) and another nitrogenous base compound, i.e., pseudocarpaine (Ogan, 1970; Tang, 1978 and Khuzhaev and Aripova, 2000) which is a stereoisomer of carpaine. This stereoisomer, however, is only isolated in very small quantity (10 %). Previous studies for carpaine isolation gave low quantity and poor quality. It, therefore, seemed worthwhile to investigate whether the low yield observed in the carpaine isolated were due to some factors in the regulation of secondary metabolism at all levels such as genes, enzymes, transport and compartmentalization or due to the extraction and refinement procedures. To establish the samples resources samples were then collected from *in vivo* and *in vitro* samples of *Carica papaya* L. var. Eksotika.

Our work in isolating carpaine from *Carica papaya* L. var. Eksotika leaves using the methods of Govindachari *et al*., (1954); Coke and Rice, (1968); Ogan, (1970); Tang, (1978) and Khuzhaev and Aripova, (2000) yielded an impure carpaine samples with lower yield. In an attempt to harvest the carpaine in a purer and higher yields,

modification to conventional acid/base extraction method were carried out. In addition, extraction was also carried out using the supercritical fluid extraction (SFE) method to determine which of the two methods is more reliable and efficient in giving carpaine in a purer and higher yield.

The amount of carpaine isolated from the various sources of plant parts using the two different techniques is shown in ***Table 5.1.***

Table 5.1: Amount and percentage of carpaine extract from *in vivo* and *in vitro* leaves of *Carica papaya* L. var. Eksotika

Samples	**Types of Extraction**	
	Acid/Base	Supercritical Fluid
In vivo leaves	0.256 g (0.051 %)	1.923 g (0.961 %)
In vitro leaves	0.003 g (0.012 %)	0.016 g (0.32 %)

In conclusion, the yield of carpaine isolated (with acid/base extraction) from *in vivo* and *in vitro* leaves was 0.256 g or 0.051 % and 0.003 g or 0.012 %. Meanwhile, the yield of carpaine isolated (with supercritical fluid extraction) from *in vivo* and *in vitro* leaves was 1.923 g or 0.961 % and 0.016 g or 0.32 %.

Our results showed improvement in the isolated yield compared to those reported for Nigerian and Solo plants 0.175 (0.012 %) (Khuzhaev and Aripova, 2000, Tang, 1978, Burdick, 1971 and Ogan, 1970) as when the yielded carpaine was up to ±1:5 and 1:50 from the respective acid/base and supercritical fluid extraction methods. In addition, the use of younger leaves compared to the previous reports where mature leaves were used

seem to enhance the quantity of carpaine produced. The evidence took place with the explanation of the nature of secondary metabolite production reported by Satoh and Flores, 1990. In nature (*in vivo*), the roots of plants produce a complex array of secondary metabolites, possibly because they live in an environment of soil, which is permeated with pathogens and predators. For this reason, they evolved capacities for synthesizing all manner of defence chemicals that may accumulate in the roots, be secreted into the rhizosphere, and later transported to the shoot. Meanwhile, in *in vitro* plants, the secondary metabolites are produced by the roots at low levels until the roots are confronted with a contamination caused pathogen, at which time they may increase the synthesis and secretion of the metabolite to try to ward off the contamination. And also for this reason, they exhibited the responses of defense mechanism towards latent bacterial contaminants which certain internal chemicals may produce and accumulated in the roots and later being transported to the shoot.

The relative amount of carpaine isolated from the different plant parts showed that in the *in vitro* samples, highest carpaine content is in the petiole and the lowest content to be in the leaves. However, in the *in vivo* samples, the highest carpaine content was found in the leaves and the lowest content in the fruit peel. Secondary metabolites such as carpaine are produced in the organelles (Yazaki, 2005 and Nishioka and Funatsu, 1999). Organelles function as transported plant cell systems (Jørgensen *et al.*, 2005 and Yazaki, 2005). The extracts in our study had been subjected to centrifugation to remove all solid particles. This presumably resulted in an increase amount of carpaine isolated since the organelles are plamolysed during the centrifugation, releasing more carpaines into the media (Knez *et al.*, 2003 and Burkart, 2002).

In this study, the isolation procedure employed ethanol/water/acetic acid (v/v/v) as extraction solvents in the conventional extraction method, while carbon dioxide (g per hour) was used in supercritical fluid extraction. More carpaine was extracted through supercritical fluid extraction than from acid/base extraction (***Table 5.1***). In SFE, extraction is carried out at low temperatures. Perhaps this helps to lessen any possible deterioration in the extracts. In addition, since carbon dioxide was used as the mobile phase, there would be no oxygen in the extraction process. This would possibly reduce significantly the chances of oxidation of the extracts during the extraction process. The more prominent advantage in using SFE is that the mobile phase is CO_2 which is gaseous. Thus, in SFE there is no residual solvent in either the extract or in the raffinate. This translates into reduction in the loss of compounds extracted during post-processing and clean-up procedures.

The purity of carpaine extracted from the leaves of Eksotika was determined by TLC (R_f value of 0.43±0.11 and 0.45±0.08 from both mode of extraction) and found to be comparable to those reported earlier (R_f value of 0.4 for carpaine and 0.15 for dehydrocarpaine II) (Coke and Rice, 1968; Burdick, 1971 and Tang, 1978). In addition, the proton NMR and mass spectrum of the carpaine isolated which is observed to be pure and identical to those reported by Tang, 1978 and by Sato and co-workers, 2003.

In our work, only one pseudo-carpaine compounds were isolated, namely dehydrocarpaine II (white needle-like crystal, m.p. 65 to 66 °C), whilst Govindachari *et al*., 1954; Coke and Rice, 1968; Ogan, 1970; Tang, 1978 and Khuzhaev and Aripova, 2000 obtained both dehydrocarpaine I and II. However, dehydrocarpaine I and

dehydrocarpaine II are not easily separable from carpaine through the conventional acid/base purification techniques such as re-crystallization. Tang, 1978 and Coke and Rice, 1968 used ethanol/water/acetic acid in a ratio of 89:10:1 (v/v/v) to soak the leaves of *Carica papaya* L. var. Solo for carpaine extract. However, they obtained a mixture of carpaine, pseudocarpaine and dehydrocarpaine I and II at ratio 31.5 (or 0.0115 % of carpaine) to 68.5 (or 0.025 % of dehydrocarpaine I and II). They obtained a mixture of carpaine, dehydrocarpaine I and dehydrocarpaine II with a higher concentration of dehydrocarpaine I and dehydrocarpaine II than that of carpaine (Khuzhaev and Aripova, 2000, Tang, 1978 and Coke and Rice, 1968). The carpaine crystals obtained by the current method are usually contaminated with two pseudocarpaines, and its purity is dependent upon the techniques used in separating them. In our work, a modification of two-phase aqueous organic extraction and two-step precipitation methods proposed by Coke and Rice, 1968 and Tang, 1978 was used for purification of carpaine from the clarified papaya crude alkaloid. Although the same solvent mixture was used, the ratio used in this study was 94.5:5:0.5 (v/v/v). This solvent system is more non-polar which presumably encourages better extraction of the non-polar carpaine. With all these modification, we were able to separate the carpaine from the dehydrocarpaine II.