

## Cell Suspension and *In Vitro* Production of Colchicine in Wild *Colchicum Hierosolymitanum* Feib

Nidal Q. Daradkeh<sup>1</sup>, Rida A. Shibli<sup>2,\*</sup>, Ibrahim M. Makhadmeh<sup>3</sup>, Feras Alali<sup>4</sup> and Tamara S. Al-Qudah<sup>5</sup>

<sup>1</sup>National Center for Agriculture Research and Extension, Baqa'a, Jordan

<sup>2</sup>Department of Horticulture and Agronomy, Faculty of Agriculture, The University of Jordan, Amman, Jordan

<sup>3</sup>Department of Plant Production, Faculty of Agriculture, Jordan University of Science and Technology

<sup>4</sup>Faculty of Pharmacy, Jordan University of Science and Technology

<sup>5</sup>Hamdi Mango Center for Scientific Research (HMCSR), The University of Jordan, Amman, Jordan

**Abstract:** Callus was induced from seeds of *Colchicum hierosolymitanum* Feib inoculated on the surface of MS media supplemented with 0.45  $\mu\text{M}$  2, 4-dichlorophenoxyacetic acid under dark conditions. Then callus was cultured on MS media supplemented with 4.52  $\mu\text{M}$  2, 4-dichlorophenoxyacetic for growth and maintenance. Friable callus from the fourth generation was transferred to liquid MS media supplemented with 0.54  $\mu\text{M}$  1-naphthaleneacetic acid to form cell suspension. Cells were successfully subcultured every 27 days on the same liquid media supplemented with 0.54  $\mu\text{M}$  1-naphthaleneacetic acid. Higher concentration (9  $\mu\text{M}$ ) of 6-benzyladenine with 0.45  $\mu\text{M}$  2, 4-dichlorophenoxyacetic acid resulted in higher cells fresh weight, while 1-naphthaleneacetic acid combinations with 6-benzyladenine had no effect on cell growth. On the other hand, the time for subculturing the cells into new fresh liquid media was determined to be after 27 days of incubation. (-) -Colchicine was identified in callus and cell suspension of *C. hierosolymitanum* by performing HPLC analysis against standard. Different ratios of  $\text{NH}_4^+ : \text{NO}_3^-$  were used to study their effect on (-)-colchicine content, the highest colchicine content of 0.070  $\text{mg g}^{-1}\text{DW}$  was obtained at 30  $\text{mM NH}_4^+$  of total nitrogen. Colchicine alkaloid was highest, 0.090  $\text{mg g}^{-1}\text{DW}$ , at 0.1 M of sucrose after 4 weeks incubation. (-) - Colchicine alkaloid was not detected in callus grown on sucrose free media. Maximum production of colchicine, 0.235  $\text{mg g}^{-1}\text{DW}$ , was obtained in callus extracts of 60 days old callus grown under dark conditions. Cell suspension had 0.012  $\text{mg g}^{-1}\text{DW}$  (-) -colchicine from suspended cells grown under dark. (-) -Colchicine content of callus incubated under dark (0.095  $\text{mg g}^{-1}\text{DW}$ ) was higher than light (0.070  $\text{mg g}^{-1}\text{DW}$ ) condition.

**Keywords:** Callus, Cell suspension, (-)-Colchicine, HPLC analysis.

### INTRODUCTION

The genus *Colchicum* belongs to the colchicaceae family. It is mainly distributed in the Mediterranean region extending as far as the mountains of Pakistan and Middle Asia [1]. The medicinal value of colchicum is due to the presence of (-)-colchicine, the main alkaloid, which was isolated from all species of colchicum. It is widely used in breeding studies and as drug to treat gout [2-4]. *Colchicum hierosolymitanum* Feib is a species found in Jordan and it is a slow propagated species because it has low germination rate [5]. It faces many threats to be extinct totally from the wild. So the use of tissue culture techniques is appropriate alternative for rapid multiplication and sustainable growth of slow propagation species like colchicum [6, 7]. Callus and suspension cell cultures are of the most valuable commercial

sources for the synthesis of medicinal substances [8]. In addition, the production of secondary metabolites by cell cultures is considered a promising alternative to intact plant [9].

(-)-Colchicine, is a phenylethyl isoquinoline derived alkaloid, and it is a poisonous, lipid-soluble alkaloid with a unique 7-membered aromatic tropolone ring [10-12]. It has a venerable history and clinically used treatment of gout [13]. More recently, (-)-colchicine has been used to treat Familial Mediterranean Fever, inflammatory dermatomes, sweet's syndrome, scleroderma and Behcet's disease [14, 15].

Several studies in the literature were reported to study (-)-colchicine content in pharmaceutical preparations, biological fluids and plant extracts [5, 16-19]. Production of colchicine alkaloid by plant tissue cultures was first investigated in suspension cultures of *Colchicum autumnale* [20, 21]. There are some studies on the *in vitro* production of colchicines from colchicum species like *Gloriosa superba*, in which colchicine content was enhanced and increased in root cultures by treatment with *p*-coumaric acid and tyramine [22]. But there are no reports on the *in vitro* study of

\*Address correspondence to this author at the Department of Horticulture and Agronomy, Faculty of Agriculture, The University of Jordan, Amman, Jordan; Tel: 962 6 535 5000; Fax: 962 6 535 5577; E-mail: r.shibli@ju.edu.jo

colchicine in *Colchicum hierosolymitanum* Feib. This study was carried out to develop a protocol for cell suspension and production of (-)-colchicine alkaloid from both callus and cell suspension cultures in *Colchicum*.

## MATERIALS AND METHODS

**Callus induction and culture.** Seeds of *Colchicum hierosolymitanum* were collected from wild grown plants in north Jordan (Erhaba, Irbid) located between 32°22'N and 35°49'E. The collected material was identified by the Genetic Resources Unit at the National Center for Agriculture Research and Extension (NCARE), Baqa, Jordan. Seeds were thoroughly washed under running tap water for 20 minutes with few drops of mild detergent. Then antiseptic surface sterilized by solution of 4.0% sodium hypochlorite plus two drops of Tween-20 for 15 minutes (under the laminar – air flow cabinet), and were then rinsed with autoclaved distilled water for three times (5 min. each). Seeds were transferred into 70% (V/V) ethanol solution for 30 seconds and then rinsed with sterile distilled water 3 times (5 min. each). In all experiments, MS media (Murashige and Skoog, 1962) [23] with 30g/L were used and pH was adjusted to 5.8 using NaOH or HCl (1.0 N each). Eventually, 8.0 g/L Difco-Bacto agar was added to the solid media.

Surface sterilized seeds were inoculated into 15x150 mm test tube, one seed in each test tube containing 10 ml of MS media (Murashige and Skoog 1962), [23] supplemented with 0.45  $\mu\text{M}$  2, 4-dichlorophenoxyacetic (2,4-D) for callus induction, then the callus was transferred to the surface of MS medium containing 4.52  $\mu\text{M}$  2,4-D for further callus growth and incubated at 24 $\pm$ 2 °C in dark inside the growth room chamber which has a standard conditions of light and temperature. Calli (Approx. 500 mg) were subculture on hormone free fresh MS media in dark to detect the effect of subculture time on callus growth. Callus color, texture and diameters were recorded every week through 60 days. Each treatment consisted of 5 completely randomized replicates (each replicate consists of 15x150 mm test tube with 10 ml of MS media and has approx. 500 mg of calli) for callus induction experiment.

**Cell Suspension Culture.** Cell suspension cultures were initiated by inoculating approximately 1.0 grams of fresh friable callus into 50 ml of MS liquid media over a rotary shaker at 100 rpm. Media for suspension culture was supplemented with (0, 0.45  $\mu\text{M}$ ) 2, 4-D or (0.0, 0.54  $\mu\text{M}$ ) 1-naphthaleneacetic acid (NAA) in combination with different levels (0.0, 2, 22, 3.11, 9  $\mu\text{M}$ ) of 6-benzyladenine (BA). Cultures were maintained in 250 ml Erlenmeyer flasks and were incubated on rotary shaker (100 rpm) under dark condition at 24 $\pm$ 2 °C inside the growth room chamber. Cells were transferred into liquid MS media supplemented with (0.54  $\mu\text{M}$ ) NAA to detect the effect of subculture time on cell growth. Data were reported every three days on cells fresh weight (mg).

**Determination of (-)-colchicine content in *Colchicum hierosolymitanum* species.** Calli and cell suspension were collected from test tubes and then were dried in shade at room temperature for one week. Next, they were ground using a mortar and pestle and resulted in a dry powder weighed approx. 0.5 g (from 5 samples) which was placed in

50 ml Erlenmeyer flasks. Petroleum ether (10 ml  $\times$ 2) was added with frequent shaking for 1 hour, followed each time by filtration using filter paper 0.45  $\mu\text{l}$ . Samples (solid residues) were dried under vacuum and then extracted with 10 ml HPLC grade dichloromethane with frequent shaking for 45 min. Ammonium hydroxide (12.5%), 0.5 ml was added, followed by vigorous shaking for 15 min, and then the mixture was left for 30 min. Calli residues were filtered and the filtrates were saved, calli residues were washed twice with 10 ml HPLC grade dichloromethane and then filtered. The collected filtrates and washes were combined. Organic phase was evaporated to dryness under vacuum at room temperature and then dissolved in 10 ml of 70% ethanol. The solution was then filtered using 0.45  $\mu\text{l}$  Teflon filters; 2 ml and transferred into 2 ml amber HPLC vials. A portion, 50  $\mu\text{l}$  was injected into HPLC. Lachrom<sup>®</sup> MERCK-HITACHI HPLC equipped with quaternary gradient L-7150 pump connected to manual injector, L-7455 Diode-Array Detector, and Hibar<sup>®</sup> MeRCK, Pre-Packed column RT 250 $\times$ 25, Lichrosorb<sup>®</sup> RP-18. The mobile phase system was an isocratic blend of acetonitrile-dionized water (25:72) with 1 ml tri-ethyleamine, 1 ml phosphoric acid. A UV monitor operated at 350 nm was used as detector. The total run time was 10 min. All samples were analyzed against an external standard constructed calibration points and quality control (QC) samples.

**Effect of nitrogen sources and concentration on callus growth and colchicine formation.** This experiment was conducted to detect the effect of nitrogen source on alkaloid formation by using HPLC method. Calli (500 mg) were subcultured on MS media with different  $\text{NH}_4^+:\text{NO}_3^-$  ratios at different concentrations. Ratios (30 mM  $\text{NO}_3^-$ , 10:20, 20:10 and 30 mM  $\text{NH}_4^+$ ) were used at 30 mM nitrogen, whereas ratios of (60 mM  $\text{NO}_3^-$ , 20: 40, 40: 20, and 60 mM  $\text{NH}_4^+$ ), were studied at 60 mM nitrogen. Control treatment was included without any exogenous nitrogen application. Callus was subcultured to media and each treatment consisted of 20 replicate in completely randomized design. Fresh and dry weights and colchicine contents were reported after 4 weeks of inoculation.

**Effect of carbohydrate source and concentration on alkaloid Production.** This experiment was conducted to detect the effect of sucrose at different concentrations 0.0, 0.1, 0.2, 0.3 M on alkaloid production. Callus was subcultured to the MS media supplemented with different concentrations and data collected every other week for colchicine content using high performance liquid chromatography (HPLC) [18].

**Effect of time of subculture on the colchicine production in callus and cell suspension.** Callus was cultured on solid MS media supplemented with 0.54 $\mu\text{M}$  NAA, callus growth was monitored over 90 days of incubation. Data were reported for fresh and dry weight and colchicine contents every 5 days for one month, then for two other months at 45, 60, 90 days. Cell suspension was cultured on liquid media supplemented with 0.54 $\mu\text{M}$  NAA. Data were reported for influence of growth time on fresh weight and dry weight of cell growth and colchicine content in *C. hierosolymitanum* during four weeks of incubation.

**Influence of light/dark incubation of callus on colchicine content.** Callus was cultured on solid MS media

**Table 1. Influence of Different levels of 2, 4-D in Combination with Different Levels of BA on *Colchicum Hierosolymitanum* Cell Growth after 30 Days of Incubation in Liquid Media**

PPGRs ( $\mu\text{M}$ ) 2,4-D: BA	Fresh Weight (g)	Color
0.00:0.00	2.13 bc*	89% brownish
00:2.22	2.34 bc	100% black
00:3.11	2.35 abc	89% brownish
00:9.00	1.92 c	100% black
0.45:00	2.50 abc	100% yellowish
0.45:2.22	2.74 ab	90% brownish
0.45:3.11	2.48 abc	90% brownish
0.45:9.00	3.04 a	80% brownish
LSD	0.695	

\*Means having different letters in each column are significantly different according to the least significant different (LSD  $\leq$  0.05).

supplemented with  $0.54\mu\text{M}$  NAA in the light and dark conditions, Data were reported for fresh and dry weight and colchicine contents in both light and dark conditions.

*Experimental Design and Statistical Analysis.* Each experiment in this study was set up as a completely randomized design (CRD) with 5 replications. Collected data were statistically analyzed using Genstat software (Rothamsted Experimental station, 2002). Means were separated according to the least significant difference (LSD) at 0.05 level of probability.

## RESULTS AND DISCUSSION

*Cell Suspension.* Calli clumps from the fourth generation grown on  $0.45\mu\text{M}$  2, 4-D provided good cell source to initiate cell suspension culture. Lee *et al.* [24] who worked on *Oryza sativa* L. used friable callus transferred to liquid medium supplemented with the same PGRs to form cell suspension culture. Higher concentration  $9\mu\text{M}$  of BA with  $0.45\mu\text{M}$  2,4-D resulted in higher cells fresh weight (Table 1). After two weeks of incubation large cell clumps forming callus grown in all treatments of 2,4-D and BA was hard and brownish in color; except  $0.45\mu\text{M}$  2,4-D alone treatment (Table 1). Sakhanokho *et al.* [25] found variation in callus color in liquid media culture from yellowish-green to black in *Gossypium barbadense* L. Lee *et al.* [24] showed that the best responses in growth of cells, no browning and aggregation of cells during suspension periods of culture depend on plant cultivar.

On the other hand, control and  $0.45\mu\text{M}$  NAA combinations with BA had no effect on cell growth (Table 2). Despite the fact that no shoot formation developed in all treatments of NAA and BA, embryogenesis occurred on PGR-free media and on  $2.22\mu\text{M}$  BA supplemented media. (Table 2). Phenolic browning appeared after two weeks of incubation except in control treatment (Table 2). *Hyoscyamus muticus* gave the highest incidence of biomass accumulation of cell suspension and alkaloid production at combination ratios between  $5.4\mu\text{M}$  BA and  $4.4\mu\text{M}$  NAA [26].

*Time of Subculturing for callus and cell suspension culture.* Callus width, length height increased with time until

**Table 2. Influence of Different Levels of NAA in Combination with Different Levels of BA on *Colchicum Hierosolymitanum* Cell Growth after 30 Days of Incubation in Liquid Media**

PGRs ( $\mu\text{M}$ ) NAA: BA	Fresh Weight (g)	Color
0.00:0.00	1.50 b*	100% yellowish
00:2.22	1.15 bc	78% brownish
00:3.11	1.04 bcd	100% black
00:9.00	0.79 cd	80% brownish
0.54:00	1.21 bc	76% brownish
0.54:2.22	1.52 b	76% brownish
0.54:3.11	0.51 d	90% brownish
0.54:9.00	3.91 a	78% brownish
LSD	0.567	

\*Means having different letters in each column are significantly different according to the least significant different (LSD  $\leq$  0.05).

reach the peak at 60 days period (Table 3). However, callus width and length had been declined after 90 days in culture (Table 3). This indicates that many factors influence on callus growth, such as depletion of essential nutrients, water loss from the medium and accumulation of toxic substances. Therefore, the healthy *C. hierosolymitanum* calli should be transferred to fresh medium after 6-8 weeks to maintain the viability and healthy growth. Gopi and Vatsala [8], who worked on *Gynmema sylvestre*, reported maximum biomass yield during 21<sup>st</sup> to 27<sup>th</sup> day on solid media.

Cells cultures were demonstrated on liquid media by recording cell fresh weight every three days. Results showed that the cells had lag phase of 9 days followed by an exponential growth phase until they reached the maximum growth (Table 4). In addition, the time for subculturing the cells into new fresh liquid media was determined to be after 27 days of incubation. Growth was declined because the cells entered a stationary phase. Cell growth declined after 27 days (Table 4) and this could be attributed to the depletion of nutrients, accumulation of toxic products and growth inhibitors [27]. Gopi and Vatsala [8] reported that maximum biomass yield on liquid media after 18<sup>th</sup> to 24<sup>th</sup> day of incubation in *Gynmema sylvestre*.

Identification of (-)-colchicine alkaloid. Effect of nitrogen sources and concentration on callus growth and alkaloid formation. The optimal nitrogen concentration in the medium was required for both callus growth and colchicine formation (Table 5, Fig 1). These results showed that low colchicine content of  $0.002\text{ mg g}^{-1}\text{DW}$  was detected on media without any source of nitrogen. In addition,  $30\text{ mM NH}_4^+$  total nitrogen resulted in highest colchicine content of  $0.070\text{ mg g}^{-1}\text{DW}$ . Yang *et al.* [28] reported that the highest flavonoid content in *Glycyrrhiza uralensis* was obtained when the ratio of  $\text{NH}_4^+/\text{NO}_3^-$  in the medium was decreased to the half, they found that  $\text{NH}_4^+$  inhibited the callus growth and flavonoid formation of *G. uralensis*.

*Effect of carbohydrate source and concentration on alkaloid production.* The presence of (-)-colchicine in callus was affected by concentration of sucrose and period of incubation on media (Table 6). (-)-Colchicine alkaloid was

**Table 3. Influence of Subculture Time on Colchicum Hierosolythanium Callus Growth Over 90 Days Incubation on Solid MS Media**

Time Subculture (Days)	Callus Width (cm)	Callus Length (cm)	Callus Height (cm)	Fresh Weight (g)	Color
5	1.14 d*	0.64 d	0.38 d	0.34 e	100% yellowish
10	1.28 d	0.76 cd	0.38 d	0.47 de	100% yellowish
15	1.24 d	0.94 c	0.44 cd	0.64 de	100% yellowish
20	1.62 c	1.32 b	0.50 cd	0.73 d	100% yellowish
25	1.76 bc	1.46 ab	0.70 bc	1.09 c	100% yellowish
30	1.86 ab	1.46 ab	0.90 ab	1.19 ab	100% yellowish
45	1.84 ab	1.64 a	1.02 a	1.27 bc	100% yellowish
60	1.96 a	1.72 a	1.02 a	1.47 ab	100% yellowish
90	1.78 b	1.52 ab	1.04 a	1.78 a	100% yellowish
LSD	0.151	0.285	0.267	0.339	

\*Means having different letters in each column are significantly different according to the least significant different (LSD  $\leq$  0.05).

**Table 4. Cell Growth of Colchicum Hierosolythanium Over 30 Days Incubation in Liquid Media Supplemented with 0.54  $\mu$ M NAA**

Parameters			
Time (Days)	Initial Weight (g)	Recorded Weight (g)	Difference (g)
3	1.05 b*	0.76 f	-0.29
6	1.05 b	0.91 e	-0.14
9	1.36 a	1.05 ed	-0.31
12	1.05b	1.23 cd	+0.18
15	1.01 b	1.29 c	+0.28
18	1.05 b	1.31 c	+0.26
21	1.01 b	1.34 bc	+0.33
24	1.01 b	1.41 bc	+0.40
27	1.45 a	1.85 a	+0.40
30	1.12 b	1.73 ab	+0.61
LSD	0.233	0.398	

\*Means having different letters in each column are significantly different according to the least significant different (LSD  $\leq$  0.05).

**Table 5. Influence of Various (NH<sub>4</sub><sup>+</sup>:NO<sub>3</sub><sup>-</sup>) Ratios on Callus Fresh Weight, Dry Weight and Colchicine Content in Callus of Colchicum Hierosolythanium after 30 days of Incubation**

Parameters			
NH <sub>4</sub> <sup>+</sup> :NO <sub>3</sub> <sup>-</sup> (mM)	Fresh Weight (g)	Dry Weight (g)	Colchicine Content (mg/g)
0.00:0.00	1.19 b*	0.13 bc	0.002
0.00:30.0	1.26 ab	0.17 a	0.005
10.0:20.0	1.39 ab	0.16 a	0.004
20.0:10.0	1.37 ab	0.15 ab	0.013
30.0:0.00	1.53 a	0.17 a	0.070
0.00:60.0	0.71 c	0.11 cd	0.030
20.0:20.0	0.78 c	0.11 cd	0.051
40.0:20.0	0.65 c	0.09 d	0.004
60.0:0.00	0.85 c	0.10 cd	0.004
LSD	0.322	0.033	

\*Means having different letters are significantly different according to the least significant different (LSD  $\leq$  0.05).







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