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Establishment and optimization of cell growth in suspension culture of *Papaver bracteatum*: a biotechnology approach for thebaine production

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Abstract: Iranian poppy (*Papaver bracteatum*) is an important medicinal plant that is the main source of the opium alkaloids codeine, morphine, and thebaine. To establish an efficient protocol for cell suspension culture and growth, the effects of different plant growth regulators (2,4-D, NAA, BAP, and kinetin) on callus induction and cell suspension culture of Iranian poppy were evaluated. The maximum percentage of callus induction (86.67%) and fresh weight of callus were obtained in MS medium supplemented with 1 or 2 mg/L 2,4-D, 0.1 or 0.2 mg/L kinetin and 15 mg/L ascorbic acid. The addition of 15 mg/L ascorbic acid to the medium was effective in reducing browning of explants and calli. In cell suspension cultures, MS medium supplemented with 1 mg/L NAA, 1 mg/L BAP, and 15 mg/L ascorbic acid gave maximum cell growth and proliferation compared to other treatments evaluated. Among the auxins, NAA was more effective than 2,4-D for growth of *P. bracteatum* cell suspension cultures. However, there was no statistically significant difference between kinetin and BAP. The thebaine yield of *P. bracteatum* cell suspension at 12 days after culture was 4.67 µg/g dry weight, which was significantly lower than that at 16 days (10.76 µg/g dry weight).

Key words: Cell suspension culture, *Papaver bracteatum*, secondary metabolites, thebaine

1. Introduction

Plants are a valuable source of a wide range of secondary metabolites, which are used as pharmaceuticals, agrochemicals, flavors, fragrances, colors, biopesticides, and food additives (Phillipson, 1990; Ramachandra Rao and Ravishankar, 2002). Plants of *Papaveraceae* are important as a commercial source of medicinal opiates and related compounds. The main alkaloid of Iranian poppy (*Papaver bracteatum*) is thebaine, which has been used for many years in the production of codeine (Milo et al., 2006; Rostampour et al., 2009).

Plant cell culture techniques provide an attractive alternative source for production of highly valuable secondary metabolites and have been used for many years as a tool for the elucidation of secondary metabolite biosynthesis (Hamill and Rhodes, 1993; Ravishankar et al., 1999; Daud and Keng, 2006). Using in vitro culture techniques, the rapid growth of callus and cell suspension cultures, from which secondary metabolites are to be extracted, can be obtained (Shibli et al., 1999; Verpoorte et al., 2002). It is known that production of secondary metabolites is affected by nutritional factors, environmental conditions, and hormone regimes. Thus,

development of appropriate nutritional and hormone regimes would increase the production of morphinanes in plant cell cultures (Roberts, 1988). In general, plant growth regulator is one of the most important factors affecting cell growth, differentiation, regeneration, and metabolite formation in plant cells and tissue cultures (Zhong et al., 1996; Okumuş et al., 2011; Verma et al., 2011). Therefore, the aim of this study was the establishment of suspension cultures of *P. bracteatum* and optimization of plant growth regulator concentrations to obtain high cell growth in cell suspension cultures of this plant.

2. Materials and methods

2.1. Plant material

The seeds of *P. bracteatum* were obtained from the Research Institute of Forests and Rangelands, Tehran, Iran. The seeds were surface-sterilized with 70% (v/v) ethanol for 45 s and 2.5% (w/v) sodium hypochlorite solution for 12 min, and then rinsed 3 times in sterile distilled water. The seeds were then germinated on MS medium (Murashige and Skoog, 1962) solidified with 8 g/L plant agar, and maintained in a growth chamber at 25 ± 2 °C with a 16-h photoperiod of cool white fluorescent light (400–500 lx).

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2.2. Callus induction and cell suspension culture initiation

For callus induction, hypocotyl–cotyledon explants of *P. bracteatum* were transferred to MS medium supplemented with different levels of auxins (2,4-D or NAA), cytokinins (kinetin or BAP), and ascorbic acid (15 and 45 mg/L) or activated charcoal (3 g/L). The cultures were maintained in the dark at 20 °C and were subcultured every 21 days. The percentage of callus induction and callus fresh weights were recorded 6 weeks after culture.

Cell suspension culture was initiated by transferring friable callus (80–100 mg) to a 50-mL Erlenmeyer flask containing 15 mL of liquid MS medium supplemented with 1 mg/L 2,4-D, 0.1 mg/L kinetin, and 15 mg/L ascorbic acid. The suspension cultures were incubated on a rotary shaker at 120 rpm and 25 ± 2 °C under 16-h photoperiods with a light intensity of 400–500 lx. Every 10 days 5 mL of fresh medium was added to the culture. The suspension cultures were established after 3 subcultures. Then the grown cells were transferred to 250-mL flasks containing 40 mL of liquid MS medium supplemented with 1 mg/L 2,4-D, 0.1 mg/L kinetin, and 15 mg/L ascorbic acid, and were subcultured every 14 days with an initial cell density of 5.7×10^4 cells per mL.

In order to determine the optimal medium for *P. bracteatum* cell suspension growth, the established suspension cells were transferred to a 250-mL Erlenmeyer flask containing 50 mL of liquid MS medium supplemented with different levels of 2,4-D or NAA (0.0, 0.5, 1, and 2 mg/L) and kinetin or BAP (0.0, 0.1, 0.5, and 1 mg/L) with 5.7×10^4 cells per mL initial cell density. The experimental design was a factorial based on completely randomized design with 2 replications.

2.3. Growth measurement

Cell growth parameters such as settled cell volume (SCV), packed cell volume (PCV), and cell number were measured every 2 days until constant growth was achieved. Fresh cell weight (FCW) and dry cell weight (DCW) were determined at the stationary phase of growth (at the end of the experiment).

For determination of SCV, the cells were transferred to 50-mL flacons, allowed to settle for 30 min, and then the fraction of the whole suspension volume occupied by the cells was determined as the settled cell volume (SCV). The packed cell volume (PCV) was determined by centrifuging 10 mL of the suspension culture in a 15-mL graduated centrifuge tube at 2000 rpm for 5 min.

Then 1 mL of the cell suspension was added to 2 mL of 8% chromium trioxide (CrO_3) solution and incubated at 70 °C for 15 min. The mixture was shaken vigorously for 15 min and the cell count was then determined under a microscope using a hemocytometer slide.

The cells were collected by filtration through a

Whatman No. 1 filter paper under vacuum, washed with 3 mL of distilled water, and dehydrated under vacuum. The filtered cells were transferred to pre-weighed aluminum foil and the fresh cell weight (FCW) was measured. The cells were then dried in a drying oven at 60 °C for 12 h and dry cell weight (DCW) was determined.

The doubling time (dt) was calculated according to the following equation (Godoy-Hernández and Vázquez-Flota, 2006):

$$dt = \frac{\ln 2}{\mu} \quad \mu = \frac{\ln X_t - \ln X_0}{t}$$

where x_0 is the initial cell density, x_t is the cell density at time t , μ is the specific growth rate, and dt is the doubling time.

The media pH and EC were directly measured using a pH and EC meter (Mi 180 Bench Meter; Martini Instrument, USA) at the end of the experiment.

2.4. Sample preparation and HPLC analysis of thebaine

The *P. bracteatum* cells subcultured to liquid MS medium supplemented with 1 mg/L 2,4-D, 0.1 mg/L kinetin, and 15 mg/L ascorbic acid at a 3.3×10^5 cells per mL initial cell density. The cell biomass of the suspension culture was harvested 12 and 16 days after culture initiation and freeze-dried. Alkaloids of 1.5–2 g dried cells were extracted by the acidic methanol method (Tisserat and Berhow, 2009) with few modifications. Eight milliliters of extraction solution consisting of 40% methanol, 59.5% water, and 0.5% acetic acid was added to samples, followed by sonication for 12 min. Sonicated cells were allowed to stand at room temperature for 12 min and then passed through a 0.22- μm filter for HPLC analysis. HPLC analysis was performed on an Agilent HPLC system (1200 Series Diode Array Detector). Sample injections of 25 μL were made in a C-18 reverse-phase Agilent column (Eclipse XDB-C18, 5 μm , 4.6×150 mm). The mobile phase for alkaloid elution was 5% methanol and 95% aqueous ammonium acetate (0.2% ammonium acetate, pH 4.6). The effluent was monitored at 280 nm. Thebaine standard curve was used to determine the thebaine concentration of samples as $\mu\text{g/g}$ dry weight. The standard of thebaine was obtained from Temad Chemical Company (Tehran, Iran).

3. Results and discussion

3.1. Callus induction

Callus initiation was observed on the shoot apex and the cut edges of the hypocotyl–cotyledon explants within 2–3 weeks in some treatments (Figures 1A and B). Percentage of callus induction and fresh weight of callus were significantly ($P < 0.01$) affected by plant growth regulator treatments. The maximum percentage of callus induction

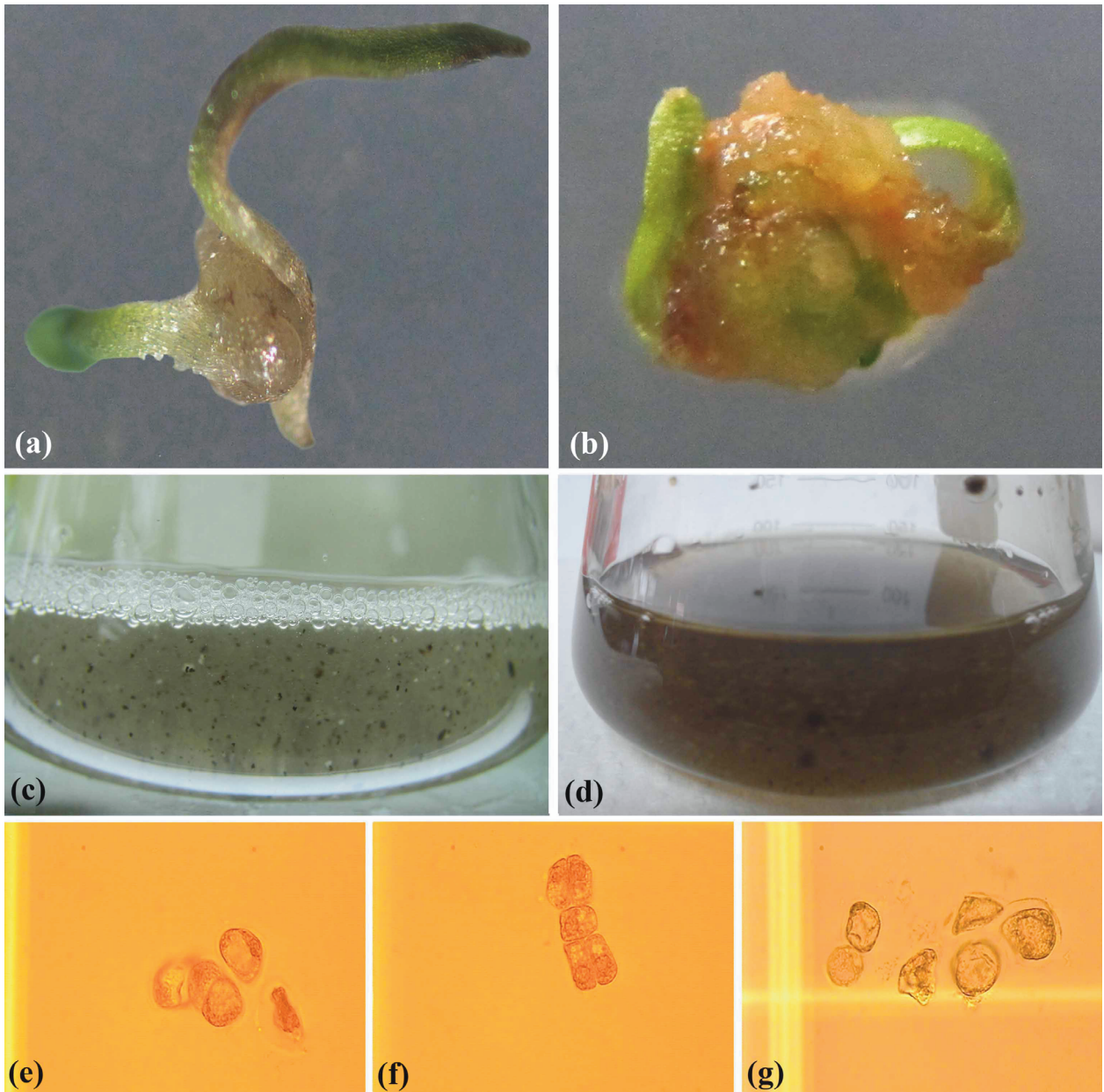


Figure 1. Callus induction and establishment of *P. bracteatum* cell suspension culture. (A and B) Callus from hypocotyl–cotyledon explant after 1 and 6 weeks of culture, respectively; (C) *P. bracteatum* cell suspension culture in early stage of growth; (D) discoloration of the suspension culture medium with increase of cell density and possible production of secondary compounds; (E, F, and G) photomicrography of *P. bracteatum* cell suspension culture (40 \times).

(86.67%) and fresh weight of callus were obtained in MS medium with 1 or 2 mg/L 2,4-D, 0.1 or 0.2 mg/L kinetin, and 15 mg/L ascorbic acid (Table 1). Ilahi and Ghauri (1994) reported that callus was induced from *P. bracteatum* seedlings cultured on MS medium supplemented with 1 mg/L NAA and 0.5 mg/L BA. The explants cultured on plant growth regulator-free MS medium and MS medium containing 3 g/L activated charcoal did not produce calli (Table 1). This may be due to the adsorption of plant growth

regulators (2,4-D and kinetin) in medium by activated charcoal (Pan and Van Staden, 1998; Toering and Pullman, 2005; Van Winkle and Pullman, 2005). The percentage of callus induction in MS medium supplemented with 1 mg/L 2,4-D, 0.1 mg/L kinetin, and with or without ascorbic acid was not significantly different. However, most of the calli that formed on ascorbic acid-free medium were brown and eventually died after 3–4 weeks. Therefore, the addition of 15 mg/L ascorbic acid to the medium was

Table 1. Effect of different plant growth regulator combinations on percentage of callus induction and fresh weight of callus in *P. bracteatum*.

Treatment	Explants forming callus (%)	Callus browning (%)	Fresh weight of callus (mg)
MS	00.00 ^d	00.00	00.00 ^d
MS + 0.5 mg/L BA + 15 mg/L AA	8.33 ^{cd}	00.00	6.50 ^{cd}
MS + 1 mg/L BA + 15 mg/L AA	8.33 ^{cd}	00.00	9.50 ^{bcd}
MS + 1 mg/L 2,4-D + 15 mg/L AA	75.00 ^{ab}	00.00	24.00 ^a
MS + 1 mg/L 2,4-D + 0.5 mg/L BA + 15 mg/L AA	78.57 ^{ab}	00.00	22.75 ^a
MS + 1 mg/L 2,4-D + 1 mg/L BA + 15 mg/L AA	30.00 ^c	00.00	19.32 ^{ab}
MS + 1 mg/L 2,4-D + 0.1 mg/L Kin + 15 mg/L AA	86.67 ^a	16.67	28.73 ^a
MS + 2 mg/L 2,4-D + 0.2 mg/L Kin + 15 mg/L AA	86.67 ^a	00.00	24.67 ^a
MS + 1 mg/L NAA + 0.5 mg/L BA + 15 mg/L AA	66.67 ^{ab}	00.00	17.78 ^{bc}
MS + 1 mg/L 2,4-D + 0.1 mg/L Kin	73.81 ^{ab}	71.67	20.29 ^{ab}
MS + 1 mg/L 2,4-D + 0.1 mg/L Kin + 3 mg/L AC	00.00 ^d	00.00	00.00 ^d
MS + 2 mg/L 2,4-D + 0.2 mg/L Kin + 3 mg/L AC	00.00 ^d	00.00	00.00 ^d
MS + 1 mg/L NAA + 0.1 mg/L Kin + 45 mg/L AA	77.78 ^{ab}	00.00	ND*
1/2MS + 1 mg/L NAA + 0.1 mg/L Kin + 45 mg/L AA	55.18 ^b	00.00	ND

Each value is the mean of 3 replicates. Values followed by different letters in each column are significantly different at $P \leq 0.05$. * Not determined; Kin: Kinetin; AA: Ascorbic acid; AC: Activated charcoal.

effective in reducing browning of explants and calli of *P. bracteatum*. Similar behavior has been previously reported in *P. bracteatum* (Rostampour et al., 2010).

The frequency of explants producing calli in the MS medium supplemented with 1 mg/L 2,4-D alone or in combination with 0.5 mg/L BAP was significantly higher than that in media containing 0.5 or 1 mg/L BAP alone (Table 1). Moreover, an increase in the concentration of BAP from 0.5 to 1 mg/L caused a significant decrease in the percentage of callus induction (Table 1). Therefore, it could be concluded that callus induction from hypocotyl-cotyledon explants of *P. bracteatum* was mainly influenced by the exogenous auxin. Auxins and cytokinins are most important for regulating growth and morphogenesis in plant tissue and organ cultures (Machakova et al., 2008). Several reports indicated that auxin acts as a very important factor in callus induction, and cytokinins facilitate its effects (Jayanthi and Mandal, 2001; Rostampour et al., 2010).

3.2. Cell suspension culture

P. bracteatum cell suspension cultures were established and culture media turned brownish with increasing cell density (Figures 1C and D). In the suspension culture,

there were many aggregates of 60–100 cells. Microscopic analysis of the cells from suspension cultures showed that *P. bracteatum* cells were globular (Figures 1E, F, and G). The results of analysis of variance indicated that growth of *P. bracteatum* cell suspension cultures as measured by cell density (number of cells per milliliter), SCV, PCV, fresh and dry weight of cells, and doubling time of cells were significantly ($P < 0.01$) influenced by type and concentration of auxin and its interaction with cytokinins. However, the effect of cytokinins on these characters was not statistically significant.

Among the auxins, 1 mg/L NAA showed a good stimulatory effect on cell growth with a cell density of 9.92×10^6 cells per mL, 17.26% SCV, 16.50% PCV, 72.61 g/L fresh weight, and 6.41 g/L dry weight of cells, which were significantly higher than those of hormone-free MS medium and media containing 0.5, 1, and 2 mg/L 2,4-D, or 0.5 mg/L NAA. On the other hand, the calculated doubling time (dt) for cell suspension cultures in MS medium supplemented with 0.5 mg/L 2,4-D was 12.03 days while MS medium supplemented with 1 or 2 mg/L NAA exhibited the minimum amount of dt (6.5 days) (Figure 2). Therefore, it could be concluded that NAA

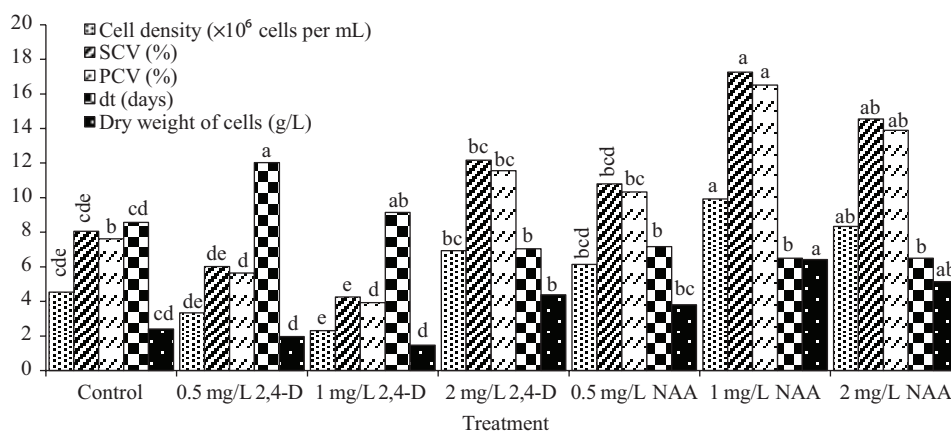


Figure 2. Effect of different types and concentrations of auxins on growth of cells in suspension culture of *P. bracteatum*; each value is the mean of 2 replicates. Values followed by different letters in each trait are significantly different at $P \leq 0.05$.

was more effective than 2,4-D for growth of *P. bracteatum* cell suspension cultures. Growth of *P. bracteatum* cell suspension cultures also varied significantly depending on the plant growth regulators and their combinations (Table 2). The maximum cell density (16.7×10^6 cells per mL), SCV (28.89%), PCV (27.69%), and fresh and dry weight of cells (118.5 g/L and 10.44 g/L, respectively) were recorded for MS medium supplemented with 1 mg/L NAA, 1 mg/L BAP, and 15 mg/L ascorbic acid. As shown in Table 2, at lower concentration of 2,4-D (0.5 mg/L), its combination with 0.5 mg/L kinetin resulted in better cell suspension growth responses compared to its combination with BAP and other levels of kinetin. On the other hand, growth of *P. bracteatum* cell suspension cultures in medium containing a moderate concentration (1 mg/L) of NAA in combination with BAP or kinetin was similar. However, in the presence of 2 mg/L 2,4-D or NAA, different levels of BAP were more favorable for cell suspension cultures growth than kinetin used at the same concentration (Table 2). Kamimura and Akutsu (1976) reported that the effect of 2,4-D on the growth of *P. bracteatum* cell suspension culture was greater than that of NAA. These differences can be attributed to differences in the genetic background of the plant materials used, which may strongly influence the in vitro response of plant cells (Wan et al., 1988; Venkatachalam and Jiayabala, 1997).

Hossain et al. (2007) reported that MS medium supplemented with 2 mg/L NAA, 0.05 mg/L BAP, and MS medium containing 2 mg/L 2,4-D and 0.05 mg/L BAP showed the best growth of suspension culture of *Solanum melongera* L. Daud and Keng (2006) also found that MS medium with 2 mg/L NAA was the most efficient medium in suspension culture of *Cyperus aromaticus*.

Contrast analysis indicated that there was a statistically significant increase in cell proliferation in treatments

containing both auxins and cytokinins compared to those containing auxins or cytokinins alone. Auxins and cytokinins are necessary for cell division at the G1-S and G2-M transitions in cultured plant cells and in planta. Auxin stimulates the acidification of the cell wall resulting in increasing extensibility, and also induces the transcription of specific mRNAs that code for proteins associated with cellular growth. Cytokinin acts directly on the cell cycle by regulating synthesis of proteins involved in the formation and operation of the mitotic spindle (Stals and Inze, 2001; Richard et al., 2002; Silveria et al., 2004). Furthermore, cytokinins may also affect the auxin-regulating systems of gymnosperms (O'Dowd et al., 1993; Wickremesinha and Arteca, 1993).

The growth curve of *P. bracteatum* cell suspension cultures in the MS medium supplemented with 1 mg/L NAA, 1 mg/L BAP, and 15 mg/L ascorbic acid and 3.3×10^5 cells per mL initial cell density is shown in Figure 3. The cells showed an initial lag phase of 2 days, a period of cellular adaptation to the new medium. Then the cells entered the growth phase, which continued up to day 14. After 14 days, the rate of cell growth decreased and cells entered the stationary growth phase. Embryogenic cell suspension culture of *Pinus taeda* in medium supplemented with 2 μ M 2,4-D, 0.5 μ M BAP, and 0.5 μ M kinetin showed an increase in SCV after 20 days of culture without subculture (Silveria et al., 2004). Falco et al. (1996) reported that in suspension culture of sugarcane, PCV and fresh and dry weight of cells increased until 24–32 days.

Correlation analysis between growth indices (cell density, SCV, PCV, fresh and dry weight of cells) with pH and EC (electrical conductivity) of culture medium showed that the pH of culture medium has a positive correlation but EC of culture medium has a negative correlation with growth indices. Therefore, with cell growth and increasing

Table 2. Average of indices measured in suspension culture of *P. bracteatum* affected by different levels of plant growth regulators.

Plant growth regulators				Number of cells per mL ($\times 10^6$)	SCV (%)	PCV (%)	FCW (g/L)	DCW (g/L)	dt (days)
2,4-D	NAA	Kinetin	BAP						
0.5	-	0.1	-	1.77	3.35	2.96	16.28	1.47	8.40
0.5	-	0.5	-	12.1	21.09	20.19	74.30	6.56	5.39
0.5	-	1	-	2.67	5.00	4.60	20.55	1.84	7.45
0.5	-	-	0.1	2.30	4.25	3.94	14.43	1.31	9.69
0.5	-	-	0.5	0.77	1.65	1.49	6.86	0.64	17.95
0.5	-	-	1	2.15	4.00	3.71	7.94	0.74	10.13
1	-	0.1	-	2.11	3.92	3.65	16.15	1.46	7.98
1	-	0.5	-	2.74	5.00	4.60	19.58	1.76	7.44
1	-	1	-	1.68	3.20	2.90	15.45	1.40	8.51
1	-	-	0.1	1.86	3.50	3.29	12.78	1.16	13.40
1	-	-	0.5	2.80	5.10	4.74	15.49	1.40	9.10
1	-	-	1	1.98	3.70	3.42	11.55	1.06	10.21
2	-	0.1	-	4.91	8.70	8.13	35.72	3.18	7.73
2	-	0.5	-	5.66	9.75	9.12	30.03	2.68	7.49
2	-	1	-	5.08	9.00	8.42	44.83	3.97	7.68
2	-	-	0.1	12.3	21.31	20.40	78.65	6.94	5.50
2	-	-	0.5	9.15	15.95	15.29	72.71	6.42	6.05
2	-	-	1	10.20	17.79	17.02	72.19	6.37	5.71
-	0.5	0.1	-	5.56	9.82	9.66	38.08	3.38	7.69
-	0.5	0.5	-	2.86	5.21	4.92	20.45	1.84	8.21
-	0.5	1	-	4.41	7.65	7.27	27.14	2.42	6.78
-	0.5	-	0.1	8.13	14.21	13.57	55.91	4.95	6.45
-	0.5	-	0.5	1.33	2.60	4.42	10.71	0.98	9.50
-	0.5	-	1	9.21	16.05	15.34	71.11	6.28	5.91
-	1	0.1	-	11.80	20.52	19.64	99.54	8.77	5.55
-	1	0.5	-	11.60	20.17	19.30	82.78	7.30	5.57
-	1	1	-	5.89	10.38	9.90	36.66	3.26	7.80
-	1	-	0.1	11.7	20.29	19.42	83.25	7.34	5.88
-	1	-	0.5	8.94	15.59	14.91	73.71	6.51	6.08
-	1	-	1	16.7	28.89	27.69	118.50	10.44	5.25
-	2	0.1	-	6.42	11.28	10.76	49.03	4.34	6.34
-	2	0.5	-	6.62	11.63	11.09	47.73	4.23	7.47
-	2	1	-	8.10	14.16	13.53	53.61	4.71	6.53
-	2	-	0.1	9.71	16.90	16.16	69.37	6.13	5.76
-	2	-	0.5	12.30	21.41	20.50	84.79	7.48	5.50
-	2	-	1	13.00	22.54	21.58	86.13	7.60	4.45
LSD ($\alpha = 0.05$)				7.15	12.03	11.77	50.59	4.11	7.75

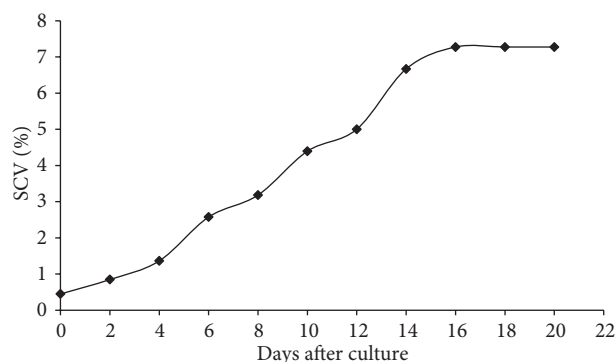


Figure 3. Batch suspension cultures of *P. bracteatum* grown in the MS medium supplemented with 1 mg/L NAA, 1 mg/L BAP, and 15 mg/L ascorbic acid.

of cell density, the pH of culture medium increases and EC of culture medium decreases. Electrical conductivity of culture medium decreases inversely to biomass gain. This is a consequence of ion uptake by cells (Ryu et al., 1990; Godoy-Hernández and Vázquez-Flota, 2006).

3.3. Thebaine content of cells

The extracted thebaine from cell suspension cultures was detected using the HPLC method with a C-18 column. Figure 4 shows the chemical structure of thebaine and HPLC spectrum of thebaine in samples obtained from cell suspension cultures. The thebaine yield of *P. bracteatum* cell suspension on day 12 after culture was 4.67 µg/g dry weight (equivalent to 0.85 mg/L), which was significantly lower than that on day 16 (10.76 µg/g dry weight, equivalent to 1.90 mg/L). The biomass yield of suspension cultures on days 12 and 16 were 43.33 g/L and 30.10 g/L, respectively. The thebaine content of *P. bracteatum* cell suspension culture reported here was little more than that previously reported by Kamimura et al. (1976) and Cline and Coscia (1988). It is known that both biotic and abiotic stresses including elicitation, varying plant growth regulators, and mineral nutrient concentrations can enhance the secondary metabolite production in plants or plant cell cultures (Verpoorte et al., 2002; Oksman-Caldentey and Inze, 2004; Gadzovska et al., 2007). Furthermore, secondary metabolite production can also be influenced

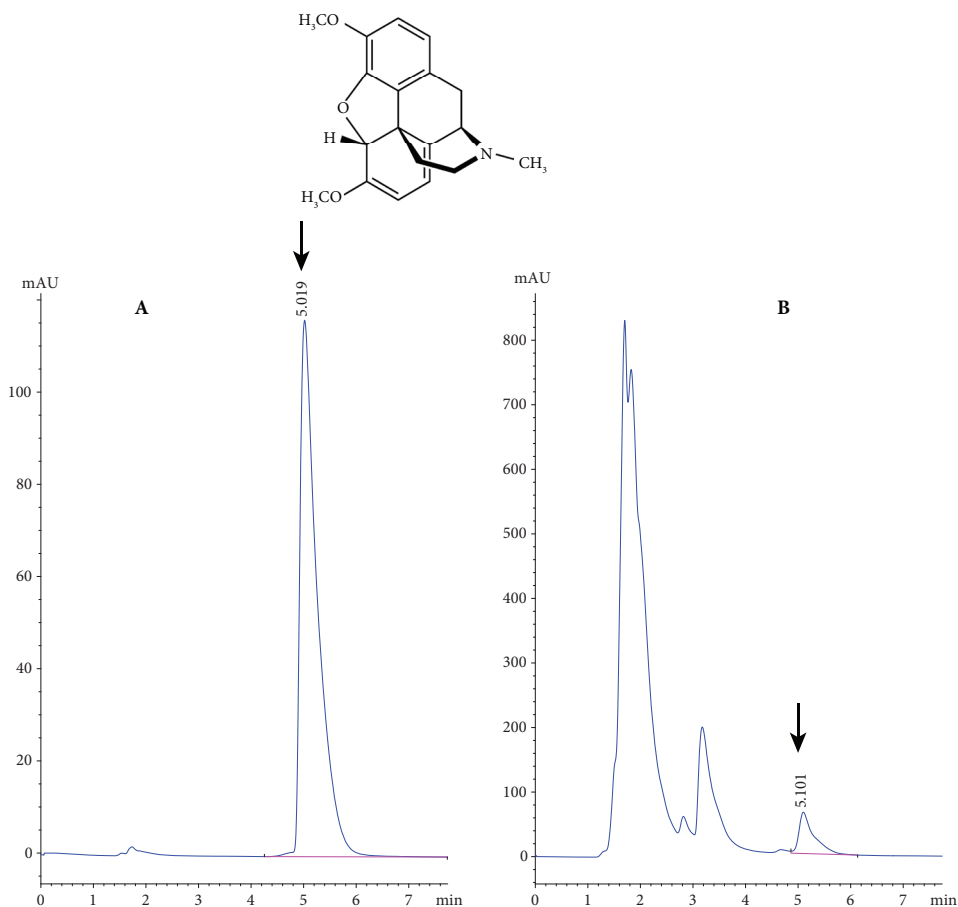


Figure 4. HPLC spectrum of standard thebaine (A), and thebaine purified from *P. bracteatum* cell culture (B). AU and arrow represent arbitrary unit and thebaine peak, respectively.

by environmental factors such as light and temperature (Bais et al., 2002; Ramachandra and Ravishankar, 2002).

In conclusion, we here describe a reliable protocol for callus induction from hypocotyl–cotyledon explants and fast-growing cell suspension cultures of *P. bracteatum*. MS

medium supplemented with 1 mg/L NAA, 1 mg/L BAP, and 15 mg/L ascorbic acid showed the maximum cell growth and proliferation, and therefore can be used to elicit the production of thebaine or other secondary metabolites, and to scale-up for mass production in bioreactors.

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