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Effect of Carbenicillin on Somatic Embryos Formation of Papaya (*Carica papaya* L. var. Eksotika I)

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ABSTRACT Embryogenic callus could be established from immature embryo of *Carica papaya* L. var. Eksotika I after 3 - 4 months of culture on Callus Induction (CI) medium. Embryogenic callus was successfully induced (100 %) on CI medium supplemented with 250 mg/L Carbenicillin plus 10 mg/L 2,4-dichlorophenoxyacetic acids (2,4-D). Transparent somatic embryos then proliferated from the light brown friable embryogenic callus. Carbenicillin that was frequently used as an antibiotic to prevent fungal contamination in *in-vitro* cultures was found to stimulate the growth of somatic embryos of *Carica papaya* L. var. Eksotika I. Somatic embryos were maintained in a medium containing either reduced or without 2,4-D. The cultures could be maintained for a period of 5 - 6 months with no apparent loss of regenerative potential. The somatic embryos germinated on Germination (G) medium supplemented with 0.2 mg/L 6-Benzylaminopurine (BAP) and 2.0 mg/L 1-Napthaleneacetic acids (NAA) produced high regeneration frequency (88.41 %) with initial development of hypocotyls followed by rapid growth of plantlets. The *in vitro* shoots were readily rooted in G medium supplemented with 0.5 mg/L Indole-3-butyric acid (IBA) with 75 % successful rate.

ABSTRAK Kalus embriogenik dapat dihasilkan daripada embrio pra-matang *Carica papaya* L. var. Eksotika I selepas aktiviti pengkulturan selama 3 - 4 bulan di atas media Induksi Kalus (CI). Kalus embriogenik berjaya dihasilkan (100 %) di atas media CI yang ditambah dengan 250 mg/L Karbenicilin dan 10 mg/L 2,4-diklorofenoksiacetik acid (2,4-D). Embrio somatik yang telus cahaya kemudiannya berkembang biak daripada kalus embriogenik yang bersifat rapuh dan berwarna coklat cerah itu. Karbenicilin yang biasaannya digunakan untuk menangani kontaminasi fungi dalam kultur *in vitro* telah didapati berupaya merangsang pertumbuhan embrio somatik daripada *Carica papaya* L. var. Eksotika I. Embrio somatik dikekalkan dalam media tanpa 2,4-D. Kultur ini dapat dikekalkan dalam tempoh masa 5 - 6 bulan tanpa kehilangan potensi perkembangan dan pertumbuhannya. Pertumbuhan embrio somatik di atas media pertumbuhan (G) yang ditambah dengan 0.2 mg/L 6-Benzilaminopurin (BAP) and 2.0 mg/L 1-Naftalinacetik acid (NAA) menghasilkan kadar pertumbuhan yang tinggi (88.41 %) dengan perkembangan hipokotil pada awalnya dan diikuti oleh pertumbuhan plantlet kemudiannya. Pucuk *in vitro* dipindahkan ke atas media G yang ditambah dengan 0.5 mg/L Indol-3-butirik acid (IBA) menghasilkan 75 % kadar kejayaan pertumbuhan.

(Somatic embryogenesis, *Carica papaya*, carbenicillin,)

INTRODUCTION

Papaya (*Carica papaya* L.) is one of the most widely grown crops in the tropical region including Malaysia. It is a multi-purpose fruit produced for food and source of compounds for medicinal use [4].

Many hybrids have been developed world wide for commercialisation, for instance Solo, Waimanalo, Subang, Setiawan and Eksotika. The

variety Eksotika was introduced to the Malaysian farmers in the late eighties [20] because of its improved fruit texture, taste and size. Various tissue culture systems have been developed in order to fulfill the demand of papaya plantlets for commercial planting [17], [19], [13] and [2]. Somatic embryogenesis via suspension cultures of papaya were carried out by many researchers for an efficient micropropagation protocol [12], [14] and [11]. Besides complementing the conventional breeding [13], it is also used for genetic transformation purposes [7]. Current protocols are reported to be slow thus increasing the chances of somaclonal variation and were further hampered by the difficulty in rooting of the *in vitro* plantlets.

The objective of this study is to improve the regeneration protocol by the incorporation of antibiotics and the use of IBA for regeneration and to ease rooting of the plantlets.

MATERIALS AND METHODS

Plant material

Seeds of immature Eksotika I were obtained from fruit randomly harvested from field grown trees, 90 - 100 days old after anthesis of female and hermaphrodite flower. The desired seed is actually three months old post anthesis, in which it will reached their final size as the ovules (3 - 4 mm long).

Media

Embryogenic callus was initiated on CI medium which consisted of half strength MS basal salts [15] enriched with myo-inositol (50 mg/L), full strength MS vitamins, adenine sulphate (45 mg/L), glutamine (100 mg/L), 2,4-D (10 mg/L), sucrose (6 % w/v) and solidified with phytagel (1.95 g/L).

Embryogenic cell suspension was maintained in liquid induction medium, LI, with similar composition as CI supplemented with 2,4-D ranging 2 - 5 mg/L and the concentration of 2,4-D would be decreased for maintaining the culture.

Germination and regeneration of embryo were carried out on modified MS, G medium supplemented with 0.2 mg/L BAP and NAA respectively. Furthermore, the 4-5 months old plantlet were transferred to maturation medium which consisted of full strength of MS with selected phytohormone, 1 mg/L Giberallic acid, GA_3, 0.5 mg/L IBA, and 3.76 mg/L Riboflavin.

The pH of all media was adjusted to 5.8 with 1.0 M NaOH or 1.0 M HCl prior to autoclaving at 121°C for 20 minutes.

Explant Culture

Harvested fruits were washed thoroughly under running tap water and sprayed intermittently with 70 % (v/v) EtOH for surface sterilisation before placing in the laminar flow cabinet. The immature seeds were taken from the immature fruit and they were cut open to remove zygotic embryos. The excised embryos were placed on solidified CI medium in Petri dishes. The cultures were placed in the dark at ± 25°C.

Observation was carried out every 14 days and the embryos were sub-cultured onto the same medium every 2 weeks for 8 weeks or until the embryogenic callus developed. The friable embryogenic callus was removed, and transferred to liquid medium with the same constituent.

Globular structures formed were sieved from the suspension cultures using a filter with 450µm pore size and cultured into fresh liquid medium. The filtrate was subsequently transferred to fresh medium with similar composition every 10 days. Cultures were maintained at ± 25°C, with a 16 hours photoperiod at 2000 lux under continuous agitation on a rotary shaker at 100 rpm.

Single cells of embryogenic suspension (5 ml) were transferred onto CI medium followed by G medium. Plantlets regenerated from the embryos were sub-cultured every two weeks for a period of 3 - 4 months until mature.

The data output can scientifically be documented based on:

i. The effect of Carbenicillin incorporation in CI medium
ii. The rate of viable immature zygotic embryos to form embryogenic callus
iii. The growth pattern of embryogenic cell suspension
iv. Values of the means of two independent experiments ±SD

RESULTS AND DISCUSSIONS

The embryogenic callus was induced from the immature zygotic embryos (Figure 2.i.) after 3

weeks of culture on CI medium. Some of the zygotic embryos plated on CI media produced masses of loose brown calli [7]. The embryogenic and non-embryogenic nature of callus was determined by double staining method using acetocarmaine and Evan's blue stains [9]. Nuclei of cells stained with intense bright red indicated positive results of embryogenic callus and conversely, blue stained cells showed otherwise. Embryogenic cells were selected and used for initiation of cell suspensions due to their higher capability of totipotency and regenerative rate.

After 8 weeks of culture, 94 % of the embryo explants developed into embryogenic callus on similar medium composition (CI) used by other reports [20] and [7] but supplemented with carbenicillin. Comparatively, the induction responses for embryogenic callus from explants were more efficient than previous reports, where 62 % and 78% were reported by [20] and [7] respectively. This indicated the dual functions of carbenicillin capabilities, first as an antibiotic with bacteriostatic property [16] and secondly as an enhancer to the growth of embryogenic callus (Table 1) when bacteria-suppressing effects of carbenicillin occured. It has been reported that endogenous bacteria contaminated cultures without the use of carbenicillin [16] As a result, the fresh weight of the callus also increased in the CI medium with 250mg/L carbenicillin. Similar observation was made by [22] on callus growth using callus induction media supplemented with 250 - 500 mg/L carbenicillin. The best response was observed in cultures placed in the dark. Other explants such as roots, shoot tips and hypocotyls were also used for plant regeneration [3] and [6]. However, zygotic tissues were reported to produce the highest number of plantlets via somatic embryogenesis and the plantlets were free of genetic off-types thus permits the cloning of adult plants [14].

Upon transfer to the agitated LI medium, large masses of single cells were produced (Figure 2.iii.). A suitable embryogenic callus for initiation of cell suspension should be easily dispersed and released into the liquid medium in a few seconds due to the mechanical effects of cell clusters breaking in the liquid.

Proliferation of globular somatic embryos from embryogenic callus became apparent after 1-2 month(s) of culture (Figure 2.ii.). The pro-embryos increased in number rapidly and produced suspension of highly uniform single embryos (Figure 2.iii.). By regular sub-culturing, the cells in fresh medium containing 2 to 5 mg/L 2,4-D, a continuous proliferation was observed for 12 months without apparent loss of regenerative potential.

Table 1. Effects of carbenicillin on growth of papaya somatic embryos culture. Growth ratio is the ratio between the fresh weight (FW) measured 4 weeks after the subculture and the starting FW. Values are the means of two independent experiments ±SD.

MEDIUM	PLATE	FRESH WEIGHT (G) INCUBATION PERIOD						GROWTH RATIO
		0	5	10	15	20	25	
CI-C$^+$	1	0.11	0.3	0.49	0.61	0.81	0.92	3.24 ± 0.31
	2	6.21	6.29	6.5	6.59	6.74	6.88	39.21 ± 0.26
	3	0.26	0.41	0.56	0.66	0.84	0.92	3.65 ± 0.25
	4	0.16	0.26	0.45	0.56	0.74	0.92	3.09 ± 0.29
	5	0.28	0.2	0.02	0.08	0.23	0.38	1.19 ± 0.13
CI-C$^-$	1	3.31	3.41	3.58	3.69	3.86	4.04	21.89 ± 0.27
	2	3.09	3.22	3.42	3.57	3.81	4.07	21.18 ± 0.37
	3	1.08	1.16	1.32	1.42	1.57	1.72	8.27 ± 0.24
	4	0.18	0.82	1.45	1.88	2.21	2.56	9.10 ± 0.89
	5	2.49	2.59	2.76	2.88	3.06	3.22	17.00 ± 0.28

Key index: CI-C$^+$ (Callus induction medium with 250 mg/l Carbenicillin)
CI-C$^-$ (Callus induction medium without Carbenicillin)

Continuous proliferation was due to profuse budding from globular pro-embryo [12] and [11]. The regenerative potential of the suspensions could be further maintained for 2 - 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. The physiological status of the cells such as nutrition, pre-treatment with the 2,4-D, environment and age of cells determined the competence of cell culture. In this work, 2,4-D was found to be the major plant growth regulator affecting the competence of the single cells in liquid culture. Cell divisions in suspension cultures were encouraged by the agitation of orbital shaker at 100 rpm served to aerate the culture and thus a higher growth rate at the exponential phase than those of callus in semi-solid medium.

Cells viability assessment was carried out by using fluoresced diacetate (FDA) stain [21]. Large numbers of cells (65 %) with dense cytoplasm fluoresced under ultraviolet (UV) microscope showing viable cells.

A non-invasive method for routine-estimation of suspension cultures which performed reproducible growth cycles was done by taking fresh weight of two different cell lines. Fresh weight (FW) was expressed as gram per 150 ml of suspension cells and the time course of FW were measured per day (Figure 1). The data obtained showed in Figure 1, that the cells were actively growing within the first period of 10 days, followed by an exponential phase from day 20 to day 30. The development of the FW during the entire growth cycle from each individual batches could be used to select batches with desired growth characteristics (Figure 1). On G medium, 88.41 % matured single embryos formed distinct structural and developmental features with organised embryo cells (shoot/root) before reaching the stage of mature plantlets. The germination rate was 20 % higher compared to cells suspensions from *Carica pubescens Lenne et Koch* and *Carica papaya* [12] and [11]. Data was shown in Table 2. In this work, maturation of somatic embryo was achieved on solidified G medium but it was also reported that vigorous embryos could also be obtained in liquid medium [10] and [8]. It was observed that for the completion of maturation process, each embryo must achieve both morphological (Figure 2.vi.) and physiological maturity. The complete process of plant regeneration encompasses a series of defined developmental stages from cell aggregate to heart shaped to cotyledonary for 8 and 3 weeks respectively. Several sub-cultures on the G medium supplemented with BAP and NAA (0.2 mg/L) produced 88 % of normal plantlets.

Rooting was initiated on G media improvised with GA_3 (1 mg/L), IBA (0.5 mg/L IBA) and riboflavin (0.37 mg/L). The formation of profuse roots and normal plantlets in this study required a specific auxin type (IBA) and concentration to suit the initial needs of the developmental tissue. In this work, 0.5 mg/L IBA was used to induce the outgrowth of axillary roots whereas 0.2 mg/L BAP and NAA promote the stimulation of shoot and root induction. For acclimatisation processes, when the first set of matured tri-lobed leaves appeared, the plantlets were transferred to poly-bags with commercial soil (Jaya Tanah Baja) for 1 - 2 months in the nursery. After reaching a desirable size (10 - 15 cm), plants are then transplanted to the field. This will enable plant physiological stabilization in order to adapt with external atmosphere.

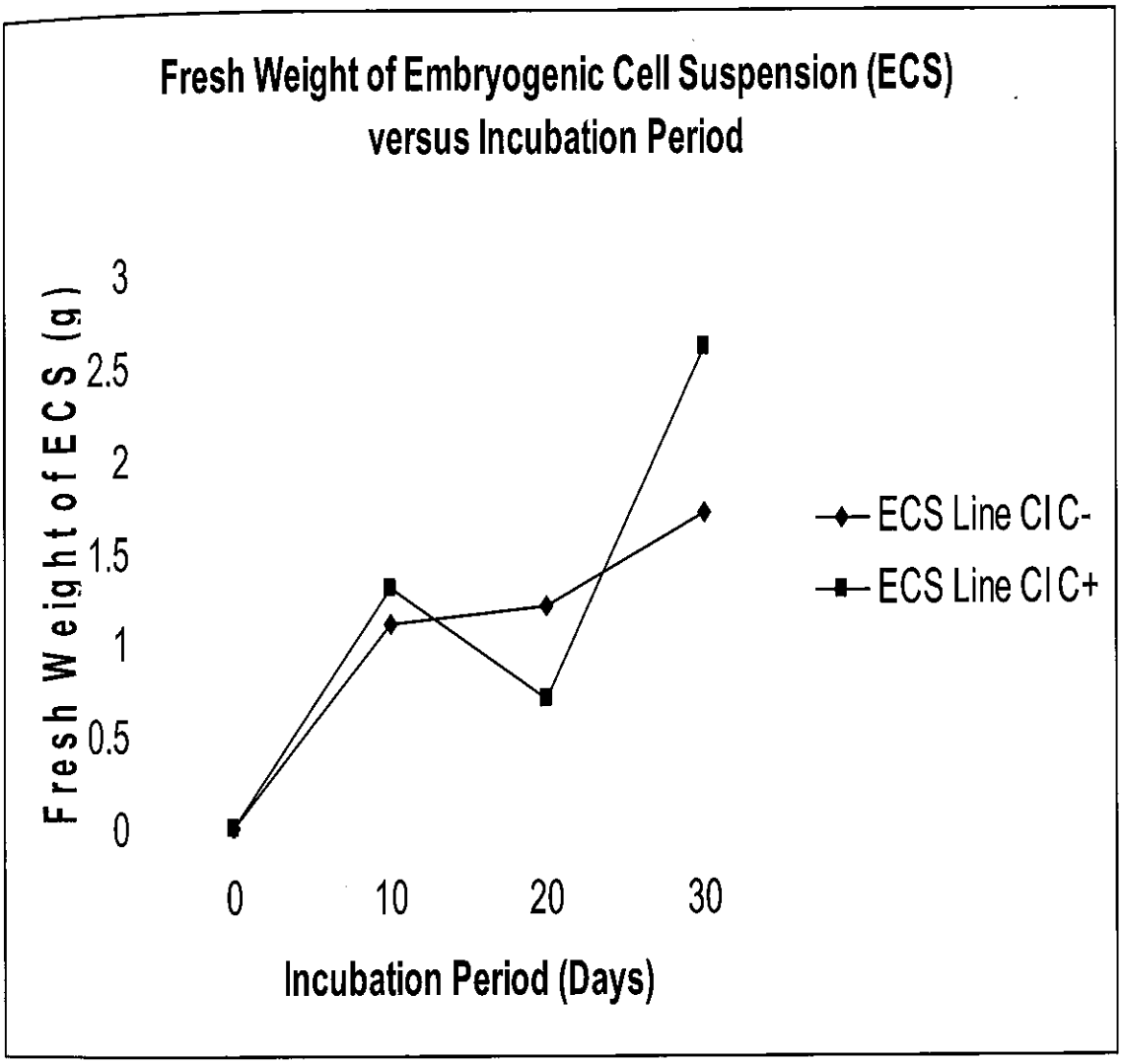

Figure 1. Time course of fresh weight (FW) of a *Carica papaya* L. var. Eksotika I suspension grown in batches of 150 ml Erlermeyer flasks

Table 2. Percentage of explants forming embryogenic callus and germinated embryos

MEDIUM	NUMBER OF EXPLANTS	PERCENTAGE (%) OF EXPLANTS FORMING EMBRYOGENIC CALLUS	NUMBER OF GERMINATED SOMATIC EMBRYOS	PERCENTAGE (%) OF GERMINATED SOMATIC EMBRYOS
Germination, G, medium	16	100	13	81.25
	20	100	20	100
	21	100	19	90.48
	24	100	21	87.50
	26	100	22	84.62
	28	100	25	89.29
	35	100	30	85.71

Average percentage = 88.41

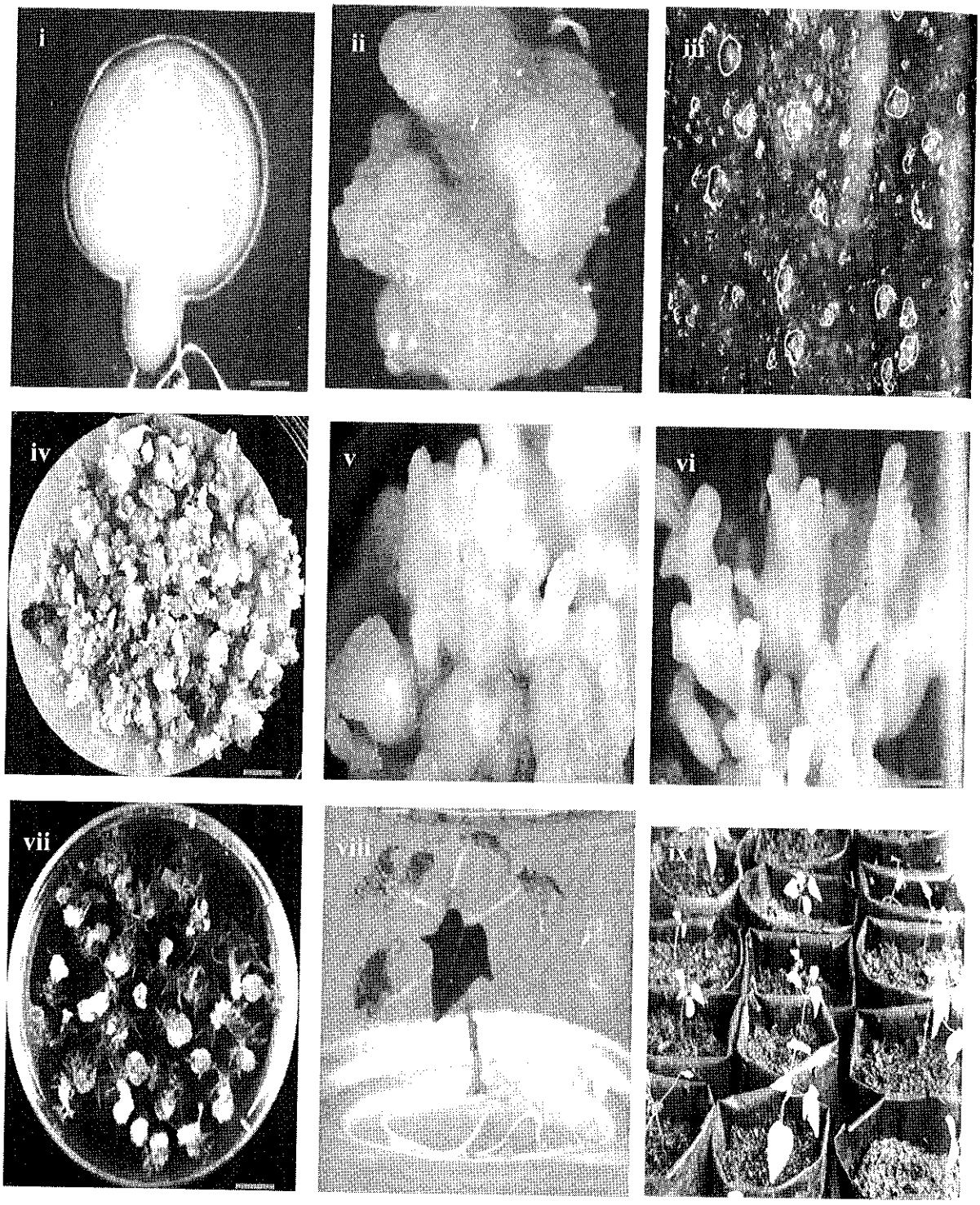

Figure 2. Immature embryo of *Carica papaya* L. var. Eksotika I (**i**); Callus induction showing globular stage (**ii**); embryogenic cell suspension (**iii**); single cells onto CI medium (**iv**); torpedo shape of somatic embryo (**v**); mature cotyledon (**vi**); shoot multiplication and maturation (**vii**); plantlet at rooting (**viii**) and vegetative acclimatization (**ix**). Bar at 0.5 cm to scale.

CONCLUSIONS

Somatic embryos were obtained from immature zygotic embryos on CI medium supplemented with carbenicillin which progressed through characteristic developmental stages (globular, heart, torpedo and mature), and became independent from the parental callus. Carbenicillin was used to eliminate contamination and was found to enhance the formation of somatic embryos simultaneously.

Batch culture technique was used to initiate single cell cultures. The cell suspensions were grown in liquid LI culture medium where early stage somatic embryos or friable embryogenic callus were best used as initial inoculum.

Germination of single cells from embryogenic suspensions culture showed that somatic embryos germinated in a highly synchronous manner where the hypocotyls first became swollen followed by rapid shoot multiplication. Concurrently, chlorophyll development became visible in the cotyledons and subsequently these shoots formed normal plants. Plants were successfully acclimatized and transferred to the nursery.

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