Induction of somatic embryogenesis and plant regeneration from immature embryos of Eksotika papaya (Carica papaya) [Induksi somatik embriogenesis dan regenerasi pokok betik Eksotika (Carica papaya) daripada embrio belum matang]

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Key words: Carica papaya, immature embryos, somatic embryogenesis, plant regeneration

Abstract
Embryogenic callus cultures were obtained on half-strength Murashige and Skoog medium, with full-strength vitamins and 2,4-dichlorophenoxy acetic acid (2,4-D). The optimum level of 2,4-D was found to be 10 mg/L where a high percentage of zygotic embryos produced embryogenic callus or sometimes-direct somatic embryos were formed on the apical dome of the immature embryos. Sucrose at 6% (w/v) was a better carbon source than maltose. Germination of somatic embryos occurred when transferred to MS medium (full strength), 3% (w/v) sucrose and a combination of naphthaleneacetic acid (NAA) (0.1 mg/L) and 6-benzylaminopurine (6-BAP) (0.1 mg/L). The transfer of the somatic embryos to hormone-free medium before germinating them was helpful in decreasing the incidence of abnormal plants. Plants were successfully rooted and transferred to vermiculite: sand: soil mixture (1:1:1). The plants were acclimatised for 2 weeks under high humidity before transferred to the glasshouse.

Introduction
Papaya is one of the most widely grown fruits in the tropics including Malaysia where it is a smallholders’ crop. It is mainly cultivated in Johore, Perak and parts of Selangor. The Eksotika papaya variety was introduced to the farmers in the late eighties and this resulted in an increase in the

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production areas. However, this variety is very susceptible to Papaya Ringspot Virus (PRSV) which affected large production areas in Johore in 1991. Although the spread of the disease is under control, there is an urgent need to develop a PRSV resistant Eksotika variety. In tandem with conventional breeding programmes to incorporate resistant genes from resistant/tolerant varieties, genetic engineering of Eksotika variety for resistance to PRSV need to be undertaken as it offers a more direct approach to the problem.

A prerequisite for an effective gene transfer system is a reliable, efficient and reproducible plant regeneration protocol. Papaya tissue culture researchers have reported plant regeneration from different explants of popular varieties, both through organogenesis and embryogenesis (Litz and Conover 1981; Chen et al. 1987; Fitch and Manshardt 1990; Hossain et al. 1993). It has been confirmed that papaya responds well to somatic embryogenesis from immature embryos (Fitch and Manshardt 1990), petioles (Yang and Ye 1992) and hypocotyls (Fitch 1993). Here we report somatic embryogenesis from immature embryos of Eksotika papaya to facilitate a transformation system for the transfer of viral resistance and shelf-life genes.

Materials and methods

**Plant material**

Immature hermaphrodite Eksotika fruits were harvested from field-grown trees. Fruits picked were between 90–100 days old after anthesis. Age of fruit was estimated roughly by its position on the tree trunk.

**Media**

The basal medium for embryogenic induction was that of Murashige and Skoog (1962)(MS). Half strength of MS salts were supplemented with myo-inositol (50 mg/L), full strength MS vitamins, adenine sulphate (45 mg/L), glutamine (100 mg/L), sucrose or maltose (4%/6%), solidified with 1% (w/v) Difco Bacto agar. The embryogenic induction medium contained various levels of 2,4-D (0, 5, 10, 15, 20 and 25 mg/L) and the germination medium was supplemented with various levels of BAP and NAA (0, 0.1, 0.01, 0.001 mg/L). The maturation medium was full strength MS without any phytohormones or further supplements. The pH of the media was adjusted to 5.8 prior to autoclaving. Embryogenic induction medium was dispensed into 9 cm Petri dishes and the germination of the embryos was carried out in 100 mL conical flasks. All experiments were conducted twice with 3 replicates per concentration.

**Culture conditions**

Immature fruits were washed thoroughly under running tap water and sprayed with 70% (v/v) ethanol in the laminar flow hood. Fruits were then cut open and immature seeds removed. These seeds were subsequently cut open and the excised zygotic embryo (10) were placed on agar-solidified medium in Petri dishes, sealed with parafilm and placed in the dark at 25–27 °C. Cultures were observed every 2 weeks for 8 weeks. Embryogenic callus was subcultured onto the same medium until somatic embryos developed, after which they were subcultured onto phytohormone free medium and placed in the light.

Three experiments were conducted. The first studied the effect of various concentrations of 2,4-D on caullogenesis. Using the optimum level of 2,4-D from the first experiment, the second experiment was conducted on the effect of carbon source (sucrose and maltose) at 4% (w/v) and 6% (w/v) on embryogenesis and the third was the effect of different concentrations of BAP and NAA on the germinability of the somatic embryos. Ten clumps of embryogenic calli was cultured on each of the levels of the phytohormone in Petri dishes, sealed with parafilm and placed in the culture room with 16 h/8 h light and dark regime. All the experiments were carried out twice with 3 replicates per treatment. Shoots with 4–5 leaves and at
least 2–3 cm high were rooted according to Drew et al. (1991) in 150 mL conical flasks for 1 month in the light and transferred to polybags containing soil:sand:vermiculite mixture (1:1:1). These bags were then covered with plastic bag to maintain high humidity and kept under ambient conditions before transferred to the glasshouse.

Results
In the first experiment in which the effect of different concentrations of 2,4-D was studied on embryogenic callus production, 10 mg/L of 2,4-D resulted in 78% of zygotic embryos producing embryogenic callus (Figure 1). Zygotic embryos on phytohormone free medium germinated or remained dormant. Levels of 2,4-D greater than 10 mg/L did not have a favourable effect on the embryogenic potential of the zygotic embryos. The optimum concentration of 2,4-D at 10 mg/L was thus used in subsequent experiments. The various stages of somatic embryo development were observed on this medium (Plate 1 A–D). Sucrose appeared to be superior to maltose where embryogenic callus production was concerned. In the embryogenic induction medium, 6% sucrose was essential for high frequency of embryogenesis (Table 1), whereas only 3% was required in the germination medium. Somatic embryos at the cotyledonary stage (Plate 1 D) were transferred to hormone-free maturation medium first before subsequent transfer to germination medium containing NAA (0.1 mg/L) and BAP (0.1 mg/L) (Table 2). This resulted in the radical and apices developing with shoots finally elongating (Plate 1 E–F). Although roots were formed, but very rarely did normal roots appear. Frequent appearance of callus and loose roots were observed and these were removed and the plantlets were rooted according to Drew et al. (1991). Although a high percentage (>30%) of the embryos germinated, abnormality was also observed in somatic embryos induced on levels of 2,4-D greater than 10 mg/L. Fasciation and hairbrush like splitting of the stem were some of the features seen in the cultures.

![Figure 1. Percentage of immature zygotic embryos that developed embryogenic callus on media containing various levels of 2,4-D](image-url)
Induction of somatic embryogenesis

Plate 1. Somatic embryogenesis from immature embryos of *Carica papaya*. Somatic embryos at different developmental stages derived from a single zygotic embryo:

(A) Globular embryogenic callus.
(B) Bi-polar torpedo shaped somatic embryo.
(C) Heart shape developmental stage of somatic embryo.
(D) Somatic embryo at the cotyledonary stage.
(E) Germination of mature somatic embryo.
(F) Regeneration of plants from somatic embryos
Discussion

The potency of 2,4-D to induce somatic embryogenesis on immature zygotic embryos of papaya has also been exhibited in other varieties of papaya (Fitch and Manshardt 1990). Ammirato (1983) who reported that many plants produce somatic embryos when their immature zygotic embryos are subjected to medium containing 2,4-D also substantiates this. The use of immature embryos to induce somatic embryos in other dicotyledonous plants proves the efficacy of this explant as a good target tissue for embryogenic material (Williams and Maheswaran 1986). Litz and Conover (1982) have also reported the superiority of 6% sucrose in induction medium in papaya. Although some workers have reported germination of somatic embryos on hormone free medium (Dhir and Yadav 1995), somatic embryos of Eksotika require NAA and BAP for faster and optimum percentage of germination and this was in consistence with the work of Yang and Ye (1992). These contrasting results might be a genotype dependent effect. The regeneration system that is reported here can be used in our non-conventional method of gene transfer, either using the biolistics or the Agrobacterium system. With this in place, gene manipulation of papaya to withstand the Papaya Ringspot Virus and to lengthen the shelf life of the fruit can proceed at a faster pace.

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References


Table 1. The effect of various concentrations of NAA and BAP on the germination of somatic embryos

<table>
<thead>
<tr>
<th>NAA (mg/L)</th>
<th>BAP (mg/L)</th>
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<tr>
<td></td>
<td>0.0</td>
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<tr>
<td>0.0</td>
<td>–</td>
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<td>0.1</td>
<td>–</td>
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<td>0.01</td>
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<td>0.001</td>
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– = no germination
+ = germination (5–10%)
++ = germination (10–30%)
+++ = germination (>30%)

Table 2. The effect of the type of carbon source and its concentration on the production of embryogenic callus

<table>
<thead>
<tr>
<th>Conc.</th>
<th>Sucrose</th>
<th>Maltose</th>
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<tbody>
<tr>
<td>3%</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>4%</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>6%</td>
<td>+++</td>
<td>+</td>
</tr>
</tbody>
</table>

– = no response
+ = poor response (0–10%)
++ = good response (10–40%)
+++ = very good response (40–80%)
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Williams, E. G. and Maheswaran, G (1986).

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