CHAPTER V

DISCUSSION

5. Discussion

*Centella asiatica (*Linn.) is a very important medicinal herb, traditionally used by ethnic people, as nervine tonic and for the treatment of asthma, hypertension, bronchitis, dropsy, skin diseases, and urethritis (Kakkar, 1988), since prehistoric times. *C. asiatica* has wound healing, anti-tumor, antibacterial, antifeedant, antituberculosis, antileprotic, and antioxidant properties (Chakraborty *et al*., 1996; Srisvastava *et al*., 1997; Shakir *et al*., 2007). *Centella* is known to accumulate large amount of pentacyclic triterpenoid saponins which forms the major store house of secondary metabolites, providing active compounds stimulating cell rejuvenation, improving physical and mental health. Several studies point out that the major bioactive compounds in *Centella* are triterpenes (asiaticoside, asiatic acid, madecassoside and madecassic acid) and phenolic compounds particularly flavonoids (Farnsworth and Bunyapraphatsara, 1992; Inamdar *et al.*, 1996; Zainol *et al*., 2003).

Major objectives of the proposed investigation was to determine the appropriate method of colchicine application; finding out the effective concentration of colchicine and treatment duration for induction of autotetraploidy; characterization of the obtained autotetraploids for their morphometric characters; establishing the ploidy level of the colchicine treated plants and quantitative estimation of saponins in the experimentally induced autotetraploids (colchiploids). Parallel experiments were also conducted to find out whether the variants produced through *in vitro* techniques (Soma-clonal variants) show any variation in the quantity of saponins.

Experimental results obtained in the present investigation entitled “Genetic Improvement of Brahmi (*Centella asiatica* (Linn) Urban) For High Yield of Saponins” were presented in the previous chapter. In this chapter, significance of various results obtained, in the present investigation, are discussed.

5.1 Effect of Colchicine on Survival Rate

Our experimental results have clearly proved that colchicine application

was very effective in inducing autotetraploidy in *Centella*. However some colchicine treated plants showed disorders in growth and change in leaf shape. Plants were necrotized in early growth stages. It seems that colchicine treatment has caused visible tissue disorders. Some plants did not survive the colchicine treatment at all. We have observed that the survival rate was lower with higher colchicine concentrations and prolonged duration of the treatment.

Similar injury to plants and some disorders due to treatment with spindle poisons, has been reported earlier by several authors (Suying *et al*., 2005; Jaskani *et al*., 1996; Jaskani *et al*., 2005; Jadrna *et al*., 2010). Decrease in plant survival rate; and slow growth response to colchicine treatments were reported in different species of *Centella* (Tanavat *et al*., 2011), *Pelargonium* (Jadrna *et al*., 2010) and Watermelon (Jaskani *et al*., 2005). Delayed sprouting and growth of shoot buds in colchicine-treated explants has been reported by Pryor and Frazier, (1968), Cohen and Yao, (1996) and Carvalho *et al*,. (2005). The slow growth may be due to the physiological disturbance caused by colchicine, resulting in reduced cell division (Eigsti and Dustin, 1954). The variable response of genotype to different concentrations of colchicine support the need for making specific determination of colchicine concentrations suited for a given cultivar.

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

As already mentioned in the results section, out of the three methods of colchicine treatment viz., Injection of stolons with colchicine, Applying colchicine to the artificially injured shoot tips and 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 stolons with colchicine and Application of colchicine to the artificially injured shoot tips were found to be least effective in inducing autotetraploidy in *Centella*.

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

From the results it is evident that the plants treated with 0.2% for 4 hours and 0.3% for 5 hours showed signs of autotetraploidy. They were monitored for 3 to 4 generations. *Centella* plants treated with 0.2% for 4 hours fared well and showed higher survival rates. From these experiments it is concluded that the best effective concentration of colchicine for *Centella asiatica*, for inducing autotetraploidy is 0.2% and effective treatment duration is 4 hours.

5.4. Impact of Induced Autotetraploidy on Morphometric Characters

Present investigation established that the selected 12 morphological characters such as 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, can be best used as a key parameters or markers for identification of putative induced autotetraploid plants.

Experimental results indicated that 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. Similar results were also reported by Mehlquist *et al*., (1947) in *Delphinium cardina* and Gupta *et al*., (2004) in *Bellis perennis*, where the autotetraploids plants obtained as a result of treatment with colchicine showed swollen hypocotyls.

A comparative study of colchicine induced autotetraploids and the diploids revealed significant morphological differences both in vegetative and reproductive characters. The autotetraploid plants exhibited distinct and highly significant morphological variations in vegetative characters like plant height, leaf length, leaf breadth, leaf size and leaf thickness, as compared to diploid *C. asiatica* plants. Fresh and dry weight of 100 leaves in autotetraploids has increased by 2-3 folds, as compared to normal diploid *Centella* plants. Similar increase in number of branches, number of leaves per plant, size of leaves and fresh and dry weight of leaves has also been reported in Autotetraploid plants of *Datura stramonium* obtained as a result of treatment with colchicine by Amiri *et al*., (2010). He has also reported that the total overall size of the tetraploid plants was greater than that of the diploid plants.

Our experimental results clearly indicate that autotetraploids of *Centella* produced as a result of treatment with colchicine, 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. Thus the autotetraploids showed almost double the size of leaves as compared to diploids. Similar increase in leaf area in the colchicine induced autotetraploids, has been reported by several investigators (Bhiravamurty and Rethy, 1984 in *Solanum nigrum*; Ozer and Sagsoz, 1991 in *Secale montanum*; Chakraborti *et al*., 1998 in *Morus alba*; Joshi and Verma, 2004 in *Vicia faba*; Jaskani *et al*., 2005 in Watermelon). Kirhara, (1951) also reported that the leaves of tetraploid plants were large, thick and dark green than the diploid plants. Koh, (2002) has also reported differences in leaf size and area as a result of treatment with colchicine. Similarly, broader leaves in Citrus (Jaskani *et al*.,1996), higher ratio of leaf width to length in *Alocasia* (Thao *et al*., 2003) and broader and thicker leaves in *B. globosa* (Rose *et al*., 2000) have been reported in tetraploid genotypes. Variation in the leaf size was observed between control and colchiploid plants under the same growing conditions. Similar increase in leaf area in colchicine induced autotetraploid plants of *Stevia rebaudiana* has also been reported by Olivera *et al*., (2004). From the above discussion it can be inferred that the most widespread effect of autotetraploidy is the increase in leaf size which can be due to the increase in overall size of leaf cells. Increased cell size is generally associated with gigantism (Gupta *et al*., 2004). This accounts for the observed overall increase in plan size.

Our experimental results indicate that the overall size of floral buds, seeds and weight of 100 seeds has increased by 2-3 folds as a result of chromosome doubling (autotetraploidization). Similar increase in the size of floral buds in *Dracocephalum moldavica* (Reza *et al*., 2010), *Datura stramonium* (Amiri *et al*., 2010) and *Bellis perennis* (Gupta *et al*., 2004), and increase in the size of seeds and weight of 1000 seeds in chickpea (Pundir *et al*., 1983) and Caraway (Dijkestra and Speckmann, 1980) as a result of induction of autotetraploidy using colchicine has been reported earlier. Some polyploid plants are known to possess larger leaves, bigger pollen and floral characters as compared to their corresponding normal diploid plants (Haig Derman,. 1947). Sari *et al*., (1999) also reported increase in

size of flowers as a result of induction of autotetraploidy employing colchicine. Some polyploid plants are known to possess large flowers and bigger pollen as compared to their corresponding normal diploid plants (Haig Derman, 1947). Autotetraploids plants of *Delphinium* obtained as a result of treatment with colchicine showed larger flowers (Mehlquist *et al*., 1947). Colchiploids of *Nicotiana alata* showed slightly bigger flowers than the control (El-Morsy Sh *et al*., 2009). Kihara, (1951) has opined that seed size and shape may be used as a criteria to identify the tetraploids, as tetraploid seeds are generally larger and thicker than diploid seeds of the same genotype.

Based on our experimental results and the above discussion, it can be concluded that the distinct differences in morphological characters, observed between the colchicine induced autotetraploid plants and the diploid, control plants, especially the size of plant, leaves, flowers, seeds, and fresh and dry weights of leaves could be used as affective morphological parameters to identify the putative autotetraploids of any plant, including *C. asiatica.* These parameters are simple, easy and faster than the chromosomal analysis.

5.5 Effect of Colchicine on Stomatal Characteristics

Our experimental results have revealed that the colchicine treated plants exhibited larger but fewer stomata in their leaves. The area of the stomata is more than double and number of stomata is less in colchicine treated plants as compared to control, untreated plants. Length and width of stomata of colchicine treated plants are higher as compared to untreated control plants. Similar significant differences in stomatal size of diploid and tetraploid plants in *Lilium* species has been reported by Iizuka and Ikeda (1968). Results obtained in *Populus tremula* plants, indicated that when polyploidy level increased, plant cells became larger and number of stomata per unit area decrease (Hommo and Valanne, 1987).

The fact that the stomata of polyploid plants are larger than those of the diploid plants, was previously reported in *Centella asiatica* (Chulalaksananukul and Chimnoi, 1999; Tanavat *et al*., 2011), *Chrysanthemum cineraiifolium* (Liu and Gao, 2007), *Lagerstroemia indica* (Zhang *et al*., 2010), *Morus alba* (Chakraborti *et*

al., 1998), *Brachiaria ruziziensis* (Ishigaki *et al*., 2009), *Bixa orellana* (Carvalho *et al*., 2005), and *Salvia coccinea* (Kobayashi *et al*., 2008).

Evan (1955) stated that stomata size and number is an accurate indicator of polyploidy level in many plants. Since, then the stomata size and stomata number, has also been used as an additional morphological parameter by many workers. Cohen and Yao (1996) induced autotetraploidy in *Zantedes hia, in vitro,* using colchicine treatment. They have selected putative tetraploid plants just by measuring stoma cell size before chromosome number count and the tetraploid condition was confirmed with the help of the chromosome counts. 91% of these putative plants were confirmed as autotetraploids after chromosome counts. Thus the stomata size was used by these authors as a most effective parameter to determine polyploidy. Mishra, (1997) has also supported that stomatal size can be used as an indicator of the ploidy level, because chromosome counting is laborious when large number of plants must be examined.

Our experimental results also indicate that the number of stomata per unit area, as indicated by the stomatal index, was low in colchicine treated tetraploid *Centella asiatica* leaves, as compared to untreated diploid ones. Similar results have also been reported in *Dracocephalum moldavica* (Reza *et al*., 2010), *Datura stramonium* (Amiri *et al*., 2010), *Bellis perennis* (Gupta *et al*., 2004) and *Lagerstroemia indica* (Zhang *et al*., 2010).

Increase in stomata size has been reported in induced autotetraploid plants (Olivera *et al*., 2004). According to these authors, the higher the ploidy number, the greater the size of the pollen and the stomata, and the lower their number per unit area. Shanti Batra (1952) reported that the autotetraploidy can induce variations in stomata size. Autotetraploids plants of *Delphinium* obtained as a result of treatment with colchicine showed large stomata (Mehlquist *et al*., 1947).

From the foregoing discussion and based on our experimental results, it can be concluded that autotetraploidy is associated with an increase in the overall size of stomata and decrease in the number of stomata per unit area. We strongly suggest that, stomatal characteristics can be definitely used as a parameter and

indicator of induction of autotetraploidy in plants.

5.6 Effect of Colchicine on Ploidy Level

Our experimental results have successfully proved that colchicine could be very effectively used for induction of autotetraploidy in *Centella asiatica*.

Colchicine was used for chromosome doubling of many crops including chickpea (Pundir *et al*., 1983), henbane (Lavania and Srivastava, 1991), hops (Roy *et al*., 2001), ginger (Adaniya and Shira, 2001) and feverfew (Saharkhiz, 2007). It was the most widely applied and best studied chemical that induces autotetraploidy (Hassawi and Liang, 1991; Poehlman, 1987).

Colchicine is a poisonous compound extracted from seeds or corms of Liliaceae member of the autumn crocus (*Colchicum autumnale* L.). Colchicine disrupts mitosis by binding to tublin, the protein subunit of microtubules and prevents the polar migration of chromosomes. The result is a cell with double the chromosome number (Tambong *et al*., 1998). Colchicine is used by several workers for doubling the chromosome number and to induce autotetraploidy in several medicinal plants. Goldy and Lyrene (1984) doubled the chromosome number of *Vaccinum* species by treating the nodes with colchicine. In different plant species, different plant parts were used to induce polyploidy. These include; seeds (Hanzelka and Kobza, 2001; Quan *et al*., 2004), flower buds (Wu *et al*., 2007), apical meristems (Lavania and Srivastava, 1991) and roots (Taira *et al*., 1991).

Chromosome doubling has beneficial effects on the tetraploid cultivars. Duplication of alleles per locus has great influence on the whole plant performance. Under tetrasomic inheritance system plant performance depends more on the level of heterozygosity, and highly heterozygous autotetraploid genotypes characterized by tetra-allelic genetic structure of loci tend to show a higher performance than the diploid genotypes (Bengham, 1980).

The ploidy level of the suspected autotetraploid plants was confirmed by

determination of their chromosome number. For this, cytological analysis was carried out on root tips of autotetraploid plants of *Centella asiatica.* Our experimental results have revealed that the diploid cells (2n) had 18 chromosomes, whereas the autotetraploid cells (4n) had 36 chromosomes. These results clearly indicate that we are successful in inducing autotetraploidy in *Centella asiatica*, employing colchicine. These results undoubtedly prove the spindle inhibiting nature of the colchicine.

Derman (1940) has reported that the concentration of colchicine and treatment duration are the two important factors that are significant and play a vital role in the process of induction of polyploidy with colchicine. Sagsoz (1982) suggested that the concentration of the colchicine and treatment duration also differ with the stage of plant growth and the plant part used for the treatment. Ladizinsky and Shefer, (1982) applied 0.02% colchicine solution for 8 hours to young *V. sativa* shoots and obtained positive results. Similar results were also reported by Joshi and Verma (2004) who obtained 50% tetraploid *V. faba* plants by applying 0.005% colchicine for 8 hours. Our current experimental results prove that in *Centella asiatica*, where stolons were used for colchicine treatment, the required concentration of colchicine for induction of autotetraploidy is 0.2% and the required duration of treatment is 4 hours.

In many of plant species, correlation between ploidy level and cytogenetic characteristics such as size of stomata cells and stomata density, were reported. In pepper, stomata density and seemed to be reliable for the estimation of ploidy level (Abak *et al*., 1998). Stomata size and changes in plant morphology were found to be useful indicators in the primary screening for new ploidy level in M1 generation of *Viola* x *Wittrockiana Gams* (Ajalin *et al*., 2002). Many domestic autotetraploid plants are larger than their corresponding diploids (Lawrence, 1980). Keeler and Davis, (1999) noted differences in the size of *Andropogon gerardii*, a species with hexaploid (2n=6x=60) cytotype, in which the biggest individuals had the highest chromosome number. Polyploidy is often accompanied by increased cell size, leading to larger reproductive and vegetative organs (Adaniya and Shira, 2001). Polyploid plants may be found in agriculture and horticulture as they often possess superior agronomic characters over their diploid counterparts. The increase in gene

dosage resulting from multiplication of chromosome sets brings about gigantisms in all characters in general. Enhancement of tolerance and adaptability are also added characteristics of polyploids. More over several interspecific and inter-generic crosses have been made fertile through polyploidy. All these facts taken together have made the induction of autotetraploidy an effective tool in the hands of agriculturalists and horticulturists.

Our experimental results indicate that doubling of chromosome sets (induction of autotetraploidy) does manifest in some immediate phenotypic effects. Prominent among them are increase in organ size, increased vigour and increase in biomass. The mechanisms by which polyploidy contributes to novel variation are not well understood, but one long-held view is that duplicate genes have relaxed constrains on their function and thus can diverge creating new phenotypes in polyploids. Although the causes of variation found in polyploids are not well understood, they are suspected to involve changes in gene expression through increased variation in dosage regulated gene expression, altered regulatory interactions and rapid genetic and epigenetic changes (Osborn, 2003).

5.7 Induction of Somaclonal Variations in *Centella asiatica*

The experimental results have revealed that the *in vitro micro*propagation studies conducted were found to be least effective in inducing notable and significant variations in *C. asiatica*. No significant difference was observed in morphological characters of any nature among the *in vitro* raised plants when compared with the mother stock. Slight morphological variations observed in the leaf morphology and shoot length were not stable. However we were successful in developing and establishing a commercially viable protocol for mass micropropagation of *C. asiatica*. This protocol can be used for clonal propagation of *C. asiatica* which will help in production of raw material with high uniformity.

Similar successful *in vitro* micropropagation studies were reported by Patra *et al*., (1998) from callus cultures *and* Banerjee *et al*., (1999) from leaf explants of *C. asiatica*. Tiwari *et al*., (2000) also developed a protocol for rapid and large scale *in vitro* clonal propagation of *Centella asiatica* by enhanced

axillary bud proliferation in nodal segment isolated from mature plants and could induce the optimum frequency (91%) of shoot formation. A rapid method for multiplication of *C. asiatica* by shoot tip culture has also been developed by Nath and Buragohain (2003). Similar results were also reported in the same species (Raghu *et al*., 2007; Karthikeyan *et al*.,2009; Deshpande *et al*., 2010; Singh *et al*., 2010; Hanumantharaya *et al*., 2011; Sholapur and Dasankoppa, 2011).

Successful micropropagation, using nodal explants, has also been reported earlier, in other species like *Rauwolfia serpentina* (Roy *et al*.,1995), *Rhinacanthus nasutus* (Johnson *et al*.,2006), *Vitex negundo* (Vadawale *et al*., 2004) and *Emblica officinalis* (Rahaman *et al*.,1999). Nath and Buragohain, (2003) have also reported a protocol for establishment of cell suspension culture for production of asiaticoside. Kim *et al*., (2007) have developed alternative strategy for production of asiaticoside using hairy root cultures by transforming leaf tissues.

Micropropagation of *C. asiatica* through tissue culture techniques is very useful especially where quick propagation of the material is the major demand. Increased demand for *Centella* has risen sharply due to the increased popularity of the new *Centella* based drugs. Because of large scale and unrestricted exploitation to meet its ever increasing demand by the pharmaceutical industry, the population of *Centella* is dwindling in nature. Furthermore, limited cultivation and insufficient attempts for its replenishment have resulted into the depletion of the wild stock. It has now been included in the list of threatened species by the International Union of Conservation of Nature and Natural Resources (IUCN) and also in the list of endangered species (Mukundan *et al*., 2004). Therefore application of tissue culture approaches especially the micro propagation will definitely aid in rapid multiplication of elite clones and germplasm conservation of this vitally important medicinal plant species.

Minor leaf morphological variations, observed in the somaclonal variants, in the present investigation, suggest that *in vitro* conditions do induce somaclonal variations in *C. asiatica* but these somaclonal variants are not notable and significant and also did not show any improvement in therapeutically active compounds. Similar results were obtained by Sholapur and Dasankoppa, (2011)

who reported that the subcultured callus did not show any improvement in the asiaticoside contents and Nath and Buragohain, (2005), who detected asiaticoside in callus and cell suspension culture, but the quantity of accumulation is less than that found in naturally grown plants. Similarly somaclonal variants, obtained from leaf explants of potato (Shepard *et al*., 1980) also did not show any increase in alkaloid contents. Evans and Sharp (1986) reported that somaclonal variations may be due molecular changes caused by mitotic crossing over in regenerated plants. This might be the reason for having minor leaf morphological variations in tissue culture propagated *C. asiatica*. Based on our experimental results and the foregoing discussion it can be concluded that the somaclonal variants and the control plants do not differ and show almost similar amount of therapeutically active compounds.

5.8. Effect of Induced Autotetraploidy on the Yield of Therapeutically Active Compounds

In the present investigation yield of triterpenioid saponins contents in colchicine induced autotetraploids and control plants of *Centella asiatica* were estimated employing High Performance Liquid Chromatography (HPLC). These results revealed that the colchicine induced autotetraploidy plant had highest 0.3 gm/gm dry wt. of medecassic acid and terminolic acid (76.47%) and it is 1.30 times higher when compared to the untreated control plants. Similarly, the asiatic acid content of the autotetraploids was 0.17 gm/gm dry wt., and that of control diploids was 0.06 gm/gm dry wt. Thus, there was 183.33% increase asiatic acid as compared to the control plants. These results clearly indicate that the endogenous level of asiatic acid has gone up by 1.5 fold due to autotetraploidization. A similar 3.26 %increase in asiaticoside content in the colchicine induced autotetraploid plants was also observed in the present investigation, but it is not statistically significant as compared to control plants of *C. asiatica*.

Thus, our experimental results clearly indicate that induction of autotetraploidy in *C. asiatica* is very affective in increasing the yield of therapeutically active compounds of this plant. Similar increase in the yield of therapeutically active compounds, as a result of induction of autotetraploidy, was

also reported by several authors but in different medicinal plants. Rawson (1944) reported an increase in the total alkaloid content in *Datura* due to induction of autotetraploidy. Bhatt and Heble (1978) reported an increase in solasodine content in fruits of a spiny *Datura* due to induction of autotetraploidy. Gottschalk (1976) has reported an increase in the content of the active substances (alkaloids) due to polyploidy induction in several plants. Krishnan (1998) reported that induced autotetraploids of *Solanum viarum* are characterized by higher solasodine content which was up to 50% higher than diploid. He also stated that chromosome doubling (autotetraploidy) appears to be the only successful avenue for genetic upgradation of solasodine content in berries of this crop. The content of alkaloid in autotetraploid plants of *Atropa belladonna* is 154% of that in the diploid plants (Jackson and Rowson, 1953). Olivera *et al*., (2004) has reported higher contents of stevioside in the autotetraploids of *Stevia rebaudiana* than diploid plants. In *Secutellaria baicalensis*, one tetraploid line exhibited an increase in baicalin of 4.6% (Gao *et al*.,2002).

It has been reported that the tetraploid plants of *Hyoscyamus muticus*, had nearly 1.5 times higher economic production potential, compared with that of its diploid counterparts (Lavania, 1988). In addition, the leaf, stem and root which can be useful parts in most medicinal plants are usually bigger in polyploids. Thus, the polyploids may increase biomass or product yields (Gao *et al*., 1996).

Manipulation of ploidy is considered as a valuable tool in genetic improvement of many plants (Dhawan and Lavania, 1996). Polyploidy generates variants that may possess useful characteristics and by doubling the gene products, they also provide a wider germplasm base for breeding studies (Thao *et al*., 2003). Chromosome duplication, employing colchicine, has been well established practice in plant breeding programs. Polyploidy has the general effect of increasing gene expression levels, on a per cell basis, in proportion to the gene dosage conferred by ploidy level, as was shown for most genes in a euploid series (triploid, and tetraploid) of maize (Gao *et al*., 2002). In *Centella asiatica,* a gene, oxidosqualene cyclase, (OC gene) has been identified as responsible for the biosynthesis of triterpene glycosides (otherwise known as triterpenoid saponins) such as asiaticoside and madecassoside (Kim *et al*., 2002). The observed increase in the

content of asiaticoside and madecassoside, in the present investigation, might be due to duplication of these OC genes due to autotetraploidization.

Induction of artificial polyploidy is useful in increasing the production of important medicinal compounds (Dhawan and Lavania, 1996). The autotetraploids obtained in the present investigation may prove useful and open new possibility for genetic breeding programme of *C. asiatica* since polyploid individuals have higher content of asiaticoside, medecassic acid, terminolic acid and asiatic acid than the wild diploid plants.