CHAPTER IV

EXPERIMENTAL RESULTS

4. Experimental Results

Experimental results obtained in the present investigation, entitled "Genetic Improvement of Bharmi (*Centella asiatica* (Linn) Urban) For High Yield of Saponins", employing the methods mentioned in the previous chapter are presented below.

In the present investigation, colchicine was used to induce autotetraploidy in *Centella asiatica*. The techniques adopted for induction of autotetraploidy using colchicine agent was described in Materials and Methods.

Methods of induction of autotetraploidy and induction of somaclonal variation were used in the present investigation for obtaining genetically improved phenotypes of *Centella asiatica,* capable of producing high yields of saponins. The concentration of colchicine and duration of treatment was determined based on plant survival rates. Morphology of the plants and stomatal characteristics of leaves were examined in order to get first idea of probable autotetraploids. Later, chromosome counts were undertaken to establish the ploidy of these plants. Saponins content of the autotetraploid and their control counterparts, was analysed using HPLC analysis method. Details of experimental results of these are as follows:

4.1. Effect of Colchicine Concentration on Survival Rate of Treated plants

The control and plants treated with colchicine of different concentrations (0.1 to 1.0%) for a fixed treatment duration of 4 hours were maintained in a greenhouse under the same environmental conditions for further growth. After 3 months of growth, plants were analyzed for the survival rates. Experimental data obtained was shown in Table. 4.1.

Highest survival rate (98.75%) of colchicine treated plants was found in plants subjected to treatment with lowest concentration of colchicine (0.1%) and lowest survival rate (1.25%) was found in plants subjected to treatment with highest concentration of colchicine (1.0%) (Table 4.1). This clearly indicates that

the rate of survival of the colchicine treated plants was inversely proportional to the rate of concentration of colchicine used in the experiments. In general all plants subjected to treatment with colchicine showed lower survival rates as compared to those of the control ones.

The survived plants were compared with the control plants for morphological parameters viz., size of the plant, size of leaves, leaf area, inter-

Table.4.1. Effect of colchicine concentration on survival rate and response of induction of autotetraploidy in *Centella asiatica*.

Colchicine Conc. (in % W/V)[a]	**Number of Running Shoots**		**Survival Rate (%)**	**Auto-tetraploids [c]**
	Treated	**Survived plants[b]**		
Control	80	80	100	0
0.1	80	79	98.75	0
0.2	80	74	92.5	8
0.3	80	62	77.5	7
0.4	80	49	61.25	0
0.5	80	41	51.25	0
0.6	80	36	45.00	0
0.7	80	19	23.75	0
0.8	80	8	10.00	0
0.9	80	7	8.75	0
1.0	80	1	1.25	0

[a] Treatment duration is 4 hours.

[b] Data was recorded after 45 days for colchicine treatment.

[c] Autotetraploidy was identified according to changes in stomata size or density, phenotypic characteristics, chromosome counting and secondary metabolites relative to diploid controls.

nodal length, thickness of leaf, leaf colour, fresh & dry weights of 100 leaves, length & width of petiole, size of seeds, weight of 100 seeds, size of flower bud, weight of 100 fresh buds and number & size of stomata per unit area.

As the autotetraploids exhibited changes in the morphological traits such as robust size, increased width to length ratio of leaves and more vigorous growth as compared to normal diploid plants, morphological characters were used as primary indicator to identify the autotetraploids of *Centella*. The morphological markers like robust size, larger leaves, long & thick petioles, larger floral buds, larger seed size and large thick stolons were used for primary identification of the autotetraploids.

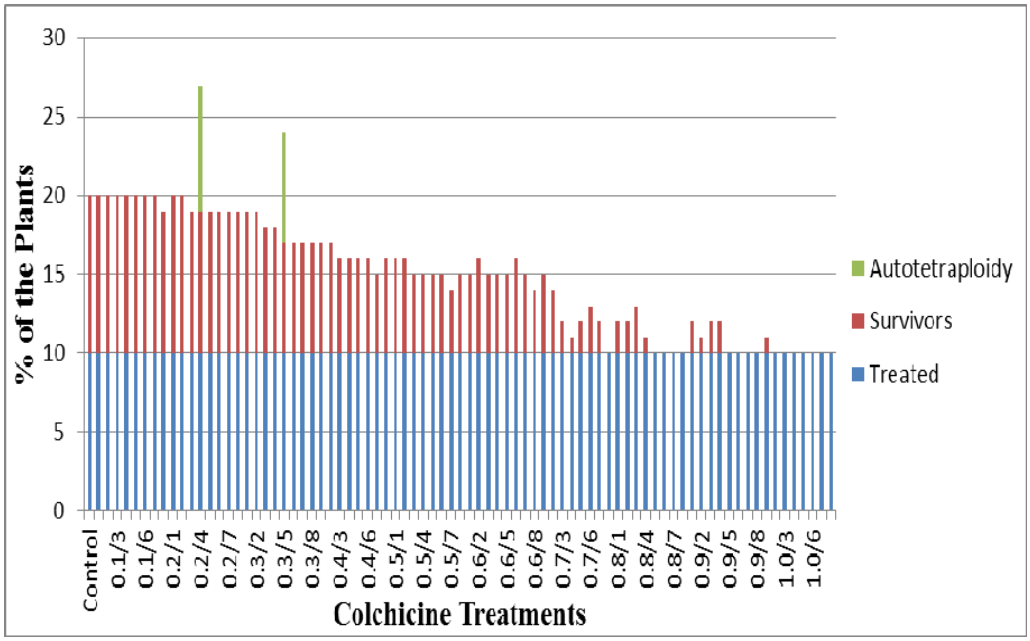


Fig. 4.1. Survival rates and response of *Centella asiatica* plants to colchicine treatment and autotetraploidy induction.

Autotetraploids showed variation in stomatal characteristics such as increase in stomata size and a reduced frequency of stomata per unit leaf area, as compared to diploid plants. So, stomatal characters were used as an important preliminary indicator of plants with the autotetraploidy level.

Chromosome counts of the promising plants were undertaken and the plants showing double the number of chromosomes as compared to control were considered as autotetraploids. Thus chromosome counts were taken as final criterion to establish the ploidy level of the treated plants. After confirmation of ploidy level, treated plants were analyzed for the quantity of saponins present in them.

4.2. Effective Method of Colchicine Treatment for Induction of Autotetraploidy in *Centella*

Out of the three methods of colchicine treatment (Injection of stolons with colchicine, artificially injured shoot tips and method of immersion of running shoot tips in colchicine), the last i.e., the method of immersion of running shoot tips in colchicine was found to be very effective method for induction of autotetraploidy in *C. asiatica*. The other two methods viz., Injection of stolon with colchicine and application of colchicine to the artificially injured shoot tips were found to be less effective in inducing autotetraploidy in *Centella*.

4.3. Effective Concentration of Colchicine and Treatment Duration for Induction of Autotetraploidy

Pilot experiments were conducted to determine the effective concentrations of colchicine and treatment duration that could induce maximum frequency of autotetraploidy in *Centella*. Different concentrations of colchicine, ranging from 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0% for 1 to 8 hours were tried. Results obtained on survival rates of *Centella asiatica* to different concentrations of colchicine and treatment durations are presented in Table 4.1 and Fig. 4.1. The treated plants showed some disorders in the growth rates and delayed appearance of running shoots. Application of higher colchicine concentration and treatment duration, made the first running shoots damage and inhibit growth and at the same time reduced the survival rates (Table 4.1 and Fig. 4.1). After colchicine treatment many plants did not survive because they caused visible tissue creation disorders.

On the basis of this investigation, it is inferred that *Centella asiatica* plants subjected to treatment with 0.4 to 1.0% for 1 to 8 hours did not show any signs of polyploidy even after hundreds of trials. Runners treated with higher concentrations of colchicine (0.4 to 1.0%) for more than 2 hours showed thick, short and swollen internodes as compared to untreated control counterparts (Fig. 4.4.). However the plants treated with 0.1 to 0.3% ranges of colchicine, especially 0.2% for 4 hours and 0.3% for 5 hours showed signs of polyploidy (Table 4.1). On the basis of change in morphological traits of plants, (ex. the first leaves appearing after treatment), 20 plants or 75% of survived plants, were suspected to be autotetraploids and they were selected for cytological examinations such as size of

Fig. 4.2. Field view of external morphology of control (Black Arrows) and 0.2% colchicine for 4 hour treated *Centella* plants (Orange Arrows).

Fig. 4.3. Field view of external morphology of control (Black Arrows) and 0.3% colchicine for 5 hour treated *Centella* plants (Yellow Arrows).

stomata and number of stomata per unit area of leaf and finally for chromosome analysis. These plants were bigger in size and showed larger leaves as compared to their control counterparts (Figs. 4.2 and 4.3).

All the colchicine treated plants were monitored for 2 to 3 generations. Only the plants treated with 0.2% colchicine concentration for 4 hours (Fig.4.2)

and 0.3% colchicine concentration for 5 hours (Fig.4.3) continued to show variations. It was further noted that the increase in colchicine concentration reduced the number of autotetraploids and it was 80% and 70% at 0.2% and 0.3% colchicine, respectively (Table- 4.1 and Fig. 4.1). During the fourth generation, only plants treated with 0.2% and 0.3% colchicine were allowed to grow in the field and all other plants with colchicine and are not showing any morphological variations were eliminated from the field.

Fig.4.4. Colchicine treated running tip showing swelling of internodes (Red Arrow) and Leaves showing the saw type margin *Centella asiatica* plants (Orange Arrow).

4.4. Establishment of Autotetraploidy in Colchicine treated C*entella* plants

Centella asiatica plants treated with 0.2% colchicine for 4 hours duration, were allowed to grow for 3 generations in the polyhouse and were later transferred to field. They were further allowed grow for another 3 generations. After their adaptation to field conditions, both the treated and untreated plants were subjected to analysis of morphometric and stomatal characters and chromosome counts.

4.5. Morphometric Traits of Colchiploids

Induction of autotetraploidy is associated with variations in morphometric traits. Significant morphological variations were observed between the colchicine

treated and control plants of *C. asiatica*. 12 important morphometric characters viz., Plant Height, Inter-nodal length, Length and width of leaves, Leaf area, Thickness of leaf, Length and Width of petioles, Fresh and Dry weights of 100 leaves and Weight of 100 seeds and buds, were analyzed in colchicine induced autotetraploid plants. The morphological differences observed between the diploid and colchicine (0.2% for 4 hour) induced autotetraploids of *C. asiatica* are presented in Table 4.2.

4.5.1. Plant Height

According to morphological observations, the plant height of the 3 month old *Centella asiatica* plants were measured as 13.44 ± 1.13 cm in diploids and 23.21 ± 1.10 cm in 0.2% for 4 hour colchicine treated autotetraploids (Table 4.2 and Fig.4.5). The difference in height of the colchiploids was statistically significant and 72.69 % higher as compared to that of diploid plants.

Fig.4.5 Morphological variations in leaf size and difference in plant height of diploid (left) and 0.2% colchicine (4 hours) treated autotetraploid (right) plants of *C. asiatica*.

4.5.2. Inter-nodal Length

The results demonstrated that the average inter-nodal length of the main stem in control diploid plants was 6.78 ± 0.54 cm and that of the colchiploids was

9.64 ± 0.38 cm. Difference in inter-nodal length between these two was 42.18% higher and statistically significant (Table 4.2 and Fig.4.5).

4.5.3. Leaf Characteristics

Leaves of colchicine treated *Centella asiatica* plants were observed to be dark green, more dissected and dented at their margins as compared to non-treated plants. The diploid and autotetraploid plants exhibited difference in length of (Table 4.2 and Fig.4.6). Mean length of leaves in diploid plants was 4.61 ± 0.40 cm where as it was 6.83 ± 0.12 cm in autotetraploids (Table 4.2). Thus the autotetraploids showed longer leaves (48.15% longer) as compared to their diploid counterparts. The difference in length of leaves is statistically significant at $P<0.01$ level.

Similarly the autotetraploids also showed difference in width of leaves. The colchiploids showed a mean leaf width of 5.66 ± 0.18 cm, where as the diploids showed a mean leaf width of 4.95 ± 0.10 cm. Thus the colchiploids

Fig.4.6. Morphological variation in leaf size and difference in plant petiole length of diploid (left) and colchiploid (0.2% for 4 hour) (right) plants of *C. asiatica.*

showed a 14.34% difference in width of leaves as compared their diploid counterparts. This difference in width of leaves is statistically significant at $p=0.01$ level.

Table.4.2. Differences in Morphometric characters of diploid and colchicine treated (0.2% for 4 hours) plants of *C. asiatica*.

Sr.No	Parameters	Control (Diploid)	Colchiploid	Difference
1	Plant height (In cm)	13.44 ± 1.13	23.21 ± 1.10**	72.69 %
2	Inter-nodal length (In cm)	6.78 ± 0.54	9.64 ± 0.38*	42.18 %
3	Length of leaf (In cm)	4.61 ± 0.40	6.83 ± 0.12*	48.15 %
4	Width of leaf (In cm)	4.95 ± 0.10	5.66 ± 0.18*	14.34 %
5	Leaf area (In cm^2)	65.81 ± 11.52	103.4 ± 12.14**	57.11 %
6	Thickness of leaf (In cm)	0.2 ± 0.10	0.6 ± 0.18**	200 %
7	Length of petiole (In cm)	7.59 ± 0.42	10.41 ± 1.12*	37.15 %
8	Width of petiole (In cm)	1.07 ± 0.14	1.74 ± 0.25**	62.61 %
9	Fresh Weight of 100 leaves (In gms)	19.00 ± 0.62	57.06 ± 0.50**	200.31 %
10	Dry Weight of 100 leaves (In gms)	3.10 ± 0.07	8.80 ± 0.16**	183.87 %
11	Fresh Weight of 100 floral buds (In gms)	2.94 ± 0.20	9.69 ± 0.20**	229.59 %
12	Dry Weight of 100 seeds (In gms)	155 ± 0.12	270 ± 0.09**	74.19%

Values are means ± standard error, statistically significant difference at P<0.01* and 0.05** as determined by t-test.

Difference leaf area was also observed between the diploid and autotetraploid plants. Autotetraploids showed larger leaves as compared to diploids. Leaf area of diploid plants was 65.81 ± 11.52 cm^2 where as the leaf area

of colchicine treated autotetraploids was 103.4 ± 12.14cm^2 (Table- 4.2 and Fig.4.6). Thus there is an increase in leaf area by 57.11% in autotetraploids as a result of diplotization with colchicine. The observed difference in leaf area is statistically significant at $p < 0.05$ level.

Thickness of leaves of diploid plants was 0.2 ± 0.10cm; while it was 0.6 ± 0.18cm in 0.2% for 4 hour colchicine treated *Centella asiatica* plants (Table 4.2). It was 200% increase in thickness of leaf for colchiploids as compare to diploid *Centella asiatica* plants. The angle of the leaf basis and the leaf thickness were also indicating statistically significant differences at $p < 0.05$ in colchiploids as compared to diploids.

4.5.4. Length and Width of Petioles

The 0.2% for 4 hour colchicine treated *Centella* plants showed longer and wide leaf petioles, as compared to those of untreated ones. Mean petiole length of diploid *Centella plant* was 7.59 ± 0.42 cm, where as it was 10.41 ± 1.12 cm in colchicine treated plants. These results indicate that the colchiploids showed 37.15 % statistically significant difference in petiole length as compared diploids. Similarly Mean width of petiole of diploid *Centella plants* was 1.07 ± 0.14cm , where as it was 1.74 ± 0.25cm in colchicine treated plants (Table 4.2 and Fig.4.6). Thus colchiploids showed a statistically significant difference of 62.61% in petiole width as compared to diploids.

4.5.5. Fresh and Dry Weight of Leaves

The fresh weight of 100 leaves of the colchicine induced autotetraploidy (0.2% for 4 hour) *C. asiatica* plants was increased several times as compared to corresponding control plants. The fresh weight of 100 leaves was 19.00 ± 0.62 gm in control and 57.06 ± 0.50 gm in colchiploids plants. Thus, there is a three fold increase in fresh weight in the colchicine induced autotetraploids as compared to those of control ones. Similar difference in dry weight of 100 leaves was also observed between the two. Diploid plants showed 3.10 ± 0.07 gm dry weight of 100 leaves, where as the autotetraploid plants showed a dry weight of 6.83 ± 0.70 gm. Thus there is an increase of 183.87 % in dry weight of 100 leaves in

Colchicine induced autotetraploids (Table 4.2). Thus, there is a 2.7 fold statistically significant (p=0.05) increase in dry weight of colchicine induced autotetraploid leaves as compared to control ones.

4.5.6. Mean Weight of Flower Buds and Seeds

The fresh weight of 100 flower buds was also increase as compare to the corresponding control *C. asiatica*. Fresh weight of 100 floral buds was 2.94 ± 0.20 gm for diploids and 9.69 ± 0.20 gm for colchicine induced autotetraploids (Fig.4.7). Thus there is an increase in fresh weight of 100 floral buds by 229.59% in colchiploids as compared to the diploid *C. asiatica* plants (Table- 4.2).

Dry weight of 100 seeds was 155 ± 0.12mg in diploids; while it was 270 ± 0.09 gm in a colchicine induced autotetraploids. Thus the colchiploids showed 74.19% increase in dry weight of 100 seeds as compared to the diploid plants

Fig.4.7. Variation in size of floral buds of diploid (left) and colchiploid (right) plants of *C. asiatica*.

Fig.4.8. Variation in size of seeds of diploid (left) and colchiploid (right) plants of *C. asiatica*.

(Table 4.2 and Fig. 4.8). This difference was found to be statistically highly significant (p=0.05).

4.6. Stomatal Characteristics of Colchiploids

Microscopic analysis of stomatal length and width of control and colchicine induced autotetraploid plants (Colchiploids) revealed that the colchiploids showed significantly larger stomata as compared to the diploid *Centella asiatica* plants. The width of stomata of diploid plants was 21.70 ± 0.41 µm, while it was 35.43 ± 2.33 µm in autotetraploid plants. Thus there was an increase of 63.27% in stomata size in autotetraploids as compared to control plants, as a result of induction of autotetraploidy. The difference in stomata width of diploid and autotetraploidy was statistically significant as inferred by the two-sample t-test at P≤0.05. Similarly, stomatal length was determined as 31.49 ± 3.09 µm for diploid and 46.34 ± 2.45 µm for autotetraploid *Centella* plants. 47.15 % increase in stomatal length was observed in colchicine induced autotetraploid plants as compared to the diploid plants (Table 4.3; Figs. 4.9 and Fig.4.10).

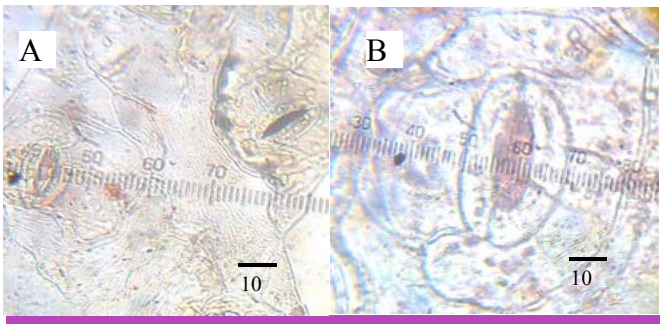


Fig.4.6. Stomata size of diploid (A) and autotetraploid (B) plants of *Centella asiatica*

However, length and width of stomata was found be significantly higher in autotetraploid plants.

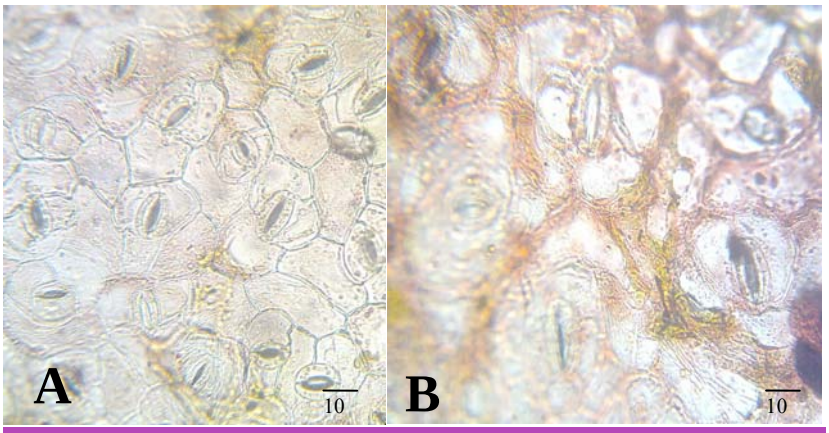
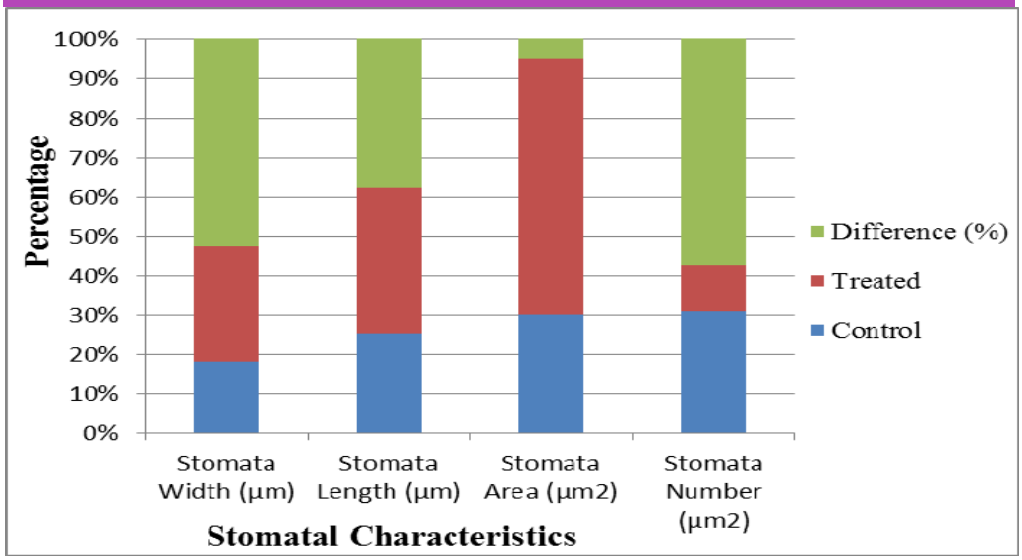


Fig.4.10. Stomata number of diploid (A) and auto-tetraploid (B) plants of *Centella asiatica*

Microscopic analysis of stomata revealed that stomatal area in autotetraploid *Centella* plants are significantly larger as compared to those of diploids. Stomatal area of diploid plants was 698.82 ± 74.48 μm^2, while it was 1500 ± 65.94 μm^2 in autotetraploids. The stomatal area of colchicine induced plants was 114% times greater than diploid *Centella asiatica* plants (Table 4.3; Fig. 4.11 and Figs.4.9 A and B). The control plants of *Centella asiatica* analysis of stomata showed that the stomatal number per unit area is larger than those of colchicine induced plants.

Fig. 4.11. Comparison of stomatal characteristics of control and colchicine induced *Centella* Plants



Study of stomatal density revealed maximum number of stomata (20.26 ± 1.19) in the per unit area of the leaves of diploid plants than autotetraploidy plants (7.62 ± 0.53). The number of stomata per unit area showed a decrease of 37.61% in colchicine induced autotetraploid *Centella* plant as compared to their corresponding control plants (Table 4.3, Figs. 4.11, 4.10 A and B). These results indicate that, the number of stomata per unit area was less in colchicine induced autotetraploids of *Centella asiatica* leaves as compared to those of control. The number, size and frequency of stomata have been useful for the comparison of polyploids particularly the diploids and autotetraploids. Hence, our results clearly indicate that autotetraploidy plants showed fewer stomata but they are large with respect width and length as compared to control plants. On the contrary diploid plants showed more number of stomata but the length and width of stomata are much less as compared to their autotetraploid counter parts.

Table.4.3. Comparison of stomatal characteristics in diploid and auto-tetraploid plants of *Centella asiatica*.

Sr. No	Stomatal Character	Control (Diploid)	Treated (Autotetraploid)	Difference (%)
1	Width of Stomata (in µm)	21.70 ± 0.41	35.43 ± 2.33*	+ 63.27%
2	Length of Stomata (in µm)	31.49 ± 3.09	46.34 ± 2.45*	+ 47.15 %
3	Stomata Area (in μm^2)	698.82 ± 74.48	1500 ± 65.94*	+ 114.32%
4	Number of Stomata	20.26 ± 1.19	7.62 ± 0.53*	- 37.61%

Values are means ± Standard error, * statistically significant difference at $P<0.05$ as determined by t-test, (+) symbol means increase and (-) decrease in stomatal difference.

4.7. Ploidy and Chromosome Number of Colchiploids

The putative autotetraploid *Centella asiatica* plants, selected on the grounds of morphometric characters and variation in stomatal characters, were then propagated in the greenhouse for further cytological and other investigations. The ploidy level of the these plants was then confirmed by the determination of their chromosome number. Cytological analysis was carried out on root tips of colchicine treated plants of *C. asiatica* to determine the true effect of colchicine in chromosome doubling. Chromosome analysis of root tips of the colchicine treated plants revealed the occurrence of tetraploid level. This clearly indicates that the diploid control plants had a chromosome number of 2n=18 (Fig.4.12A), whereas the chromosome number of the colchicine treated plants is 2n=36 (Fig.12B). From these cytological observations it is confirmed that the tetraploid plants were obtained by the treatment with 0.2% and 0.3% colchicine solutions, with

immersion times of 4 and 5 hours respectively. These results indicated that the optimum tetraploid induction was performed with a lower concentration of colchicine and minimum immersion time.

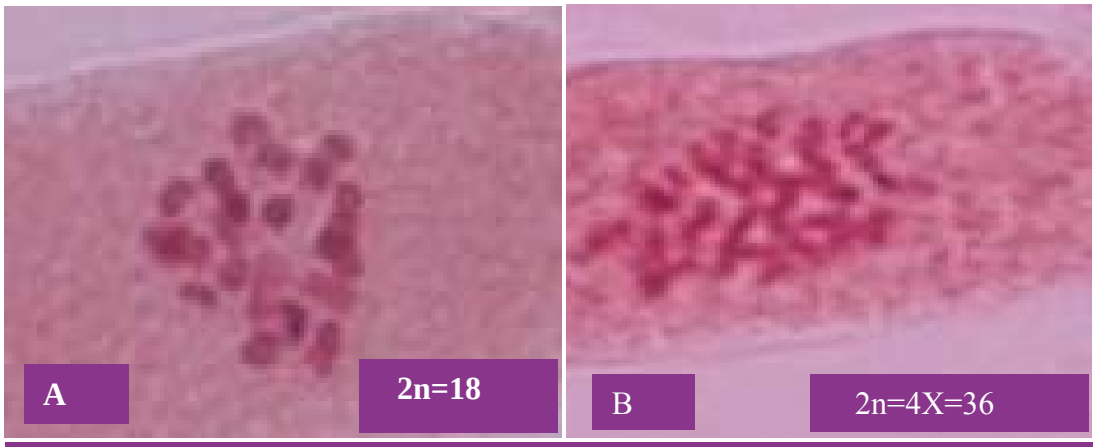


Fig.4.12. Chromosome number of diploid (A) and tetraploid plants (B) of *Centella asiatica*

4.8. Yield of Triterpenoid Saponins in Colchiploids

Yield of triterpenoid saponin compounds in colchiploids and control diploid plants of *Centella asiatica* were estimated using the technique of High Performance Liquid Chromatography (HPLC). Compounds of these triterpenoid saponins (medecassic acid, terminolic acid, asiatic acid and asiaticoside) varied greatly in colchiploid and diploid plants of *C. asiatica.* These results are presented in table 4.4.

The HPLC analysis of diploid and autotetraploid *Centella* plants indicated that the colchicine induced autotetraploid plants showed higher levels of triterpenoid saponins as a whole as compared to diploid *C. asiatica* plants. Results revealed that the colchicine induced autotetraploid plants showed highest medecassic acid and terminolic acid (0.3 gm/gm dry wt.) contents which is 1.30 times (76.47%) higher than the diploids as compared to the untreated control plants (Table 4.4). These results clearly indicate that the endogenous levels of medecassic acid and terminolic acid have gone up by 1.30 fold due to autotetraploidization.

Table.4.4. Triterpenoid saponin contents of diploid and autotetraploid plants of *Centella asiatica.*

Sr.No	Parameter	Diploids (%w/w)	Auto-tetraploids (%w/w)	Difference (%)
1	Madecassoside + Asiaticoside B (gm/gm. dry weight)	2.97	2.61	- 12.12 %
2	Asiaticoside (gm/gm. dry weight)	0.92	0.95	+ 3.26 %
3	Medecassic acid + Terminolic acid (gm/gm. dry weight)	0.17	0.3	+ 76.47 %
4	Asiatic acid (gm/gm. dry weight)	0.06	0.17	+ 183.33 %

(+) Symbol means increase and (-) decrease in triterpenoid saponins difference.

HPLC analysis of control and colchicine induced autotetraploid plants revealed differences in their asiatic acid content (Figs. 4.13 and 4.14). The asiatic acid content of the colchicine induced autotetraploid plants was 0.17 gm/gm dry wt., and that of control diploid plants was 0.06 gm/gm dry wt. (Table 4.4). Thus the autotetraploids produced more than double the quantity of asiatic acid (+283.33%) as compared to the corresponding normal diploid *C. asiatica*. These results also clearly indicate that the endogenous level of asiatic acid has 2.8 fold due to autotetraploidization.

The colchicine induced autotetraploid plants of *Centella* showed a non–significant increase of 3.2% in asiaticoside over that of the diploid *Centella asiatica* plants. The asiaticoside content was 0.92 gm/gm. dr. wt. in control plants where as it was 0.95 gm/gm dr.wt. in colchicine induced autotetraploids *Centella asiatica* (Table 4.4). The diploid and colchicine induced autotetraploid plants did

not show any significant difference in the contents of medecassoside and asiaticoside B contents (Table 4.4 and Figs. 4.13 and 4.14).

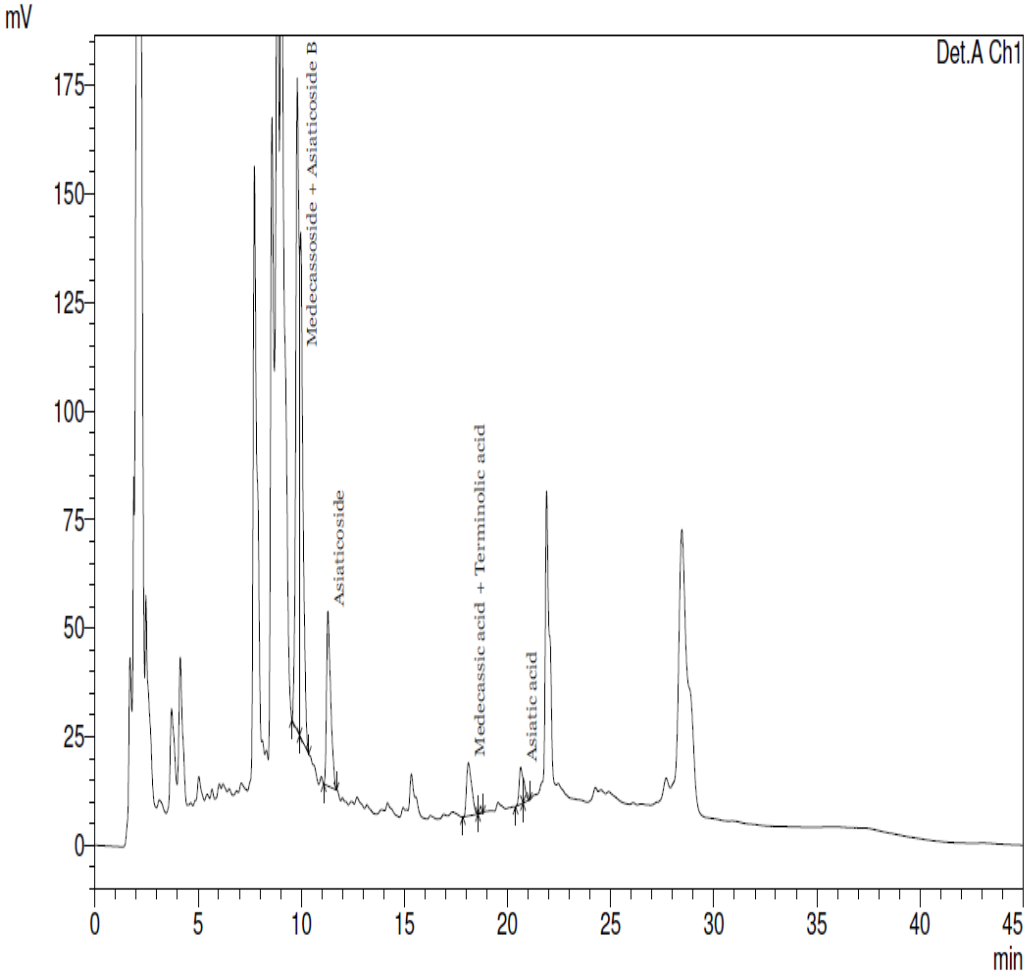


Figure 4.13. HPLC Chromatogram of the control plant extracts of *Centella asiatica* used for the quantitative determination of triterpenes

Table 4.5. HPLC analysis of peak area and retention time for madecassoside + asiaticoside B, asiaticoside, madecassic acid + terminolic acid and asiatic acid in diploid *C. asiatica* plant.

Peak #	**Ret. Time**	**Name of Chemicals Compounds**	**Area**	**Area %**	**Height %**
2	9.970	Medecassoside+Asiaticoside B	1264740	33.185	34.866
3	11.291	Asiaticoside	554844	14.558	12.055
4	18.102	Medecassic acid + Terminolic acid	210401	5.521	3.682
6	20.637	Asiatic acid	92925	2.438	2.554

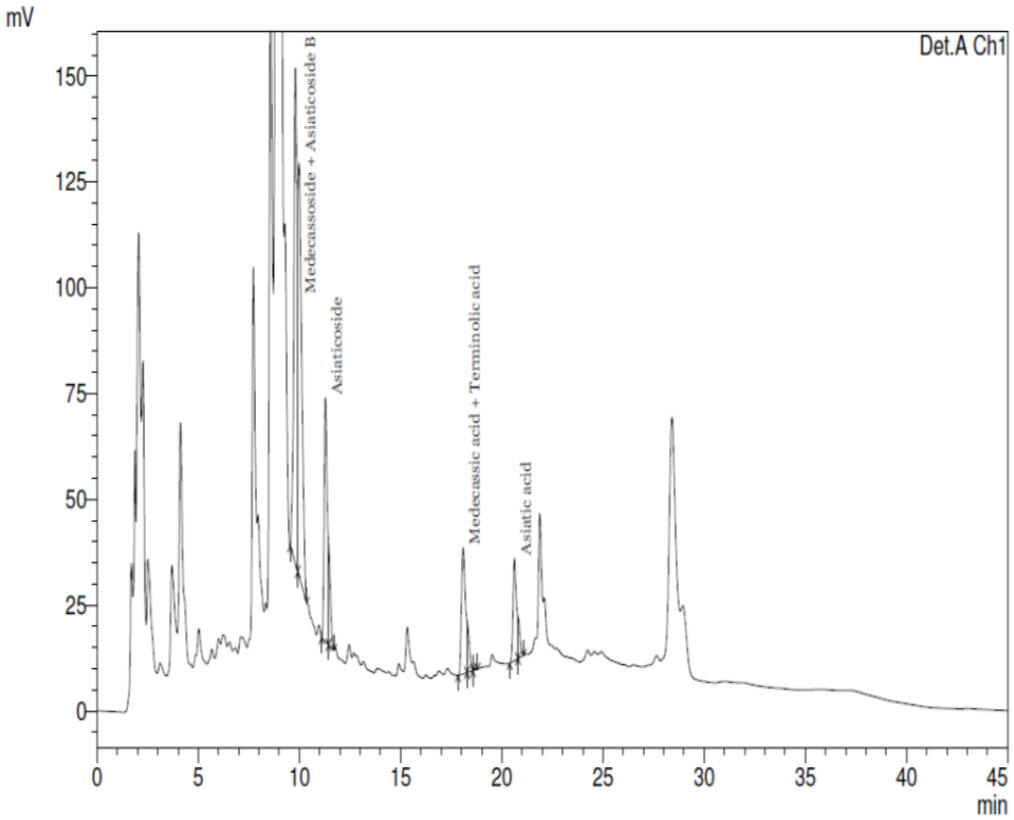


Figure.4.14. HPLC Chromatogram of the colchicine induced autotetraploid plant extracts of *Centella asiatica* used for the quantitative determination of triterpenes.

Table.4.6. HPLC Analysis of peak area and retention time for madecassoside + asiaticoside B, asiaticoside, madecassic acid + terminolic acid and asiatic acid in colchicine induced autotetraploid to the *Centella asiatica*

Peak #	**Ret. Time**	**Name of Chemicals Compounds**	**Area**	**Area %**	**Height %**
2	9.976	Medecassoside+Asiaticoside B	1249312	29.585	26.266
3	11.278	Asiaticoside	612914	14.514	15.493
5	18.087	Medecassic acid + Terminolic acid	402504	9.532	8.015
8	20.620	Asiatic acid	281025	6.655	6.482

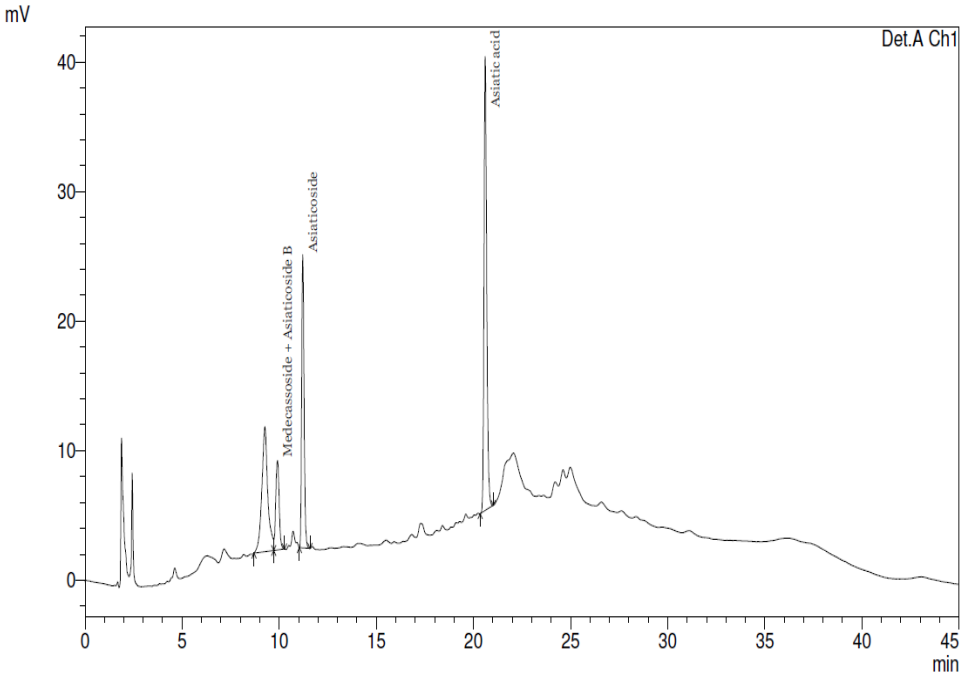


Figure.4.15. HPLC Chromatogram of the standard used for the quantitative determination of triterpenes.

Table.4.7. HPLC Analysis of peak area and retention time for madecassoside + asiaticoside B, asiaticoside and asiatic acid in standard to the *Centella asiatica*.

Peak #	**Ret. Time**	**Name of Chemicals Compounds**	**Area**	**Area %**	**Height %**
2	9.910	Medecassoside+Asiaticoside B	84895	10.371	9.297
3	11.210	Asiaticoside	192178	23.477	30.483
4	20.603	Asiatic acid	337090	41.181	47.228

4.9. Induction of Somaclonal Variations

For induction of somaclonal variations, young leaf explants of *C. asiatica* were grown aseptically on MS medium supplemented with different combinations of growth hormones as per methods described in chapter 3 and are presented in Tables 4.8, 4.9 and 4.10. These are briefly described below.

4.9.1. Induction of Somaclonal Variations through Leaf explant Cultures

Fig.4.16. Leaf explants developed within week of inoculation

For initiation and establishment of callus from *Centella asiatica,* leaf explants were cultured on MS medium having different combinations and concentrations of BAP and 2-4 D and IAA. Response of Centella leaf explants to these different combinations of growth hormones varied. The leaf explants of *C. asiatica* developed callus within 12-14 days of inoculation. Callus initiation started at cut margins and later extend to the entire surface of explant within 18-24 days (Figs. 4.16 and 4.17).

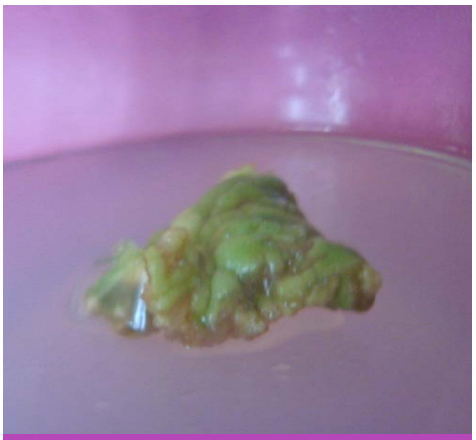


Fig.4.17. Leaf explant of *Centella asiatica* with primary callus

Green, compact and embryogenic calli were observed with 40-90% response at different concentrations of BAP, 2, 4-D and IAA. The best callogenic response from leaf explants was obtained on MS medium fortified with combination of 4 mg/l BAP and 2 mg /l 2, 4-D. It was observed that 86.66% response was seen only after a period of 16-19 days after inoculation (Table.4.8 and Fig 4.18A). In this combination of growth hormones, profuse greenish callus proliferation was

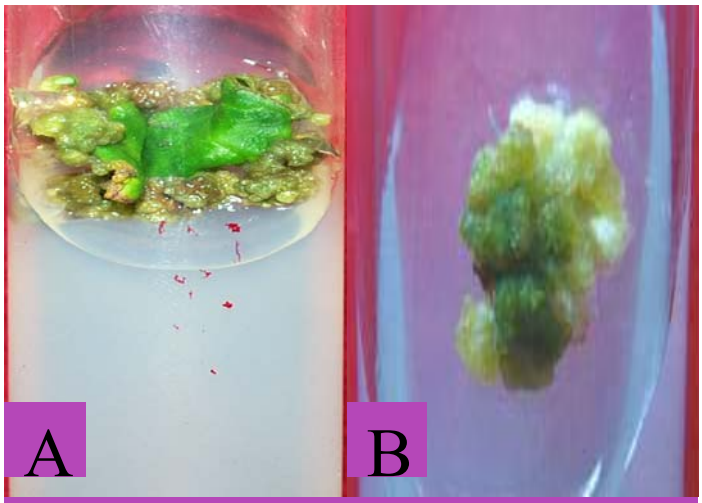


Fig.4.18. (A) Compact callus initiation through leaf culture (B) Brownish callus formation on 2mg/l 2-4 D +4mg/l BAP

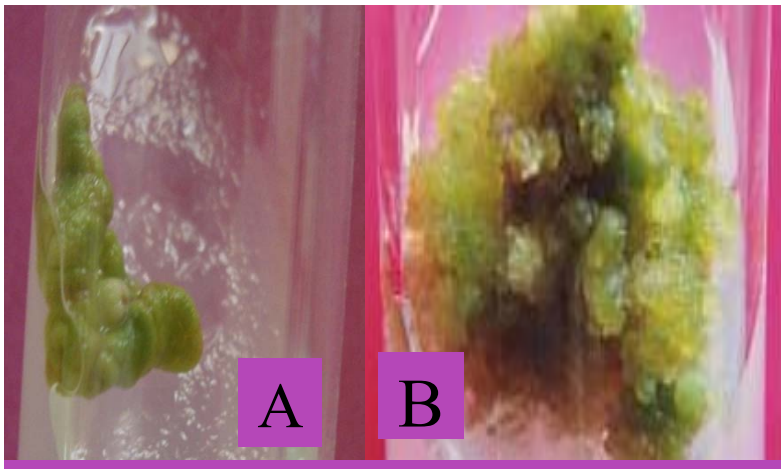


Fig.4.19. (A) Callus Induction on MS medium supplemented with 1.5mg/l IAA and 3 mg/l BAP (B) Initiation of embrogenic callus after 21-25 days

noticed within 10-12 days of primary culture and after 20-25 days of sub-culture. Most of these calli turned to brownish green (Fig. 4.18 B).

The MS medium supplemented with 3 mg /l BAP and 1.5

Table 4.8. Effect of combination of 2,4 D / IAA + BAP on proliferation rate and percent induction of callus in *Centella asiatica* leaf explants.

Sr. No	Plant Hormones			Total Number of Cultures	Number of Cultures responded	Proliferation rate	Percent Induction of callus (%)
	2,4-D mg/l	IAA mg/l	BAP mg/l				
1.	0.5		1.0	15	4	+	26.66
2.	1.0		2.0	15	6	++	40.00
3.	1.5		3.0	15	9	+++	60.00
4.	2.0		4.0	15	13	++++	86.66
5.	2.5		5.0	15	5	+	33.33
6.	3.0		6.0	15	5	+	33.33
7.		0.5	1.0	15	7	++	46.66
8.		1.0	2.0	15	9	+++	60.00
9.		1.5	3.0	15	11	++++	73.33
10.		2.0	4.0	15	9	+++	60.00
11.		2.5	5.0	15	7	++	46.66
12.		3.0	6.0	15	4	+	26.66

+ = Very less callus, ++ = Less callus, +++ = Moderate callus, ++++ = Optimum callus

mg/l IAA also showed rapid response of callus formation, but the growth of the callus was slow (Fig. 4.19 A). This combination of medium showed 73.33% response for callus induction. The callus formed was compact and embryogenic with variation in colour (Fig.4.19 B). The media containing low concentration of IAA and 2,4-D, in combination with high/low concentration of BAP showed inhibitory effect on the growth of callus (Table.4.8).

4.9.2. Shoot Initiation

Fig.4.20 Initiation of shoots from leaf callus

For induction of somaclonal variations, the proliferated greenish callus obtained from the leaf explant of *C. asiatica*, was isolated and subcultured on MS medium supplemented again with different concentrations and combination of IBA and Kinetin for shoot initiation. Performance of the sub-cultured calli containing embryoids was assessed for growth, morphology and other parameters like leaf colour, leaf shape, leaf margin, petiole length etc. These parameters were compared externally to the plants sub-cultured on the MS basal medium containing low hormone concentrations and plants were reared in a polyhouse. From such plants, number of shoots and height of the explants were recorded.

Results have revealed that satisfactory shoot initiation response was observed in almost all media. Maximum number of shoot formation (17.72 ± 0.86) was observed on MS medium fortified with 1mg/l IBA and 3mg/l kinetin. This combination of medium promoted rapid growth of shoots from callus (Fig.4.21). Higher concentration of kinetin (MS+ 1mg/l IBA + 4mg/l Kinetin) has inhibited shoot initiation (Table.4.9). Next effective combination of the medium, which promoted

Fig.4.21 Multiple Shoot Initiation form leaf callus

Fig.4.22. Multiple Shoot initiation from leaf callus

rapid growth of shoot formation within 15-18 days (14.24±0.45), is the MS medium supplemented with 1mg/l IBA and 2mg/l Kinetin (Table 4.9). Callus, sub-cultured on MS medium fortified with 1mg/l IBA and 1mg/l Kinetin showed slow growth of shoot initiation but produced shoots with maximum length (3.56 ± 0.65) as compared to media with other combinations.

MS media supplemented individually with IBA or Kinetin, induced callus in 15-17 days. This was followed by the formation of multiple shoots (Fig.4.22). Among the two hormones, Kinetin was found to be superior and more effective than IBA, in inducing shoot development as well as shoot multiplication from leaf explants (Table 4.9). Out of various concentrations of Kinetin studied, MS medium supplemented with 2.0 mg/l Kinetin proved to be optimum for growth and multiplication of shoots from sub-cultured callus (Table-4.9). Lower concentration of Kinetin (0.5 and 1.0 mg/l) produced stunted shoots from sub-cultured callus. The mean formation of shoots in the presence of Kinetin was high as compared to IBA but the shoots formed were found to be thin and weak. Among the combinations of IBA and Kinetin, MS medium supplemented with 1mg/l IBA and 3mg/l Kinetin produced maximum number of shoots (17.72±0.86) which were healthy. Next effective combination of growth hormones, that produced maximum number of shoots (14.24±0.45), is the MS medium supplemented with 1mg/l IBA and 2mg/l Kinetin (Table 4.9). After the 25 to 35 days of shoot initiation and elongation, the newly formed shoots were transferred to a rooting medium and allowed to grow further.

Fig.4.23 Induced Leaf Morphological variation

4.9.3. Root Induction in Regenerated Plants

Table 4.9. Effect of various concentrations and combinations of IBA and Kinetin on mean number and length of shoots produced *in vitro* from *Centella asiatica* leaf explants.

Sr.No	Growth Regulator		Mean Number of Shoots	Mean Length of Shoots
	IBA mg/l	Kinetin mg/l		
1.	0.5		1.06±0.53	1.76±0.43
2.	1.0		2.11±0.56	2.01±0.12
3.	1.5		6.34±0.31	2.10±0.23
4.	2.0		4.23±0.36	2.88±0.54
5.		0.5	3.18±0.28	3.00±0.43
6.		1.0	4.87±0.38	3.01±0.55
7.		1.5	7.21±0.42	2.12±0.12
8.		2.0	13.78±0.11	2.22±0.34
9.	1.0	1.0	6.12±0.77	3.56±0.65
10.	1.0	2.0	14.24±0.45	2.89±0.12
11.	1.0	3.0	17.72±0.86	2.75±0.97
12.	1.0	4.0	8.35±0.37	2.12±0.31

The values represent the mean ± S. E, three independent experiments. At least 15 cultures were raised for each experiment.

Profuse and rapid root initiation was obtained from the micro-shoot explants of *C. asiatica* raised on MS medium and supplemented with 1.5mg/l IBA and 1.5 mg/l NAA individually (Table-4.10). However, the frequency and number of roots varied with the type and concentration of the auxin used (Table- 4.10). Among these auxins, IBA (1.5mg/l) produced maximum number of roots (3.24±0.65) within 20 to 30 days of incubation. This is followed by NAA (1.5mg/

l), which produced good number of roots (1.30±0.46). Minimum number of primary roots per shoot were observed in NAA at a concentration of 0.5 mg/l (Table 4.10). IBA was highly effective in increasing the length of primary roots regenerated from micro shoots. Reduction in length of primary roots was observed in MS media supplemented with different concentrations of NAA. The shortest primary roots were observed in NAA at a concentration of 0.5 mg/l .

Table 4.10 Effect of various concentrations and combinations of NAA and IBA on induction and mean length of roots from *C. asiatica* leaf explants.

Sr.No	Plant Hormones		Mean Number of Roots	Mean Length of Roots
	NAA mg/l	IBA mg/l		
1.	0.5		0.78±0.22	0.43±0.04
2.	1.0		1.00±0.33	0.76±0.11
3.	1.5		1.30±0.46	1.00±0.76
4.	2.0		1.21±0.75	1.12±0.33
5.	2.5		1.16±0.11	1.03±0.22
6.	3.0		0.98±0.45	1.08±0.76
7.		0.5	1.78±0.88	1.32±0.55
8.		1.0	3.11±0.45	2.11±0.67
9.		1.5	3.24±0.65	2.45±0.89
10.		2.0	2.11±0.76	2.13±0.43
11.		2.5	0.0±0.0	0.0±0.0
12.		3.0	0.0±0.0	0.0±0.0

The values represent the mean ±S. E, three independent experiments. At least 15 cultures were raised for each experiment.

4.9.4. Transplantation to the Cultured Plants

After 30 to 35 days of rooting, the well rooted plantlets of *Centella* were transferred to plastic cups, which, contained a mixture of non-sterilized black cotton soil and agropeat. They were transferred to a polyhouse for hardening. Subsequently the rooted plantlets were removed from the semi-solid agar based medium, washed properly in tap water to remove any traces of agar and transferred

to field. After proper acclimatization, the plantlets showed 75% survival rate. After 40 to 50 days, when the plants were fully mature, they were compared with the naturally growing diploids for various morphological parameters.

The *in vitro* studies were found to be least effective in inducing significant variations (somaclonal variations) in morphological characters in *C. asiatica*. Whenever a slight morphological variation of any nature was observed among the *in vitro* raised plantlets, it was compared with the control mother stock. The variations observed in the leaf morphology, shoot length etc., were not stable. *In vitro* propagated plants did not show any significant difference in total alkaloid contents as compared to control plants.

An efficient *in vitro* regeneration method was developed for rapid propagation of *Centella asiatica* using leaf segments as explants. The tissue culture techniques developed in this study can be useful for micro propagation and also for the conservation of germplasm of this medicinally valuable plant which can enhance the rate of multiplication and can reduce the time period involved and also the cost of production.