

# Sweet Potato (*Ipomoea batatas* L.): Suspension Culture for cell Aggregation

## Research Article

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### Abstract

Cell aggregation in suspension culture of sweet potato (*Ipomoea batatas* L.) was established from petiole-derived callus. The highest rate (55%) of petiole induced callus on agar-gelled Murashige and Skoog (MS) medium containing 10  $\mu$ M 2,4-Dichlorophenoxyacetic acid (2,4-D) within 30 days of culture. These calli were used for preparation of suspension culture in liquid medium containing same concentration of 2,4-D that was used for callus induction. A growth curve with lag, exponential and stationary phases of cell growth was obtained when total amount of cell was plotted against the culture periods. Cells underwent divisions, resulting in free cells multiplication and cell aggregates formation after further 30 days of culture. The initial amount of cell during suspension culture showed effect in the cell proliferation, cell size and cell aggregate development. The additional amount of cell and cell aggregates formation were the highest when 5 ml Sedimented Cell Volume (SCV) of cells was cultured. The cell proliferation reduced and high number of elliptical cell was found in suspension when culture maintained for longer period without transferring to fresh medium.

**Keywords:** Callus; Cell aggregate; Petioles; Sweet potato; Suspension culture

## Introduction

Sweet potato (*Ipomoea batatas* L.) is one of the major food crops in the world. It is an important source of carbohydrates. Since sweet potato is a vegetatively propagated plant, the molecular breeding based genetic transformation is an important option to improve this plant. The successful genetic transformation cannot be achieved unless efficient plant regeneration protocol is established. Few reports were published on plant regeneration procedures of sweet potato through organogenesis and somatic embryogenesis on gelled medium. The ability of regeneration into plantlets using micropropagation system from apical meristems, lateral buds and nodal segments of sweet potato [1,2]. The callus induction and adventitious shoot formation procedure from storage root sections of sweet potato was established in vitro [3]. The clonal propagation of

sweet potato through both adventitious and axillary procedures was established by Villordon and Labonte [4]. The embryogenic callus and efficient in vitro plant regeneration system were characterized by rapid and continuous production of somatic embryos [5-8]. The report on plant regeneration system using cell suspension culture has not been established, although one report on genetic transformation using somatic embryogenesis through cell suspension culture for sweet potato was published [9]. One report is insufficient for genetic transformation by somatic embryo genesis in suspension culture. Besides, lacking of detail information on somatic embryogenesis has remained in the protocol of Xing et al. [9]. Therefore, more detail research on cell proliferation, cell aggregate formation and their appearance in suspension culture of sweet potato is necessary for genetic transformation studies.

The aim of the present study is to establish efficient cell suspension culture for sweet potato. Herein, proliferation of cell and development of cell aggregates in suspension culture were studied in various media and culture conditions.

## Materials and Methods

### Basal medium preparation and culture conditions

MS basal medium was used for callus induction and cell suspension culture. All media were adjusted to pH  $5.7 \pm 0.1$  and autoclaved at  $121^\circ\text{C}$  for 20 min [10]. The medium was solidified with 0.8% (w/v) agar (Type M, Sigma), which was used for callus induction and the liquid medium was applied for cell suspension culture. The medium without Plant Growth Regulators (PGRs) received as control for all experiments. The cultures were maintained at  $27 \pm 1^\circ\text{C}$  under a 16-h photoperiod ( $35 \mu\text{mol m}^{-2} \text{s}^{-1}$ ).

### Induction of callus and initiation of cell suspension culture

Leaves with petiole of field grown plant were collected between 10:00 to 11:00 am in the daytime with bright sunlight. Petioles were separated from leaves and washed thoroughly under running tap water for 30 min. Finally they were disinfected with 2% (v/v) solution of NaOCl for 10 min under laminar airflow followed by washing thrice with autoclaved distilled water. The disinfected petioles were sliced (approximately 1 cm in length) and cultured on MS medium containing either different concentrations of 2,4-Dichlorophenoxyacetic acid (2,4-D) at 5, 10 and  $15 \mu\text{M}$  alone or in combinations with kinetin (KIN) at  $0.5 \mu\text{M}$ .

Proliferated 30-day-old calli (1 g, fresh weight) on gelled MS medium containing  $10 \mu\text{M}$  2,4-D were cultured in 300 ml Erlenmeyer flask containing 50 ml liquid MS medium fortified with  $10 \mu\text{M}$  2,4-D. After 15 days of suspension culture, cells were proliferated in suspension cultures, which were used for further experiments.

### Cell growth in batch culture

Cell proliferation in suspension culture was examined by batch culture. The amount of proliferated cells was determined by Sedimented Cell Volume (SCV) ml. For measuring of cell amount by SCV, suspension dispensed into sterile 100 ml measuring cylinders and allowed to sediment for 30 min. For batch culture, 5 ml SCV of cells from 15-day-old suspension culture were cultured initially in 300 ml Erlenmeyer flasks containing 50 ml MS liquid medium fortified with  $10 \mu\text{M}$  2,4-D. They were routinely transferred at one-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, cell size and cell aggregates development

Cells from fifteen-day-old suspension were cultured initially at different volumes, 1, 3, 5, 10, 15, and 20 ml SCV in 300 ml Erlenmeyer flasks containing 50 ml of MS liquid medium supplemented with  $10 \mu\text{M}$  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. In above

all suspension culture, the number of cell aggregates was measured using hemacytometer. For obtaining the number of cell aggregates,  $10 \mu\text{l}$  of liquid cultures were used. The number of cell aggregates was counted using the manual counter in each grid and the total number was calculated per  $10 \mu\text{l}$  suspension and then total number of cell aggregates was counted from 1 ml suspension. For measurement of cell diameter from all suspensions, cells were observed under light microscope and photographs were taken. Radial and tangential diameters were measured by Image J software and average of radial and tangential diameters of each cell denoted as cell diameter.

### Effect of subculture duration on cell size

Fifteen-day-old 5 ml SCV cells were cultured in MS containing  $10 \mu\text{M}$  2,4-D. Cell amount and appearances were observed in 6-week-old culture without transferring to the fresh medium and in 6-week-old culture with regularly transferring at 7-day-interval. The types and size of cells were compared between these two conditions of cell suspension culture.

### Statistical analysis

For callus induction, ten internode segments were cultured in one Petri dish, which was considered as one replication. To observe the effect of initial cell amount on the cell proliferation, cell size and cell aggregates number, four Erlenmeyer flasks were used and each flask was considered as one replication. From each flask, amount of cell (ml) SCV, diameters (twenty cells) and number of cell aggregates were measured. 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. The Least Significance Difference (LSD) test was used to distinguish differences among the mean value of treatments at 5% ( $p \leq 0.05$ ) level.

## Results and Discussion

### Callus induction

All variants of PGRs in MS medium were responsible for the callus induction except PGR-free MS medium. Of all PGR treatments examined, the callus induction frequency was the highest (55%) on MS medium containing  $10 \mu\text{M}$  2,4-D within 30 days of culture (Table 1). On this medium, callus induction was first observed 7 days after culture at the cut sides of petiole segments. After 30 days of culture, callus proliferation was surrounded throughout the explant. The

**Table 1:** Callus induction from internode explants of sweet potato on MS medium supplemented with different concentrations and combinations of PGRs after 30 days of culture.

PGRs (mM)		Percentages of explant that induce callus (Mean $\pm$ SE)	Callus color
KIN	2,4-D		
0	0	0	-
0	5	$30 \pm 3.2\text{b}$	White
0	10	$55 \pm 6.8\text{a}$	Pale-yellow
0	15	$15 \pm 1.4\text{c}$	White
0.5	5	$20 \pm 3.1\text{bc}$	White
0.5	10	$25 \pm 3.5\text{b}$	White
0.5	15	$10 \pm 1.1\text{cd}$	White

**Note:** Means within column followed by same letters are not significantly different from each other by the LSD test at the 5% level ( $p \leq 0.05$ ). RG: Rapid growth; SG: Slow growth.

induced callus color was pale-yellow (Figure 1A). White calli also induced on the rest of other PGRs with lower frequencies than that of 10  $\mu$ M 2,4-D. The significant differences ( $p \leq 0.05$ ) were observed in the frequencies of explant inducing callus among the PGR treatments examined here (Table 1). Alves et al. reported that the embryogenic callus was induced from lateral bud of sweet potato on MS medium fortified with 10  $\mu$ M 2,4-D [7]. The medium containing 0.5-2.0 mg/l 2,4-D assisted to induce pale- to bright-yellow calli from leaf, shoot-tip, stem and root explants, which were responsible for embryos development [6]. Root discs of sweet potato cv. Leucorhiza produced callus and then developed meristemoids and shoots on various levels of Kn and 2,4-D combination [11]. From the above discussion, it is clarified that 2,4-D was essential for rapid callus induction, which is responsible for embryo or organ development of sweet potato. The trends of callus induction in the previous report is supported the present study, in which the best callus induction obtained from the petiole explants on medium containing 2,4-D alone and that could form proembryogenic aggregates.

### Cell growth by batch culture

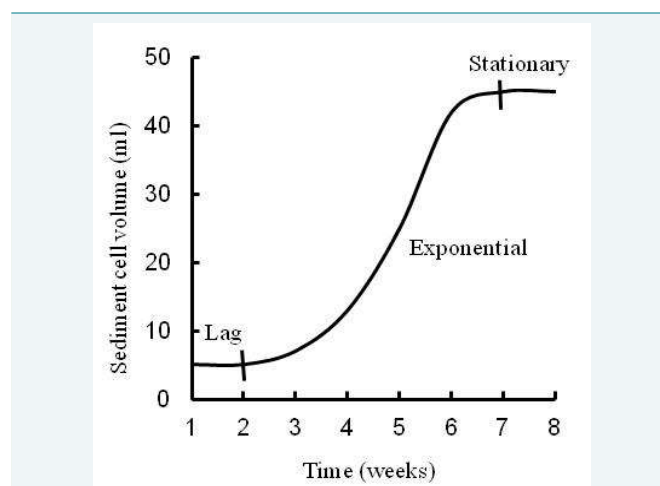
The induced callus (1 g, fresh weight) on agar-gelled MS medium containing 10  $\mu$ M 2,4-D were cultured in liquid MS medium containing 10  $\mu$ M 2,4-D for 15 days (Figure 1B). The cells of this suspension culture (5 ml SCV) were used for cell growth study in the batch culture. When the cell amount (SCV) is plotted against the culture period, a growth curve was obtained. The increase cell amount in the suspension culture was not observed from initial day to end of 2<sup>nd</sup> weeks of culture, indicating lag phase of cell growth. The cell growth gradually increased from 3<sup>rd</sup> to end of 7<sup>th</sup> week, which was indicated exponential growth phase. The subsequent one week (from 7<sup>th</sup> to end of 8<sup>th</sup> week) did not show cell growth, which is denoted as stationary phase (Figure 2). The biomass growth in the batch culture follows this growth curves with lag, exponential and stationary phase has been report by Razdan [12]. Krogstrup stated that lag phase was not clear in the growth curve but the cultures show a linear growth until day 26-28 and then cell proliferation eventually deteriorate in the cell suspension culture for *Picea sitchensis* [13].

### Effects of cell initial cell amounts on cell proliferation, cell size and cell aggregates development

The initial amount of cell during suspension culture showed an important role in the cell proliferation, cell size and cell aggregate

development in liquid MS medium containing 10  $\mu$ M 2,4-D. The additional amount of cell (SCV) was gradually increased in the suspension culture with increasing initial amount of cell increased from 1 to 5 ml SCV and then it was gradually decreased when initial amount of cell from 10 to 20 ml (Table 2). The highest amount of additional cell was  $8.2 \pm 1.3$  ml SCV, which was found in the suspension when initial cell amount was 5 ml SCV.

When initial amounts of cell were 1, 3 and 5 ml SCV in cell suspension cultures, majority free single cells (3/4 of total cells) in the suspension culture were large and spherical. The similar diameters of cell (178, 180 and 193  $\mu$ m) were found in suspension when initial amounts of cell were 1, 3 and 5 ml SCV, respectively (Table 2). In rest of suspension cultures where initial amounts of cell were 10, 15 and 20 ml SCV, multiplied majority single cells (3/4 of total cells) were small and spherical, and the diameters of cell were similar 47, 45 and 42  $\mu$ m, respectively. The diameter of cell differed significantly in the amount of cell during initiation of culture (Table 2).

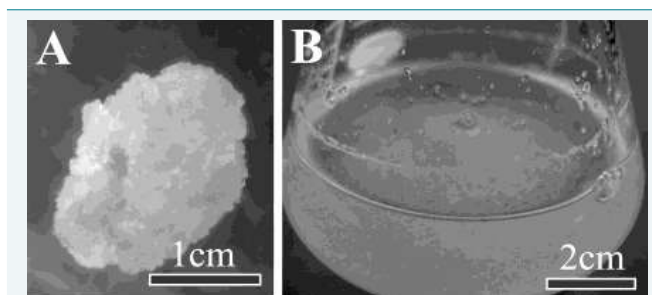


**Figure 2:** Growth curve of cell in the batch culture showing different cell growth phase.

**Table 2:** Effect of initial amounts of cell on cell proliferation, cell size and cell aggregates development on MS medium containing 10  $\mu$ M 2,4-D after 4 weeks of culture.

SCV in initial cell culture (ml)	SCV of additional amount of cell (ml) (Mean $\pm$ SE)	Cell structure		Number of cell aggregates per milliliter culture (Mean $\pm$ SE)
		Morphology of majority spherical cells (3/4 of total cells)	Average cell diameter (mm) (Mean $\pm$ SE)	
1	1.3 $\pm$ 0.1d	Large	178 $\pm$ 13.7a	0.1 $\times$ 10 <sup>4</sup> $\pm$ 254d
3	3.1 $\pm$ 0.3bc	Large	180 $\pm$ 18.3a	0.3 $\times$ 10 <sup>4</sup> $\pm$ 295d
5	8.2 $\pm$ 1.3a	Large	193 $\pm$ 12.8a	1.5 $\times$ 10 <sup>4</sup> $\pm$ 405a
10	5.5 $\pm$ 1.0b	Small	47 $\pm$ 6.2b	0.9 $\times$ 10 <sup>4</sup> $\pm$ 325b
15	4.2 $\pm$ 0.4b	Small	45 $\pm$ 3.5b	1.0 $\times$ 10 <sup>4</sup> $\pm$ 373b
20	2.3 $\pm$ 0.6d	Small	42 $\pm$ 5.1b	0.7 $\times$ 10 <sup>4</sup> $\pm$ 290bc

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

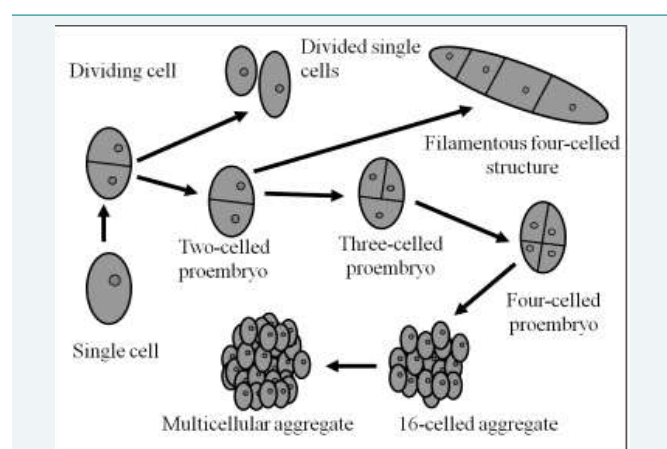


**Figure 1:** Callus induction and cell suspension culture of sweet potato. A. The induced callus from petiole explant on agar-gelled MS medium supplemented with 10  $\mu$ M 2,4-D after 30 days of culture. B. Cell suspension culture in liquid MS medium containing 10  $\mu$ M 2,4-D after 15 days of culture.

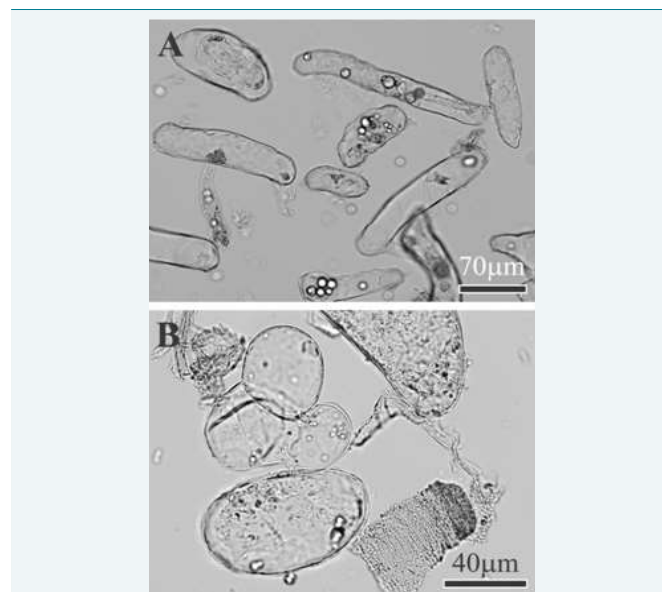
After 30 days of culture, free cells and cell aggregates were observed in cell suspension culture. In the suspension culture, cells underwent mitosis divisions, resulting in free cells multiplication and early proembryonic structure like two-, three-, four-, octa-, 16-celled early proembryonic structure and multicellular aggregate (proembryo) formation (Figure 3). The filamentous four-celled embryonic structure was also found in this suspension culture (Figure 3). In this experiment, two-celled, three-celled, four-celled early proembryos and Multicellular aggregates (proembryos) were observed (Figures not shown). The number of cell aggregates was gradually increased when initial cell amount was increased from 1 to 5 ml SCV. Decrease in the number of cell aggregates was found when initial cell amount was increased from 10 to 20 ml SCV in liquid medium (Table 2). The initial cell amount used in a given culture system can have dramatic effects on cell proliferation [14]. The cell size and cell aggregate formation in the cell suspension culture was influenced by the initial cell amount [15].

#### Effect of subculture duration on cell size

For long time maintenance of cell suspension culture, polyphenols are accumulated in the liquid medium, which restricted cell division and cell enlargement [16]. In the present study, cell amount was lower (32 ml SCV) in a medium for long time (6 weeks) culture without subculturing to fresh medium than regular subculture to fresh MS medium (45 ml SCV) (Table 3). The reduction of cell amount in suspension without subculturing to fresh medium might be occurred with the extensive accumulation of polyphenols. When cell culture was maintained for a long time (6 weeks) without subculturing to fresh MS medium containing  $10 \mu\text{M}$  2,4-D, the most of the cells shape was elliptical and large-sized (range 50-210  $\mu\text{m}$  in diameter) (Figure 4A). The spherical cells were also observed in this culture condition but they were smaller size (range 30-100  $\mu\text{m}$  in diameter) than that of elliptical cells. In this culture, elliptical cell number was higher than spherical cell (elliptical cell: spherical cell was 1: 0.35). On the other hand, when cell culture regularly transferred to fresh medium at 7-day-interval, the number of spherical cell was higher than elliptical cell (elliptical cell: spherical cell was 1: 7) (Table 3). The diameters of elliptical cells (range 40 - 150  $\mu\text{m}$  in diameter) were



**Figure 3:** A diagrammatic figure of cell division and cell aggregates formation in the suspension culture.



**Figure 4:** Cell size of sweet potato in liquid MS medium containing  $10 \mu\text{M}$  2,4-D. A. Elliptical cells in suspension culture after six weeks of culture when suspension maintained without subculturing. B. Spherical cells in suspension culture after six weeks of culture when suspension maintained by subculturing at 7-day-interval.

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

Subculture durations	Amount of cell in SCV (ml)	Elliptical cell : spherical cell	Range of elliptical cell diameter (mm)	Range of spherical cell diameter (mm)
Six-week-old culture maintained without transferring to fresh medium	32	01:00.4	50 - 210	30 - 100
Six-week-old culture maintained by transferring to fresh medium at 7-day-interval	45	1:07	40 - 150	30 - 80

higher than spherical cells (range 30 - 80  $\mu\text{m}$  in diameter) (Figure 4B). In the present experiment, the higher number of elliptical cells in 6-week-old culture without subculturing may form at the presence of PGR. The contrast trend was reported by Häsler, who observed that elliptical and spherical cells formed in ten-year-old sugar beet leaf-derived callus when callus was maintained on agar-gelled MS medium supplemented with  $0.1 \text{ mg l}^{-1}$  2,4-D and  $0.1 \text{ mg l}^{-1}$  BAP, while only spherical cells were found in ten-year-old callus grown on PGR-free MS medium.

#### Conclusion

The present study established cell suspension culture of sweet potato. For establishment of more efficient plant regeneration procedure in comparison with previously established protocol by Xing et al., the knowledge in cell suspension culture established here will be cooperative [9]. The present study will also be helpful to establish single cell origin of plant as well as suitable genetic modification processes for sweet potato.



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