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ASSESSING PHENOTYPIC, BIOCHEMICAL, AND MOLECULAR DIVERSITY IN *PENTAS LANCEOLATA* (FORSSK) DEFLERS

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Pentas lanceolata, the Egyptian star cluster belongs to Rubiaceae, include many cultivars with variable flower colour. An initial attempt was made to differentiate the cultivars using morphological characters of leaves and flowers. The cultivars show more or less uniformity in these characters except size of the leaves and flower colour. As the second phase of the study, molecular analysis was carried to differentiate the cultivars. Among DNA based molecular markers, Random Amplified Polymorphic DNAs (RAPDs) provide an excellent tool for studying genetic relationships. Genetic diversity among the cultivars of *Pentas* was attempted by DNA fingerprinting using 12 RAPD primers. Good quality DNA was exracted from young tender leaves of *Pentas lanceolata* cultivars by CTAB method with minor modification like detergent concentration (2% PVP and 3% CTAB). The quality of DNA was checked by Agarose Gel Elctrophoresis. The isolated DNA was quantified using UV spectrophotometer at 260 nm and 280 nm and the quantified DNA was subjected to PCR amplification. 81 unambiguous, readable and reproducible RAPD bands were produced using 12 RAPD primers. The bands obtained were having sizes raning from 250 to 5000 bp. Of the 81 badns, 31 (38.2%) were polymorphic and shared between five individuals, while 50 (61.7%) were monomorphic in the five cultivars. Thus the present study showed low to moderate genetic diversity among the cultivars. As the last phase of the study pigment concentrations, protein and phenol content were also showed variations. Red cultivar showed higher content in protein, phenols and chlorophyll pigments.

Keywords : Cultivars; DNA, Genetic divesity; Morphological; PCR; *Pentas lanceolata; RAPD.*

Introduction

Characterization of plant genetic diversity has long been based on morphological traits, which often have notable advantages and relevance to characters of importance to germplasm users. However, they can also have serious limitations. Many are controlled by multiple alleles and loci, making it difficult to relate patterns of phenotypic variation to their genetic bases, and their expression may be strongly affected by the environment[1]. Still, most ivnestigations of variation within and among populations have been based on morphological traits, although many of those traits are environmentally influenced, and genotype-by environment interactions are common[2].

Chemotaxonomy has been used to elucidate systematic relationships at various taxonomic levels, and, although pool of phytochemicals can be produced in plants, relatively few are typically responsible for characteristic colour, aromas or flavours[3] and even fewer are generally used to define chemotypes. Chemical profiles of compounds are widely used to help to establish systematic relationships among plant populations, but these secondry metabolites are most useful in taxonomic classification only when other factors, such as environmental conditions, plant development, and extraction methods, are carefully standardized[4]. Seiler *et al.*[5] detected wide variation in oil concentration and fatty-acid composition among six wild sunflower species. In contrast to morphological and biochemical traits, molecular markers based on DNA polymorphisms are generally not affected by environment. They are widespread within plant genomes, and techniques are improving to make them more reliable and efficient[6]. The choice of an appropriate molecular marker depends on many considerations, with no single approach optimal for studying intraspecific variation or for solving the needs of *ex situ* germplasm conservation[7], such as the identification of duplicate accessions and gaps in genebank collections or the development of effective regeneration systems. Important classes of DNA-based molecular markers, which have been used for genetic-

Table 1. Arbitrary primers of P1-P12 and their sequences used for the RAPD analysis of *Pentas lanceolata* genotypes.

Code	5'-3'	M.W	pmoles	µg/tube
RAPD-01	ACCGCGAAGG	3062	4864	15
RAPD-02	GGACCCAACC	2982	5267	16
RAPD-03	GTCGCCGTCA	3004	5743	17
RAPD-04	TCTGGTGAGG	3099	5194	16
RAPD-05	TGAGCGGACA	3077	4801	15
RAPD-06	ACCTGAACGG	3037	4990	15
RAPD-07	TTGGCACGGG	3084	5267	16
RAPD-09	CTCTGGAGAC	3028	5302	16
RAPD-10	GGTCTACACC	2988	5533	17
RAPD-11	AGCGCCATTG	3028	5302	16
RAPD-12	CACCGTATCC	2948	5785	17

Table 2. Ingredients in the PCR mixture.

Parameter	Tested Range
Primer	100ng
dNTP	100µg
$MgC1_2$	2mM
10X PCR buffer	2.5 µl
Taq polymerase	2U
DNA	50 ng
ddH_2O	Xµl
Total	25µl

diversity assesment, cultivar fingerprinting, and phylogenetic studies, include AFLPs, as well as restriction fragmentlength polymorphisms (RFLP), random amplified polymorphic DNA (RAPD), and single sequence repeats (SSR)[8,9]. Despite their utility for elecidating genetic relationships within plant species, molecular markers have not yet been applied to questions of infraspecific classification or fingerprinting in *Pentas* cultivars. *Pentas* flower colour research program was initiated in the late 1970s, flower colour polymorphisms appeared to be a natural starting point of genetical, biochemical and molecular polymorphism in plants.

Pentas lanceolata belongs to Rubiaceae, commonly called as star cluster plant, has a unique system due to its attractive flower colour. Based on the variation in the flower colour (red, pink, rose, violet and white), the present study has been attempted to reveal the polymorphism of the plant at morphological, biochemical and molecular levels.

Material and Methods

Plant material : For the whole research work, the healthy *Pentas lanceolata* plants were collected and maintained in the garden.

Methodology : The experimental part of the study was categorized into three sections *viz.* molecular, biochemical and morphological.

Morphology variation studies : Morphological studies are carried out by analyzing the height of the plant, leaf size, floral variations like length of the corolla, variation in stigma, leaf epidermal hair and the stomata type among the cultivars. Micrometric techniques were used to calculate the length of the cells.

Biochemical :

Extraction and estimation of protein : Protein content of the leaves of the cultivars of *Pentas* were estimated by Bradford method[10]. Absorbancy was read at 595 nm against a blank.

Estimation of total phenol : Total phenol content of *Pentas*

Table 3. Morphological variation observed among the cultivars of *Pentas lanceolata.*

Variety	Leaf width (cm)	Leaf length (cm)	Corolla tube length (cm)	Color of stigma	Epidermal hair length (μm)
Red	4.9	14.5	2.5	Red	873
Rose	5.2	13.5	2.7	Purple	1040
Pink	4.5	10.3	1.3	Violet	675
Violet	3.5	8.2	1.5	Purple	390
White	4.2	7.5	2.0	White	650

Table 4. Pigment, Protein and Phenol variation observed among the cultivars of *Pentas lanceolata.*

Variety	Chlorophyll *a* (mg/g)	Chlorophyll *b* (mg/g)	Total Chlorophyll (mg/g)	Total carotenoids (mg/g)	Total Protein (mg/g)	Total Phenol (mg/g)
Red	14.92	6.07	20.99	2.49	2.75	6.69
Rose	8.22	4.86	13.08	3.92	1.87	4.79
Pink	6.47	5.99	12.36	5.44	2.37	2.96
Violet	6.08	5.38	11.46	3.62	0.98	2.83
White	8.66	3.63	12.29	3.68	1.29	3.43

Table 5. Percentage of genetic distance between accessions.

Accessions	Percentage of genetic distance
Red and Rose	53
Red and Violet	85.7
Red and Pink	93.4
Red and White	96.5
Rose and Violet	96.15
Rose and Pink	93.44
Rose and White	89.29
Violet and Pink	58.33
Violet and White	81.48
Pink and White	60.87

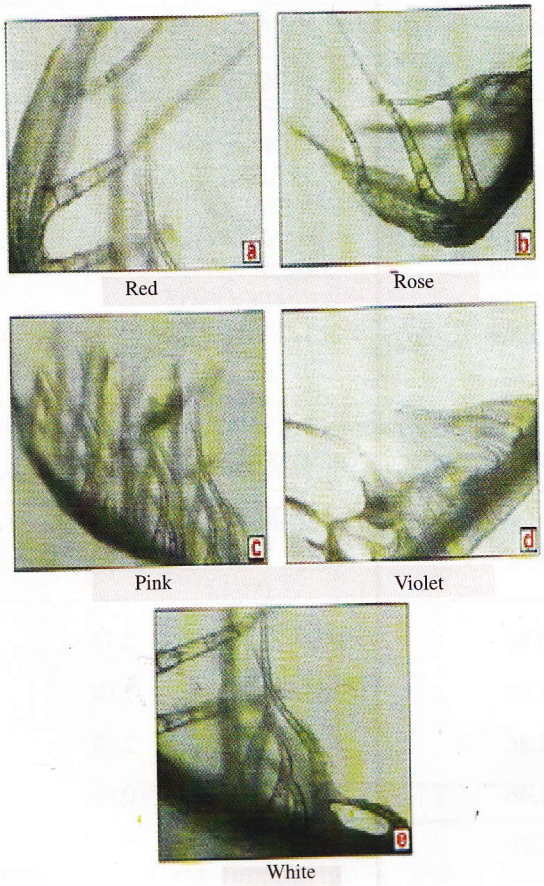

Fig.1a,b,c,d & e. Epidermal hair in *P. Lanceolata.*

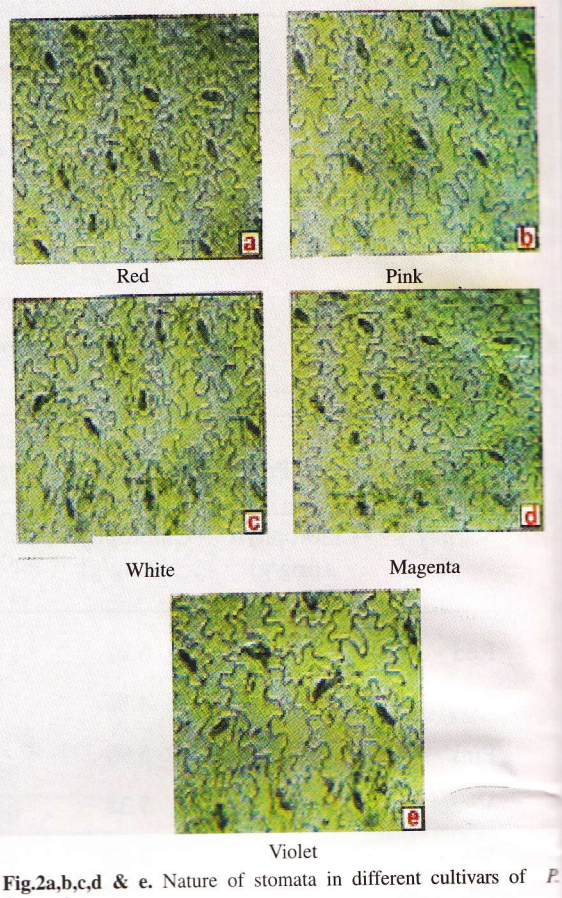

Fig.2a,b,c,d & e. Nature of stomata in different cultivars of *P. Lanceolata.*

leaves was estimated by the method of Mayr *et al.*[11], with the absorbance at 650 nm.

Estimation of total chlorophylls and carotenoids : Total chlorophylls and carotenoids were estimated by the method of Armon[12] and the optical density was measured at 480, 645, 652 and 663 nm for quantifying pigment levels.

Molecular

Random Amplification of Polymorphic DNA (RAPD)-Isolation and purification of DNA : Genomic DNA was extracted and purified by CTAB method[13] with some modifications. 1g of tissue was taken, finely chopped and ground using liquid Nitrogen. The tissue was made into a slurry using the extraction buffer containing 1M Tris-HCl (pH 7.5), 5M NaCl, 0.5M EDTA (pH 8), 1% CTAB and 1% b-Mercapto ethanol pre heated at 65°C. An aliquot of the purified DNA was dissolved in 3ml of TE buffer and the absorbance was measured at 260 nm and 280 nm to check the purity of the DNA. The DNA content was determined using the standard curve of DNA. Twelve Oligo nucleotide 10mer primers were used for the random amplification of the genomic DNA (Table 1).

Optimization of PCR assay for RAPD : Critical factors, which influence the optimization of the DNA amplification during PCR reaction, include the quality and quantity of the genomic DNA, annealing temperature, concentrations of $MgCl_2$, dNTPs and Taq polymerase and the number of cycles during PCR amplification (Table 2). Standardization of these factors is crucial for the elemination of defects associated with PCR amplification such as smear on running gel, nonspecific bands and false amplification. The reactions were carried out in a DNA thermocycler (MJ Research Inc. USA) using 20 ml reaction mixture. Reactions without DNA were used as negative control.

Agarose Gel Electrophoresis : Agarose gel was casted in a Genei mini model horizontal gel apparatus by melting agarose in IX TAE buffer, pH 8.0 (100mM Tris-acetate and 10mM EDTA). Ethidium bromide (0.5mg/ml) was added to the buffer after sufficient cooling, which fluoresces DNA. Molecular weight was determined from the GeneRuler™ DNA ladder mix[14]. The polymorphic DNA bands that showed consistency in repeated experiments were screened according to their presence ('1') or absence ('0') in each of the genotypes. Percentage of genetic distance between the genotypes was estimated by the pair wise comparison method of Nie and Li[15]. After calculation of all pair wise similarities between varieties,

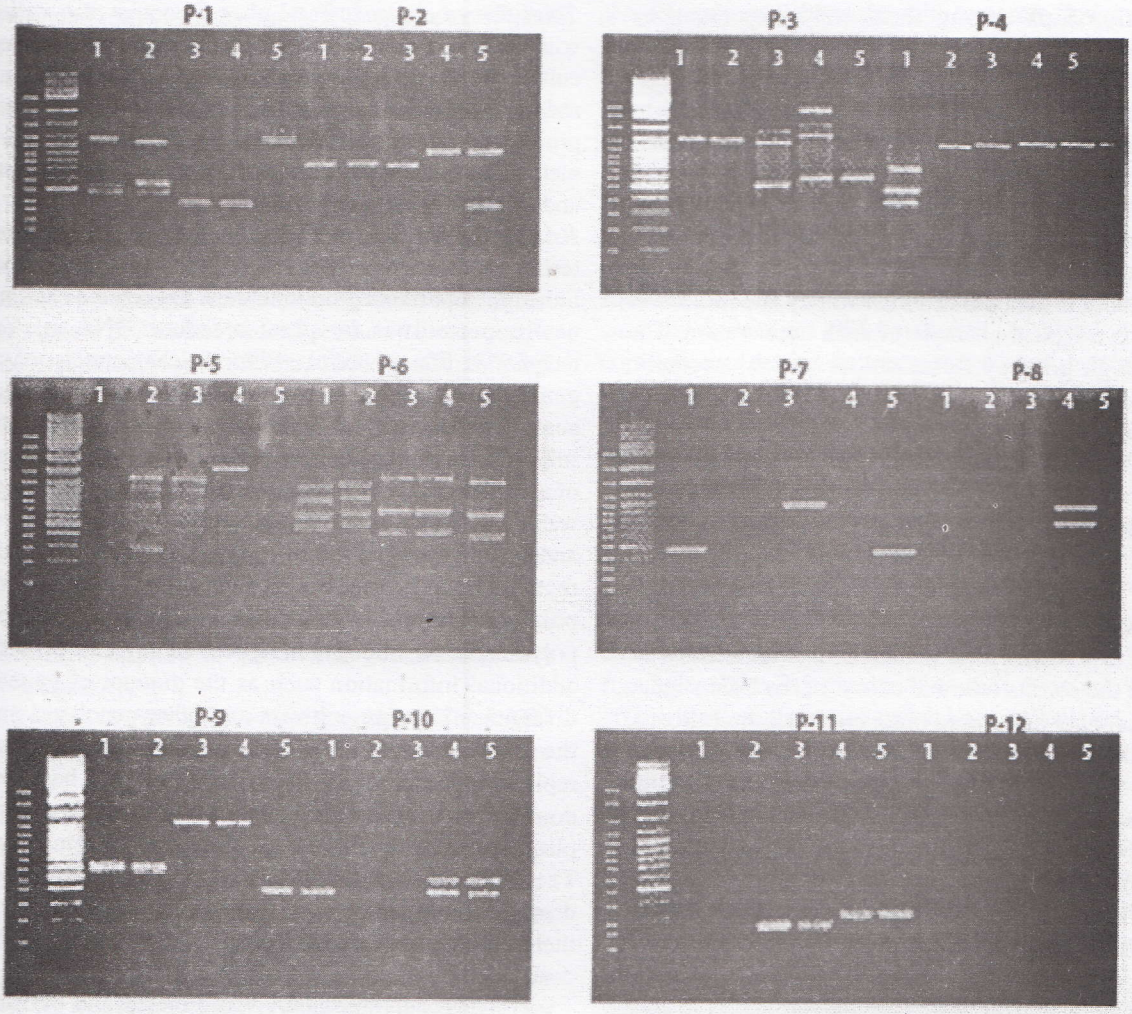

Fig.3. RAPD profile generated with operon primers; Lane M-marker and Lane 1,2,3,4 and 5 represent 5 genotypes of *P. lanceolata*

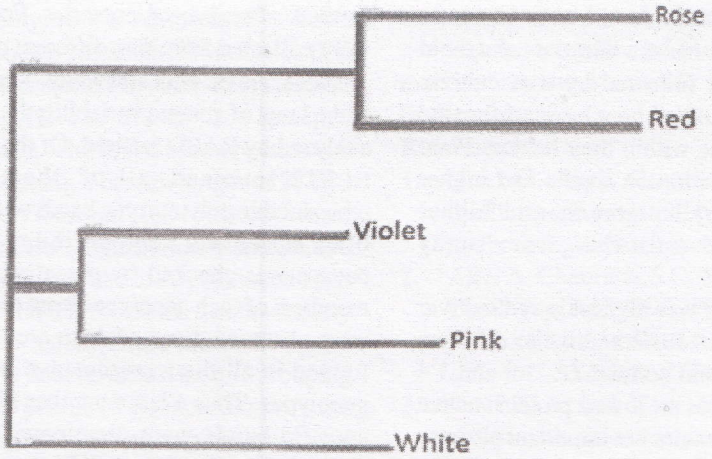

Fig.4. Dendrogram constructed using GENSTAT cluster analysis of RAPD data generated by 15 primers for the 5 genotypes of *P. lanceolata*

the relationships among them were expressed by performing cluster analysis using the software GENSTAT. It is then graphically represented as a dendrogram.

Results and Discussion

Exploitation of genetic variability is of major importance in basic genetic studies and in plant improvement programs. The collection of these genetic resources and the assessment of genetic diversity within and between species should have high priority. The introduction of flower colour in the field of Biochemistry and Molecular Biology is a typical example of such acceleration. The intense high potency floral colour and its aesthetic properties have made this nature's gift a subject of significant, scientific and commercial interest.

Morphological : Leaf size was analyzed in terms of width and length in each cultivar. Red cultivar possess longer leaves whereas, the rose possess broader leaves than white, pink and violet cultivars (Table 3).

The height of the cultivars also showed variations *i.e.,* red cultivar grows much taller (6-7 feet) than other cultivars (3-4 feet). Floral variations are restricted to the length of the corolla tube and colour of the bifid stigma. Dense coronary hairs were observed in all the cultivars. The epidermal hair also showed variation in terms of number of cells (Fig. la-e). Stomata are similar in the cultivars-paracytic type (Fig. 2a-e). Hair density was remarkable in white and red cultivars compared to others (Table 3).

Photosynthetic pigments : Plant employ chlorophylls *a* and *b* and a variety of carotenoids to caputre light for photosynthesis[16,17]. Concentration and ratios of photosynthetic pigments are correlated to their radiance experienced by the plants in their natural habitat. The five cultivars showed varied response in the chlorophyll and carotenoid content. Chlorophyll a and b was higher in red cultivar followed by rose. Meanwhile, the carotenoid content was higher in violet followed by rose cultivar (Table 4). *Pentas* cultivars studied here have acclimated to the levels of light available within their habitat. Plant from the habitat with low radiation inputs had higher concentration of chlorophyll pigments and higher chlorophyll to carotenoid ratio than from sunny environment.

Total protein : Protein profile was highest in red cultivar (2.8mg/g) followed by violet. Rose and white cultivars have more or less same protein content *i.e.,* 1.9 and 1.4 mg/g respectively. Pink possess the lowest protein content (0.98 mg/g) (Table 4). The proteins are important nitrogen intermediates related to plant metabolism or act as stress proteins to environmental stresses[18].

Total phenol content : Total phenol content also varied with cultivars. Significant level was observed in the red cultivar (6.7 mg/g) followed by rose (4.8 mg/g). Pink showed the lowest level (2.8 mg/g). The over all higher profile of phenols suggest the active phase of secondary metabolism and inturn the resistance of the plants to pests and pathogens[12] (Table 4).

RAPD Analysis : Development of molecular marker technology and consequent identification of marker loci linked to important agronomic traits has created exciting new opportunities for plant breeders. DNA marker techniques offer powerful tool for the characterization of genetic variability, genotypic identification, genetic analysis and selection and breeding programmes in plants. Important advantages of molecular markers include lack of sensitivity to changes in environmental conditions, as well as a nearly unlimited potential number of markers and speed of the marker assays as compared with field tests. In many species, random amplification of polymorphic DNA has proven useful for revealing polymorphism among genotypes[19]. They can provide additional information such as the amount of genetic divergence between cultivars and other genotypes and the amount of genetic variability between seedling replicates of genotypes. So the present study has been done to reveal the genetic polymorphism of *P. lanceolata* plants with different flower colour using RAPD markers. The RAPD data was subjected to cluster analysis for detecting the genetic polymorphism of the cultivars and their phylogenetic status was also detected from the dendrogram[7].

The genomic DNA was isolated and purified from the leaf samples collected from *P. lanceolata* plant cultivars. Each cultivar was properly identified from the gardens of registered nurseries. For the study the plants were collected from five different cultivars of Kerala *i.e.* red, rose, violet, pink and white. The DNA polymorphism at the level of genetic variability in *Pentas* genotypes was analyzed by RAPD method. Of the 12 RAPD primers P1 to P12 screened, all of them produced distinct reproducible polymorphic bands within the five genotypes of *P. lanceolata.* Reproducibility of the amplification pattern was checked by repeating the reactions in five members of each genotype. Even though diagnostic bands were observed, most of them are faint or not repeatedly formed in all the representative individuals of the five genotypes. Thus a large number of potentially genotype specific bands were eleminated from consideration. Fig. 3 represents the amplification pattern obtaned with the 12 primers for the five genotypes. The RAPD profile

shows a total number of 81 bands with appropriate band size range of 250 to 5000 bp. PCR reactons were optimized at annealing temperature 36°C for 40 cycles. All the 12 primers in the five genotypes were considered as a single reaction[20].

Identification of P. lanceolata genotypes based on specificity of primers : Based on the screening of the RAPD profile of the five genotypes, it is possible to categorize the primers into five groups. The primer 2 produced two specific bands (400 bp and 1000 bp) for white cultivar of *Pentas*. Primer 3 produced three specific bands in pink cultivar at 450 bp, 1200 bp and 1500 bp. Primer 4 generated three specific bands for red cultivar at 300 bp, 400 bp and 600 bp. Primer 5 generated a single prominent band for violet cultivar at 1500 bp. Primer 5 produced six prominent bands for rose cultivar at 250 bp, 400 bp, 500 bp, 700 bp, 800 bp and 1000 bp. No specific single primer is available for distinguishing all the cultivars. Thus the RAPD data of the primers indicates the discriminatory power in amplification and it can be successfully applied to reveal the genetic diversity between genotypes[21].

Dendrogram of Pentas lanceolata genotypes : The RAPD data of the five genotypes was extended further for statistical analysis in order to measure the genetic distances among them. The bands were scored according to their presence ('1') or absence ('0') and were arranged as per the molecular size. A pair wise genetic distance was done among the five genotypes by a statical method by Nie and Li[7]. It is evident from the percentage of genetic distance that genetic dissimilarity was found between the plants of the five cultivars. RAPD data was further extended for statistical analysis in order to measure the genetic distances among the genotypes. The distance matrix between the genotypes was represented in Table 5. From the genetic distance calculated from the RAPD data, a dendrogram was prepared by GENSTAT cluster analysis software (Fig.4). The cluster tree grouped basically into three clusters. The first cluster contains two cultivars, rose and red, having a branch length of 27.073 and 25.927 respectively. Violet and pink cultivars formed another cluster, with branch lengths 24.850 and 33.890 respectively. White cultivar is found to be positioned apart from both these clusters and it is estimated that this variety is distant with other varieties. Thus, the dendrogram prepared from the RAPD data reflects a unique grouping of genotypes. The RAPD profile of *P. lanceolata* provides sufficient insight to categorize the *Pentas* genotypes based on its gentic relatedness[15].

The biochemical and molecular data of the present study on *P. lanceolata* with respect to flower colour demonstrates the genetic and physiological potential of the crop as a ornamental. The RAPD profile would be helpful in detecting specific primers for identifying the cultivars. More extensive molecular evidence at gene expression level is warranted to establish this hypothesis.

In conclusion, the evidence from RAPD analysis indicates the existence of a moderate degree of genetic diversity in *Pentas* cultivars. Even though this study showed close realtionships among a few genotypes studied, there were still duplicates. Although a good correlation observed between genetic and biochemical data, future studies involving a large number of morphological traits with molecular marker should have important implications for germplasm management. The development of *Pentas* cultivars with superior floral colour properties will be very important for promoting commercial production. RAPD makers have proved to be effective for characterizing the genetic basis of *Pentas* for assessing genetic diversity and relatedness between cultivars. The result of this study is of critical importance for *Pentas* breeding programs as well as informed and efficient management of germplasm collections. Thus, molecular techniques answer many new evolutionary and taxonomic questions, which were not previously possible with only phenotypic methods. Molecular techniques such as DNA barcoding, random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), microsatellites and single nucleotide polymorphisms (SNP) have recently been used for plant diversity studies. These techniques differ in their resolving power to detect genetic differences, type of data they generate and their applicability to particular taxonomic levels. Similarly, other DNA markers such as SNP markers are best for characterizing and conserving the gene bank materials and the AFLP and microsatellite markers are more suitable for diversity analysis and fingerprinting.

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