

Full Length Research Paper

Efficient somatic embryo production of Limau madu (*Citrus suhuiensis* Hort. ex Tanaka) in liquid culture

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Effects of N6-benzylaminopurine (BAP) concentration, initial cell density and carbon sources and concentrations for producing cell suspension and somatic embryos of Limau madu (*Citrus suhuiensis* Hort. ex Tanaka) were investigated using cell suspension culture. Cells were first inoculated into Murashige and Skoog (MS) medium (1962) supplemented with 4.4 to 13.3 μM BAP. Growth rate of cells was at its maximum (6.69 mg day^{-1}) in media supplemented with a lower concentration of BAP (6.7 μM). Embryogenic cell at 2 mg ml^{-1} was found to be the most effective inoculum size for the highest growth rate (3.35 mg day^{-1}) of cell proliferation within a period of 15 to 30 days after inoculation (DAI). This inoculum size resulted in 31.75% faster embryo growth than those with inoculum densities of 4 to 6 mg ml^{-1} . Sucrose (88, 117 and 146 mM), glycerol (16, 22 and 27 mM) and combinations of sorbitol and galactose (146:0, 110:36, 73:73, 36:110 and 0:146 mM) were tested for their effects on embryogenic cell proliferation and somatic embryo induction. Results indicates that sucrose at 146 mM induced cell proliferation (7.65 mg day^{-1}) and produced a higher quantity of cells than glycerol at 27 mM (2.33 mg day^{-1}) and a combination of sorbitol and galactose at 73:73 mM (4.64 mg day^{-1}), but failed to induce somatic embryos. Glycerol in different concentrations was ineffective in cell proliferation and somatic embryo induction. At optimal BAP concentration (6.7 μM), a small amount of embryogenic cells (100 mg in 50 ml) can be multiplied profusely in sucrose-containing medium. A large number of somatic embryos (951) were induced in a medium containing 110 mM sorbitol and 36 mM galactose as the most effective carbon source for inducing somatic embryos without BAP.

Key words: Limau madu, cell suspension, sucrose, sorbitol, galactose.

INTRODUCTION

Somatic embryogenesis in *Citrus* is one of the techniques that make possible genetic advances in *Citrus*, facilitating the recovery of plants from irradiated cells, callus transformation, protoplast fusion and somatic hybridization. Efficiency of plant recovery through somatic embryogenesis is therefore very important and should be

well defined for specific elite genotypes. In *Citrus*, somatic embryogenesis has been extensively studied, both in terms of media components and of the structure of the medium, as well as using either solid or semi-solid medium. However, only a few studies (Gavish et al., 1991, 1992; Vu et al., 1993; Cabasson et al., 1995, 1997) have focused on the improvement of induction of somatic embryos of *Citrus* in liquid culture. One early study showed that the rate of growth and differentiation in liquid culture of *Citrus* is slower than that of other plants (Gavish et al., 1991), but a subsequent study showed that by using temporary immersion, high somatic rates can be achieved (Cabasson et al., 1997).

Somatic embryogenesis of *Citrus* genotypes commonly begins using explants originating from nucellar and somatic

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Abbreviations: PEDC, Pro-embryogenic derived callus; PEM, pro-embryogenic masses; ME, malt extract; MS, Murashige and Skoog; DAI, days after inoculation; FW, fresh weight; DW, dry weight; GR, growth rate.

tissue (Carimi and De Pasquale, 2003). Carbohydrate source and composition of the culture are crucial for inducing somatic embryogenesis expression in *Citrus* (Ben-Hayyim and Neumann, 1983; Gavish et al., 1991). Types of hormone and their concentrations are also specific in somatic embryogenesis expression in *Citrus*. The mechanism for induction and expression of somatic embryogenesis in *Citrus* differs greatly from that of carrot, which is often used as the model system for plant embryogenesis. For example, in the case of carrot cultures, the application of auxin is necessary to induce somatic embryogenesis and to maintain the growth and competence of the callus cultures. However, in the *Citrus* system, when the expression of somatic embryogenesis is to be induced, cultures are transferred to a medium lacking auxin (Komamine et al., 1992).

Cytokinin is the hormone most frequently used in somatic embryogenesis of *Citrus*. Cytokinin activity is a property of two classes of compounds: the N6-substituted adenine derivatives and the synthetic phenyl urea derivatives (Mok et al., 1982, 1987). A range of different cytokinin concentrations have been used to induce somatic embryogenesis in floral explants of *Citrus* and in non-ovular explants (Carimi et al., 1995, 1999; Fiore et al., 2002; Siragusa et al., 2007) and have been shown to induce higher embryogenic potential and shoot regeneration of *Citrus*.

The successful performance of all the stages of somatic embryogenesis has been reported, from the growth of embryogenic cells to embryo maturation in solid medium, but these techniques are difficult to automate as well as expensive to produce, rendering them less suitable for large-scale production. The use of larger-scale liquid cultures and automation has the potential to resolve the problems of manual handling of the various stages of "micro-propagation" while decreasing production costs significantly. Of the various forms of *in vitro* plant tissue culture, cell suspension culture is most amenable to large-scale production of plantlets due to its superior culture homogeneity.

The objectives of the present study were to determine the best concentration of N6-benzylaminopurine (BAP) for proliferating embryogenic cells of Limau madu through cell suspension cultures, to obtain the best inoculum density for fast growth of embryogenic cells and somatic embryo, to select the best carbon source(s) for fast embryogenic cells proliferation and somatic embryo induction in liquid medium, and to identify the best concentration of carbon source for embryogenic cells proliferation and somatic embryo induction in cell suspension.

MATERIALS AND METHODS

Plant materials and explanting

Open pollinated, immature fruits of Limau madu at different ages

(80 to 120 days after anthesis) were harvested from mature plants at Desaru Fruit Farm, Desaru, Johor Bahru, Malaysia. The fruits were surface sterilized by immersion for 5 min in 70% (v/v) ethanol, followed by immersion for 20 min in 20% (w/v) sodium hypochloride, and finally by rinsing three times in autoclaved distilled water for 5 min per rinse in a laminar air flow cabinet (LAF). Immature seeds located along the part of segment wall adjacent to the fruit axis were carefully excised and transferred to a Petri dish containing semisolid (0.25% gelrite) Murashige and Skoog (MS) (1962) medium without growth regulators. The integuments (testa and tegmen) were then removed with the aid of a dissecting microscope under aseptic conditions in a LAF. Nucellus segments were directly cultured as explants for callus induction.

Callus induction and cell suspension culture

For callus induction the MS basal salts and vitamins (Duchefa M220) medium was used as the basic medium, supplemented with 500 mg L⁻¹ malt extract (ME), 146 mM sucrose, 0.27% gelrite and 13.3 μM BAP. The pH of the medium was adjusted to 5.7 ± 0.1 with 0.5 M potassium hydroxide before autoclaving at 121°C and 15 psi (103 kPa) for 15 min. Petri dishes (90 × 15 mm) were filled with 25 ml of medium and sealed with parafilm M. Five nucellus tissues were cultured on each Petri dish. Explants were transferred to the same fresh media every 15 days. Explants and callus proliferation were maintained under the environment of a growth room with temperature of 25 ± 2°C and a 16 h photoperiod under cool white light at 35 μmolm⁻²s⁻¹ provided by Osram cool-white fluorescent lamps.

The friable embryogenic callus formed was subcultured at two weeks interval into 50 ml liquid MS media in 100 ml flask for initiation of cell suspensions. Cultures were maintained on a shaker at 50 rpm in a growth room. 15 days after starting culture, liquid media were refreshed by reducing the existing media and topping-up with fresh 50 ml medium, after which cultures were mechanically agitated at 70 rpm. Liquid media were thus refreshed biweekly until the cultures attained the growth coefficient of three to six times more of the initial inoculum obtained within 15 days.

Effects of different BAP concentrations on cell suspension cultures

About 300 mg stabilized embryogenic cells from previous cultures were transferred into 50 ml of the BAP treatment medium in 100 ml flasks. Five flasks each of six concentrations of BAP (4.4, 5.6, 6.7, 7.8, 8.9 and 13.3 μM) were inoculated with 300 mg cells. This experimental design had a total of five samples for each treatment. Cell suspensions were sub-cultured at two-week intervals. Flasks were observed for fresh cell biomass and dried cells every 15 days for 60 days. Cells were dried on filter paper for 2 h to observe fresh weight (FW) of cell biomass and then were kept in a dryer at 50 to 60°C for 48 h for observation of dried cells (DW).

Effects of initial cell density on growth of cell suspension cultures

To select the suitable density of cells for cell proliferation, embryogenic cells at three levels of density; 2, 4 and 6 mg ml⁻¹, were inoculated into 50 ml MS media supplemented with 6.7 μM BAP, 500 mg/L ME and 146 mM sucrose. Cell suspensions were sub-cultured at two-week intervals under the same culture conditions and the same growth parameters were measured as indicated earlier.

Effects of carbon sources on undifferentiated cell growth and somatic embryogenesis

Embryogenic cells in the amount of 300 mg in 50 ml medium were transferred into the carbon sources and concentration treatment medium. Three types of sugar; sucrose (88, 117, 146 mM), glycerol (16, 22, 27 mM) and a combination of sorbitol and galactose in ratios of 146:0, 110:36, 73:73, 36:110 and 0:146 mM (100:0, 75:25, 50:50, 25:75 and 0:100%) were individually tested on 6 mg ml⁻¹ cells per flask. Culture medium was supplemented with 6.7 µM BAP during undifferentiated cell growth, but was BAP-free during somatic embryogenesis.

Liquid culture conditions

The cells were incubated on the shaker up to 125 rpm, and the media for each treatment were refreshed every 15 days for 90 days. These cultures were placed in an incubation room at 26°C under continuous illumination with cool white fluorescent lamps with an intensity of 35 µmolm⁻²s⁻¹.

Experimental design, data collection and statistical analysis

All experiments followed a completely randomized design with five replications per treatment and five samples for each replication. The cells were harvested every two weeks and measurements of the total biomasses produced in the period of culture were expressed as fresh weight and dry weight. Absolute growth rate as well as the number of globular, heart-shaped, torpedo-shaped and cotyledonary stages formed in each treatment were observed with the aid of a stereo microscope and recorded regularly for each subculture. Absolute growth rates were determined based on the difference between final and initial dry weights within the period of culture ($DW_f - DW_i / t$). Analysis of variance (ANOVA) was applied to data and comparison of means was made with Duncan's multiple range test (DMRT) using statistical analysis system (SAS). Values of $P \leq 0.05$ were considered significant.

RESULTS

Friable embryogenic calli of nucellar origin were obtained and proliferated from seeds of Limau madu. We inoculated the most friable embryogenic callus, which was subcultured regularly every 15 days, on solidified MS medium supplemented with 13.3 µM BAP and 500 mg l⁻¹ malt extract.

Effects of different BAP concentrations on cell suspension

Fresh and dry weights of embryogenic cells increased constantly during the culture period. The highest multiplication rate was observed in the period of 15 to 30 days after inoculation (DAI) (Figure 1A) and thereafter reduced. Cell growth capacity was found to be affected by the concentration of BAP ($R^2 = 0.84$; $CV = 7.89$). In the present study, we found that 6.7 µM (1.5 mg L⁻¹) of BAP was the best concentration for growth and proliferation of cells in liquid media. At 6.7 µM of BAP, the maximum growth rate (GR) of 6.69 mg day⁻¹ was reached at 45 days after

culture (Figure 1C), while at concentration of 13.3 µM BAP, the GR reached only 2.49 mg day⁻¹. Figures 1A and B shows two different groups in terms of cell growth for fresh and dry weight, respectively. The first group showed growth at medium concentration of 5.6 to 8.9 µM BAP (1.25 to 2.0 mg L⁻¹), growing faster than the second group which consisted of treatments represented by 4.4 µM (1 mg L⁻¹) and 13.3 µM (3 mg L⁻¹) BAP. The fresh weight of cells in the treatment with 6.7 µM BAP was 65.65% higher than that of 13.3 µM BAP and was significantly different from the other treatments, showing the efficient nutrient absorption in liquid media. Higher concentrations of BAP in liquid culture exerted a negative effect on embryogenic cell growth. Our study shows that the proliferation capacity of cells was reduced from 65 to 20% as BAP concentration increased from 6.7 to 13.3 µM BAP.

In the present study, friable embryogenic callus was used from solid culture which had been habituated in sucrose-containing medium supplemented with 13.3 µM BAP and 500 mgL⁻¹ malt extract. This study found that the addition of lower concentrations of BAP (5.6 to 7.8 µM) for embryogenic cell proliferation in liquid effectively increases the quantity of cells higher than in media with 13.3 µM BAP, although that is the rate of BAP supplementation that has been found to be the optimal concentration for certain genotypes of citrus somatic embryogenesis in solid medium (Carimi and De-Pasquale, 2003; Agisimanto, 2006). Cytokinin represented by BAP induced profuse embryogenic cell proliferation. After six to 10 subcultures such callus cultures are usually considered habituated and continue to proliferate in the absence of these growth regulators. Our experiment shows that an addition of 6.7 µM BAP is always superior to addition of other concentrations of BAP.

Effect of initial cell density on cell growth in cell suspension

Cell density is an important factor for achieving the maximum rate of multiplication in liquid culture. In our study, 2, 4 and 6 mg ml⁻¹ embryogenic cells were inoculated respectively, into 50 ml liquid medium which was refreshed every 15 days. The growth followed a sigmoid curve after three months of culture in basic medium supplemented with 6.7 µM BAP and 146 mM sucrose (Figures 2A and B). The total amount of cells harvested after culturing in liquid medium was significantly affected by the level of initial cell density ($R^2 = 0.82$; $CV = 6.12$). Embryogenic cells at initial density of 2 mg ml⁻¹ grew slower in the first 15 DAI compared to 4 and 6 mg ml⁻¹, but grew faster in the period of 15 to 45 DAI (Figure 2A). The growth rate of cultures inoculated with 2 mg ml⁻¹ inoculum was statistically different from that of 6 mg ml⁻¹ but not significantly different from that of 4 mg ml⁻¹. In the early period of culture, within 30 DAI, doubling the inoculum density increased total GR from 8.79 mg day⁻¹ at the 2 mg ml⁻¹ density to 10.88 and 12.04 mg day⁻¹ at the 4 and 6 mg ml⁻¹ inoculum densities, respectively. The cells

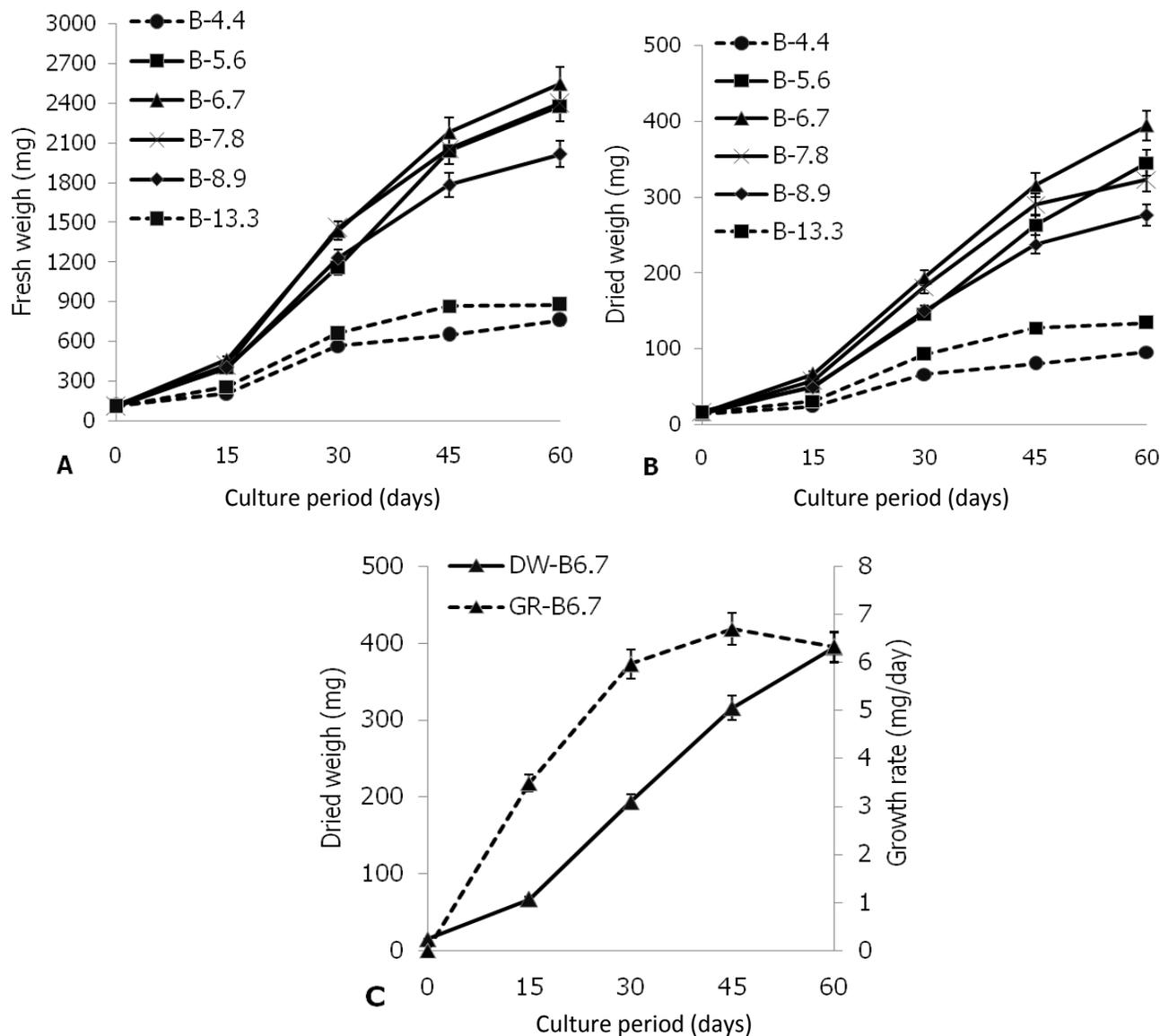


Figure 1. The kinetics growth of Limau madu (*Citrus suhuiensis* Hart. ex Tanaka) cell cultures maintained in 50 ml MS medium supplemented with 146 mM of sucrose and different concentrations of BAP (μM). A and B, Effects of BAP 4.4 μM (B-4.4), 5.6 μM (B-5.6), 6.7 μM (B-6.7), 7.8 μM (B-7.8), 8.9 μM (B-8.9) and 13.3 μM (B-13.3) on fresh and dried cell weight of biomass; C, growth rate of cell suspensions at 6.7 μM BAP (B-6.7). Initial density of embryogenic cells was 2 mg ml^{-1} . Bar represents standard errors.

in the 2 mg ml^{-1} inoculum culture grew 11-fold from initial cells, higher than at 4 and 6 mg ml^{-1} , where cell growth was only 7- and 6-fold, respectively. At the end of observation (90 DAI), the culture with inoculum density of 2 mg ml^{-1} had multiplied at a rate 2.4 times higher than that with 6 mg ml^{-1} (Figures 2A and B). The maximum GR reached 12.04 mg day^{-1} with 6 mg ml^{-1} inoculum at the second subculture (30 DAI); even the lower initial cell inocula grew faster in early stages of growth. However, the total amount of cells produced in the 6 mg ml^{-1} inoculum culture was still higher than that in other treatments (Figure 2C).

At the time of inoculation, a plant cell culture needs a

minimum inoculation concentration as the level of nutrient per cell is important for the initial cell division. There is a critical inoculum size for better growing of cells in liquid medium. Inoculum size can influence fresh cell biomass (Sinlaparaya et al., 2007) and somatic embryogenesis (Mavituna and Buyukalaca, 1996). Result of this study shows that higher inoculum density (6 mg ml^{-1}) reaches higher GR than lower inoculum densities (2 to 4 mg ml^{-1}). An inoculum size of 4 mg ml^{-1} fresh weight was used by Cabasson et al. (1995). A lower cell density (2 mg ml^{-1}) supported slower cell growth and reached maximum GR at 45 days of culture, while culturing at 6 mg ml^{-1} cell

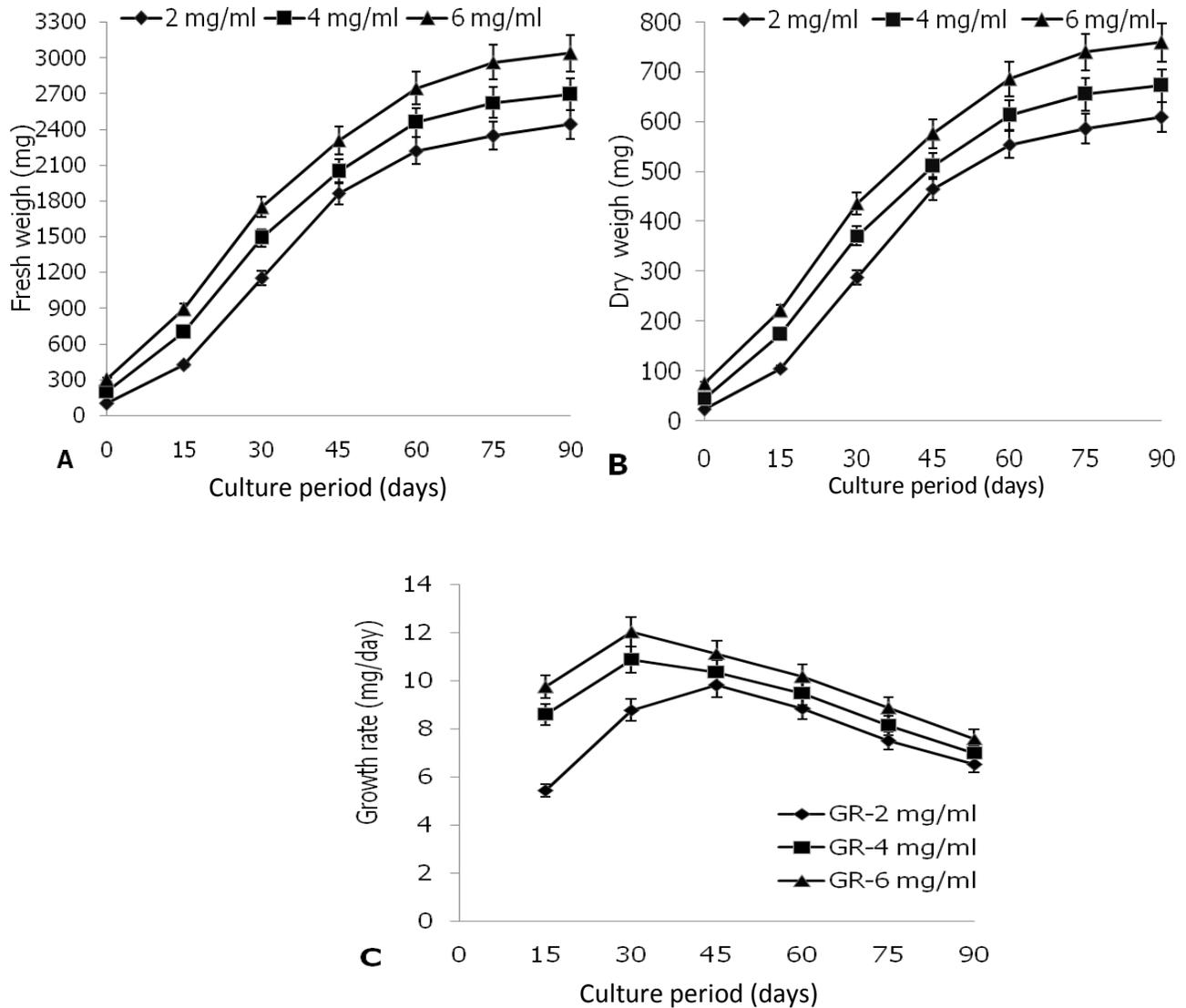


Figure 2. The kinetics growth of Limau madu (*Citrus suhuiensis* Hart. ex Tanaka) cell cultures inoculated with \blacklozenge , 2 mg ml⁻¹; \blacksquare , 4 mg ml⁻¹ and \blacktriangle , 6 mg ml⁻¹ cells in 50 ml MS medium supplemented with 146 mM of sucrose and 6.7 μ M BAP for A, fresh; and B, dry weight of cell biomass and C, growth rate in the period of culture for cell proliferation on shaken liquid culture. Bar represents standard errors.

density reached its maximum within 30 days of culture.

Effects of carbon source on undifferentiated cell growth and somatic embryogenesis

Carbon sources were expressed differently in supporting cell growth and morphogenesis (Table 1), showing three different groups of cell growth (Figures 3B and C) in sucrose (solid line), sorbitol/galactose (dotted line) and glycerol-containing (dashed line) media. Carbon sources were statistically different for all stages of cell growth, that is, undifferentiated stage ($R^2 = 0.96$; CV = 6.2) at 0 to 30 DAI, somatic embryo induction period ($R^2 = 0.99$; CV =

2.84) at 30 to 60 DAI and somatic embryo development ($R^2 = 0.99$; CV = 5.85) at 60 to 90 DAI. Fresh and dry weights, shown in Figures 3A and B respectively, showed that inoculation of 4 mg ml⁻¹ cells in liquid culture, which had been refreshed regularly every 15 days, had increased the quantity of cells significantly up to 45 DAI, and thereafter growth rates were slower up to 90 days of culture. Dry weight was clearly affected by particular carbon sources. For the period of the undifferentiated stage (0 to 30 days), the treatment with sucrose was superior to those with glycerol and sorbitol/galactose (Figure 3C), indicating the capacity of sucrose to support callus proliferation. Furthermore, the population of embryogenic cells in the early period of culture (0 to 30 days) in

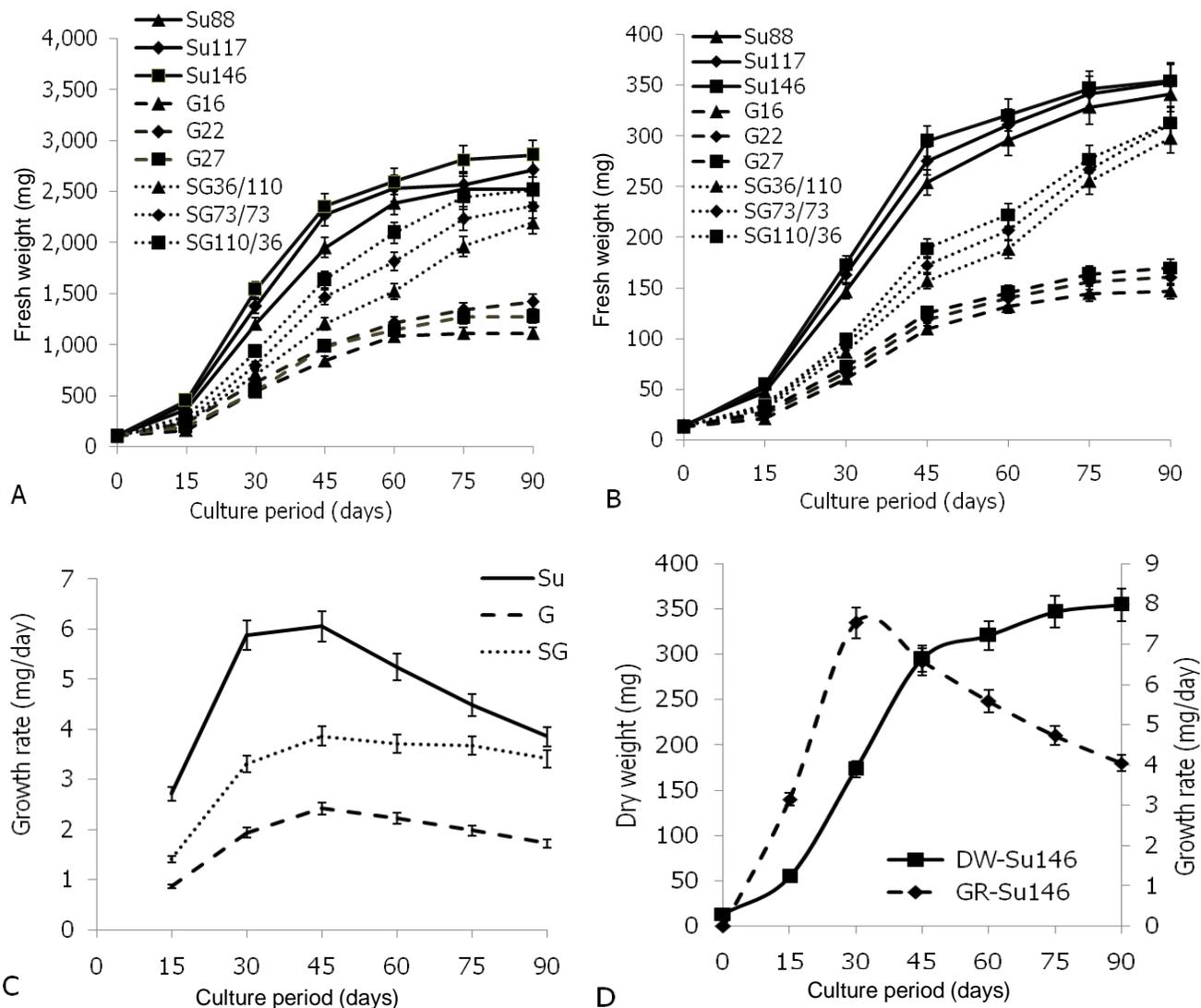


Figure 3. The kinetic growth of cell biomass of Limau madu (*Citrus suhuiensis* Hart. ex Tanaka). Effects of different concentrations of sucrose (mM) (sucrose, solid line), glycerol (mM) (galactose, dashed line) and sorbitol/galactose ratio (mM) (SG, dotted line) on biomass of, (A) fresh and (B) dried cells. (C) Mean growth with addition of sucrose (solid line), glycerol (dashed line) and sorbitol/galactose (SG) at different ratios (dotted line); (D) Optimal dry weight (DW) and growth rate of cells cultured in 146 mM sucrose (GR-Su146). Bar represents standard errors.

Table 1. Effects of carbon source and concentration on proliferation of embryogenic cells of Limau madu (*Citrus suhuiensis* Hart. ex Tanaka) in liquid culture.

Carbon (mM)	Undifferentiated cell (0 to 30 DAI)	SE (31 to 60 DAI)	SE (61 to 90 DAI)
Glycerol 16	Yellowish, friable	Invisible	Invisible
Glycerol 22	Yellowish, friable	Invisible	Invisible
Glycerol 27	Yellowish, friable	Invisible	Invisible
Sorbitol/Galactose 36:110	White, friable,	Invisible	Visible
Sorbitol/Galactose 73:73	White, friable,	Visible	Visible
Sorbitol/Galactose 110:36	White, friable,	Visible	Visible
Sucrose 88	White yellowish, friable,	Invisible	Invisible
Sucrose 117	White yellowish, friable,	Invisible	Invisible
Sucrose 146	White yellowish, friable,	Invisible	Invisible

SE, somatic embryo.

Table 2. Effects of sorbitol/galactose ratio on somatic embryogenesis of Limau madu (*Citrus suhuiensis* Hart. ex Tanaka).

Sorbitol/Galactose ratio (mM)	Undifferentiated growth		Somatic embryogenesis	
	FW (mg)	GR (mg day ⁻¹)	FW (mg)	GR (mg day ⁻¹)
146:0	508.82 ^d	2.32 ^c	389.99 ^d	1.52 ^c
110:36	928.35 ^a	3.36 ^a	2517.13 ^a	3.09 ^{ab}
73:73	942.33 ^a	3.22 ^a	2366.30 ^b	3.34 ^a
36:110	819.71 ^b	2.71 ^b	1026.47 ^c	2.70 ^b
0:146	612.76 ^c	1.99 ^d	237.19 ^e	1.90 ^c

Data were subjected to ANOVA at 5% level. Each value represents average of five replicates; means in the column followed by the same letters are not significantly different at $p \leq 0.05$ using Duncan's multiple range test. FW, Fresh weight; GR, growth rate.

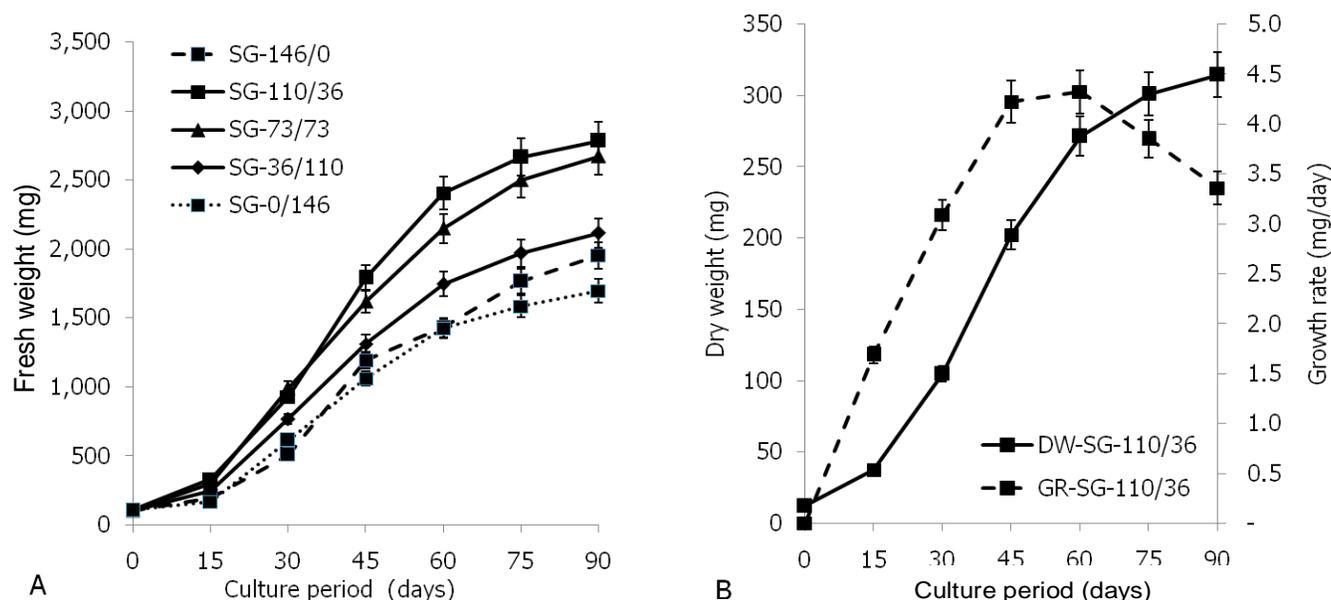


Figure 4. (A) Fresh weight of Limau madu (*Citrus suhuiensis* Hart. ex Tanaka) cell cultures with different ratios of sorbitol (S) and galactose (G) (mM). (B) Optimal dry weight (DW) and growth rate (GR) of cells cultured in 110 mM sorbitol and 36 mM galactose (SG-110/36; solid line ■). Callus proliferated during the first 30 days, and somatic embryos were induced during the second period of 30 days. Somatic embryos developed in the last 30 days. Bar represents standard errors.

sucrose-containing medium increased dramatically up to twelvefold, while the combination of sorbitol/galactose reached sevenfold and glycerol fivefold increase in inoculum density. In this period the GR of cells reached on average of 5.88, 1.94 and 3.31 mg day⁻¹ in sucrose, glycerol and sorbitol/galactose media, respectively. The highest GR of cells (7.53 mg day⁻¹) was supported by 146 mM sucrose at 30 days of culture (Figure 3D).

As carbon source for somatic embryo induction, sorbitol in combination with galactose was shown to be significantly superior to sucrose and glycerol, as well as to sorbitol or galactose individually (Tables 1 and 2, Figure 4A). The most effective ratio of sorbitol to galactose for somatic embryogenesis was 110 and 36 mM, respectively, which is equal to 75% sorbitol and 25% galactose in medium (Figure 4A). Growth with this sorbitol/galactose combination was significantly ($R^2 = 0.96$; $CV = 6.2$) different from that of the other treatments except for

treatment with the combination of 73 mM sorbitol and 73 mM galactose (50% sorbitol and 50% galactose). However, cell proliferation and morphogenesis in media having 73 mM sorbitol and 73 mM galactose increased at a lower rate compared to that of 110 mM sorbitol and 36 mM galactose (Table 2). Treatment of 110 mM sorbitol and 36 mM galactose continued to support the most profuse multiplication and somatic embryo development in the period of 30 to 60 DAI (Figure 5) and thereafter GR was reduced (Figure 4B). Cells were induced and converted to somatic embryos at a rate of 38 to 90% across all combinations of sorbitol and galactose but only 20 and 14% formed embryos for individual sorbitol and galactose, respectively. Once FEC was transferred into regeneration medium, it took five to seven days to reach the globular stage and a further 15 to 20 days to complete the formation of somatic embryos. The results of this protocol are summarized as in Figure 6.

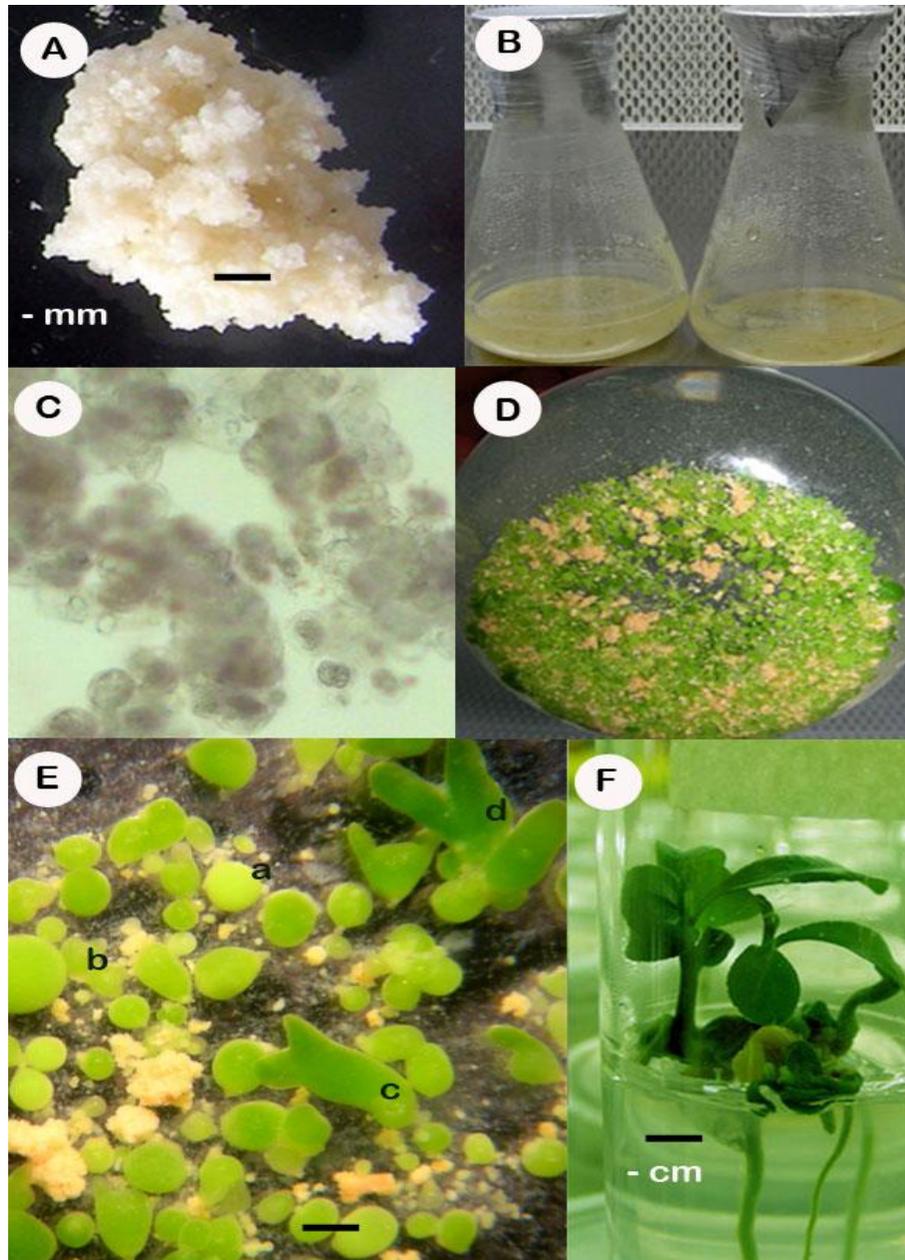


Figure 5. Friable embryogenic masses, callus and somatic embryo performance of Limau madu (*Citrus suluensis* Hart. ex Tanaka) in liquid medium cultured under optimal components of medium. A to C, Friable embryogenic masses, somatic embryogenesis and somatic embryos in liquid culture. D and E, Somatic embryos at different stages: a, globular stage; b, heart stage; c, torpedo stage; d, cotyledon. F, Plantlet in solid medium. (A to E bar= 1 mm, F bar= 1 cm).

Somatic embryogenesis is the process by which somatic cells, which are originally non-zygotic, differentiate into somatic embryos that have no vascular connection with the original tissues. Somatic embryos are morphologically similar to zygotic embryos that are bipolar and bear typical embryonic organs, the radicles, hypocotyls and cotyledon (Arnold et al., 2002). Somatic embryogenesis is an *in vitro*-applied method that offers

opportunities for clonal plant propagation and plant regeneration in mutagenesis experiments. It may result in virus-free plants and organisms that are vigorously growing, normally developed and genetically true-to-type. Somatic embryogenesis in the *Citrus* genotype has been reported initially by *in vitro* culture of *Citrus* ovules (Maheswari and Ranganswamy, 1958). Thereafter, reports have highlighted the embryogenic potential of nucellus-

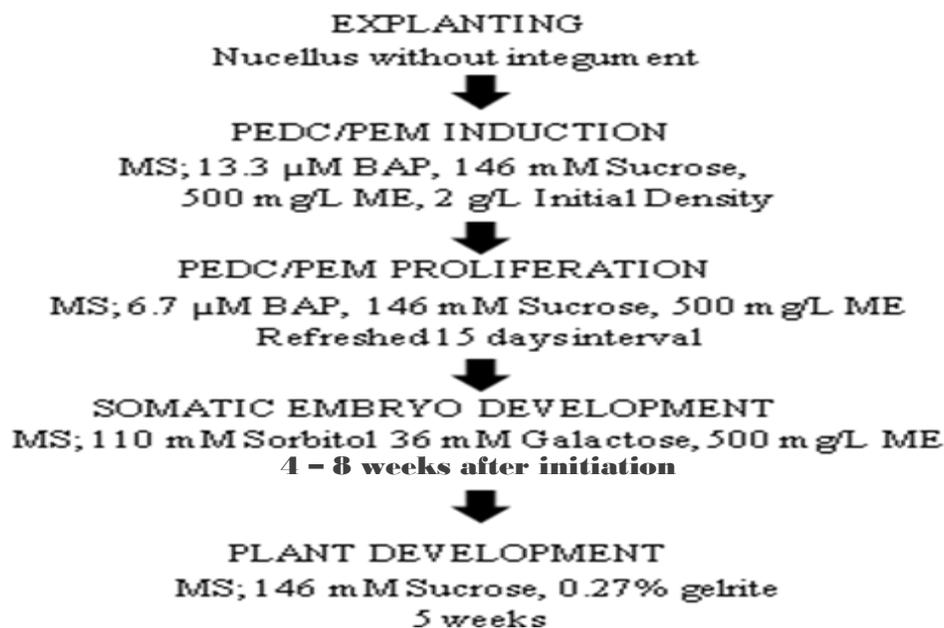


Figure 6. Protocol developed for somatic embryogenesis of Limau madu (*Citrus suhuiensis* Hart. ex Tanaka). PEDC, Pro-embryogenic derived callus, PEM, pro-embryogenic masses; ME, malt extract; MS, Murashige and Skoog.

derived callus as a natural phenomenon (Litz et al., 1985). However, there have been only a few reports of efficient somatic embryogenesis in liquid culture. Regeneration of citrus has been found to be obtainable in liquid from callus cultures via somatic embryogenesis (Kochba et al., 1978; Cabasson et al., 1995; Tomaz et al., 2001; Kayim and Koc, 2006). Several factors usually influence the process, although somatic embryogenesis is considered widespread in polyembryonic varieties like citrus. Somatic embryogenesis in citrus is induced either by the lack of growth regulators and/or by changing the carbohydrate composition in the culture medium (Kochba et al., 1978; Ben-Hayyim and Neumann, 1983; Cabasson et al., 1995; Kayim and Koc, 2006).

The stimulatory effects of carbohydrates on callus growth and embryogenesis were investigated in the present study. In the case of Limau madu, sucrose supported embryogenic cell proliferation and optimally increased the growth rate to a greater degree than did the other carbon sources tested, both individually and in combination. However, sucrose was inferior in supporting somatic embryo induction. Cells failed to form somatic embryos with extended sucrose application. This was similarly reported for several genotypes of *Citrus* (Kochba et al., 1982; Perez et al., 1999; Komai et al., 1996; Tomaz et al., 2001). Moreover, sorbitol and galactose combinations recently tested on somatic embryogenesis of Limau madu were shown to be efficient for supporting somatic embryo induction and development than glycerol. It was also found that the ratio of sorbitol to galactose influenced the rate of somatic embryo induction. Sorbitol

in higher proportion in the sorbitol/galactose combination provided better conditions for cell growth and differentiation. After transfer into regeneration medium, somatic embryos were initiated in a period of 14 to 21 days culture in responsive cultures and completed in another 14 days. Contrary to previous reports (Ben-Hayyim and Neumann, 1983; Gavish et al., 1991; Kayim and Koc, 2006), our study shows that the use of glycerol in liquid culture of Limau madu resulted in lower cell proliferation and somatic embryo induction, and required a longer period (four months) to complete somatic embryogenesis compared to sorbitol-galactose medium.

Several carbohydrate sources, either in the form of monosaccharides or disaccharides such as glucose, galactose, lactose, maltose, sucrose, glycerol, sorbitol and mannitol, have been employed as carbon sources for somatic embryogenesis of *Citrus sinensis* L. Osbeck, cv. Caipira, Valencia, *Citrus reticulata* Blanco, cv. Cleopatra, line I and Cleopatra, line II and *C. limonia* L. Osbeck, cv. Rangpur lime (Tomaz et al., 2001) and *Citrus deliciosa* cv willow-leaf mandarin (Cabasson et al., 1995) and *C. sinensis* Osbeck cv Washington Navel, *Citrus clementina* Hort. Ex. Tanaka cv Clementina mandarin, *C. limon* (L.) Burm.f. cv Kutdiken and Zagara Bianca (Kayim and Koc, 2006). Calli were habituated in sucrose-containing medium for proliferation and thereafter transferred to galactose as a substitute of sucrose for inducing somatic embryogenesis. Tomaz et al. (2001) found that galactose as sole carbon source was effective for induction of somatic embryos in sweet orange cv Caipira and Mandarin cv Cleopatra line 1 and reached maximum

number of somatic embryos (110 to 194 embryos from 50 mg callus) at 110 to 150 mM galactose after four weeks of culture, while Cabbason et al. (1995) observed visible embryos after 18 days of culture with galactose producing 1000 ± 160 somatic embryos per g callus. However, sucrose did not stimulate somatic embryogenesis on those tested species. In the present study, however, sucrose induced globular stages but then failed to continue further development following the globular stages. In addition, Kayim and Koc (2006) found that lactose and galactose were always superior to glycerol and sorbitol in cell proliferation but did not stimulate somatic embryo formation in individual application. However, in our study, galactose interacts significantly with sorbitol in giving the best somatic embryogenesis in Limau madu. Kayim and Koc (2006) as well as Kochba et al. (1978), assumed that the galactose has its own effects on physiological processes rather than as a source of energy, since galactose was effective in lower quantity. In many cases of citrus somatic embryogenesis, galactose has efficiently promoted somatic embryogenesis with its ability to inhibit auxin synthesis and transport, thereby inducing somatic embryos by modifying the endogenous auxin balance.

Conclusion

An efficient protocol for somatic embryogenesis of Limau madu in liquid culture was developed. Rates of somatic embryogenesis in Limau madu were improved by changing the medium's carbon source from the typical sucrose to a combination of sorbitol and galactose to modify endogenous auxin concentration and at the same time reducing BAP concentration. At optimal BAP concentration (6.7 μ M), a small amount of embryogenic cells (100 mg in 50 ml) can be multiplied profusely in sucrose-containing medium. A large number of somatic embryos (951) were induced in a medium containing 110 mM sorbitol and 36 mM galactose without BAP. This protocol is therefore useful for producing large numbers of elite genotypes from a minimal amount of starting material.

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