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Ontogeny of embryogenic aggregates in suspension culture of diploid watermelon [*Citrullus lanatus* (Thunb.)]

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Abstract

Cell suspension culture from leaf-derived callus of diploid watermelon [*Citrullus lanatus* (Thunb.)] was established. The callus obtained on agar-gelled Murashige and Skoog (MS) medium containing 2.5 mgl^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) was used for suspension culture. The growth of cell in the suspension culture was the highest in liquid MS medium containing same concentration (2.5 mgl^{-1}) of 2,4-D as used for callus induction. Cells in suspension culture underwent division as result both free cells and cell aggregates were formed. In first 15 days, profuse number of early proembryogenic structures (two-, three- and four-celled) was observed while after more 15 days of culture, multi-celled proembryogenic structure was greater in suspension. The initial amount of cell in suspension culture was effective for cell proliferation and cell aggregation. As initial amount of cells, 4 ml sedimented cell volumes (SCV) could proliferate and aggregate at a highest level after 30 days of culture. Three typical phages (lag, exponential and stationary phases) in S-shaped growth curve were found in the batch culture. Among the cell growth phages, duration of exponential phage is important for the high production of cell and biosynthesis of cell compounds.

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Introduction

Watermelon is an important tropical and subtropical cucurbitaceous vegetable. It is grown worldwide and ranked sixth in the world production of fruit crops. China has been the number one producer of watermelon since 1986. It is an economically important crop, a valuable alternative source of water and is often consumed as a cool dessert. It is a good source of fiber, which is important for keeping digestive tract operating properly by preventing constipation, hemorrhoids and diverticular diseases of human (Park and Russell, 1991). Huge seeds consisted in watermelon fruit resulting problematic for eating. Therefore, plant breeders developed seedless triploid watermelon (Compton *et al*., 1996a). Besides, diseases and pests attach during cultivation are also problematic. Furthermore, growers of watermelon want elite cultivars to high yield, resistant to disease, pest, and drought. By conventional breeding, it is so far to achieve in elite watermelon. Therefore, the introduction of new characters into watermelon by means of genetic manipulation is of great potential value, in which *in vitro* propagation system is essential as prerequisite for genetic transformation system. There are a number of reports on *in vitro* adventitious shoot regeneration of watermelon on gelled medium from excised cotyledons (Blackmon and Reynolds, 1982; Srivastava *et al*., 1989; Adelberg *et al*., 1990; Compton and Gray, 1991, 1993a, 1994; Dong and Jia, 1991; Compton *et al*., 1994a, 1996a, 1996b; Compton, 1997; Jaworski and Compton 1997; Hao and Wang, 1998) and from leaf segments (Sultana *et al*., 2004). Many researchers (e.g., Ahad *et al*., 1994, Sultana and Bari, 2003) established micropropagation system for watermelon from different explantts. The plant regeneration of watermelon by somatic embryogenesis in gelled medium has been reported by Tabei (1997) and Compton and Gray (1993b). Using regeneration system, genetic transformation methods have been established (Compton e*t al*., 1994b; Choi *et al*., 1994; Tabei, 1997). As goal for efficient plant regeneration, the cell suspension culture is not reported so far although suspension culture established for synthesis of biochemical from cell. Our research motto is to establish efficient plant regeneration system of watermelon in suspension culture.

In the present study, we established cell suspension culture and observed cell morphology and ontogeny of cell aggregates of watermelon.

Materials and methods

Plant material

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

Medium preparation and culture conditions

As a basal medium, MS (Murashige and Skoog, 1962) medium was used for all experiments studied here. The pH of media was adjusted to 5.7 ± 0.1 and 0.8% (w/v) agar (Type M, Sigma) was added to medium solidification before autoclave at 121 ^{o}C for 20 min under 1.1kg cm^{-2} pressure. The medium without plant growth regulators (PGRs) used as control for all experiments. The cultures were maintained in a growth chamber controlled with 27 ± 1^{o}C and a 16-h photoperiod (35 μmol m^{-2} s^{-1}).

Cell suspension culture

Callus was achieved followed by our previous protocol on organogenesis of watermelon (Sultana *et al*., 2004). The induced callus (5 g fresh weight) on MS medium fortified with 2.5 mgl^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) was transferred to a 300-ml Erlenmeyer flask containing 50 ml of liquid MS medium fortified with either 0.1 and 2.5 mgl^{-1}2,4-D alone or in combinations with 0.5 mgl^{-1} 6-benzylaminopurine (BAP). The flasks were sealed with Aluminum foil, wrapped with parafilm and then they were placed on a rotary shaker (90 rpm). Callus cells were proliferated in suspension cultures for 15 days. 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).

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

Proliferated cells from initial suspension at different amounts (2, 4, 6 and 8 ml SCV) were transferred in 300 ml Erlenmeyer flasks containing 50 ml of MS liquid medium supplemented with 2.5 mgl^{-1} 2,4-D. For this experiment, initially established suspension was first filtered several times to avoid cell aggregates and then transferred to fresh medium routinely in a week. After 15 days and 30 days of culture, cell suspension observed under light microscope. Additional cell amount was measured by SCV ml. The number of cell aggregates was also counted after 30 days of culture in all suspension using hemacytometer followed by method that used for cell suspension culture of sweet potato (Sultana and Rahman, 2011).

Determination of cell proliferation in batch culture

Initially proliferated cells within 15 days were used to examine cell growth by batch culture. Therefore, 4 ml SCV cells were cultured in 300 ml Erlenmeyer flasks containing 50 ml MS liquid medium fortified with 2.5 mgl^{-1} 2,4-D and routinely transferred to a fresh medium at one-week-interval. The cultures were maintained up to 8 weeks. Total amount of cell (SCV ml) per flask measured prior to transfer in each transfer.

Statistical analysis

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

Results and discussion

Cell suspension culture

Significant differences ($p \leq 0.05$) in cell proliferation were observed among the PGR treatments (Fig. 1). Cell growth (18 ml SCV per flask) was the highest in liquid MS medium supplemented with 2.5 mgl^{-1}2,4-D after 15 days of culture. The cell growth in liquid MS medium containing rest other PGRs slowed (bellow 10 ml SCV per flask) (Fig. 1). The establishment of efficient cell suspension culture is a difficult process. The callus induction as well as cell multiplication in suspension for watermelon showed the best responses in same PGR condition, which usually not found in many species. The finding of the present study was in agreement with the observation of callus induction and cell suspension culture for sweet potato (Sultana and Rahman, 2011). The contrary trend described for callus induction and suspension culture of *Atropa belladonna* and *Atropa belladonna* (Thomas and Street 1969).

Table 1. Effect of initial amounts of cell on cell proliferation and cell aggregates formation after 30 days of culture.

Initial amounts of cell (SCV ml)	Additional amount of proliferated cell (SCV ml) (Mean ± SE)	Number of cell aggregates (proembryogenic structures) per milliliter suspension (Mean ± SE)
2	2.1 ± 0.5c	0.3 x 10^3 ± 135c
4	7.5 ± 0.9a	1.1 x 10^3 ± 300a
6	5.2± 0.4b	0.5 x 10^3 ± 215b
8	3.4 ± 0.4c	0.3x 10^3 ± 150c

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

Effects of cell initial cell amounts on proliferation and aggregation of cell

With increasing initial amount of cell from 2 ml to 4 ml SCV, the additional amount of cell (SCV) was gradually increased in the suspension. The highest amount of additional cell (8.2±1.3 ml SCV) was obtained in the suspension after 30 days of culture when initial cell amount was 4 ml SCV (Table 1).

The gradual decrease in the cell growth was observed when initial amount of cell increased from 6 to 8 ml. The additional amount of cell differed significantly ($p \leq 0.05$) in the amounts of cell during initiation of culture (Table 1). Bhojwani and Razdan (1996) reported that the initial cell amount used in a given culture system can have dramatic effects on cell proliferation. On the other hand, Ogita *et al.* (2000) investigated that the initial amount of cell influenced cell aggregation in the cell suspension culture of Japanese conifers.

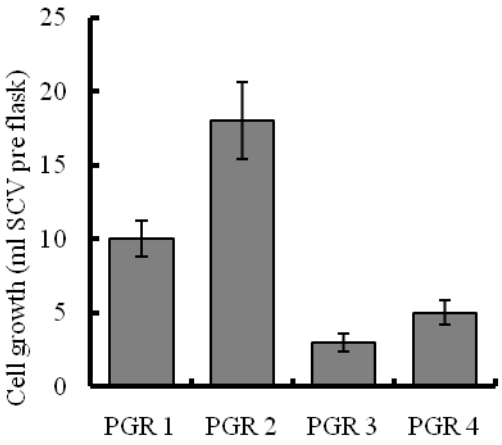

Fig. 1. The cell proliferation in liquid MS medium containing different PGR treatments after 15 days of culture. PGR 1, 0.1 mgl^{-1} 2,4-D; PGR 2, 2.5 mgl^{-1}2,4-D; PGR 3, 0.1 mgl^{-1} 2,4-D + 0.5 mgl^{-1} BAP; PGR 4, 2.5 mgl^{-1} 2,4-D + 0.5 mgl^{-1} BAP.

In cell suspension culture after 30 days of culture, the both free cell and cell aggregate were observed. Cells in the suspension culture underwent mitosis division as a result free cells and cell aggregates were formed. Different cell aggregates were found in suspension culture of watermelon such as, two-, three- and four-celled, which are early proembryogenic structure (Fig. 2A). The filaments cell aggregates (four-celled) also observed in the cell suspension (Fig. 2B). By the increasing of culture period, the early proembryogenic turned into multi-celled proembryogenic structure by mitosis cell division (Fig. 2C). The number of early proembryogenic structure in suspension was high in 15-day-old culture, however, opposite trend was found in the culture after 30 days. The formation of proembryogenic structures was highest in suspension of 4 ml SCV initial cells among the treatments tested (Table 1). The proembryogenic structures differentiation from liquid cultures reported in some plants, i.e. sweet potato (Sultana and Rahman, 2011), *Cajanus cajan* (Anbazhagan and Ganapathi, 1999), *Arachis hypogaea* (Eapen and George, 1993) and *Macrotyloma uniflorum* (Mohamed *et al.*, 2004).

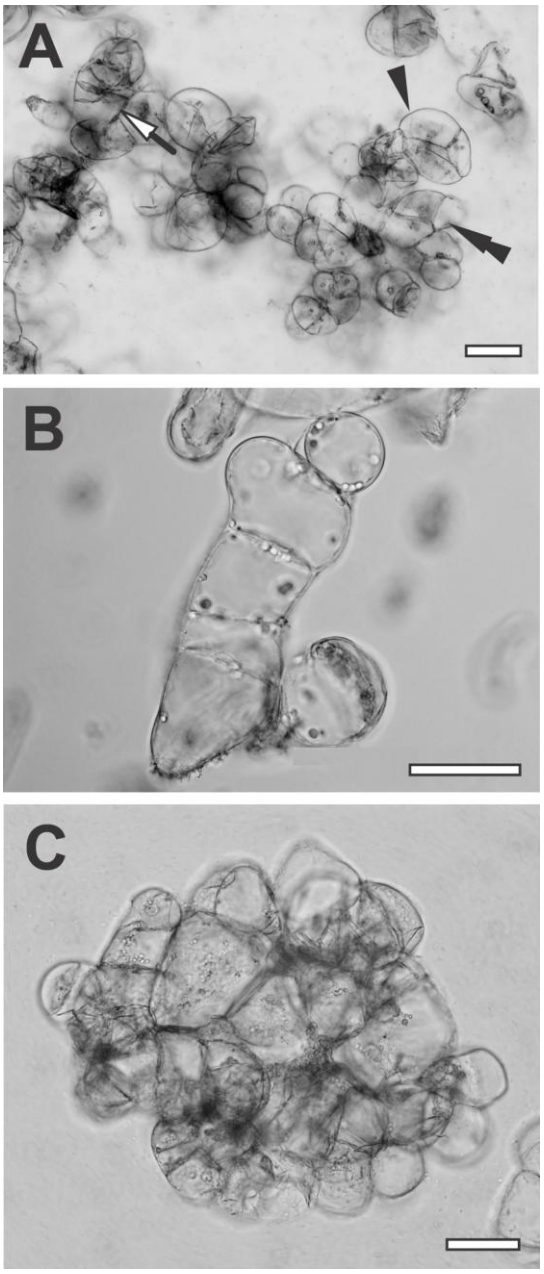

Fig. 2. Ontogenesis of cells aggregation of watermelon in liquid MS medium containing 2.5 mgl^{-1} 2,4-D. A. Early proembryogenic structures of cell aggregates, two-celled structure (Arrow head),

three-celled structure (Double arrow head), four-celled structure (Arrow). B. A filamentous four-celled structure. C. A multi-celled proembryogenic structure of aggregate. Bar, 150 μm.

Cell growth by batch culture

The cell (4 ml SCV) initially differentiated from callus were cultured in liquid MS medium containing 2.5 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 watermelon (Fig. 3). The growth curve indicated three typical phages. 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. In exponential growth phage, the cell growth drastically increased from 3rd to end of 6th week. The last phage was stationary phase from 7th to end of 8th week where cell growth was very slow (Fig. 3). Three typical phages of cell growth in the batch culture have been reported previously by Razdan (1993) and Sultana and Rahman (2011). In contrast, a linear growth observed until day 26 – 28 although lag phase was not clear in the cell suspension culture for *Picea sitchensis* (Krogstrup, 1990).

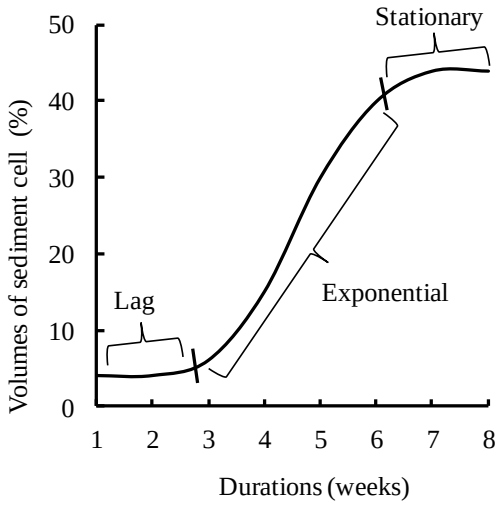

Fig. 3. S-shaped curve of cell growth in the batch culture of watermelon showing three typical growth phases.

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

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