International Journal of Biosciences (IJB)
ISSN: 2220-6655 (Print) 2222-5234 (Online)
Vol. 2, No. 3, p. 97-105, 2012
http://www.innspub.net

RESEARCH PAPER OPEN ACCESS

Cells structure and morphogenesis of embryogenic aggregates in suspension culture of bitter melon (*Momordica charantia* L.)

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Received: 03 March 2012
Revised: 10 March 2012
Accepted: 10 March 2012

Key words: Bitter melon, callus, cell aggregate, cell suspension, morphogenesis, proembryogenic structures.

Abstract

Cell suspension culture from leaf-derived callus of Bitter melon (*Momordica charantia* L.) was established. The callus could be induced from leaf segments on agarified Murashige and Skoog (MS) medium containing 1.0 mgl^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), which consequently used for suspension culture. In the establishment of cell suspension culture, the highest performance in the growth of cells was observed in liquid MMS (addition of 0.5 mgl^{-1} folic acid and 0.05 mgl^{-1} biotin to MS medium) medium containing 2,4-D (1.5 mgl^{-1}) when callus was subcultured. The S-shaped growth curve with three typical phages (lag, exponential and stationary phases) was found in the batch culture. Cells in suspension culture underwent division as result both free cells and cell aggregates were formed. The number of cell aggregation affected by the initial amount of cells. When the initial amounts of cell were increased from 1 to 5 ml sedimented cell volume (SCV), the number of cell aggregates was increased gradually. The number of aggregates decreased with further increasing initial amount of cells from 5 ml SCV. Aggregate formation at a highest level when 5 ml (SCV) initial cells subcultured. The duration of subculture was found to be effective for cell proliferation in suspension and proliferated cell structure. The cell growth in culture was lower when suspension maintained up to 8 weeks without subculturing. The elliptical cell structure was mostly found in suspension maintained up to 8 weeks without subculturing. The most of cells were spherical-shaped and cell growth was high in suspension when suspension culture changed to fresh medium at 7-week-interval. The established system on the cell suspension culture might be used for establishing an efficient somatic embryogenesis method of bitter melon.

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Introduction

Bitter melon (*Momordica charantia* L.) belonging to the family Cucurbitaceae, grows in tropical areas of Asia, Amazon, east Africa, and the Caribbean. It is cultivated throughout the world for use as a vegetable and medicine (Thiruvengadam *et al.*, 2010). Bitter melon plants, fruits and roots contain high levels of inorganic elements (calcium, iron, potassium and phosphorus), organic elements (beta carotene, proteins and vitamins), and good sources of dietary fiber (Paul and Raychaudhuri, 2010; Sultana and Miah, 2003). The biologically active proteins (namely, momordin, α- and β-momorcharin, cucurbitacin, and MAP30) are effective anti-human immunodeficiency (HIV), anti-tumor, anti-diabetic, and anti-rheumatic properties. It is also functioned as febrifuge medicine for jaundice, hepatitis, leprosy, hemorrhoids, psoriasis, snakebite, and vaginal discharge (Singh *et al.*, 1998; Bourinbaiar and Lee-Huang, 1996; Beloin *et al.*, 2005).

The reports on genetic improvement of bitter melon by conventional plant breeding methods has been published (e.g., Dhillon *et al.*, 2005; Pandey and Singh, 2001), but there many barriers in this conventional breeding occurred due to their cross-pollination. Therefore, as another option is molecular breeding and this has advanced and opened a new avenue for this crop improvement. Among key tools for the genetic transformation, the availability of an efficient *in vitro* regeneration system is one of them. Thus, plant regeneration protocol from individual cells or explants is essential for the application of genetic engineering to bitter melon.

There are several investigations have been reported on the plant regeneration of *M. charantia* from different explants such as, micropropagation from nodal and shoot tips (Wang *et al.*, 2001; Sultana and Miah, 2003; Huda and Sikdar, 2006); organogenesis from stem segments (Tang *et al.*, 2011a; Tang *et al.*, 2011b), from leaf segments (Thiruvengadam *et al.*, 2010), from cotyledon segments (Islam *et al.* 1994) and from nodal and root segments (Munsur *et al.*, 2009); and somatic embryogenesis from leaf explants (Thiruvengadam *et al.*, 2006; Paul *et al.*, 2009). The both micropropagation and organogenesis of shoot have established using shoot apices, and nodal and internodal explants (Agarwal and Kamal, 2004). Malik *et al.* (2007) reported that various explants of leaf, stem, and cotyledon induced different types of callus in *M. charantia* but that none of these produced any shoots. We (Sultana *et al.*, 2005) also investigated the effects of sucrose, pH and agar levels in the medium for micropropagation of shoots using the nodal segments. *Agrobacterium*-mediated b-glucuronidase expression was detected in explants of immature cotyledonary nodes in *M. charantia* (Sikdar *et al.*, 2005).

The reports on somatic embryogenesis in cell suspension culture have not been established extensively. A report on somatic embryogenesis through cell suspension culture for bitter melon was published (Thiruvengadam *et al.*, 2006). One report is insufficient for efficient plant multiplication and genetic transformation by somatic embryogenesis in suspension culture since somatic embryogenesis in suspension is very efficient technique for genetic engineering. Besides, lacking of detail information on somatic embryogenesis in suspension for bitter melon has remained in the protocol of Thiruvengadam *et al.* (2006). Therefore, further studies for more detail research on cells proliferation, cell aggregate formation and their appearance in suspension culture of bitter melon are being necessitated.

The objective of the present study was to establish efficient cell suspension culture for bitter melon from the leaf-derived callus. In the present study, the growth of cells, cells structure and development of cell aggregates in suspension culture were studied under the various media and culture conditions.

Materials and Methods

Plant materials

Seeds of bitter melon were collected from local market and used for further investigation.

Preparation of basal medium and conditions of in vitro culture

MS (Murashige and Skoog, 1962) basal medium were used for callus induction and MMS (additionally used 0.5 mgl^{-1} folic acid and 0.05 mgl^{-1} biotin to MS medium) for the establishment of cell suspension culture. The callus induction was carried out on agarified medium. The medium was solidified with 0.8% (w/v) agar (Type M, Sigma). The liquid medium was applied for cell suspension culture. All media were adjusted to pH 5.7 ± 0.1 and autoclaved at 121 ^{o}C for 20 min. The medium without plant growth regulators (PGRs) received as control for all experiments. The cultures were maintained at 27 ± 1^{o}C under a 16-h photoperiod (35 µmol m^{-2} s^{-1}), 16-hour light and 8-hour dark.

Callus induction

Leaves of ten-day-old seedling were used as plant materials in the present study. The seeds collection, disinfection and *in vitro* germination procedures were described in our previous report (Sultana and Miah, 2003). The leaves of aseptically growing seedling were harvested and sliced into approximately 1 cm x 1 cm pieces and placed on MS medium containing different concentrations of 2,4-Dichlorophenoxyacetic acid (2,4-D) at 0.5, 1.0 and 2.0 mgl^{-1} alone or combinations of 6-Benzylaminopurine (BAP) (1.0, 5.0 and 10.0 mgl^{-1}) and 1-Naphthaleneacetic acid (NAA) (0.1 and 0.5 mgl^{-1}) for callus induction. Data on percentage of explants that induced callus were recorded after 4 weeks of culture initiation.

Initiation of cell suspension culture

The induced callus (5 g fresh weight) on MS medium fortified with 1.0 mgl^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) was transferred to each 300-ml Erlenmeyer flask containing 50 ml of liquid MMS medium fortified with 1.0, 1.5, 2.0 and 3.0 mgl^{-1} 2,4-D alone. The flasks were sealed with Aluminum foil, wrapped with parafilm and then they were placed on a rotary shaker (95 rpm). Callus cells were proliferated in suspension cultures for 2 weeks. The cell growth in each PGR treatment was measured. For measurement of cell growth, the proliferated cells in an Erlenmeyer flask were dispensed in a sterile 100 ml measuring cylinders and allowed to sediment for 30 min after that measured total cell amount by sedimented cell volume (SCV) as milliliter (ml).

Cell growth by batch culture

Cell proliferation in suspension culture was examined by batch culture. The total amount of proliferated cells at the end of each subculture was determined by SCV (ml). For batch culture, 5 ml SCV of cells collected from 2-week-old suspension culture using callus and they were subcultured in 300 ml Erlenmeyer flasks containing 50 ml MMS liquid medium fortified with 1.5 mgl^{-1} 2,4-D. The cultures were routinely transferred at 1-week-interval to a fresh medium and total amount (ml) SCV of cell per flask measured prior to transfer in each subculture. The suspension cultures were maintained up to 8 weeks.

Effect of initial cell amounts on cell proliferation and cell aggregates development

Cells from 2-week-old suspension were subcultured initially at different volumes, 1, 3, 5, 10, 15, and 20 ml SCV in 300 ml Erlenmeyer flasks containing 50 ml of MMS liquid medium supplemented with 1.5 mgl^{-1} 2,4-D. The cultures were transferred into fresh medium routinely in a week. After 4 weeks of culture, the proliferated cells in an Erlenmeyer flask were dispensed in a sterile 100 ml measuring cylinders and additional cell amount measured with SCV (ml). In above all suspension cultures, the number of cell aggregates was measured using hemacytometer. For counting the number of cell aggregates, 10 µl of liquid cultures were used. The number of cell aggregates was

counted using the manual counter in each grid of hemacytometer and the total number was calculated per 10 µl suspension and then total number of cell aggregates was counted from 1 ml suspension.

Effect of subculture duration on cell size

Two-week-old 5 ml SCV cells (on MMS containing 1.5 mgl^{-1} 2,4-D) were subcultured in MMS containing 1.5 mgl^{-1} 2,4-D. Cell structure in suspension culture were observed after 8-week-old culture without transferring to the fresh medium and in 8-week-old culture with regularly transferring at 7-day-interval under the light microscope. The structures of cell were compared between these two conditions of cell suspension culture.

Statistical analysis

All experiments were repeated at least three times. The experiments were conducted with a completely randomized design and the data were analyzed by analysis of variance with Tukey's multiple comparison test using JMP Statistical Discovery Software (SAS Institute, Cary, NC, USA). The least significance difference (LSD) test was used to distinguish differences among the mean value in treatments at 5% ($p \leq 0.05$) level.

Results and discussion

Callus induction

The MS medium containing different concentrations of 2,4-D and combinations of BAP and NAA were applied for the callus induction from the leaf slices of bitter melon. All treatments of PGRs in MS medium were performed for the callus induction except PGR-free MS medium. The callus induction was achieved from the highest 65% leaf segments on MS medium containing 1.0 mgl^{-1} 2,4-D within 4 weeks of culture among all PGR treatments tested (Table 1). On this medium condition, callus induction at the cut sides of leaf segments was first observed after one week of culture initiation. After 4 weeks of culture, callus mass induction increased and spread throughout the explant. The induced callus color on MS medium containing 1.0 mgl^{-1} 2,4-D was greenish-yellow and showed embryogenic competent. On the rest of other PGRs that used in the callus induction, white calli induced with lower frequencies than that of 1.0 mgl^{-1} 2,4-D. The frequencies of explant that induced callus differed significantly ($p \leq 0.05$) among the PGR treatments examined (Table 1). Thiruvengadam *et al.* (2006) reported that the embryogenic callus was induced from leaf segments of bitter melon on MS medium fortified with 1.0 mgl^{-1} 2,4-D, which is very similar trend in the present study. The best callus induction obtained from the leaf explants on medium containing 1.0 mgl^{-1} 2,4-D was used for the formation of proembryogenic aggregates by subsequent cultures. The contrast of our result for callus induction of bitter melon was reported by Paul *et al.* (2009), who observed that embryogenic callus induction was best on MS media supplemented with 0.5 mgl^{-1} NAA and 5 mgl^{-1} BAP.

Table 1. Callus induction from internode explants of bitter melon on MS medium supplemented with different concentrations of 2,4-D and combinations of BAP and NAA after 4 weeks of culture.

PGRs (mgl^{-1})		Percentages of explant that induced callus (Mean ± SE)	Callus color
	0	0	
2,4-D	0.5	40 ± 3.2b	White
	1.0	65 ± 6.8a	Greenish-yellow
	2.0	20 ± 1.4bc	White
	1.0+0.1	20 ± 3.1bc	White
	1.0+0.5	30 ± 3.5bc	White
BAP+NAA	5.0+0.1	30 ± 1.1bc	White
	5.0+0.5	45 ± 1.1b	White
	10.0+0.1	30 ± 1.1bc	White
	10.0+0.5	35 ± 1.1b	White

Cell suspension culture

The induced greenish-yellow callus on agarified MS medium containing 1.0 mgl^{-1} 2,4-D was used for this experiment. Differences ($p \leq 0.05$) in cell proliferation were significant among the PGR treatments examined

(Fig. 1). The highest cell growth (average 25 ml SCV per flask) was observed in liquid MMS medium supplemented with 1.5 mgl^{-1} 2,4-D after 2 weeks of culture. The rest other concentrations of 2,4-D in MMS liquid medium slowed lower (bellow 20 ml SCV per flask) cell growth than that of 1.5 mgl^{-1} 2,4-D (Fig. 1). It is a difficult process to establish an efficient cell suspension culture. In the present study, the suspension culture was established in same auxin type (2,4-D) that used for callus induction but the concentration of 2,4-D was higher than that of callus induction concentration. In case of auxin type and concentration, the finding of the present study was in agreement with the observation of cell suspension culture (Thiruvengadam *et al.*, 2006) but the composition of basal medium was different between present study and observation of Thiruvengadam *et al.* (2006). Sultana and Rahman (2011, 2012) reported that the callus induction as well as cell multiplication in suspension showed the best responses in same PGR condition.

Table 2. Effect of initial amounts of cell on cell proliferation and cell aggregates development on MMS medium containing 1.5 mgl^{-1} 2,4-D after 4 weeks of culture.

Initial cell amount that cultured (SCV ml)	SCV of additional amount of cell (ml) (Mean ± SE)	Number of cell aggregates per milliliter culture (Mean ± SE)
1	2.5 ± 0.3c	0.2 x 10^3 ± 50d
3	3.5 ± 0.6c	0.4 x 10^3 ± 95d
5	8.5 ± 2.5a	2.0 x 10^3 ± 75a
10	6.5 ± 2.0b	1.5 x 10^3 ± 90b
15	4.0 ± 0.6c	1.5 x 10^3 ± 80b
20	2.5 ± 0.2c	1.0 x 10^3 ± 75c

Means within column followed by same letters are not significantly different from each other by the LSD test at the 5% level ($p \leq 0.05$)

Cell growth by batch culture

The cells (5 ml SCV) from initially established suspension culture using callus on MMS medium fortified with 1.5 mgl^{-1} 2,4-D were subcultured in liquid MMS medium containing 1.5 mgl^{-1} 2,4-D. When the total amount of cells plotted against the culture duration, an S-shaped growth curve was obtained in batch culture of bitter melon (Fig. 2). In the growth curve, three typical growth phages of cell were observed. The lag phage of growth curve found from initial day to end of 2nd weeks of culture where amount of cell was not change in the suspension culture, indicating the cells were not multiplied. In exponential growth phage, the cell growth drastically increased from 3rd to end of 6th week, indicating rapid multiplication occurred. The stationary phase was last phage of cell growth curve, which observed from 7th to end of 8th week where cell growth was very slow (Fig. 2). Three typical phages of cell growth in the batch culture have been reported previously by Razdan (1993) and Sultana and Rahman (2011, 2012). In contrast, a linear growth observed until days 26 – 28, but lag phase did not find out in the cell suspension culture for *Picea sitchensis* (Krogstrup, 1990).

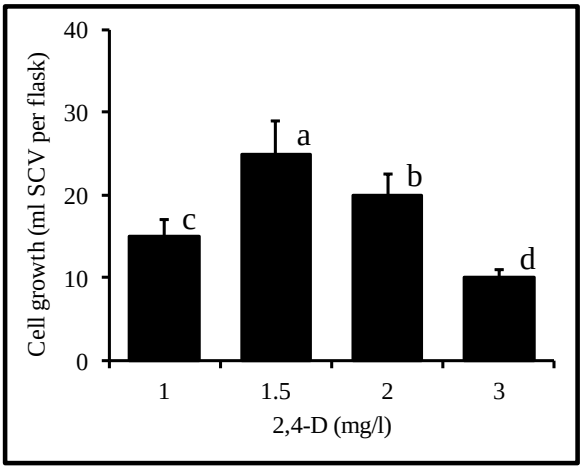

Fig. 1. The cell proliferation of bitter melon in liquid MMS medium containing different concentrations of 2,4-D after 2 weeks of culture. Mean values in bar followed by same letters are not significantly different from each other by the LSD test at the 5% level ($p \leq 0.05$).

Effects of cell initial cell amounts on cell proliferation and cell aggregates development

The amounts of cell (1, 3, 5, 10, 15 and 20 ml SCV) induced in the suspension culture were used in the present experiment. These amounts of cell were subcultured in liquid MMS medium containing 1.5 mgl^{-1} 2,4-D. The initial amount of cell in suspension culture showed an important role in the cell proliferation and cell aggregate development. The additional amount of cell (SCV) was gradually increased in the suspension culture with increasing initial amount of cell from 1 to 5 ml SCV after 4 weeks of culture. It was then gradually decreased when initial amount of cell upgraded from 10 to 20 ml (Table 2). The highest amount of additional cell was 8.5 ± 2.5 ml SCV in the suspension when initial cell amount was 5 ml SCV. The additional amount of cell (SCV) differed significantly ($p \leq 0.05$) in the amount of cell during initiation of culture (Table 2). The high amount initial cells during suspension culture reduced the cell growth (Sultana and Rahman, 2011; 2012).

Table 3. Effect of subculture duration on proliferated cell amount and cell size after 6 weeks of suspension culture.

Subculture condition	Amount of proliferated cell (SCV ml)	Elliptical cell: spherical cell
Eight-week-old culture maintained without transferring to fresh medium	25	1 : 0.20
Eight-week-old culture maintained by transferring to fresh medium at 7-day-intervel	40	1 : 8

Both free cells and cell aggregates were observed in cell suspension culture after 4 weeks of culture. In the suspension culture, single cells underwent mitosis divisions, resulted in free single cells multiplication and early proembryogenic structure like two-, three- and four-celled early proembryogenic structures and finally these early proembryogenic structures developed into a multicellular proembryogenic aggregate (Fig. 3). The number of cell aggregates was gradually increased when initial cell amount was increased from 1 to 5 ml SCV. The number of cell aggregates was reduced in suspension when initial cell amount was raised from 10 to 20 ml SCV in liquid medium (Table 2). The number of cell aggregate differed significantly ($p \leq 0.05$) in the amount of cell during initiation of culture (Table 2). The initial cell amount used in a given culture system can have dramatic effects on cell proliferation (Bhojwani and Razdan, 1996). The cell size and cell aggregate formation in the cell suspension culture was influenced by the initial cell amount (Ogita *et al.*, 2000).

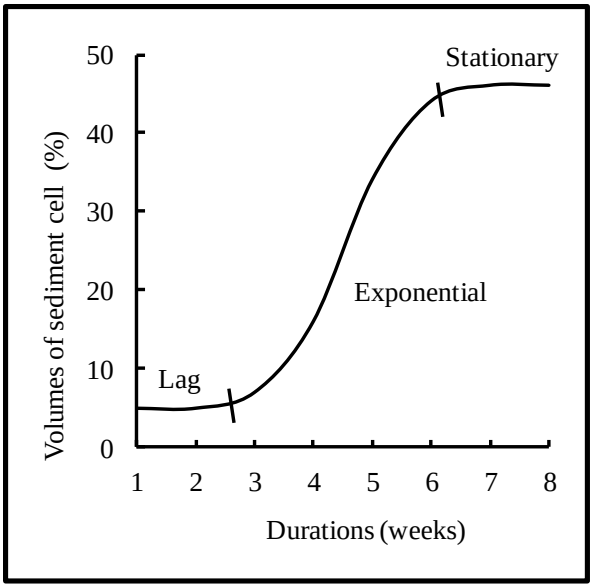

Fig. 2. S-shaped curve of cell growth in the batch culture of better melon showing three typical growth phases.

Effect of subculture duration on cell size

The cell amount was lower (25 ml SCV) in a suspension for long time (8 weeks) culture without sub-culturing to fresh medium than regularly sub-culturing to fresh MS medium (40 ml SCV) (Table 3). The reduction of cell amount in suspension without sub-culturing to fresh medium might result in extensive accumulation

of polyphenols. For long time maintenance of cell suspension culture, polyphenols are accumulated in the liquid medium, which restricted cell division and cell enlargement (Davies, 1972).

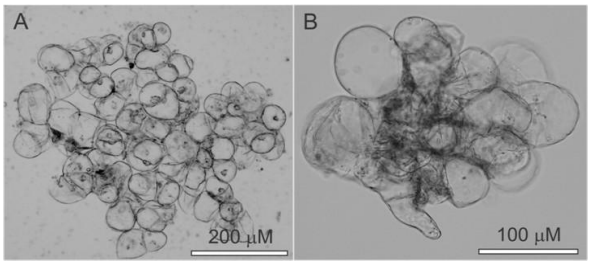

Fig. 3. Morphogenesis of cells aggregates for bitter melon in liquid MMS medium containing 1.5 mgl^{-1} 2,4-D. A. Early proembryogenic structures of cell aggregates (two-, three-, four- celled structure). B. A multi-celled proembryogenic structure of aggregate.

In the present study, when cell culture was maintained for a long time (8 weeks) without subculturing to fresh MMS medium containing 1.5 mgl^{-1} 2,4-D, the most of the cells shape was elliptical and large-sized (Fig. 4B). The spherical cells were also observed in this culture condition but they were smaller in size than that of elliptical cells. In this culture condition, elliptical cell number was higher than spherical cell (elliptical cell : spherical cell was 1 : 0.20).

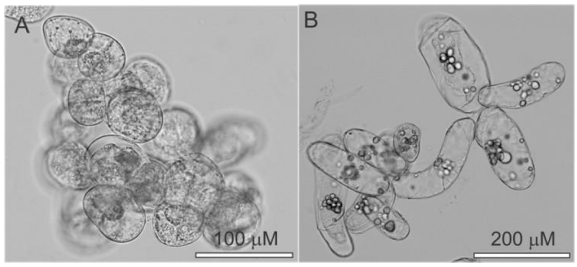

Fig. 4. Cell size of bitter melon in liquid MMS medium containing 1.5 mgl^{-1} 2,4-D at two subculture conditions. A. Spherical cells in suspension culture after 8 weeks of culture when suspension maintained by subculturing at 7-day-intervel. B. Elliptical cells in suspension culture after 8 weeks of culture when suspension maintained without subculturing.

On the other hand, when cell culture regularly transferred to fresh medium (MMS+1.5 mgl^{-1} 2,4-D) at 7-day-intervel, the number of spherical cell was higher than elliptical cell (elliptical cell : spherical cell was 1 : 8) (Table 3, Fig. 4A). In the present experiment, the higher number of elliptical cells in 8-week-old culture without sub-culturing could form at the presence of PGR. The present study is in agreement with the observation of cell suspension culture for sweet potato (Sultana and Rahman, 2011). The contrast trend was reported by Häsler *et al.* (2003), who observed that elliptical and spherical cells formed at a same ratio in ten-year-old sugar beet leaf-derived callus when callus was maintained on agar-gelled MS medium supplemented with 0.1 mgl^{-1} 2,4-D and 0.1 mgl^{-1} BAP, while only spherical cells were found in ten-year-old callus gown on PGR-free MS medium.

In conclusion, cell suspension culture of bitter melon was established in the present study. The medium and culture conditions for the free cells and different stages of proembryogenic structures in the suspension were optimized. The present study will accelerate the establishment of somatic embryogenesis through cell suspension culture and also will be helpful to establish single cell origin of bitter melon plant.

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