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Improved protocol for high frequency plant regeneration through somatic embryogenesis in *Carica papaya*

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An efficient *in vitro* method has been established for the production of whole plantlets of *Carica papaya* L. *var*. Eksotika, *C. papaya* (E) using immature zygotic embryo (IZE) as explants excised from fruit 90 to 100 days (d) post-anthesis. Proliferating callus cultures were obtained by placing IZE explants on half-strength Murashige and Skoog (MS) medium supplemented with carbenicillin and 2,4-dichlorophenoxyacetic acid (2,4-D) either alone or in combination. MS medium augmented with 10 mg/L 2,4-D and 250 mg/L carbenicillin was found to be effective in providing the highest frequency (77.5%) of embryogenic callus formation. All of the somatic embryos (100%) germinated after 3 months where only 90.4% produced normal hypocotyls when continuously cultured in 0.2 mg/L 6-benzyl amino purine (BAP) and naphthalene acetic acid (NAA). Subsequently, complete plants were regenerated (97.3%) when plantlets formed well-developed shoots and roots in solid MS medium augmented with 0.5 mg/L indole-3-butyric acid (IBA). Regenerated plantlets were successfully acclimatised and subsequently transferred to the field. Histo-anatomical studies on the different stages of somatic embryogenesis were carried out in order to understand the morphogenic events and growth responses of regenerated plants. This is a complete protocol on regenerable embryogenic cell suspension cultures in *C. papaya*.

Keywords: Carpaine; Eksotika; histology; papaya; somatic embryogenesis

Abbreviations: 2,4-D- 2,4-Dichlorophenoxyacetic acid; BAP- 6-Benzyl amino purine; CI- callus induction medium; G- germination medium; GA_3- gibberellic acid; GE- globular embryo; h- hours; IBA- Indole-3-butyric acid; IZE- immature zygotic embryo; LM- liquid multiplication medium; MS- Murashige and Skoog medium; NAA- Naphthalene acetic acid; PCT- pro-cambial traces; PEM- pro-embryogenic mass; R- regeneration medium; SR- suspensor region; TO- torpedo embryo.

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Papaya (*Carica papaya* L.) is a member of the *Caricaceaea* (Fernando *et al*., 2001) and is one of the most important fruit crops in the tropics and subtropics grown commercially (Tsay and Su, 1985; Reuveni *et al*., 1990). It gains its economical value because of the global demand and continuous fruit production throughout the year (Hossain *et al*., 1993). The fruit is cultivated for its nutritive food value as it is an important source of certain vitamins such as ascorbic acid, vitamin A, macro and micromineral elements such as Na, K, Ca, Fe, Cu and Zn (Hernández *et al*., 2006; Wall, 2006). Papaya also plays an important role in pharmacology where the plant compound is used in the treatment of dyspepsia of spleen and liver, digestive disorders and skin blemishes. Consumption of papaya aids in digestion because of the presence of proteolytic enzymes, papain and chymopapain compounds (Yamamoto and Tabata, 1989).

Traditionally, papaya-planting materials have been obtained through seeds and conventional breeding for cultivar improvement. However, this may lead to low productivity because of inaccuracy in selection of planting material, and the shortage of good planting material from selected superior varieties. Alternatively, various tissue culture systems have been developed for micropropagation to complement conventional breeding of papaya. Considerable interest has been directed towards the genetic improvement and metabolic engineering of this crop through biotechnology such as somatic hybridization, genetic transformation, and metabolomics (Lai *et al*., 1998; de Almeida *et al*., 2000; Yu *et al*., 2001). Plant regeneration through tissue culture especially somatic embryogenesis is a pre-requisite for many successful applications of biotechnology such as artificial seeds, micropropagation and transgenic plants.

To date, information on the histo-anatomy of papaya somatic embryos is scarce in comparison to other crops. Therefore, this is important to understand and manipulate the development and regeneration process of somatic embryos cells (Sharma and Millam, 2004). Limitations to regeneration of somatic embryos may hamper crop improvement using biotechnology approaches. In the present study, the developmental process in somatic embryogenesis of *Carica papaya* L. *var*. Eksotika, *C. papaya* (E) leading to a comparatively high yield of regeneration frequency was conducted.

Materials and Methods

Induction of callus and development of somatic embryos

Immature hermaphrodite Eksotika fruits were randomly harvested from field-grown trees. Fruits were collected between 90 to 100 d after anthesis of hermaphrodite flowers producing the desired seeds, where the final sizes of the ovules were about 3 to 4 mm long. Harvested fruits were washed thoroughly under running tap water and sprayed intermittently with 70% (v/v) ethanol (EtOH) for surface disinfestations. The immature seeds were excised from the immature fruit and were cut open to remove the immature zygotic embryos (IZE). The excised embryos were placed on callus induction (CI) medium consisting of half-strength Murashige and Skoog (1962) (MS) basal salts and enriched with 50 mg/L myo-inositol, full-strength MS vitamins, 45 mg/L adenine sulphate, 100 mg/L glutamine, 250 mg/L carbenicilin, 6% w/v sucrose, 10 mg/L 2,4-D, solidified with 0.195% Phytagel. Meanwhile, CI medium without 250 mg/L carbenicilin is for control experiments. The medium was adjusted to pH 5.8 prior to autoclaving and was dispensed into 9 cm Petri dishes. The cultures were placed in the dark at ±25^{o}C. Observations were carried out every 14 d, and the embryos were sub-

cultured onto fresh medium every 14 d for 8 weeks or until the embryogenic callus developed.

Cell suspension cultures for proliferation of embryogenic callus

Cell suspensions were multiplied in liquid multiplication (LM) medium with similar composition as CI medium, but reducing the concentration of 2,4-D to 2 mg/L. For maintaining the cultures, the concentration of 2,4-D was successively reduced 0.5 mg/L every 14 d. Cultures were kept without plant growth regulators either until 4 to 5 months or as required for regeneration. The pH of the medium was adjusted to 5.8 prior to autoclaving. Ten milliliters of LM medium was initially dispensed into 100 mL Erlenmeyer flasks with about 500 mg embryogenic callus as the inoculum. Subsequently, 10 mL of LM medium was added to the cultures after 2 weeks. Somatic embryos containing mostly the globular structures were sieved from the suspension cultures using a 450 µm pore size filter. The filtrate was transferred to 10 mL of the conditioned and fresh medium respectively. For the following sub-culture (2 weeks), all medium was removed and replaced with 20 mL of fresh medium. Subsequently, the cultures were sub-cultured every 2 weeks by adding 10 mL of LM medium until the volume reached 50 mL. Cultures were maintained at ± 25^{o}C, with a 16 photoperiod at 2000 µmol m^{-2} s^{-1} under continuous agitation on a rotary shaker at 100 rpm.

Histo-anatomy and ultrastructure analysis

Samples were examined after 14 d of culture for histo-anatomy analysis of callus, and 21, 40, and 60 d of culture at different developmental stages. Samples were fixed in a Formalin-Aceto-Alcohol (FAA) 50 solution of formalin: glacial acetic acid: ethanol (EtOH) in a ratio of 5: 5: 90 (v/v/v), and were vacuumed to remove the air from the solution. The samples were kept for 48 h followed by washing with 50% (v/v) EtOH overnight. The mixtures were then dehydrated through nine graded series of alcohol solution. At each step, the samples were treated for 12 h for dehydration process. After the samples were cleaned in fresh Tertiary butyl alcohol (TBA) for 12 h in step nine for the dehydration process, a few chips of aerated wax were added and then left overnight at room temperature. Samples soaked with TBA wax were placed into an oven at 49°C for 2 h. The TBA wax was then added up and left in the oven for another 2 h. Half of the solution was decanted and replaced with 49°C wax mixture with TBA and left overnight, and on the next day replaced with fresh solution for 5 to 7 h followed by replacement with paraplast. After about 12 h, 25 to 30 atmospheric pressure suction was applied for one-half for at 58°C, and the process was repeated to remove air within the samples. Subsequently, samples were embedded in paraffin wax. For sample sectioning, a rotatory microtome (Leica, RM2125) was used following standard procedures. The samples were subjected through a series of staining solution in fuchsin-astra blue combination prior to completely mount microscopy (Fernando *et al*., 2001). Slides were dried overnight and were prepared for observation under the binocular microscope, Carl ZEISS, DSM940A.

Germination, regeneration, and rooting of plantlets

The somatic embryos were cultured on germination (G) medium consisted of MS basal salts supplemented with 108 mg/L myo-inositol, 3% (w/v) sucrose, 0.2 mg/L BAP and NAA respectively, and solidified with 0.195% phytagel for 4 weeks to form the radical shoots. Elongated shoots were transferred to regeneration (R) medium consisting of full-strength MS medium with 1 mg/L gibberellic acid (GA_3), 0.5 mg/L IBA,

and 3.76 mg/L riboflavin for plant maturation within 4 weeks. Finally, the shoots were subjected to rooting medium consisting of full-strength MS medium with 2.0 mg/L IBA for complete plant formation within 8 weeks. Cultures were maintained at ±25°C, with a 16 photoperiod at 2000 µmol m^{-2} s^{-1}. The pH of all media was adjusted to 5.8 prior to autoclaving. Observations were made twice a week for 4 months.

Statistical analysis

In this study, Student *t*-test was used to determine the significant differences between the treated and the control mean values of samples. The standard normal distribution of the means of a random sample, $t = (x-\mu) / Sx$ was applicable if reliable estimate of S sample size was small. As Sx is estimated from a small sample, it is subject to sampling error and will depart more or less widely from the true x. The accuracy of the estimate increases as the number of degrees of freedom on which it is base increases (Murdoch and Barnes, 1998; Dawson *et al*., 1986; Gomez and Gomez, 1984).

Results and Discussion

Callogenesis

Visual observations were made fortnightly for 8 weeks on all immature embryo explants excised from *C. papaya* (E) placed on CI medium. It was observed that explants placed on CI medium produced initial slimy callus after 5 d in culture. The growth rate of the callus increased rapidly after the first subculture as indicated by the weight of the callus. After 14 d of culture on CI medium, friable callus was induced. Embryogenic callus was observed after 21 d of culture on the same medium. Some of the explants produced masses of loose brown calli (Drew and Miller, 1989) which later became necrotic. The similar medium composition was also reported used by other authors (Fitch and Manshardt, 1990; Vilasini *et al*., 2000). The percentage for embryogenic callus induced from IZE explants was more efficient in this study (average of 77.5% in culture medium supplemented with 250 mg/L carbenicillin) (Table 1) compared to previous reports, where only 4% and 47% were reported by Monmarson *et al*., 1995 and Fitch and Manshardt, 1990 respectively. In this study, it was also observed that carbenicillin enhanced the production of embryogenic callus significantly compared to CI medium without carbenicillin (Mohamad Fhaizal *et al*., 2006). Yu *et al*., (2000) also observed better callus growth on media supplemented with 250 to 500 mg/L carbenicilin than medium devoid of the antibiotic. This indicated the dual functions of carbenicillin capabilities, first as an antibiotic with bacteriostatic property (Naumann, 1969) and secondly as an enhancer to the production of embryogenic callus.

Development of somatic embryos

In this study, CI medium was proved capable for inducing friable callus and somatic embryos. In CI medium, somatic embryos were strongly proliferated on the surface of the friable callus in the presence of 2,4-D and carbenicillin. Somatic embryos were first observed after 60 d of culture. After 120 d, about 20-50 somatic embryos were produced from each IZE explants. The callus continuously produced somatic embryos after 150 d of culture. Incorporation of 2,4-D into the media to induce somatic embryos from IZE explants has also been demonstrated in other varieties of papaya such as Kapoho and Taiwan (Fitch and Manshardt, 1990; Malabadi *et al*., 2011). However, the percentage of somatic embryos formation was more efficient in this study (Table 1) compared to previous reports; i.e. showing that the induction of somatic embryogenesis using IZE of *C. papaya* (E) was recorded at 60 to 90%. In this study, it was also observed that in presence of carbenicillin

in combination with 2, 4-D, the compounds enhanced the production of somatic embryos significantly compared to CI medium without carbenicillin (Mohamad Fhaizal *et al*., 2006; Yu *et al*., 2000). In contrast, BAP and NAA supplemented MS medium were also using the same explants (Chen *et al*., 1987; Litz and Conover, 1983) but less percentage of somatic embryos was induced.

Histo-anatomical studies

Various stages of somatic embryo development were studied. During the initial stage, meristematic cells known as pre-embryonic mass (PEM) divided to form pro-embryos (Fig 1a). Somatic embryos arose from a clump of pre-embryonic meristematic cells, which later developed, into globular-shaped embryos (Fig 2a). The histological analysis showed small sized cells with dense cytoplasmic contents, large nuclei with prominent enlarged nucleoli and lacked vacuoles (Fig 2b). Cells that accumulated starch acquired embryogenic characters and became isolated in polysaccharide mucous-like substances visible as protrusions in embryos (Fig 2a). At the end of the first subculture, high frequency embryogenesis was expressed by cleavage divisions within some cells. This was characterized by the multiplication of embryogenic cells which developed into true somatic embryos during the third and fourth sub-culture**.** During the development, the globular-shaped embryos were attached to the original undifferentiated cells through the suspensor group of cells (Fig 2a). Suspensor cells were basal to somatic embryos attached to the pre-embryonic complex (Fig 3a and 3b). This connection was important for the nutrient supply to the embryo. No vascular connection between the embryos with the intact cells was observed. Therefore, somatic embryos were easily separated from the original undifferentiated cells. Somatic embryos also had both shoot and root poles surrounded by distinct layers of protoderm (Fig 2b). Protoderm developed at the early stage of the embryo forming the unicellular outer layer of embryo sac to protect the embryo cells (Fig 2b). Protoderm was primarily meristematic tissue, which then formed epidermal cells. The globular embryo elongated to form an oblong embryo, which later developed into heart-shaped tissue (Fig 3a). The oblong embryo had elongated pre-cambial trace (Fig 3a) observed as a dense band of cells in the embryo (Fig 3b). The cells in this region were highly developed and compact. The heart-shaped embryo subsequently elongated and became a torpedo-shaped embryo (Fig 4a). The developed pre-meristematic cells showed the initial shoot and root apex formation (Fig 4b). Subsequently it developed into the vascular tissue, which consisted of xylem and phloem cells. During the late torpedo-stage, the initiation of the development of leaf primordia indicated that the embryos developed into cotyledonary stage (Fig 5b). Shoot initials at an early stage were dome-shaped from the apical region of embryo axis with densely stained cells and subsequently developed into a typical apical meristem covered with leaf primordia. Finally, the advanced stage of somatic embryo was observed (Fig 5a). At this stage, most of the structures were developed and clearly observed.

Germination, regeneration and rooting

Consistently, 100% shoot germination (Table 2) was observed when somatic embryos were cultured on G medium supplemented with 0.2 mg/L BAP and 0.2 mg/L NAA in light. In this study, shoots were obtained within 3 months of initial culture. When normal somatic embryos germinated, hypocotyls raised cotyledons and shoot apex (Fig 6). However, germination of abnormal somatic embryos produced aberrant cotyledons and the shoot apex became stunted. The somatic

embryos germinated in a highly synchronous manner; where the hypocotyl became swollen followed by rapid shoot multiplication. Chlorophyll development became obvious in the cotyledons and the shoots underwent the first stage of plant regeneration (Fig 7). On the same medium, averages of 90.4% of shoots were formed (Table 3). Maturation of somatic embryos was achieved on G medium. However, liquid medium for somatic embryos maturation was also reported by Gupta and Timmis, 1999; Gorbatenko and Hakman, 2001. Several sub-cultures on the G medium supplemented with BAP and NAA followed by culturing on R medium consisting of GA_3, IBA and riboflavin produced an average of 97.3% complete plantlets (Table 4). The rooting for rhizogenesis of the new *in vitro* derived shoots was induced in medium containing 2.0 mg/L IBA, where a minimum percentage of 50% formation of profuse roots and normal plantlets (Fig 8) was achieved. Acclimatization was carried out for 2 months for the purpose of hardening the vegetative structures and later to the field where the conditioning of regenerants were essential for adaptation (Fig 10). For acclimatization, when the first set of matured tri-lobed leaves appeared (Fig 9), the plantlets were transferred to polybags with soil for 1 to 2 months in the nursery. After reaching a desirable size (10 to 15 cm), plants were then transplanted to field.

Table 1. Response of immature zygotic embryos on callus induction and somatic embryo formation. Values represent three replicates per treatment in two difference experiments.

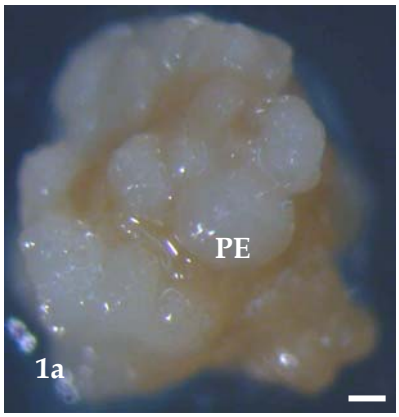
Medium	No. replications	No. explants	No. explants producing callus	Percentage of callus clumps forming somatic embryos	Mean ± SD
**CI medium (control)	1	47	31±11.3	66.0±11.3	70.8±8.6
	2	32	21±7.8	65.6±7.8	
	3	31	25±4.2	80.7±4.2	
*CI medium	1	30	28±1.4	93.3±1.4	77.5±15.4
	2	30	23±4.9	76.7±4.9	
	3	24	15±6.4	62.5±6.4	

CI medium supplemented with* and without** (control) 250 mg/L carbenicillin. In each replicates, the values with different letters are significantly different ($p \leq 0.05$). Mean separation at $p \leq 0.05$ as determined by *t*-test followed by the variability observed for quantitative characters as determined by ± standard deviation (SD).

Table 2. Effects of BAP and NAA on germination of somatic embryo, measured by the number of somatic embryos producing cotyledonary-stage embryos.

Medium	No. of somatic embryos (Clumps)	No. of germinated somatic embryos (Clumps)	Percentage germinated somatic embryos (%)
*G medium	7	7	100
	8	8	100
	9	9	100
	7	7	100
	10	10	100

*Germination medium supplemented with 0.2 mg/L BAP and NAA.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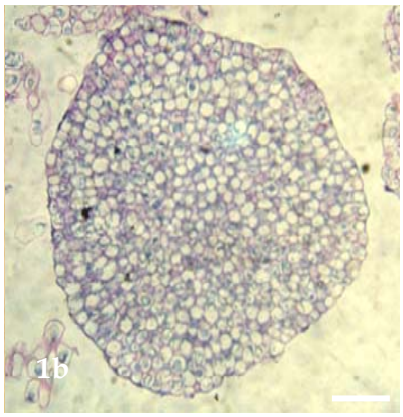
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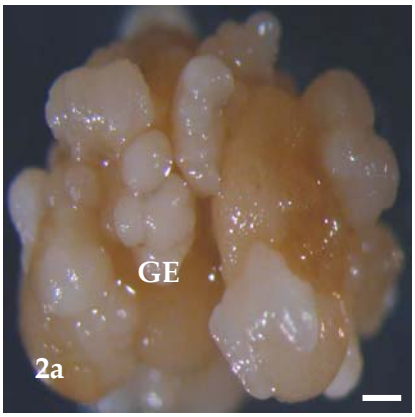
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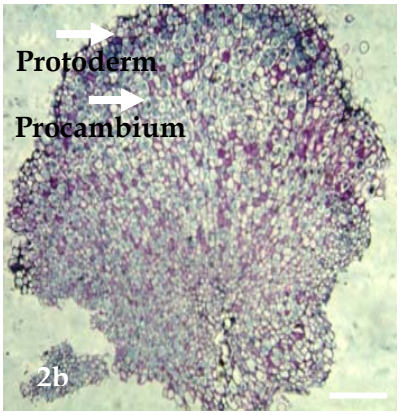
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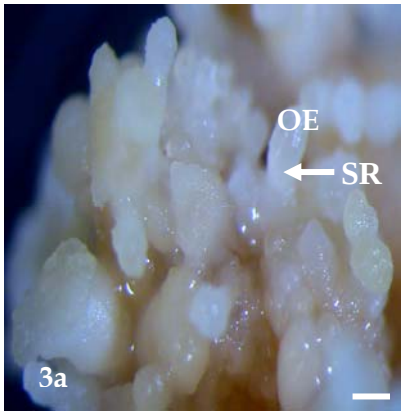
Procambium
2b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SR
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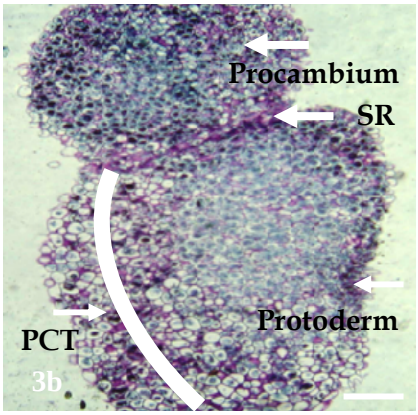
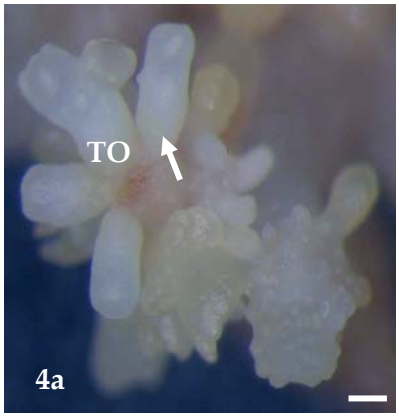

Procambium
SR
PCT
Protoderm
3b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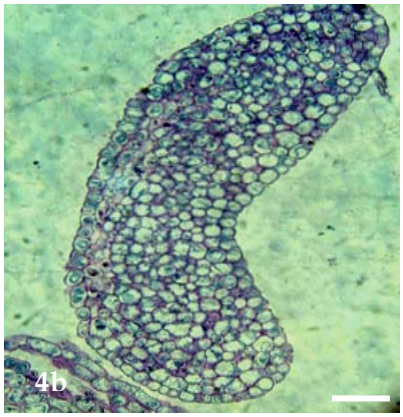
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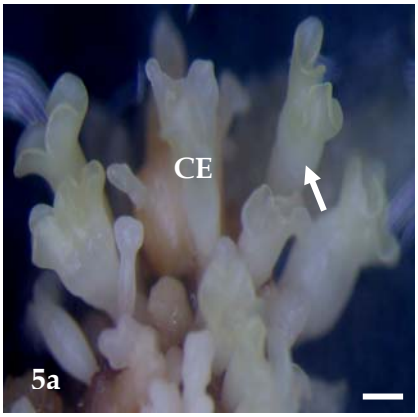
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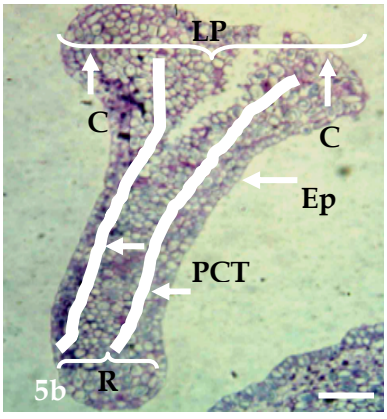
LP
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5b

Fig 1a-5a: Phases of embryogenesis after 14, 21, 40, and 60 d of culture in MS medium containing 10 mg/L 2,4-D, (10x). Fig 1b-5b: Longitudinal sections of the embryogenic cells (10x). C: Cotyledon, CE: Cotyledonary embryo, GE: Globular embryo, LP: Leaf primordial; OE: Oblong embryo, PCT: Pro-cambial traces, PE: Pro-embryo, PEM: Pro-embryo mass, R: Root, SR: Suspensor region, TO: Torpedo embryo, Ep: Epidermis. (Fig 1a and 1b) Morphological appearance of high frequency pro-embryo (Scale: Bar at 1 mm) cluster of callus mass of *Carica papaya* L. *var*. Eksotika and dedifferentiation of cells (Scale: Bar at 50 µm) within the callus and accumulation of starch. (Fig 2a and 2b) Direct origin of globular embryo (Scale: Bar at 1 mm) formed on a high frequency embryogenic callus and internal cleavage results in globular embryo. (Fig 3a and 3b) Oblong- and heart-shaped embryo (Scale: Bar at 1 mm) formed after 21 d of culture from the superficial cells of the complexes and small cells (Scale: Bar at 50 µm) arranged compactly with dense cytoplasm (arrow). (Fig 4a and 4b) Torpedo-shaped embryo (Scale: Bar at 1 mm) linked to the pre-embryonic complex by its basal portion (arrow) and pronounced development of the apical caulinar meristem (arrow). (Fig 5a and 5b) Multicotyledonary cylindrical embryo (Scale: Bar at 1 mm) in an advance embryo structure and histological appearance of a somatic embryo (Scale: Bar at 50 µm).

Table 3. Effects of BAP and NAA on well-germinated somatic embryo showing cotyledonary leaves.

Medium	No. of somatic embryos (Clumps)	No. of plantlets with shoots and root initials (Clumps)	Percentage of plantlets with shoots and root initials (%)	Mean ± SD
*G medium	20	20	100	90.4±5.8
	21	19±1.4	90.5±1.4	
	24	21±2.1	87.5±2.1	
	26	22±2.8	84.6±2.8	
	28	25±2.1	89.3±2.1	

*Germination medium supplemented with 0.2 mg/L BAP and NAA. In each replicates, the values with different letters are significantly different ($p \leq 0.05$). Mean separation at $p \leq 0.05$ as determined by *t*-test followed by the variability observed for quantitative characters as determined by ± standard deviation (SD).

Table 4. Mean number of well-germinated somatic embryo showing cotyledonary leaves from immature embryos-derived calli maintained on R medium 4 weeks.

Medium	No. of somatic embryo (Clumps)	No. of plantlets with shoots and root initials (Clumps)	No. of complete regenerated somatic embryo (Clumps)	Completely regenerated somatic embryo (%)	Mean±SD
*R medium	25	25	24±0.7	96±0.7	97.3±2.5
	25	23±1.4	23	100	
	25	19±4.2	18±0.7	94.7±0.7	
	25	25	24±0.7	96±0.7	
	25	25	25	100	

*Regeneration medium supplemented with 1 mg/L GA_3, 0.5 mg/L IBA, and 3.76 mg/L riboflavin. In each replicates, the values with different letters are significantly different ($p \leq 0.05$). Mean separation at $p \leq 0.05$ as determined by *t*-test followed by the variability observed for quantitative characters as determined by ± standard deviation (SD).

Fig 6-10: The developmental stages of somatic embryos. Mature cotyledonary-stage (6); Mature shoots (7); A plant with well-developed roots (8); Plantlets transferred into poly bag for acclimatization (9); and a plant grown in soil (10). (10x) Scale: Bar at 1.0 cm.

Conclusion

Results demonstrated that the histo-anatomy analysis showed the friable calli produced numerous somatic embryos originating from multiplication of cells in the IZE of *C. papaya* (E) cultured in two-simple medium. Although somatic embryogenesis was easily expressed for IZE of Eksotika, further studies are necessary to define the requirements and enhance embryo formation from other sources of explants, since this was a promising multiplication technique for the *C. papaya* (E) with elite characteristics. This technique has the potential to be applied in other papaya species for crop improvement.

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