CHAPTER VI

SUMMARY AND CONCLUSION

6.Summary and Conclusion

Plant based drugs play an important role in traditional and conventional medicine throughout the world. In recent times, interest in the use of herbal products and the focus on plant research has grown dramatically in the western world as well as in developed countries. Medicinal herbs as a potential source of therapeutic aid has attained a significant role today in health system all over the world, not only in the diseased condition but also as potential material for maintaining proper health. Hence it has become imperative to establish breeding procedures for these medicinally important plants for improving the yield of therapeutically active compounds.

The present investigation entitled "Genetic Improvement of Brahmi (*Centella asiatica* (Linn) Urban) For High Yield of Saponins" was undertaken with an objective of inducing autotetraploidy in the medicinally important plant, Brahmi, employing techniques of Ploidy Breeding, so that the newly produced genotypes yield higher quantities of saponins.

6.1. Introduction

Centella asiatica (Linn) Urban, is commonly known as Indian pennywort in English, Brahmi or Mandookaparni in Hindi and Karivana in Marathi, belongs to the family Apiaceae (Previously known as Umbelliferae). *Centella asiatica* is found throughout the tropical and subtropical regions of India.

Centella asiatica is small creeping herb with shovel shaped leaves emerging alternately in clusters at stem nodes. Leaves are rounded to reniform, 2 to 5 centimeters wide, horizontal, more or less cupped, rounded at the tip, and heart shaped at the base. Petioles are erect and long. Inflorescence is an umbel and each umbel consisting of 3-4 white to pink, sessile flowers. The fruit is minute, ovoid, white or green and reticulate, each with 9 sub similar longitudinal ridges (Tiwari *et al*., 2011).

Centella asiatica is one of the most important medicinal plant. The plant is used in Ayurveda and is popular as a nerve tonic to promote relaxation and to

enhance memory. It is also used for the treatment of asthma, bronchitis, kidney troubles, leucorrhoea and skin disease with antibacterial, anti-filarial, anti-stress and wound-healing properties (Kakkar, 1998 and Chakraborty *et al*.,1996).

The plant has also high nutritional value since it is rich in carotenoids and vitamins B and C (Paramageetham *et al*., 2004 and Chew Shio Heong *et al*., 2011). It is commonly used as porridge for feeding pre-school children in Sri-Lanka in order to combat nutritional deficiencies (Cox *et al*., 1993). The herb contains a blend of compounds including at least 3 saponins (Asiatic acid, madecassic acid and asiaticoside) that appear to have antioxidant benefits and ability to stimulate collagen synthesis for tissue regeneration. They probably enhance formation of collagen in bones, cartilage and connective tissue (Tiwari *et al*., 2011).

Kurian and Sankar, (2007) has reported that *Centella asiatica* to be one among the best selling herbal drugs in Europe and USA, exported from India. The annual requirement of *Centella asiatica* was around 12,700 tones of dry biomass valued at rupees 1.5 billion (Ahmad, 1993). In the year 2001 and 2002 was also increased annual demand 3,822.5 metric tonnes (Sursh Wagh, 2006). The national medicinal plants board, government of India, has projected demand of *Centella asiatica* in the year 2004-05 it was 6,621.8 metric tonnes with an annual growth rate of 20.1 % per annum. Nowadays, the large scale and unrestricted exploitation of this natural resources to meet its increasing demand of the user industry has created a deficient in supply of sufficient quantities of genuine plant material. In view of the international market demand of the *Centella asiatica*, it is necessary either to increase the area under cultivation of *Centella* or develop high yielding varieties.

There have been several reports which clearly indicate that secondary metabolite production in medicinal plants can be increased by induction of autotetraploidy using colchicine (Rawson, 1944; Jackson and Rawson, 1953; Bhatt and Heble, 1978; Dhawan and Lavania, 1996; Gao, 1996; Krishnan, 1998).

The present investigation was undertaken with a major objective of developing autotetraploid genotypes of Brahmi, employing the techniques of Ploidy Breeding, especially induction of autotetraploidy using colchicine, which is quick, reliable and very effective in doubling the chromosome number of plants.

Such newly developed genotypes of *Centella* are expected to produce higher yields of major saponins compounds viz. asiaticoside, madecassoside, asiaticoside-B, madecassic acid and asiatic acid. So this was verified by carrying out HPLC analysis of saponins contents of diploid and autotetraploid *Centella* plants.

Other objectives of the investigation include, study of differences in morphology of phenotypic traits, stomatal characters and ploidy level of the diploid and autotetraploid plants of *Centella*. An attempt has also been made to induce somaclonal variations so that they can be assessed for their suitability for use in induction autotetraploidy breeding and induction of somaclonal variations programmes aimed at genetic improvement of *Centella*. It is undertaken with the following objectives:

1. To find out the optimum concentration of colchicine and treatment duration for induction of autotetraploidy in *Centella asiatica*,

2. To verify that autotetraploids of *Centella asiatica* plants have been obtained,

3. Induction of somaclonal variations in *Centella* through *in vitro* techniques and if successful, isolate the somaclones showing desired traits,

4. Propagation of control, autotetraploid as well as somaclonal elite clones for several generations in the field and analyzing them for morphometric traits like plant height, leaf area, inter-nodal length, petiole length, fresh and dry weight of 100 leaves and number of stomata per unit area,

5. Quantitative estimation of major saponins viz., asiaticoside, madecassoside, asiaticoside B, madasiatic acid and asiatic acid in the control and induced autotetraploid plants as well as elite clones obtained through somaclonal variation.

The objectives, if realized, would go a long way in creating a novel and superior phenotypes of *Centella asiatica* capable of producing higher yield of medicinal compounds. These superior and high yielding lines of *Centella* can be sup-

plied to the local farmers, who can be benefitted by cultivating them. Therefore, the present investigation can immensely benefit the farmers, ayurvedic and homeopathic manufacturing concerns.

6.2. Material and Methods

Wild genetic stocks of *Centella asiatica* were obtained from Dhanwantari Udyan (Medicinal Garden) of Mahatma Phule Krishi Vidyapeeth (MPKV) Rahuri, District Ahmednagar, Maharashtra state, India. These plants were initially propagated under greenhouse conditions and later shifted to field conditions. The plants were planted at 30 X 25 cm spacing between plant to plant and row to row. After proper acclimatization, these plants were used as experimental plant material. Two different techniques were employed to achieve the objective. They are 1. Induction of autotetraploidy and 2. Induction of somaclonal variations.

An antimitotic agent, colchicine was used in the present investigation to induce autotetraploidy in the selected plant material. Stolons of *Centella asiatica* were treated with different concentrations of colchicine (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 %) for different time intervals (1 to 8 hours) at room temperature. Untreated stolons served as control. Running shoots (stolons) of at least ten experimental plants were selected for the colchicine treatment.

The colchicine treated plants were grown in greenhouse. After attaining an age of at least 45 days, morphometric studies were conducted on these as well as control plants.

Since changes in morphometric traits, are the best primary indicators to identify the promising autotetraploid plants (Blakeslee and Avery, 1937; Kirhara, 1951; Jaskani *et al*., 1996), both the control and colchicine treated plants were screened for 12 morphometric traits 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.

Similarly, stomatal characteristics were also used as one of the initial criterion to identify the promising autotetraploids of *Centella*. Leaves having similar size, from colchicine treated and untreated plants of 3-4 months old, were used

for the stomatal study. Studies were carried out following the method suggested by Topho and Ghosh (1997) using mechanical peelings or scraping of the epidermis from fresh leaves.

The chromosome number and ploidy level of the promising autotetraploids (assessed by the above mentioned morphometric and the stomatal traits), and also the diploids was studied from the root tip cells following the Aceto - Orcein - HCl squash method as suggested by Sharma and Sharma(1980).

Leaf explants of *Centella asiatica* were used for induction of somaclonal variations. Earlier work as per the recommendations of Karthikeyan *et al*., (2009), Raghu *et al*., (2007), Aziz *et al*., (2007), Mukundan *et al*., (2006), Nath and Buragohain (2005), Tiwari *et al*., (2000), Banerjee *et al*., (1999) were reported in this species. Murashige and Skooge (1962) medium was used and supplemented with different concentrations and combinations of auxins and cytokinins were used as a nutrient medium.

After 10-14 days of culture on rooting media, the rooted plantlets were transplanted to pots or trays for hardening prior to their final transfer to soil. After planting, plantlets are thoroughly watered and kept in polyhouse under humidity range of approximately 80%. These plantlets are sprinkled with water time to time as per the requirement. After hardening and acclimatization, these plantlets were cultivated on seed beds containing a mixture of loamy and black cotton soil. Such somaclonal variants propagated on large scale in the polyhouse were screened for morphological traits and compared with their control counter parts growing naturally in the field.

Induction of artificial polyploidy may prove useful in increasing the production of important medicinal compounds. For that reason, colchicine induced autotetraploid plants (often referred as colchiploids) and untreated diploids were analyzed for the active medicinal compounds viz., major saponins.

Major saponins contents were estimated employing HPLC analysis method (Tiwari *et al*., 2010). High Performance Liquid Chromatographic system equipped with LC8A pump, SPD-M 10Avp Photo Array Director in combination with Class

LC 10A software, was used for the analysis. Samples for HPLC analysis were prepared from the dried leaves of *Centella asiatica.*

6.3. Experimental Results

The experimental results obtained on "Genetic Improvement of Bharmi (*Centella asiatica* (Linn) Urban) For High Yield of Saponins" are described below.

6.3.1. 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 running 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*.

6.3.2. Effect of colchicine concentration on survival rate

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 (0.1%). 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. Our experimental results also indicated that all plants subjected to treatment with colchicine in general, showed lower survival rates as compared to those of the control ones.

6.3.3. Effective Concentration of Colchicine and Treatment Duration for Induction of Autotetraploidy in *Centella*

From the results it is evident 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. Plants of *Centella asiatica* treated with 0.2% colchicine for 4 hours and 0.3% colchicine for 5 hours showed signs of polyploidy. On the basis of change in morphometric traits and high survival rate (75%), the 0.2% of colchicine was adjudged as effective concentration of colchicine and 4 hours as

effective period of treatment for *Centella* plants.

6.3.4. Establishment of Autotetraploidy in Colchicine treated *Centella* plants

Centella asiatica plants treated with 0.2% colchicine for 4 hours duration (here after called as colchicine treated plants), 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 traits and chromosome counts.

(i) Variations in Morphometric Traits

The colchicine treated plants showed a mean increase of 72.69% in plant height, 42.18% increase in inter-nodal length, 48.15% increase in leaf length, 14.34% increase in leaf width, 57.11% increase in leaf area, 200% increase in leaf thickness, 37.15% increase in petiole length, 62.61% increase in petiole width, 200.31% increase in fresh weight and 183.87% increase in dry weight of leaves, 229.59% increase in fresh weight of buds and 74.19% increase in dry weight of seeds, as compared untreated control ones. Thus colchicine treatment has resulted in an overall increase of all morphometric traits. The colchicine treated plants were robust in size, vigorous in growth and showed thick, dark green coloured and large leaves.

(ii) **Variation in Stomatal Traits**

The colchicine treated plants showed lower frequency of stomata (37.61%) as compared the diploid plants. But stomata were larger in colchicine treated plants. The mean area of the stoma in colchicine treated plants was 140.32% higher as compared to that of control. Width and length of stomata are higher in colchicine treated plants as compared to control plants. This clearly indicates that treatment of Centella with 0.2% colchicine for 4 hours has increased overall stomata size but decrease stomata number per unit area of leaf. Thus the promising

autotetraploids showed larger but fewer stomata as compared to their control counterparts.

(iii) Effect of Colchicine on Ploidy and Chromosome Number

Root tips of control plants of *C. asiatica*, squashed and stained with Aceto-Orcein-HCl reagent revealed the presence of 18 chromosomes. On the other hand the root tips of 0.2% colchicine treated plants, allowed to reproduce vegetativealy for three consequent generations, squashed and stained with Aceto-Orcein-HCl reagent revealed the presence of 36 chromosomes. This clearly indicates that the colchicine treated plants were autotetraploids and possessed 4 sets or 36 chromosomes. These experimental results have clearly indicate that, the 0.2% colchicine for 4 hours was very effective in inducing autotetraploidy in *Centella asiatica*.

6.3.5. Induction of Somaclonal Variations

For induction of somaclonal variations, young leaf explants of *C. asiatica* were grown aseptically on MS medium supplemented with different concentrations and combinations of growth hormones. They were analyzed for induction of callus, induction of shoot and induction of root medium. These are very useful in induction of somaclonal variation for *Centella asiatica* and also in the analysis of effective growth hormones.

6.3.5.1. Induction of Callus

Leaf explants of *Centella asiatica were* cultured on MS medium supplemented with different combinations and concentrations of BAP and 2-4 D or IAA. The best callogenic response was obtained on MS medium supplemented with a combination of 4 mg/l BAP and 2 mg /l 2, 4-D.

6.3.5.2. Induction of Shoots

In the present investigation, maximum shoot initiation response (17.72±0.86) was observed on MS medium fortified with 1mg/l IBA and 3mg/l kinetin. Higher concentration of kinetin (MS+ 1mg/l IBA + 4mg/l Kinetin) resulted in inhibition of shoot initiation. Callus, sub-cultured on MS medium fortified with

equal concentration of IBA and Kinetin (1mg/l IBA+1mg/l Kinetin) resulted in slow initiation of shoots but this combination was also found to produce maximum length of the shoots (3.56±0.65 cm) as compared to other combinations.

Our experimental results indicate that, Kinetin was superior and more effective than IBA, in inducing shoot formation and shoot multiplication from leaf explants. MS medium supplemented with 2.0 mg/l Kinetin was found to be optimum for growth and multiplication of shoots.

6.3.5.3. Induction of Roots

Sub-culturing of micro-shoots on MS media supplemented with 1.5mg/l IBA and 1.5 mg/l NAA individually resulted in profuse and rapid root initiation. IBA at a concentration of 1.5mg/l has resulted in production of maximum number of roots (3.24±0.65) within 20 to 30 days of incubation.

The *in vitro* studies were found to be least effective in inducing variations (somaclonal variations) in *C. asiatica*. In our experimental results, *in vitro* propagated plants did not show any significant variation in morphometric traits. Similarly *In vitro* propagated plants and the control plants did not show any significant difference in total alkaloid contents.

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 the germplasm of this medicinally important plant which can enhance the rate of multiplication and can reduce the time period and cost of production.

6.3.6. Biochemical Analysis of Autotetraploids of *Centella asiatica*

The colchicine induced autotetraploids (colchiploids) of *C. asiatica*, were analyzed for the quantity of major saponins like madecassoside, asiaticoside B, asiaticoside, madecassic acid, terminolic acid and asiatic acid.

High performance liquid chromatographic (HPLC) analysis of the autotetraploid leaves revealed presence of higher quantities of medecassic acid + terminolic acid (0.3 gm/gm dry wt.) as compared to the untreated control plants (0.17 gm/gm dry wt.). These results clearly indicate that the endogenous levels of medecassic acid and terminolic acid have gone up by 1.30 fold due to autotetraploidization. Similarly, the asiatic acid content of the colchiploids plants was 0.17 gm/gm dry wt., and that of control diploid plants was 0.06 gm/gm dry wt. Thus, there was 1.10 times increase in control of asiatic acid in the colchicine induced autotetraploidy plants. These results also clearly indicate that the endogenous level of asiatic acid has 1.10 fold due to autotetraploidization.

The control and autotetraploid plants did not show any significant increase in asiaticoside as a result of autoteraploidization. HPLC analysis of medecassoside and asiaticoside-B has revealed that there is no difference in the contents of these two saponins between the diploid and autotetraploid plants of *Centella asiatica*. This indicates, autotetraploidization did not result in increase of these two saponins.

Finally, it can be concluded that, we are successful in realizing our objective of inducing autotetraploidy in *Centella asiatica* plants, using 0.2% colchicine for 4 hours. The induced autotetraploids were morphologically distinct and easily differentiated from the diploids. The autotetraploid plants showed a significant increase in morphometric traits like plant height, inter-nodal length, length & width of leaves, leaf area, leaf thickness, length & width of petiole , fresh & dry weight of leaves, fresh weight of buds and dry weight of seeds, as compared to untreated control plants. The autotetraploids were robust in size, vigorous in growth and showed large, thick, dark green coloured leaves.

Similarly the autotetraploids showed large and fewer stomata as compared to diploid plants. Diploid control plants had a chromosome number of 2n=18 whereas the autotetraploids had a chromosome number of 2n=36.

The somaclonal variants produced through *in vitro* propagation, were very much similar to those of diploid plants. Thus our studies have established that it is not possible to get autotetraploids through somaclonal variations.

The induced autotetraploids (colchiploids) showed 1.30 times high levels of medecassic acid, terminolic acid and asiatic acid.

Thus the present investigation, establishes that it is possible to improve the quantity of therapeutically active compounds through polyploidy breeding of medicinal plants.