MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF SELECTED COLEUS SPECIES AND MUTANTS OF *Coleus forskohlii* Briq.

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in

PLANTATION MEDICINAL AROMATIC AND SPICE CROPS

BENGALURU **JULY, 2011**

AFFECTIONATELY DEDICATED TO

My parents, sister
&

My Adorable Chairman

Dr. M. VASUNDHARA

DIVISION OF HORTICULTURE
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GKVK, BENGALURU-560 065

CERTIFICATE

This is to certify that the thesis entitled **"Morphological and molecular characterization of selected Coleus species and mutants of *Coleus forskohlii* Briq."** submitted in partial fulfillment of the requirements for the award of the degree of **MASTER OF SCIENCE (Horticulture)** in **PLANTATION MEDICINAL AROMATIC AND SPICE CROPS** of the University of Agricultural Sciences, Bengaluru, is a bonafide record of research work done by **Mr. PRASANNA HEGDE, ID. No. PHK 924** during the period of his study in the university under my guidance and supervision and that no part of this thesis has been submitted for the award of any degree, diploma, associateship, fellowship or other similar titles.

Bengaluru
July, 2011

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Morphological and molecular characterization of selected Coleus species and mutants of *Coleus forskohlii* Briq.

ABSTRACT

Coleus is an important medicinal plant containing a diterpinoid called forskolin in its tuberous roots. In the present study 3 Coleus species, 11 mutants and a variety of *C. forskohlii* were characterized through morphological and molecular markers.

In morphological study entries differed significantly for different morphological and yield characters. MV5 was superior with respect to plant height (60 and 120 DAP), number of branches (at 180 DAP), fresh and dry root weight, number of roots, root length, total biomass and harvest index. Aisiri (MV7) recorded maximum number of branches (60 days and 120 DAP) and a forskolin content of 0.74%. SV2 recorded highest forskolin content of 0.76%. Among the various species under the study *Coleus vettiveroides* differed from others for both morphological and yield characters.

In the molecular diversity study 20 RAPD primers generated 293 bands, of which 13 were polymorphic. Different Coleus species and mutants under study differed from *Coleus vettiveroides* in dendrogram, generated by using banding pattern of decamers. Principle component analysis with 2 dimensional and 3 dimensional figures also showed the same clustering as of the dendrogram. In the distance matrix higher genetic distant of 50% was observed in between SV1 and *Coleus aromatics var. variegated* and least distance of 7% in MV9 and K-8. SCAR was developed from the primer RAPD-4. Thus, it can be concluded that although the mutants were morphologically alike, a narrow genetic difference was existed between them.

Signature of student

(PRASANNA HEGDE)

Signature of major advisor

(M. VASUNDHARA)

ಆಯ್ದ ಕೋಲಿಯಸ್‌ನ ಜಾತಿ ಮತ್ತು ಕೋಲಿಯಸ್ ಫೋರ್ಸ್ಕೊಲಿಯ ಪರಿವರ್ತಕಗಳಲ್ಲಿ ಸ್ವರೂಪ ಮತ್ತು ಗುಂಪಿಗೆ ಸಂಬಂಧಿಸಿದ ಗುಣ ಪರಿಶೀಲನೆ

ಸಾರಾಂಶ

ಕೋಲಿಯಸ್ ಒಂದು ಪ್ರಮುಖ ಔಷಧಿ ಬೆಳೆಯಾಗಿದ್ದು, ಬೇರುಗಳಲ್ಲಿ ಫೋರ್ಸ್ಕೊಲಿನ್ ಎಂಬ ಡೈಟರ್ಪಿನೊಯ್ಡ್ ಇರುತ್ತದೆ. ಪ್ರಸ್ತುತ ಅಧ್ಯಯನದಲ್ಲಿ ಕೋಲಿಯಸ್‌ನ ಮೂರು ಜಾತಿಗಳು, ಕೋಲಿಯಸ್ ಫೋರ್ಸ್ಕೊಲಿಯ ಹನ್ನೊಂದು ಪರಿವರ್ತಕಗಳು ಹಾಗೂ ಒಂದು ವಾಣಿಜ್ಯ ತಳಿಯ ಸ್ವರೂಪ ಮತ್ತು ಅಣು ಗುರುತಿನಿಂದ ವಿಶ್ಲೇಷಿಸಲಾಯಿತು.

ಪರಿವರ್ತಕ ಎಮ್‌ವಿ–5 ಯು ಗಿಡದ ಎತ್ತರ (ನೆಟ್ಟ 60 ಮತ್ತು 120 ದಿನಗಳ ನಂತರ), ಕೊಂಬೆಗಳ ಸಂಖ್ಯೆ (ನೆಟ್ಟ 180 ದಿನಗಳ ನಂತರ), ಕಾಂಡದ ತಾಜಾ ಮತ್ತು ಒಣ ತೂಕ, ಪ್ರತಿ ಗಿಡದಲ್ಲಿ ಬೇರುಗಳ ಸಂಖ್ಯೆ, ಬೇರಿನ ಉದ್ದ, ಒಟ್ಟು ಜೀವರಾಶಿ, ಕೊಯ್ಲಿನ ಸೂಚ್ಯಂಕಗಳಂತಹ ಲಕ್ಷಣಗಳಲ್ಲಿ ಅತ್ಯುತ್ತಮವಾಗಿತ್ತು. ಪರಿವರ್ತಕ ಎಮ್‌ವಿ–7 (ಐಸಿರಿ ತಳಿ)ಯು ಅತಿ ಹೆಚ್ಚಿನ ಕೊಂಬೆಗಳ ಸಂಖ್ಯೆಯನ್ನು ದಾಖಲಿಸಿತು (ನೆಟ್ಟ 60 ಮತ್ತು 120 ದಿನಗಳ ನಂತರ) ಮತ್ತು 0.74 % ನಷ್ಟು ಫೋರ್ಸ್ಕೊಲಿನ್ ಅಂಶವನ್ನು ಹೊಂದಿದ್ದು, ಸೊಮಕ್ಲೊನ್ ಎಸ್‌ವಿ–2ನ ಫೋರ್ಸ್ಕೊಲಿನ್ ಅಂಶ (0.76%) ದೊಂದಿಗೆ ಸಾಮ್ಯತೆ ಇತ್ತು. ಅಧ್ಯಯನಕ್ಕೆ ಬಳಸಿದ ವಿವಿಧ ಜಾತಿಗಳಲ್ಲಿ *ಕೋಲಿಯಸ್ ವೆಟಿವೆರೊಯ್ಡಸ್*‌ನ ಇಳುವರಿ ಮತ್ತು ಗುಣಸ್ವರೂಪಗಳು ಉಳಿದವುಗಳಿಗಿಂತ ಬೇರೆಯಾಗಿತ್ತು.

ಅನುವಂಶೀಯ ವೈವಿಧ್ಯತೆಯನ್ನು ಅರಿಯಲು 20 ಆರ್‌ಎಪಿಡಿ ಪ್ರೈಮರ್‌ಗಳನ್ನು ಬಳಸಲಾಯಿತು. ಇವು ಉತ್ಪತ್ತಿಸಿದ 293 ಪಟ್ಟಿಗಳಲ್ಲಿ, 13 ಪಟ್ಟಿಗಳು ಬಹುರೂಪತೆಯನ್ನು ಪ್ರದರ್ಶಿಸಿದವು. ಕೋಲಿಯಸ್‌ನ ವಿವಿಧ ಜಾತಿಗಳು ಮತ್ತು ಪರಿವರ್ತಕಗಳು ತೋರ್ಪಡಿಸಿದ ಗುರುತಿನ ಪಟ್ಟಿಗಳ ಪದ್ಧತಿಯ ಆಧಾರದ ಮೇಲೆ ಡೆಂಡ್ರೊಗ್ರಾಮ್ ಮತ್ತು ತತ್ವ ಘಟಕ ವಿಶ್ಲೇಷಣೆಯಿಂದ 2ಡಿ ಮತ್ತು 3ಡಿ ಚಿತ್ರಗಳನ್ನು ಅಭಿವೃದ್ಧಿಪಡಿಸಲಾಯಿತು. ಈ ಎರಡೂ ವಿಶ್ಲೇಷಣೆಗಳು *ಕೋಲಿಯಸ್ ವೆಟಿವೆರೊಯ್ಡಸ್*‌ವು ಉಳಿದ ಪರಿವರ್ತಕಗಳು ಮತ್ತು ಜಾತಿಗಳಿಗಿಂತ ವಿಭಿನ್ನವಾಗಿವೆ ಎಂದು ತೋರ್ಪಡಿಸಿದವು. ದೂರದ ಮೆಟ್ರಿಕ್ಸ್‌ನ್ನು ಲೆಕ್ಕಾಚಾರ ಮಾಡಿದಾಗ ಎಸ್‌ವಿ–1 ಮತ್ತು *ಕೋಲಿಯಸ್ ಅರೊಮ್ಯಾಟಿಕಸ್ ವರ್. ವೆರಿಗೇಟೆಡ್*‌ನ ನಡುವೆ ಅತಿ ಹೆಚ್ಚಿನ ಅಂದರೆ 50% ನಷ್ಟು ಸಾಮ್ಯತೆ ಕಂಡು ಬಂದಿತು. ಅತಿ ಕಡಿಮೆ, ಅಂದರೆ 7% ನಷ್ಟು ಸಾಮ್ಯತೆಯನ್ನು ಕೆ–8 ಮತ್ತು ಪರಿವರ್ತಕ ಎಮ್‌ವಿ–9 ಗಳು ತೋರಿದವು. ಎಸ್‌ಸಿಏಆರ್‌ನ್ನು ಆರ್‌ಎಪಿಡಿ –4 ನಿಂದ ಅಭಿವೃದ್ಧಿಪಡಿಸಲಾಯಿತು. ಪ್ರಸ್ತುತ ಅಧ್ಯಯನದಲ್ಲಿ ಬಳಸಿದ ಪರಿವರ್ತಕಗಳು ನೋಡಲು ಒಂದೇ ತೆರನಾಗಿ ಇದ್ದರೂ, ಅವುಗಳ ನಡುವೆ ಅತಿ ಸೂಕ್ಷ್ಮವಾದ ಅನುವಂಶೀಯ ವ್ಯತ್ಯಾಸ ಇರುವುದು ಕಂಡುಬಂದಿತು.

ವಿದ್ಯಾರ್ಥಿಯ ಸಹಿ
(ಪ್ರಸನ್ನ ಹೆಗಡೆ)

ಪ್ರಧಾನ ಮಾರ್ಗದರ್ಶಕರ ಸಹಿ
(ಎಮ್. ವಸುಂಧರ)

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INTRODUCTION

I. INTRODUCTION

Plants constitute one of the major sources of drug in traditional as well as modern medicine. These plants provide raw materials for pharmaceutical industries, thus are the valuable source of income by earning foreign exchange for a large number of small and marginal farmers in developing countries like India, China, Brazil etc.

India's biodiversity coupled with varied agro climatic zones plus a competitive workforce, highly intelligent scientific force and rich business community make the country the best choice for growing medicinal plants during recent years. Due to the cumulative derogatory effect by the use of synthetic chemicals and antibiotics, a definite trend in adoption of plant-based medicament is seen. Further, field of medicinal plants is attaining tremendous importance because of growing demand for natural products obtained from plants such as phytochemicals, steroids, alkaloids etc. Many of the medicinal plants are still collected from the wild, as they have not been commercially exploited. However, their availability from natural sources and their indiscriminate and destructive collection have made them vulnerable and several medicinal plants have been included in the endangered list (Vishwakarma *et al.*, 1988). Hence much importance is now being given for the commercial cultivation of medicinal plants. *Coleus forskohlii* is one such plant which is attaining economic importance in recent times.

The genus *Coleus,* an important member of the family Lamiaceae (formerly Labiatae) consists of about 150 species, most of which are annual or perennial herbs (Patil *et al.,* 2001). Important members of the genus *Coleus* include species like *Coleus amboinicus (C. aromaiicus), Coleus forskohlii* Briq. (*C.barbatus* Benth.), *Coleus malabaricus* Benth., *Coleus parviflorus* Benth., *Coleus rotundifolius (C. tuberosus* Benth.),

Coleus scutelliroides Benth. *(C. blumei* Benth.), *Coleus spicatus* Benth. *(C. canises), Coleus vettiveroides* Jacob., (Syn. *Plectranthus tomentosus), Coleus zeylanicus* etc.

The genus *Coleus* is important from the point view of pharmaceutical industry as revealed by Ayurvedic uses. C *forskohlii* is the only naturally occurring species to have tuberous roots (Shah *et al.,* 1980). *C. forskohlii* is the most important species, popularly known as 'Garmai' in Gujrathi, 'Patharchur' in Hindi, 'Maimul' in Marathi and 'Makandiberu or Manganiberu' in Kannada. It is one of the most significant potential medicinal crops of future as its pharmacological properties have only been discovered recently. Its tuberous roots are found to be a rich source of forskolin, which is a very good drug for hypertension, glaucoma, asthma, congestive heart failure and certain types of cancers. The plant is distributed all over the tropics and subtropics of India. It is cultivated mainly in Rajasthan, Maharashtra, Karnataka and Tamil Nadu for its tuberous roots (Patil and Hulamani, 2001).

Over the past decade, extensive investigations on *Coleus forskohllii* for its pharmacological effects has led to the isolation of its active compound, a diterpine named as 'Forskolin' (Ammon and Muller 1985). This compound possesses several biological and pharmacological properties. It shows positive hypotensive activity, inhibits thrombolyte aggregation and decreases intracular pressure. It is also an amoebicidal and antidiarroeal agent. These properties made the pharmaceutical industry to recognize the plant as one with great medicinal and economic importance (Shah et al., 1980). So far, *C. forskohlii* is the only known source of forskolin (De Souza and Shah, 1988). Though almost all parts of the plant are found to have traces of forskolin, tuberous roots are the main source of the compound and are therefore preferred for extraction.

The root juice is anthelmintic and given to children suffering from constipation (Singh *et al.*, 1980). *Kotas*, the native tribals of Trichigadi in Nilgiris, South India consider decoction of tubers roots as a tonic (Abraham *et al.*, 1988). Roots are eaten for cough and 1-3 teaspoonfuls of root decoction is recommended for treatment of asthma in Maharastra and is also believed to have blood-purifying action (Shah *et al.*, 1980).

The root extracts of *Coleus forskohlii* were found to contain a diterpene, forskolin, which is exclusive to this species (Shah, *et al.*, 1980). It is used for the treatment of abdominal colic, heart diseases, respiratory disorders, painful menstruation, insomnia and certain types of cancers (Ammon and Muller, 1985). These therapeutic properties of *Coleus forskohlii* made it as a taxon of importance in modern medicine. The novel feature of forskolin is its unique mechanism of generating cyclic AMP (Adenosine Monophosphate) in the cells through direct activation of the catalytic unit of adenylate cyclase enzyme (Seamon and Dally, 1981).

Like any other crop, *C. forskohlii* also shows a large variation with respect to plant growth, tuberous root yield and in the forskolin content. Most of the economic characters including yield are polygenically controlled and are highly influenced by environmental factors. Since tubers constitute the economic yield in this crop, higher tuber yield alone can fetch good returns to the farmers. Therefore, an evaluation of genotypes for higher tuber yield is important.

The availability of wide genetic diversity both for forskolin content as well as for tuber yield besides other characters render *C. forskohlii* an ideal crop species for genetic improvement (Vishwakarma *et al.,* 1988; Hegde and Krishnan, 1994). Presently, the classification of *Coleus* is mainly based on morphological characters. The morphological characteristics are key issues in characterizing the genotypes or

accessions, but most of the times these characters are highly influenced by environmental conditions. Hence, the data obtained by such evaluations are not easily understood at genetic level, often resulting in maintenance of duplicate accessions or genotypes. Moreover, such evaluation studies demand higher cost and efforts.

Characterizing the genetic diversity existing in genotypes or accessions of a species is of prime importance especially to plant breeders. In this regard, molecular markers have an unique advantage to study the diversity at DNA level. Among the various DNA markers available, Randomly Amplified Polymorphic DNA (RAPD) markers are found to be more suitable for the diversity analysis, since they are faster, cheaper and are able to detect even minute variation at molecular level.

The arbitrary marker techniques are sensitive to changes in the reaction conditions. In order to bridge the gap between the ability to obtain linked markers to a gene of interest in a short time and the use of these markers for map-based cloning approaches and for routine screening procedures, SCAR marker technique was developed and applied. The SCARs are PCR-based markers that represent genomic DNA fragments at genetically defined loci that are identified by PCR amplification using sequence specific oligonucleotide primers (Paran and Michelmore, 1993; McDermott *et al.*, 1994). Derivation of SCARs involves cloning the amplified products of arbitrary marker techniques and then sequencing the two ends of the cloned products. The sequence is thereafter used to design specific primer pairs of 15–30 bp which amplify single major bands of the size similar to that of cloned fragment.

Therefore the present study is an attempt to evaluate and characterize the available species and mutants of *Coleus forskohlii* based on their morphology, yield attributes, forskolin content and molecular diversity. The investigation is titled as “Morphological and molecular

characterization of selected *Coleus* species and mutants of *Coleus forskohlii* Briq."

The objectives of the study are as follows:

1) Screening for the morphological characters in *Coleus* species and mutants of *Coleus forskohlii*.
2) Characterization of selected Coleus species and mutants of *Coleus forskohlii* using molecular markers.
3) Estimation of forskolin content in mutants of *Coleus forskohlii*.
4) Development of varietal specific SCAR marker for the variety Aisiri (mutant MV7).

REVIEW OF LITERATURE

II. REVIEW OF LITERATURE

Characterizing the diversity existing in various species of a genus and genotypes of a species is of prime importance. Diversity is observed for morphology, yield attributes and genetic variation. In this chapter an effort has been made to review the existing literature on evaluation of *Coleus* species and accessions based on morphological and yield attributes and through RAPD assisted characterization. As the information available on the above mentioned characters in *Coleus* is limited, the work done on other related medicinal and aromatic plants is also reviewed.

The research work undertaken to evaluate and characterize various genotypes of a species in medicinal and aromatic plants have been presented under the following headings:-

2.1 Botany

2.2 Cytology

2.3 Genetic variation

2.4 Chemistry and pharmaceutical applications

2.5 Genetic diversity assessment using RAPD markers

2.6 Development of SCAR markers

2.1 BOTANY

The genus *Coleus* (Lamiaceae) was first described by De Loureiro in 1970. The generic name is derived from the Greek word 'Koleos' meaning sheath. The species has four didynamous, declinate stamens, whose filaments unite as a sheath at their base. *Coleus* is closely allied to the genus *Plectranthus*, whose species lack monodelphous stamens. Because two genera are very similar, some taxonomists do not consider them to

be distinct and extend the genus *Plectranthus* to include all *Coleus* species (Morton, 1962; Launert, 1968; Codd, 1971)

Several modern taxonomists (Keng, 1969; Blake, 1971; Cramer, 1978) follow Loureiro's classification and consider *Coleus* as separate genus.

***Coleus amboinicus* (Syn. *C. aromaticus*)**

Coleus amboinicus is a highly aromatic, semi-woody perennial, growing to a height of 60-90 cm, grown in tropical countries. It is concluded that plant can be cultivated commercially, particularly in USA and that the use of a standard common name, such as 'country borage', will improve its identification and widen its use.

Morton (1992) reported that in the *C. forskohlii* plants, stem is fleshy with 30-90 cm height, leaves are 2.5-5 cm long, simple, opposite, petioled, broadly ovate or cordate, crenate, fleshy and very aromatic. Flowers are shortly pedicelled, 3 mm long, pale purplish in dense whorls at distant intervals in a long slender raceme. Upper calyx is ovate, acute, membranous, lower acuminate. Corolla is pale purplish, short tubed, throat inflated, lips are short. Stamens are exserted. Fruits are orbicular or ovoid nutlets.

Coleus forskohlii

C. forskohlii Briq. (Syn. *Coleus barbatus* Benth.) is an aromatic perennial plant grows to a height of 60-70 cm and is highly branched. Leaves are 7.5-12.5 cm in length and 3-5 cm in width. Inflorescence is a receme, which is 15-30 cm in length. Flowers are stout, 2-2.5 cm in size and usually perfect with hairy calyx inside. Upper lip of calyx is broadly ovate. The blue or liliac corolla is bilabiate. Lower lobes are elongated and concave. The ovary consists of four parts. Stigma is two lobed. Roots

are tuberous, fasciculate, 20 cm long and 0.5-2.5 cm across, conical fusiform, straight or anglish within and with a strong aroma (Bailey, 1942).

Based on anatomical studies on tuberous roots in *C. forskohlii*, Abraham *et al*. (1998) reported the presence of yellowish to reddish brown cytoplasmic vesicles in cork cells which store secondary metabolites.

2.2 CYTOLOGY

Reddy (1952) studied chromosome number in *Coleus* species. *C. forskohlii* Briq. was reported as a diploid with n = 14. However, Riley and Hoff (1961) in their studies on chromosome numbers, in some South African dicotyledons, reported that C. *forskohlii* Briq. was a diploid with basic number n = 16.

Bir and Saggoo (1985) studied meiosis in central and south Indian collections of *C. forskohlii* Briq. and reported that central Indian collections were reported to have basic number of n = 17, while south Indian collection had n = 15. The authors concluded that variability in basic chromosome number of various members of the family could possibly be the result of aneuploidy at generic level which ultimately leads to morphological variations. This could also hold true for the above intraspecific variations in basic number recorded for *C. forskohlii.*

Determination of the nuclear DNA content and total chromosome length in eight morphologically distinct diploid clones (2n = 30) of *C. forskohlii* collected from different regions and two induced autotetraploids were compared by Bahl and Tyagi (1989). The study revealed intraspecific nuclear DNA variations to the extent of 8.973 to 5.910 µg. The autotetraploid showed double the amount of DNA than diploid progenitors. Duncan's multiple range tests revealed six distinct groups

among diploids with some over lapping. Linear regression analysis of total chromosome length and DNA amounts of 10 genotypes showed a positive relationship.

In order to understand the evolutionary relationship and aid in the preparation of linkage groups for use in genetic and cytogenetic investigations, Bahl and Tyagi (1988) conducted detailed pachytene analysis of the chromosome compliment in *C. forskohlii* and described the morphology of 15 chromosome pairs. Based on length, centromere position, extent of differentiation into euchromatic and heterochromatic regions, size, number and position of chromosomes, the entire pachytene compliment was identified. Chromosome length varied from 50.45 µ to 150 µ and arm ratio from 0.09 to 0.9. Chromosomes 9 and 13 were readily identified based on the presence of nucleolus organizing region in short arm.

2.3 GENETIC VARIATION

Coleus forskohlii

Limited studies on genetic variation in *C. forskohlii* have been undertaken. Vishwakarma *et al.* (1988) screened 38 genotypes collected from diverse sources for root yield and forskolin content. The forskolin content showed a wide range of variation from 0.01 to 0.44 per cent. Maximum forskolin content was recorded in IH-l genotype. The dry root yield ranged from 16.6 to 203.3 g per plant. The forskolin yield per plant varied from 0.004 to 0.803 g. Collection KM-2, recorded high root yield of 200.7 g per plant and forskolin content of 0.4 per cent.

Hegde (1992) studied the genetic variability present in *C. forskohlii.* Higher heritability was observed for total dry matter content, fresh weight of roots, harvest index and root diameter. Total dry matter content, fresh weight of roots and harvest index recorded high phenotypic variability.

Vegetatively propagated diploid and induced autotetraploid plants were evaluated and data were recorded for 12 quantitative traits. The 2 types differed for all measured characters except ratio of dry weight of shoot: root, harvest index and growth rate. At one or more growth stages, the diploid registered higher values for total dry matter, dry weight of leaves, stem and tubers, number of leaves/plant, leaf area/plant, leaf area index, leaf area ratio, specific leaf area, leaf area duration and relative growth rate (121-150 days), while the autotetraploid registered higher leaf: stem ratio, leaf weight, specific leaf weight, net assimilation rate and relative growth rate (51-90 days). In general, the physiological changes in the autotetraploid contributed to low biological yield (Hegde and Krishnan, 1994).

Hegde and Krishnan (1998) evaluated 13 entries consisting of 7 tuber bearing and 6 non-tuber bearing accessions of C. *forskohlii* collected from across India. Data on 10 morphological and agronomic characters were recorded after 190 days and harvesting was performed at 160, 190 and 230 days. Significant differences between accessions were noted and non-tuberous accessions were superior to tuberous accessions for numbers of branches and leaves. Among the tuberous types tested total dry matter values ranged from 82.6 to 1093 g.

Patil and Hulamani (2001) studied the performance of six diverse genotypes of *C. forskohlii* for growth, yield and essential oil content. Among these genotypes, C2 excelled the rest of the accessions for morphological characters like plant spread at 120 days, number of branches per plant at 150 days, number of leaves per plant at 60 days, and lamina length and petiole length at all the crop-growth stages. Among the yield parameters evaluated accession C2 recorded the highest length of tubers (15.44 cm), diameter of tubers (1.68 cm), number of tubers per plant (41.37), fresh weight of tubers per plant

(534.75 g), volume of tubers (487.04 cc), harvest index (45.94%) and dry weight of tubers (66.64 g).

Patil and Hulamani (2001) studied the effects of planting stock, i.e. cuttings obtained from the terminal, middle and basal portions of shoots, in *C. forskohlii* for different morphological characters. Plants raised from terminal cuttings were tallest (61.35 cm), exhibiting the highest values for the number of branches (100.61), east-west plant spread (53.68 cm), number of leaves (392.99) and stem diameter (2.59 cm). The yield and essential oil content were not significantly affected by the source of cuttings.

Pharmacognostical study of roots of *C. forskohlii* procured from different geographical zones of India was carried out (Srivastava *et al.,* 2002*),* there was remarkable variation in the percentages of coleonol, sugars, starch and protein in different samples. For instance, the percentage of coleonol is higher in Salem sample (3.11 %) and Vijayawada sample (2.202%) as compared to Agrakhal (1.139%) and Tarikhet sample (0.7319%). Similarly, there was an almost four fold increase in percentage of sugar and starch in southern samples while the protein percentage was almost twice as high in northern samples.

C. parviflorus

Fifty morphologically different mutants of *C. parviflorus* were evaluated and maintained as mutant clones. Two early mutants were identified which were harvested at 100 days after planting as compared with 150-160 days taken normally. The early mutants formed tubers mainly near the base of the plant which made for harvesting easier (Vasudevan and Jos, 1988).

Twelve *Coleus* (*C. parviflorus* [syn. *C. rotundffolius])* mutants, induced in cultivar CP 11 by treatment with 1-4 kR gamma rays, were

raised from node cuttings and evaluated for 12 morphological, yield and quality traits. Compared to untreated plants, the mutants showed reduced canopy spread and top yield (biomass), and fewer tubers/plant, whereas higher values were noted for harvest index, yield/plant and tuber size uniformity. Quality, traits varied in both the positive and negative directions. The highest tuber yielding mutants were CPM-33 and CPM-49, giving 423 and 372.5 g/plant of tuber yield respectively (Vasudevan and Jos, 1992).

2.4 CHEMISTRY AND PHARMACEUTICAL APPLICATIONS

Coleus amboinicus

C. *amboinicus* is used as a herb extensively in India. Leaves are used as a remedy for cholera (Chatterjee *et al.,* 1958).

Brieskorn and Riedel, (1977) reported about the presence of flavones like salvigeriin, 6-methoxygenkwanin, quercetin, chrysoeriol, luteolin and apigenin, eriodyctiol and the flavanonol taxifolin in the leaves of *C. amboinicus*.

The polyphenol contents of *C. aromaticus (Plectranthus amboinicus),* can be therapeutically valuable inhibiting significant antitumour and antimuragenic properties (Annapurni and Priya, 1999). Leaf extracts from this species have also been found to have anti-bacterial properties. Its leaves are carminative, used in the treatment of urinary diseases, colic and dyspepsia. *C. amboinicus* known as Pashan bheda in Sanskrit and Patharchur in Hindi is used as a major ingredient in Ayurveda for kidney stones, conjunctivitis, spastic colon (Parra, 1999).

Coleus blumei

Presence of a long chain hydrocarbon, a sterol, flavonoid and a triterpenoid compound has been reported from the leaves of a purple

variety of *C. blumei.* The ethyl acetate soluble fraction of the ethanol extract showed antibacterial and anti-tumour activities (Garcia *et al.,* 1973).

C. *blumei* leaves are used for treatment of dyspepsia, potential source of rosmarinic acid-an antioxidant compound (Molgaard and Ravn, 1988). Rosmarinic acid is an ester of caffeic acid and 3.4-dihydoxy phenyl lactic acid. It is commonly found in *C. blumei.* It has a number of interesting biological activities, antiviral, antibacterial, anti-inflammatory and antioxidant. The presence of rosmarinic acid in medicinal plants, herbs and species has beneficial and health promoting effects (Petersen and Simmonds, 2003).

Coleus forskohlii

Tuberous roots of *C. forskohlii* are used as condiments in India. Plant is commonly cultivated in native gardens at Bombay for roots which are pickled and eaten (Anon, 1950). Since ancient times, preparations of *Coleus* species have been used for medical treatment in Ayurvedic traditional medicines. The major uses are the treatment of heart diseases, abdominal colic, respiratory disorders, painful micturation, insomnia and convulsions (Ammon and Muller, 1985).

Different *Coleus* species have been used as medicine in Africa, Arab and Brazil. With the isolation of the labdane diterpene forskolin (= coleonol), which has become an important research tool in studying the role of the enzyme adenylate cyclase and cyclic-AMP (Adenosine Monophosphate) in cellular physiology this particular crop has gained worldwide attention. The compound eventually has became a useful drug in treating hypertension, glaucoma, asthma and certain cancers (Valdes *et al.,* 1987).

Pharmacological studies of *C.barbatus* (Bhakuni *et al.,* 1971; DeSouza, 1977) revealed cardiovascular activity in the root extract. Coleonol from C. *forskohlii* roots was characterized as 6, 9-dihydroxy-11-oxomanoyl oxide (Jauhuri *et al.,* 1978). The root extract of *C. barbatus* was found to contain a diterpene, forskolin which was exclusive to this species (Shah *et al.,* 1980). The therapeutical properties of forskolin especially in the treatment of glaucoma, congestive cardiomyopathy, asthma and certain cancers contributed to the emergence of *C. barbatus* as a taxon of importance in modern medicine (Vishwakarma *et al.,* 1988).

The blood pressure lowering and antispasmodic effects of root extracts of *C. forskohlii* was reported by Dubey *et al.* (1974) based on the extensive screening of Indian plants for biological activity. The active principle was named as Coleonol by CDRI scientists, while a group of scientists at Hoechst India Ltd., Bombay, named it 'forskolin'.

Coleonol, a diterpene possessing hypotensive and spasmolytic activities isolated from the roots, has been assigned the hydroxy-keto-manol-oxide acetate structure on the basis of chemical and spectral evidences and X-ray crystallographic data (Tandon *et al.,* 1977).

Several papers have been published describing the use of forskolin as a 'tool to study the role of the enzymes adenylase cyclase and cyclic-AMP. Forskolin directly activates almost all hormone- sensitive adenylate cyclases in intact cells, tissue and even solubelised preparation of adenylate cyclase. The unique feature of this activation is that the site of action for forskolin is the catalytic subunit of the enzyme or a closely associated protein (Seamon and Daly, 1981).

This action of forskolin proved the potential use of the molecule, not only as an invaluable research tool for understanding cyclic-AMP dependent physiological processes but also as a potential therapeutic

agent for diseases like cardiac insufficiency, hypertension, glaucoma, thrombosis, asthma and met static condition (Seamon, 1984).

For the assay of forskolin, thin layer, gas-liquid and high-performance liquid chromatographic methods were developed (Inamdar *et al.,* 1984). Occurrence of forskolin was found to be unique and restricted to C. *forskohlii* growing in different parts of India.

Shah *et al.* (1980) reported that forskolin could not be detected in six other *Coleus* species and six taxonomically related *Plectranthus* species. Studies carried out using one hundred samples belonging to species of *Coleus, Orthosiphon* and *Plectranthus* of the sub family Ocimoideae also revealed the absence of forskolin in them. The available evidence clearly indicates that the Indian herb C. *forskohlii* is the only known natural source of the diterpenoid forskolin (De Souza and Shah, 1988; De Souza, 1991).

Forskolin was studied as a bronchodilator for its potential use in the treatment of asthma. An advantage of forskolin over conventional bronchodilators is it has little or no systemic effect (Bruka, 1986). The effect of forskolin on aqueous humor dynamics and intraocular pressure (IPO) was first described by Caprioli and Sears (1983). They demonstrated that use of forskolin lowered the IOP in rabbits, monkeys and in humans also. The reduction in IOP was associated with a reduction in aqueous inflow and no change in outflow facility, indicating a potential for forskolin as a therapeutic agent in the treatment of glaucoma. Detailed pharmacological studies established that forskolin lowered normal or elevated blood pressure in different animal species through a vasodilatory effect and it had a positive inotropic action on the heart muscle.

The isotopic and atomic 14C/3H ratios obtained for polyoxygenated diterpenes isolated from the tuberous roots of C. *forskohlii,* which had been fed with [2-14C,2-3H2J-,[2-14C,4R-3H1J-,[2-14C,5-3H2] mevalonic acid, revealed that 8,13-epoxy-labd-14-en-ll-one was the first mono-oxygenated labdane to be formed on the biosynthetic pathway leading from the labdane diterpene skeleton. Subsequent sequential addition of oxygen gave 1, 9dideoxyforskolin, 9-deoxyforskolin and forskolin, along with many other diterpenes. Forskolin was the last compound to be formed in the biogenetic sequence (Akhila *et al.,* 1990). Though patented forskolin drugs are yet to be marketed, forskolin, as a biochemical compound is marketed by Sigma Chemical Company, USA (Anon., 1992) by virtue of its antihypeliensive, positive inotropic and adenyl cyclase activating properties.

Further, potential role of forskolin is foreseen in vascular diseases in improving graft potency through the bonding of forskolin to the inner surface of arterial synthetic graft materials such as polytetrafluoroethylene implants (Christenson *et al.,* 1989). The development by Hoechst India Ltd., of second generation forskolin analogs, namely DELTAS-6-deoxy-7 -ethylaminocarbonyl forskolin (HIL568) as a potential antiglauco maagent, 6-(3-dimethyl aminopropionyl) forskolin hydrochloride (NKH 477) as a potential anti thrombotic agent is described (Morton, 1992). Alcoholic extracts of *C.forskohlii* was found to inhibit passive cutaneous anaphylaxis (PCA) (Gupta *et al.,* 1993). The medicinal plant *C. forskohlii* has recently become important as the only natural source of forskolin (Prudent *et al.,* 1995).

C. forskohlii is cultivated in Maharashtra and Gujarat for its edible roots but the plant is also valued for its hypotensive activity, the chief active principle being forskolin (coleonol). Forskolin also acts as a

bronchodilator, and stimulates adenylate cyclase activity and acid and pepsinogen secretion by gastric glands (Abraham *et al.,* 1998).

C. forskohlii an ancient root drug recorded in the Ayurvedic Materia Medica under the Sanskrit name "'Makandi or Marjini". Drug is described as multiple fragrant tuberous roots, bitter, sharp .and sweet to taste. The drug is claimed to improve appetite, facilitate indigestion, increase vitality, useful in anemia, inflammation, flatulence, dropsy, fever with rigors, infestation of worms, spleenomegaly dysentery, abdominal problems, colitis and piles (Anon., 2001).

Coleus zeylanicus

C. *zeylanicus* (syn. *Plectranthus zeylanicus)* is grown in India Sri Lanka etc. and used to treat diarrhoea. An ethanolic extract of the plant afforded two new abietane- type diterpenoids characterized as 7 beta-acetoxy 6 betahydroxyroleanone and 7 beta, 6 beta-dihydroxyroyleanone. The known stereoisomer, 7 alpha-acetoxy-6 beta-hydroxyroyleanone was isolated from the same plant (Mehrotra *et al.,* 1989).

2.5 GENETIC DIVERSITY ASSESSMENT USING RAPD MARKERS

Andrographis paniculata

Padmesh *et al.* (1999) used RAPD analysis to determine intraspecific variability in *Andrographis paniculata*, source of a major antipyretic and hepatoprotective drug used in traditional medicine in India. The accessions collected from different parts of India and South East Asia when subjected to molecular analysis revealed moderate variation within the species. Cluster analysis resulted in 5 major groups based on geographical distribution that generally reflected expected trends between the genotypes. There were also important exceptions like AP-48, an accession from Thailand showing close resemblance to AP- 38 collected from Tamil Nadu and AP- 29 from Assam significantly diverse

from the rest of the native genotypes. The results indicated that RAPD could be effectively used for genetic diversity analysis in wild species of prospective value, as it is reliable, rapid and superior to those based on pedigree information.

Acorus calamus

In the present study *A. calamus* accessions based on RAPD marker, ploidy level and β-asarone content were characterized and correlated on the basis of β -asarone content and ploidy level. Of the 40 random primers used, 6 primers generated polymorphism. Genetic relatedness among accessions evaluated by a similarity matrix based on Dice's coefficient ranged from 0.72 to 0.97. A phonetic dendrogram based on UPGMA analysis grouped accessions into two clusters. *A. calamus* accessions were found to be both triploid and tetraploid. β -asarone content was found to be ranging between 6.92–8.0%. The study clustered the accessions as per their ploidy level, β -asarone content and geographical locations (Ahlawat *et al.*, 2010).

Allium sativum

Twenty-three accessions of *Allium sativum* (garlic) from different geographical parts of India and two accessions from Argentina were used for RAPD profiling and bioactivity evaluation. RAPD profiling of all accessions was done by PCR using MAP, OPT, OPJ and OPO random primer kits (each primer kit contained 20 different primer sequences). In RAPD analysis, a total of 2998 bands were observed; out of which, 2459 (82.02%) bands were polymorphic. Similarity and variation among the garlic accessions was observed by cluster analysis and a dendrogram was constructed and later compared with the dendrogram constructed from the morphological characters (Shasany *et al.*, 2000).

***Panax* spp.**

Seven wild *Ginseng* (*Panax ginseng*) populations collected from different sites were subjected to RAPD analysis. Results revealed that the level of genetic variation in wild ginseng was much higher than that in garden ginseng. Based on the pair wise distances of all samples, it could be concluded that environment plays more important role than genetic factors in morphological differences of wild ginseng. Cluster analysis showed that genetic variation among *Panax ginseng* samples was narrower than that between *Panax ginseng* and *P.quinequefolium*. Analysis showed that wild ginseng can be used for breeding purpose (Xiaojun *et al.*, 1999).

In the course of searching for high quality *Panax ginseng*, Tochiku *et al.* (2001) found a useful RAPD primer, which showed 725 base pair band for a selected elite strain Aizu K-11 (now called Kaishusan) including its cultured tissues. While the other strains did not showed this particular band. They sequenced the particular DNA fragment amplified and designed primers to improve electrophoretic profiles, based on the sequence.

Genetic diversity within Canadian grown North American ginseng (*P.quinequefolium*) was evaluated using RAPD markers. 15 primers that produced 35 polymorphic bands were used to screen over 600 plant samples. 10 samples from a seed lot and 58 samples from 3 natural ginseng populations were also included for comparison. Genetic distance values within cultivated populations ranged from 0.21- 0.34. Distance values within 3 natural populations were either similar (0.33) or lower (0.12, 0.19) compared with cultivated populations, indicating that populations under cultivation have not undergone a reduction in overall genetic diversity. However, RAPD marker was polymorphic only in natural populations. Monotonic multidimensional scaling and chi2

analysis indicated that natural populations were genetically distinct from cultivated populations. Individual plants originating as seeds from the same mother plant had much lower genetic diversity (mean of 0.18) compared with individual field grown plants chosen at random from the same farm. Segregation of some RAPD markers was observed among the progeny, indicating that parental plants had some degree of heterozygosity and that a level of out crossing may be present. Estimates of the component for genetic diversity between populations (GST) were 18% and 28% for cultivated and natural populations, respectively. Much of the variation was detected within and not between the populations. These results imply that North American ginseng is a heterogeneous mix of genetic material and that the observed genetic diversity in cultivated populations in Canada results largely from the mixing of different seed lots. In addition, heterozygosity within the parent plants and cross pollination appears to contribute to genetic variation in this species (Schluter and Punja, 2002).

Artemissia annua

RAPD analysis of selected chemo types of *Artemissia annua* indicated the distinct variation amongst the accessions. Furthermore, the detection of highly polymorphic profiles suggests the existence of very high levels of genetic improvement for higher artemisinin content. UPGMA analysis of RAPD and phytochemical trait data indicates that wide phytochemical diversity is observed within the genetic diversity. These results further support the prospects for selection and breeding of lines with higher artemisinin content (Sangwan *et al.*, 1999).

Chamamoile

In order to evaluate the genetic diversity of different chamomile landraces based on morphological and molecular markers, 20 landraces were collected from different area of Iran and five accessions were taken

from Europe for RAPD analysis. Results showed that the yield, the number of flowers in plant, and the essential oil content had maximum variance coefficient. The flower diameter and plant height had minimum variance coefficient. According to the cluster analysis on both morphological and molecular markers, 25 populations were classified into 5 clusters. From 29 primers that were used, 369 bands were detected and of which 314 (85.44%) bands were polymorphic. Genetic Jaccard's similarity coefficient was estimated in the range of 0.15–0.63, and with a mean of 0.35. Results showed that the genetic diversity was not according to the geographical diversity (Solouki *et al.*, 2008).

***Tagetus* spp.**

RAPD analysis was carried out to assess the extent of genetic diversity in six accessions each of *Tagetus minuta* and *T. petula* collected from different geographical parts of India. In case of *T. minuta* only 7 per cent polymorphism was observed and all the six accessions showed 95-100 per cent similarity. While, in case of *T. petula,* 70 per cent polymorphism was observed. Relatively much higher variation was observed among the accessions of this species (Darokar *et al.,* 2000).

Podophyllum hexandrum

Sultan *et al.* (2008) carried out RAPD analysis, In order to determine the genetic diversity with respect to morphological characteristics and phytochemical variation in different accessions of *Podophyllum hexandrum*. Twelve accessions grown in gene bank repository were subjected to RAPD analysis and were assessed for podophyllotoxin and podophyllotoxin β-D-glycoside content by HPLC. RAPD analysis revealed a high degree of genetic diversity among the accessions used in the study. There was also high diversity in the concentration of marker compounds in the collected samples as revealed by HPLC analysis. Similarity measurement using UPGMA followed by

cluster analysis resulted in formation of many groups based on geographical distribution that generally reflected expected trends between the genotypes. Accession PSH-B from Keller was significantly diverse from the rest of the native genotypes with respect to phytochemical characters, morphological characters and also at molecular level. RAPD data analysis was found to be significant predictor of phytochemical markers in cultivated *P. hexandrum* germplasm. Individual regressions of podophyllotoxin and podophyllotoxin b-D-glycoside by RAPD analysis against HPLC has been found to determine linear values.

***Desmodium* spp.**

The identification of *Desmodium gangeticum* was carried out using genomic approach. Authentic samples of *D. gangeticum*, *D. velutinum* and *D. triflorum* were analyzed and compared to commercial samples of various origin. Of the total twenty primers used, eleven gave 223 RAPD fragments. RAPD profiles of three species showed very low similarity index (0.21–0.39), whereas market samples showed high similarity of 0.82–0.89 with authenticated *D. gangeticum* (Irshad *et al.*, 2009).

***Pelargonium* sp.**

RAPD patterns from thirty four cultivars of *Pelargonium* were scored to study their relationship. Twenty three selected primers generated multiple bands and a varying number of minor bands, leading to ninety six markers, 79 per cent of them being polymorphic. Seventeen markers were specific to *Pelargonium X haderaefolium.* The research demonstrated that, RAPD marker analysis could be used successfully to study the genetic relationship among and within *Pelargonium spp.* (Renou *et al.,* 1997).

Barcaccia *et al.*(1999) studied the genetic diversity in *Pelargonium peltatum* using RAPD markers. Results suggested that the *Pelargonium* plants assayed with RAPD markers belonged to two genetically different cultivars. It was also found that RAPD fingerprints were reproducible under strictly controlled experimental variables, even though changes in banding patterns were documented according to DNA template amount and quality.

Four accessions (BSPI-l, BSPI-2, BSPI-3 and BSPI-4) of Rose scented geranium *(P. graveolens)* were analyzed and compared with the three cultivars Bipuli, Hemanti and Kunti for essential oil yield and quality related traits and their RAPD profiles. The crop yield and essential oil parameters and DNA profiles of the four new accessions and three cultivars revealed that the accessions BSP-l, BSP-2 and BSP-3 were Bipuli x Kunti hybrids and BSP-4 was a Bipuli x Hemanti hybrid (Ritika *et al.,* 2001).

Considering the commercial importance of Rose-scented geranium *(P. graveolens),* the available genotypes were investigated through RAPD analysis. Among the 120 primers used in this study to differentiate the accessions, OPA 03, OPA 06, OPA 08, OPA 15, OPA 18, OPA 19, OPB 02, OPB 03, OPB 05, OPB 16, OPO 02, OPO 15, OPO 16, OPO 18, OPQ 06, and OPQ 07 were more informative with regards to differentiating the three cultivars Algerian, Bourbon, and Kelkar. Among these primers, OPA 18, OPA 19, OPB 02, OPB 03, OPB 05, OPB 16, OPO 02, OPO 15, OPO 16, OPO 18, OPQ 06, and OPQ 07 generated unique fragments at least for one genotype. The primers OPB 03 and OPB 05 differentiated all the three genotypes when used individually for amplification. In this investigation, Algerian, Kelkar, and the Bourbon could be distinguished at the genetic level during early growth stage through genotype-specific RAPD profiles. This will be helpful for maintaining the genetic purity of

the available germplasm and for quality control in commercial fields (Shasany *et al.,* 2002).

Harish (2001) studied the genetic diversity among 14 accessions of Scented geranium. Among the 40 primers used in this study, the primers OPF2, OPF-4, OPG-11, OPG-18, OPX-9, OPX-11, OPX-13, OPX-14 and OPX-15 proved much more useful in differentiating the accessions. The linkage distances among the accessions has revealed by distance matrix, ranged from 15-164, both the principle component analysis and dendrogram analysis showed that the distinctive nature of the accession Kelkar, which was introduced to India from Egypt by Kelkar, a prominent perfumery house of India. The variation among the accessions as shown in principle component analysis was found to be 63.6%.

Hypericum *perforatum*

RAPD analysis of individual plants of *Hypericum perforatum* was carried out. From a wild population, 10 offsprings were investigated by genomic analysis. The results obtained revealed that the collections included significantly distinct genotypes. Whereas, seven of the individuals displayed identical fingerprints, representing the dominant genotype of this accession. A deviating but identical finger print was shown by two plants and third genotype was represented by another plant. Additionally, genome characterization of progenies from individual plants of further four accessions was performed. The results showed that most of the fingerprints from each accession indicated an identical mode of reproduction for *Hypericum perforatum*. (Arnholdt *et al.*, 2002).

Coleus forskohlii

Kavitha *et al.* (2010) reported the genetic analysis of 37 diverse *Coleus forskohlii* genotypes by using 25 RAPD primers, which yielded 117 bands, of which 60(51.28%) were polymorphic providing an average of

3.75 bands per primer. The number of bands per primer varied from 1 (OPZ8 & 16) to 7 (OPZ 11). The result indicated that RAPD could be used for genetic diversity analysis in *C.forskohlii* using higher number of primers.

Ocimum spp.

Morphological, chemical and genetic differences of 12 accessions of *Ocimum gratissimum* were studied to determine whether volatile oils and flavonoids can be used as taxonomic markers and to examine the relationship between RAPD marker and these chemical markers. Euginol, thymol, and geraniol were the major volatile oil constituents found in *O. gratissim*. A distinct essential and flavone chemo type (producing geraniol and a mixture of the flavones ocirsim artin, isothym, xanthomicrol and luteolin) were found in an accession genetically more distinct from the other two groups when analyzed by molecular markers. Cluster analysis of RAPD markers showed that there were 3 groups genetically distinct and highly correlated (r=0.814) to volatile oil constituents (Vieira *et al.*, 2001).

Various in vitro cultures were established from shoot tips of *Ocimum americanum* seedlings. Rosmarinic acid content of the in vitro produced plants as well as parent plant were determined by HPLC analysis and subjected to RAPD analysis. RAPD analysis revealed 64 scorable bands from four primers, including six polymorphic bands. The band pattern revealed differences between the parent plant and the in vitro regenerated plants. Certain band changes were found in *O. americanum* plants regenerated in vitro, suggesting the existence of genetic variation that might affect the biochemical synthesis of plants derived from tissue culture (Rady and Nazif 2005).

Marjoram sp.

3 progenies of marjoram pollinator lines were examined by means of RAPD assays. Single plant analysis as well as an analysis with bulked DNA samples was used. The RAPD fingerprints of single plants illustrated the heterogeneity inside the accessions in the first year and the enhancement of the homogeneity in the next progenies caused by selfing (Klocke *et al.*, 2002).

Melissa spp.

Various samples of *Melissa officinalis* sub spp. *officinalis* and *Melissa officinalis* sub spp. *altissima* were investigated using RAPD markers. Results showed that RAPD analysis is a fast and reliable method of distinguishing sub species on the pharmaceutical market that have been previously classified according to the distribution pattern of compounds present in the lemon balm oil (Wolf *et al.*, 1999).

Mint spp.

Khanuja *et al.* (2000) reported molecular analysis through RAPD profiling of *Mentha spicata,* genotype. Among different genotypes CIMAP/C-33 was found to share about 60 per cent genomic similarity with other genotype CIMAP/ C-32 of *M. spicata* indicating the probability of these two types as hybrids with one common parent. RAPD profile showed polymorphic bands in the hybrid in *M. arvensis* (CIMAP/C-l7) and *M. spicata* (CIMAP/C-33).

Fifteen elite accessions of *M. spicata* were investigated for the level of genetic diversity in morphological and oil quality characteristics. RAPD profiling of the accessions was carried out using 80 random decamer primers. From the polymorphism observed for DNA bands, the pooled similarity matrix was developed. The graphic phenogram of the genetic association among the 15 accessions was generated using UPGMA

cluster analysis using the similarity indices of the matrix. These genetic and morpho-chemical clusters were compared for relatedness and differences. It was observed that RAPD analysis for the phylogenetic relationship was a better indicator of descendancy and origin among the germplasm accessions (Shasany *et al.,* 2002).

Satureja hortensis

Genetic diversity of 28 accessions of *Satureja hortensis* collected from different parts if Iran were evaluated by using RAPD markers. Statistical analysis showed significant differences for many characters like plant height, flowering time, fresh and dry weight of the plant etc. in the accessions. Molecular analysis of diversity was carried out using 18 random primers. 18 primers produced totally 218 bands, of which 181 bands were polymorphic. A dendrogram was prepared on the basis of a similarity matrix using UPGMA algorithm. Dendrogram results separated 28 accessions into three groups. Finally they concluded that RAPD approach is best suited for fingerprinting and assessing genetic relationship among *S. hortensis* accessions with greater accuracy (Hadin *et al.*, 2008).

Scutellaria spp.

Nine accessions of three species of medicinal plants in the genus *Scutellaria* (*S. galericulata, S. lateriflora* and *S. baicalensis;* collectively known as skullcap) were analyzed by RAPD. 10 arbitrary primers produced 92 fragments, and 8 of the primers yielded 23 species-specific fragments. 6 fragments were specific for *S. galericulata*, 7 for *S. lateriflora* for and 10 for *S. baicalensis*. Primer A02 produced 5 species-specific fragments: one was specific for *S. galericulata*: two for *S. lateriflora* and two for *S. baicalensis*. Primer A06 also produced species-specific fragments: one was specific for *S. galericulata*: one for *S. lateriflora* and one for *S. baicalensis*. RAPD markers that were generated with these two

primers rapidly identified members of three species of *Scutellaria*. The consistency of the identifications made with these species-specific RAPD markers were demonstrated by the observation that each respective marker was generated from 3 accessions of each species, all with different origins. Furthermore, cluster analysis using 92 RAPD fragments produced a dendrogram of genetic relatedness that was in good agreement with the taxonomic designations of the 3 species (Hosokawa *et al.*, 2000).

Neem

Farooqui *et al.* (1998) analyzed the RAPD variation amongst provenances of Neem. The similarities in RAPD profiles amongst the different accessions were more than that expected due to the out-crossing nature of neem and further, more than expected similarities were not due to random chance. These results suggest that neem has a narrow genetic base.

Opium Poppy

An F_2 population obtained from a cross between a low morphine poppy plant and a high morphine plant from the Hungarian poppy variety 'Cosmos' was investigated by both AFLP and RAPD markers. The segregation of six morphological traits was detected during the vegetative phase of plant harvested capsules. Totally 125 molecular markers were detected, 77 as AFLP and 48 as RAPD markers. A total of 87 marker loci (66% of the total number) were placed in 16 linkage groups, which is five times more than the haploid chromosome number of Opium Poppy (n=11). Linkage analysis and map construction were performed by MAP MARKER 3 software. 11 major linkage groups contained 4-12 loci, whereas 5 minor groups comprised 2-3 loci. (Straka *et al.*, 2002).

Phytolacca dodecandra

The genetic relationships among 10 wild types of Endod (*Phytolacca dodecandra*) were studied using morphological and molecular markers. A total of 18 morphological characters, 194 AFLP and 42 RAPD primers were used to determine genetic proximity between these types. Cluster and principal component analysis performed on the AFLP and RAPD markers demonstrated the presence of distinct separation of E56 loci but not that of E44 loci from the others. Metrices correspondence tests demonstrated the presence of greater correspondence between AFLP and RAPD data (r=0.842) but not between the morphology that of AFLP and RAPD. This indicates the correspondence was more between the two DNA markers systems than either of them with morphological traits (Semagn, 2002).

Piper longum

RAPD fingerprints of 20 micro propagated plants of *Piper longum* and the mother plants were analyzed by PCR of genomic DNA using 10 random decamer primers. 18 plants formed a major cluster along with the mother plant. The other two were molecular off-types as they showed less than 80% similarity to mother plant (Parani *et al.*, 1997).

Piper longum, a medicinally important plant, showing a dioeciously flowering pattern was investigated for the molecular basis of genotypic differentiation between the male and female plants, using RAPD markers. Polymorphism in the genomic DNA of plants of 25 female and 6 males was analyzed by RAPD markers, using 40 decamer random oligonucleotide primers. 2 RAPD bands consistently appeared only in the plants showing male genotype, suggesting that the male associated nature of these DNA markers in dioecious *Piper longum* (Banerjee *et al.*, 1999).

Three female varieties of *Piper longum*, one each from Assam and Calicut and one released variety, Vishwam was subjected to RAPD analysis. Compared to the Assam variety, Calicut and Vishwam were genetically closer (95% similarity). Among themselves, Assam variety showed 75% and 73% genetic similarity with Vishwam and Calicut varieties respectively (Philip *et al.*, 2000).

Bacopa monnieri

A collection of 24 *Bacopa monnieri* accessions from different agro-climatic zones of India and an introduction from Malaysia maintained in the field gene bank at CIMAP was analyzed for RAPD variation. Among the 40 random primers tested, 29 primers generated one or more polymorphic bands. The number of polymorphic bands generated was primer dependent, ranging from a minimum of 2 to a maximum of 8. Similarity matrices were generated from the RAPD data on the basis of Neil's estimates of similarity indices and dendrogram was constructed based on UPGMA clustering. All the accessions were found to be in the range of 0.8-1.0 of similarity, which is indicative of a narrow genetic base among the various accessions with a medium level of polymorphism. It was possible to differentiate individual accessions, showing differences in morphological and growth properties at DNA level. Observed levels of genetic variation were attributed to interplay of sexual and vegetative modes of reproduction and similarity of local environment in habitats of *Bacopa monnieri* (Darokar *et al.*, 2001).

***Digitalis* spp.**

RAPD markers were used to assess levels and patterns of genetic diversity of *Digitalis obscura*, an outstanding cardenolide producing plant. 50 plants from 6 Spanish natural populations were analyzed by 6 arbitrary primers produced totally 96 bands. The analysis of molecular variance (AMOVA) with distances among individuals correlated for the

dominant nature of RAPDs showed that most of the variation (84.8%) occurred among individuals within the population, which is expected for an out crossing population. Of the remaining variance, 9.7% was attributed to differences among populations within regions (Nebaucer *et al.*, 1998).

Fifteen wild-growing plants of *Digitalis obscura* collected in three different regions were characterized according to their capacities to biosynthesize cardenolides and to proliferate in vitro. Selected genotypes were differentiated using RAPD markers. Once an elite genotype with high-yielding properties was isolated, it was micro propagated. Further, the identity of the donor plant and the regenerants was confirmed by RAPD analysis (Gavidia *et al.*, 1996).

Plumbago zeylanica

Clones of *Plumbago zeylanica* were micro propagated using nodal culture. Application of RAPD in assessing the genetic integrity of the micro propagated plants was evaluated by PCR. 20 arbitrary decamer primers were used to amplify genomic DNA from *in vitro* and *in vivo* plant material to assess the genetic fidelity. All RAPD profiles from micro propagated plants were monomorphic and similar to those of field grown mother plants. No polymorphism was detected within the micro propagated plants (Rout and Das, 2002).

Citronella spp.

An extensive RAPD analysis to quantify and assess the similarities among the accessions of C*ymbopogan winterianus* was carried out. Considerable similarity was observed between the cultivars CW5 and CW3, because CW5 was an induced mutant from CW3. CW8 was more similar to CW4 in comparison to CW1 from which it was selected. CW6

derived from CW3 formed an out-group in the major cluster with more similarity towards CW4 (45%) and CW3 (43%) (Khanuja *et al.,* 2000).

Eight accessions of *C. winterianus* were analyzed for similarity and genetic distances at the molecular level via RAPD profiling using 20 random primers. More than 50 per cent divergence was observed for all the *C. winterianus* accessions in relation to *C. nardus* accession CN2. The clustering based on similarity matrices showed a major cluster. Of the six accessions, consisting of two sub- clusters, CN2 got linked out along with *C. winterianus* accessions CW2 and CW6. On the other hand, CW2 and CW6 demonstrated distinct identities compared to CN2 at the DNA level (Shasany *et al.,* 2000).

***Jamarosa* spp.**

Among 100 somaclones of jamrosa screened for morphological characters, a wide range of variation was recorded for several traits, including plant height, tiller number, herb weight, oil content and total oil yield. Qualitative analysis of essential oil was carried out for 45 somaclones which performed better or equal to the donor parent. Eleven somaclones selected on the basis of total oil yield and qualities were further evaluated in replicated trial. Five superior somaclones showing a total oil yield twofold than the donor and possessing quality oil containing a high geraniol content of 84% were selected for further analysis. Out of the five superior somaclones two improved somaclones which showed relative stability in oil yield and quality were subjected to RAPD analysis. Changes in RAPD banding pattern in the improved somaclone as compared to donor parent revealed occurrence of gross genetic changes (Nayak *et al.*, 2003).

Veteveria zizanioides

Four elite genotypes of *V. zizanioides* namely BDP-1, BMH-l, MBR-5 and KS-l were analyzed by RAPD profiling. Among 12 decamer primers used, molecular variation in terms of polymorphic bands was observed in case of 9 primers. The genotype KS-1 was grouped with BDP-1, which also possesses a similar khus note in its essential oil. The other two accessions were clustered together with the smell of kesar (BMH- 1) and rose (MBR- 5). A divergence of 29-35 per cent was estimated to exist in the form of DNA polymorphism among these two subgroups, providing scope for further genetic improvement using a marker assisted approach (Shasany *et al.,* 1998).

Rosa damascena

The genetic relationships among forty one *Rosa damascena* accessions from various cultivation areas of Iran and one accession from Bulgaria were analyzed using 31 RAPD primers. Each primer exhibited 3–12 banding patterns for a total of 343 bands, of which 184 bands were polymorphic. UPGMA cluster analysis based on similarity values revealed 10 groups at the distance of 0.85. The Bulgarian genotype grouped with the majority of the Iranian genotypes in a main cluster. Results of molecular variance analysis (AMOVA) indicated that the major proportion (65.7%) of the total genetic variation was within collecting provinces rather than between them (*Kiani et al.*, 2010).

***Aconitum* spp.**

The RAPD technique and phytochemical analysis, based on the investigation of flavanoid composition, were used to study *Aconitum vulparia*, *A. paniculatum*, *A. napellus* subsp. *Tauricum* and *A. napellus* subsp. *neomontanum*. Twenty primers were screened for the genetic analysis of which 14 were selected providing 51 polymorphic bands. The

phenogram based on UPGMA clustering of Jaccard coefficient revealed a clear division between yellowish and blue *Aconitum* plants and inside this second group *A. paniculatum* is clearly separated from all populations belonging to *A. napellus* group (Fico *et al.*, 2003).

***Houttuynia* spp.**

The genetic relationships among 70 accessions of *Houttuynia* species were tested using RAPD and ISSR markers. The results showed that the polymorphism of *Houttuynia* germplasm was high at the DNA level. The genetic variation between the cultivated and the wild *Houttuynia cordata* accessions was insignificant. The results of cluster analysis by using UPGMA method showed that the groups based on ISSR marker was correlated with chromosome numbers and thus many accessions with the same chromosome numbers could be classified together. Analysis based on RAPD marker was more related to geographic distribution. But the research work could not separate *Houttuynia emeiensis* accessions completely from *H. cordata* accessions, as it was closely related to *H. cordata* cytotype A with the chromosome number of 36. Within *H. cordata*, the genetic similarities between each pair of cytotypes C, D, E, F, G, H, I, J, K and L were higher, but the genetic similarities between each of them to the cytotype A were relatively lower (Wu *et al.*, 2006).

Thymus caespititius

RAPD and ISSR markers were used as molecular markers and combined with the chemical analysis of the volatiles, aiming at characterizing *Thymus caespititius*. Thirty-one individuals were analyzed using 17 RAPD and 11 ISSR primers, which generated 199 and 127 polymorphic bands respectively. Volatiles were isolated by distillation–extraction and analyzed by GC and GC–MS. Molecular data obtained from both markers discriminated assessments from Corvo and Flores

which clustered separately from plants collected in Graciosa, Portugal. Volatile data grouped plants in a similar way, giving rise to two groups, depending on their oil type; plants collected on Corvo and Flores yielded carvacrol-rich volatiles while those from Graciosa yielded both carvacrol and a-terpineol-rich oils (Trindade *et al.*, 2009).

***Alpinia* spp.**

The rhizomes of *Alpinia jianganfeng* are used as a traditional Chinese medicine, Jian Gan Feng, to cure rheumatism in Guangdong. The rhizomes of other species of the genus *Alpinia* such as *A.japonica, A.suishaensis,* and *A.nanchuanensis* are also used as Jian Gan Feng in south west China. However, the identification of original plants of the crude drug is difficult. The internal transcribed spacers and the 5.8S coding region of nuclear ribosomal DNA of the four species were sequenced and analyzed. The results revealed that DNA markers can determine the genetic diversity efficiently and they can be used for the molecular identification of these medicinal plants (Zhao *et al.*, 2002).

***Curcuma* spp.**

RAPD markers were applied to detect the genetic relationships and diversity among 33 accessions of *Curcuma* species in. A total of 115 bands were amplified by 21 primers, among which 106 bands (92.17%) were found to be polymorphic. A total of 3 to 8 polymorphic bands were amplified by each polymorphic primer, with an average of 5.48 bands per primer. The data of 115 RAPD bands were used to generate Jaccard's similarity coefficients and to construct a dendrogram by means of UPGMA. The results show that the genetic similarity coefficient of these six species is relatively large, from the cluster diagram, while the genetic relationships are not associated with their geographical distributions (Zou *et al.*, 2011).

Zingiber officinale

Forty nine ginger clones cultivated in North-eastern India were subjected to genetic diversity analysis sing RAPD markers. Thirty random primers were used for the RAPD analysis and the primers generated totally 109 bands, of which 101 bands were polymorphic. Jaccard's genetic similarity, cluster analysis and principal component analysis identified five clusters. Cluster V included four clones traditionally cultivated in the north-eastern states of India and they were known for production of high quality ginger. Specific bands for these clones were also identified (Sajeev *et al.*, 2010).

2.6 DEVELOPMENT OF SCAR MARKERS

Cannabis sativa

Mandolino *et al.* (1999) identified 400-bp RAPD marker generated by a primer of random decamer sequence associated with the male sex phenotype in 14 dioecious cultivars and accessions of hemp (*Cannabis sativa* L.). The primer OPA 8 generated most of polymorphic bands among all the individual plants tested, and 1 of which, named $OPA8_{400}$, present in all male plants and absent in female plants. Screening of 167 plants belonging to different genotypes for the association of the $OPA8_{400}$ marker with the sex phenotype revealed that in 3 cases 400-bp band was present in plants phenotypically female; on the contrary, in male plants the band was never missing, while in monoecious plants it was never present. Despite this sex-specific association, the sequences corresponding to $OPA8_{400}$ were present in both staminate and carpellate plants, as revealed by Southern blotting and hybridization with the cloned RAPD band. The RAPD marker was sequenced and specific primers were constructed. These primers were generated on the same genotypes used for RAPD analysis, a SCAR marker 390 bp in length and male-specific.

Echinacea purpurea

Adinolfi *et al*. (2007) converted a RAPD marker of 750 bp for *E. purpurea* into a SCAR marker. SCAR-PCR revealed the amplicon of 330 bp only in *E. purpurea* and not in the other two species (*E. pallida* and *angustifolia*), giving evidence for differences in medicinal *Echinacea* spp. genome and confirming a greater similarity between *E. pallida* and *angustifolia*.

Curcuma longa

Dhanya *et al.* (2011) developed SCAR markers to detect adulterants in traded turmeric powder. Two putative RAPD markers, 'Cur 01' and 'Cur 02', generated by random primers OPA 01 and OPE 18 were identified as *C. zedoaria*/*C. malabarica* specific by comparative RAPD analysis of genuine turmeric and market samples of turmeric powder, *C. zedoaria* and *C. malabarica*. These specific RAPD markers were cloned and sequenced. Two pairs of SCAR primers were designed from the RAPD markers 'Cur 01' and 'Cur 02', respectively. Six market samples of turmeric powder and four simulated standards besides the genuine samples were analyzed using the specific SCAR markers.

Anuntalabhochai *et* al. (2007) developed a SCAR marker for the Curcuma variety Patumma. Starting with a set of 11 decamer primers, polymorphic bands ranging from 100 to 2500 base pairs were used to examine 20 Curcuma varieties from which banding patterns of interest were selected for conversion to reproducible SCAR markers. In particular, one SCAR marker amplified a region 600 bp in length which was conserved in all Patumma varieties and hybrids and as an independent test did not amplify an additional series of 24 distinct Curcuma varieties. Since new varieties of *Curcuma* are often dissimilar from their progenitors, this genomic analysis allowed a cost-effective morphologically independent characterization of *Curcuma* hybrids.

Ipomoea mauritiana

Vidari is an Ayurvedic herbal drug used as aphrodisiac, galactagogue and is also used in the preparation of *Chyavanaprash*. Tubers of *Ipomoea mauritiana*, *Pueraria tuberosa*, *Adenia hondala* and pith of *Cycas circinalis* are all traded in the name of Vidari, creating issues of botanical authenticity of the Ayurvedic raw drug. DNA-based markers have been developed to distinguish *I. mauritiana* from the other Vidari candidates. A putative 600-bp polymorphic sequence, specific to *I. mauritiana* was identified using RAPD technique. Furthermore, sequence SCAR primers (IM1F and IM1R) were designed from the unique RAPD amplicon. The SCAR primers produced a specific 323-bp amplicon in authentic *I. mauritiana* and not in the allied species (Devaiah *et al.,* 2011).

Spica prunellae

To develop appropriate molecular markers to distinguish genuine herbal medicine *Prunella asiatica*, two putative markers, which are specific for *P. asiatica* were identified by RAPD. Two sequence SCAR markers were further developed from the two RAPD markers above. The amplification using *P. asiatica* specific SCAR marker showed that *P. vulgaris, P. asiatica* and *P. hispida* could be regarded as one species in the taxonomy. The amplification of another SCAR marker was closely linked to region specific. The results showed that newly developed molecular marker method could be considered as a convenient and reliable method for the genuineness identification *of Spica prunellae* (Xiao *et al.,* 2011).

MATERIAL AND METHODS

III. MATERIAL AND METHODS

The present investigation was carried out to characterize selected species and mutants of *Coleus forskohlii* in relation to their morphology, yield potential, forskolin content and molecular diversity. Morphological evaluation and forskolin estimation was conducted at Division of Horticulture, UAS, GKVK, Bangalore. Diversity analysis by using molecular markers was carried out in the Department of Biotechnology, UAS, GKVK, Bangalore.

3.1 SELECTION OF PLANTING MATERIAL

The present study of comprises of nine induced mutants, two somaclonal mutants, a variety K-8 and three different species of *Coleus,* details of which are given in Table 1.

3.2 LOCATION

The investigations for morphological characters were conducted at Sugandhavana, Medicinal and Aromatic section, Division of Horticulture, UAS, GKVK, Bangalore.

3.3 EXPERIMENTAL DETAILS

3.3.1 Evaluation of Coleus species and mutants of *Coleus forskohlii* for morphological characters

Season of planting : August, 2010

Location : Sugandhavana, UAS, GKVK, Bangalore

Treatment details :

Experimental design : Completely Randomized Design (CRD)

Number of treatments **:** 15

Number of replications **:** 3

Table 1. Different species of Coleus and mutants of *Coleus forskohlii* used for morphological evaluation

Sl. No.	Mutant/ Species
1	MV1
2	MV2
3	MV3
4	MV4
5	MV5
6	MV6
7	MV7
8	MV8
9	MV9
10	SV1
11	SV2
12	K-8
13	*Coleus aromaticus*
14	*Coleus aromaticus var. variegated*
15	*Coleus vettiveroides*

Plate 1 General view of the experiment (*Coleus forskohlii*)

Plate 2A Mutants of *Coleus forskohlii* used in the study (MV1, MV2, MV3, MV4)

Plate 2B Mutants of *Coleus forskohlii* used in the study (MV5, MV6, MV7, MV8)

Plate 2C Mutant, somaclones and a variety of *Coleus forskohlii* used in the study (MV9, SV1, SV2, K-8)

Plate 2D Different species of Coleus used in the study

3.4 METHODOLOGY

3.4.1 Preparation of potting mixture

Potting mixture for planting the cuttings was prepared by mixing soil and sand in the ratio of 45:55, well mixed and filled to the pots for planting the cuttings.

3.4.2 Preparation of planting material

The mutants of *Coleus forskohlii* (9 mutants and 2 somaclones) and a variety K-8 along with other three *Coleus* species maintained in the Division of Horticulture are utilized to multiply propagating material; tip cuttings of 10 cm length with 2-3 nodes of each accession were made. The cuttings were planted directly in pots filled with pot mixture after dipping in Bavistin solution (2 g/l) to avoid infection of fungal wilt. Pots were kept in polyhouse.

3.4.3 Irrigation

Plants were irrigated immediately after planting. Initially plants were irrigated daily for better establishment and once the plants got established they were irrigated once in four days depending on soil moisture condition and prevailing weather.

3.4.4 Weeding

Pots were kept free of weeds through hand weeding.

3.4.5 Plant protection

Pests were not noticed during the crop season. When crop showed of wilt two months after transplanting, care was taken by drenching once with Streptocyclin (2.5 g in 5 litres) at 100 ml per plant, alternatively with Cephalaxin drenching 50 mg/ l at 100 ml per plant at 15 days of interval.

3.4.6 Harvesting

Coleus plants were harvested 180 days after transplanting. Prior to the day of harvesting, the pots were lightly irrigated and next day the plants were uprooted and the tuberous roots were separated from foliage and then the roots were thoroughly washed in running water and kept in shade for recording observations.

3.5 OBSERVATIONS RECORDED

Observations on the growth parameters were recorded at different stages of growth (60, 120 and 180 days after planting). Further, observations on yield parameters were recorded at the time of harvest (180 days after planting).

3.5.1 Growth parameters

3.5.1.1 Plant height (cm)

The longest shoot arising from the ground was measured from its tip and expressed in centimeters.

3.5.1.2 Number of branches per plant

Number of branches per plant was recorded by counting total number of branches arising from the main stem at different stages of crop growth (60, 120 and 180 days after planting).

3.5.1.3 Plant spread (cm^2)

The shoot area occupied by a single plant in the East – West and North – South directions was measured and expressed in cm·

3.5.1.4 Number of leaves per plant

At 180 days, the total number of leaves present on the second branch from the bottom of the plant was counted and the same was multiplied with total number of branches.

3.5.1.5 Leaf area per plant (cm^2)

The observation on leaf area was recorded for each plant at harvest. The length of the leaf was measured from the base to the tip and the width was measured at the widest portion of the lamina of the fully opened leaf in the plant. The leaf area was computed as product of leaf length and width and multiplied with the factor 0.8 to calculate the actual leaf area. The average of eight such selected plants leaf area were multiplied with total number of leaves in order to arrive at the total leaf area per plant. The factor was arrived by measuring the actual leaf area of the whole plant using leaf area meter and also by plotting the same leaves on a graph and then taking measurements.

3.5.2 Yield parameters

3.5.2.1 Number of tuberous roots per plant

Total number of tuberous roots per plant was counted from each of the selected plants and the mean was worked out after harvest and recorded.

3.5.2.2 Tuberous root length (cm)

The longest root length was recorded from each of the eight selected plants and the mean was worked out after harvest and recorded and expressed in centimeter.

3.5.2.3 Tuberous root diameter (cm)

The diameter of the tuberous roots at the thickest portion was measured using Vernier callipers. The mean diameter of selected tuberous roots per plant was recorded and expressed in centimeter.

3.5.2.4 Fresh weight of tuberous roots (g)

Fresh weight of tuberous root was recorded after thorough washing with running water to remove the soil and roots weighed using electronic balance and expressed in grams.

3.5.2.5 Dry weight of tuberous roots (g)

The dry weight of the tuberous roots per plant was recorded after the tuberous roots were chopped in to small pieces, then they were kept in hot air oven. The temperature maintained was 55±5^{0}C. The moisture content of the root pieces was brought down to 8-10 per cent and then weighed using electronic balance.

3.5.2.6 Fresh weight of shoot (g)

The fresh weight of the above ground portion of plant viz., leaf and stem was recorded separately and taken as fresh weight of shoot per plant and expressed in grams.

3.5.2.7 Dry weight of shoot (g)

The shoot sample of five plants was weighed separately and dried at constant temperature of 55±5℃ till a constant dry weight of the sample was reached. Later the dry weight of shoot was recorded by using electronic weighing machine and expressed in gram.

3.5.2.8 Total biomass

Total biomass was calculated by adding total dry weight of leaves, stem, roots and tuberous roots of respective plants and the mean values was taken as total biomass of plant and expressed in grams.

3.5.2.9 Root to shoot ratio

Root to shoot ratio was calculated as a ratio of dry weight of roots to dry weight of shoots.

3.5.2.10 Dry matter content (%)

Dry matter content in five plants was recorded by taking dry weight of roots/ tuberous roots by fresh weight of roots/ tuberous roots and the mean dry matter content was calculated and expressed as percentage.

$$\text{Dry matter (\%)} = \frac{\text{Dry weight of roots}}{\text{Fresh weight of roots}} \text{ X } 100$$

3.5.2.11 Harvest Index

Harvest index is expressed as a ratio of economic yield (dry weight of roots) over biological yield (total dry weight of plant) multiplied by 100. Then the mean harvest index value was calculated.

$$\text{Harvest Index} = \frac{\text{Economic yield (Dry weight of roots per plant)}}{\text{Biological yield (Total dry weight of plant)}}$$

3.6 STATISTICAL ANALYSIS AND INTERPETATION OF DATA

The data on growth and yield parameters was subjected to two way analysis of Variance as outlined by Sundararaj *et al.* (1972). Wherever the F-test was significant, comparison of treatment means, critical difference (C.D.) values were calculated at 5 per cent probability level.

3.7 MOLECULAR CHARACTERIZATION USING RAPD MARKERS (GENETIC DIERSITY ANALYSIS)

Studies on genetic variation in *Coleus* species and mutants of C. *forskohlii* have been limited (Vishwakarma *et al.,* 1988). It is often difficult to differentiate accessions morphologically or even through chemotype profiling. Hence, RAPD markers are very handy to measure the degree of genetic relatedness among the various *Coleus* species and accessions of C. *forskohlii.*

3.7.1 Plant material

The plant material of different *Coleus* species and mutants of *C. forskohlii* used in RAPD analysis were obtained from Sugandhavana, Department of Horticulture, UAS, GKVK, Bangalore.

In the present study various mutants of *C. forskohlii* were used for characterization by using RAPD marker. These mutants of *C. forskohlii* were evolved by treating with different intensities of γ radiations. Further, these mutants were selected based on their morphological characters, yield characters and also based on forskolin content in their tuberous roots.

Further, these mutants were evaluated under different agro climatic conditions in Karnataka and these superior mutants which gave good results over check (K-8) are maintained in the Department of Horticulture.

3.7.2 DNA isolation

3.7.2.1 Sample preparation

Young and healthy leaves of *Coleus* species and different mutants of C. *forskohlii* were collected from the potted plants and also from field grown plants in paper bags. Mainly the upper pair of leaves in the plant

was considered for the collection. The collected leaves were washed thoroughly with double distilled water and later air dried to remove moisture weighed using electronic weighing machine. Two grams of this leaf tissue was taken for DNA extraction.

3.7.2.2 Protocol for extraction of genomic DNA

a. Two grams of fresh leaf sample was taken in a mortar and crushed well using liquid nitrogen.

b. The ground tissue was transferred to a 50 ml sterile centrifuge tube by adding 15ml of CTAB buffer (Extraction buffer- 2 per cent C-TAB, 1.4 NaCl, 20 mM EDTA (Disodium) & 10mM Trisbase (pH 8). Then 50 µl of β- mercaptaethanol was added to each tube and the extract was mixed well by inverting the tubes several times. The tubes were incubated in a water bath maintained at 65 ^{0}C for an hour with constant stirring at intervals of 15 minutes.

c. After an hour, 15 ml of chloroform isoamyl alcohol (24:1) was added to the incubated sample and mixed well by inverting. The tubes were then centrifuged at 6000 rpm for 20 minutes.

d. The aqueous upper phase was carefully transferred using 1ml cut tips into fresh sterile centrifuge tubes. To this supernatant 0.7 volume (10.5 ml) of cold Isopropanol was added.

e. The tubes were carefully inverted and kept for 5 min in ice. DNA precipitation was seen in the form of strands. The tubes were then centrifuged at 6000 rpm for 20 minutes and sedimentation of DNA as a hard pellet was seen.

f. Further the supernatant was decanted gently and the tubes were inverted on a clean filter paper.

g. The pellet was washed twice by suspending in one ml of 70 per cent ethanol for 5 to 10 minutes and the DNA was centrifuged at 6000 rpm for two minutes.

h. Ethanol was drained off slowly and the pellet is vacuum dried in a desicator for 5 to 10 minutes. The pellet is then dissolved in 500 µl of TE buffer by flicking the tubes. (TE buffer = 0.1mM Tris + 0.05mM EDTA).

i. To remove the RNA 5µl of RNase (10mg/mL) was added into the DNA solution and was incubated at 37°C in a water bath for 1 hour.

j. DNA solution was cleaned once again by washing with equal volume (500 µl) of Phenol: Choloroform: Isoamyl alcohol (25:24:1) by invert mixing several times and centrifuging at 6000 rpm for 15 minutes to separate the two phases.

k. The aqueous upper phase was transferred into a clean 1.5 ml eppendorf tube and twice the volume of 100 per cent ethanol was added to precipitate the DNA.

l. The DNA pellet was washed with 70 per cent ethanol twice and after removing ethanol the pellet was dried.

m. Finally, DNA pellet was dissolved in 500 µl of TE buffer and stored at -20°C until use.

n. Physical integrity of DNA was verified by electrophoresis on 1% agarose gel.

o. The extracted DNA was quantified using spectrophotometer

3.7.3 Quantification of DNA

The genomic DNA was quantified spectrophotometrically both at 260 nm and 280 nm wavelengths. The absorbance at 260 nm allows the calculation of DNA concentration in the sample. An OD at 260 nm

Table 2. List of RAPD primers used for DNA amplification

Sl. No.	Primer code	Sequence
1	OPB-10	CTGCTGGGAC
2	OPG- 12	CAGCTCACGA
3	OPE-16	GGTGACTGTG
4	OPA-03	AGTCAGCCAC
5	OPA-07	GAAACCGGTG
6	OPG-09	CTGACGTCAC
7	OPG-10	AGGGCCGTCT
8	OPB-14	TCCGCTCTGG
9	OPN-20	GGTGCTCCGT
10	OPA-15	TTCGAGCCAG
11	OPN-09	TGCCGGCTTG
12	OPB-11	GTAGACCCGT
13	OPAR-18	CTACCGGCAC
14	OPN-13	AGCGTCACTC
15	OPI-10	ACAACGCGAG
16	OPX- 11	GGAGCCTCAG
17	OPX-6	ACGCCAGAGG
18	OPV-11	AGACGATGGG
19	OPV-3	ACAGCCTGCT
20	OPV-14	GGTCGATCTG

Table 3. Amplification conditions for RAPD

Sl. No.	Condition	Temperature	Duration
1	Initial denaturation	94^0 C	3 min.
2	Denaturation	94^0 C	1 min.
3	Annealing	37^0 C	1 min.
4	Extension	72^0 C	3 min.
6	Final extension	72^0 C	10 min.
7	Final hold	4^0 C	-

Step 2, 3 and 4 is repeated 35 times.

corresponds to 50 µg of double stranded DNA. A pure sample of DNA shows the ratio of OD 260/280 as 1.8. Ratios less than 1.8 indicate contamination in the isolation either with phenol or with proteins. The values higher than this indicate the presence of RNA in the isolation.

3.7.4 DNA amplification by Polymerase Chain Reaction (PCR)

The PCR procedure as described by Williams *et al.* (1990) was followed with minor modifications. A single decamer primer of arbitrary sequence was used for RAPD analysis of each amplification using PCR. Amplification was carried out in 25 µl reaction mixer containing template DNA 1 µl, primer 2µl. $MgCl_2$ 2 µl, Taq DNA Polymerase 0.5 µl , 10x buffer 2.5 µ1 with dNTPs 2µl and sterile water 15 µl. Totally 20 primers were screened for *Coleus* DNA amplification and are listed in Table 2. Amplification conditions are listed in Table 3.

3.7.5 Estimation of genetic relatedness of different *Coleus* species accessions using RAPD markers.

Amplified RAPD fragments were separated on 2 per cent agarose gel containing Ethidium bromide. The reaction volume of 25µl along with sample buffer was loaded into the wells. Electrophoresis was conducted at 50V for one to one and half hours. The agarose gels were viewed under UV light and were photographed in a gel documentation system (GEL DOC).

3.7.6 Data analysis

The RAPD bands were scored for its presence as '1' and absence as '0' at each position. The data were analyzed for dendrogram, distance matrix and principle component analysis based on minimum variance algorithm (Ward, 1963) and squared Euclidean distances (Sokal and Smith, 1973).

3.8 Cloning of PCR product

The PCR amplified DNA fragment was cloned into the plasmid vector pTZ57R/T using InsT/A clone PCR product cloning kit (Cat#K1214, MBI, Fermentas) following the manufacturer's instruction. The T/ A cloning method is suitable for cloning of PCR fragments amplified with primers that carry dG or dC at their 5' ends. The pTZ57R/T vector is a derivative of pUC19 with size of 2.88 Kb. The vector has been pre-cleaved with *Eco* 321 and treated with terminal deoxynucleotidyl transferase to create 3' - ddT overhangs at both ends which allows ligation of Taq-amplified PCR products with 3' dA overhangs into the vector. It has a versatile multiple cloning sites flanked by *EcoRI* and *HindIII,* which permits easy excision of the DNA insert and subcloning into other vectors as well as sequencing using standard M13 primers. The vector also contains the ampicillin resistant gene for antibiotic selection and the *lacZ* gene that allows blue/white selection of recombinant colonies by α-complementation.

A. Reagents for ligation

Plasmid vector pTZ57R/T	0.055µg/ µl
10X Ligation Buffer	400mM (Tris-Cl, 100mM $MgCl_2$,100mM)
T4 DNA Ligase	5 U/ µl
Deionized water	7.0µl
T4 DNA ligase	0.5µl

Procedure

A total reaction volume of 15µl was set up for ligation. Following components were mixed in a 0.5 ml tube

Plasmid vector pTZ57R/T	1.5µl
Purified PCR fragment	3.0µl
10X ligation buffer	3.0µl

Deionised water	7.0µl
T4 DNA ligase	0.5µl

Ligation was carried out at 4^0C for 16 hrs. The ligation mixture was used for transformation.

Preparation of Competent Cells for Transformation: Competent cells of *Escherichia coli* strain DH5α was prepared by calcium chloride method as described by (Sambrook and Russel, 2001).

Protocol

Overnight grown culture of DH5α cells was inoculated into 30 ml of LB media. The cells were grown at 37°C on a rotary shaker at 200 rpm till OD_{600} reached 0.3 to 0.4. The cells were then aseptically transferred to a sterile chilled centrifuge tube and immersed in ice for 15min. Then the cells were pelleted by centrifugation at 5000 rpm for 5 min at 4°C. The pelleted cells were resuspended in 15 ml ice cold 0.1M $CaCl_2$ and incubated on ice for 30 min. The cells were again recovered by centrifugation at 5000 rpm for 5 min at 4^0C. The pellet obtained was resuspended in 6ml of 0.1M $CaCI_2$ and 1ml of 100% glycerol. The cells were then stored in chilled 1.5 ml microcentrifuge tubes in aliquots of 200µl and quickly frozen by keeping it in liquid nitrogen and stored in -80^0C freezer.

B. Transformation

The tube containing competent cells was removed from -80^0C freezer and kept briefly in ice for thawing. 10µl of ligation mixture was added to 100µl of competent cells and incubated in ice for 30 min. The cells were subjected to heat shock at 42^0C for 2.5 min and snap chilled for 2 min on ice. 800µl of sterile LB broth was added to this and cells were grown at 37°C for 2 hr in shaking incubator at 120 rpm. After

incubation the cells were spun down and dissolved in 50µl of LB media and plated on LB agar plates containing ampicillin (50mg/ml) and X-gal/ IPTG and incubated overnight at 37^0C.

C. Screening of Clones

The T- vectors used in cloning were compatible for blue-white screening when plated on X-gal and IPTG. The recombinant clones with a foreign DNA insert ligated at the 3' T overhangs disrupt the reading frame of *lacZ* and produce white colonies. Whereas the self ligated (circularized) vector produces blue colonies due to enzymatic activity of *lacZ* on the substrate X-gal.

Colony PCR: PCR was performed to directly analyse the positive transformants using the gene specific primers as follows.

i) 25µl reaction volume PCR cocktail was prepared consisting of PCR buffer, dNTPs, primers, water and Taq DNA polymerase.

ii) White colonies were selected and resuspended individually into 25µl of the PCR cocktail and simultaneously the colonies were patched onto separate plate to preserve it for future.

PCR was performed as described earlier. Amplified products were resolved on 1.5% agarose gel, After confirmation of colony PCR with our insert size. The putative transformants which appear as white coloured colonies were picked from the plate and grown in 3-5 ml LB broth containing ampicillin (50mg/ml) incubated at 37^0C overnight in a rotary shaker at 120rpm for the plasmid DNA isolation. By using the Plasmid DNA isolation Kit (column based, Cat. No.1001, 1002).

D. Plasmid DNA isolation by column based kit:

Take 1.5 to 3ml of a saturated overnight bacterial cultured inoculated by white colonies before night in a standard bench top micro centrifuged for 30sec at 11,000rpm, decant the supernatant. Resuspended the cell pellet in 250µl buffer A1 by vigorously vortexing.

Sample lysis step: Add 250µl of buffer A2, then mixed gently by inverting the tube 6-8 times (do not vertex). Incubated at room temperature for 2-3 min.

Neutralization step: Add 350 µl of buffer A3 and mixed gently by inverting the tubes 6-8 times (do not vertex). Centrifuged for 5 min. at 11,000 rpm at room temperature.

Binding step: For each preparation, one Bioserve column was taken and placed in a 2ml collection tubes. Transferred the cleared lysate to the column and centrifuged at 6000 rpm for 1 min.

Wash step-1: Place the Bioserve column back into the 2ml collection tube, then add 500µl of buffer AW. Centrifuged for 1 min at 11,000rpm.

Wash step-2: Place the Bioserve column back into the 2ml collection tube, then add 600µl of buffer A4. Centrifuged for 1 min at 11,000 rpm.

Drying step: Spun the column with cap open at 11,000rpm for 2min (drying) and left the caps open for 2min before proceeding to the elution step. (Residual ethanolic washing solution might inhibit enzymatic reactions, which are removed by this centrifugation step completely).

Elution of plasmid DNA: Place the Bioserve column in a fresh of 1.5ml micro centrifuge tube and add 25µl of buffer AE or nuclease free water. Keep it at room temperature for 5 min, then centrifuged at 11,000rpm for

2 min (referred elution procedures). The resulting eluate contains plasmid DNA which can be used for downstream processing reactions.

Visualization of plasmid DNA by agarose gel electrophoresis:

Electrophoresis of plasmid DNA from clones was done in 1% agarose gel according to the procedure outlined (Sambrook and Russel, 2001). Plasmid DNA from blue colony containing only pTZ57R/T plasmid without the DNA insert (control) was also loaded along with plasmid DNA from white colonies carrying the cloned insert in the plasmid vector. The clones were selected based on the lower mobility of the plasmid DNA due to the larger size compared to that of the control plasmid. The purity and approximate concentration of the purified plasmid DNA was also determined by visual estimation on agarose gel and documentation using gel documentation system. The plasmid DNA from the clones was subjected to other conformation tests for the presence of the cloned DNA fragment such as restriction digestion analysis and PCR.

Plasmid PCR: PCR was performed to directly analyse the positive transformants.

PCR mixture for plasmid PCR:

Template DNA (25 ng/µl)	: 1 µl
Taq buffer- A (10X)	: 5 µl
dNTP's (10mM)	: 2 µl
Primer (5 pM) F	: 2 µl
R	: 2 µl
Taq DNA polymerase (1U/µl)	: 0.5µl
Water	: 37.5 µl
Total reaction volume	**50 µl**

PCR conditions for RAPD primers

Step 1	94° C for 5 min	
Step 2	94° C for 1 min	40 cycles
Step 3	70°C for 1 min	
Step 4	72° C for 2 min	
Step 5	72° C for 7 min	
Step 6	4^0 C for ever	

Amplified products were resolved on 2 % agarose gel.

E. Plasmid DNA Sequencing and SCAR primers designs:

The plasmids were sequenced by Chromous Biotech Pvt. Ltd. in both reverse and forward directions with M-13 universal primers using ABI3700 DNA analyzer (Applied Biosystems, USA). The sequence is there after used to design specific primer pairs of 16-24 bp which amplify single major bands of the size similar to that of cloned fragment. From the sequence of RAPD-4 (AACGCGCAAC) forward and reverse primers were designed.

3.8 ESTIMATION OF FORSKOLIN CONTENT IN THE MUTANTS OF *Coleus forskohlii*

High Performance Liquid Chromatographic (HPLC) analysis was carried out in the Department of Horticulture, to estimate the forskolin content in tuberous roots of *C. forskohlii* mutants.

3.8.1 Procedure

I. CHROMATOGRAPHIC SYSTEM: Waters HPLC system

Pumps: Waters 515, Binary gradient operated under isocratic conditions.

Detector: Waters 2487 dual absorbance detector.

CHROMATOGRAPHIC CONDITIONS

a. Mobile phase: Millipore water : HPLC grade acetonitrile
Ratio : 55 : 45

b. Flow rate: 1.8 ml/ min
Pump A - 0.99 ml/ min, Pump B - 0.81 ml/ min.

c. Detection wavelength: 220 nm

d. Column: Symmetry C 18 column {4.6mm x 250mm}

e. Injection volume: 20 microlitre.

II. STANDARD CURVE FOR FORSKOLIN

a. Reference – standard

Forskolin of pure form (99.5%) obtained from M/s Natural Remedies Pvt. Ltd. Co., Bengaluru was the reference compound.

b. Stock solution

The above reference compound - 20mg in quantity was dissolved in 1 ml of analytical grade acetonitrile solvent to make a stock solution of 20,000 ppm or a concentration of 20 mg/ ml.

c. Preparation of different dilutions

From the stock solution, different dilutions/ concentrations were made (Table 4).

d. Injection of standards

The above concentration of forskolin in acetonitrile was clarified using sample clarification kit and was injected into injection port with 20 microlitre loop being attached to the HPLC unit. The run time was fixed at 30 minutes. A sequence of injections were made from higher concentration to lower concentration of standards.

Table 4. Details of different concentrations of forskolin prepared from stock solution

Sl. No.	Amount of stock solution (ml)	Acetonetrile added (ml)	Total volume (ml)	Final concentration (mg/ ml) or ppm
1	0.05	0.45	0.5	2000
2	0.10	0.40	0.5	4000
3	0.15	0.35	0.5	6000
4	0.20	0.30	0.5	8000
5	0.25	0.25	0.5	10000

e. Calibration

A particular peak with retention time of twelve minutes was identified as the standard concentration peak of forskolin. By the repeated injections of the standards it was possible to get the peak at a particular retention time. Hence, that was considered as the standard peak. Then, the standard curve was prepared by entering component name, concentration and units.

f. Procedure for estimation of forskolin in root samples

i. Dried roots (tuberous roots) were made into fine powder by using electric blender.

ii. Ten grams of the finely powered root was weighed and packed in silver films.

iii. Ten grams of tuberous root powder was taken in a extraction thimbles and placed inside the Soxhlet extraction unit.

iv. To the root sample 250 ml of analytical grade methanol was added.

v. The apparatus was kept on the mantle connected to the electric source and the extraction process was initialized.

vi. When 3-4 siphonings of extraction were over and a clear solution appeared, the extraction process was stopped.

vii. The root powder along with the extraction thimbles was discarded.

vii. For obtaining a concentrate of the extract, was taken in a round bottom flask and the flask was fitted to a rotary evaporator.

vi. The evaporation procedure was continued until a minimum amount of the extract remained in the flask.

viii. The volume of the remaining extract was made up to 25 ml by adding methanol.

g. Clarification of samples

The sample solutions before injection were clarified. The extract was taken in a funnel, inside of which a cellulose filter paper was kept. The concentrated extract was poured into the funnel and obtained a clarified extract in the beaker kept at the bottom of the funnel. The clarified extract was transferred to a volumetric flask and kept in a cool place.

h. Mobile phase preparation

Acetonitrile (HPLC grade) and millipore water were used for mobile phase prepation. The filtered (clarified) solvents were connected to two different pumps of HPLC and the flow rate was adjusted to 1.8 ml.

i. Injection of sample solution

The root extracts of the samples thus prepared after clarification were injected (20 µl) port with the help of 25 µl syringe. Before injection the syringe was thoroughly rinsed with methanol and then with the sample. The excess fluid was collected in a bottle. The run time was adjusted for 30 minutes. After injection the syringe was thoroughly rinsed with methanol and used for further injections.

For each sample the injections were repeated at least three times and the peaks were identified with reference to the standard curve.

j. Calculation

Forskolin content was calculated by using the formula:

$$\frac{\text{HPLC reading X Concentration of standard X \% purity of standard forskolin (mg/ ml)}}{\text{Area of standard forskolin X Concentration of sample (mg/ ml)}}$$

EXPERIMENTAL RESULTS

IV. EXPERIMENTAL RESULTS

Present study on "Morphological and molecular characterization of selected *Coleus* species and mutants of *Coleus forskohlii* Briq." was carried out with eleven *Coleus forskohlii* accessions and three different *Coleus* species during 2010-2011. The results of the study are presented in this chapter under the following headings:-

4.1 Evaluation of *Coleus* species and mutants of *Coleus forskohlii* for morphological characters.

4.2 Evaluation of *Coleus* species and mutants of *Coleus forskohlii* for yield attributes.

4.3 Assessment of genetic relatedness in *Coleus* species and mutants of *Coleus forskohlii.*

4.4 Estimation of forskolin in mutants of *Coleus forskohlii.*

4.1 Evaluation of *Coleus* species and mutants of *Coleus forskohlii* for morphological characters

4.1.1 Plant Height (cm)

Significant differences were recorded for plant height among different *Coleus* species and mutants of *Coleus forskohlii* at different stages of growth. Data on plant height has been presented in Table 5.

4.1.1.1 60 days after planting

Among the mutants and species evaluated, mutant MV5 recorded significantly superior plant height of 27.70 cm and was *on par* with *C. aromaticus var. variegated* and *C. vettiveroides* which recorded a plant height of 27.30 and 27.50 cm respectively. Least plant height was recorded in SV2 (21.86 cm).

Table 5. Plant height (cm) of different *Coleus* species and mutants of *C. forskohlii* at different stages of growth

Mutants / species	60 days	120 days	180 days
MV1	22.33	41.43	65.33
MV2	25.66	42.26	57.30
MV3	23.06	42.40	54.86
MV4	25.46	52.10	71.23
MV5	27.70	53.83	73.53
MV6	22.56	43.26	56.63
MV7	21.93	51.56	74.26
MV8	22.50	47.07	71.26
MV9	22.40	46.30	68.13
SV1	24.20	53.53	72.56
SV2	21.86	43.33	65.30
K-8	22.03	44.46	66.46
Coleus aromaticus	22.56	43.20	68.73
C. aromaticus var. variegated	27.30	48.50	74.86
C. vettiveroides	27.5	47.5	72.90
Mean	23.94	46.71	67.56
F Test	*	*	*
S.E.m±	0.28	0.55	0.81
C.D.	0.81	1.61	2.34

* Significant at 5% level

4.1.1.2 120 days after planting

After 120 days of planting also mutant MV5 maintained its trend and recorded a plant height of 53.83 cm which was highest amongst the mutants and species evaluated. This was *on par* with plant height of SV1 (53.53 cm). Least plant height was recorded in mutant MV1 (41.43 cm).

4.1.1.3 180 days after planting

At the time harvest *Coleus aromaticus var. variegated* showed maximum plant height of 74.86 cm, which was *on par* with the plant height of mutant MV5 (73.53 cm), mutant MV7 (74.26 cm), somaclone SV1 (72.56 cm) and with the species *C. vettiveroides* (72.9 cm). Least plant height of 54.86 cm was recorded in the mutant MV3.

4.1.2 Number of branches

Data on number of branches at different stages of plant growth has been presented in Table 6.

4.1.2.1 60 days after planting

Plants exhibited significant differences for number of branches. After 60 days of plating mutant MV7 recorded maximum number of branches (15.10). Least number of branches of 9.93 was recorded both in somaclone SV2 and accession K-8.

4.1.2.2 120 days after planting

Data with respect to number of branches was found to be significant at 120 days after planting. Mutant MV7 maintained its superiority with respect to number of branches over others after 120 days of planting also by recording maximum number of branches (34.30). Which was *on par* with mutant MV1, which recorded 33.9 branches per plant. Least number of branches was recorded in mutant MV6 (29.10).

Table 6. Number of branches in different *Coleus* species and mutants of *Coleus forskohlii* at different stages of growth

Mutants/ species	60 days	120 days	180 days
MV1	12.03	33.90	53.56
MV2	12.63	30.46	48.73
MV3	11.83	29.33	47.4
MV4	11.76	30.56	54.43
MV5	14.36	29.46	55
MV6	10.60	29.10	45.46
MV7	15.10	34.30	49.33
MV8	11.80	29.10	45.40
MV9	11.36	25.83	41.60
SV1	11.43	29.03	51.90
SV2	9.93	26.06	45.26
K-8	9.93	26.36	42.73
Coleus aromaticus	10.60	23.30	41.36
C. aromaticus var. variegated	12.83	28.70	49.33
C. vettiveroides	12.50	27.23	47.30
Mean	11.91	28.85	47.92
F- Test	*	*	*
S.E.m±	0.20	0.47	0.58
C.D.	0.60	1.36	1.68

* Significant at 5% level

Table 7. Plant spread (cm^2) in different *Coleus* species and mutants of *Coleus forskohlii* at harvest (180 days after planting)

Mutants/ species	Plant spread
MV1	3061.86
MV2	2777.10
MV3	2882.96
MV4	3086.26
MV5	3082.60
MV6	2868.63
MV7	3055
MV8	2821.96
MV9	3023.26
SV1	2539.23
SV2	2682.80
K-8	3041.36
Coleus aromaticus	2688.53
C. aromaticus var. variegated	3141.86
C. vettiveroides	2877.53
Mean	2908
F- Test	*
S.E.m±	47.78
C.D.	138

* Significant at 5% level

4.1.2.3 180 days after planting

Results were significant at 180 days after planting also. Mutant MV5 recorded maximum number of branches of 55 after 180 days of planting, which was *on par* with mutant MV1 (53.56) and mutant MV4 (54.43). Least number of branches was recorded in *Coleus aromaticus* (41.36 branches/ plant).

4.1.3 Plant spread (cm^2)

Data on the plant spread in different *Coleus* species and mutants of *Coleus forskohlii* has been presented in the Table 7. Among the various species of *Coleus* and mutants of *C. forskohlii*, the species *Coleus aromaticus var. variegated* recorded the maximum plant spread of 3141.86 cm^2 at 180 days after planting and it was *on par* with the mutant MV1 (3061.86 cm^2), mutant MV4 (3086.26 cm^2), mutant MV5 (3082. 60 cm^2), mutant MV7 (3055 cm^2), mutant MV9 (3023.26 cm^2) and with accession K-8 (3041.36 cm^2). Whereas, least plant spread of 2539.23 cm^2 was recorded in the somaclone SV1.

4.1.4 Number of leaves per plant

Significant results were obtained with respect to number of leaves per plant and the data has been presented in Table 8. After 180 days of planting mutant MV3 recorded 909 leaves/ plant, which was highest among the mutants of *C. forskohlii* and the other *Coleus* species in the study. Least number of leaves was recorded in *Coleus vettiveroides* (175 leaves/ plant).

4.1.5 Leaf area

Data on the leaf area at the time of harvest is presented in Table 9. Various mutants of *C. forskohlii* and species of *Coleus* recorded

Table 8. Number of leaves/ plant in different *Coleus* species and mutants of *Coleus forskohlii* at harvest (180 days after planting)

Mutants/ species	Number of leaves/ plant
MV1	783
MV2	814.90
MV3	909
MV4	808.90
MV5	860.23
MV6	775.10
MV7	845.20
MV8	718.83
MV9	802.80
SV1	747.63
SV2	810.56
K-8	667.16
Coleus aromaticus	453.63
C. aromaticus var. variegated	364.96
C. vettiveroides	175
Mean	702.46
F- Test	*
S.E.m±	9.59
C.D.	27.71

* Significant at 5% level

Table 9. Leaf area/ plant (cm^2) in different *Coleus* species and mutants of *Coleus forskohlii* at harvest

Mutants/ species	Leaf area/ plant
MV1	9951.36
MV2	10029.06
MV3	10431.41
MV4	11637.68
MV5	10259.12
MV6	9526.41
MV7	10501.58
MV8	9457.58
MV9	10082.35
SV1	9521.26
SV2	10180.08
K-8	9142.50
Coleus aromaticus	10964.73
C. aromaticus var. variegated	7610.55
C. vettiveroides	7524.12
Mean	9838.92
F- Test	*
S.E.m±	201.01
C.D.	580.58

* Significant at 5% level

significant difference for leaf area. Mutant MV4 recorded highest leaf area of 11637.68 cm^2). Least leaf area was recorded in K-8 (9142.5 cm^2).

4.2 Evaluation of *Coleus* species and mutants of *Coleus forskohlii* for yield attributes

4.2.1 Fresh weight of shoot (gm)

Significant results were obtained with respect to fresh weight of shoot at harvest. Data has been presented in Table 10. At the time of harvest *Coleus aromaticus* recorded maximum shoot weight of 452.86 g/plant and this was *on par* with *C. vettiveroides* (441.83 g/ plant). Least fresh weight of shoot was recorded in mutant MV3 293.73 g/ plant.

4.2.2 Dry weight of shoot (gm)

Data on the dry weight of shoot is presented in the Table 11. Significant results were obtained as, maximum dry shoot weight of 38.23 g/ plant was recorded in *Coleus aromaticus*. Lowest dry shoot weight of 24.33 g/ plant was recorded in mutant MV3.

4.2.3 Fresh weight of root (gm)

All mutants and species showed a significant difference for the fresh weight of root. Data of which is presented in the Table 12. Maximum fresh root weight was recorded in mutant MV5 (764.69 g/ plant) but was *on par* with MV7 (749.23 g/ plant). Least fresh root weight was recorded in *C. aromaticus var. variegated* (244.4 g/ plant).

4.2.4 Dry weight of root (gm)

Among the various mutants and species under study, mutant MV5 recorded maximum dry root weight of 189.7 g/ plant. Followed by MV7 (177.4 gm/ plant). Least dry weight of root was recorded was in *C. aromaticus var. variegated* (83.43 g/ plant).

Table 10. Fresh shoot weight/ plant (g) in different *Coleus* species and mutants of *Coleus forskohlii* at harvest

Mutants/ species	Fresh shoot weight/ plant
MV1	365.96
MV2	396.16
MV3	293.73
MV4	364.16
MV5	378.8
MV6	347.6
MV7	403.83
MV8	374.8
MV9	408.83
SV1	358.73
SV2	342.43
K-8	395.83
Coleus aromaticus	452.86
C. aromaticus var. variegated	410.2
C. vettiveroides	441.83
Mean	382.36
F- Test	*
S.E.m±	7.73
C.D.	22.34

* Significant at 5% level

Table 11. Dry shoot weight/ plant (g) in different *Coleus* species and mutants of *Coleus forskohlii* at harvest

Mutants/ species	Dry shoot weight/ plant
MV1	28.80
MV2	32.93
MV3	24.33
MV4	28.53
MV5	31.16
MV6	26.93
MV7	34.03
MV8	30.66
MV9	33.96
SV1	27.33
SV2	26.23
K-8	32.50
Coleus aromaticus	38.23
C. aromaticus var. variegated	35.30
C. vettiveroides	36.70
Mean	31.13
F- Test	*
S.E.m±	0.46
C.D.	1.3

* Significant at 5% level

Table 12. Fresh root weight/ plant (g) in different *Coleus* species and mutants of *Coleus forskohlii* at harvest

Mutants / species	Fresh root weight/ plant
MV1	528.52
MV2	445.61
MV3	473.37
MV4	661.40
MV5	764.69
MV6	589.38
MV7	749.23
MV8	544.52
MV9	398.34
SV1	702.31
SV2	458.81
K-8	422.62
Coleus aromaticus	301.43
C. aromaticus var. variegated	244.40
C. vettiveroides	308.96
Mean	506.24
F- Test	*
S.E.m±	10.47
C.D.	30.25

* Significant at 5% level

Table 13. Dry root weight/ plant (g) in different *Coleus* species and mutants of *Coleus forskohlii* at harvest

Mutants/ species	Dry root weight/ plant
MV1	127.26
MV2	126.33
MV3	140.26
MV4	154.57
MV5	189.70
MV6	126.03
MV7	177.40
MV8	132.79
MV9	110.34
SV1	171.02
SV2	106.69
K-8	114.60
Coleus aromaticus	87.90
C. aromaticus var. variegated	83.43
C. vettiveroides	99.13
Mean	129.83
F- Test	*
S.E.m±	3.06
C.D.	8.86

* Significant at 5% level

4.2.5 Number of roots per plant

Significant results were obtained for number of roots per plant (Table 14). Among various mutants and species, MV5 recorded maximum number of roots per plant of 18.73. This was *on par* with17.90 roots/ plant in the mutant MV7. Least number of roots was found in the plants of the species *C. aromaticus var. variegated* (9.23).

4.2.6 Length of tuberous roots (cm)

Data on the root length of different *Coleus* species and mutants of *Coleus forskohlii* has been presented in Table 15. Significant and maximum root length was recorded in mutant MV5 (34 cm). Least root length was recorded in *C. vettiveroides* (22.4 cm).

4.2.7 Root diameter (cm)

Data on the root diameter of different *Coleus* species and mutants of *Coleus forskohlii* has been presented in Table 16. Significant and maximum root diameter was recorded in mutant MV4 (3.14 cm) and was *on par* with the mutant MV5 and also MV7 both of which recorded a root diameter of 3.08 and 3.05 cm respectively. Least root diameter of 1.35 cm was recorded in *C. aromaticus var. variegated.*

4.2.8 Total biomass (gm)

Significant results were obtained for total biomass in different *Coleus* species and mutants of *C. forskohlii*. Data on the total biomass is presented in the Table 17. Maximum biomass of 220.87 g was recorded in mutant MV5. Least biomass of 118.73 g was recorded in species *C. aromaticus var. variegated*.

Table 14. Number of roots/ plant in different *Coleus* species and mutants of *Coleus forskohlii* at harvest

Mutants/ species	Number of roots/ plant
MV1	13
MV2	13.86
MV3	13.43
MV4	13.63
MV5	18.73
MV6	12.13
MV7	17.90
MV8	12.43
MV9	11.60
SV1	16.16
SV2	11.90
K-8	12.13
Coleus aromaticus	11.50
C. aromaticus var. variegated	9.23
C. vettiveroides	13.10
Mean	13.34
F- Test	*
S.E.m±	0.32
C.D.	0.95

* Significant at 5% level

Table 15. Root length/ plant (cm) in different *Coleus* species and mutants of *Coleus forskohlii* at harvest

Mutants/ species	Root length/ plant
MV1	25.83
MV2	23.90
MV3	23.50
MV4	30.93
MV5	34
MV6	27.56
MV7	31.16
MV8	27.30
MV9	25.76
SV1	31.23
SV2	26.87
K-8	25.40
Coleus aromaticus	25.30
C. aromaticus var. variegated	23.73
C. vettiveroides	22.40
Mean	26.99
F- Test	*
S.E.m±	0.52
C.D.	1.52

* Significant at 5% level

Table 16. Root diameter/ plant (cm) in different *Coleus* species and mutants of *Coleus forskohlii* at harvest

Mutants/ species	Root diameter /plant
MV1	2.61
MV2	2.68
MV3	2.36
MV4	3.14
MV5	3.08
MV6	2.59
MV7	3.05
MV8	2.35
MV9	2.55
SV1	2.96
SV2	2.57
K-8	2.60
Coleus aromaticus	1.36
C. aromaticus var. variegated	1.35
C. vettiveroides	1.53
Mean	2.45
F- Test	*
S.E.m±	0.05
C.D.	0.14

* Significant at 5% level

Table 17. Total biomass (g) in different *Coleus* species and mutants of *Coleus forskohlii*

Mutants/ species	Total biomass
MV1	156.06
MV2	159.26
MV3	164.59
MV4	183.10
MV5	220.87
MV6	152.96
MV7	211.43
MV8	163.46
MV9	144.31
SV1	198.35
SV2	132.93
K-8	147.10
Coleus aromaticus	126.13
C. aromaticus var. variegated	118.73
C. vettiveroides	135.13
Mean	160.96
F- Test	*
S.E.m±	3.02
C.D.	8.74

* Significant at 5% level

4.2.9 Root to shoot ratio

Data on root to shoot ratio of different *Coleus* species and mutants of *Coleus forskohlii* has been presented in Table 18. Maximum and significant root to shoot ratio of 6.2 was recorded in somaclone SV1 and was *on par* with mutant MV5 which recorded a root to shoot ratio of 6.1. Least root to shoot ratio of 2.6 was recorded in the accession K-8.

4.2.10 Dry matter content (%)

Significant difference for dry matter content was observed in different *Coleus* species and mutants of *Coleus forskohlii*. Data on dry matter content has been presented in Table19. Maximum dry matter content was recorded in *C. aromaticus var. variegated* (32.4%) and this was *on par* with *C. vettiveroides* which recorded a dry matter content of 32%. Least dry matter content was recorded in the mutant MV6 (21.3%).

4.2.11 Harvest index (%)

Data on significant harvest index values is presented in Table 20. Maximum harvest index was recorded in the mutant MV5 (0.857%) and this was *on par* with mutant MV1 (0.826%), MV3 (0.853%), MV4 (0.841%), MV6 (0.838%), MV7 (0.851%) and somaclone SV1 (0.862%). Least harvest index was recorded in *C. aromaticus* (0.716%).

4.3 Assessment of genetic relatedness in *Coleus* species and mutants of *Coleus forskohlii*

4.3.1 Extraction of genomic DNA and optimization of PCR conditions

Genomic DNA was extracted from fresh young leaves of *Coleus* species and different mutants of *C. forskohlii* as described in chapter 3. The extraction method yielded a good amount of DNA. The quantity of DNA per µl of fresh leaf tissue and relative ratio of DNA to proteins is

Table 18. Root to shoot ratio in different *Coleus* species and mutants of *Coleus forskohlii*

Mutants/ species	Root to shoot ratio
MV1	4.3
MV2	3.7
MV3	5.7
MV4	5.2
MV5	6.1
MV6	4.6
MV7	5.1
MV8	4.4
MV9	3.2
SV1	6.2
SV2	3.9
K-8	2.6
Coleus aromaticus	4
C. aromaticus var. variegated	3.9
C. vettiveroides	3.5
Mean	4.2
F- Test	*
S.E.m±	0.1
C.D.	0.3

* Significant at 5% level

Table 19. Dry matter content (%) in different *Coleus* species and mutants of *Coleus forskohlii*

Mutants/ species	Dry matter content
MV1	23.4
MV2	27.5
MV3	29.9
MV4	23.3
MV5	24.7
MV6	21.3
MV7	23.6
MV8	24.3
MV9	27.7
SV1	24.3
SV2	23.2
K-8	27
Coleus aromaticus	29.1
C. aromaticus var. variegated	34.1
C. vettiveroides	32
Mean	26.2
F- Test	*
S.E. m±	0.6
C.D.	1.9

* Significant at 5% level

Table 20. Harvest Index (%) in different *Coleus* species and mutants of *Coleus forskohlii*

Mutants/ species	Harvest Index
MV1	0.826
MV2	0.807
MV3	0.853
MV4	0.841
MV5	0.857
MV6	0.838
MV7	0.851
MV8	0.803
MV9	0.764
SV1	0.862
SV2	0.802
K-8	0.778
Coleus aromaticus	0.716
C. aromaticus var. variegated	0.725
C. vettiveroides	0.749
Mean	0.805
F- Test	*
S.E. m±	0.01
C.D.	0.04

* Significant at 5% level

Plate 3 Tuberous roots of superior mutants and somaclones of *Coleus forskohlii* (MV5, MV7, SV1 and SV2)

given in Table 21. The recovery of DNA varied widely ranging from 384.8 to 2611.3 ng/µl of leaf tissue. The ratio of DNA to proteins ranged from 1.23 to 1.85.

The PCR conditions were optimized for informative fingerprint profiles of *Coleus* species and different mutants of *C. forskohlii*. Amplification conditions for each cycle of PCR consisted of the following three steps which were repeated 35 times for RAPD.

1. Denaturation 2. Annealing 3. Extension

The optimum amplification conditions are mentioned in chapter 3 (Table 3). For RAPD analysis, concentration of 1 µl of template DNA, 2 µl $Mgcl_2$, 2µl dNTPs, 2.5µl of Tag buffer, Taq DNA polymerase 0.5 µl and 2 µl of primer was found optimum by which, quality amplification banding patterns were obtained.

4.3.2 Primer selection

During the study, 20 random 10 base long primers (Operon Technologies, USA) were screened for the diversity analysis. List of primers are presented in Table 22.

4.3.3 RAPD analysis of Amplified Fragments

The study aimed at determining the genetic relatedness among the Coleus species and various mutants of *C. forskohlii*. The amplified bands, both monomorphic and polymorphic DNA obtained by Polymerase Chain Reaction (PCR) analysis allows the comparison of the genetic material of *Coleus* species and various mutants of *C. forskohlii.* Bands (both monomorphic and polymorphic) were scored and were used in the diversity analysis. The RAPD gel profiles for three *Coleus* species and eleven mutants and a variety of *C. forskohlii* obtained using 5 decamer primers are presented in Fig. 1 to Fig. 5.

Table 21. DNA concentration (ng/µl) in the leaf samples and relative ratio of DNA to proteins

Mutants/Species	DNA Concentration	260/280 nm Ratio
M1	643.4	1.34
M2	1158.2	1.71
M3	623.0	1.48
M4	829.7	1.30
M5	391.2	1.23
M6	852.0	1.30
M7	744.6	1.57
M8	1959.3	1.78
M9	497.5	1.63
S1	384.8	1.33
S2	1611.6	1.69
K-8	2611.3	1.85
C. aromaticus	465.8	1.58
C. Vetteveroides	521.1	1.34
C. aromaticus var. variegated	1298.0	1.50

4.3.4 Dendrogram and Principle Component Analysis

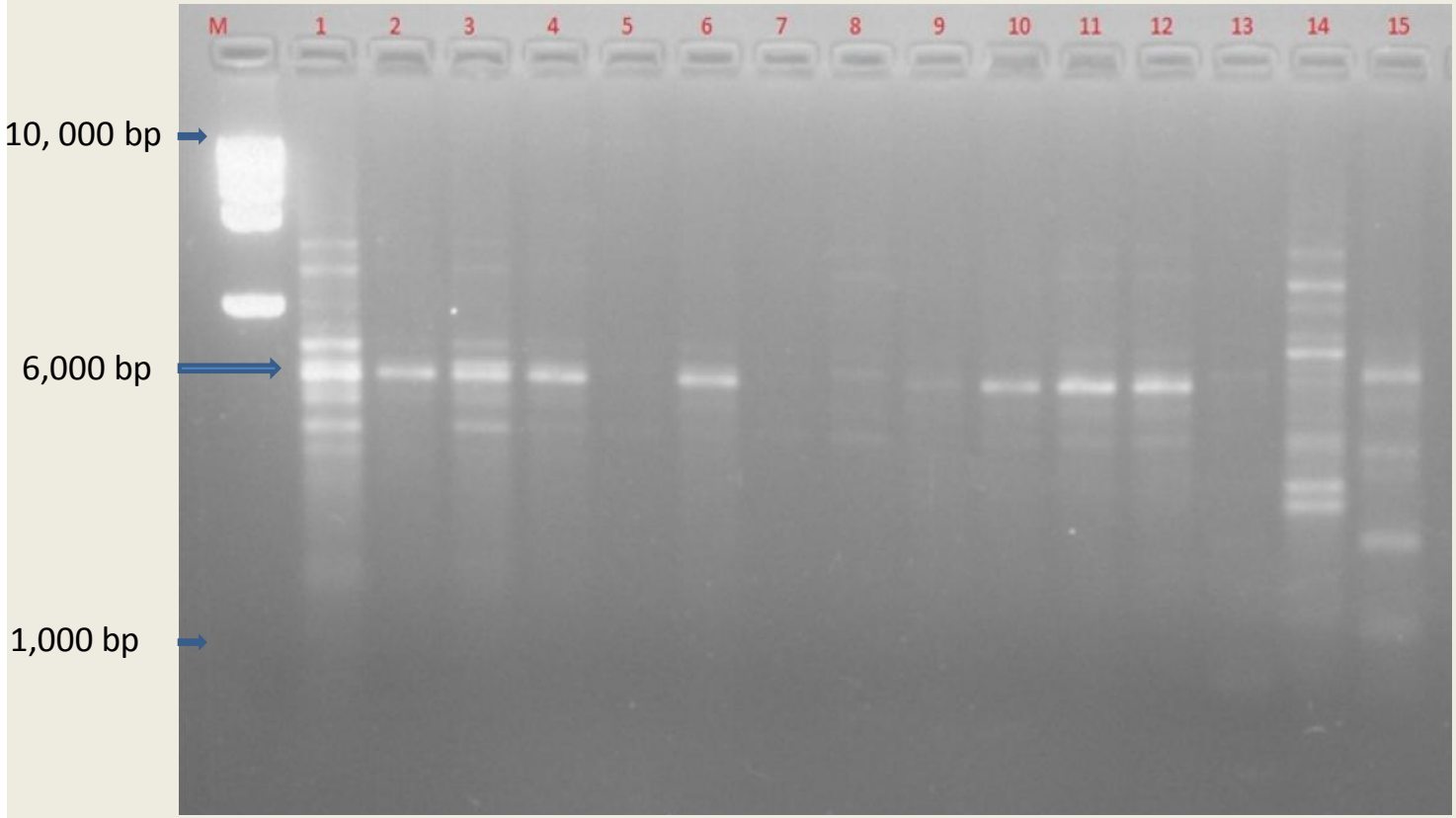
Five arbitrary oligoncleotide primers amplified 293 markers, out of which 13 were polymorphic. Each primer amplified on an average 2.6 polymorphic bands. The total number of bands produced by each primer ranged from 24 to 93 with an average of 58.6 bands per primer. The details regarding the primers used for amplification and the bands produced by them are presented in Table. 22. The primers OPA 15, OPB 14, OPG 10, OPI 18, and OPN 20 proved more useful in differentiating the *Coleus* species and mutants of *C. forskohlii*. Linkage among the three *Coleus* species and eleven mutants and a variety of *C. forskohlii* revealed by cluster analysis using UPGMA method is presented in Fig. 6.

The linkage distance among different Coleus species and mutants of *C. forskohlii* as revealed by distance matrix ranged from 0 to 50, suggesting a narrow genetic base among the mutants of *C. forskohlii*.

Different *Coleus* species and mutants of *C. forskohlii* used in the study were grouped into two major clusters. Cluster 1 comprises of two *Coleus* species (*C. aromatics* and *C. aromaticus var. variegated*) and different mutants of *C. forskohlii* (M1, M2, M3, M4, M5, M6, M7, M8, M9, S1, S2 and K-8). It can be further divided into two groups. Group 1 included mutants like S1, M1, M6, M7, M2, M4 and one *Coleus* species, that is *C. aromaticus var. variegated*. Among these S1 and M1 were closely related with a linkage distance of 15. Mutants M6 and M7 were related with a linkage distance of 17.5. These two were related together with a linkage distance of 22.5. Mutants M2 and M4 were related with a linkage distance of 17.5 and these were in turn related to M6-M7 with a linkage distance of 27.5. A linkage distance of 28 was seen between this group and the species *C. aromaticus* var. *variegated*.

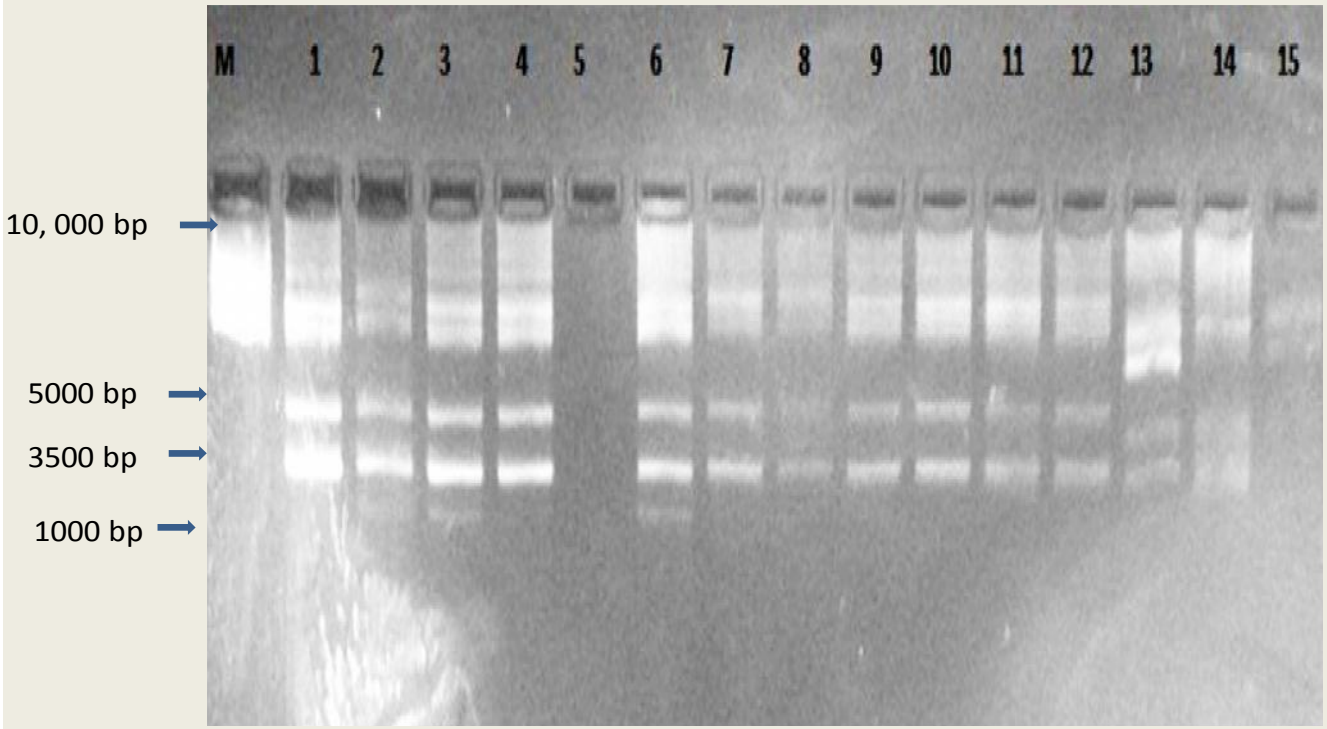
Table 22. Nature and number of bands produced by different primers

Serial No. of Primer	Primer code	Sequence	Number of polymorphic bands	Number of monomorphic bands	Total number of bands
1	OPA 15	TTCGAGCCAG	4	89	93
2	OPB 14	TCCGCTCTGG	0	24	24
3	OPG 10	AGGGCCGTCT	2	66	68
4	OPI 18	ACAACGCGAG	5	29	34
5	OPN 20	GGTGCTCCGT	2	72	74



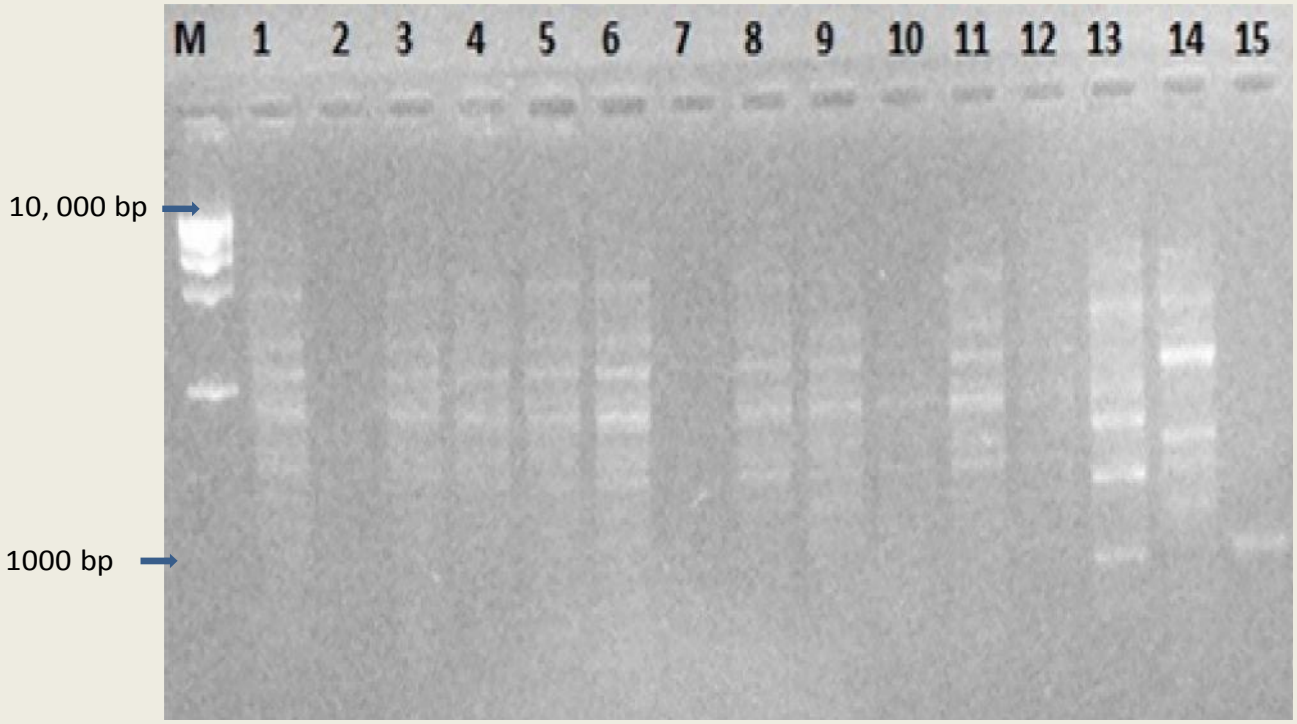
M- 1 kb ladder **1**- MV1 **2**- MV2 **3**- MV3 **4**- MV4 **5**- MV5
6- MV6 **7**- MV7 **8**- MV8 **9**- MV9 **10**- SV1
11- SV2 **12**- K8 **13**- *Coleus aromaticus* **14**- *C. aromaticus var.* variegated **15**- *C. vettiveroides*

Fig. 2 PCR amplification in primer OPB 14



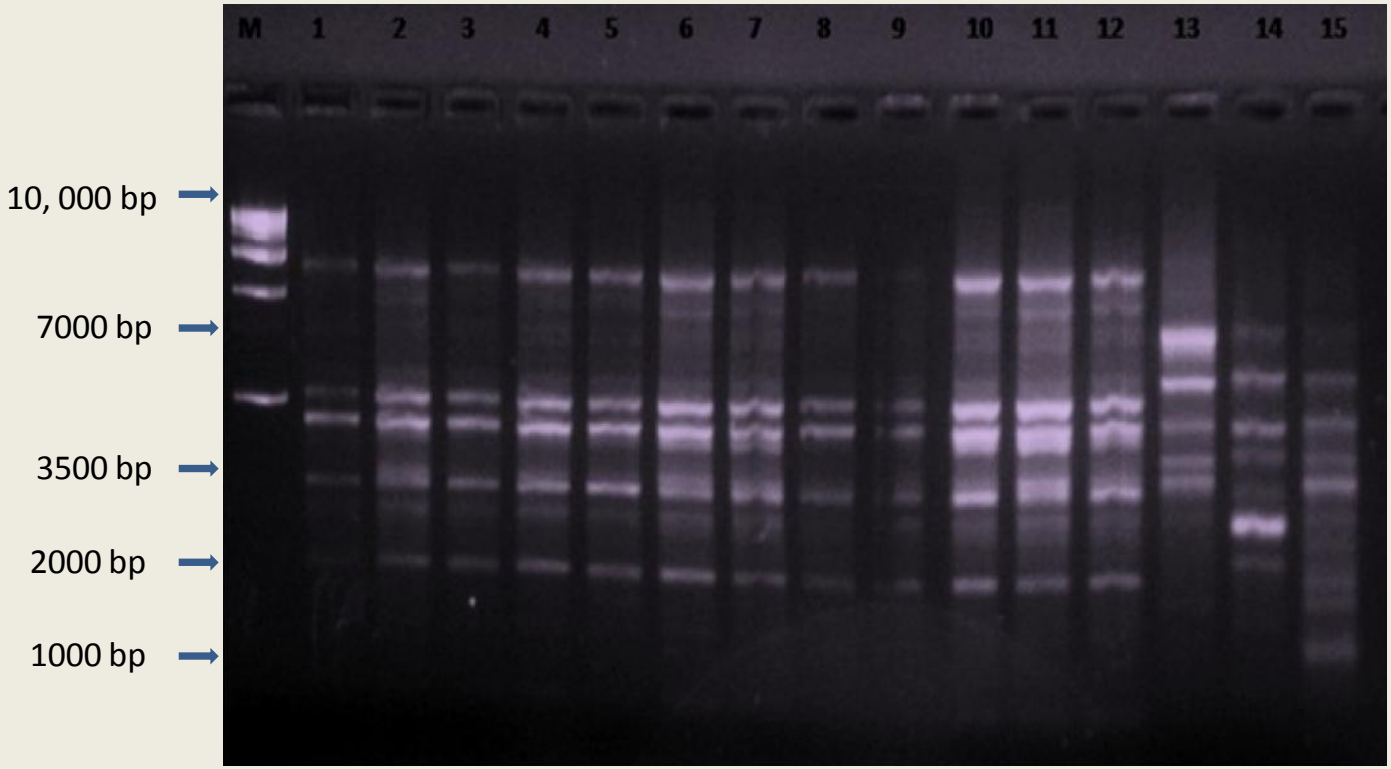
M- 1 kb ladder **1**- MV1 **2**- MV2 **3**- MV3 **4**- MV4 **5**- MV5
6- MV6 **7**- MV7 **8**- MV8 **9**- MV9 **10**- SV1
11- SV2 **12**- K8 **13**- *Coleus aromaticus* **14**- *C. aromaticus var.* variegated **15**- *C. vettiveroides*

Fig. 3 PCR amplification in primer OPG 10



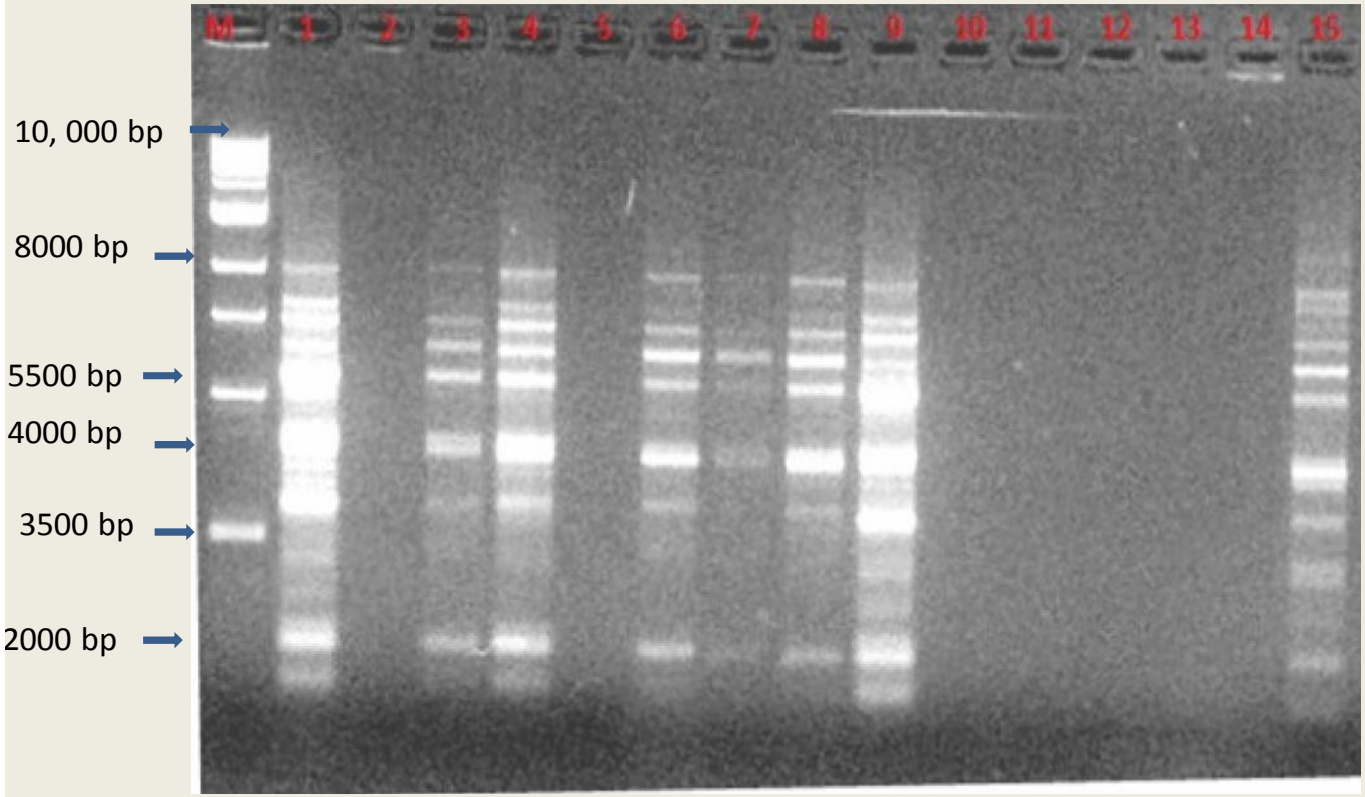
M- 1 kb ladder **1**- MV1 **2**- MV2 **3**- MV3 **4**- MV4 **5**- MV5
6- MV6 **7**- MV7 **8**- MV8 **9**- MV9 **10**- SV1
11- SV2 **12**- K8 **13**- *Coleus aromaticus* **14**- *C. aromaticus var.* variegated **15**- *C. vettiveroides*

Fig. 4 PCR amplification in primer OPI 18



M- 1 kb ladder **1**- MV1 **2**- MV2 **3**- MV3 **4**- MV4 **5**- MV5
6- MV6 **7**- MV7 **8**- MV8 **9**- MV9 **10**- SV1
11- SV2 **12**- K8 **13**- *Coleus aromaticus* **14**- *C. aromaticus var.* variegated **15**- *C. vettiveroides*

Fig. 1 PCR amplification in primer OPA 15



M- 1 kb ladder **1**- MV1 **2**- MV2 **3**- MV3 **4**- MV4 **5**- MV5
6- MV6 **7**- MV7 **8**- MV8 **9**- MV9 **10**- SV1
11- SV2 **12**- K8 **13**- *Coleus aromaticus* **14**- *C. aromaticus var.* variegated **15**- *C. vettiveroides*

Fig. 5 PCR amplification in primer OPN 20

The second group consisted of *C. aromatics* and mutants (S2, M3, M5, M8, M9 and K-8) of *C. forskohlii*. Among these, mutant M9 and accession K-8 were closely related at a linkage distance of 7.5. To these M8 was related with a linkage distance of 12.5. Further, M5 was related to M8 at a linkage distance of 21. Somaclone SV2 and the mutant M3 were related with a linkage distance of 24, share a linkage distance of 16.5 with M8 and *C. aromaticus* with a linkage distance of 28.5. Another major cluster consists of the species *C. vetteveroides*, which was related to the other group with a linkage distance of 38.5, indicating that it was totally different from other *Coleus* species and various mutants of *C. forskohlii* at the genetic level.

Principle Component Analysis as revealed by RAPD marker is presented in Table 23 (Fig. 7 and Fig. 8). PCA clearly revealed the relatedness between the mutants and also different species.

As shown in the dendrogram, PCA also revealed the species *Coleus vettiveroides* is totally different from rest of the entries in the study. It divided all the species and mutants under study into seven clusters. It clearly revealed that *C. aromaticus* and *C. aromaticus* var. *variegated* are closely related and thus grouped in single cluster. Among the mutants of *Coleus forskohlii*, mutant MV5 was totally different from others thus forming a separate cluster. As revealed by the dendrogram, PCA also clustered the mutant MV3 and somaclone SV2 in a single cluster. Accession K-8, mutants MV9 and MV8 made a different cluster. Further, mutants MV1, MV2, MV7 and somaclone SV1 formed a separate cluster. Mutants MV6 and MV4 were grouped in a single cluster. Finally species *Coleus vettiveroides* formed a separate group indicating, it is totally different from rest of the species and mutants of *C. forskohlii*.

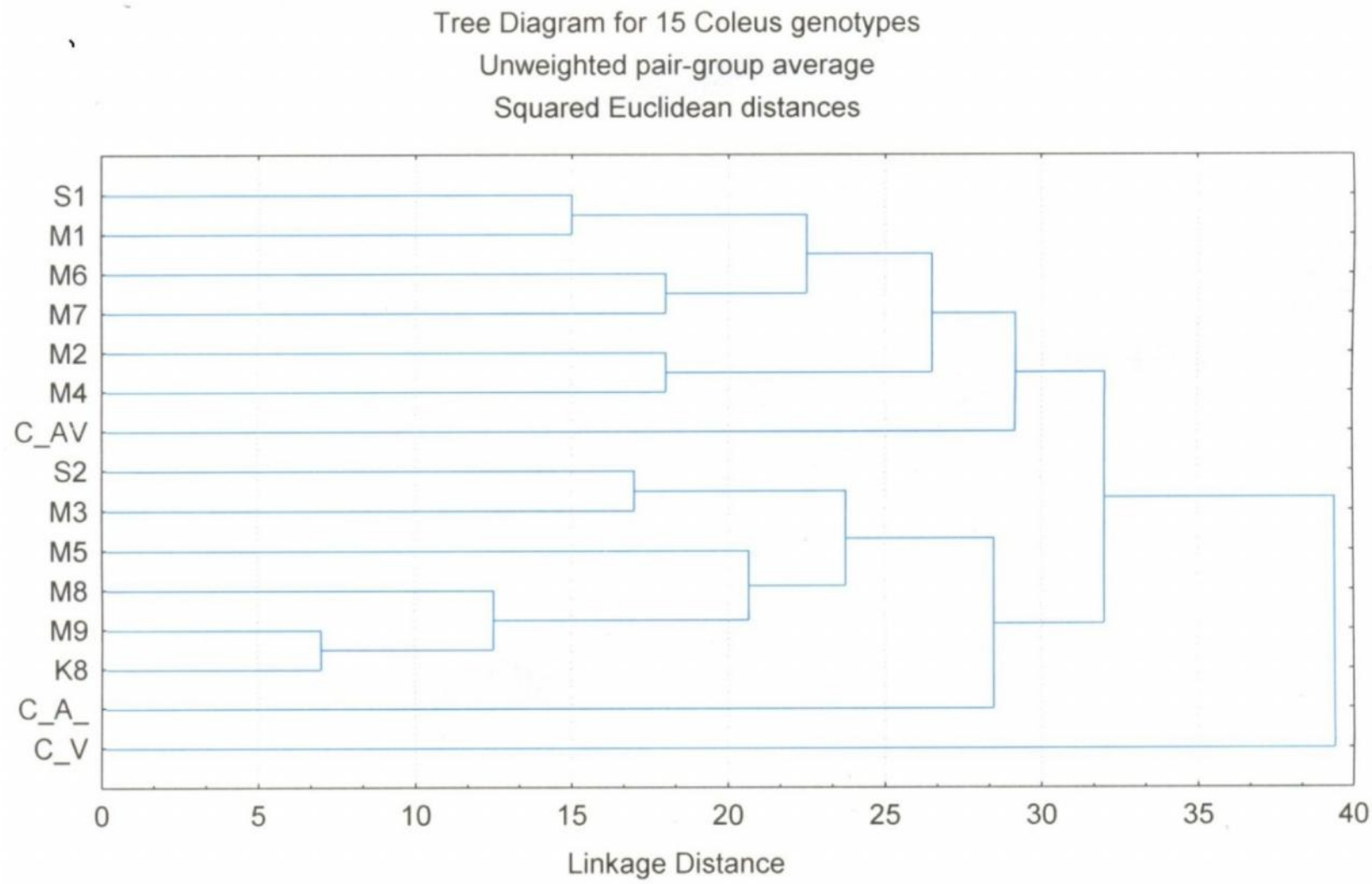


Fig. 6 Dendrogram of different Coleus species and mutants of Coleus forskohlii

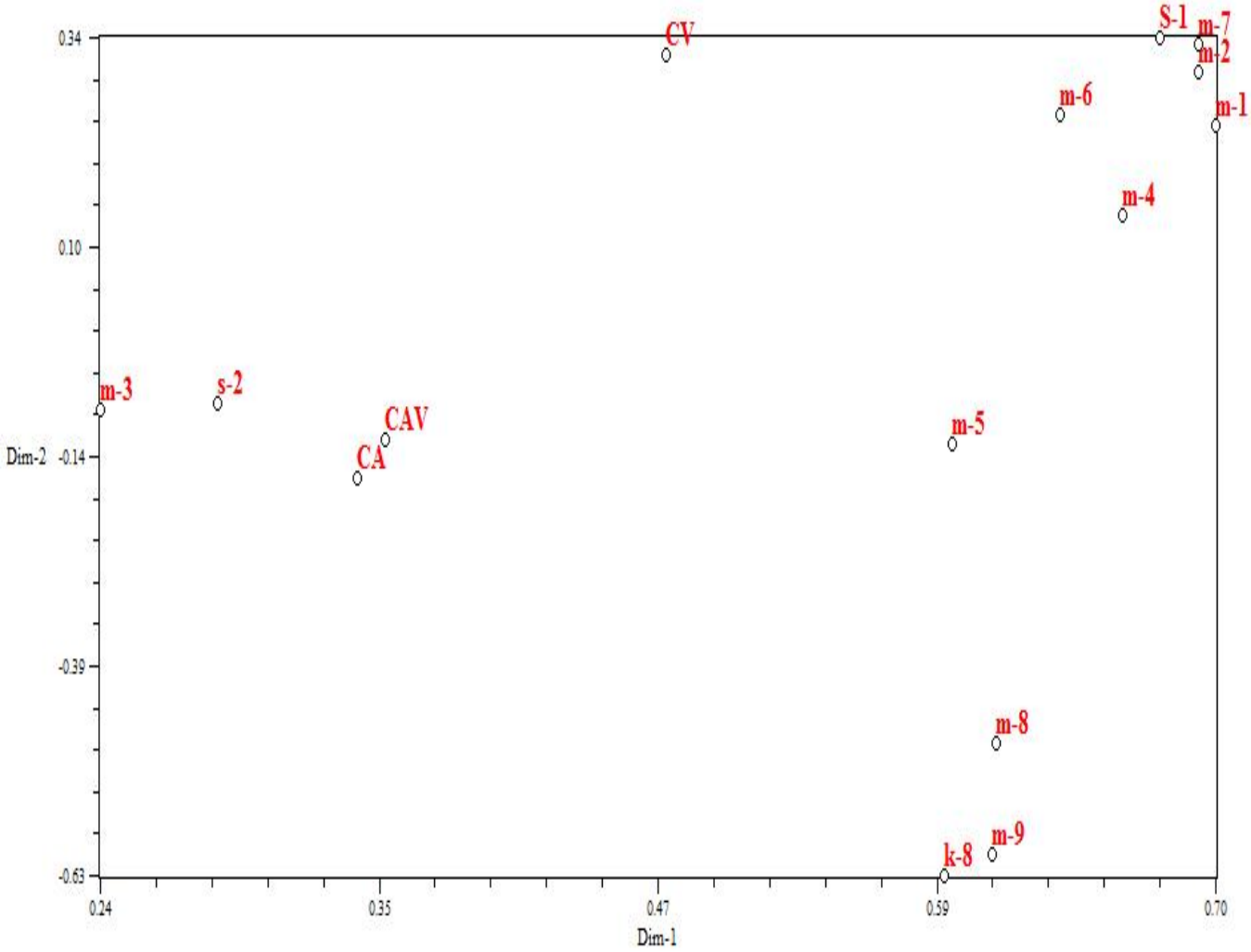


Fig. 7 Principle Component Analysis of different Coleus species and mutants of *Coleus forskohlii* (2D diagram)

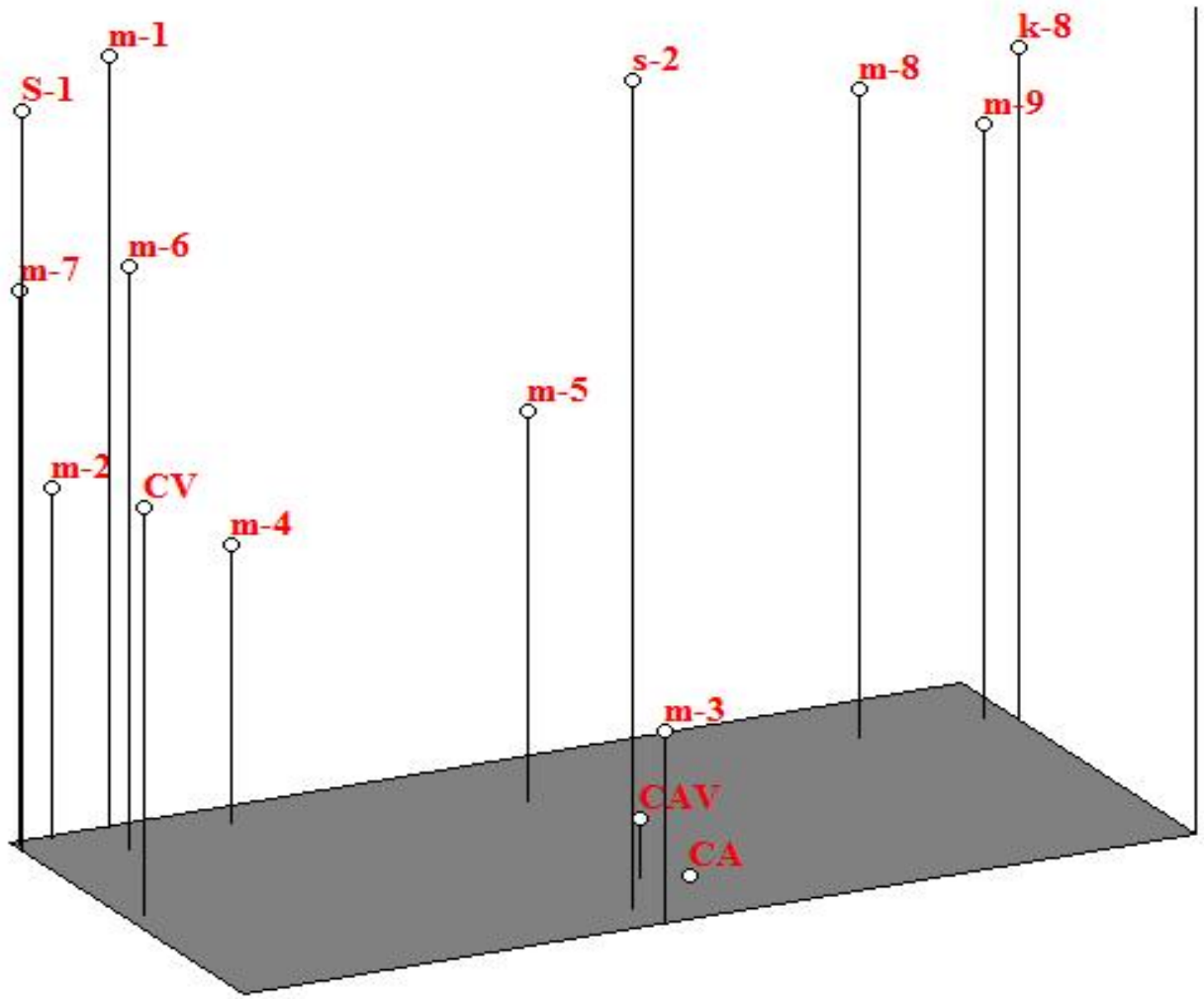


Fig. 8 Principle Component Analysis of different Coleus species and mutants of *Coleus forskohlii* (3D diagram)

Table 23. Principle Component Analysis as revealed by RAPD marker

	SV1	**SV2**	**MV1**	**MV2**	**MV3**	**MV4**	**MV5**	**MV6**	**MV7**	**MV8**	**MV9**	**K8**	***C.a.***	***C. a. v***	**C.v.**
SV1	1														
SV2	0.20	1													
MV1	0.65	0.29	1												
MV2	0.46	0.14	0.48	1											
MV3	0.06	0.05	0.08	0.16	1										
MV4	0.35	0.1	0.36	0.55	0.14	1									
MV5	0.21	0.1	0.29	0.36	0.12	0.39	1								
MV6	0.43	0.05	0.46	0.36	0.14	0.35	0.35	1							
MV7	0.55	0.10	0.45	0.52	0.12	0.41	0.35	0.5	1						
MV8	0.23	0.17	0.28	0.26	0.07	0.25	0.48	0.30	0.34	1					
MV9	0.28	0.14	0.31	0.21	0.17	0.31	0.26	0.26	0.24	0.53	1				
K8	0.24	0.18	0.3	0.22	0.12	0.3	0.32	0.21	0.19	0.55	0.74	1			
C. a.	0.12	0.09	0.12	0.21	0.11	0.22	0.16	0.09	0.15	0.15	0.25	0.16	1		
C. a. v.	0.1	0.04	0.18	0.24	0.08	0.21	0.20	0.14	0.13	0.17	0.20	0.15	0.26	1	
C. v.	0.3	0.08	0.26	0.32	0.06	0.34	0.20	0.34	0.34	0.14	0.12	0.15	0.17	0.07	1

C.a. - *Coleus aromaticus*

C. a. v. - *Coleus aromaticus var. variegated*

C. v. - *Coleus vettiveroides*

4.3.5 Distance Matrix

By using the RAPD data distance matrix was generated, which revealed genetic distance between various species and mutants of *C. forskohlii*. Highest distance of 50 was observed between somaclone SV1 and species *C. aromaticus* var. *variegated*. Thus indicating they are genetically distinct. But among the various mutants of *C. forskohlii* least genetic distance of 7 was observed between accession K-8 and MV9. Further, the study revealed mutants MV7 and MV5 which gave *on par* results for many of the morphological and yield characters are related with a genetic distance of 18.

4.3.6 Development of SCAR marker

The RAPD-4 produced band only in the variety Aisiri (mutant MV-7) and not in the check K-8 and these were eluted and purified using Gel Elution Kit. The purified DNA product was cloned into pTZ57R/T vector by T/A cloning kit (Cat#K1214, MBI, Fermentas). The transformed colonies were selected by LB medium with ampicillin 50mg/ml, X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and IPTG (isopropyl-β-D-thiogalactopyranoside) based on blue/white colony selection. The transformed white colonies were further confirmed by colony PCR with M13 universal primers. Further isolated plasmid DNA by the spin column based Plasmid DNA isolation kit from overnight culture prepared by the inoculation of single transformed white colony in LB broth containing antibiotic Ampicillin 50 mg/ml.

4.3.6.1 DNA Sequencing and sequence Analysis

The plasmids were sequenced by Chromous Biotech Pvt. Ltd. in both reverse and forward directions with M-13 universal primers using ABI3700 DNA analyzer (Applied Biosystems, USA). The contigs the forward and reverse sequence of M-13 primer using sequence

Table 24. Distance matrix as revealed by RAPD marker

	SV1	**SV2**	**MV1**	**MV2**	**MV3**	**MV4**	**MV5**	**MV6**	**MV7**	**MV8**	**MV9**	**K8**	***C.a***	***C.a.v.***	**C.v.**
SV1	0														
SV2	35	0													
MV1	15	24	0												
MV2	28	35	23	0											
MV3	42	17	33	30	0										
MV4	35	36	30	19	29	0									
MV5	40	27	29	26	22	23	0								
MV6	26	33	21	28	24	27	22	0							
MV7	20	33	23	20	28	25	24	18	0						
MV8	39	24	30	33	25	32	15	25	25	0					
MV9	38	29	31	40	24	31	28	30	34	15	0				
K8	37	22	28	35	21	28	21	29	33	12	7	0			
C. a.	49	28	42	37	23	34	31	39	37	32	29	30	0		
C. a. v.	50	39	43	40	32	41	34	42	46	37	38	37	31	0	
C. v.	35	32	34	31	29	28	31	25	27	36	41	34	34	49	0

C.a. - *Coleus aromaticus*

C. a. v. - *Coleus aromaticus var. variegated*

C. v. - *Coleus vettiveroides*

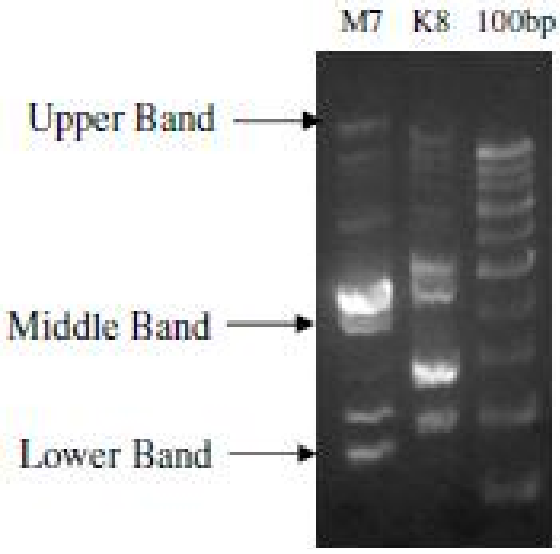


Fig . 9 RAPD profile of the two entries (K-8 and Aisiri) taken for development of SCAR marker

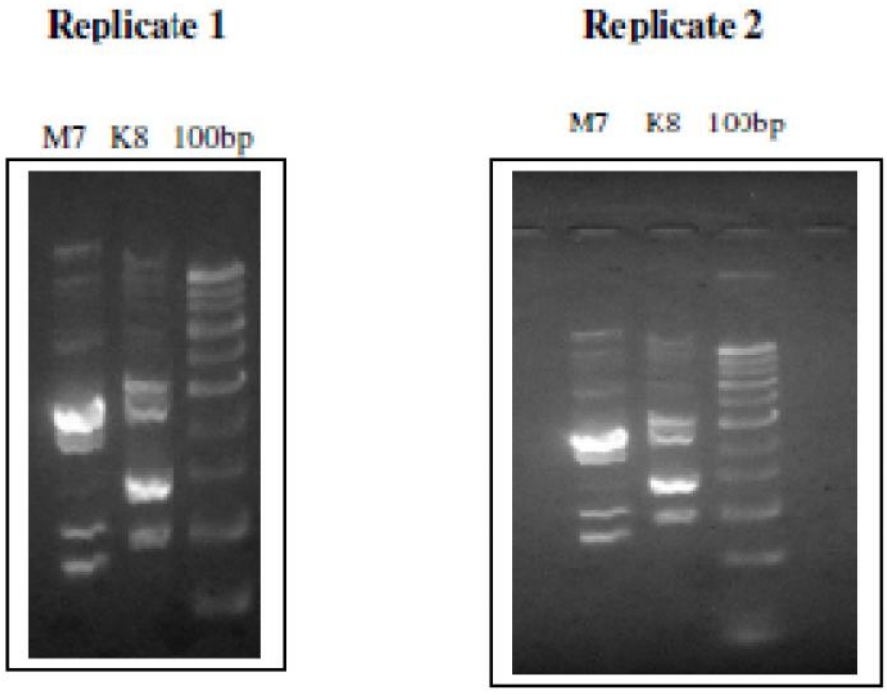


Fig. 10 Repeatability of the bands for the RAPD-4

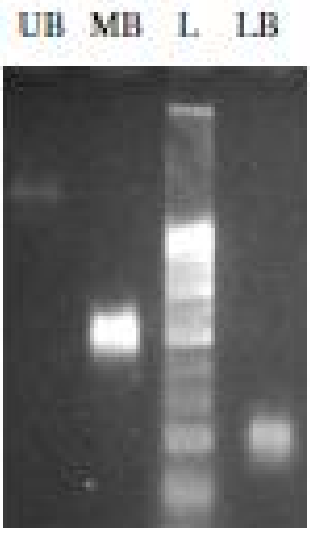


Fig. 11 Gel eluted bands in Aisiri (mutant MV7)

UB: Upper Band

MB: Middle Band

LB: Lower Band

L: 100 bp Ladder

>LB_M13R
GGCAAGTGGAAGTGCCCCCCAAGCTTAACGCGCAACACAATAGCCGGCTTCTCTTTCATCGCGTCTCCAAGGC
TTTGCGGACATCACTGACGGTCAGCCACTGGGAATCGTGATACAACCCCGCGGTCACCTTTGCTACTTGCGGC
GCATATGCCAACGAACTGGCCAGAATCTCCCGTGCTGGCCCCTGGGCCACGATTTCTCCTCGAGCCATCTCCA
CTACTCGATCCGCGCACAGTGCAGCGAACTCCACGTCGTGGGTGGCAATGACCACTGCGTGTCCGAGATCGCG
CAAGTGTTTCACCGACGCCGCCAGTGCCTCCTTGCCCTTATAATCCAGCCCGCGGGTTGGTTCATCACAGAGG
AGCACTCGGTTGCGCGTTAAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCCCGGGTACCGAGCTCGAAT
TCCACGGGCCCGTTTTG

Blue: Genomic DNA sequence
Black: Vector sequence

Fig. 12 Lower band clone sequence taken for SCAR marker development

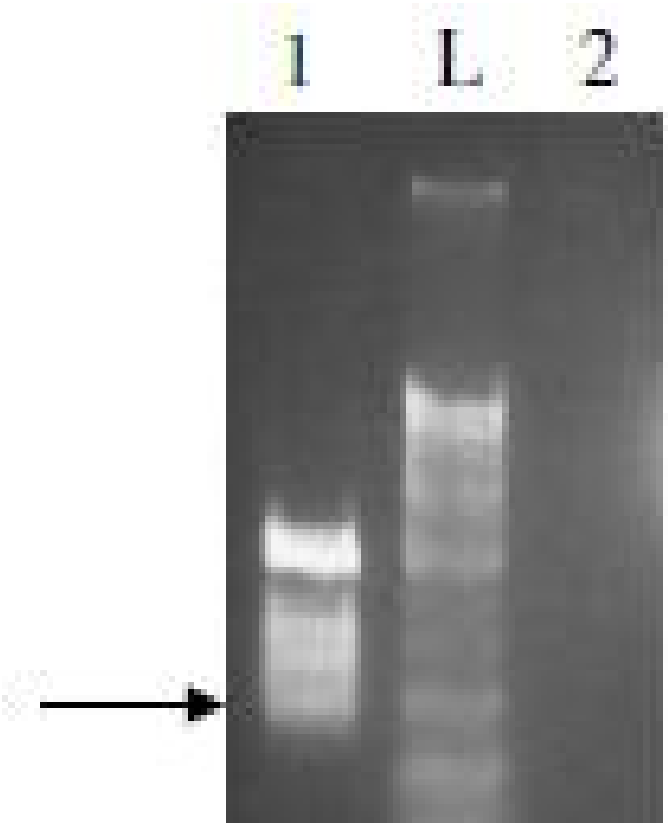


Fig. 13 PCR amplification by using SCAR marker amplification in the variety Aisiri (mutant MV-7)

1: Aisiri (mutant MV-7)

L: 100 bp DNA Ladder

2: K-8

Table 25. Forskolin content (%) in mutants of *Coleus forskohlii*

Mutants/ species	Forskolin content
MV1	0.54
MV2	0.44
MV3	0.46
MV4	0.63
MV5	0.75
MV6	0.71
MV7	0.74
MV8	0.64
MV9	0.69
SV1	0.72
SV2	0.76
K-8	0.52
Mean	0.66
F- Test	*
S.E.m±	0.01
C.D.	0.04

*Significant at 5% level

chromatograms with the help of Bio-edit programme. The contig sequences were subjected to BLAST (Altschul *et al.,* 1990) in the NCBI website.

4.3.6.2. Designing the SCAR primer

The sequence is there after used to design specific primer pairs of 16-24 bp which amplify single major bands of the size similar to that of cloned fragment. From the sequence of RAPD-4(AACGCGCAAC) designed forward and reverse primers.

4.3.6.3. Validation of SCAR primer

The SCAR from RAPD-4 (LBFP1: AACGCGCAACACAATAG LBRP1: AACGCGCAACCGAGTGC) amplifies a discriminating band in the variety Aisiri (mutant MV-7) but not in the check K-8. This is the first report on development of such varietal specific SCAR markers in *C. forskohlii.* Adinolfi *et al.* (2007) in three medicinal *Echinacea* species, Anuntalabhochai *et al.* (2007) and Dhanya *et al.* (2011) in turmeric, Devaiah *et al.* (2011) in *Ipomoea mauritiana* reported about the development of SCAR marker.

4.4 Estimation of forskolin content in mutants of *Coleus forskohlii*

Significant results were obtained for the forskolin content in the mutants of *Coleus forskohlii*. The data is presented in Table 23. Maximum forskolin content was recorded in the somaclone SV2 (0.76%) which was *on par* with mutant MV4 (0.63%), MV5 (0.75%), MV6 (0.71%), MV7 (0.74%), MV8 (0.64%), MV9 (0.69%) and with somaclone SV1 (0.72%). Least forskolin content was recorded in the mutant MV2, which had 0.41% of forskolin in its tuberous roots.

DISCUSSION

V. DISCUSSION

Presence of forskolin in *Coleus forskohlii* which is exclusive to this particular species and the recognition of its use as a plant of high medicinal and economic importance have rendered a high profile to this crop. Though interspecific variations has been recorded in this species, a systematic study has not been undertaken to know the extent of variation present among different *Coleus* species and accessions of *C. forskohlii* for morphological, growth, yield and forskolin content as parameters. Hence there is scope for evaluating the genetic relatedness among different *Coleus* species and accessions of *C. forskohlii*.

Evaluation of germplasm lines and different species along with a popular variety is the first step towards the selection of good genotypes. Besides yield and its components, quality components of produce are also equally important to make the produce more acceptable to the users. Genetic diversity assessment through molecular markers offer a unique advantage to study the diversity at DNA level and are able to detect even minute variations at molecular levels. In the present study an attempt has been made to evaluate various species of *Coleus* and mutants of *C. forskohlii* using RAPD markers. The results obtained have been discussed in this chapter under separate headings.

5.1 Morphological attributes of *Coleus* species and mutants of *C. forskohlii*

Bir and Saggoo (1982) reported that variability in basic chromosome number in the members of the species could be the result of aneuploidy, which ultimately leads to morphological variations. As the mutants used in the present study are evolved by treating with different dosage of gamma irradiation (gamma rays are known to cause chromosomal breakage), the exposure of cells to gamma rays could have

brought ionization. Mutation caused by these irradiations could have lead to variable chromosome number and thus leading to wide variations in morphological characters.

5.1.1 Plant height (cm)

Among different *Coleus* species and mutants of *C. forskohlii,* mutant MV5 recorded maximum plant height of 27.7 cm and 53.83 cm at 60 and 120 DAP respectively. But it did not maintain the same trend at other stages of growth. It could be due to differential growth rate existing in the species. At the time of harvest (180 DAP) *C. aromaticus var. variegated,* recorded significantly highest plant height of 74.86 cm. The reduction in plant height among the mutants might be due to physiological and chromosomal damage caused by irradiation and also non tuberous nature of the *C. aromaticus var. variegated*, which has supported good vegetative growth. Hegde and Krishnan (1994) recorded wide variation for morphological characters among various autotetraploid and diploid accessions of *C. forskohlii* and concluded that physiological changes in the autotetraploid contributed to low biological yield. The results are in line with the results obtained by Mune Gowda (2005) in various tuberous and non tuberous accessions of *Coleus forskohlii*.

5.1.2 Number of branches

Results are significant at all stages of growth. Mutant MV5 recorded maximum number of branches (14.36) at 60 days after planting. But it did not maintain the same trend, as mutant MV7 recorded maximum number of branches of 34.3 at 120 days after planting. But at 180 DAP (at the time of harvest) mutant MV5 again recorded maximum number of branches of 55. Superiority of mutants over other non tuberous species with respect to number of branches might be due to the effect of gamma irradiation on the plant cells, which may have caused an increased activity of enzymes involved in the

biosynthesis of the growth hormones like cytokinin, auxin etc. (Vagera *et al.,* 1976), encouraging the mutant plants to produce more number of branches and also in exhibiting more vigorous growth. Similar results were reported by Reddy (1952), Hegde and Krishnan (1998) in tuberous and non tuberous accessions of *Coleus forskohlii*. Kathiresan (2005) recorded similar results in mutants of *Coleus forskohlii*.

5.1.3 Plant spread (cm^2)

Among various species of *Coleus* and mutants of *C. forskohlii*, the *Coleus* species *C. aromaticus var. variegated* registered maximum plant spread of 3141.86 cm^2. The result obtained can be mainly attributed to the genetic factor of the plants or due to varietal trait as it is governed by the genetic makeup of the plant and also due to the interaction effect with the environment. Least plant spread was recorded in the plants of somaclone SV2 (2539.23 cm^2). The reduction in plant spread may be due to the effect of gamma radiation on the mutants, which may has caused inhibition of translocation of assimilates or impairment of water uptake. Similar observations were recorded by Patil *et al.*, (2001) and by Manjunatha Swamy (2008) in *Coleus*.

5.1.4 Number of leaves per plant

Mutant MV3 recorded maximum number of leaves per plant of 909. The results could be due to the fact that it had significantly more number of branches as compared to other mutants. Hence, it has got maximum number of leaves due to the production of more of growth promoting hormones in the plant cells. Least number of leaves was recorded in the species *C. vettiveroides* (175 leaves/ plant). This may be due to the fact that these non tuberous species mature early as compared to the mutants of *Coleus forskohlii* as evident from observations for number of leaves per plant taken at 180 days after planting, by which time they would have shed their leaves. These results

are in conformity with the results of Hegde and Krishnan (1998) in a study of variability in different tuberous and non tuberous accessions of *C. forskohlii*.

5.1.5 Leaf area per plant (cm^2)

The leaf area was calculated at the time of harvest. Among various *Coleus* species and mutants of *C. forskohlii* maximum leaf area was found to be in the mutant MV4 (11637.68 cm^2). As this particular mutant had *on par* results for number of branches and also for number of leaves in the plant, it has put forth good vegetative growth. Genetic makeup of the mutant which has promoted cell elongation could have resulted in higher leaf area as compared to other mutants and species under study. Lowest leaf area was recorded in *C. vettiveroides* (7524.12 cm^2). This variation in leaf area could be largely attributable to varietal differences as this particular species had recorded least number of leaves, thus contributing to poor development of the plant. Hegde and Krishnan (1994) and Patil *et al.* (2001) reported similar results in Coleus. Virk *et al.* (1988) made similar observations in Perwinkle.

5.2 Yield attributes of *Coleus* species and mutants of *C. forskohlii*

5.2.1 Fresh and dry shoot weight (gm)

Coleus aromaticus recorded highest fresh and dry shoot weight of 452.86 gm and 38.23 gm respectively. This result obtained could be due to the fact that as *Coleus aromaticus* is a non tuberous species of *Coleus,* hence it was able to use all available nutrients for a luxurious shoot growth. While, the least fresh and dry shoot weight was recorded in mutant MV3 (293.73 gm and 24.33 gm respectively), Which is due to the fact that it had recorded the least plant height and also number of leaves, leading to poor vegetative growth, producing less fresh and dry

weight of shoot. Similar results were reported in *C. aromaticus* by Morton (1992) and Reddy (1952).

5.2.2 Fresh and dry weight of root (gm)

Among the different species of *Coleus* and mutants of *C. forskohlii*, mutant MV5 recorded highest fresh and dry weight of root (tuberous root) of 764.69 gm and 189.7 gm respectively. This result can be attributed to the superiority of mutant over others. This might be the impact of irradiation which may have caused alteration in physiology of the plant, leading to increased source-sink activity. Lowest fresh and dry weight of root was recorded in *C. aromaticus var. variegated* (244.4 gm and 83.3 gm respectively), which is a non tuberous species, where the root weight was less as compared to the mutants of *Coleus forskohlii*. Similar results were obtained by Mune Gowda (2005) and Manjunatha Swamy (2008) in *Coleus.* Vishwakarma *et al*. (1988) recorded wide variation in root weight of *C. forskohlii* accessions.

5.2.3 Number of roots per plant

Maximum number of roots per plant was recorded in the mutant of *C. forskohlii* MV5 (18.73 roots per plant). Kathiresan (2005) reported an increase in root length in plants which were irradiated with different intensities of gamma radiations. This result can be attributed to the effect of mutagens on the physiological characters which has made the plants to put forth maximum number of roots coupled with maximum root weight. Least number of roots was recorded in *C. aromaticus var. variegated* (9.23 roots per plant). This result can be correlated with the non tuberous nature of the species, which has resulted in decreased number of roots per plant. Nanaiah (1993) and Manjunatha Swamy (2008) observed parity for various characters in the accessions of *C. forskohlii*.

5.2.4 Root length per plant (cm)

Among different mutants of *C. forskohlii* and different species of *Coleus* maximum root length of 34 cm was recorded in the mutant MV5 after 180 days of planting. As the mutant MV5 has recorded maximum root weight and also maximum number of roots due to the effect of mutagens, source-sink activity was increased leading to increased root length per plant. Least root length of 22.4 cm was recorded in the plants of the species *C. vettiveroides*. The result could be due to the fact that the species *C. vettiveroides* was inferior for many of the shoot parameters, resulting in less accumulation of reserve food material in the roots, hence, the plants were inferior for many of the root characters. Similar results were obtained by Manjunatha Swamy (2008) in *Coleus forskohlii* and Virk *et al*. (1988) in *Periwinkle*.

5.2.5 Root diameter (cm)

Among the mutant and species under the study, mutant MV5 recorded maximum root diameter of 3.08 cm. this result might be due to the effect of gamma radiation on the plant cells, which may have caused an increased activity of enzymes involved in the biosynthesis of the growth hormones like cytokinin, auxin etc. (Vagera *et al.,* 1976), causing elongation of cells in plants leading to maximum root diameter in this particular mutant. Incidentally, this particular mutant had recorded maximum root weight which may be the other reason for higher root diameter. Further, the variation among the mutant may be due to inherent difference and potentiality of mutant to produce maximum tuberous root diameter. Least root diameter of 1.35 cm was recorded in *C. aromaticus var. variegated*. This result can be correlated with the non tuberous nature of the species, which has resulted in decreased root diameter. These results are in line with the results obtained by Manjunatha Swamy (2008) and Hegde *et al*. (2005) in *Coleus*.

5.2.6 Total biomass (gm)

Mutant MV5 recorded maximum total bio-mass (220.87 gm) at harvest. The result can be attributed to the fact that mutant MV5 had recorded maximum root weight and had also shown average shoot weight, thus resulting in maximum total biomass. Least biomass was recorded in the species *Coleus aromaticus* var. variegated. This result could be attributed to the fact that *Coleus aromaticus* var. variegated is a non tuberous species and thus had recorded least dry root weight, thus resulting in least total biomass. Similar results were reported by Mune Gowda (2005) in *Coleus* species.

5.2.7 Root to shoot ratio

Among different species of *Coleus* and mutants of *C. forskohlii* maximum root to shoot ratio of 6.2 was recorded in the somaclone SV1. As the somaclone SV1 had recorded *on par* results for both shoot and root parameters, resulting in maximum root to shoot ratio. Least root to shoot ratio of 2.6 was recorded in the accession K-8. The result could be attributed the fact that the accession K-8 had recorded inferior results for both shoot and root characters as compared to the mutants resulting in least root to shoot ratio. Mune Gowda (2005) obtained similar results in *Coleus* species.

5.2.8 Dry matter content (%)

Maximum dry matter content of 34.1% was recorded in the *Coleus* species *C. aromaticus var. variegated*. The result obtained is due to the fact that *C. aromaticus var. variegated* a non tuberous species of *Coleus* recorded less difference between the fresh and dry weight roots, thus resulting in highest dry matter accumulation. Least dry matter content was recorded in the mutant MV6 (21.3%). This may be due to the fact that this particular mutant had recorded more difference between the

fresh and dry weight of root, resulting in least dry matter content. Vasudevan and Jose (1988) reported inferiority of *Coleus parviflorus* mutants over untreated plants with respect to many of the yield characters. Similar results were obtained by Mune Gowda (2005) in different tuberous and non tuberous accessions of *Coleus forskohlii*.

5.2.9 Harvest index (%)

Harvest index is one of the important characters deserving improvement through breeding programme. Among the different *Coleus* species and mutants of *C. forskohlii*, the mutant MV5 recorded a maximum harvest index of 0.857%. As mutant MV5 had recorded maximum weight of root and also shoot, harvest index was found to be maximum in that. Least harvest index was recorded in *Coleus aromaticus* (0.716%). Least dry root weight was recorded in species *Coleus aromaticus,* as it's a non tuberous species, accounting to lower harvest index in plants. Hegde (1992) observed similar results for autotetraploids of *C. forskohlii*.

5.3 Genetic diversity as revealed by RAPD markers

Genus *Coleus* contains more than 150 species, which include both annuals and perennials. Most of the aromatic, essential oil and drug yielding species and accessions are included under this genus (Bailey 1942 and Vishwakarma *et al.*, 1988). Genetic variation is a prerequisite for any successful crop improvement programme. Morphological characters and forskolin content have been used over the years for improvement of *C. forskohlii* accessions. However, these methods have certain limitations as they could be influenced by environmental factors and developmental stages. Cultivar identification can become very difficult by relying upon morphological or botanical characters alone.

Thus, DNA marker technology appears to be useful for assessing the genetic relatedness and to distinguish the species and cultivars. RAPD could be effectively used for genetic diversity analysis as it is reliable, rapid and superior to those based on pedigree information (Padmesh *et al.*, 1999). Further, DNA polymorphism generated by RAPD technique require a minute amount of template DNA, it is simple and is capable of detecting a high level of genetic variation. Hence, RAPD approach is best suited for fingerprinting and assessing genetic relationship with greater accuracy (Hadin *et al.*, 2008). Shasany *et al.* (2002) reported that RAPD analysis was a better indicator of descendancy an origin among the germplasm accessions.

The present study was carried out using three *Coleus* species and eleven mutants and a variety of *C. forskohlii* for RAPD analysis. The results obtained on polymorphic amplification products, discriminating power of primers and grouping of different accessions and species are discussed below.

5.3.1 Extraction and amplification of genomic DNA of *Coleus* species and different mutants of *C. forskohlii*

Wide variation was observed for DNA recovery among three species of *Coleus*, eleven mutants and a variety of *C. forskohlii*, which ranged from 384.8 ng/ µl to 2611.3 ng/ µl of leaf tissue (Table 17). This variation could be due to the morphological variations and because of differences in amounts of interfering co extractives. Further, the DNA to protein ratio varied considerably (1.23 to 1.85) among the *Coleus* species and mutants of *C. forskohlii*, which is due to the differences in the DNA content. A study conducted by Bahl and Tyagi (1989) for diploids and autotetraploids of *Coleus forskohlii* revealed that autotetraploids showed double the amount of DNA than diploid progenitors, suggesting

chromosomal aberrations as a cause for such difference in the experimental material used in the present study.

5.3.2 Dendrogram and Principle Component Analysis in amplified fragments of *Coleus* species and mutants of *C. forskohlii*

In the present study three *Coleus* species, eleven mutants and a variety of *C. forskohlii* were grouped using data from five oligonucleotide primers. RAPD profiles were generated that allowed *Coleus* species and mutants of *C. forskohlii* to be distinguished from each other.

The cluster analysis (Dendrogram) grouped different *Coleus* species and mutants of *C. forskohlii* used in the study into two major clusters. Cluster 1 consisted of a non tuberous species *C. vetteveroides*, indicating that it was totally different (both genetically and morphologically) from other *Coleus* species and mutants of *C. forskohlii*. Another cluster (Cluster 2) comprised of two *Coleus* species (*C. aromatics* and *C. aromaticus var. variegated*) and mutants of *C. forskohlii* (MV1, MV2, MV3, MV4, MV5, MV6, MV7, MV8, MV9, SV1, SV2 and K-8). The results can be attributed to the factors like geographical distribution, morphological characters, ploidy level, monoclonal material etc. As the mutants used in the study were regenerated from tissue culture difference in banding pattern was observed. This result is supported by the study carried out by Ahlawat *et al.* (2010) on *Acorus calamus,* in which they clustered the accessions based on their ploidy level, β-asarone content and geographical locations. Shasany *et al.* (2000) clustered the *Allium sativum* accessions based on morphological characters. Sultan *et al.* (2008) clustered *Podophyllum hexandrum* accessions based on their geographical distribution. Reddy and Nazif (2005) observed difference between the parent plant and *in vitro* regenerated plants in *Ocimum americanum*.

Second cluster is further divided into two subgroups. Sub group 1, which included mutants like SV1, MV1, MV6, MV7, MV2, MV4 and one *Coleus* species, that is *C. aromaticus var. variegated*. Among these SV1 and MV1 were closely related, MV6 and MV7 were related to each other and MV2 and MV4 were related closely to each other. A close linkage was also seen between second cluster and the species *C. aromaticus* var. *variegated*.

The second sub group in the cluster consisted *Coleus* species like *C. aromatics* and mutants (SV2, MV3, MV5, MV8, MV9 and K-8) of *C. forskohlii.* Among these MV9 and K-8 were closely related. While, MV8 and then MV5 linked this group SV2 and MV3. Thus, RAPD marker revealed that although the mutants of *C. forskohlii* are morphologically alike, they are genetically different, but they are linked. As these mutants are evolved from same mother plant, they had a narrow genetic base and this was successfully revealed by the RAPD markers. The study also revealed the genetic relatedness among the different species of Coleus. Results are in line with those obtained by Kavitha *et al.* (2010) in *Coleus forskohlii*, in which its reported about clustering of accessions based on the banding pattern that they had observed for different tuberous and non tuberous accessions of *C. forskohlii*. Similar results were also reported by Vieira *et al.* (2001) in *Ocimum gratissimum*, Shasany *et al.* (1998) in *Veteveria zizanioides*, Kiani *et al.* (2010) in *Rosa damascena* and Zou *et al.* (2011) in *Curcuma* spp.

The pooled RAPD binary data was utilized for cluster analysis and Principle Component Analysis (PCA) using the statistical programme called STATISTICA for the 15 Coleus genotypes. The genetic dissimilarity value in the distance matrix ranged from 0 to 50, suggesting a narrow genetic base within the mutants and species of Coleus. The highest genetic distance of 50 was observed between SV1 and the species Coleus

aromaticus var. variegated, indicating that these are genetically more distinct. The primary reason for narrow genetic base may be the parent material from which they have been evolved.

5.3.3 Development of SCAR marker

In order to develop SCAR markers in *C. forskohlii* mutants, putative markers identified by arbitrary marker analysis (RAPD) was used. The arbitrary marker technique is sensitive to changes in the reaction conditions. In order to bridge the gap between the ability to obtain linked markers to a gene of interest SCAR marker technique was developed and applied. The SCARs are PCR-based markers that represent genomic DNA fragments at genetically defined loci that are identified by PCR amplification using sequence specific oligonucleotide primers. Out of the twelve putative RAPD markers used, RAPD-4 which has shown very clear, consistent co segregation with the variety Aisiri (mutant MV-7) and not in the check K-8 was selected for converting it to SCAR marker. RAPD-4 primer linked to the variety Aisiri (mutant MV-7) was developed in to SCAR based on isolated, cloned and sequenced female specific fragments.

SCAR from RAPD-4 (LBFP1: AACGCGCAACACAATAG LBRP1: AACGCGCAACCGAGTGC) amplifies a band at around 200 bp in the variety (mutant MV-7) but not in check K-8. In the present study RAPD markers proved to be a good tool for diversity analysis in various Coleus species and also in different mutants of *C. forskohlii* at the same time, they were a useful starting point for the development of SCAR primers.

There are several reports of development of SCAR markers from the RAPD primers in various other crops. In papaya, Urasaki *et al.* (2002) developed a 450 bp RAPD marker fragment in to the male and hermaphrodite specific sex SCAR, named as PSDM (Papaya Sex

Determination Marker). Mandolino *et al.* (1999) identified 400-bp RAPD marker associated with the male sex phenotype in 14 dioecious cultivars and accessions of hemp (*Cannabis sativa* L.). The primer OPA8 generated most of polymorphic among all the individual plants tested, and 1 of which, named $OPA8_{400}$, present in all male plants and absent in female plants. A SCAR marker 390 bp in length and male-specific was then developed. Adinolfi *et al.* (2007) converted a RAPD marker of 750 bp for *E. purpurea* into a SCAR marker. SCAR-PCR revealed the amplicon of 330 bp only in *E. purpurea* and not in the other two species (*E. pallida* and *angustifolia*).

Dhanya *et al.* (2011) developed SCAR markers to detect adulterants in traded turmeric powder. RAPD primers OPA 01 and OPE 18 were identified as *C. zedoaria*/*C. malabarica* specific by comparative RAPD analysis. Two pairs of SCAR primers ('Cur 01' and 'Cur 02') were designed from the RAPD markers. Anuntalabhochai *et* al. (2007) developed a SCAR marker for the Curcuma variety Patumma. Eleven decamer primers were used to examine 20 Curcuma varieties from which banding patterns of interest were selected for conversion to the more reproducible SCAR markers. A SCAR marker amplified a region 600 bp in length which was conserved in all Patumma varieties and hybrids was identified and converted into a SCAR marker.

Devaiah *et al.* (2011) identified a putative 600-bp polymorphic sequence, specific to *I. mauritiana* using RAPD technique. Furthermore, sequence SCAR primers (IM1F and IM1R) were designed from the unique RAPD amplicon. The SCAR primers produced a specific 323-bp amplicon in authentic *I. mauritiana* and not in the allied species. Xiao *et al.* (2011) developed a SCAR marker to distinguish genuine herbal medicine of *Prunella asiatica*. Two putative markers, which are specific for *P. asiatica* were identified by RAPD.

5.4 Forskolin content in mutants of *C. forskohlii*

In the present study among the various mutants of *C. forskohlii* highest forskolin was estimated in the somaclone SV2 (0.76%), least forskolin content of 0.44% was recorded in the mutant MV2.

Even though somaclone SV2 recorded *on par* results for various morphological and also for the yield characters yielded maximum forskolin content in their tuberous roots. Thus, it revealed that forskolin content in the roots are independent of other characters like dry and fresh weight of root. Similar results were obtained by Kathiresan (2005) where he has reported, that variations observed in root characters like root length, root girth, root width and number of roots had no correlation with forskolin content. Srivastava *et al.* (1986) has reported that tuberous types had more forskolin content than the non- tuberous types. A major reason for variation for forskolin content may be the source from which these mutants have been collected. Vishwakarma *et al.* (1988) observed the variation in forskolin content in the tuberous roots of 38 genotypes collected from diverse sources.

SUMMARY

VI. SUMMARY

In the present study, entitled "Morphological and molecular characterization of selected *Coleus* species and mutants of *Coleus forskohlii* Briq." three *Coleus* species, eleven mutants and a variety of *C. forskohlii* were selected for evaluation and genetic variability studies. The field and laboratory investigations were carried out during 2010-2011 at Department of Horticulture and laboratory investigations pertaining to diversity analysis using molecular markers were carried out in Department of Biotechnology, UAS, GKVK, Bangalore.

With respect to the morphological characterization observations were recorded on seventeen morphological and yield characters. Morphological characters like plant height, number of branches, number of leaves, plant spread, leaf area and yield characters like fresh shoot weight, dry shoot weight, fresh root weight, dry root weight, number of roots per plant, root length, root diameter, root to shoot ratio, total biomass, dry matter content, harvest index, forskolin content were calculated. The analysis of variance revealed significant differences for all the characters.

Diversity analysis was carried out by using RAPD marker. By using the RAPD data a dendrogram (tree diagram) was constructed to analyze linkage distances between the mutants and species. Further, Principle Component Analysis and Distance matrix was calculated to check the genetic distance among different species and mutants of *Coleus forskohlii*.

Salient features of the present study are summarized below under the following headings.

6.1 Growth and yield attributes of different *Coleus* species and mutants of *C. forskohlii*

- Mutant MV5 was superior with respect to many of the morphological characters like, plant height (60 and 120 days after planting), number of branches (180 days after planting) and yield characters like fresh and dry root weight, number of roots per plant, root length and total biomass, harvest index. Further, it recorded *on par* results the characters like plant height (180 days after planting), plant spread, root to shoot ratio, root diameter and forskolin content.
- Mutant MV7 recorded superior results for number of branches (60 days and 120 days after planting) and *on par* results for the characters like plant height (180 days after planting), plant height, fresh and dry weight of root, number of roots per plant, root diameter, harvest index and forskolin content. Thus, suggesting this as the second best mutant under study. But in the stability studies conducted earlier revealed mutant MV7 as the better performer with respect to both yield and morphological characters [Manjunatha Swamy (2008)].
- Although somaclone SV2 recorded highest forskolin content in its tuberous roots, it was inferior for many of the characters like plant height (60 days after planting),number of branches (60 days after planting), total biomass. Thus, indicating that forskolin content is not related to the superiority of the morphological characters.
- Somaclone SV1 recorded superior results for root to shoot ratio, *on par* results for the characters like plant height (120 and 180 days after planting), harvest index and forskolin content in the roots. But it exhibited inferior results for plant spread.

- Among the different species under the study, *Coleus vettiveroides* was distinct and it was totally different from the mutants under the study, for both morphological and yield characters.
- *Coleus aromaticus var. variegated* recorded maximum recorded maximum plant height (180 days after planting), maximum plant spread and maximum dry matter content.
- *Coleus aromaticus* recorded maximum fresh and dry shoot weight.

6.2 Genetic relatedness as revealed by RAPD markers

- Dendrogram based on the banding patterns of the different *Coleus* species and the mutants of *C. forskohlii* revealed distinct level of genetic diversity existing among them.
- Dendrogram analysis also revealed the distinctive nature of the mutants MV5 and MV7, which were reported to produce highest fresh and dry tuber yield and also maximum forskolin content in their tuberous roots.
- Principle Component Analysis and Distance Matrix which were calculated by using RAPD data, grouped the mutant MV5 in a separate cluster. Thus it can be concluded that mutant MV5 is the best performer as compared to other mutants under study.
- It revealed that the superior mutants MV5 and MV7 are genetically closely related.
- It also separated the species *Coleus vettiveroides* from the other species and mutants under the study.
- Further, it can be concluded that even though, mutants and a variety under the present study have the morphological resemblance to each other but they are genetically distinct and unique.

- Thus, utility of RAPD markers in assessment of genetic relatedness in *Coleus* has been successfully demonstrated.
- It was very clear from the study that, the mutants which are similar in morphology may not be genetically similar always.
- From RAPD-4 [specific to the variety Aisiri (mutant MV-7)]was converted to SCAR marker through cloning. The primer developed SCAR marker showed RAPD-4 F-R amplification with the plants of the variety Aisiri (mutant MV-7) but not in the individuals of check K-8. It showed clear co segregation with the mutant MV-7. It is the first report of development of SCAR marker for varietal identification in *C. forskohlii.*

6.3 Future line of work

- In the present study, three *Coleus* species were evaluated for morphological, yield attributes and also for genetic diversity. Still there is scope for evaluating the remaining species for morphological, yield, presence of essential oils and also for the presence of major chemical compounds for their commercial utilization.
- As the IPR (Intellectual Property Rights) is in force, characterization of these mutants by using advanced markers like SSR (Single Sequence Repeats) markers or SNP (Single Nucleotide Polymorphism) markers may be useful.
- Molecular evaluation of mutants for high forskolin content and also for resistance to major problems like wilt and nematode is a need.

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VII. REFERENCES

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*Originals not seen

APPENDIX

APPENDIX-I

Composition of reagents used in DNA isolation

1. Extraction buffer	18.6g EDTA (20 mM) and 3.03g Tris HCL (100mM). Dissolve both these in small quantities of water, mix and adjust pH to 8.0, to this add 1.5 M NaCl (29.22g) and 2 % w/w CTAB (Cetyl Trimethyl Ammonium Bromide 10gm) by heating to 60^0 C. Make up the volume to 300 ml and stored at 37^0 C. Add 0.2% of β-mercapto ethanol (0.5g) just before use.
2. TAE buffer (50X)	For 100ml buffer = 1M Tris buffer-24.12ml, Glacial Acetic acid 5.71 ml, 0.5M EDTA (pH 8.0) 10 ml. make up the volume 100 ml in distilled water and sterilize. Prepare working solution 1X.
3. TE buffer	For 100 ml TE= 1M Tris 0.2 ml and 0.5M EDTA 1ml. Dissolve separately, mix and make up the volume to 100 ml, adjust the pH to 8.0 and sterilize.
4. Chloroform - isoamyl alcohol	24:1 v/v
5. NaCl	5M 29.22g in 100ml and sterilize)
6. Absolute alcohol	99.9% Ethanol; stored at -20^0 C
7. RNAase	10mg/ml; dissolve RNAase in 10mM Tris HCL + 15mM NaCl, pH 5.0, boil for 5 minute and cool to room temperature
8. PVP	Pinch
9. Ethidium bromide	10 mg/ml

APPENDIX-II

Abbreviations

µl	Micro Litre
ML	Milli Litre
ng	Nano Gram
µg	Micro Gram
mg	Milli Gram
kg	Kilo Gram
cm	Centi Meter
M	Molar
µM	Micro Molar
pM	Pico Molar
mM	Milli Molar
g	Gram
OD	Observance Difference
rpm	Rotation Per Minute
CTAB	Cetyl Trimethyl Ammonium Bromide
PCA	Principle Component Analysis
PCR	Polymerase Chain Reaction
RAPD	Randomly Amplified Polymorphic DNA
SSR	Simple Sequence Repeats
SNP	Single Nucleotide Polymorphism