Chapter - V

DISCUSSION
DISCUSSION

Plants produce an array of natural products, the so-called secondary metabolites, which play a variety of roles and are also important to man as a source of pharmaceuticals, fragrances, agrochemicals and food additives. The screening of natural products is one of the earliest steps in drug discovery. Medicinal chemists are now routinely expected to be able to improve the potency and/or specificity of a lead compound, perhaps by several orders of magnitude, and thus take a lead compound and turn it into a drug. Herbal products are often questioned for quality control and assurance. Extracts standardized to active constituents and marker compounds have definite advantage over the crude drugs. The active constituents are usually secondary metabolites, derived from biosynthetic pathways present within the plant tissue.

India has been the traditional grower and exporter of medicinal plants and their products. We have perhaps one of the richest ethno-botanical traditions in the world. Over 7,000 species of plants of diverse habits from orchids ferns to trees, grasses, shrubs and climbers, are used by local communities in different eco-systems (Bhojvaid, 2003). The country exported a total of 42,000 tones of medicinal plant raw material to other countries during the year 2000-2001 (Anon., 2001). However, India’s share is very poor, due to lack of quality control and standardization measures. If India’s share has to be increased, a great strategic plan, particularly in standardization and quality control of herbal drugs should be introduced which may boost up the export to Rs.10,000 crores by 2010 (Mehrotra, 2003).

Due to changing ecological factors in the forest and unscrupulous collection of medicinal plants, the wealth of medicinal plant is getting depleted. The collection of these plants from forest cannot cope up with the ever increasing and changing demand from the pharmaceutical industries. Hence, in order to provide regular and sustained supply of medicinal plants, it is essential to domesticate and systematically cultivate these plants on a large scale. The availability of quality planting material is to be ensured. There is also a need for retrieval and documentation of medicinal plants.
In general, the active principles or precursor are synthesized in the leaves, translocated, biosynthesized, and stored in root. There is inter-relationship between the morphology and the metabolite accumulation. Therefore, the medicinal plants have to be characterized at morphological, biochemical as well as genetic level for the evaluation of best quality variety and for the identification of unknown species.

In the present study, two important medicinal plants, *Curcuma* (Haldi) and *Chlorophytm* (Safed musli) were selected for their systematic characterization at morphological, genetic and biochemical level and the biotechnological tools were applied for enhancement of lead compounds.

Turmeric (*Curcuma*) is an important spice crop grown in India. The turmeric genotypes perform differently in different agro-climatic conditions. A considerable variability exists in this crop with regard to morphological and biochemical characters. Exploitation of already existing variability in available germplasm is very important to identify superior cultivars for local conditions. Keeping this objective in view, present study was conducted with 22 *Curcuma* genotypes.

5.1 Morphological Characterization of *Curcuma* genotypes

Higher yields and quality are normally reflected through good growth and it is generally governed by the genetic constitution of the cultivars and environmental conditions under which the crop is raised. In the present study, twenty-two genotypes from different locations and forest area were collected and planted in the field for three successive years 2002, 2003 and 2004. Significant differences for the growth characters i.e., plant height, number of leaves, length and breadth of the leaf were recorded among the genotypes. The morphological difference was evident, since they were grown under identical conditions; it is the genetic factor that expressed the morphological differences among genotypes.

Such variations in the growth parameters among different cultivars of turmeric were also observed by other researchers (Rao et al.; 2004, Sit et al.; 2004) Philip; 1981). The analysis of variance revealed that the mean squares for almost all the quantitative characters were significant at 5% level. This is the indication of the sufficient variability present in the genotypes for number of characters studied.
Any successful hybridization program for varietal improvement depends mainly on selection of parents having high genetic variability so that the desirable character combinations could be selected for crop yield and quality improvement. Cluster analysis was employed to estimate genetic divergence. A wide range of diversity was recorded in terms of genetic distance and on the basis of the observations on seven characters, 22 genotypes formed 4 clusters. Cluster I and II included 6 and 4 genotypes respectively. Cluster III had only one genotype while cluster IV had maximum number of genotypes (11). The highest inter-cluster distance was recorded for cluster IV and the maximum intra-cluster distance showed by cluster III and IV and minimum distance was observed between cluster II and IV.

The genotypes in cluster II showed highest mean performance for plant height, number of leaves per tiller and leaf length. Cluster IV showed highest mean performance for leaf breadth, yield per plant and dry recovery. Only one genotype included in cluster III and showed maximum mean value for number of tillers per clump. The genotype of cluster II showed maximum mean value for leaf length.

These observations suggest that intercrossing of genotypes from different cluster showing good mean performance might help in obtaining high yield with better growth parameters. The genotypes from above different clusters may be utilized as parents in hybridization program to isolate desirable segregant for yield and growth traits.

5.2 Molecular Characterization of Curcuma genotypes

To efficiently evaluate and utilize a large collection of plants, one needs a structured germplasm. The underlying structure of a germplasm collection can be revealed through the use of marker to group individuals, varieties or accessions, into a limited number of entries based on their degree of similarity. Because similar genotypes are more likely to share common characteristics, a limited number of genotypes can efficiently represent a much larger group. The existing ambiguities about identification of genotypes can cause several problems in cultivation of best genotype. Molecular markers offer a promising alternative to morphological markers currently used to identify and characterize the genotypes. These molecular markers are useful to fingerprint varieties, establish phylogenetics, determine similarity among inbreds and mapping entire genome.
Randomly Amplified Polymorphic DNA (RAPD) markers represent amplification products from a polymerase chain reaction (PCR) utilizing arbitrary primers and genomic DNA. Most variation among genotypes for RAPDs probably arises from base pair substitutions or insertion or deletions that modify the primer site, or insertion in the genomic sequence that separates the primer sites to a distance that will permit amplifications. Each of these changes results in presence/absence of a particular fragment. Comparison of the results of cluster analysis based on morphological and DNA based marker system has distinct advantages. The morphological markers are limited in number and they do not reliably reflect genetic relationship, because of their interaction with environment and largely unknown genetic control of traits. In contrast DNA fingerprinting (RAPD) proved to be quite reliable and powerful tool in characterizing individual genotypes. Moreover, RAPD markers represent genetic variation at the DNA level, allowing an estimation of the degree of relatedness between individuals without the influence of environment.

In the present study, twenty-two genotypes from different locations and forest area were collected and characterized using PCR based DNA fingerprinting technique called RAPDs in order to assess variability. In the RAPD analysis the twenty-two genotypes formed two major groups. Genotype T21 (Wild local 1) and T22 (Wild local 2) formed one group, which showed only 0.45 similarity, index value with another genotypes. These two genotypes were collected from the forest area. Similar type of study was reported by Chen et al. (1999) for two Curcuma species. In his study based on morphological and chemical data of the two different species were combined into one but RAPD analysis solved the problem of certifying the medicinal plant Curcuma.

5.3 Estimation of lead compound (Curcuminoids)

The turmeric (Curcuma) is an important spice valued for the characteristics yellow colour and flavour. Curcuminoids constitutes the major colouring matter in turmeric. The three major constituents were identified as diferuloyl methane (curcumin) and its two analogs, viz. p-hydroxy-cinnamoyl-feruloyl methane and p,p- dihydroxy-dicinnamoyl- methane.

In the present study curcuminoids were separated and analyzed by HPLC method. The alcoholic extracts were purified through column chromatography and identified by
thin layer chromatography. Finally the HPLC separation was performed on a C (18) column. Twenty-two different genotypes of *Curcuma* were analyzed to estimate the percentage of these three curcuminoids. The total curcuminoid percentage ranged from 0.069 to 6.16. The variation was observed in curcumin and its analogs. The highest curcuminoid content was recorded in T22 (Wild local 2) and T21 (Wild local 1), which were collected from forest area of Chhattisgarh. Tonnesen *et al.* (1995) used the HPLC method for separation and estimation of curcumin and its structural isomers.

### 5.4 *In-vitro* regeneration of *Curcuma* genotypes

Biotechnology with its apparently limitless potential offers new and exciting opportunities to address the various problems in medicinal plant cultivation. One of the important applications of biotechnology in medicinal plants is micropropagation and rapid clonal multiplication to generate good quality planting material. *Curcuma* is mainly propagated by vegetative part (rhizome) because seed germination rate is very low. Since pathogens fungi, bacteria and virus are readily transmitted through seeds and vegetative parts of plants, it is important to develop micropropagation techniques and to make available the disease free *Curcuma* germplasm for commercial use.

Attempts have been made in this study to develop a technique for more rapid and more convenient clonal propagation of *Curcuma*. Two different genotypes T20 (Local Raipur) and T16 (Ama haldi) were selected for the standardization of *in-vitro* regeneration protocol. The result of surface sterilization treatment indicated that the duration of treatment and percentage of sterilant affected the fungal contamination. The concentrations and combinations of sterilant and duration of treatment have been standardized. Application of bavistin (antifungal agent) and Sodium hypochlorite and mercuric chloride was found beneficial in reducing the fungal contamination. Earlier, Salvi *et al.* (2002) and Mukhri and Yamaguchi (1986) also done similar type of study and found Sodium hypo chlorite and Mercuric chloride was suitable for sterilization to get contamination free cultures.

No response was observed when rhizome part and leaf segments were used as explants. Young sprouted buds were found as the best explants for micropropagation. Salvi *et al* (2002) also reported that young buds were the best explants for *in vitro* regeneration protocol of *Curcuma*. 
Using young sprouted buds as explants, different concentrations and combinations of phytohormones were used in MS medium and their effect on shoot initiation and multiplication was examined. The effect of different concentrations and combinations of 2,4-D, BAP and KIN was observed over the explants. Shoot initiation percentage and the mean number of shoots were found higher in 2.0 mg/l BAP + 2.0 mg/l KIN for both Curcuma genotypes.

The shoot multiplication rate was maximum (9 shoots / explant), when the combination of NAA and BAP (1.5 mg/l and 2.0 mg/l) was used. These favorable results are in commensurate with the finding of Sunitibala et al., (2001). She reported maximum multiplication of Curcuma plantlets in MS medium supplemented with 1.0 mg/l NAA and 2.0 mg/l BAP.

The shoots regenerated from the subculture medium were further used to study rooting. The half strength MS medium with 500 mg/l charcoal exhibited good response for rooting. This is in contrary with the earlier report of Rout et al., (1995). Sit and Tiwari (1997) and Rout et al., (1998), they reported that IBA was essential for root induction. The in-vitro regenerated plants could sustain themselves easily on their own roots and showed normal growth.

Standardized medium was tested for all remaining genotypes. Among the twenty genotypes only seven genotypes responded in the same medium. This shows that in-vitro regeneration technology can be successfully employed for Curcuma plantlets, if careful selection for proper combination and concentration of phytohormones and explant is made for specific genotypes.

5.5 Assessment of in-vitro regenerated plantlets of Curcuma

Curcuma, although an important spice and medicinal plants, but it has not received much attention from tissue culturist and only a few reports are available on in-vitro multiplication. In this study the effect of in-vitro regeneration on the curcuminoid content was observed. The results showed that in one selected genotype the curcuminoid percentage was significantly higher but in another case decrease in curcuminoid content was recorded. The results of the study clearly demonstrates the reliability and enormous scope of in-vitro regeneration technology, but with due consideration for the specific genotype.
Safed musli

*Chlorophytum* (Liliaceae) commonly known as safed musli is an endangered species valued for the dried fasciculated storage roots. These are reputed to have aphrodisiac properties. Due to large scale and indiscriminate collection of wild material and insufficient attempts either to allow its replenishment or its cultivation, *Chlorophytum* is rapidly disappearing. The natural regeneration of this herb is through tuberous roots that have become scarce in nature. Though the importance of this valuable crop is known, yet very few attempts have been made. The present study was conducted for the systematic characterization of *Chlorophytum* genotypes at morphological, genetic and biochemical level.

5.6 Morphological characterization of *Chlorophytum* genotypes

*Chlorophytum* genotypes are generally distinguished on the basis of morphological features. The comparative study was performed for five different genotypes of *Chlorophytum* for successively three years 2002, 2003 and 2004. Significance of variance for all the quantitative characters indicated presence of considerable variability. The mean sum of square of almost all the quantitative characters was found significant for each genotype at five percent level. On the basis of cluster analysis five genotypes were grouped into two clusters. Four genotypes were in cluster II and only one genotype formed one separate cluster I. The results indicated that this M3 genotype was different from all other genotypes with respect to morphology. Among the qualitative characters variation in colour was observed in different genotypes. Cluster II showed highest mean value for number of tuber, leaf length, length of solitary scape, number of flowers, plant height, number of leaves, dry recovery and yield. Genotypes of cluster I showed highest mean value for leaf breadth only. This variation in cluster can be used for hybridization program for crop improvement.

5.7 Molecular characterization of *Chlorophytum* genotypes

DNA based marker system offer a more reliable alternative to detect genetic polymorphism used for cultivars identification and to study genetic diversity among genotypes. We screened 64 RAPD markers on five different *Chlorophytum* genotypes. One seventy-seven polymorphic bands were obtained among a total of 272 amplified
bands. Similarities were calculated among the genotypes by NTSYS software. In dendrogram genotypes split into two major clusters. As expected the dendogram showed clustering in good accordance with the classification previously established on the basis of morphology. DNA fingerprinting can be further exploited to develop species-specific molecular marker for identification of unknown species and also useful to address intraspecific questions concerning the genetic relatedness between individuals.

5.8 Estimation of lead compound (Saponin)

Research studies on Chlorophytum indicated that saponin is responsible for medicinal properties. Saponin estimated for all the collected five genotype. Matured finger root used for the extraction. After peeling of skin root fingers were dried at 60 degree followed by Sun drying for 3-4 days. Saponin extracted by 85% ethanol. Extracted material purified through column chromatography. After identification of saponin fraction by thin layer chromatography, extracted and purified sample subjected to HPLC for the estimation of saponin. Saponin content varies from 10.75 to 28.28%. The highest saponin content reported in accession number M1. High heritability (79.87%) in sapogenine content confirmed less influence of environment, further suggesting the presence of additive gene effect in expression of this economically important trait with aphrodisiac property was reported by Bhagat and Jadeja (2003). Studies on saponin indicated that content in Chlorophytum were significantly affected by the environment. Over the year analysis indicated that saponin content can be improved by sacrificing the root yield through selection. (Jat and Sharma, 1996). Saponin compound present in Bupleurum falcatum, the coefficient of variability values for silksaponin in the extract are below 4%. (Park et al., 2000). Sapogenine content also estimated by biochemical method based on LB reaction in Chlorophytum. The sapogenine content varied with solvent system used for extraction. (Joshi et al., 2000). In the present study huge variation was recorded regarding saponin content in different genotypes.

5.9 In-vitro regeneration of Chlorophytum genotypes

Due to large scale and indiscriminate collection of the wild material and insufficient attempts either to allow its replenishment or its cultivation Chlorophytum is rapidly disappearing from nature. The natural regeneration of this herb is through tuberous roots. Seed germination is only 14-16 percent. Thus an in-vitro method for conservation and
multiplication of this crop is very useful. High frequency in-vitro regeneration technology can be further exploited for obtaining somoclones with higher medicinal value. In this experiment, strong treatment has been given to explants to protect them from contamination. Shoot initiation was observed only in stem disc (crown) explant. No response had found in leaf as well as root segment. Growth regulators NAA, 2,4-D, BAP, 2iP and KIN either alone or in combination tested in the present study. Response has been recorded by Stem disc (crown) on the medium containing different concentrations of BAP. BAP in higher amount was found best for shoot initiation in comparison to 2,4-D, KIN, 2iP. MS medium supplemented with 5.0 mgL⁻¹ BAP was best for shoot initiation. Mean number of shoot initiated in MS medium supplemented with 5.0 mgL⁻¹ BAP is 7.8 in M1 and 6.6 in M2. More than 80% response has been found in 5.0 mgL⁻¹ BAP treatments. These cultured shoots were used for sub culturing. MS medium supplemented with 5.0mgL⁻¹ BAP used as a parent media. Different concentrations and combinations of growth regulators tried for multiple shoot formation. Average response of 75% with 6-8 mean numbers of shoots was found in auxin and cytokinin ratio. When sub culturing was done on to the medium of same composition (5.0mgL⁻¹ BAP) resulted in an increase in size and extra ordinary response was found. 100% response with more than 15 mean numbers of shoots in sample 1 and 17 in sample 2 generated from the MS medium supplemented with 5.0 mgL⁻¹ BAP. 75 % response reported when BAP concentration increased more than 5.0 mgL⁻¹ BAP. Healthy roots were found when shoots transferred to half strength MS medium supplemented with 500 mgL⁻¹ charcoal. Plantlets developed through tissue culture were successfully transferred in soil and sand (1: 1) with high rate of survivability (80%).

Tissue culture has been used to accelerate plantation development, to shorten breeding cycle and to rapid multiplication. The best use of micro propagation technique is to overcome dormancy problem. Plant tissue culture has been successfully used to micro-propagate medicinal plants. Plantlet regeneration has been reported through apical meristem in C. comosum. Plantlet regeneration and bulbi! formation has been reported through leaf and stem explant in Curculigo orchiodes (Suri et al., 1999).
5.10 Assessment of in-vitro regenerated plantlets of Chlorophytum

In-vitro regeneration in standardized medium was tested for all collected genotypes of Chlorophytum. All genotypes did not respond similar in the standardized media, which indicated that in-vitro potential was genotype specific. Micropropogated plants were field evaluated and compared with conventionally grown plants. Increase in number of tubers, length of solitary scape, leaf breadth, number of leaves were recorded in in-vitro regenerated plantlets of M1 in comparison with conventionally grown plants of M1. In contrary, comparatively less number of tubers, number of leaves, number of flowers, plant height, leaf breadth, leaf length and yield was recorded in in-vitro regenerated plantlets of M2. It has been found that all the phenotypic characters showed variation due to genetic and environmental interaction. The saponin content recorded were comparatively low in in vitro regenerated plantlets of Chlorophytum genotypes. The saponin content in in-vitro regenerated plants was 25.65 % as compared to conventional grown, M1 genotype, which was 28.28 %. Similarly, in in-vitro regenerated plants of M2 it was 9.1 % as compared to conventional grown M2, which was 10.75 %. This clearly indicated that micropropagation is not very effective in enhancement of saponin content, but it is very useful to break the dormancy and to get disease free planting material in large amount through out the year.