



CALLUS INDUCTION AND CELL CULTURE OF CASTOR (*RICINUS COMMUNIS* L. CV. SHABJE)

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Abstract

Context: Tissue culture is an important tool in plant biotechnology that allows for an increase in biomass or metabolite production by utilizing several techniques in callus or cell cultures. Ricin is a toxic protein that can be extracted from the castor bean as secondary metabolite. The ricin has been used experimentally in medicine to kill cancer cell. We developed callus and cell culture technique for the possible extraction of ricin from the cell suspension culture of *Ricinus communis*.

Objectives: The present investigation aimed to develop the cell culture technique of *R. communis* L. cv. Shabje and tried to establish a protocol for cell suspension culture of castor for possible extraction of ricin from cell extract.

Materials and Methods: The hypocotyl explants of *R. communis* L. cv. Shabje were used as experimental materials. Cultured on Murashige and Skoog medium supplemented with different concentrations and combinations of BAP, NAA, 2,4-D and IAA for callus induction. For cell culture, the media were used without agar with different concentrations and combinations of these hormones.

Results: For callus induction the combination of BAP 2.0 mg/l + 0.5 mg/l NAA showed the best performance but in case of cell culture the combination of BAP 2.0 mg/l and 0.2 mg/l NAA showed the best result.

Conclusion: The present investigation clearly established and demonstrated the method of obtaining cell suspension culture and important secondary metabolite ricin could be obtained from cell suspension culture of *R. communis* L. holding promises to explore cell culture industry for ricin production.

Key words: Castor, hypocotyl, embryogenic callus, cell culture and regeneration.

Introduction

On a global scale, medicinal plants are mainly used as crude drugs and extracts. Several of the more potent and active substances are employed as isolated compounds obtained from medicinal plants, including many alkaloids such as morphine (pain killer), codeine (antitussive), quinine papaverine (phosphodiesterase inhibitor), ephedrine (stimulant), ajmaline (antirhythmic), (antimalarial), reserpine (antihypertensive), galanthamine (acetylcholine esterase inhibitor), scopolamine (travel sickness), berberine (psoriasis), caffeine (stimulant), capsaicin (rheumatic pains), colchicines (gout), yohimbine (aphrodisiac), pilocarpine (glaucoma), and various types of cardiac glycosides (heart insufficiency) (Wilken *et al.* 2005). Extraction from the *in vitro* tissues is much simpler than extraction from organized complex tissues of a plant. Cell cultures in this respect are very useful for the secondary metabolites they produce. Some of these metabolites that are valuable source include flavors, natural sweeteners, industrial feed stocks, perfumes and commercial

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insecticides. These products do not perform vital physiological functions like amino acids or nucleic acids, but they are produced to ward off potential predators, attract pollinators, or combat infectious diseases. Another useful metabolite produced by plants includes Shikokin, which is a chemical used as both a dye and a pharmaceutical. Plant cell suspension culture is valuable also for studying the biosynthesis of these secondary metabolites. Although there are limitations of cell culture systems in producing secondary metabolites, they are favored over conventional cultivation methods. This is because of their ability to produce useful compounds under controlled conditions as well as their capability of using this technique to produce chemicals to meet market demands.

Plant tissue and cell culture is an important tool in plant biotechnology that allows for an increase in biomass or metabolite production by utilizing several techniques in callus or morphogenetic cultures (Ramachandra and Ravishankar 2002, Dornenburg and Knorr 1995). Among others, these techniques include bioreactor scale-up, hairy transformed roots, micropropagation, elicitation, precursor compound addition and genetic engineering (Mulabagal and Tsay 2004, Smetanska 2008, Dornenburg and Knorr 1995). High *in vitro* multiplications have been reported for *R. communis* (Ahn *et al.* 2007) that produced ricin which is mostly known for its potential as a biological weapon. Castor oil is a triglyceride of fatty acids and almost 90 percent of its fatty acid content consists of ricinoleic acid. Ricinoleic acid is not found in any other substance except castor oil (Williams 1995). Ricin has been used experimentally in medicine to kill cancer cell. Ricin is a secondary metabolite toxic protein abundantly produced from castor bean and it can also be obtained from callus and cell culture of *R. communis*. *As Calophyllum brasiliense* (Cambes) produces calanolide secondary metabolites that are active against human immunodeficiency virus type 1 reverse transcriptase which was isolated by callus culture (Bernabé-Antonio *et al.* 2010). Acridone and furoquinoline alkaloids and coumarins have been isolated from four week old calli in *Ruta* species (Alfred Baumert 1992). The production of the steroidal sapogenin and diosgenin by callus cultures of *Trigonella foenum-graecum* L. (Fenugreek) was reported by Oncina *et al.* in 2000. In addition, plant cell culture also offers an attractive alternative source to whole plant for the production of high-value secondary metabolites (Ravishankar *et al.* 1999, Dornenburg and Knorr 1995, Scragg 1997, Alfermann and Petersen 1995, DiCosmo and Misawa 1995, Stockigt *et al.* 1995, Ravishankar and Venkataraman 1990). Plant cell culture systems represent a potential renewable source of valuable medicinal, flavors, essences and colorants that cannot be produced by microbial cells or chemical syntheses. However, only a few cultures produce these compounds in commercially useful amounts. The low productivities are associated with our poor understanding of the biochemistry of these systems. Recent advances in molecular biology, enzymology, physiology and fermentation technology of plant cell cultures suggest that these systems will become a viable source of important natural products.

In Bangladesh *R. communis* is widely grown all over the country holding great potential for producing castor oil as a source of green energy and also for commercial production of toxic protein ricin. We have developed the standard protocol for *in vitro* regeneration of *R. communis* and under the present investigation we have made efforts to develop the standard protocol for wide scale callus culture and cell culture of *R. communis* towards isolation of active protein ricin from these cultures.

Materials and Methods

The hypocotyl explants of *R. communis* L. cv. Shabje, were used as experimental materials in the present investigation. Seeds were collected from the research field of the Institute of Biological Sciences, Rajshahi University, Rajshahi, Bangladesh. Seeds were washed thoroughly under running tap water and then treated

with 1% Savlon and 2-3 drops of Tween-80 for about 10 min. This was followed by successive three washing with distilled water to make free the seeds from Savlon and Tween-80. Surface sterilization was carried out with 0.1% HgCl₂ for 6-7 min followed by gentle shaking. After this treatment, the seeds were rinsed 4-5 times in sterile distilled water to make free the seeds from HgCl₂. Sterilized seeds were partially decorticated and aseptically germinated in glass bottle containing 50 ml of autoclaved (121° C temperature for 20 minutes at 1.1 Kg/cm² pressure) MS (Murashige and Skoog 1962) medium, fortified with BAP (1.0 mg/l), 30 gm/l sucrose and 0.8 gm/l agar.

Callus culture: Hypocotyl segments as explants were taken from 8 days old *in vitro* grown seedlings of the plant. The explants were cultured in 9 cm petri dish and placed horizontally on the callus induction medium. The MS medium supplemented with 3% sucrose and different concentrations of NAA, 2, 4-D, IAA and BAP in combination were compared for the induction of callus. The data for callus initiation were scored after 4 weeks of culture.

Cell culture: Rapidly proliferating friable calli subcultured for 18 days, were aseptically transferred to MS liquid medium supplemented with 2.0 mg/l BAP + 0.05 mg/l NAA, 2.0 mg/l BAP + 0.2 mg/l NAA and 2.0 mg/l BAP + 0.5 mg/l NAA in three lots in 250 ml flasks. The flasks were placed on a rotary shaker (100 rpm). After 4 days, the liquid medium containing cells and micro calli were filtered through a 500 micron mesh sieve. The filtrates containing cell were maintained in the laboratory. To observe the growth efficiency, flasks containing the liquid medium with cell culture were kept in an orbital shaker. Cell growth was measured by weighting the cells in 5 ml liquid medium taken after every two days. On the other hand, to obtain callus, some cells were distributed to petri dishes (4 cm) containing the fresh semi solid medium at 25°C in dark for 35-42 days of incubation. Micro calli were appeared in the plates initiating induction of callus from cell aggregates.

Results

Callus induction: The surface sterilized seeds exhibited 95% germination after 10 days of inoculation on MS medium supplemented with 1.0 mg/l BAP. For callus induction, hypocotyl explants were taken from *in vitro* grown seedlings and cultured on MS media supplemented with cytokinin (BAP 0.5 mg/l – 3.0 mg/l) in combination with auxins (NAA 0.1mg/l – 1.0 mg/l, 2,4-D 0.1 mg/l – 1.0 mg/l and IAA 0.1 mg/l – 1.0 mg/l). Different concentrations took different time periods for callus induction. Data on response after inoculation, percentage of explants responded and natures of callus were recorded and the results are presented in Table 1. When the explants were cultured on MS media containing different combinations and concentrations of BAP (0.5 mg/l – 3.0 mg/l) and NAA (0.1mg/l – 1.0 mg/l) highest rate 100% callus induction was obtained in both the combinations of 2.0 mg/l BAP+ 0.5 mg/l NAA and 2.0 mg/l BAP + 0.8 mg/l NAA. The induced calli were green in color, compact and nodular (Fig. 1 B). When the explants were cultured on MS media containing different combinations and concentrations of BAP (0.5 mg/l – 3.0 mg/l) with 2, 4-D (0.1mg/l – 1.0 mg/l) highest rate 91.5% callus induction was obtained in 2.0 mg/l BAP + 0.8 mg/l 2,4-D and the induced calli were green in color and compact in structure (Fig. 1.C). When the explants were cultured on MS medium containing different combinations and concentrations of BAP (0.5 mg/l – 3.0 mg/l) with IAA (0.1mg/l – 1.0 mg/l) highest rate 54.5% was obtained in 2.0 mg/l BAP + 0.5 mg/l IAA and the induced calli were green and compact (Fig. 1. D). The experiments in our investigation also demonstrated that combined effect of BAP and NAA showed the best performance in callus induction in *R. communis* L. On the other hand, the embryogenic calli were green and compact while the non embryogenic calli were large, white and friable.

Table 1. Effect of different growth regulators on callus induction of *Ricinus communis* L cv Shabje from hypocotyl explant (Each treatment consisted of 20 explants).

Effect of different concentrations and combinations of BAP and NAA in MS medium on callus induction				Effect of different concentrations and combinations of BAP and 2,4-D in MS medium on callus induction				Effect of different concentrations and combinations of BAP and IAA in MS medium on callus induction			
Conc. of growth regulators (mg/l)	Response after days	% of explants responded	Nature of callus	Conc. of growth regulators (mg/l)	Response after days	Response after days	Nature of callus	Conc. of growth regulators (mg/l)	Response after days	Response after days	Nature of callus
BAP+ NAA				BAP+ 2,4-D				BAP+ IAA			
0.5+0.1	13	18.5	WG	0.5+0.1	14	8.2	WG	0.5+0.1	15	8.9	WG
0.5+0.2	9	37.4	GF	0.5+0.2	11	20.1	WG	0.5+0.2	14	10.1	WG
0.5+0.5	8	44.6	GF	0.5+0.5	10	36.3	GF	0.5+0.5	13	21.0	GF
0.5+0.80	7	52.4	GF	0.5+0.8	9	42.2	GF	0.5+0.8	12	23.2	GF
0.5+1.0	7	28.7	GF	0.5+1.0	9	39.7	GF	0.5+1.0	12	25.3	GF
1.0+0.1	12	22.8	WG	1.0+0.1	13	10.3	WG	1.0+0.1	14	12.1	WG
1.0+0.2	8	47.5	GF	1.0+0.2	10	28.3	WG	1.0+0.2	13	22.0	WG
1.0+0.5	7	63.6	GF	1.0+0.5	9	46.5	GF	1.0+0.5	12	28.5	GF
1.0+0.8	6	78.5	GC	1.0+0.8	8	66.2	GF	1.0+0.8	11	34.2	GF
1.0+1.0	6	51.3	GC	1.0+1.0	8	59.1	GF	1.0+1.0	11	38.5	GF
1.5+0.1	11	29.1	WG	1.5+0.1	12	14.5	WG	1.5+0.1	13	14.5	WG
1.5+0.2	7	55.2	GF	1.5+0.2	9	36.0	WG	1.5+0.2	12	32.1	WG
1.5+0.5	6	81.1	GF	1.5+0.5	8	57.3	GF	1.5+0.5	11	43.2	GF
1.5+0.8	5	91.0	GC	1.5+0.8	7	73.1	GC	1.5+0.8	10	47.2	GC
1.5+1.0	5	60.2	GC	1.5+1.0	7	65.8	GC	1.5+1.0	10	45.1	GC
2.0+0.1	10	31.9	WG	2.0+0.1	11	15.9	GF	2.0+0.1	12	16.2	WG
2.0+0.2	6	85.5	GF	2.0+0.2	8	41.3	GF	2.0+0.2	11	41.0	GF
2.0+0.5	5	100.0	GCN	2.0+0.5	7	71.2	GC	2.0+0.5	10	54.5	GC
2.0+0.8	4	100.0	GCN	2.0+0.8	6	91.5	GC	2.0+0.8	9	53.4	GC
2.0+1.0	4	77.3	GCN	2.0+1.0	6	82.6	GC	2.0+1.0	9	52.3	GC
2.5+0.1	11	30.2	WG	2.5+0.1	12	14.1	GF	2.5+0.1	13	16.5	WG
2.5+0.2	7	58.1	GF	2.5+0.2	9	45.2	GF	2.5+0.2	12	36.3	GF
2.5+0.5	6	81.3	GC	2.5+0.5	8	61.0	GC	2.5+0.5	11	50.2	GC
2.5+0.8	5	89.1	GC	2.5+0.8	7	80.4	GC	2.5+0.8	10	51.5	GC
2.5+1.0	5	62.0	GC	2.5+1.0	7	72.6	GC	2.5+1.0	10	50.4	GC
3.0+0.1	11	20.9	WG	3.0+0.1	12	15.0	GF	3.0+0.1	13	15.1	WG
3.0+0.2	8	37.2	GF	3.0+0.2	9	41.6	GF	3.0+0.2	12	30.1	GF
3.0+0.5	7	63.2	GC	3.0+0.5	8	52.2	GF	3.0+0.5	11	39.2	GF
3.0+0.8	6	72.0	GC	3.0+0.8	7	68.5	GF	3.0+0.8	10	38.0	GF
3.0+1.0	6	55.7	GF	3.0+1.0	7	56.3	GF	3.0+1.0	10	35.1	GF
WG – whitish green, GF – green friable, GC – green compact, GCN – green compact nodular.				WG – whitish green, GF – green friable, GC – green compact.				WG – whitish green, GF – green friable, GC – green compact.			

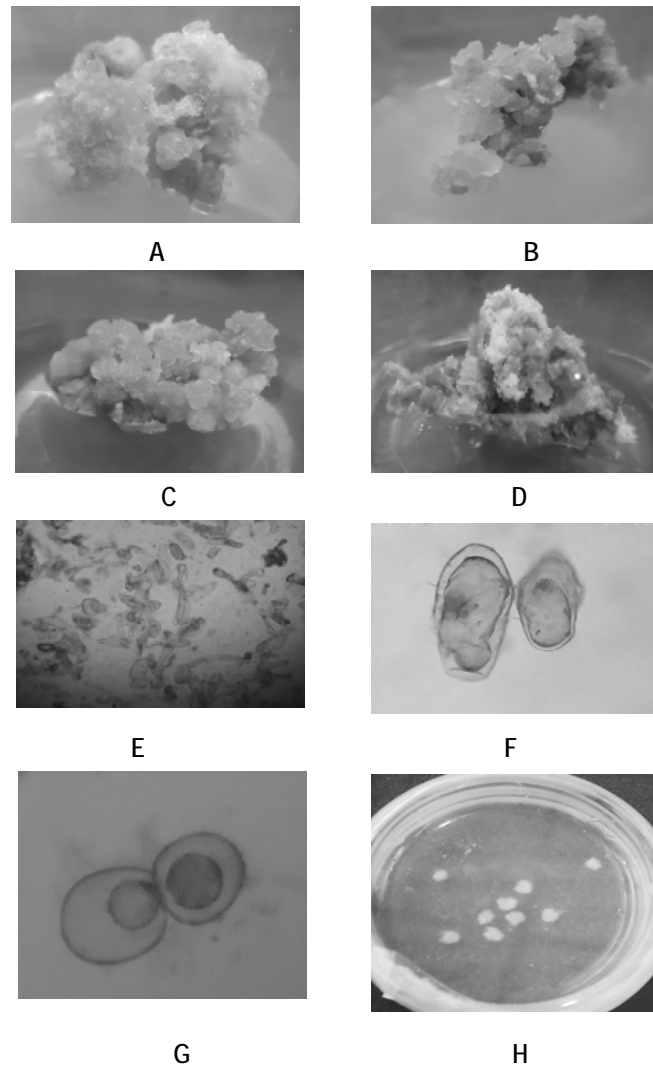


Fig. 1. A. Three weeks old whitish green callus. B. Four weeks old green compact nodular organic callus. C. Five weeks old green compact callus. D. Six weeks old green compact callus. E. Isolated cells. F. Isolated single cells. G. Cell division. H. Callus induction from single cells.

Cell culture:

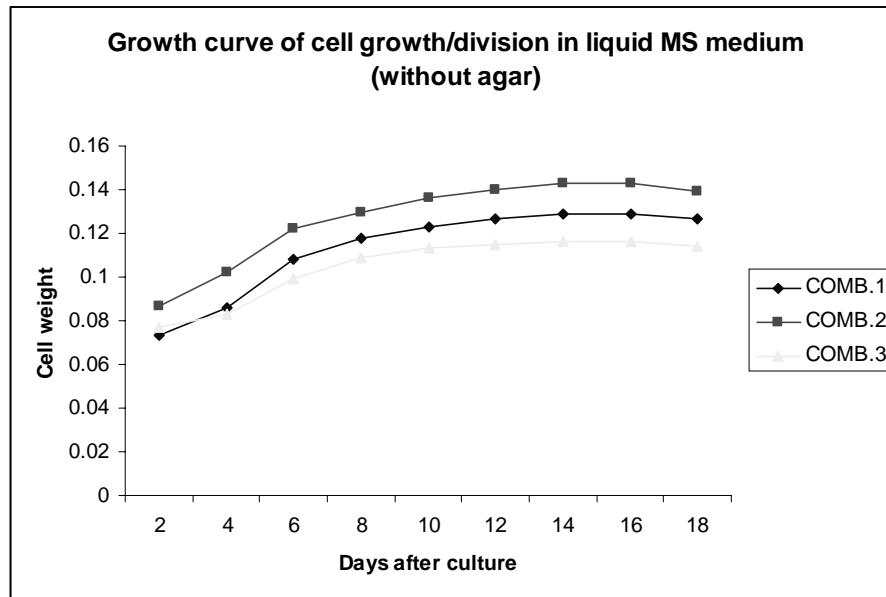
Rapidly proliferating greenish friable embryonic calli (about 1-2 gm fresh weight) grown in solid medium were considered as the better candidate for initiation of cell suspension culture in castor. Embryonic calli in solid media containing 2.0 mg/l BAP + 0.5 mg/l NAA were used to initiate cell suspension in liquid medium. Cell culture experiment was started in three separate lots in three 250 ml flasks in liquid MS medium contain 2.0

mg/l BAP + 0.05 mg/l NAA, 2.0 mg/l BAP+ 0.2 mg/l NAA and 2.0 mg/l BAP+ 0.5 mg/l NAA. The flasks were placed on a rotary shaker (100 rpm) in dark and within 4 days cells in the liquid medium appeared distinctly visible and under microscope they were found in different growth stages (Fig.1. E, F and G). Cell weight was recorded nine times with every 2 days intervals. The summarized results are shown in the Table 2. Cells were found to grow continuously in three flasks with three media combinations of BAP and NAA. But when the cells were cultured on the MS medium having 2.0 mg/l BAP + 0.2 mg/l NAA, the rate of cell growth found the highest than that of MS medium having 2.0 mg/l BAP + 0.05 mg/l NAA and 2.0 mg/l BAP+ 0.5 mg/l NAA but in all cases, the cells continued to grow until 14 days. The peak period of cell growth was observed from 4th day to 6th day.

The mean value of three combinations were calculated and plotted in a graph and presented in Fig. 2 The results presented in the graph indicated that three media combinations showed the similar trend of cell growth and their growths were found highest in between 4-6 days. After 10 days their growth found to be stationary in the artificial medium and under given environmental condition of the laboratory.

Table 2. Cell weight of *R. communis* L. cv. Shabuje growing after different periods.

Times	Combination of growth regulators											
	2.0 mg/l BAP + 0.05 mg/l NAA				2.0 mg/l BAP + 0.2 mg/l NAA				2.0 mg/l BAP + 0.5 mg/l NAA			
	Fla.1 (mg/)	Fla.2 (mg/)	Fla.3 (mg/)	Mean (gm)	Fla.1 (mg/)	Fla.2 (mg/)	Fla.3 (mg/)	Mean (gm)	Fla.1 (mg/)	Fla.2 (mg/)	Fla.3 (mg/)	Mean (gm)
After 2 days	0.056	0.084	0.079	0.073	0.089	0.074	0.098	0.087	0.067	0.089	0.075	0.077
After 4 days	0.068	0.097	0.093	0.086	0.103	0.092	0.111	0.102	0.072	0.096	0.081	0.083
After 6 days	0.092	0.118	0.114	0.108	0.125	0.118	0.123	0.122	0.086	0.114	0.097	0.099
After 8 days	0.104	0.126	0.124	0.118	0.133	0.126	0.131	0.130	0.098	0.122	0.107	0.109
After 10 days	0.110	0.130	0.129	0.123	0.139	0.132	0.137	0.136	0.103	0.125	0.111	0.113
After 12 days	0.115	0.134	0.132	0.127	0.144	0.135	0.141	0.140	0.106	0.126	0.113	0.115
After 14 days	0.117	0.135	0.134	0.129	0.149	0.135	0.145	0.143	0.108	0.126	0.114	0.116
After 16 days	0.117	0.135	0.134	0.129	0.150	0.135	0.145	0.143	0.108	0.126	0.114	0.116
After 18 days	0.116	0.133	0.132	0.127	0.145	0.134	0.141	0.140	0.108	0.124	0.113	0.115



COMB.1= 2.0 mg/l BAP+0.05 mg/l NAA
 COMB.2= 2.0 mg/l BAP+0.2mg/l NAA
 COMB.3= 2.0 mg/l BAP+0.5 mg/l NAA

Fig. 2. Growth curve of cell growth / division in liquid MS medium.

Discussion

Under the present investigation in castor, embryogenic calli were induced when explants were cultured on MS medium containing auxin with cytokinin (BAP). The explant showed highest percentage (100%) of embryogenic callus when NAA was used with BAP in MS medium supplemented with 2.0 mg/l BAP + 0.5 mg/l NAA and 2.0 mg/l BAP + 0.8 mg/l NAA. Similar results were reported by Kumari *et al.* (2008) in *R. communis* L. and they obtained callus induction from cotyledon explants using combination of BAP 2.0 mg/l and 0.8 mg/l NAA. Vila *et al.* (2003) also reported similar results in *Melia azedarach*, they obtained callus cultures using combination of BA (4.4 μ M) and NAA (0.46 μ M).

The embryogenic calli were then placed on a shaker to allow the cell aggregates to disperse to form smaller clumps of single cells equally distributed throughout the liquid media. The cells were found continuously growing until one of the factors became limiting causing cell growth to slow. Better performance of the isolated cells depends on the condition of embryogenic calli from which they were derived and the duration of their subculture. Under the present investigation in castor, cells were cultured on the MS media having 2.0 mg/l BAP + 0.2 mg/l NAA, the rate of cell growth found highest than that of MS media having 2.0 mg/l BAP + 0.05 mg/l NAA and 2.0 mg/l BAP+ 0.5 mg/l NAA. But in all cases, the cell continued to grow until 14 days. The peak period of cell growth was observed from 4th day to 6th day. Similar result was obtained in brinjal and cell growth highest peak within 4-6 days of incubation (Hossain *et al.* 2007) was noticed. Similar peak period of cell growth was obtained in *Abrus precatorius* where cells attained their highest peak within 6 – 8 days of growth (Bari *et al.* 2009). Similar results were also reported in sugar beets, using different concentrations and

combinations of BAP and 2,4-D, the growth patterns of cell suspension cultures were examined during a range of culture periods (0, 3, 5, 7, 9, 11, 13 and 15 days). In all lines, the growth rates of cells were initially slow but as the culture proceeded, they increased significantly and accumulated great amounts of biomass over a period of 15 days in Sugar Beet (Songül *et al.* 2002). The time required to establish the cell suspension culture varies greatly and depends on the tissue of the plant species and the medium composition. The use of fine suspension culture offers the opportunity to confirm single cell origin of somatic embryos and the asymmetry of the first cell division, which starts the process.

From the foregoing discussion it may be concluded that the auxins NAA, 2,4-D and IAA have role in callus induction but 2.0 mg/l BAP + 0.5 mg/l NAA and 2.0 mg/l BAP + 0.8 mg/l NAA concentrations proved to be most suitable combinations for induction of embryonic calli of *R. communis*. Under this investigation NAA in combination with BAP proved to be suitable media composition for enhancing cell growth of *R. communis* in artificial medium. The present experiments clearly demonstrated that in *R. communis*, cell begins to grow only within 4 days of culture proving its potential for developing cell culture industry for production of important toxic protein ricin as secondary metabolite. In regards of other medicinal plants *R. communis* proved suitable plant species for developing cell suspension culture in artificial medium and the present findings open up a new potential venture for obtaining secondary metabolites ricin as a alternative source from its cell .

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