Chapter 5

ASIATICOSIDE IN CALLUS AND CELL CULTURE

5.1 Introduction

An important development in the field of plant cell and tissue culture has been the demonstration of the totipotency of plant cells. The concept of cellular totipotency is inherent in the Cell Theory of Sehleiden and Schwann, proposed during 1838-1839, and popularized by Virchow in 1858 with his famous aphorism "omnis cellula e cellula". However, no attempts were made to obtain any experimental evidence to support the idea of totipotency until 1878, when Vochting succeeded in dissecting plants into smaller and smaller fragments and keeping the fragments viable and growing. The famous botanist Gottlieb Haberlandt, presented a prophetic paper entitled "Experiments on the Culture of Isolated Plant Cells" before a meeting of the Mathematics and Natural Sciences Section of the Vienna Academy of Sciences in Berlin on February 6, 1902 and introduced the idea of plant cell, tissue and organ culture. But, the first successful culture of excised roots for indefinite periods of time was achieved by White (1934). A few years later, Gautheret, Nobecourt and White (1939) independently succeeded in cultivating cambial tissues of carrot and tobacco for prolonged periods (Vasil and Vasil, 1972).

Extensive cell proliferation and the formation of a relatively undifferentiated and homogeneous mass of cells called callus, has been obtained in a variety of plants. One of the common methods used to obtain such calli, which are capable of indefinite growth with periodic sub culturing, is to grow explants from various parts of a plant in a nutrient agar medium supplemented with an auxin and a cytokinin, or very often an auxin like 2, 4-D alone. Cells which proliferate under the influence of the plant growth substances are large, thin-walled, vacuolated, differentiated, and mature parenchyma cells. These must undergo dedifferentiation in order to become meristematic and mitotically active. During this process, particularly in the presence of auxins, many changes are noticeable in cells of the explant. These include enlargement of nuclei and nucleoli, appearance of vacuoles in nucleoli, an increase in the density of nucleohistones around the nucleoli and increased synthesis of proteins, DNA and particularly ribosomal RNA (Chrispeels and Hanson, 1962; Key and Shannon, 1964; Gifford and Nitsch, 1969; Vasil, 1971; Yeoman, 1970; Vasil and Vasil, 1972). Increased differentiation of the granular zone in the nucleolus and the formation of ribosomal precursor particles or the ribonucleoprotein particles takes place during cell proliferation induced by the auxin herbicide, 2,4-D (Vasil, 1971). Once a continuing callus culture has been obtained, its segments are transferred into a

liquid nutrient medium and incubated on a gyratory shaker. Viable and growing cell suspensions can be maintained in liquid media by transferring small aliquots of the cell suspension to fresh media every 2 to 4 weeks. Such suspension cultures yield large populations of cells and can be used as convenient sources for obtaining secondary metabolites.

Recently, the production of secondary metabolites using plant cells has been a subject of extended research. Cell cultures have a higher rate of metabolism than intact differentiated plants because the initiation of cell growth in culture leads to fast proliferation of cell mass and to a condensed biosynthetic cycle. This is the most important advantage of plant cell cultures as model systems for the study of biosynthetic pathways, as secondary metabolite formation can take place within a short (about 2-4 weeks) cultivation time (Dornenburg and Knorr, 1995). The plant cell culture technology is now sufficiently advanced to allow for large quantities of relatively homogeneous, undifferentiated cells to be produced. A number of investigations have been made for production of important metabolites through cell culture. Cell cultures of several species of *Taxus* for Taxol production, *Panax ginseng* for Ginsenosides, *Lithospermum erythrorhizon* for shikonin, *Vinca minor* for Vincamine and *Rauwolfia serpentina* for Ajmaline and Raucaffricine production are being used for commercial production of important secondary metabolites (Dicosmo and Misawa, 1995; Dornenburg and Knorr, 1995). Thus, large scale production with plant cell tissue culture for the generation of pharmaceuticals seems to be of continued commercial interest. Plant cell and tissue culture systems are complementary and may provide competitive metabolite production systems when compared to whole plant extraction (Dicosmo and Misawa, 1995).

Taking into account the importance of callus and cell culture for secondary metabolite production, the present study was carried out to study the influence of growth regulators on induction and proliferation of callus and also on the accumulation of Asiaticoside in callus and cell culture of *Centella asiatica*. The potential of different explants of *C. asiatica* for induction and proliferation of callus using different concentrations of cytokinin and auxin individually and in combinations, has also been investigated.

5.2 Materials and methods

5.2.1 Induction of callus

At the beginning of this experiment, leaf, stem and fruit explants were inoculated on MS medium containing several concentrations of auxins (IAA, NAA and 2,4-D) and cytokinins (BA and Kin) alone and in combination. The cultures were incubated in the culture room under controlled conditions, as already described in Chapter 3. The cultures were examined after a period of 28 days, to select the optimum concentration of growth regulators for callus growth.

5.2.2 Growth kinetics of callus

The callus obtained on all the culture treatments was harvested after 28 days of culture incubation. The average fresh weight of the callus was recorded and it was followed by its drying in an oven at 60°C until the callus reached a constant weight. Callus formation, colour and compactness of the calli were judged visually. Increase in biomass was determined on the basis of fresh and dry weight of the callus. The moisture percentage was calculated by employing the formula given below:

Moisture percentage = [(fresh weight-dry weight)/fresh weight] x 100

5.2.3 Callus proliferation and maintenance

Four weeks after incubation, the combinations of plant growth regulators that induced maximum proliferation of callus were selected. The callus cultures were maintained in the nutrient medium supplemented with the selected growth regulator combinations. Obtained callus was subcultured by transferring approximately 500 mg of callus in freshly prepared MS medium supplemented with the same concentration of plant growth regulator combinations at four weeks interval. The cultures were incubated in culture room under controlled conditions of temperature, light and humidity, as already described in Chapter 3.

5.2.4 Establishment and growth kinetics of cell culture

The cell culture was established from the callus line showing optimum accumulation of Asiaticoside. For initiation of the cell culture, the type and concentration of growth regulators was selected based on the results of Asiaticoside production in callus culture. Therefore, the leaf derived callus maintained on MS medium

supplemented with 9.05µM 2, 4-D and 17.74 µM BA was found to be most suitable for this purpose. Cell cultures were established from the leaf derived callus. About 100 mg of fresh callus was inoculated in 35 ml liquid MS medium enriched with phytohormones supporting optimum Asiaticoside production in the callus. The cultures were incubated on a rotary incubator shaker at 85 rpm, under controlled conditions of temperature(25±2ºC) and photoperiod(16 h light and 8 h dark).

For study of growth kinetics of the cell culture, the cells were harvested every 2 day interval, beginning from 4th day to 35 th day of culture incubation period. For this, the cells were separated from the liquid medium by wire sieve. The cells were then centrifuged at 5000 rpm for 5 min. and the remaining medium was pipetted out. The cell pellet was dried by pressing gently between the sheets of filter paper to remove moisture completely. Fresh weight of the pellet was then determined with the help of electronic balance. The cells were then kept in an oven at 60ºC for drying till constant weight was obtained and dry weight of the cells was then taken.

5.2.5 Asiaticoside extraction and estimation

Asiaticoside extraction from callus and cell pellets was carried out as per the procedure already explained in Chapter 3. For Asiaticoside analysis in spent cell culture medium, the spent medium from the replicate treatment flasks was pooled together and dried under vacuum to get the dry residue. HPTLC analysis of callus, cell biomass and spent cell culture medium was carried out for quantification of Asiaticoside. The method has already been dealt with in Chapter 3.

5.2.6 Statistical analysis

All the experiments were performed in triplicate and each experiment was repeated thrice. The data was analysed by using statistical methods already described in Chapter 3.

5.3 Results

5.3.1 Induction and maintenance of leaf callus

Callus cultures were initiated from the leaf explants cultured on MS basal medium supplemented with auxins (IAA, NAA and 2,4- D) and cytokinins (BA and Kin) alone and in combination. The responses towards callus formation depended

upon the type and concentration of the Plant Growth Regulator used. The results have been tabulated in Table 5.1 and Plate 5.1.

Inclusion of low levels of Auxins in culture medium resulted in direct root formation from the leaf explants, as described in Chapter 3. Addition of 1.73-2.31µM IAA resulted in slight callus formation. However, at higher concentration of IAA (2.89 µM), moderate callus induction was observed (122 ± 2.8 mg DW). The callus formation was observed during second week of incubation. The callus produced was creamish, soft and friable. Incorporation of NAA in MS medium resulted in the formation of straw coloured, soft callus. Among all the concentrations of NAA tried, maximum callus formation was achieved on 5.37 µM NAA and 184 ± 1.3 mg DW callus was formed. The inclusion of NAA provided better results as compared to IAA. But, addition of 2,4-D in the medium was found to be most effective for callus formation. Leaf explants inoculated on MS medium fortified with 9.05 µM 2, 4-D produced 184 ± 1.3 mg DW callus. The callus produced was cream coloured, soft and friable. When the 2,4-D level was increased to 18.10 µM, a decrease in callus biomass was observed. Further increase in 2,4-D concentration resulted in the formation of watery and straw coloured callus.

Inoculation of leaf explant on MS medium containing Cytokinin resulted in shoot formation. However, callus formation was also quite prevalent in this case. Addition of 9.2 µM Kinetin produced 137 ± 1.6 mg DW callus biomass. Incorporation of BA was found to be more favourable for callus formation from leaf explant and addition of 17.74 µM BA resulted in the formation of 193 ± 2.3 mg DW callus. The callus formed was compact and green in colour.

Conjugation of 2,4-D and BA increased the callogenic potential of leaf explant. On the contrary, the result for callus formation in the case of 2,4-D and Kinetin were not found encouraging and scanty callus was formed. Incorporation of 9.05 µM 2,4-D along with 4.44-17.74 µM BA yielded the best results for callus formation from the leaf explants of *C. asiatica*. The callus formed was compact, dry and green in colour. MS medium supplemented with 9.05µM 2,4-D along with 17.74 µM BA was found to be the most effective medium for extensive callogenesis from the leaf explant. The callus biomass obtained in this case was 239 ± 1.1 mg DW.

The growth of callus was measured by recording its fresh weight and the dry weight. As far as the growth kinetics are concerned, the growth curve was in exponential phase upto the seventh week of culture. The callus cultures attained

Table 5.1. Influence of auxins and cytokinins on Asiaticoside production in leaf derived callus of *C.asiatica*.

MS+PGR (µM)				Moisture %	D.W (mg)	Asiaticoside mg/g DW
2,4-D	**NAA**	**BA**	**Kin**			
4.52				97.6	159 ± 2.3^{i}	0.041 ± 0.028^{o}
9.05				97.1	184 ± 1.3^{e}	0.068 ± 0.016^{m}
18.10				98.2	145 ± 2.1^{l}	0.055 ± 0.018^{n}
	5.37			89.4	163 ± 1.7^{h}	0.022 ± 0.021^{s}
	10.74			92.3	153 ± 1.9^{j}	0.026 ± 0.013^{r}
	21.58			96.8	141 ± 2.3^{m}	0.031 ± 0.022^{q}
		4.44		95.2	149 ± 1.9^{k}	0.131 ± 0.031^{g}
		8.87		95.6	183 ± 3.9^{e}	0.189 ± 0.012^{c}
		17.74		93.7	193 ± 2.3^{c}	0.167 ± 0.086^{d}
			4.65	98.2	131 ± 3.7^{o}	0.017 ± 0.015^{u}
			9.29	97.3	137 ± 1.6^{n}	0.069 ± 0.026^{i}
			18.58	98.6	126 ± 1.4^{p}	0.019 ± 0.014^{t}
4.52		4.44		98.4	162 ± 2.6^{h}	0.112 ± 0.063^{h}
		8.87		97.2	165 ± 1.3^{g}	0.080 ± 0.067^{k}
		17.74		98.4	158 ± 3.0^{i}	0.083 ± 0.024^{i}
9.05		4.44		95.7	191 ± 1.6^{d}	0.111 ± 0.031^{e}
		8.87		98.3	196 ± 1.4^{b}	0.192 ± 0.012^{b}
		17.74		97.4	239 ± 1.1^{a}	0.201 ± 0.015^{a}
18.10		4.44		96.8	167 ± 1.5^{f}	0.081 ± 0.015^{j}
		8.87		98.2	121 ± 1.9^{q}	0.137 ± 0.016^{f}
		17.74		98.7	116 ± 2.2^{r}	0.035 ± 0.051^{p}

Values are mean ± SE of 21 replicates. Data scored after four weeks of culture incubation. Mean values with in column followed by same letters did not differ at 5% probability by DMRT.

D.W:dry weight

PLATE 5.1

Influence of auxin and cytokinins on callogenesis in leaf explant

a. Callus formation on MS+ 9.05µM 2, 4-D

b. Callus formation on MS+ 17.74µM BA

c. Leaf derived callus on MS + 9.05µM 2, 4-D + 17.74 µM BA

PLATE 5.1

a

b

c

maximum growth on dry weight basis during fourth week of culture incubation after which the dry weight became constant. The callus exhibited the signs of browning after the 7th week of culture incubation.

This experiment reveals that MS medium supplemented with 9.05 µM 2,4-D and 17.74 µM BA was best suited for prolific callus growth from leaf explant. Thus, this combination was selected for maintenance of callus cultures. The callus cultures were maintained on the said medium for around 35 months with regular sub culturing on fresh medium every 28 days.

5.3.2 Induction and maintenance of stem callus

The stem segments of *C. asiatica* were cultured on MS medium containing varied concentrations of Auxins (IAA, NAA and 2,4-D) and Cytokinins (BA and Kinetin) alone and in combination and the results have been depicted in Table 5.2 and Plate 5.2.

Among all the Auxins employed, 2,4-D was found to be most effective for callus induction from stem explants of *C. asiatica*. 4.52 µM 2,4-D was found to be the most effective concentration for callogenesis and produced 88 ± 1.6 mg callus on dry weight basis.

IAA and NAA when employed alone produced scanty callus. However, when 2,4-D was incorporated with IAA and NAA, an increase in callus formation was observed. 11.56 µM IAA in combination with 4.52 µM 2,4-D produced 86 ±1.5 mg DW callus. Addition of 10.74 µM NAA along with 4.52 µM 2,4-D led to the production of 68 ± 1.7 mg DW callus. The callus formed was off-white, soft and friable. But, these combinations were not as effective as 4.52 µM 2,4-D alone, towards the production of callus from stem explant.

The Cytokinins BA and Kinetin were also tested for their efficiency towards the production of callus. BA was found to be more effective than Kinetin for the same. 4.44-8.87 µM BA produced 97 ±2.0 mg DW of callus. The callus produced was green, hard and friable. 9.29 µM Kinetin produced 80 ± 2.4 mg DW of callus when used along with 4.52 µM 2,4-D. But, this combination produced less amount of callus as compared to the usage of 4.52 µM 2,4-D alone. Also, the callus produced was feeble, straw coloured and jelly like in appearance.

Incorporation of BA in MS medium containing 2,4-D increased the callogenic potential of stem explants. Addition of 4.44 µM BA in MS medium

Table 5.2. Influence of auxins and cytokinins on Asiaticoside production in stem derived callus of *C.asiatica*.

MS+PGR (µM)					D.W (mg)	Moisture %	Asiaticoside mg/g DW
2,4-D	NAA	IAA	BA	Kin			
4.52					98.5	88 ± 1.6^{h}	0.079 ± 0.031^{k}
9.05					96.4	71 ± 1.8^{m}	0.059 ± 0.015^{0}
18.01					98.5	63 ± 2.6^{o}	0.063 ± 0.028^{n}
			4.44		96.3	97 ± 2.0^{f}	0.091 ± 0.023^{g}
			8.87		98.5	97 ± 1.5^{f}	0.092 ± 0.015^{f}
			17.74		98.7	94 ± 1.8^{g}	0.084 ± 0.036^{j}
		11.56			98.4	86 ± 1.5^{i}	0.063 ± 0.019^{n}
	10.74				88.9	68 ± 1.7^{n}	0.077 ± 0.016^{l}
				9.29	89.8	80 ± 2.4^{j}	0.075 ± 0.015^{m}
4.52			4.44		98.7	113 ± 2.4^{d}	0.089 ± 0.021^{h}
			8.87		97.9	177 ± 1.2^{a}	0.159 ± 0.013^{a}
			17.74		98.5	145 ± 1.4^{b}	0.146 ± 0.017^{b}
9.05			4.44		98.5	118 ± 1.9^{c}	0.129 ± 0.012^{c}
			8.87		98.2	78 ± 1.8^{k}	0.109 ± 0.027^{d}
			17.74		98.4	102 ± 1.0^{e}	0.086 ± 0.041^{i}
18.01			4.44		98.2	74 ± 1.1^{l}	0.095 ± 0.081^{e}

Values are mean ± SE of 21 replicates. Data scored after four weeks of culture incubation. Mean values with in column followed by same letters did not differ at 5% probability by DMRT.
D.W: dry weight

PLATE 5.2

Influence of auxin and cytokinins on callogenesis in stem explant

a. Callus formation on MS+ 4.52µM 2, 4-D

b. Callus formation on MS+ 8.87µM BA

c. Stem derived callus on MS+ 4.52µM 2, 4-D + 8.87µM BA

d. Fruit explant on MS + 0.90 µM 2,4-D + 1.78 µM BA

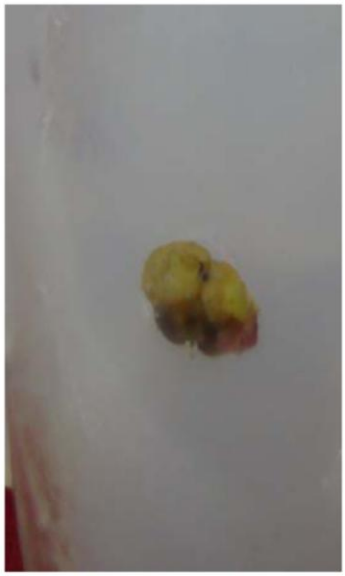
e. Pigmentation in callus induced from fruit

PLATE 5.2

a

b

c



d

e

containing 4.52 µM 2,4-D produced 113 ± 2.4 mg DW callus. However, fortification of 4.52 µM 2, 4-D with 8.87 µM BA produced the best results for callus formation and produced 177± 1.2 mg DW callus. The stem explants cultured on MS medium containing 4.52-18.01 µM 2,4-D in combination with 4.44-17.74 µM BA gave 100% response towards callus formation. But, a decrease in callogenesis was observed with an increase in 2,4-D concentration. As a result of this experiment, it was concluded that, among all the tested concentrations and combinations of phytohormones, maximum callus induction was observed from the stem explants cultured on MS medium supplemented with 4.52 µM 2, 4-D and 8.87 µM BA. The explants cultured on this combination produced 177 ± 1.2 mg DW callus and this combination was selected for maintenance of stem callus.

5.3.3. Callus induction from fruit

The fruits of *C. asiatica* were also inoculated after surface sterilization in MS medium for callus production. Very low concentration of 2,4-D along with BA resulted in callus formation from the fruit explant. This is the first report of callus induction from the fruit of *C. asiatica*. 0.90 µM 2,4-D in combination with 1.78 µM BA was found to produce callus from the fruit. The callus was green and friable and also exhibited pink colouration occasionally (Plate 5.2). The average dry weight of this callus was 123 ± 3.1 mg. This callus was also multiplied by sub culturing on the same medium and analysed for Asiaticoside content.

5.3.4 Morphogenesis in callus

The callus derived from the leaf, stem and the fruit explants were tested for their potential for morphogenesis by sub culturing them on MS medium containing various concentrations and combinations of plant growth regulators (Plate 5.3).

Leaf callus cultured on MS medium containing Auxins started turning pale during the beginning of the second week. On treatment with Auxins, root induction was observed from the callus cultured on different culture media. Among the tried Auxins (IAA, NAA and 2,4-D), NAA was found to be most favourable for rhizogenesis. Incorporation of 1.07-2.15 µM NAA to the parental medium led to the initiation and formation of moderate number of roots from the callus during the second week of culture incubation. Tiny, delicate, white hair-like outgrowths were observed coming out of certain parts of callus which later on developed into roots. On

PLATE 5.3

Organogenesis in callus

a. Rhizogenesis in leaf callus on MS+ 2.15 µM NAA

b. Rhizogenesis in callus on MS+ 2.15 µM NAA+ 0.93µM Kin

c. Rhizogenesis in stem callus on MS+ 2.15 µM NAA

d. Root formation in callus on MS+ 2.15 µM NAA+ 0.93µM Kin

e. Leaf derived callus on MS+ 8.87 µM BA +0.54 µM NAA

f. Stem derived callus on MS+ 9.29 µM Kin+ 0.54 µM NAA

PLATE 5.3

a

b

c

d

e

f

the other hand, treatment of callus with IAA and 2,4-D produced only sparse, thin roots. Moreover, the callus also turned brown during the 4th week. Conjugation of low levels of Cytokinins BA (0.88-2.66 µM) and Kinetin (0.93-2.79 µM) with NAA resulted into an increase in the number of roots formed from the callus. 2.15 µM NAA along with 0.93 µM Kinetin was found to be the best combination for root initiation from the leaf callus. The effect of Auxins was more or less same in case of stem and the fruit derived callus, the difference being in the callus quality which was found to be light green and more compact. In addition, the number of roots formed was also quite less in stem derived callus, as compared to the leaf derived callus.

Treatment of the leaf, stem and fruit derived callus with Cytokinins did not result in the formation of shoot. Presence of 8.87-17.74 µM BA and 9.29-18.58 µM Kinetin resulted in the formation of green, hard and compact callus. When low concentration of NAA (0.26-0.54 µM) was used along with the above concentrations of BA and Kinetin, the callus became more compact with concentrated green areas at few places in leaf callus (Plate 5.3). More so, light brown coloured small outgrowths were observed in case of stem derived callus (Plate 5.3). These neither got converted into roots, nor into shoots, when subcultured on different concentrations and combinations of Auxins and Cytokinins. These outgrowths turned brown and perished eventually. The fruit callus also did not show any sign for caulogenesis when cultured on different concentrations and combinations of Cytokinins.

5.3.5 Asiaticoside content of callus

Results for the accumulation of Asiaticoside in leaf and stem derived callus of *C. asiatica* have been summarized in Tables 5.1 and 4.2 respectively. The callus obtained from fruit did not show the presence of Asiaticoside. On the other hand, all the cultures derived from both leaf and stem explants revealed the presence of Asiaticoside. But, its quantity varied depending upon the type of explant used as well as the type and concentrations of Auxins and Cytokinins employed.

In case of the leaf derived callus obtained on MS medium fortified with low level of Auxin 2, 4-D (4.52 µM), 0.041 ± 0.028 mg/g DW Asiaticoside was produced. An increase in 2,4-D concentration to 9.05 µM also led to an increase in Asiaticoside concentration and 0.068 ± 0.016 mg/g DW Asiaticoside was recorded. However, further increase in 2,4-D level to 18.10 µM led to a decrease in Asiaticoside accumulation and 0.053 ± 0.018 mg/g DW Asiaticoside was produced. IAA and NAA

were found to be less effective towards Asiaticoside production in leaf derived callus, as compared to 2, 4-D. NAA was found to be more effective than IAA and the leaf callus obtained on 21.58 µM NAA was found to possess 0.031 ± 0.022 mg/g DW Asiaticoside.

Leaf derived callus obtained on Cytokinins was found to yield better results for the production of Asiaticoside. Callus initiated on MS containing 9.29 µM Kinetin produced 0.069 ±0.026 mg/g DW Asiaticoside. But, the callus initiated on BA was found to be superior in Asiaticoside content among all the PGRs employed. Leaf callus produced on 8.87 µM BA was found to contain maximum content, i.e. 0.189 ± 0.012 mg/g mean Asiaticoside content. BA concentrations ranging from 4.44 - 17.74 µM were found to most effective concentrations for Asiaticoside production.

Among the different concentrations and combinations of Auxins and Cytokinins used in conjugation, incorporation of BA (4.44 - 17.74 µM) in MS medium containing 2,4-D (4.52 - 18.10 µM) resulted in an improvement in Asiaticoside accumulation in leaf derived callus. Best results for Asiaticoside production were obtained on MS + 9.05 µM 2,4-D + 17.74 µM BA where the leaf derived callus was found to possess 0.201 ± 0.015 mg/g DW Asiaticoside.

Accumulation of Asiaticoside was also detected in all the callus cultures derived from stem explants but, the quantity of Asiaticoside in leaf derived callus was more than the stem derived callus. Nevertheless, stem derived callus initiated on MS containing 4.52 µM 2,4-D in combination with 8.87 µM BA was found to produce the most optimum concentration of Asiaticoside. The Asiaticoside content recorded in the stem derived callus on the said medium was 0.159 µM 0.013 mg/g DW. It was 2.0 times more than the Asiaticoside accumulated in callus obtained on MS + 4.52 µM 2,4-D and 1.72 times higher than that obtained on MS + 8.87 µM BA.

This experiment reveals that usage of leaf explant as callus source is more effective than the stem explant for Asiaticoside production. The Asiaticoside content also varied depending upon the type of PGR used. Leaf explant cultured on MS medium containing BA and 2,4-D resulted in an increased production of Asiaticoside. Optimum Asiaticoside content was detected in 4 week old leaf callus cultured on MS medium supplemented with 9.05 µM 2,4-D in combination with 17.79 µM BA. Moreover, although Asiaticoside was found to be present in both leaf and stem derived callus cultured on different combinations of Plant Growth Regulators, the

content of Asiaticoside was found to be less as compared to *in vitro* raised intact leaf biomass.

5.3.6 Growth and Asiaticoside accumulation in cell culture

The leaf derived callus cultured on MS medium supplemented with 9.05 µM 2, 4-D and 17.74 µM BA yielded the best results for Asiaticoside production and also for biomass production. Therefore, this combination and concentration of growth regulators was selected for the establishment of cell culture (Plate 5.4).

100 mg leaf derived callus was inoculated on MS liquid medium containing 9.05 µM 2, 4-D and 17.74 µM BA and kept for incubation on a shaker. The growth of the cultures was monitored on a daily basis with periodic harvesting. The changes in dry weight and Asiaticoside accumulation in cell culture were recorded. The growth curve of the cell culture was seen with an initial lag phase upto 8 days of incubation. This was followed by a steep rise in the growth rate during the third week. This represented the log phase. The cells entered the stationary phase of growth eventually. A decline in the growth of cells was observed after fourth week during which the cells exhibited a change in colour from light green to straw colour, followed by senescence. It was noted that the cells in the suspension culture grew with a higher rate as compared to the cells in callus. 358 ± 6.4 mg dry weight biomass was obtained from the cell culture.

Analysis of cell culture for Asiaticoside content showed no encouraging results for first 8 days of culture incubation. An increase in Asiaticoside content was recorded from 8^{th} day onwards. Maximum Asiaticoside accumulation was recorded on day 21^{st}, after which a decline in Asiaticoside content was recorded. 0.426±0.016 mg/g DW Asiaticoside content was recorded to be present in the biomass harvested on day 21. Analysis of the spent liquid medium for Asiaticoside content showed no positive results for the presence of Asiaticoside.

5.4 Discussion

5.4.1 Effect of auxins alone and in combination with cytokinins on callogenesis

Callus cultures were initiated from the leaf, stem and the fruit explants of *C. asiatica* on MS medium supplemented with Auxins and Cytokinins, alone and in combination in the present study. The explants failed to produce callus in the absence of exogenously provided growth regulators in MS medium. Media supplemented with

PLATE 5.4

Established callus and cell culture

a. Leaf derived callus proliferation on MS + 9.05µM 2, 4-D + 17.74 µM BA

b. Stem- derived callus proliferation on MS+ 4.52µM 2, 4-D + 8.87µM BA

c. Cell biomass obtained from leaf derived cell culture

PLATE 5.4

a

b

c

growth regulators significantly influence the callus development, somatic embryogenesis and plant regeneration in *Centella asiatica* (Martin, 2004).

Inclusion of low levels of Auxins in culture medium resulted in direct root formation from the leaf explants. Similar observations in *C. asiatica* have also been made earlier by Aziz *et al*., (2007). The inclusion of higher levels of NAA provided better results as compared to IAA but, addition of 2,4-D in the medium was found to be most effective for callus formation. 2,4-D has been reported to be an effective hormone for callus formation in other members of *Apiaceae* (Sugano and Hayashi, 1967). Leaf explants inoculated on MS medium fortified with 9.05 µM 2, 4-D produced 184 ± 1.3 mg DW callus. The callus produced was cream coloured, soft and friable. Inoculation of leaf explant on MS medium containing Cytokinin resulted in shoot formation. But, addition of 9.2 µM Kinetin produced 137 ± 1.6 mg DW callus biomass. Incorporation of BA was found to be more favourable for callus formation from leaf explant and addition of 17.74 µM BA resulted in the formation of 193 ± 2.3 mg DW callus. The callus formed was compact and green in colour. Incorporation of 2, 4-D along with BA yielded the best results for callus formation from the leaf explants of *C. asiatica*. The callus formed was compact, dry and green in colour. MS medium supplemented with 9.05µM 2, 4-D along with 17.74 µM BA was found to be the most effective medium for extensive callogenesis from the leaf explant. The callus biomass obtained in this case was 239 ± 1.1 mg DW. On the other hand, best result for callus induction from leaf explant of *C. asiatica* were found with the MS medium supplemented with 1.0 mg dm^{-3} BA and 1.0 mg dm^{-3} NAA by Nath and Buragohain (2005).

In the experiment, the results for callus formation in the case of 2, 4-D and Kinetin were not found encouraging and scanty callus was formed from the leaf explant. In contrast, Paramageetham *et al*., (2004) have reported the production of abundant somatic embryos in *C. asiatica*, when cultured on MS medium with 9.29 µM kinetin in combination with 2.26 µM 2,4-D. Granular, white, shiny clusters of callus developed after 1 week of culture, and then formed heart and cotyledonary stage embryos on the same medium after 4 weeks. Difference in plant race and genotype might account for the difference in responses of *in vitro* cultures towards the different combinations and concentrations of plant growth regulators.

Among all the Auxins employed, 2,4-D was found to be most effective for callus induction from stem explants of *C. asiatica*. 4.52 µM 2, 4-D was found to be

the most effective concentration for callogenesis and produced 88 ± 1.6 mg callus on dry weight basis. IAA and NAA when employed alone produced scanty callus. The Cytokinin BA was found to be more effective than Kinetin for the same. 4.44-8.87 µM BA produced 97 ±2.0 mg DW of callus. The callus produced was green, compact and friable. Incorporation of BA in MS medium containing 2,4-D increased the callogenic potential of stem explants. Fortification of 4.52 µM 2, 4-D with 8.87 µM BA produced the best results for callus formation from stem explant and produced 177± 1.2 mg DW callus. The stem explants cultured on MS medium containing 4.52-18.01 µM 2, 4-D in combination with 4.44-17.74 µM BA gave 100% response towards callus formation. Furthermore, it was observed that, callus formed on the leaf explant proliferated very fast and the stem explants produced callus at a relatively slower rate. Similar observations have also been reported earlier (Patra *et al*., 1998). In tune with present results, callusing was observed on leaf and stem explants of *C. asiatica*, cultured on MS medium supplemented with BAP in combination with 2,4-D by Paramageetham *et al*., (2004) and Aziz *et al*., (2007). In *Falcaria vulgaris*, an important medicinal plant belonging to the family Apiaceae, the maximum callus from leaf explant was induced in medium containing 2, 4-D in combination with BA (Hamideh *et al*., 2012).

Callus cultures have been reported to be initiated from other explants of Apiaceae members. Mature zygotic embryos responded for callogenesis, only on medium containing 0.1 mg/l BAP in *Cuminum cyminum* (Beiki *et al*., 2011). The highest callus induction was obtained from embryo on B5 medium supplemented with 0.2 mg/l NAA and 0.2 mg/l BAP in *Cuminum setifolium* (Safarnejad, 2011). Callus was also induced from the fruit of *C. asiatica*. 0.90 µM 2, 4-D in combination with 1.78 µM BA was found to produce callus from the fruit. The callus was green and friable and also exhibited pink colouration occasionally. The average dry weight of this callus was 123 ± 3.1 mg. However, the frequency of the fruit explants responding towards callogenesis was only 8.16% since the rate of survival of explants was extremely low due to fungal and bacterial contamination.

As a result of this investigation, it can be concluded that, Auxins are important factor for callus induction and Auxin when supplemented with Cytokinin boosts the callus growth and proliferation. The leaf explants were found to be more responsive for callogenesis as compared to the stem explants and optimum callus

formation was achieved from leaf explant on MS medium supplemented with 9.05µM 2,4-D along with 17.74 µM BA.

5.4.2 Morphogenesis in callus culture

Regeneration of whole plants from cells, tissues, or segments from different parts of the plant body is a relatively common phenomenon in most plant groups and is an important means of vegetative propagation in nature as well as in horticulture and agriculture. Buds formed from cells or groups of cells in roots of *Cichorium* and C*onvolvulus,* leaves of *Bryophyllum* and *Kalanehoe,* inflorescences and flowers of *Agave,* and from epidermal cells in the leaves of *Begonia* or the hypocotyl of *Linum* develop into normal adult plants. Buds, shoots, and roots also develop readily in tissue cultures derived from various tissues and organs of a wide variety of flowering plants (Vasil and Vasil, 1972).

The results of the effects of various concentrations and combinations of plant growth regulators have been studied in the present study on *C. asiatica*. Among the tried Auxins, NAA was found to be most favourable for rhizogenesis. Incorporation of 1.07-2.15 µM NAA to the parental medium led to the initiation and formation of tiny, delicate, white hair-like outgrowths coming out of certain parts of callus which later on developed into roots. On the other hand, treatment of callus with IAA and 2, 4-D produced only sparse, thin roots. Conjugation of low levels of Cytokinins BA and Kinetin with NAA resulted into an increase in the number of roots formed from the callus. The effect of Auxins was more or less same in case of stem and the fruit derived callus, the difference being in the callus quality which was found to be light green and more compact. In addition, the number of roots formed was also quite less in stem derived callus, as compared to the leaf derived callus. Rhizogenesis from the callus cultured on plant growth hormones provides evidence to indicate that such differentiation and organogenesis is under hormonal control. The induction of callus growth and subsequent differentiation and organogenesis is accomplished by the differential application of growth regulators and the control of conditions in the culture medium. With the stimulus of endogenous growth substances or by addition of exogenous growth regulators to the nutrient medium, cell division, cell growth and tissue differentiation are induced (Tripathi and Tripathi, 2003).

Treatment of the leaf, stem and fruit derived callus with Cytokinins did not result in the formation of shoot. Presence of 8.87-17.74 µM BA and 9.29-18.58 µM

Kinetin resulted in the formation of green, hard and compact callus. However, when low concentrations of Auxins were used along with the above concentrations of BA and Kinetin, the callus became more compact with concentrated green areas at few places in leaf callus. Also, light brown coloured small outgrowths were observed in case of stem derived callus. These neither got converted into roots, nor into shoots, when sub cultured on different concentrations and combinations of Auxins and Cytokinins. These outgrowths turned brown and perished eventually. The fruit callus also did not show any sign for caulogenesis when cultured on different concentrations and combinations of Cytokinins. Contrary to present results, shoot regeneration has been reported from leaf derived and stem derived callus of *C. asiatica* (Patra *et al*., 1998; Banerjee *et al*., 1999). Shoot regeneration occurred from callus on MS medium containing BAP and low concentrations of 2, 4-D (Aziz *et al*., 2007). But, no such responses were observed in this experiment. The plant genotype and the interaction of explants with the plant growth regulators might be responsible for such differences. Nonetheless, regeneration via callus culture has been reported in various medicinal plants like *Plumbago rosea* (Satheesh and Bhavanandan, 1988), *Dioscorea alata* (Mantell and Hugo, 1989), *Cephaelis ipecacuanha* (Rout *et al*., 1992) and *Asparagus cooper* (Ghosh and Sen, 1994).

5.4.3 Asiaticoside production in callus culture

Saponins are a vast group of glycosides, widely distributed in higher plants. A number of different plant species synthesize triterpenoid saponins as part of normal growth and development with the most predominant group being pentacyclic triterpene derivatives and their sapogenins (Haralampidis *et al*., 2002). The triterpenoid saponin Asiaticoside, found in *C. asiatica* is a pharmacologically active compounds having complex structure, making chemical synthesis an economically uncompetitive option. Plant cell culture has been used in attempts to increase the production of bio-active secondary metabolites of pharmaceutical interest (Gaines, 2004). Considering this, a primary investigation was carried out to assess the biosynthetic potential of *C. asiatica* callus culture obtained on medium enriched with different concentrations and combinations of plant growth regulators.

Results for the accumulation of Asiaticoside in leaf and stem derived callus of *C. asiatica* have been depicted in Tables 5.1 and 5.2 respectively. The callus obtained from fruit did not show the presence of Asiaticoside. On the other hand, all

the cultures derived from both leaf and stem explants revealed the presence of Asiaticoside. But, its quantity varied depending upon the type of explant used as well as the type and concentrations of plant growth regulators employed.

In case of MS medium fortification with Auxins, NAA and IAA were found to be less effective towards Asiaticoside production in the leaf derived callus, as compared to 2, 4-D. Leaf derived callus obtained on Cytokinins was found to yield better results for the production of Asiaticoside. But, the callus initiated on BA was found to be superior in Asiaticoside content among all the PGRs employed. Leaf callus produced on 8.87 µM BA was found to contain 0.189 ± 0.012 mg/g mean Asiaticoside content. Moreover, incorporation of BA (4.44 - 17.74 µM) in MS medium containing 2, 4-D (4.52 - 18.10 µM) resulted in an improvement in Asiaticoside accumulation in leaf derived callus. Best results for Asiaticoside production were obtained on MS + 9.05 µM 2, 4-D + 17.74 µM BA where the leaf derived callus was found to possess 0.201 ± 0.015 mg/g DW Asiaticoside.

Accumulation of Asiaticoside was also detected in all the callus cultures derived from stem explants but, the quantity of Asiaticoside in leaf derived callus was more than the stem derived callus. Stem derived callus initiated on MS containing 4.52 µM 2,4-D in combination with 8.87 µM BA was found to produce the most optimum concentration of Asiaticoside. The Asiaticoside content recorded in the stem derived callus on the said medium was 0.159 µM 0.013 mg/g DW. It was 2.0 times more than the Asiaticoside accumulated in callus obtained on MS + 4.52 µM 2,4-D and 1.72 times higher than that obtained on MS + 8.87 µM BA.

Callus is an undifferentiated tissue which has the ability to develop into any plant organ whether it is a root, shoot or leaf, under the suitable growth hormone concentrations. This might be the reason behind the presence of Asiaticoside in the callus samples, albeit in low concentrations. The detection of Asiaticoside in callus contrasts with the findings of Kim *et al*., (2004) and Aziz *et al*., (2007), who failed to detect Asiaticoside in undifferentiated cells of *C. asiatica*, but is supported by Nath and Buragohain (2005) and James *et al*., (2008) who stated that callus cultures did, in fact, synthesise Asiaticoside. The accumulation of secondary products in plant cell cultures depends on the composition of the culture medium, and on culture conditions (Stafford *et al*., 1986). Ravishankar and Grewal (1991) reported that the influence of media constituents and nutrient stress influenced the production of diosgenin from callus cultures of *Dioscorea deltoidea.* Parisi *et al*. (2002) obtained high yields of

proteolytic enzymes from the callus tissue culture of garlic (*Allium sativum* L.) on MS medium supplemented with NAA and BAP.

This experiment reveals that usage of leaf explant as callus source is more effective than the stem explant for Asiaticoside production. The Asiaticoside content also varied depending upon the type of PGR used. Leaf explant cultured on MS medium containing BA and 2, 4-D resulted in an increased production of Asiaticoside. Optimum Asiaticoside content was detected in 4 week old leaf callus cultured on MS medium supplemented with 9.05 µM 2,4-D in combination with 17.79 µM BA where the leaf derived callus was found to possess 0.201 ± 0.015 mg/g DW mean Asiaticoside content. Moreover, although Asiaticoside was found to be present in both leaf and stem derived callus cultured on different combinations of Plant Growth Regulators, the content of Asiaticoside was found to be less as compared to *in vitro* raised intact leaf biomass.

5.4.4 Asiaticoside production in cell culture

The development of alternative and complimentary methods to whole plant extraction for the production of clinically important secondary metabolites is an issue of considerable socio-economic importance. Plant cell and tissue culture systems are complementary and may provide competitive metabolite production systems when compared to whole plant extraction (Dicosmo and Misawa, 1995). In the present study, cell cultures of *C. asiatica* were established and analysed for Asiaticoside production.

The leaf derived callus cultured on MS medium supplemented with 9.05 µM 2, 4-D and 17.74 µM BA yielded the best results for Asiaticoside and biomass production. Therefore, this combination of growth regulators was selected for the establishment of cell culture. Under the described experimental culture conditions, cell suspensions of *C. asiatica* exhibited an initial lag phase up to 8 days of incubation. This was followed by a steep rise in the growth rate during the third week. This represented the log phase. The cells entered the stationary phase of growth eventually. A decline in the growth of cells was observed after fourth week during which the cells exhibited a change in colour from light green to straw colour, followed by senescence. But, Bouhouche *et al*., (1998) have reported a latent phase between 0 and 3 days and an exponential growth phase from 3 to 12 days and a stationary phase from 12 to 14 days under the same conditions. However, Nath and

Buragohain (2005) found an initial lag phase up to day 10 of incubation for *C. asiatica* cell suspensions followed by a rise in growth rate until the third week.

It was also observed that the cells in the suspension culture grew with a higher rate as compared to the cells in callus. 358 ± 6.4 mg dry weight biomass was obtained from the cell culture. Analysis of the spent liquid medium for Asiaticoside content showed no positive results for the presence of Asiaticoside. Similar findings in case of spent medium analysis for Asiaticoside presence were obtained by James *et al*., 2008. Analysis of cell culture for Asiaticoside content showed that, maximum Asiaticoside accumulation was on day 21st, where 0.426±0.016 mg/g DW Asiaticoside content was recorded to be present in the cell biomass harvested. This was 2.12 times more as compared to the Asiaticoside produced in the callus. The Asiaticoside in the suspension-cultured cells was found to be 2.60 times higher amount than in callus samples by Nath and Buragohain (2005). But, Asiaticoside was undetectable in undifferentiated cells of Korean *C. asiatica* as reported by Kim *et al*., (2004).

Differences between varieties in medicinal plants of the same species (chemotypes) are common and variation in secondary metabolites has been observed with identical phenotypes and growth conditions, depending on plant origin (Aziz *et al*., 2007). Significant differences in active constituents have therefore also been observed between samples of *C. asiatica* originating from different countries (Das and Mallick, 1991). Moreover, the biosynthesis of major secondary metabolites is often either tissue or organ specific (Aziz *et al*. 2007). This also seems to be the case in *C. asiatica* where Asiaticoside, could not be detected in undifferentiated cells of a Korean chemotype (Kim *et al*., 2002a). In contrast, detectable levels of the Asiaticoside in cultured cells (callus and cell suspensions) were reported in South African chemotypes (James *et al*., 2008). In the study carried out by Aziz *et al*. 2007, Asiaticoside was not detected in undifferentiated callus. Two morphologically distinct phenotypes of *C. asiatica* in South Africa were analysed in relation to the level of Asiaticoside produced in undifferentiated cultured cells and leaves (James *et al*., 2008). In both cases Asiaticoside was detected in undifferentiated cells (callus and cell suspensions). The reasons for variability in the Asiaticoside content can be due to climate, seasonal and geographical conditions, harvesting times and storage conditions. Furthermore, the differences can also be attributed to genomic diversity and variation in the oxidosqualene cyclase and other genes involved in the

biosynthesis (Haralampidis *et al*., 2002; Kim *et al*., 2007), as well as the presence and activity of enzymes involved in the attachment of the sugar residues to the aglycones (James *et al*., 2008).

From the present investigation, it is clear that *C. asiatica* cell cultures are capable of producing secondary metabolite of interest. However, Asiaticoside accumulation in cell cultures was comparatively less than the content in the *in vitro* raised shoot biomass. The cell cultures were initiated from the leaf derived callus and maintained on MS liquid medium supplemented with 9.05 µM 2, 4-D and 17.74 µM BA. Moreover, Asiaticoside content in the cell culture was found to be 2.12 times more as compared to the Asiaticoside produced in the callus.