



Full Length Article

Initiation and Maintenance of Cell Suspension Cultures of Two Citrus Species for Protoplast Isolation

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Abstract

Cell suspension cultures were initiated from six month old ovule-derived embryogenic calli of two *Citrus* species named *Citrus aurantifolia* (Christm.) Swingle cultivar Sweet lime and *Citrus reticulata* Blanco cultivar Kinnow. The growth rate of cells was observed on two different media MT basal medium + 0.5 g L⁻¹ malt extract (EME) and MT containing half strength NH₄NO₃ and KNO₃, supplemented with 1.55 g L⁻¹ glutamine and 0.75 g L⁻¹ KCl (H+H). Kinnow and Sweet lime cells showed a sigmoidal growth pattern on (H+H) and (EME) liquid media, when PCV was plotted against time. The logarithmic phase of cells started from 4th to 10th day and after that a stationary phase observed in cells with elongated vacuoles. This indicated that citrus cells active phase started soon after subculture and ends after two weeks. These embryogenic suspension cultures were later used for protoplast isolation which is a key step towards somatic hybridization. © 2017 Friends Science Publishers

Keywords: Somatic hybridization; Embryogenesis; Callus culture; Protoplast fusion; Liquid medium

Introduction

Cell suspension cultures are capable of regenerating plants by the process of somatic embryogenesis and were first reported in carrot 25 years ago (Zimmerman, 1993). Somatic embryos were first observed to arise from cultured carrot (*Daucus carota*) tissues in suspension cultures by Takeda *et al.* (2003). Such embryogenic cell suspensions have since been established in many species of angiosperms.

In *Citrus*, embryogenic cell suspension cultures have been established from unfertilized ovules and been used as target tissues for Agrobacterium-mediated transformation (Dutt and Grosser, 2010). Several economically important metabolites have been produced from cell cultures of citrus. A limonoid produced from “pera” orange [*Citrus sinensis* (L.) Osbeck] cell suspensions have received considerable attention because of its anti-cancer actions (Gerolino *et al.*, 2015). Suspension cultures offer several distinct advantages over stationary cultures. When grown in liquid medium, cells and embryos are bathed by the culture medium and evenly exposed to nutrients and hormones. This allows more precise manipulation of media components, handling of cells and embryos and control of development. In stationary cultures gradients develop from the medium to the top of the tissues. Uniform tissue response and control of development become more difficult. In addition in suspension cultures proembryogenic clusters and embryos

usually separate from each other and float freely in the medium. Cells can easily be sieved, centrifuged or otherwise manipulated. Large number of cells can be moved easily from one vessel to another and through the various treatments whereby cells grow into embryos and then into plantlets (Hippolyte *et al.*, 1992; Soomro and Memon, 2007; Yann *et al.*, 2012). Embryogenic suspension cultures present an excellent tool for both theoretical studies and practical applications. For example, selection schemes with embryogenic cultures have given rise to temperature sensitive carrot variants for studies of differentiation and stable salt tolerant citrus embryos and plants (Kobayashi, 1992).

The biosynthetic potentiality of cell cultures has long been of interest. Embryogenic suspension cultures and population of somatic embryos may be another alternative for the production of important chemicals. Somatic embryos of celery produce the same flavor compounds present in the mature plants but absent in celery callus cultures (Jiménez, 2001). Somatic embryos of Cacao produce the same lipids, including cacao butter, as their zygotic counterpart (Jain *et al.*, 2012). The synthesis of storage proteins in legume somatic embryos may be possible. That substantial population of somatic embryos can be raised in a small volume of liquid medium offers the distinct possibility that somatic embryogenesis can be used for large scale clonal propagation.

Suspension cultures of somatic embryos in which

embryos separate and float freely in the medium, appear to be especially amenable to mechanical handling fluid drilling. Mahto and Sharma (2004) suggested liquid cultures a way to deliver large quantities of somatic embryos from the cultured flask to the field. This could be achieved even more readily if development could be synchronized and staged. The results seen with the effects of different suspension culture techniques suggest that this is possible. Embryos are natural organs of perennation many of which typically become dormant. Because of their innate properties, somatic embryos may prove useful for long term storage such as in germplasm banks. Cold storage, dry storage, or cryogenic preservation may play a role here.

If dormancy could be induced in somatic embryos, they could be incorporated into artificial seeds by coating or encapsulation (Nakano *et al.*, 2004). However, a number of problems have to be solved, including the induction, maintenance and breaking of dormancy but the prospects are intriguing. Our working hypothesis for the research reported herein was to study the active growth period of citrus cells in suspension phase on two different media formulation for long term storage of cell lines. Later, these cell lines would be used to make somatic hybrids.

Materials and Methods

Initiation and Maintenance of Cell Suspension Cultures

For cell suspension cultures, citrus embryogenic calli were initiated from ovules of two *Citrus* species named *Citrus aurantifolia* (Christm.) Swingle cultivar Sweet lime and *Citrus reticulata* Blanco cultivar Kinnow. Friable, fast growing calli (2.0 g fresh weight) was transferred to 150 mL Erlenmeyer flasks each containing 20 mL each of MT basal medium + 0.5 g/L malt extract (EME) and MT containing half strength NH_4NO_3 and KNO_3 , supplemented with 1.55 g/L glutamine and 0.75 g/L KCl (H+H) liquid media (Table 1). Cultures were incubated on a rotary shaker (125 rpm) at $26 \pm 2^\circ\text{C}$ under 16 h of photoperiod. Two weeks after culture, 20 mL more fresh liquid media was added to these flasks. These cultures were maintained by subculture every two weeks to 40 mL aliquots of both media shaking at 125 rpm, under the same incubation conditions.

During the first stage of cell suspension initiation, sub culturing was performed at 4–5 d intervals, by allowing cells to settle for 5 min and pouring off 50% of spent culture medium. The later was replaced with an equivalent volume of fresh liquid medium. Initially, a higher proportion of fresh medium was added than had been previously removed to increase culture volumes slowly. In order to keep the cell clumps small, the suspension was passed through a 500 μm nylon sieve (Wilson Sieves, Hucknall, Nottingham, UK). Retained cell clumps were either broken up with a spatula and re-cultured or if large and non-friable, discarded. After 4 passages (28 d; 7 d each), cultures were transferred

Table 1: Composition of H+H and EME media for cell suspension cultures

Component (mg/L)	H+H	EME
NH_4NO_3	825	1650
KNO_3	950	1900
KH_2PO_4	170	170
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	370	370
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	440	440
Na_2EDTA	37.3	37.3
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	27.8	27.8
KCl	750	—
$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	22.3	22.3
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	8.6	8.6
H_3BO_3	6.2	6.2
KI	0.83	0.83
$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	0.25	0.25
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.025	0.025
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	0.025	0.025
Glutamine	1550	—
Thiamine HCl	10	10
Pyridoxine HCl	10	10
Nicotinic acid	1	1
Myo-inositol	100	100
Malt extract	500	500
Sucrose	50,000	50,000
Agar	8,000	8,000

to 250 mL Erlenmeyer flasks. Suspensions were routinely sub-cultured every 14 d, by transfer of a 2 mL settled cell volume (SCV) to 38 mL of fresh respective callus induction liquid medium and 10 mL of conditioned medium.

Growth Rate of Cell Suspension Cultures

Growth rate of Kinnow suspension cultures was determined by measuring packed cell volume, fresh cell weight, dry cell weight and growth index on two different media such as (EME) and (H+H) after the cell suspension cultures contain a homogeneous cell population. The growth parameters were determined as a two days interval and were plotted against time. For all growth parameters three readings were recorded from a single flask, and the values in the graphs are the means of three observations.

For packed cell volume (PCV), selected flasks were incubated under conditions described earlier but without subculture. From day 0 and thereafter daily, 10 mL of cell suspension was transferred to a 15 mL graduated plastic centrifuge tube and PCV of the suspension was measured for a period of 20 days. Data were recorded and plotted against time (Godoy-Hernandez and Vazquez-Flota, 2006). For fresh weight measurement, the weight of filter paper was pre-determined. Then whole contents of the flask were filtered through it. The fresh weight was calculated by subtracting the weight of filter paper from filtered fresh cells weight (Evans *et al.*, 2003). While dry weight was determined by drying the filtered fresh cells in an oven at 60°C for 48 h. The wet and dry weights were plotted against time to produce the growth curve (Evans *et al.*, 2003).

The growth index was calculated as the ratio of the

accumulated and the initial biomass. The accumulated biomass corresponds to the difference between the final and the initial masses (Godoy-Hernandez and Vazquez-Flota, 2006; Yann et al., 2012).

Statistical Analysis

Data were analyzed using Sigma Plot v.12 Statistical Software, in order to determine the relationship between subculture days and growth parameters i.e. packed cell volume, fresh cell weight, dry cell weight and growth index of Kinnow and Sweet lime. The values in the graphs were shown with the standard errors for three observations.

Results

Maintenance of suspension cultures was done by the addition of fresh medium to cells at regular intervals in order to keep the cells alive and to prevent browning and loss of viability. Fig. 1A–F shows the amount of callus which was transferred from solid to liquid medium. Callus cells were transferred to suspension and a batch culture of suspension was initiated.

Measurement of Growth Kinetics of Kinnow Cells

Kinnow suspension cells showed an increase in PCV and FCW on both media when plotted against alternate day's interval (Fig. 2a and b). Cells showed an increase in volume of packed cells after 4th day of subculture. Similar results were obtained from fresh cell weight of cultured cells when bathed in the liquid medium of EME. The dry cell weight was also higher in case of EME media (Fig. 2c).

Measurement of Growth Kinetics of Sweet Lime Cells

The cell suspension of sweet lime showed an ascending order response to packed cell volume on both H+H and EME media. The packed cell volume increased gradually from 4th days of subculture to 10th days of subculture (Fig. 3a). Sweet lime cells exhibited a swirl order increase in fresh weight of cells on both media from day 4 to 8 and then showed a parallel trend from 12th day to onward (Fig. 3b). Dry weight of sweet lime cells increased from 5th to 12th day (Fig. 3c). The increase in cell biomass was also observed from day 4th to 10th (Fig. 3d).

Discussion

The present study demonstrated that the cell culture of Kinnow on H+H and EME liquid media showed a sigmoidal growth pattern, when PCV was plotted against time of two days intervals. The cells showed maximum PCV from 4th to 10th day. In case of cells fresh weight on both media the S shaped sigmoidal curve represented more fresh weight on EME medium. A rise in FCW was also observed from 4th to 10th day. These findings indicated that cells start their logarithmic or exponential phase from fourth

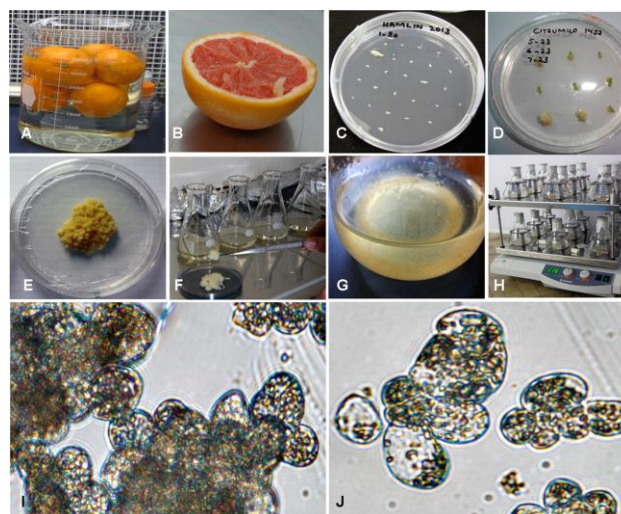


Fig. 1: A: Fruit sterilization, B: Ovule extraction, C: Ovule culture on semi-solid media, D: Callus induction from ovules, E: Callus proliferation, F: Transfer of callus from solid to liquid media, G: Homogeneous cell population, H: Batch culture on a rotary shaker at 125 rpm, I: Cells at 4th day of subculture in logarithmic phase: cells more round and compact), J: Cells at 14th day of subculture in exponential phase: elongated cells showing large vacuole)

day of subculture Grosser and Gmitter Jr (2011).

Kinnow cells showed similar trend between dry weight (DCW) and growth index (GI) on EME media as compared to H+H. The reason was that EME media had sucrose and it plays an important role in cell enlargement. George and Debergh (2008) reported that by increasing the sucrose concentration up to 6% and addition of sodium succinate up to 15 mM enhanced the cell volume 2.7 times and dry weight up to 2.8 times. It also enhanced the cell enlargement and maximum number of cells per culture.

Obviously, Kinnow cells expressed significant response to EME media for all growth parameters. The sigmoid growth pattern in all cases showed that cells started their logarithmic phase of growth at 4th day of subculture and cells remained more active during that period. This growth phase remained active up till 10th day of subculture and after that cells enter in to the stationary phase of growth and a linear growth pattern was noted. A plausible reason is that cells consume nutrients more actively and oxygen concentration is high at beginning as compared to the late phase of subculture (J.W. Grosser).

In Sweet lime sigmoidal curve represents the similarity for PCV, FCW and DCW. The maintenance of cell suspension cultures offers useful information for further studies of cell cultures. A similar behavior was observed among FCW and DCW and PCV curves, with a growth peak around 4th to 10th days followed by a decrease in growth. The logarithmic phase started from 4th to 10th days and after that a straight line represents the stationary phase (Grosser and Gmitter Jr, 2011).

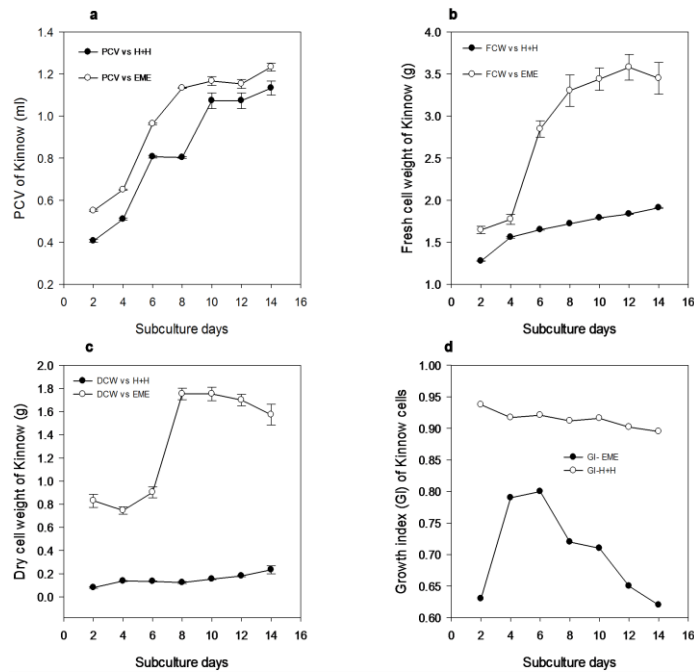


Fig. 2: Growth kinetics of Kinnow cell suspension cultures a) Growth determined by packed cell volume (PCV) (mL), b) fresh cell weight (FCW) (g), c) dry cell weight (DCW) (g) and d) Growth index (GI) on two different suspension media H+H and EME. Bars represent means \pm standard error (SE) of three replications

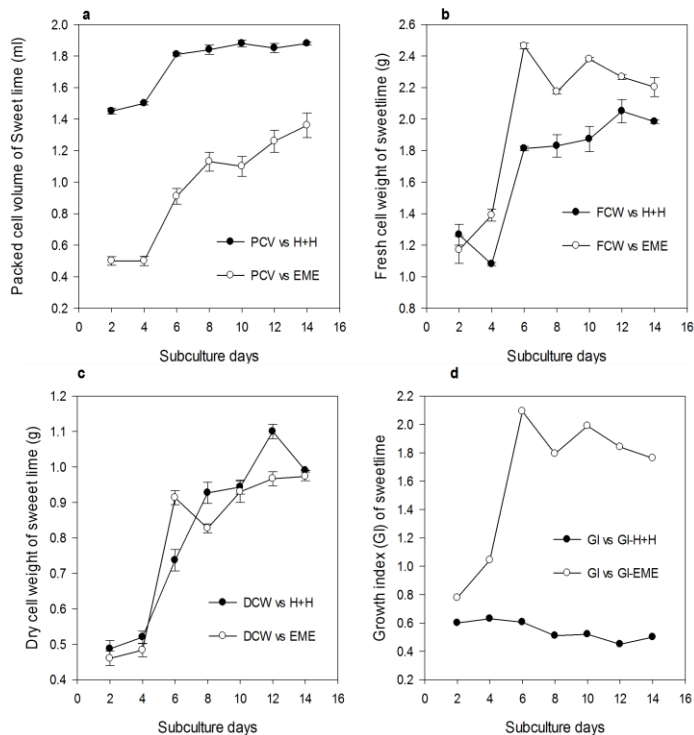


Fig. 3: Growth kinetics of Sweet lime cell suspension cultures a) Growth determined by packed cell volume (PCV) (mL), b) fresh cell weight (FCW) (g), c) dry cell weight (DCW) (g) and d) Growth index (GI) on two different suspension media H+H and EME

The growth measurements were necessary to decide the timings for the replacement of medium during cell culture maintenance. These measurements were taken into account to decide the ideal timing for medium replacement during cell suspension maintenance. After a certain time period, cell embryogenic potential decreased which consequently increased the number of non-embryogenic cells. These non embryogenic cells were elongated in shape with large vacuole under microscopic observation. The appearance of this kind of cells increases the PCV although no increase in embryogenic cell numbers was observed. This indicated a gradual increase in FCW, DCW and PCV but no increase in embryogenic cell number, and even though by 14th day cell vacuolization could be observed (Fig. 1J).

If the cell cultures are maintained without shifting them onto new media after 15 days, the cell quality will be decreased. It will also reduce the chance for cell recovery. Citrus cells can retain the embryogenic competence state for years (Dutt and Grosser, 2010; Omar et al., 2016).

Conclusion

The research described here allows the initiation and maintenance of citrus cell suspension cultures for long term storage of cell lines. The citrus cell suspension cultures can be initiated from embryogenic callus of undeveloped ovules for stable cell lines. The logarithmic phase of cells starts from 4th day and ends at the 14th day of subculture. In other words, citrus cells complete their growth cycle in a period of two weeks after that inclusion fresh liquid media is necessary to keep them alive for embryogenic competence. These lines can be further utilized for protoplast fusion, genetic transformation and as a tool for basic studies of plant biochemistry and molecular biology.

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References

- Dutt, M. and J.W. Grosser, 2010. An embryogenic suspension cell culture system for Agrobacterium-mediated transformation of citrus. *Plant Cell Rep.*, 29: 1251–1260
- Evans, D.E., J.O.D. Coleman and A. Kearns, 2003. Cell suspension cultures. In: *Plant Cell Culture*, pp. 90–91. D.E. Evans, J. O. D. Coleman and A. Kearns (eds.). BIOS Science, New York, USA
- George, E.F. and P.C. Debergh, 2008. Plant propagation by tissue culture. In: *Micropropagation: Uses and Methods*, pp. 29–64. E.F. George, M.A. Hall and G. Jan De Klerk (eds.). Springer, Dordrecht, The Netherlands
- Gerolino, E.F., T.P.C. Chierito, A.S. Filho, E.R. Souto, R.A.C. Goncalves and A.J.B. DeOliveira, 2015. Evaluation of limonoid production in suspension cell culture of *Citrus sinensis*. *Braz. J. Pharmacog.*, 25: 455–461
- Godoy-Hernandez, G. and F.A. Vazquez-Flota, 2006. Growth measurements: Estimation of cell division and cell expansion. In: *Plant Cell Culture Protocols*, pp. 55–57. V.M. Lovola-Vargas and F.A. Vazquez-Flota (eds.). Humana Press, New Jersey, USA
- Grosser, J.W. and F.G. Gmitter Jr., 2011. Protoplast fusion for production of tetraploids and triploids: Application for scion and rootstock breeding in citrus. *Plant Cell Tiss. Org. Cult.*, 104: 343–357
- Hippolyte, I., B. Marin, J.C. Baccu and R. Jonard, 1992. Growth rosmarinic acid production in cell suspension cultures of *Salvia officinalis*. *Plant Cell Rep.*, 11: 109–112
- Jain, S.M., P.P.K. Gupta and R.J. Newton, 2012. *Somatic Embryogenesis in Woody Plants*, Vol. 3. S.M. Jain, P.P.K. Gupta and R.J. Newton, (eds.). Springer, Dordrecht, The Netherlands
- Jiménez, V.M., 2001. Regulation of *in vitro* somatic embryogenesis with emphasis on the role of endogenous hormones. *Rev. Bras. Fisiol. Veg.*, 13: 196–223
- Kobayashi, S., 1992. The production of novel cultivars of fruit trees using protoplast fusion. *Res. J. Food Agric.*, 15: 16–20
- Mahto, V. and V.P. Sharma, 2004. Rheological study of a water based oil well drilling fluid. *J. Petroleum Sci. Eng.*, 45: 123–128
- Nakano, M., K. Mizunashi, S. Tanaka, T. Godo, M. Nakata and H. Saito, 2004. Somatic embryogenesis and plant regeneration from callus cultures of several species in the genus *Tricyrtis*. *In Vitro Cell. Dev. Biol. Plant*, 40: 274–278
- Omar, A.A., M. Dutt, F.G. Gmitter Jr and J.W. Grosser, 2016. Somatic Embryogenesis: Still a Relevant Technique in Citrus Improvement. In: *In Vitro Embryogenesis in Higher Plants, Methods in Molecular Biology*, Vol. 1359, pp. 289–327. M.A. Germana and M. Lambardi (eds.). Springer, New York, USA
- Soomro, R. and R.A. Memon, 2007. Establishment of callus and suspension culture in *Jatropha curcas*. *Pak. J. Bot.*, 39: 2431–2441
- Takeda, H., T. Kotake, N. Nakagawa, N. Sakurai and D.J. Nevins, 2003. Expression and function of cell wall-bound cationic peroxidase in asparagus somatic embryogenesis. *Plant Physiol. Biochem.*, 131: 1–10
- Yann, L.K., N.B. Jelodar and C. Lai-Keng, 2012. Investigation on the effect of subculture frequency and inoculum size on the artemisinin content in a cell suspension culture of *Artemisia annua* L. *Aust. J. Crop Sci.*, 6: 801–807
- Zimmerman, J.L., 1993. Somatic Embryogenesis: A Model for early development in higher plants. *Plant Cell*, 5: 1411–1423

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