GENETIC VARIABILITY ANALYSIS IN INDIAN INDIGO (*Indigofera tinctoria* L.) USING RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) TECHNIQUE

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2004

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Thesis submitted in partial fulfilment of the requirement for the degree of

Master of Science in Horticulture

Faculty of Agriculture
Kerala Agricultural University, Thrissur

2004

Department of Plantation Crops and Spices
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DECLARATION

I hereby declare that this thesis entitled "**Genetic variability analysis in Indian indigo (*Indigofera tinctoria* L.) using Random Amplified Polymorphic DNA (RAPD) Technique**" is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar title, of any other university or society.

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CERTIFICATE

Certified that this thesis entitled "**Genetic variability analysis in Indian indigo (*Indigofera tinctoria* L.) using Random Amplified Polymorphic DNA (RAPD) Technique**" is a record of research work done independently by Mrs. Neema.M (2002-12-10) under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to her.

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Dedicated
to
my Grand mother

ACKNOWLEDGEMENT

With deep and heartfelt gratitude, I express my sincere indebtedness to ***Dr. B.R. Reghunath,*** *Associate Professor, Department of Plantation Crops and Spices and Chairman of the Advisory Committee, for his timely help, technical advice, constructive criticism, inspiring guidance and encouragement for the successful completion of the research work and preparation of the manuscript.*

My heartfelt gratitude to ***Dr. B.K. Jayachandran,*** *Associate Professor and Head, Department of Plantation Crops and Spices and member of the Advisory Committee for his valuable comments, kind concern and encouragement throughout the study.*

I am particularly grateful to ***Dr. K. Rajmohan,*** *Associate Professor and Head, Department of Pomology and Floriculture and member of the Advisory Committee for his valuable suggestions and wholehearted approach throughout the course.*

I wish to express my profound thanks and gratitude to ***Dr. K.B. Soni,*** *Associate Professor, Department of Plant Biotechnology and member of the Advisory Committee for her utmost help and valuable suggestions at various stages of the course of study.*

I extend my sincere thanks to Dr. G.R. Sulekha, Associate Professor and Dr. P.C. Jessykutty, Assistant Professor, Department of Plantation Crops and Spices, for their kind concern and ever- willing help.

I convey my deep sense of gratitude to Dr. L. Rajamony, Dr. AbdulVahab, Dr.I. Sreelathakumari and Dr. V.A Celine Department of Olericulture, Dr. M.M. Viji, Dr. R.V. Manju, and Dr. Roy Stephan, Deparment of Plant Physiology, Dr.Chandramony and Dr.V.S. Radha Devi, Dapartment of Plant Breeding and Genetics, Dr. Meena Kumari, Department of Plant Pathology for their sincere help rendered during the course of investigation.

I am obligated to each and every one who helped me in the collection of neelayamari seeds and special thanks to Dr. P.K. Warrier, Kottakkal, Mr. Biju Jacob, Thrissur, Mr. Somarajan Vaidyan, Nellimoodu and Mrs. Sabeena, Koduvazhanoor for the same.

I remember with gratitude, the immense help given by Mr. Gopinathan, Farm Supervisor, for his help in the fieldwork. I also thank all the labourers who helped me in raising the crop successfully.

I must thank specially Ms. Anitha, Mr. Pradeep and Mr. Satheesh for their selfless help and moral support rendered during molecular analysis. I also thank Mrs. Susheela and all the Research Assistants, Department of Plant Biotechnology for their help and co-operation during the work.

I thankfully acknowledge Dr. George Thomas, Rajiv Gandhi Center for Biotechnology, Thiruvananthapuram for his help in providing gel documentation facilities.

I express my indebtedness to Mr. C.E. Ajithkumar, Junior Programmer, Department of Agricultural Statistics for statistical analysis of the experimental data.

My sincere thanks to Sri. Biju, Ardra for his prompt computerized type setting and preparation of the thesis.

I am thankful to the Library staff, College of Agriculture, Vellayani and Kariyavattom Campus for helping me in collecting the literature. I am also thankful to Mr. Jayakumar for his help in taking photographs.

My heartfelt thanks to my seniors Mrs.Thangaselva Bai, Mrs. Sarada S. and Mrs. Deepa S Nair for giving necessary suggestions and encouragement throughout the course of study.

I wish to express my sincere thanks to my classmates Resmi D.S, Krishna Priya, Sheena, Juliya Mathew and Palani Kumar for their help on various occassions during my study. My heartfelt thanks to Aswathy Vijayan, Nishana, H. and Simi, S. for their co-operation and help.

I am unable to express my deep sense of gratitude to my friends Resmi J, Manuel Alex and Suresh for their selfless help, constant encouragement and support throughout the course of study and research.

I sincerely acknowledge Kerala Agricultural University for granting me the KAU Junior Research Fellowship.

I am thankful to Mrs. Sainaba Samad and her family for their moral support rendered during my studies.

I remember with gratitude the co-operation, encouragement and sincere support rendered by my in laws Mrs. Aisha Beevi and Mr. Basheer and also Sherin during the course of study.

From deep within my heart, I owe my indebtedness to my uncles Mr. Shafeekh, Mr. Najeeth and Mr. Firoze for their constructive suggestions, deep concern and prayers with out which this work would not have seen light. I also thank all my Aunts and cousins for the same.

Words fail to express my indebtedness to my father Mr. Mohammed Naina, mother Mrs. Aysha, brother Sabu, sister Silishi, brother in law Mr. Noushad and little Nikku for their unconditional love, prayers, moral support and encouragement without which this work would not have been a success.

I thank my husband Mr. Shahir Basheer for his wholehearted support, constant inspiration, abundant love and patience that helped me a lot in completing the study. I also thank him for bearing all the inconveniences during the study.

Above all I humbly bow my head before God Almighty for all the blessings showered upon me to complete this endeavor successfully.

Neema .M

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LIST OF ABBREVIATIONS

%	-	Per cent
°C	-	Degree Celsius
µg	-	Micro gram
µl	-	Micro litre
AFLP	-	Amplified fragment length polymorphic DNA
bp	-	Base pair
CD	-	Critical difference
CIMAP	-	Central institute of medicinal and aromatic plants
cm	-	Centimeter
d.f.	-	Degrees of freedom
DNA	-	Deoxy ribonucleic acid
dNTPs	-	Deoxy nucleotide tri phosphates
EDTA	-	Ethylene diamino tetra acetic acid
et al.	-	And others
Fig.	-	Figure
g	-	Gram
GCV	-	Genotypic coefficient of variation
i.e.	-	That is
M	-	Molar
mM	-	Milli molar
N	-	Normality
ng	-	Nanogram
nm	-	Nanometer
No.	-	Number
NS	-	Non significant
OD	-	Optical density
PCR	-	Polymerase chain reaction
PCV	-	Phenotypic coefficient of variation
PIs	-	Plant introductions
RAPD	-	Random amplified polymorphic DNA
RFLP	-	Restricted fragment length polymorphic DNA
rpm	-	Rotations per minute
SE	-	Standard error
Tris HCL	-	Tris (hydroxy methyl) aminomethane hydrochloride
UPGMA	-	Unweighted pair group method for arithmatic average
USDA	-	United states department of agriculture
viz.	-	Namely

INTRODUCTION

1. INTRODUCTION

Indian indigo (*Indigofera tinctoria* L.) known as neelayamari in Malayalam, is a leguminous deciduous sub shrub of the south-eastern Asia. The shoot of the plant contains 'indigotin', a deep blue dye in the form of a glycoside namely 'indican'. Plant derived dyes like indigotin have been shown to be useful in dying clothes which do not produce "dermatitis", an allergic condition of the skin often caused by synthetic dyes (Sertoli *et al*., 1994). Historically, indigo is the oldest blue dye utilized by man and was commercially much exploited till synthetic colouring materials were evolved.

The plant besides utilized as a source of blue dye, is also valued in Ayurveda as an important ingredient of hair tonics like 'Neelibhringadi thailam'. Neelayamari is also reported to be useful in the treatment of myelocytic leukemia, inflammatory skin conditions and in the glandular inflammation of the lymph nodes and tonsils. The root of the plant is used for the treatment of hepatitis. An infusion of the root is given as an antidote in case of snake bite and poisoning by arsenic (Nadkarni, 1976).

Lack of authentic varieties of medicinal plants is a major drawback that hinders quality standardization of pharmaceutical preparations made out of them. Screening of existing germplasm for evolving superior genotypes and releasing authentic varieties of medicinal plants would help in maintaining the uniformity of raw materials used in pharmaceutical industries. Natural variations in DNA sequence, that can be detected using marker systems, are now days reliably utilized for differentiating various accessions and their different traits. Molecular marker assisted breeding has proved to have the potential to enhance the pace and efficiency of genetic improvement of crop plants. Random Amplified Polymorphic DNA (RAPD) is a technique useful for genomic analysis as it detects high level

of polymorphism in a much shorter time. The amount of DNA used in RAPD reactions is very low and hence needs only small amount of tissue. The simplicity, reliability and not affected nature by environmental and tissue sources, all add to the utility of this technique.

The present study titled ‘Genetic variability analysis in Indian indigo (*Indigofera tinctoria* L.) using Random Amplified Polymorphic DNA (RAPD) Technique’ is aimed at morphologically describing, cataloguing and evaluating the growth and yield parameters of various accessions of *Indigofera tinctoria* collected from different parts of the state of Kerala, and analyzing the genetic variability of the accessions using RAPD technique. The results of the study would help in providing better insight into the morpho-taxonomical traits of the plant leading to precise genetic cataloguing.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

The growing popularity of indigenous systems of medicine such as Ayurveda and Unani is resulting in increased demand for the raw drugs required for preparation of various medicines. The major components of raw drug *i.e*., medicinal plants, is extracted from forest areas (40%) or from non-forest areas (50%) and from limited cultivated lands (10%). Due to increased demand for medicinal plants, the rate of extraction from the natural source is higher than that of their regeneration. Thus medicinal plant germplasm collection and their biodiversity conservation assume greater significance in the present scenario.

Indigofera tinctoria is a leguminous medicinal plant having high relevance in the plant based dye industry. The leaves of the plant is highly praised as a major ingredient in hair tonics and the plant finds place in many of the authentic Ayurvedic books such as *Ashtangahridaya* and *Charakasamhitha*. An infusion of the root is given as an antidote in arsenic poisoning. The leaf extract is applied to reduce swellings in the body due to the bites and stings of venomous insects and reptiles (Nadkarni, 1976). Han (1995) revealed that indirubin from *Indigofera tinctoria* exhibited anticancerous properties. Zang (1996) had mentioned about the antitumour drugs obtained from *Indigofera tinctoria*.

The present work is aimed at determining the genetic variability of *Indigofera tinctoria* (Indian indigo) using RAPD technique. Works on genetic variability analysis on *Indigofera tinctoria* is rather scanty. Hence relevant literature on variability analysis in medicinal plants in general, and in some important leguminous crops is reviewed in this chapter.

2.1 GERMPLASM COLLECTION AND CONSERVATION

A detailed study of evaluation of genetic stock of *Clitoria ternatea* for yield, alkaloid content and nitrogen fixing potential was made by Nair (2000).

Samuel (2000) conducted a similar study in *Mucuna pruriens*. She had also estimated L-dopa content in seeds and assessed nitrogen fixing potential when raised as an intercrop in coconut garden.

Sarada (2000) discussed the need for evolving suitable strategies for sustainably utilizing the medicinal plant resources, occurring as indigenous or naturalized within the oil palm plantations and their vicinity.

Sunitha (1996) compiled information on collection, description and performance evaluation of sixteen herbaceous leguminous medicinal plant species including *Indigofera tinctoria,* and worked out the feasibility of raising these plant species as intercrops in coconut garden.

2.2 MORPHOLOGICAL STUDIES

2.2.1 Mean Performance

Kulkarni and Karadge (1991) reported that plant height was found to increase throughout the growth stages in moth bean (*Phaseolus aconitifolium* Jacq.).

A study made by Anitha (1997) showed that a mean of 147 days was needed for 50 per cent flowering in *Indigofera tinctoria*.

While evaluating the genetic stock of *Mucuna pruriens,* Samuel (2000) noticed that the fresh and dry weight of leaves increased considerably during the earlier growth stages, but declined towards later stages.

Nair (2000) and Resmi (2001) observed that in *Clitoria ternatea*, the performance of different accessions differed during various growth stages. In the same work it was also noted that the leaf area and root yield increased during the pre flowering and flowering stage, but decreased during the pod maturation stage. The shoot yield was found to increase through out the growth stages.

Mathew (2002) studied the floral biology and anthesis in *Moringa oleifera* and reported that the anthesis occurred during morning hours.

2.2.2 Variability

Variability Studies in Medicinal Plants

Singh and Khanna (1991) reported that the coefficient of variability for the agronomic characters studied in opium poppy was low due to the narrow genetic base of the genotypes. Singh *et al*. (2000) reported that significant variation among genotypes were recorded for the characters such as plant height, number of leaves per plant and number of branches per plant in opium poppy. The estimates of PCV were higher than those of GCV. The yield was positively and significantly associated with number of leaves per plant and number of branches per plant.

Sankaranarayanan *et al*. (1992) reported that in senna high genotypic coefficient of variation estimates were found for total leaf yield and leaf yield at 90 days.

Misra *et al*. (1998) reported that the highest phenotypic and genotypic coefficients of variation were recorded for dry root yield followed by plant canopy and the lowest for plant height in *Withania somnifera*.

Twenty two morphometrically diverse genotypes of *Andrographis paniculata* collected from North India were evaluated for genetic variability and the results revealed considerable amount of genetic variability among genotypes. Highest phenotypic and genotypic coefficient of variations were recorded for dry biomass yield, leaf: stem ratio followed by plant height and lowest for leaf length (Misra *et al*., 2000).

Fifteen fenugreek genotypes were studied for the presence of 12 quantitative characteristics for effective selection of the important characters for higher yield by Dash and Kole (2001). The variability in 29 M-lines of fenugreek (*Trigonella foenum-graecum*) was estimated by Kaushik and Dashora (2001). Significant variability was recorded for plant height and seed yield per plot.

Kalamani and Gomez (2001) while conducting a study on the genetic variability analysis in *Clitoria ternatia* recorded that seed weight, leaf breadth, number of leaves per plant, leaf length and plant height showed high PCV and GCV estimates. The root length, girth and yield, and also nodule number and yield of 13 accessions of *Clitoria ternatea* intercropped under shade in a coconut garden, were examined by Nair and Reghunath (2002) during the pre-flowering, flowering, seeding and seed maturation stages of the plant and came to the conclusion that the parameters measured increased from pre-flowering up to the seeding stage and decreased during the seed maturation stage.

The work on the evaluation of *Trigonella foenum-graecum* and *Trigonella corniculata* for leaf yield and its components done by Varalakshmi (2002) revealed that the most promising lines in terms of yield and dry matter content were IIHR-4 that produced the tallest plant and IIHR-9, where the leaf height was highest. Kumar and Choudhary (2003) assessed the magnitude of genetic variability in 12 diverse fenugreek (*Trigonella foenum-graecum*) genotypes. Sufficient genetic variation was found for plant height, plant girth, number of branches per plant, number of leaves per plant and root weight.

A study on the genetic variation and relationship between root yield and biochemical traits of 13 genotypes of safed musli by Bhagat and Jadeja (2003) showed that GCV was lower than PCV for root yield.

Variability studies in legumes

Very narrow differences between GCV and PCV were observed for days to 50 per cent flowering and days to maturity in black gram, indicating that effect of the environment on these traits is low. Seed yield had significant positive association with plant height, number of clusters per plant, number of pods per cluster, number of seeds per pod, biomass, pod length and pod yield (Savithramma *et al*., 1999). According to Gupta *et al*. (2001) in black gram (*Vigna mungo*) the highest variability was recorded for

the yield followed by pods per plant and plant height. The lowest variability was recorded for 100 seed weight.

A study made by Jeena and Arora (2001) in chickpea revealed that plant height and pods per plant and secondary branches per plant showed significant positive correlation with yield.

Reddy *et al*. (2002) studied variability and correlation in sixty genotypes of faba bean (*Vicia faba* L).

An experiment was conducted by Kalia *et al.* (2003) on twenty four faba bean cultivars to determine the genetic variability in the available germplasm.

Solanki *et al*. (2003) reported that in moth bean highly significant variation was observed for plant height, number of primary branches per plant, fodder yield and seed yield per plant. The phenotypic coefficient of variation (PCV) was higher than the genetic coefficient of variation (GCV) for all the characters.

2.2.3 Correlation

Correlation studies in medicinal plants

Sankaranarayanan *et al*. (1992) reported that in senna, leaf yield was positively correlated with numbers of branches, length of leaves and leaves per plant and pod yield was significantly correlated with plant height and number of branches.

In *Catharanthus roseus*, the values of genotypic correlation were higher than the phenotypic and environmental values. Number of primary branches showed highly significant positive genotypic association with total fresh leaf yield, whereas stem width showed highly significant negative genotypic association with number of primary branches. Significant positive genotypic associations were observed between plant height, stem width and total dry leaf yield, whereas the association between total fresh leaf yield and total dry leaf yield was non-significant (Dwivedi *et al*., 1999).

Saini *et al*. (1999) recorded data on ten quantitative traits in 75 indigenous and exotic genotypes of opium poppy and observed a wide range of phenotypic and genotypic variations of all kind. Shukla *et al*. (2003) conducted correlation and path coefficient analysis in 22 selections of opium poppy for seed yield. Seed yield showed positive and significant correlation with plant height, leaves per plant and stem diameter.

Mishra *et al*. (2001) observed large differences among 32 accessions of periwinkle collected from different geographical areas of India, Madagascar, Singapore and Malaysia. Strong correlation was observed between leaf area and leaf yield.

Krishnamoorthy and Madalageri (2002) conducted studies to assess the range of variability and character associated with growth, yield and yield attributing traits in fifteen genotypes of ajowan (*Tachyspermum ammi*).

Mulas *et al.* (2002) observed a positive correlation between leaf width and shoot fresh weight in rosemary (*Rosmarinus officinalis* L.) cultivars.

Lal *et al*. (2003b) observed tremendous variability in quantitative traits such as days to flower, leaf length, main stem diameter, branches per plant among genetic stocks in curry neem (*Murraya koenigii*).

Correlation studies in legumes

Tambal *et al*. (2000) observed that in lentil (*Lens culinaris*) selection for the number of pods per inflorescence couldn't be recommended for increasing seed yield.

In black gram, correlation studies made by Gupta *et al.* (2001) showed that significant correlation was found between yield and pods per plant but not between yield and seeds per pod. A strong positive correlation existed between the number of pods per plant and the number of branches per plant.

Venkateswarlu (2001) noticed that in green gram, pods per plant and seeds per pod had maximum positive direct effect on seed yield. Days to

maturity, clusters per plant, plant height, 100 seed weight and seeds per pod exhibited high indirect effect on seed yield and pods per plant.

Haritha and Sekhar (2002) reported that in mung bean, highly significant positive correlation of grain yield was observed with pods per plant and pods per cluster.

Singh and Mishra (2002) studied the correlation among the parents, F_1s and F_2s of 10 x 10 diallele cross in *Pisum sativum* and found that the seed yield per plant was positively and significantly associated with pods per plant and primary branches per plant.

Correlation studies on winged bean by Dhandannavar et *al*. (2003) showed that positive and highly significant correlation was observed between total green pod yield, total dry matter accumulation and leaf area. Green pod yield had negative correlation with number of days before flowering.

2.3 MOLECULAR MARKERS

The varietal distinctness procedure involves expensive, space and time consuming measurements of phenotypic traits. Moreover for most species and traits, interactions between genotypes and environment complicate the cultivar evaluation. Molecular markers could provide a distinctness procedure that helps increasing the reliability of decisions and saving time. The molecular markers are useful in taxonomic studies, linkage mapping, germplasm conservation and clonal identification (Bretting and Widrlechner, 1995). The important applications of molecular markers include verification of identity and labeling of plants in production and marketing, commercial variety protection, assessment of seed purity and marker assisted plant breeding (Henry, 1998). Shashidhara *et al*. (2003) reported that molecular markers and isoenzyme analyses are rapidly being developed in recent years for tree improvement and are increasingly used for diversity analysis, germplasm characterization and identification of core population.

The potential of molecular markers in augmenting the information

available on plant genetic resources is applicable to several aspects of *ex situ* gene bank work. Molecular analyses offer insight into the diversity present within collections and even into the evolution of certain cultivated plant species (Dehmer and Forsline, 2003).

Molecular biology offers an assortment of techniques that can be used for the authentication of medicinal plants (Techen *et al*., 2004).

Protein markers and DNA markers have the widest application among the molecular markers.

2.3.1 Protein Markers

2.3.1.1 Isozymes

Isozymes are variant forms of an enzyme usually detectable through electrophoresis due to differences in their net electrical charges.

Pooler and Simon (1993) employed morphological and isozyme assay method for intra specific classification of 110 garlic cultivars.

Sebastian *et al*. (2000) observed wide variation among cultivated types of *Piper nigrum* through isoenzyme analysis.

Reis and Frederico (2001) studied the genetic diversity in cowpea using isozyme electrophoresis.

2.3.2 DNA Markers

DNA based markers clearly allow the direct comparison of the genetic material of two individual plants avoiding any environmental influence on gene expression. According to Lavi *et al*. (1998) the two main purposes for applying DNA markers are identification and linkage analysis.

Among the molecular markers used, DNA markers are more suitable and ubiquitous to most of the living organisms (Joshi *et al*., 1999). DNA markers are generally classified as hybridisation based markers and polymerase chain reaction (PCR) based markers.

2.3.2.1 Hybridization based DNA Marker Technique

The hybridization based DNA marker technique is those that use labeled nucleic acid molecules as hybridization probes (Anolles *et al*. 1991). The most important hybridisation based DNA marker technique is restriction fragment length polymorphism (RFLP).

2.3.2.1.1 Restriction Fragment Length Polymorphism

Restriction fragment length polymorphism analysis involves digesting the subject genome with restriction enzymes, separating the fragments electrophoretically and then preferentially visualizing the fragments containing particular homologous sequence by hybridizing them to a specific DNA probe.

Mess *et al*. (1998) used RFLP technique for conducting a phylogenetic study in 29 *Allium* species and seven related genera.

Banerjee *et al*. (1999a) carried out genetic variability analysis in chick pea (*Cicer arietinum*) accessions using RFLP and the analysis using 26 sub-genomic clones on 10 chick pea accessions in 130 probe enzyme combinations detected polymorphism with only two clones.

To analyze the quantitative trait loci (QTLs) related to flowering time in soybean, Yamanaka *et al*. (2000) constructed a soybean linkage map using an F_2 population derived from a cross between two varieties, Misuzudaizu and Moshidou Gong 503. The most effective QTL, FT1, accounted for approximately 70 per cent of the total variation and appeared to correspond to E1, the locus for flowering time and maturity on the classical genetic map.

Pupilli *et al.* (2000) reported that RFLP analysis could be used for the identification of alfalfa ecotypes.

2.3.2.2 *Polymerase Chain Reaction (PCR) based DNA Marker Technique*

The PCR technique uses an *in vitro* enzymatic reaction to specifically amplify a multiplicity of target sites in one or more nucleic acid molecules

(Anolles and Trigiano, 1997).

Among the PCR based DNA marker techniques the important ones are amplified fragment length polymorphism (AFLP), microsatellites, random amplified polymorphic DNA (RAPD) etc.

Amplified Fragment Length Polymorphism (AFLP)

Amplified fragment length polymorphism is based on PCR amplification of restricted fragments generated by specific restriction enzymes and oligonucleotide adapters of a few nucleotide bases (Vos *et al*., 1995).

Morphological descriptors, quantitative, phytochemical analysis and DNA finger printing using AFLP were utilized to define the extent of variation existing between Hawaiian cultivars of *Piper methysticum* and extremely narrow genetic base was observed among the accessions (Lebot *et al*., 1999).

AFLP analysis was used for the molecular assessment of germplasm diversity in several medicinal plants such as *Withania somnifera* (Negi *et al*., 2000), *Papaver somniferum* (Saunders *et al*., 2001) and *Aloe* species (Darokar *et al*., 2003).

Massawe *et al*. (2002) reported that AFLP analysis provide sufficient polymorphism to determine the amount of genetic diversity and to establish genetic relationships in bambara groundnut landraces.

AFLP analysis was used for ascertaining clonal fidelity in medicinal trees like neem (Singh *et al*., 2002).

2.3.2.2.2 Microsatellites

Microsatellites consists of tandemly arrayed di-tri-tetra nucleotide repeats, which are hyper variable and ubiquitously distributed through eukaryotic genome. Microsatellite DNA marker, which can be directly amplified by PCR have been developed using the unique sequence that flank microsatellites (Tantz, 1989; Weber and May, 1989).

Highly polymorphic microsatellites became the markers of choice for linkage mapping and population studies in chickpea (Sharma *et al*. 1995).

Microsatellites could be useful and powerful tools for assessing genetic variation and genetic relationships in tetraploid alfalfa (Mengoni *et al*., 2000) and in common bean (Solis *et al*., 2002).

2.3.2.2.3 Random Amplified Polymorphic DNA

RAPD technique utilizes single short oligonucleotide primers of arbitrary sequence for the amplification of DNA segments distributed randomly throughout the genome, using polymerase chain reaction (PCR). In this reaction the primers of arbitrary sequence bind to perfect or imperfect sites in the genomic DNA, so that a subset of them will lie in inverted orientation to each other. As a result a number of bands are amplified through the action of DNA polmerase. The reaction products are analyzed on agarose gels.

Polymerase chain reaction in conjugation with random primers was used for finger printing genomes (Welsh and Mc Clelland, 1990) for identification of genome specific markers (Williams *et al*., 1990) and for population biology studies (Astley, 1992).

2.3.2.2.3.1 RAPD for Variability Studies

Variability studies in medicinal plants

Bradley *et al*. (1996) studied the usefulness of RAPD markers for determining the distribution of genetic variability within and among 20 taxonomic units of Australian garlic cultivars.

RAPD profiles of 34 accessions or provenances of neem were generated with 200 decamer random primers, of which 49 resulted in reproducible amplification products. The similarities in RAPD profiles amongst the different accessions was more than that expected due to the out crossing nature of neem and the results suggest that neem may have a narrow genetic base (Farooqui *et al*., 1998).

RAPD analysis was done by Padmesh *et al*. (1999) to determine intraspecific variability in *Andrographis paniculata*. It was found that AP-48, an accession from Thailand showed close resemblance to AP-38 collected from Tamil Nadu and AP-29 from Assam was significantly diverse from the rest of the native genotypes.

Sangwan *et al.* (1999) worked on the detection of highly polymorphic profiles (97 polymorphic markers out of a total of 101 markers) in *Artemesia* by RAPD analysis and suggested the existence of very high levels of genetic variation in the Indian population despite geographical isolation and the work opens out a strong possibility of further genetic improvement for superior artemisinin content.

Khanuja *et al*. (2000) used a set of 60 random primers to analyze 11 accessions from six taxa of *Mentha* developed by CIMAP. A total of 630 bands could be detected as amplified products upon PCR amplification, out of which 589 were polymorphic (93.5%).

Shasany *et al.* (2000) reported that RAPD profiling of 23 accessions of *Allium sativum* (garlic) from different geographical parts of India and two accessions from Argentina showed a total of 2998 bands out of which, 2459 (82.02%) bands were polymorphic and 52 (1.73%) were unique bands. Low level of diversity was found among the accessions.

The genetic diversity of recognized varieties of Asparagus was carried out using RAPD markers (Raimondi *et al*., 2001).

Zhang *et al*. (2001) applied RAPD technique to distinguish *Lycium barbarum* from other closely related species of the same genus and obtained distinctive DNA finger prints corresponding to different *Lycium* species and higher similarity values between intra-species than inter-species. Darokar *et al*. (2001) examined narrow genetic base among various geographically distinct accessions of *Bacopa monnieri* by RAPD analysis. Low level of polymorphism was observed and the range of similarity was 0.8-1.0.

RAPD analysis in two morphologically distinct varieties of *Digitalis* by Sales *et al*. (2001) showed restricted population variability.

A comparison between the chemical markers such as volatile oil and flavanoids, and molecular markers like random amplified polymorphic DNA was made by Vieira *et al.* (2001) in tree basil (*Ocimum gratissimum*). Cluster analysis of RAPD markers showed that there were three groups which are genetically distinct and highly correlated (r = 0.814) to volatile oil constituents.

The genetic variation among the 21 accessions of various species of *Aloe* was evaluated by Darokar *et al*. (2003) based on molecular analysis by RAPD. The phylogenetic tree generated by RAPD classified the accessions into three major clusters representing *Aloe vera*, *Aloe arborescens* and *Aloe saponaria*.

A collection of *Phyllanthus amarus* (*P. niruri*) was made from various parts of India by Jain *et al*. (2003) to determine the extent of genetic variability using RAPD and 65 per cent variation among the accessions was found.

Lal *et al*. (2003a) made a detailed investigation on the genetic diversity of 51 *Vetiveria zizanioides* gene bank accessions in CIMAP, using RAPD and observed a 40 per cent genetic diversity.

Shasany *et al*. (2003) detected significant diversity among *Asparagus racemosus* accessions (51.7 %) and also within the species (48 %).

RAPD analysis was used to assess genetic diversity in basil (Vieira *et al.*, 2003). Means of genetic similarities within *Ocimum* spp. indicated that the domesticated species have the lowest similarity.

Variability studies in legumes

RAPD is used and has great potential for use in gene mapping, gene tagging, genetic diversity studies, phylogeny and evolution studies in soybeans (Yong *et al*., 1997).

Mignouna e*t al*. (1998) reported that 95 accessions of three cultivar groups of cowpea (*Vigna unguiculata*) from diverse geographical origin analysed by RAPD using 9 out of 120 tested random decamer primers showed a high degree of genetic diversity.

Ahmad (1999) reported that a methodology based on random-primed DNA amplification that can be used for studying *Cicer* (chick pea) phylogeny and its improvement.

Duarte *et al*. (1999) studied the genetic divergence of 27 common bean (*Phaseolus vulgaris*) cultivars from different races using RAPD markers and found that these markers were efficient in separating cultivars according to domestication centres.

RAPD analysis was undertaken by Qiang *et al*. (2000) to characterize genetic variation in three forms of *Vigna angularis* namely cultivated, wild and weedy forms.

2.3.2.2.3.2 RAPD for Cultivar Identification

Echt *et al*. (1994) constructed a genome map of cultivated alfalfa using segregating RFLPs and RAPDs in a diploid backcross population generated from non-inbred parents. It was found that the combined use of RFLPs and RAPDs was an effective method for developing an alfalfa genome map.

Graham *et al*. (1994) reported that RAPD was applied to the identification of Australian navy bean (*Phaseolus vulgaris*) varieties. A total of 296 markers were generated from 29 primers. Analysis using RAPD-PCR molecular markers allowed distinction of varieties for plant variety rights and had provided information on the genetic relationships of these varieties for breeding purposes.

The genomic DNAs of 42 *Pisum sativum* genotypes, representing four wild and cultivated sub-species were used as templates in RAPD reactions by Samec and Nasinec (1996). Amplification with eight decamer primers generated 149 polymorphic products.

Laucou *et al*. (1998) reported that a genetic linkage map of *Pisum sativum* was constructed based primarily on RAPD markers that were carefully selected for their reproducibility and scored in a population of 139 recombinant inbred lines (RILs).

Thompson *et al*. (1998) assessed the diversity of 18 soyabean ancestors and 17 selected plant introductions (PIs) maintained in the USDA Soybean Germplasm Collection. All methods of cluster analysis identified distinct groups of ancestors or PIs. The genotypes within the distinct PI clusters may possess useful genetic diversity that could be exploited by soyabean breeders to increase yield.

Hosokawa *et al*. (2000) analysed nine accessions of three species of medicinal plants in the genus *Scutellaria* (*S. galericulata, S. lateriflora* and *S. baicalensis*; known collectively as skullcap) by RAPD to distinguish between members of these three species.

Two genetic linkage maps of mung bean (*Vigna radiata*) derived from the cross Berken X ACC 41 are reported by Lambrides *et al*. (2000). The F2 map constructed from 67 individuals consisted of 110 markers (52 RFLP and 56 RAPD) that grouped into 12 linkage groups.

A work to find the genetic difference among *Piper longum* varieties was made by Philip *et al*. (2000).

Chowdhury *et al*. (2002) used twenty-two RAPD markers to determine genetic relationships in six chickpea (*Cicer arietinum* L.) cultivars and breeding lines.

The molecular characterisation of seven accessions of *Indigofera tinctoria* done by Jose (2002) revealed that certain distinct and specific bands were produced by the primers OPX-13, OPX-16 and OPX-20.

RAPD analysis distinguished different *Panax* spp. as well as the morphological variants of *P. notoginseng* by producing cultivar specific bands (Cui *et al*., 2003).

2.3.2.2.3.3 Taxonomic Studies

Bullitta (1995) examined the potential use of RAPDs for taxonomic studies, and factors influencing DNA isolation and RAPD patterns in *Trifolium* spp. Twenty primers previously never used in *Trifolium* were selected for production of strong reproducible bands. Reproducible amplification products were obtained from different varieties and species belonging to the genus. RAPD markers revealed polymorphisms that appeared to be useful for taxonomic studies at the population and species level.

Wolff and Richards (1998) identified some intermediate forms while analysing the two sub-species in *Plantago major* through RAPD analysis.

2.3.2.2.3.4 Mutations and RAPD

The RAPD assay and related techniques like the arbitrarily primed polymerase chain reaction (AP-PCR) have been shown to detect genotoxin-induced DNA damage and mutations. Random mutations occurring in mismatch repair-deficient strains did not cause any changes in the banding patterns whereas a single base change in decamer primers produced substantial differences (Atienzar *et al*., 2002).

2.3.2.2.3.5 Sex Determination and RAPD

RAPD technique was utilized to find the genotypic differentiation between the male and female *Piper longum* plants by Banerjee *et al*. (1999b).

Sex of nutmeg seedling at an early age can be determined using RAPD markers (Shibu *et al*., 2000).

Steck *et al*. (2001) noticed that RAPDs might be used in addition to cytological studies to confirm the mode of reproduction by apomixis versus self-pollination, haploid parthenogenesis or cross-fertilization in the medicinal plant *Hypericum perforatum.*

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The study titled 'Genetic variability analysis in Indian indigo (*Indigofera tinctoria* L.) using Random Amplified Polymorphic DNA (RAPD) technique' was carried out at the Department of Plantation Crops and Spices and at the Department of Plant Biotechnology, College of Agriculture, Vellayani during the period 2002-2004. The work was conducted in two phases:

PHASE I Morphological studies

PHASE II Molecular analysis

3.1 PHASE I MORPHOLOGICAL STUDIES

3.1.1 Materials

Experimental materials consisted of 20 accessions of *Indigofera tinctoria* collected from different parts of Kerala state. Details of the accessions and their sources are given in Table1. The experimental field was laid out at the garden area, College of Agriculture, Vellayani (Plate 1).

3.1.2 Methods

Seeds were sown on earthen seed pan after subjecting to acid scarification (Sy *et al.*, 2001). Seedlings were transplanted after three weeks at five leaf stage to 250 gauge polythene bags of size 40 x 20 cm filled with potting mixture consisting of cowdung, sand and soil in the ratio 1:1:1. The experiment was laid out in CRD with three replications. Fifteen plants were maintained in each accession. The crop was raised as per Package of Practices Recommendations of Kerala Agricultural University (KAU, 2002). The twenty accessions of *Indigofera tinctoria* collected are shown in Plate 2.

Table 1. Sources and date of collection of *Indigofera tinctoria* accessions

Sl. No.	Accession No.*	Source of germplasm	Date of collection/ renewal
1	IT 1 (MP- 97)	TBG&RI, Palode, Thiruvananthapuram Dist.	22.05.01/ 15.09.03
2	IT 2 (MP- 99)	Thomas Mathew, Puthenchantha, Mundakkayam, Kottayam Dist.	13.07.01/ 15.09.03
3	IT 3 (MP-101)	AICRP on Medicinal and Aromatic Plants, Vellanikkara, Thrissur Dist.	28.07.01/ 15.09.03
4	IT 4 (MP- 104)	Mathewkutty Theruvapuzha , Secretary, 'Vrikshabandhu' Pala, Kottayam Dist.	04.08.01/ 15.09.03
5	IT 5 (MP- 111)	Aromatic and Medicinal Plant Research Station, Odakkali, Eranakulam Dist.	20.08.01/ 15.09.03
6	IT 6 (MP-143)	Biju Jacob, Muthipeedika house, Ollur, Thrissur Dist.	18.08.03
7	IT 7 (MP-144)	College of Horticulture, Department of Plantation Crops & Spices, Vellanikkara, Thrissur Dist.	23.08.03
8	IT 8 (MP-145)	College of Agriculture, Instructional Farm, Vellayani, Thiruvananthapuram Dist.	29.08.03
9	IT 9 (MP-146)	Y.Somarajan Vaidyan, Yesudasan Vaidyan Memorial Sidha Vaidyasala, Nellimoodu, Thiruvananthapuram Dist.	06.09.03
10	IT 10 (MP-147)	Kuzhipallam Botanical Garden, Kazhivoer road, Nellimoodu, Thiruvananthapuram Dist.	14.09.03
11	IT 11 (MP-148)	Mohammed, Kunnuvila, Kollam Dist.	19.09.03
12	IT 12 (MP-149)	Sree Narayana stores, T.C-39/286, Chalai, Thiruvananthapuram Dist.	22.09.03
13	IT 13 (MP-150)	Padmanabha Pillai and Sons, Ayurveda College Jn., Thiruvananthapuram Dist.	22.09.03
14	IT 14 (MP-151)	Sabeena, Koduvazhanoor, Nagaroor, Thiruvananthapuram Dist.	23.09.03
15	IT 15 (MP-152)	Aysha,Thekkaebhagom house, Varkala, Thiruvananthapuram Dist.	30.09.03
16	IT 16 (MP-153)	Kottakkal Aryavaidyasala, Kottakkal, Malappuram Dist.	05.10.03
17	IT 17 (MP-154)	Prabhakaran, Kalluparambil Veedu, Palapur, Thiruvananthapuram Dist.	08.10.03
18	IT 18 (MP-155)	Dr. Hareendran Nair, Pankajakasthuri, Vellanadu, Thiruvananthapuram Dist.	10.10.03
19	IT 19 (MP-156)	Anil Kumar, Maanthoppil house, Pattambi, Palakkad Dist.	20.10.03
20	IT 20 (MP-157)	Lekshmi Agencies, Krishnan Kovil Jn., Neyyatinkara, Thiruvananthapuram Dist.	3.11.03

* Number in parenthesis indicates original accession number as per the Accession Register of the Dept of Plantation Crops and Spices, College of Agriculture, Vellayani

Plate 1. General view of experimental field one month (top) and three months (bottom) after transplanting

IT 1

IT 2

IT 3

IT 4

Plate 2. Accessions of *Indigofera tinctoria*

IT 5

IT 6

IT 7

IT 8

Plate 2. Continued

IT 9

IT 10

IT 11

IT 12

Plate 2. Continued

IT 13

IT 14

IT 15

IT 16

Plate 2. Continued

IT 17

IT 18

IT 19

IT 20

Plate 2. Continued

3.1.3 Observations Recorded

The following observations were recorded at pre-flowering, flowering and at pod maturation stages.

3.1.3.1 Growth and Yield Observations

3.1.3.1.1 Growth Characters

Plant height (cm)

Height of the plant from the collar region to the tip was measured using measuring tape.

Plant spread (cm)

The distance covered by the plant was measured using a measuring tape in the North-South and in East-West directions from the axis and the average was recorded.

Height at first branching (cm)

The height at which first branch is produced was measured from the ground level to the position from where the branch is produced using a measuring scale.

Number of branches

The total number of branches of the plant was counted.

Girth of stem (cm)

Measurement was taken round the stem at a height of 3 cm from the soil surface using a tag and the length of the tag was taken as the stem girth.

Length of leaf (cm)

The average length of leaves selected at random from the observational plant was recorded.

Breadth of leaves (cm)

The average breadth of leaves selected at random from the observational plant was recorded.

Number of leaves (cm)

The total number of leaves produced by the observational plant was taken.

Leaf area (cm^2)

The leaf area was calculated by adopting punch method. Fifty punches were made. The discs as well as leaves were dried in hot air oven at 70˚C and their respective dry weights were recorded. From the data of leaf dry weight leaf area per plant was computed and recorded (Watson, 1952).

Root length (cm)

The length of the longest root was measured using a measuring scale.

Root girth at collar region (cm)

The girth of the root at the collar region was measured using a tag and the length of the tag was recorded.

3.1.3.1.2 Yield and Yield Attributes

Fresh weight of leaves (g)

The total weight of leaves of the observational plants in each accession was recorded.

Dry weight of leaves (g)

The leaves were oven dried at 70 ± 5˚C to a constant weight and the dry weight was recorded.

Fresh weight of shoots (g)

Weight of the stem and leaves of the observational plants from each accession was recorded.

Dry weight of shoots (g)

The shoots were oven dried at 70 ± 5˚C to a constant weight and the dry weight of shoot was recorded.

Fresh weight of pods (g)

The fresh weight of the pods of each accession during harvesting stage was recorded.

Dry weight of pods (g)

The pods with seeds were oven dried at 70 ± 5˚C to a constant weight and the dry weight of pods was recorded.

Fresh weight of seeds (g)

The fresh weight of the seeds of each accession during harvesting stage was obtained.

Dry weight of seeds (g)

The seeds were oven dried at 70 ± 5˚C to a constant weight and the dry weight of seeds was recorded.

Shell weight (g)

The weight of the dry shell obtained from the observational plants after removing the seeds was recorded.

Fresh weight of roots (g)

Fresh weight of the roots of each accession during the three stages of observation *viz*. pre flowering, flowering and pod maturation stage was recorded.

Dry weight of roots (g)

The roots obtained from the three stages of observation were oven dried at 70 ± 5˚C to a constant weight and the dry weight was recorded.

3.1.3.1.3 Root Nodule Characters

Number of root nodules

The root nodules were detached from the plant root system and the number of nodules present was observed during pre flowering and flowering period (Plate 3).

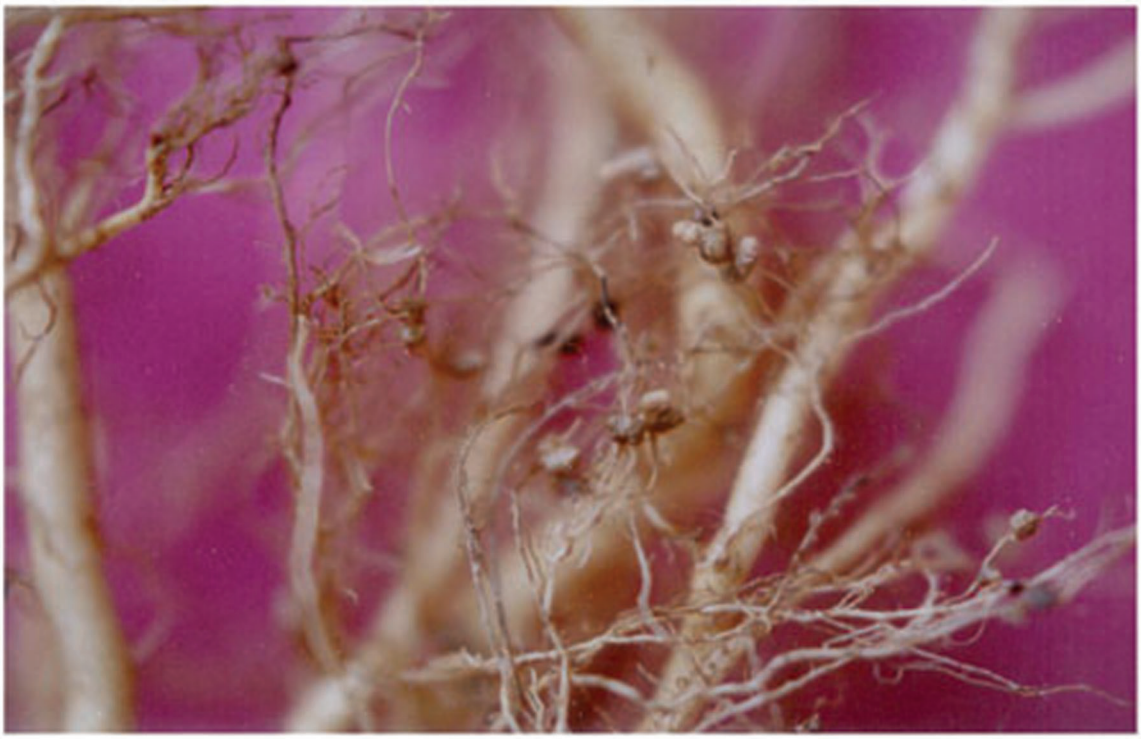


Plate 3. Root nodule of *Indigofera tinctoria*

Number of effective root nodules

Root nodules obtained were cut into half and those showing pinkish colouration was taken as effective root nodules.

Fresh and dry weight of root nodules (g)

Fresh weight of root nodules of each observational plant was taken using an analytic electronic balance. Root nodules were then dried in hot air oven at 70˚C until constant weight was obtained.

3.1.3.1.4 Flowering and Seeding Behaviour

Number of days for flowering

Number of days taken for the appearance of first flower from the date of sowing was computed for the observational plants and recorded the mean value.

Time of anthesis

Observations were made during the flowering stage of the crop. After preliminary observations on the commencement of anthesis, (which revealed that the flowers opened in the morning), ten inflorescence of each accession were tagged at 6.00 a.m. and observations were made at two hour intervals. Later, precise observations were made as per Mathew (2002).

Time of anther dehiscence

Flower buds were tagged in group of ten at the time of anthesis. They were observed with a hand lens at two hour interval for anther dehiscence. Appearance of longitudinal splits in the pollen sac indicated the commencement of anther dehiscence. When more than three anthers in a flower liberated pollen grains it was reckoned as having completed anther dehiscence. The observation was repeated for three days with another group of flowers and the average was worked out (Mathew, 2002).

Number of pollen grains produced per anther

Pollen production per flower was estimated using a haemocytometer. Mature flower buds were collected just before opening. One or two anthers are crushed in one ml distilled water and was placed on the haemocytometer using a micropipette and observed through the low power (10×10 x) of the compound microscope. The number of pollen grains present in four corner squares and one center square is counted. The pollen count obtained is multiplied by 5 to get the total number of cells present in all 25 squares. The number of pollens present was obtained by multiplying the number of cells counted in all 25 squares by 10^4 (Gunasekaran, 1992).

Statistical Analysis

Quantitative parameters of twenty accessions of *Indigofera tinctoria* were recorded from the observational plant at three different stages of growth *viz*. preflowering, flowering and pod maturation stages. Analysis of variance as proposed by Panse and Sukhatme (1967) was worked out for all the traits to find out whether there was significant difference between the accessions in respect of various traits. The mean values for all the accessions for each of the characters were worked out and compared using critical difference and the significance was tested using F-test. The variability that existed in the population for various characters were apportioned using the estimates of coefficient of variation (Singh and Chaudhary, 1985). The phenotypic, genotypic and the environmental coefficient of variations with respect to each character were estimated. Phenotypic, genotypic and environmental correlation coefficients were also worked out. The selection index developed by Smith (1936) using the discriminant function of Fisher (1936) was used to discriminate the genotypes based on fifteen characters.

PHASE II MOLECULAR CHARACTERIZATION OF *INDIGOFERA TINCTORIA*

3.2.1 Isolation of Genomic DNA

For the isolation of genomic DNA tender leaves from the *Indigofera tinctoria* plant was used. The isolation was done following a modified method given by Murray and Thompson (1980).

About 1g of the plant leaf was taken as the base material for isolation after removing the midrib. The leaves were collected in the early morning and washed thoroughly in running tap water and then twice rinsed with distilled water. The leaves were then placed in tissue paper for removing the entire water content. After this the leaves were placed in pre-chilled porcelain container and liquid nitrogen was poured over the leaf material. The leaves were ground well to a fine powder and then transferred to 1ml extraction buffer (0.7 N NaCl, 1% CTAB, 50 mM Tris- HCl (pH 8.0), 10 mM EDTA) placed in 1.5 ml polypropylene centrifuge tube. To this added 5µl 0.2% β-mercaptoethanol and 1% polyvinyl pyrollidone. This was mixed gently by tapping and is placed in the water bath preset at 60˚C for one hour. The tubes were then centrifuged at 15,000 rpm for 10 minutes. The supernatant was taken and to this equal volume of Phenol: Chloroform: Isoamyl alcohol (25:24:1) was added and again subjected to centrifugation at 15,000 rpm for 10 minutes at 4˚C. After collecting the upper phase the Phenol: Chloroform: Isoamyl alcohol (25:24:1) extraction was repeated until the interphase disappeared. To the aqueous phase collected equal volume of Chloroform: Isoamyl alcohol (24:1) solution was added and the two phases was mixed gently. Centrifugation was done at 15000 rpm for 10 minutes at 4˚C. To the supernatant collected $1/10^{th}$ volume of 3.0 M sodium acetate and two volumes of cold absolute ethanol were added and were kept in refrigerated condition at 4˚C for 30 minutes. Centrifugation of the resultant was done at 10000 rpm for 10 minutes to pellet the DNA. The aqueous phase is drained out and the pellet was washed with 70 % cold ethanol and

centrifugation at 10,000 rpm for 5 minutes at 4˚C. The excess ethanol was drained out and kept in hybridization incubator for drying. It was then dissolved in 0.5 µl of 1x Tris EDTA buffer (10 mM Tris-HCl, 1mM EDTA, pH 8.0) and stored at 4˚C.

3.2.2 Quantification of DNA

Reliable quantification of DNA concentration is important for many applications in molecular biology including amplification of target DNA polymerase chain reaction. DNA quantification was carried out with the help of UV-VIS Spectrophotometer (Spectronic Genesis 5). The buffer in which the DNA was already dissolved was taken in a cuvette and used for the calibration of the spectrophotometer at 260 nm as well as 280 nm wavelength. The optical density of the DNA sample dissolved in the buffer was recorded at both 260 nm and 280 nm. Since an OD of 1.0 at 260 nm represents 50 µg / ml of DNA, the DNA concentration was estimated by employing the following formula:

$$\text{Amount of DNA (µg / ml)} = \frac{A_{260} \times 50 \times \text{dilution factor}}{1000}$$

where, A_{260} = absorbance at 260 nm

The quantity of DNA could be judged from the ratio of the OD values, recorded at 260 and 280 nm. The A_{260} / A_{280} ratio between 1.7 and 1.9 indicates best quality of DNA.

3.2.3 Agarose Gel Electrophoresis

Agarose gel electrophoresis was carried out in a horizontal gel electrophoresis unit supplied by the Bangalore Genei. Required amount of agarose was weighed out (0.9% for visualizing the genomic DNA and 1.4% for visualizing the amplified products) and melted in 1x TAE buffer (0.04M Tris acetate, 0.001M EDTA, pH 8.0) by boiling. After cooling to about 50˚C, ethidium bromide was added to a final concentration of 0.5 mg ml^{-1}. The mixture was poured immediately to a preset template with appropriate comb.

After solidification, the comb and the sealing tapes were removed and the gel was mounted in an electrophoresis tank filled with 1x TAE buffer. The DNA sample was mixed with required volume of gel loading buffer (6.0x loading dye *viz*. 40% sucrose, 0.25% bromophenol blue). Each well was loaded with 20 µl of sample. One of the wells was loaded with 5 µl of molecular weight marker along with required volume of gel loading buffer. Electrophoresis was carried at 50 volts until the loading dye reached $3/4^{th}$ length of the gel. The gel was visualized using an ultra-visible (uv-vis) transilluminator (Appligene, Oncor, Trans).

3.2.4 Random Amplified Polymorphic DNA (RAPD) Analysis

RAPD analysis was performed by the method recommended by Williams *et al*. (1990), with required modification. Forty arbitrarily designed decamer primers supplied by Operon Inc., CA, USA were used.

Genomic DNA (40 ng) was amplified in a 25 µl reaction mixture containing 2.5 µl 10x PCR buffer, 2 µl $MgCl_2$ (Magnesium chloride), 1 µl primer, 2 µl each of deoxy nucleotides (dNTPs) and 0.6U of Taq DNA polymerase (Bangalore Genei Pvt. Ltd; Bangalore). Amplification was carried out in a programmable thermal controller (MJ Research Inc. USA) set for the following programme. An initial denaturation at 94˚C for 4 minutes followed by 40 cycles of denaturation at 94˚C for 1 minute, annealing at 35˚C for 1 minute and extended at 72˚C for 1 minute 30 seconds. The synthesis step of the final cycle was extended further by 10 minutes. Finally the products of amplification were cooled to 4˚C. A negative control containing water instead of template DNA was included in each reaction set. The amplified products along with DNA molecular weight marker supplied by Bangalore Genie were separated by electrophoresis using 1.4 per cent agarose gel, stained with ethidium bromide and visualized on a UV transilluminator. The number of monomorphic bands, number of polymorphic bands and intensity of bands were recorded. Those primers, which when used for amplification, produced the maximum number of bands

were used to amplify the DNA of all the twenty *Indigofera tinctoria* accessions. The photograph of the amplified profile obtained in all the accessions using A-10, B-3, B-5, and B-10 primers, were taken with the help of a gel documentation system. The RAPD bands were represented as '+' for presence and '-' for absence and were recorded. The PCR was repeated in order to check the reproducibility.

3.2.5 Data Analysis

The data obtained were subjected to cluster analysis by UPGMA method using NTSYS software to estimate the similarity indices and genetic relatedness among the accessions. The reproducible bands were scored for the presence '+' or absence '-' for all the accessions studied. A genetic similarity matrix was constructed using the Jaccard's coefficient method (Jaccard, 1908).

$$Sj = a / (a + b + c)$$

where,

a : number of bands present in both accessions

b : number of bands present in 1st accession but not in the 2nd

c : number of bands present in the 2nd accession but not in the 1st

Association between the various accessions was found out from the dendrogram.

RESULTS

4. RESULTS

The experimental data recorded during the course of investigation of the present study titled 'Genetic variability analysis in Indian indigo (*Indigofera tinctoria* L.) using Random Amplified Polymorphic DNA (RAPD) Technique' were subjected to morpho-molecular analysis and the results are presented here under in the following heads:

4.1 Morphological studies in *Indigofera tinctoria*

4.2 Genetic variability in *Indigofera tinctoria*

4.3 Molecular characterization in *Indigofera tinctoria*

4.1 MORPHOLOGICAL STUDIES IN *INDIGOFERA TINCTORIA*

Mean performance of growth parameters

Analysis of variance showed significant differences among the 20 accessions for all the characters studied at all the stages except for root girth at collar region during the pre flowering stage. The mean values of the different characters studied at different stages of plant growth are presented in Tables 2, 3 and 4.

4.1.1 Mean Performance of Shoot Characters

Plant height

Plant height at the pre flowering stage found to vary from 21.87 (IT 12) to 49.93 cm (IT 14). At the flowering stage it ranged from 75.97 (IT 4) to 123.07 cm (IT 15). At the pod maturation stage the minimum height was observed for the accession IT 4 and maximum for IT 20, which ranged from 132.70 to 221.40 cm.

Plant spread

Mean plant spread exhibited a range of 40.67 (IT 14) to 69.20 cm (IT 1) in the pre flowering stage. The accession IT 16 showed the maximum plant

Table 2. Growth parameters of *Indigofera tinctoria* at pre flowering, flowering and pod maturation stages of plant growth

Sl. No.	Accession No.	Plant height (cm)			Plant spread (cm)			Height at first branching (cm)	No. of branches			Girth of stem		
		A	B	C	A	B	C	A	A	B	C	A	B	C
1	IT 1	42.07	104.07	184.20	69.20	95.27	120.43	8.23	14.67	39.67	60.33	2.30	3.13	6.93
2	IT 2	33.23	90.53	176.00	58.33	83.30	96.13	4.77	11.00	33.33	58.67	3.20	2.83	6.67
3	IT 3	33.10	94.10	171.67	48.50	103.7	140.90	6.47	8.67	27.67	54.67	2.50	3.10	6.53
4	IT 4	32.60	75.97	132.70	52.00	51.67	110.43	6.00	13.67	37.33	64.67	2.30	3.30	6.20
5	IT 5	29.27	92.03	164.90	58.67	81.60	112.43	5.43	12.67	31.00	59.67	2.37	3.57	6.40
6	IT 6	26.03	111.6	172.20	56.00	91.63	143.10	5.20	12.67	35.67	58.67	2.43	4.20	7.27
7	IT 7	30.13	91.00	200.30	45.17	63.53	114.37	6.53	12.00	35.00	58.00	2.37	3.30	6.20
8	IT 8	42.43	103.17	191.10	54.33	91.47	117.50	7.23	10.00	31.00	55.33	2.70	4.10	7.17
9	IT 9	36.13	101.07	174.43	52.50	93.60	105.53	7.53	13.33	39.67	63.33	2.53	2.73	5.77
10	IT 10	43.20	102.93	182.83	46.33	85.50	106.53	8.27	10.67	38.33	68.67	2.43	3.03	5.50
11	IT 11	37.53	87.73	176.27	53.50	86.47	123.50	6.37	13.00	41.33	71.33	2.37	3.20	6.33
12	IT 12	21.87	95.17	142.40	61.33	89.30	125.37	4.20	7.67	30.67	52.67	2.13	3.43	6.30
13	IT 13	24.07	113.53	161.47	51.00	100.57	115.67	5.20	16.00	45.67	75.00	2.20	2.73	6.07
14	IT 14	49.93	99.17	145.60	40.67	75.97	113.63	5.07	9.67	39.33	65.33	2.67	4.53	7.20
15	IT 15	29.37	123.07	188.17	42.67	91.63	116.60	8.67	7.33	29.33	58.33	2.43	3.47	5.77
16	IT 16	33.20	105.7	182.37	42.83	113.4	118.10	5.57	13.33	44.00	74.67	3.37	3.47	6.33
17	IT 17	36.13	103.27	178.17	58.83	89.33	126.47	6.27	13.33	32.00	59.67	2.13	3.57	6.57
18	IT 18	33.47	121.87	204.33	68.83	98.47	135.67	5.30	10.67	32.33	61.33	2.33	3.33	6.37
19	IT 19	34.50	93.33	218.90	66.17	83.30	137.63	5.47	9.33	30.67	60.00	1.90	3.17	7.00
20	IT 20	33.17	94.17	221.40	48.83	101.7	140.93	5.40	11.33	35.67	63.00	1.67	2.87	5.67
Mean		34.07	100.17	178.47	53.79	88.57	121.05	6.16	11.55	35.48	62.17	2.42	3.35	6.4
F ratio		64.37**	291.11**	867.19**	125.21**	5157.5**	3816.12**	119.88**	7.88**	23.17**	24.66**	27.81**	20.58**	30.2**
CD		2.41	1.92	2.23	2.16	0.55	0.60	0.32	2.36	2.00	3.58	0.21	0.3	0.27

A: Pre-flowering stage
B: Flowering stage
C: Pod maturation stage

** Significant at 1% level

Contd…

Table 2. Continued

Sl. No.	Accession No.	Length of leaf (cm)	Breadth of leaf (cm)	No. of leaves			Leaf area (cm^2)		
		A	A	A	B	C	A	B	C
1	IT 1	10.87	4.40	129.67	533.67	442.67	2037.13	7187.40	5883.47
2	IT 2	10.37	4.53	111.00	451.67	406.67	1511.57	6195.73	5367.20
3	IT 3	11.47	4.30	104.00	428.33	373.67	1503.20	5963.73	5238.90
4	IT 4	11.33	4.53	121.00	480.33	410.00	1705.53	6490.97	5676.10
5	IT 5	10.20	4.23	116.33	464.67	386.00	1930.40	6242.77	5517.80
6	IT 6	10.47	4.47	115.33	444.00	384.67	1814.63	6148.23	5344.10
7	IT 7	11.20	4.47	116.67	450.00	380.00	1842.70	6214.10	5300.50
8	IT 8	10.73	4.47	103.00	425.67	379.00	1617.77	5907.43	5090.90
9	IT 9	10.50	4.40	126.67	498.00	427.00	1864.87	6811.90	5747.73
10	IT 10	10.53	4.33	101.33	379.33	344.67	1623.83	5234.63	4593.73
11	IT 11	9.70	4.10	120.00	509.00	452.33	1816.70	6836.73	6318.93
12	IT 12	10.40	4.37	70.33	320.33	272.00	1098.20	4440.00	3667.13
13	IT 13	11.17	4.43	141.00	579.67	499.67	1756.90	7769.70	6987.10
14	IT 14	10.47	4.30	102.00	422.33	372.00	1601.87	5835.20	5078.10
15	IT 15	11.23	4.17	65.33	301.00	209.33	1029.23	4052.90	2805.03
16	IT 16	10.50	4.47	129.33	501.33	409.67	2037.17	6767.07	5384.40
17	IT 17	11.27	4.27	129.33	514.67	410.33	2017.80	6886.90	5383.93
18	IT 18	9.70	3.87	99.67	425.00	333.67	1198.00	5841.87	4537.10
19	IT 19	10.17	3.80	95.33	361.33	270.67	1511.13	4996.63	3726.47
20	IT 20	9.83	4.13	110.67	480.67	402.33	1792.70	6627.10	4542.97
Mean		10.61	4.30	110.4	448.55	378.32	1665.57	6122.55	5109.58
F ratio		102.42**	8.43**	132.66**	318.57**	132.43**	5.11**	77.72**	42.87**
CD		0.15	0.20	4.7	11.19	16.69	371.32	294.27	413.25

A: Pre-flowering stage
B: Flowering stage
C: Pod maturation stage

** Significant at 1% level

Table 2. Continued

Sl. No.	Accession No.	Root length (cm)			Root girth at collar region (cm)			Shell weight (g)
		A	B	C	A	B	C	C
1	IT 1	26.03	27.33	35.40	2.40	3.37	7.43	27.30
2	IT 2	24.17	28.17	32.90	3.33	4.10	6.93	21.8
3	IT 3	26.70	31.43	36.27	2.63	3.27	7.17	17.63
4	IT 4	32.00	37.53	42.43	2.43	3.47	6.77	32.63
5	IT 5	35.90	43.77	49.60	2.53	3.70	6.77	39.67
6	IT 6	36.90	42.47	48.77	2.57	4.67	7.60	26.53
7	IT 7	38.43	45.83	51.33	2.60	3.73	6.60	48.00
8	IT 8	29.77	36.27	41.63	2.90	4.43	7.37	58.97
9	IT 9	32.83	38.47	44.83	2.73	3.77	6.50	51.13
10	IT 10	36.90	43.23	48.27	2.73	3.50	6.07	27.77
11	IT 11	31.20	36.43	42.37	2.63	4.70	6.60	31.03
12	IT 12	43.73	49.47	53.70	2.40	4.57	7.13	24.50
13	IT 13	43.13	50.13	54.33	2.43	3.90	6.37	34.90
14	IT 14	43.13	48.23	53.30	3.0	5.13	7.57	24.47
15	IT 15	33.63	38.83	44.93	2.67	4.00	6.27	25.57
16	IT 16	34.60	40.20	43.87	3.57	4.10	6.70	47.37
17	IT 17	29.23	36.73	41.17	2.33	4.37	6.87	26.33
18	IT 18	27.90	33.70	38.70	2.53	3.80	6.70	33.93
19	IT 19	32.60	37.73	42.37	3.77	4.80	7.23	38.03
20	IT 20	36.00	41.37	44.87	1.87	4.27	6.33	18.87
Mean		33.79	39.37	44.55	2.95	4.03	6.85	32.82
F ratio		46.80**	49.31**	105.4**	0.91	26.2**	24.51**	32.41**
CD		2.41	2.62	1.71	4.23	0.32	0.26	5.67

A: Pre-flowering stage
B: Flowering stage
C: Pod maturation stage

** Significant at 1% level

Table 3. Yield parameters of *Indigofera tinctoria* at pre flowering, flowering and pod maturation stages

Sl. No.	Accession No.	Leaf weight (g)						Shoot weight (g)					
		A		B		C		A		B		C	
		F	D	F	D	F	D	F	D	F	D	F	D
1	IT 1	42.53	21.70	155.13	76.53	127.33	62.70	128.43	67.95	346.50	171.43	809.73	408.67
2	IT 2	36.70	18.60	135.53	66.70	118.47	59.26	98.63	52.20	264.80	130.50	714.43	358.98
3	IT 3	34.13	17.53	128.57	63.60	109.07	54.40	69.33	36.83	175.63	85.68	395.17	197.96
4	IT 4	39.73	20.30	144.07	70.60	118.53	57.60	113.83	60.33	298.77	146.15	623.87	313.20
5	IT 5	38.10	19.50	139.37	68.50	113.70	57.70	105.40	54.57	271.33	132.69	685.67	344.80
6	IT 6	37.93	19.33	133.23	65.50	112.00	56.93	126.40	66.87	339.30	166.10	801.87	402.17
7	IT 7	38.43	19.80	135.07	66.20	110.03	56.47	93.70	49.57	250.57	123.18	643.43	322.70
8	IT 8	32.6	17.37	128.73	63.00	109.47	54.23	64.97	34.27	160.87	79.62	398.07	200.38
9	IT 9	41.57	19.87	149.53	72.67	124.10	61.23	122.93	65.03	334.20	164.40	684.60	343.20
10	IT 10	34.03	17.30	113.83	55.77	96.50	49.13	116.50	61.63	315.97	155.37	606.27	304.67
11	IT 11	39.40	20.07	152.70	72.93	132.37	67.30	129.40	68.75	351.00	172.13	643.13	322.77
12	IT 12	23.07	11.70	96.10	47.30	77.70	39.07	88.93	47.09	233.97	114.92	516.87	260.42
13	IT 13	46.43	23.60	113.93	82.83	145.20	74.43	133.33	70.54	361.93	177.00	717.00	360.79
14	IT 14	35.17	17.07	126.70	62.17	109.80	54.10	118.67	62.79	323.90	159.12	696.97	350.59
15	IT 15	21.50	10.97	90.37	43.43	60.03	29.90	77.40	40.95	189.70	72.48	535.10	259.77
16	IT 16	39.17	21.70	150.43	72.33	117.77	57.33	131.37	69.51	356.37	175.82	794.63	399.16
17	IT 17	42.50	21.50	154.37	73.03	118.93	55.30	121.50	64.28	331.50	162.66	641.53	322.17
18	IT 18	31.03	15.33	127.47	62.37	96.43	48.33	74.67	39.51	183.03	91.15	475.47	238.96
19	IT 19	31.53	16.10	108.37	53.23	78.47	39.70	61.80	32.69	157.50	76.63	423.10	212.21
20	IT 20	37.43	19.10	114.13	70.60	114.9	55.50	108.90	57.62	285.43	141.25	597.77	299.70
Mean		36.15	18.42	131.38	65.47	109.54	54.53	104.31	55.15	276.61	134.92	620.23	311.16
F ratio		13.32**	4.80**	43.66**	65.76**	32.24**	89.26**	2152.04**	4792.2**	19441.9**	7533.55**	89528.61**	19035.83**
CD		4.81	4.14	8.17	3.42	9.98	3.00	1.48	0.52	1.45	1.20	1.22	1.34

A: Pre-flowering stage
B: Flowering stage
C: Pod maturation stage

F: Fresh weight
D: Dry weight

** Significant at 1 per cent level

Contd…

Table 3. Continued

Sl. No.	Accession No.	Pod weight (g)		Seed weight (g)		Root weight (g)					
		C		C		A		B		C	
		F	D	F	D	F	D	F	D	F	D
1	IT 1	131.37	45.47	44.50	16.17	31.47	11.30	55.00	25.57	51.37	22.33
2	IT 2	103.03	36.33	37.60	13.97	33.2	12.33	50.43	23.37	46.80	20.27
3	IT 3	86.57	29.37	31.53	11.97	34.27	13.37	57.27	25.80	54.50	26.87
4	IT 4	156.23	55.03	54.10	20.63	37.27	15.23	59.23	28.60	55.80	22.80
5	IT 5	190.27	66.13	66.90	25.47	45.63	22.27	71.23	46.00	66.73	42.67
6	IT 6	128.33	44.20	44.13	16.97	44.00	19.57	67.20	41.90	56.27	39.23
7	IT 7	230.77	80.03	80.10	31.17	45.50	21.33	70.07	40.67	64.83	37.13
8	IT 8	280.93	98.07	96.20	37.27	39.07	15.43	59.60	27.67	54.97	24.20
9	IT 9	243.17	85.23	82.07	32.00	40.97	16.33	60.00	33.10	57.57	27.37
10	IT 10	133.30	46.27	43.57	17.03	43.53	18.33	71.27	44.43	67.20	40.97
11	IT 11	147.37	51.70	51.57	20.33	47.43	20.37	69.20	43.87	64.53	41.20
12	IT 12	101.60	35.27	34.53	13.73	48.43	22.30	77.03	47.10	72.50	44.77
13	IT 13	168.50	58.20	44.93	22.07	50.03	25.43	75.37	42.33	71.47	38.73
14	IT 14	80.20	26.33	24.00	9.17	42.27	18.03	69.50	36.57	63.17	33.17
15	IT 15	113.30	42.65	40.23	13.47	36.23	14.07	59.23	27.87	54.70	25.53
16	IT 16	219.67	78.90	75.77	29.13	41.37	15.10	61.53	31.50	53.77	27.17
17	IT 17	191.13	43.97	43.50	16.93	35.27	11.07	54.53	25.63	48.67	24.10
18	IT 18	160.03	56.43	54.80	20.93	35.30	13.60	55.37	23.17	43.00	21.50
19	IT 19	176.40	62.73	63.60	24.30	37.77	16.60	59.57	30.33	54.87	25.20
20	IT 20	80.97	31.43	28.60	11.13	43.20	19.30	62.83	36.23	58.53	32.13
Mean		156.16	53.69	52.11	20.19	40.63	17.07	63.27	34.09	58.06	30.87
F ratio		47.57**	487.99**	116.03**	209.6**	117.39**	223.55**	98.25**	86.14**	48.93**	51.32**
CD		23.6	2.57	5.2	1.52	1.43	0.77	2.17	2.52	3.27	3.24

A: Pre-flowering stage
B: Flowering stage
C: Pod maturation stage

F: Fresh weight (g)
D: Dry weight (g)

** significant at 1% level

Table 4. Root nodule characteristics of *Indigofera tinctoria* at pre-flowering and flowering stages

Sl. No.	Accession No.	No. of root nodules		No. of effective root nodule		Root nodule weight (g)			
						A		B	
		A	B	A	B	F	D	F	D
1	IT 1	22.00	41.00	7.00	18.33	0.3317	0.0150	0.6167	0.2767
2	IT 2	23.00	73.33	8.00	31.67	0.3467	0.1567	1.0967	0.5000
3	IT 3	22.67	65.00	7.00	30.67	0.3233	0.1567	1.0633	0.4400
4	IT 4	18.00	67.67	8.00	33.67	0.2667	0.1200	1.0167	0.4600
5	IT 5	18.33	76.33	7.67	34.33	0.2800	0.1167	1.1467	0.5167
6	IT 6	18.67	62.67	8.00	24.67	0.2833	0.1267	0.9433	0.4267
7	IT 7	18.00	56.00	6.33	22.33	0.2700	0.1500	0.8433	0.3467
8	IT 8	32.33	96.67	11.67	42.33	0.4833	0.2000	1.4667	0.6600
9	IT 9	26.00	65.33	12.67	26.33	0.3433	0.1433	0.9833	0.4433
10	IT 10	15.00	36.33	7.00	11.33	0.2700	0.1333	0.5467	0.2467
11	IT 11	22.00	60.67	9.00	31.00	0.3500	0.1533	0.9133	0.4133
12	IT 12	20.33	49.00	8.00	20.00	0.3200	0.1500	0.7400	0.3333
13	IT 13	23.00	61.67	6.00	33.67	0.3600	0.1500	0.9267	0.4200
14	IT 14	20.00	52.67	4.33	18.67	0.3033	0.1633	0.7900	0.3567
15	IT 15	29.67	80.67	8.33	39.33	0.4533	0.2267	1.2100	0.5367
16	IT 16	41.00	117.00	14.33	57.67	0.6000	0.2433	1.7567	0.7967
17	IT 17	26.33	66.67	8.33	19.33	0.4000	0.1767	1.0033	0.4567
18	IT 18	30.00	74.67	10.00	18.33	0.4133	0.1600	1.1200	0.5067
19	IT 19	15.67	45.00	7.00	16.33	0.2533	0.1300	0.6667	0.3033
20	IT 20	22.33	71.67	7.33	28.67	0.3633	0.1633	1.0767	0.4867
Mean		23.22	66	8.3	27.92	0.35	0.1585	1.00	0.45
F ratio		13.70**	49.08**	4.95**	57.66**	30.20**	4.31**	51.33**	62.75**
CD		1.70	7.60	3.00	4.08	0.004	0.004	0.11	0.004

A: Pre-flowering stage
B: Flowering stage
C: Pod maturation stage

F: Fresh weight (g)
D: Dry weight (g)

** Significant at 1%

spread of 113.40 cm and IT 4 showed the minimum plant spread of 51.67 cm during the flowering stage. In the pod maturation stage the variation between the plant spread ranged from 96.13 cm in accession IT 2 to 143.10 cm in IT 6.

Height at first branching

Among the accessions, the height at first branching ranged from 4.20 cm (IT 12) to 8.67 cm (IT 15).

Number of branches

The maximum number of branches during the pre flowering, flowering and pod maturation stages was observed for the accessions IT 13 (16, 45.67 and 75), while the minimum number of branches during these stages was observed for IT 15 (7.33), IT 3 (27.67) and IT 12 (52.67) respectively.

Girth of stem

The girth of stem during pre flowering stage varied between 1.67 cm (IT 20) and 3.37 cm (IT 16). During the flowering stage the range observed was between 2.73 cm (IT 9) and 4.53 cm (IT 14). In the pod maturation stage, the stem girth varied between 5.50 cm (IT 10) and 7.27 cm (IT 6).

Length of leaves

Length of leaves was found to vary between 9.70 cm (IT 18) to 11.47 cm (IT 3).

Breadth of leaves

Mean leaf breadth exhibited a range of 3.8 cm (IT 19) to 4.53 (IT 2) cm.

Number of leaves

The number of leaves produced was greatest for the accession IT 13 for pre flowering, flowering and pod maturation stage (141, 579.67 and

499.67) and minimum for the accession IT 15 (65.33, 301 and 209.33) during the three stages.

Leaf area

Among the 20 accessions studied the maximum leaf area was found for accession IT 16 (2037.17 cm^2) and the minimum for IT 15 (1029.23 cm^2) in the pre flowering stage. In the flowering and pod maturation stage the maximum leaf area was for IT 13 (7769.70 and 6987.10 cm^2), while the minimum leaf area was for IT 15 (4052.90 and 2805.03 cm^2).

4.1.2 Mean Performance of Root Characters

Root length

The accession IT 12 produced the longest root (43.73 cm) while the accession IT 2 produced the shortest root (24.17 cm) in the pre flowering stage. In the flowering stage, root length was maximum in IT 13 (50.13 cm) and minimum in IT 1 (27.33 cm). In the pod maturation stage the maximum and minimum root length was observed for the accessions IT 13 (54.33 cm) and IT 2 (32.90 cm) respectively.

Root girth at collar region

At the pre flowering stage mean root girth at the collar region exhibited a range of 1.87 (IT 20) to 3.77 cm (IT 19). In the flowering and pod maturation stages the maximum root girth at the collar region was found in IT 14 (5.13 cm) and IT 6 (7.60 cm) and minimum in IT 2 (3.10 cm) and IT 10 (6.07 cm) respectively.

Shell Weight

Maximum shell weight was recorded for the accession IT 8 (58.97 g) while the minimum shell weight was recorded for IT 3 (17.63 g).

4.1.3 Mean Performance of Yield Characters

The leaf yield, shoot yield, root yield and pod yield are shown in Fig. 1, 2, 3 and 4 respectively.

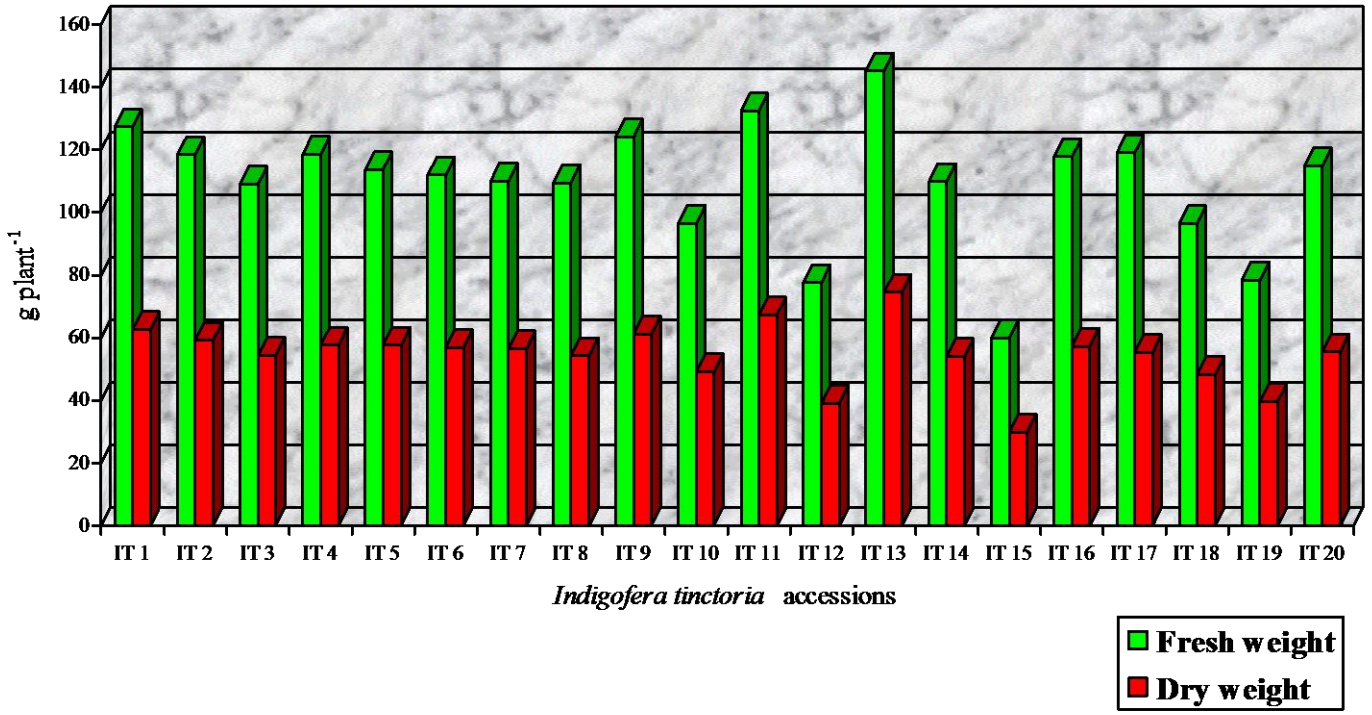


Fig. 1. Leaf yield of 20 accessions of *Indigofera tinctoria* at pod maturation stage

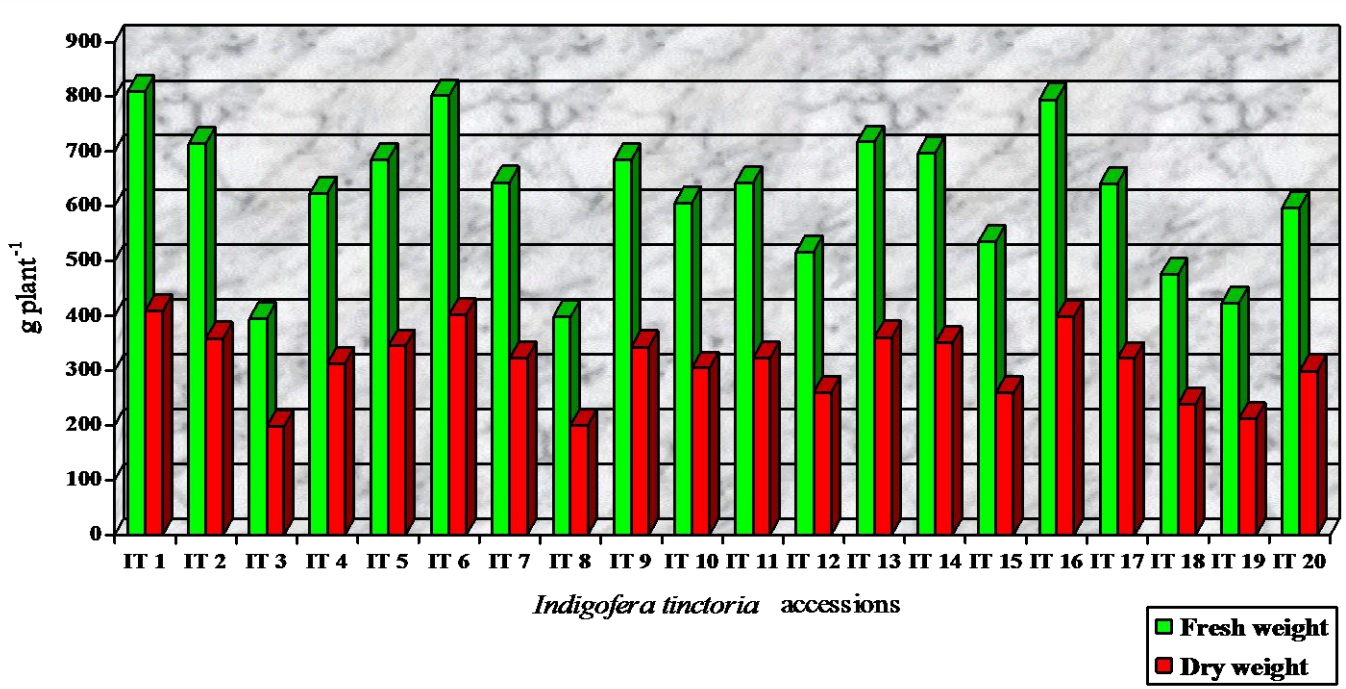


Fig. 2. Shoot yield of 20 accessions of *Indigofera tinctoria* at pod maturation stage

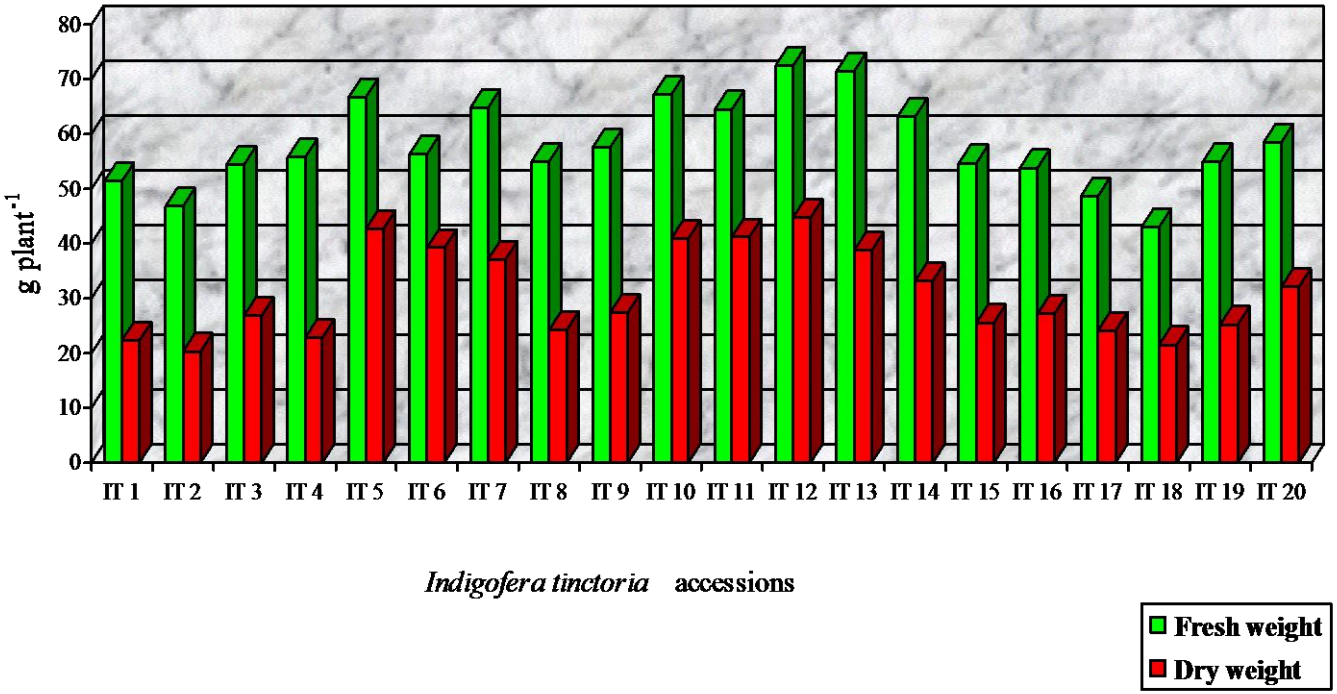


Fig. 3. Root yield of 20 accessions of *Indigofera tinctoria* at pod maturation stage

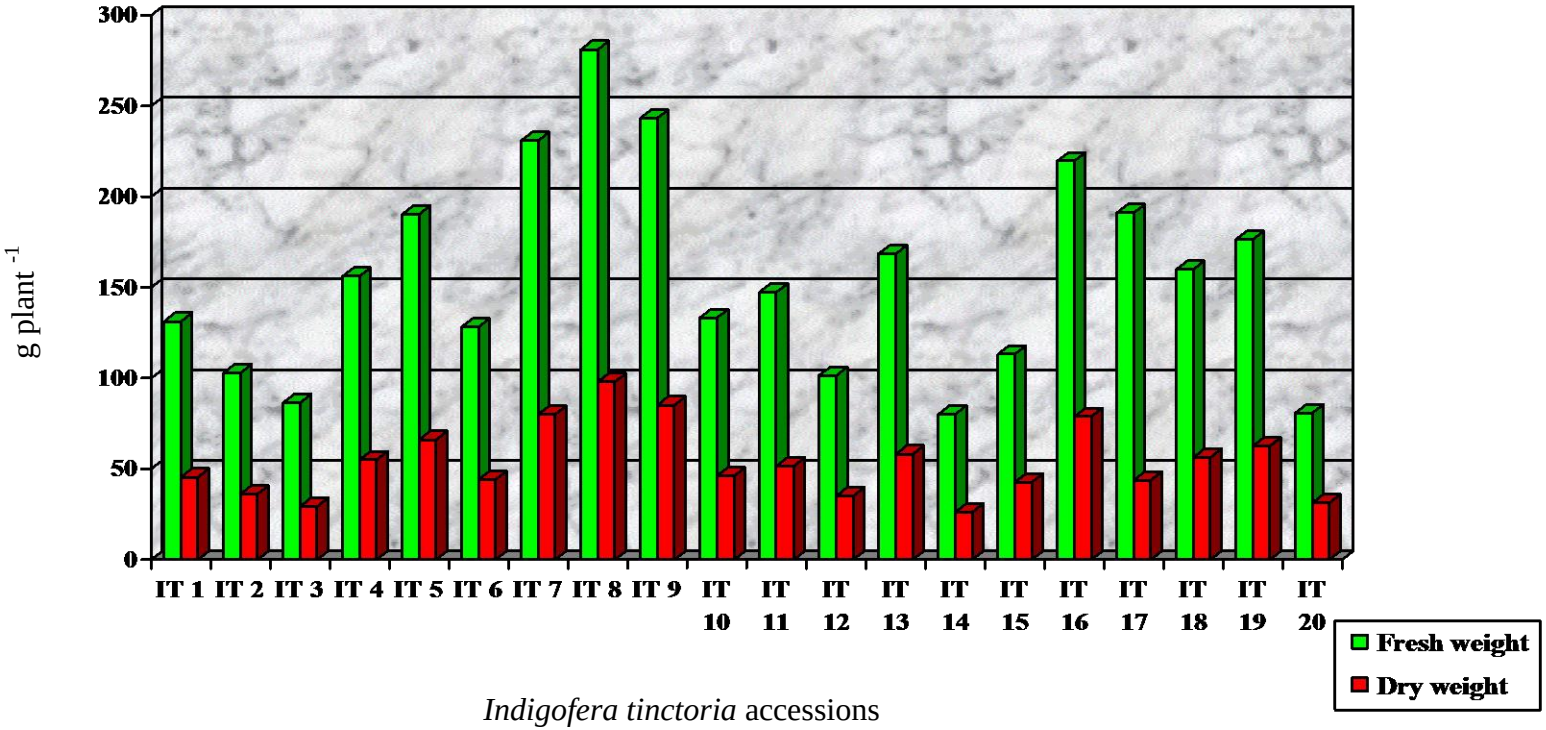


Fig. 4. Pod yield of 20 accessions of *Indigofera tinctoria*

Fresh weight of leaves

Range in the fresh weight of leaves was from 21.50 g (IT 15) to 46.43 g (IT 13) in the pre flowering stage, 90.37 g (IT 15) to 155.13 g (IT 1) in the flowering stage and 60.03 g (IT 15) to145.2 g (IT 13) in the pod maturation stage.

Dry weight of leaves

Range in the dry weight of leaves was from 10.97 g (IT 15) to 23.6 g (IT 13) in the pre flowering stage, 43.43 g (IT 15) to 82.83 g (IT 13) in the flowering stage and 29.9 g (IT 15) to 74.43 g (IT 13) in the pod maturation stage.

Fresh weight of shoots

Among the twenty accessions, the fresh weight of shoot was greatest for the accession IT 13 for pre flowering and flowering (133.30, 361.93 g) stages and for IT 1 (809.73 g) in the pod maturation stage. The shoot fresh weight was minimum for the accession IT 19 in the pre flowering and flowering stage (61.80 and 157.50 g) and for IT 3 (395.17 g) in the pod maturation stage.

Dry weight of shoots

Dry weight of shoot was found to vary between 32.69 g (IT 19) to 70.54 g (IT 13) in the pre flowering stage. In the flowering stage the range was between 72.48 (IT 15) and 177 g (IT 13) and in the pod maturation stage the range shown was between 197.96 (IT 3) and 408.67 g (IT 1).

Fresh weight of pods

The fresh weight of the pods was highest for the accession IT 8 (280.93 g) and lowest for the accession IT 14 (80.20 g) during the pod maturation stage.

Dry weight of pods

Among the twenty accessions maximum pod dry weight was recorded for the accession IT 8 (98.07 g) and the accession IT 14 recorded the minimum dry pod weight of 26.33 g.

Fresh weight of seeds

The fresh weight of the seeds was highest for the accession IT 8 (96.2 g) and lowest for the accession IT 14 (24 g).

Dry weight of seeds

The highest dry seed weight was recorded for the accession IT 8 (37.27 g) and lowest for the accession IT 14 (9.17 g).

Fresh weight of roots

At the pre flowering stage the mean fresh root weight of the twenty accessions exhibited a range of 31.47 to 50.03 g with the maximum in accession IT 13 and minimum in accession IT 1. In the flowering and pod maturation stages the maximum root fresh weight was found in IT 12 (77.03 and 72.50 g respectively) and minimum in IT 2 (50.43 g) and IT 18 (43.0 g) respectively.

Dry weight of roots

Dry weight of shoot was found to vary between 11.07g (IT 17) to 25.43 g (IT 13) in the pre flowering stage. In the flowering stage the range was between 23.17 (IT 18) and 47.10 g (IT 12) and in the pod maturation stage the range shown was between 20.27 (IT 2) and 44.77 g (IT 12).

4.1.5 Root nodule Characters

Number of root nodules

The number of root nodule produced was greatest in the accession IT 16 for pre flowering and flowering stage (41 and 117) and minimum for the accession IT 10 (15 and 36.33) during these stages.

Number of effective root nodules

The highest number of effective root nodule during the flowering and pre flowering stage was produced by the accessions IT 16 (14.3 and 57.67) and the lowest number was found in the accession IT 14 (4.33) during the pre flowering stage and in IT 10 (11.33) during the flowering stage.

Fresh weight of root nodule

The highest fresh weight of the root nodule during the pre flowering and flowering stages was observed for the accessions IT 16 (0.600 g and 1.757 g) while the lowest fresh weight of the root nodule was observed for IT 19 (0.253 g) and IT 10 (0.547 g) respectively.

Dry weight of root nodule

Dry weight of root nodule was found to vary between 0.117 g (IT 5) to 0.243 g (IT 16) in the pre flowering stage. In the flowering stage the range was between 0.247 g (IT 10) and 0.797 g (IT 16).

4.1.6 Floral Characters

Floral characters such as number of days for 50 per cent flowering, time of anthesis, time of anther dehiscence and number of pollen grains per anther is shown in Table 5.

Number of days for 50 percent flowering

Number of days for 50 per cent flowering ranged from 109.63 to 125.27 days. The accession IT7 flowered early while the accession IT 14 flowered last.

Time of anthesis

Time of anthesis was between 9.10 a.m. (IT 7) to 11.10 a.m. (IT 8).

Time of anther dehiscence

Time of anther dehiscence was between 3.10 am (IT 6) and 5.15 am (IT 7).

Table 5. Floral characteristics of *Indigofera tinctoria*

Sl. No.	Accession No.	No. of days for 50% flowering	Time of anthesis (a. m.)	Time of anther dehiscence (a. m.)	Pollen grains anther $^{-1}$
1	IT 1	123.75	10.55	4.55	130000
2	IT 2	121.63	10.45	4.45	114000
3	IT 3	120.63	10.30	4.30	129000
4	IT 4	121.63	10.05	4.50	108000
5	IT 5	123.38	10.55	4.55	126000
6	IT 6	124.38	10.05	3.10	62000
7	IT 7	109.63	9.10	5.15	110000
8	IT 8	121.38	11.10	5.05	106000
9	IT 9	122.63	10.50	4.51	108000
10	IT 10	121.63	10.50	4.45	124000
11	IT 11	122.88	9.10	3.15	100000
12	IT 12	122.63	10.15	4.15	168000
13	IT 13	121.88	10.15	4.15	76000
14	IT 14	125.27	10.50	4.50	12000
15	IT 15	121.94	10.40	4.40	96000
16	IT 16	123.38	10.15	4.15	118000
17	IT 17	125.18	9.55	3.55	196000
18	IT 18	121.63	9.20	3.25	137000
19	IT 19	122.89	9.15	3.15	154000
20	IT 20	124.38	9.20	3.20	136000

Number of pollen grains produced per anther

Number of pollen grains produced per anther ranged from 12,000 in accession IT 14 to 196000 in accession IT 17.

4.2 GENETIC VARIABILITY IN *INDIGOFERA TINCTORIA*

4.2.1 Variability Studies

The mean, range, phenotypic, genotypic and environmental variances, phenotypic and genotypic coefficients of variations of plant characters such as plant height, plant spread, number of branches, number of leaves, leaf area, root length, fresh weight of leaves, fresh weight of shoots, fresh weight of pods, fresh weight of roots and number of effective root nodule are presented in Table 6.

Leaf area exhibited the highest phenotypic and genotypic variance followed by shoot fresh weight. A close association between phenotypic and genotypic variance was observed for characters such as plant height, plant spread, root length and fresh weight of shoot. Wide variations were found in number of leaves, leaf area and leaf and pod fresh weight.

The highest phenotypic and genotypic coefficient of variation was observed for the number of effective root nodule (39.453 and 38.448 respectively) followed by fresh weight of pods (37.220 and 36.076) respectively. The lowest PCV (10.414) and GCV (9.819) were exhibited by number of branches followed by plant spread (10.804 and 10.800) (Fig. 5).

4.2.2 Correlation Analysis

The phenotypic, genotypic and environmental correlation coefficients were estimated for 15 characters *viz.* plant height, plant spread, number of branches, number of leaves, leaf area, root length, fresh weight of leaves, fresh weight of shoots, fresh weight of pods, fresh weight of roots and number of effective root nodule (Tables 7, 8 and 9 respectively).

Table 6. Range, mean, phenotypic (σp^2), genotypic (σg^2) and environmental (σe^2) variances and coefficients of variation for different characters in *Indigofera tinctoria*

Sl. No	Characters	Range	Mean ± SEm	σp^2	σg^2	σe^2	PCV	GCV
1	Plant height (cm)	132.7 – 221.4	178.47 ± 0.7792	527.853	526.031	1.8219	12.87334	12.8511
2	Plant spread (cm)	96.13 – 143.1	121.05 ± 0.2116	171.020	170.886	0.1344	10.80364	10.79943
3	No. of branches	52.67 – 75	62.17 ± 1.2539	41.910	37.194	4.7168	10.41366	9.810175
4	No. of leaves	209.33 – 499.67	378.32 ± 5.8409	4586.356	4484.006	102.3500	17.90105	17.70018
5	Leaf area (cm^2)	2805.03 – 6987.1	5109.58 ± 144.5877	937993.100	875276.3	62716.8000	18.9546	18.30996
6	Root length (cm)	32.9 – 54.33	44.55± 0.5980	38.407	37.334	1.0729	13.9104	13.71474
7	Fresh weight of leaves (g)	60.03 – 145.2	109.54 ± 3.4901	417.123	380.580	36.5438	18.64488	17.70018
8	Fresh weight of shoots (g)	395.17 – 809.73	620.23 ± 0.4282	16413.950	16413.4	0.5500	20.65624	20.6559
9	Fresh weight of pods (g)	80.2 – 280.93	156.16 ± 8.2553	3378.059	3173.609	204.4500	37.21971	36.07581
10	Fresh weight of root (g)	43 – 72.5	58.06 ± 1.1448	66.750	62.818	3.9320	14.07141	13.65067
11	No. of effective root nodule	11.33 – 57.67	27.92 ± 1.6980	121.305	115.205	6.1000	39.45261	38.44785

Table 7. Phenotypic correlation coefficients among yield and its components

Characters		X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11
Plant height (cm)	(X1)	1.0000										
Plant spread (cm)	(X2)	0.3883**	1.0000									
No. of branches	(X3)	-0.0818	-0.2357	1.0000								
No. of leaves	(X4)	-0.1935	-0.2027	0.5025**	1.0000							
Leaf area (cm^2)	(X5)	-0.3042**	-0.2377	0.4866**	0.9432**	1.0000						
Root length (cm)	(X6)	-0.3124**	-0.0693	0.2040	-0.0871	-0.0346	1.0000					
Fresh weight of leaves (g)	(X7)	-0.2122	-0.2005	0.4889**	0.9955**	0.9479**	-0.0823	1.0000				
Fresh weight of shoots (g)	(X8)	-0.2817*	-0.3463**	0.4616**	0.5646**	0.5291**	0.1698	0.5632**	1.0000			
Fresh weight of pods (g)	(X9)	0.1833	-0.2472*	0.0868	0.2079	0.2478*	0.0427	0.2069	-0.0245	1.0000		
Fresh weight of roots (g)	(X10)	-0.3689**	-0.1756	0.2227	0.0632	0.1283	0.8023**	0.0606	0.0943	-0.0516	1.0000	
No. of effective root nodules	(X11)	-0.0777	-0.1676	0.2505*	0.1707	0.1660	-0.0998	0.1767	0.1107	0.3120**	-0.0879	1.0000

** Significant at 1 % level
* Significant at 5 % level

Table 8. Genotypic correlation coefficients among yield and its components

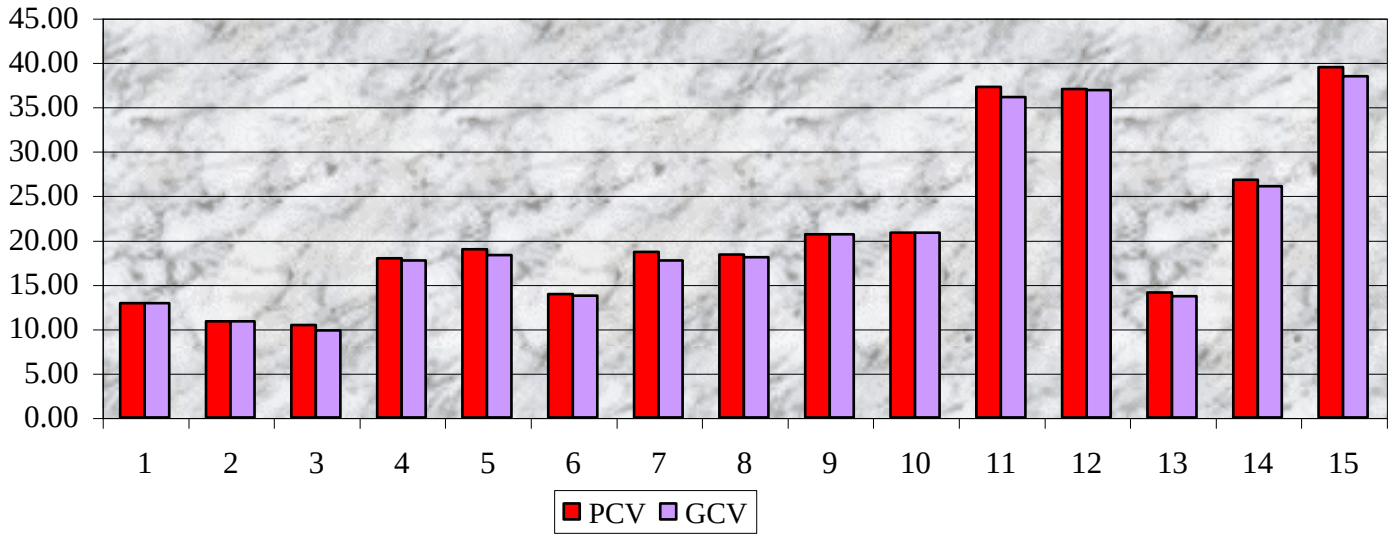
Characters		X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11
Plant height (cm)	(X1)	1.0000										
Plant spread (cm)	(X2)	0.3890	1.0000									
No. of branches	(X3)	-0.0829	-0.2502	1.0000								
No. of leaves	(X4)	-0.1974	-0.2035	0.5341	1.0000							
Leaf area (cm^2)	(X5)	-0.3163	-0.2454	0.5127	0.9760	1.0000						
Root length (cm)	(X6)	-0.3157	-0.0704	0.2245	-0.0860	-0.0267	1.0000					
Fresh weight of leaves (g)	(X7)	-0.2131	-0.2009	0.5217	0.9996	0.9840	-0.0831	1.0000				
Fresh weight of shoots (g)	(X8)	-0.2821	-0.3464	0.4912	0.5685	0.5457	0.1722	0.5658	1.0000			
Fresh weight of pods (g)	(X9)	0.1931	-0.2563	0.1068	0.2232	0.2820	0.0387	0.2208	-0.0258	1.0000		
Fresh weight of roots (g)	(X10)	-0.3803	-0.1795	0.2408	0.0602	0.1273	0.8426	0.0592	0.0969	-0.0535	1.0000	
No. of effective root nodules	(X11)	-0.0811	-0.1718	0.2722	0.1771	0.1640	-0.1002	0.1821	0.1133	0.3252	-0.0859	1.0000

** Significant at 1 % level
* Significant at 5 % level

Table 9. Environmental correlation coefficients among yield and its components

Characters		X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11
Plant height (cm)	(X1)	1.0000										
Plant spread (cm)	(X2)	0.0593	1.0000									
No. of branches	(X3)	-0.2089	-0.0722	1.0000								
No. of leaves	(X4)	0.3405	-0.2084	0.1011	1.0000							
Leaf area (cm^2)	(X5)	0.1553	0.0316	0.2309	0.1064	1.0000						
Root length (cm)	(X6)	-0.1684	0.0260	-0.0712	-0.1524	-0.2269	1.0000					
Fresh weight of leaves (g)	(X7)	-0.0752	-0.2226	0.0258	0.6406	-0.0965	-0.0419	1.0000				
Fresh weight of shoots (g)	(X9)	-0.2279	0.0976	0.0112	-0.0890	-0.1058	-0.0322	-0.1503	1.0000			
Fresh weight of pods (g)	(X11)	-0.2627	0.1904	-0.1262	-0.2519	-0.2925	0.1427	-0.2705	0.3356	1.0000		
Fresh weight of roots (g)	(X13)	0.0538	-0.1541	0.0311	0.1887	0.1446	-0.1699	0.1509	-0.0088	-0.0195	1.0000	
No. of effective root nodules	(X15)	0.1247	0.0597	0.0041	-0.0518	0.2058	-0.0930	-0.0268	-0.0758	0.0708	-0.1287	1.0000

** Significant at 1 % level
* Significant at 5 % level



1: Plant height
2: Plant spread
3: No. of branches
4: No. of leaves
5: Leaf area
6 : Root length
7 : Fresh weight of leaves
8 : Dry weight of leaves
9 : Fresh weight of shoots
10: Dry weight of shoots
11: Fresh weight of pods
12: Dry weight of pods
13: Fresh weight of roots
14: Dry weight of roots
15: No. of effective root nodule

Fig. 5. PCV and GCV of selected characters of *Indigofera tinctoria*

Phenotypic correlation

A high positive correlation was observed between plant height and plant spread (0.3883) followed by fresh weight of pods (0.1833), whereas fresh weight of roots (-0.3689), root length (-0.3124) and leaf area (-0.3042) showed negative correlation with plant height.

Plant spread has negative correlation with all characters studied.

Number of branches per plant exhibited high positive correlation with number of leaves per plant (0.5025), fresh weight of leaves (0.4889), leaf area (0.4866), and fresh weight (0.4616) of shoots. It was also positively correlated with root length, fresh weight of pods, fresh weight of roots and number of effective root nodules per plant.

Number of leaves per plant was positively correlated with fresh weight of leaves (0.9955) followed leaf area (0.9432), and fresh weight (0.5646) of shoots, whereas root length (-0.0871) was negatively correlated with number of leaves per plant.

Correlation analysis of leaf area showed a positive correlation with fresh weight of leaves (0.9479) and fresh weight of shoots (0.5291).

Root length was found to have positive correlation with fresh weight of roots (0.8023), but showed negative correlation with fresh weight of leaves (-0.0823) and number of effective root nodule per plant (-0.0998).

High positive correlation was found between fresh weight of leaves and fresh weight of shoots (0.5632). Fresh weight of shoots was positively correlated with dry weight of shoots (0.9994).

Fresh weight of pods exhibited a high positive correlation with number of effective root nodules (0.3904).

Genotypic Correlation

Plant height showed positive genotypic correlation with plant spread (0.3890), fresh weight of pods (0.1931). It had negative correlation with fresh weight of roots (-0.3803), leaf area (-0.3163) and root length (-0.3157).

Plant spread has negative correlation with all characters studied.

A high positive correlation was recorded for number of branches per plant with number of leaves per plant (0.5341), leaf area (0.5127), fresh weight of shoots (0.4912). It was also positively correlated with root length, fresh weight of pods, fresh weight of roots and number of effective root nodules.

Number of leaves per plant showed high positive genotypic correlation with fresh weight of leaves (0.9996) followed by leaf area (0.9760) and fresh weight of shoots (0.5685).

Leaf area showed high positive genotypic correlation with fresh weight of leaves (0.9840) and fresh weight of shoots (0.5457), but it was negatively correlated with root length (-0.0267). Root length had high positive correlation with fresh weight of roots (0.8426). It was negatively correlated with number of effective root nodules and fresh weight of leaves.

Fresh weight of leaves recorded high positive correlation with fresh weight of shoot (0.5658).

Fresh weight of shoots exhibited high positive correlation with number of effective root nodule per plant.

Fresh weight of roots exhibited negative genotypic correlation with number of effective root nodule per plant (0.1664).

Environmental correlation

Environmental correlation coefficients were found to be negligible among yield and its component characters except for the correlation between fresh weight of shoots and pod fresh weight (0.3356).

4.2.3 Selection Index

A discriminant function analysis was carried out for isolating superior accessions of *Indigofera tinctoria*. The characters selected for the analysis are plant height (X_1), plant spread (X_2), number of branches (X_3), number of

leaves (X_4), leaf area (X_5), root length (X_6), fresh weight of leaves (X_7), dry weight of leaves (X_8), fresh weight of shoots (X_9), dry weight of shoots (X_{10}), fresh weight of pods (X_{11}), dry weight of pods (X_{12}), fresh weight of roots (X_{13}), dry weight of roots (X_{14}) and number of effective root nodule (X_{15}).

The selection index worked out was as follows:

$I = 6.741908\ X_1 + 4.057361\ X_2 + 7.054427\ X_3 - 4.464126\ X_4 + 0.002647484\ X_5 - 10.31294\ X_6 + 26.0696\ X_7 + 74.6475\ X_8 + 9.787668\ X_9 - 16.65474\ X_{10} + 2.634035\ X_{11} + 3.339388\ X_{12} - 4.378023\ X_{13} + 7.412608\ X_{14} - 5.666589\ X_{15}$

The scores obtained for the accessions based on selection index are presented in Table 10.

Based on selection index IT 13 ranked first (24227.74), followed by IT 11 (18552.48) and IT 9 (21000.63). The minimum scores were obtained for IT 15 (9504.365) followed by IT 19 (12310.56).

4.3 MOLECULAR CHARACTERISATION IN *INDIGOFERA TINCTORIA*

4.3.1 Isolation of Genomic DNA

Tender leaves of *Indigofera tinctoria* yielded good quantity of DNA.

4.3.2 Quantification of DNA

The DNA yield from 20 accessions of *Indigofera tinctoria*, estimated using UV-Vis spectrophotometer ranged from 0.51 (IT 5) to 5.85 µg / µl (IT 3). The purity of DNA A_{260} / A_{280} ratio ranged from 1.53 (IT 3) to 2.01 (IT 10). The results are detailed in Table 11.

4.3.3 Agarose Gel Electrophoresis

The quality of the isolated DNA was assessed by gel electrophoresis. The genomic DNAs were run on 0.7 per cent agarose gel stained with ethidium bromide and visualized using ultraviolet transilluminator. Agarose

Table 10. Selection index scores and ranks of *Indigofera tinctoria* accessions

Sl. No.	Accession No.	Selection index scores	Ranks
1	IT 13	24227.74	1
2	IT 11	21986.24	2
3	IT 9	21000.63	3
4	IT 1	20821.89	4
5	IT 16	19795.86	5
6	IT 4	19502.88	6
7	IT 5	19404.40	7
8	IT 6	19396.08	8
9	IT 2	18867.57	9
10	IT 17	18801.94	10
11	IT 7	18552.48	11
12	IT 14	17737.62	12
13	IT 8	17458.48	13
14	IT 20	16561.39	14
15	IT 3	16452.88	15
16	IT 10	15789.64	16
17	IT 18	15330.32	17
18	IT 12	12597.43	18
19	IT 19	12310.56	19
20	IT 15	9504.365	20

Table 11. Quantitative and qualitative characters of DNA isolated from twenty accessions of *Indigofera tinctoria*

Sl. No.	Accession No.	260 nm	280 nm	Ratio $\frac{260}{280}$	DNA yield ($\mu g\ \mu l^{-1}$)
1	IT 1	0.032	0.018	1.78	0.96
2	IT 2	0.143	0.075	1.92	4.29
3	IT 3	0.195	0.128	1.53	5.85
4	IT 4	0.077	0.046	1.68	2.31
5	IT 5	0.017	0.01	1.72	0.51
6	IT 6	0.169	0.088	1.92	5.07
7	IT 7	0.094	0.05	1.86	2.82
8	IT 8	0.13	0.079	1.65	3.9
9	IT 9	0.04	0.021	1.87	1.2
10	IT 10	0.038	0.019	2.01	1.14
11	IT 11	0.096	0.058	1.65	2.88
12	IT 12	0.029	0.016	1.79	0.87
13	IT 13	0.042	0.022	1.95	1.26
14	IT 14	0.083	0.053	1.57	2.49
15	IT 15	0.02	0.012	1.69	0.6
16	IT 16	0.056	0.032	1.75	1.68
17	IT 17	0.072	0.039	1.83	2.16
18	IT 18	0.056	0.029	1.94	1.68
19	IT 19	0.073	0.046	1.58	2.19
20	IT 20	0.08	0.048	1.68	2.4

gel at 1.4 per cent concentration was used for RAPD analysis. Smearing of the bands indicated sheared DNA. In such cases, the isolation procedure was repeated to obtain a single crisp band of DNA.

4.3.4 Random Amplified Polymorphic DNA (RAPD) Analysis

RAPD analysis was performed by the method recommended by Williams *et al.* (1990) with required modification. Forty decamer primers were screened for their efficiency using the DNA isolated from IT 15 as the representative sample. Out of this, twenty-two yielded amplification products. The primers such as OPA-2, OPA-3, OPA-5, OPA-7, OPA-8, OPA-13, OPA-14, OPA-16, OPA-18, OPA-19, OPB-1, OPB-6, OPB-7, OPB-13, OPB-15, OPB-16, OPB-18 and OPB-20 yielded no amplification. The total number of bands, number of faint bands and number of intense bands produced by the forty decamer primers are listed in Table 12.

The primers produced a total of 44 bands, of which the highest number of RAPDs was produced by the primers OPA-10 (6 bands), OPB-3 and OPB-5 (4 bands) and OPB-10 (3 bands). The amplification profiles of the DNA of twenty accessions of *Indigofera tinctoria* using the selected primers, OPA-10, OPB-5, OPB-10 and OPB-3 are presented in Fig. 6, 7, 8 and 9 respectively.

Based on the reproducibility of the bands, level of polymorphism, number and intensity of bands produced the primers OPA-10, OPB-3, OPB-5 and OPB-10 were selected for further PCR amplification of twenty accessions of *Indigofera tinctoria.* The reproducibility of the selected primers was confirmed, by repeating the PCR reaction at least twice and the data obtained from these primers were used for further statistical analysis. Nucleotide sequence of the selected primers and total number of informative RAPD markers amplified with them in the accessions of *Indigofera tinctoria* used in the study is given in Table 13.

Table 12. Primer associated banding patterns in DNA sample of accession IT 15

Sl. No.	Primers	Total number of bands	Number of intense bands	Number of faint bands
1	OPA-01	2	1	1
2	OPA-02	0	0	0
3	OPA-03	0	0	0
4	OPA-04	1	1	0
5	OPA-05	0	0	0
6	OPA-06	1	1	0
7	OPA-07	0	0	0
8	OPA-08	0	0	0
9	OPA-09	1	0	1
10	OPA-10	6	4	2
11	OPA-11	1	0	1
12	OPA-12	1	1	0
13	OPA-13	0	0	0
14	OPA-14	0	0	0
15	OPA-15	2	1	1
16	OPA-16	0	0	0
17	OPA-17	1	0	1
18	OPA-18	0	0	0
19	OPA-19	0	0	0
20	OPA-20	2	1	1
21	OPB-1	0	0	0
22	OPB-2	2	0	2
23	OPB-3	4	2	2
24	OPB-4	2	1	1
25	OPB-5	4	3	1
26	OPB-6	0	0	0
27	OPB-7	0	0	0
28	OPB-8	2	0	2
29	OPB-9	2	1	1
30	OPB-10	3	3	0
31	OPB-11	2	1	1
32	OPB-12	1	0	1
33	OPB-13	0	0	0
34	OPB-14	1	1	0
35	OPB-15	0	0	0
36	OPB-16	0	0	0
37	OPB-17	1	1	0
38	OPB-18	0	0	0
39	OPB-19	2	0	2
40	OPB-20	0	0	0

Table 13. Nucleotide sequence of primers and total number of informative RAPD markers amplified with them in the accessions of *Indigofera tinctoria* used in the study

Sl. No.	Primer	Sequence	No. of informative RAPD markers
1	OPA-10	GTGATCGCAG	8
2	OPB-3	CATCCCCCTG	5
3	OPB-5	TGCGCCCTTC	7
4	OPB-10	CTGCTGGGAC	6

The highest number of scorable bands (8) was obtained from the primer OPA-10. Four bands were monomorphic for all the accession. The accessions IT 3, IT 6, IT 8, IT 10, IT 16, IT 18 and IT 20 produced seven bands each. Six bands were produced by the accessions IT 7, IT 13, IT 14 and IT 15. A total of five bands were produced by the accessions IT 9, IT 12, IT 17 and IT 19. The accessions IT 1, IT 3, IT 5 and IT 11 produced four bands each (Plate 4).

The primer OPB-5 produced a total of seven scorable bands, of which one band was monomorphic to all the accessions. Almost all the accessions produced a total of four bands each except IT 8 and IT 20 which produced a total of three bands and IT 6, IT 18 and IT 20 which produced two bands each. A polymorphic banding pattern was observed in the accession IT 13 (Plate 5).

Primer OPB-10 could amplify a total of six scorable bands for twenty accessions of *Indigofera tinctoria*. The accessions IT 3, IT 7, IT 10, IT 15 and IT 18 produced three bands each, while the accessions IT 1, IT 2 and IT 20 produced four bands each. All the other accessions produced five bands each. Of the six scorable bands, three were monomorphic and the rest were polymorphic (Plate 6).

The primer OPB 3, from a total of five scorable bands produced three monomorphic bands. The accession IT 20 produced five bands, whereas the accessions IT 1, IT 2, IT 3, IT 12, IT 18 and IT 19 produced three bands each. All the other accessions gave four bands each (Plate7).

The amplification profiles of the DNA of twenty accessions of Indigofera tinctoria are presented in Fig. 6, 7, 8 and 9 respectively.

4.3.5 Statistical analysis

Reproducible bands were scored for their presence (+) or absence (-) for all the accessions studied. The data obtained were subjected to cluster analysis by UPGMA method using NTSYS software to estimate the similarity indices and genetic relatedness among the accessions. A genetic

Fig. 6. Representation of the amplification profile of the DNA of the twenty accessions of *Indigofera tinctoria* using the primer OPA – 10

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
-	-	+	-	-	+	-	+	+	+	-	+	+	-	-	+	-	+	+	+
+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
-	-	+	-	-	+	+	+	-	+	-	-	-	+	+	+	+	+	-	+
-	-	+	-	-	+	+	+	-	+	-	-	-	+	+	+	+	+	-	+
+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Fig. 7. Representation of the amplification profile of the DNA of the twenty accessions of *Indigofera tinctoria* using the primer OPB – 5

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
+	+	-	+	+	-	+	-	+	+	+	+	-	+	+	-	+	-	-	-
+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
+	+	+	+	+	-	-	+	+	+	+	+	-	+	+	-	+	-	-	+
-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-

Fig. 8. Representation of the amplification profile of the DNA of the twenty accessions of *Indigofera tinctoria* using the primer OPB – 10

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
-	-	-	+	+	+	-	+	+	-	+	-	-	+	-	+	+	-	+	-
+	+	-	+	+	+	-	+	+	-	+	-	+	+	-	+	+	-	+	+
-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Fig. 9. Representation of the amplification profile of the DNA of the twenty accessions of *Indigofera tinctoria* using the primer OPB – 3

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
+	+	-	+	+	-	+	+	+	-	+	+	+	+	+	+	-	-	+	+
-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
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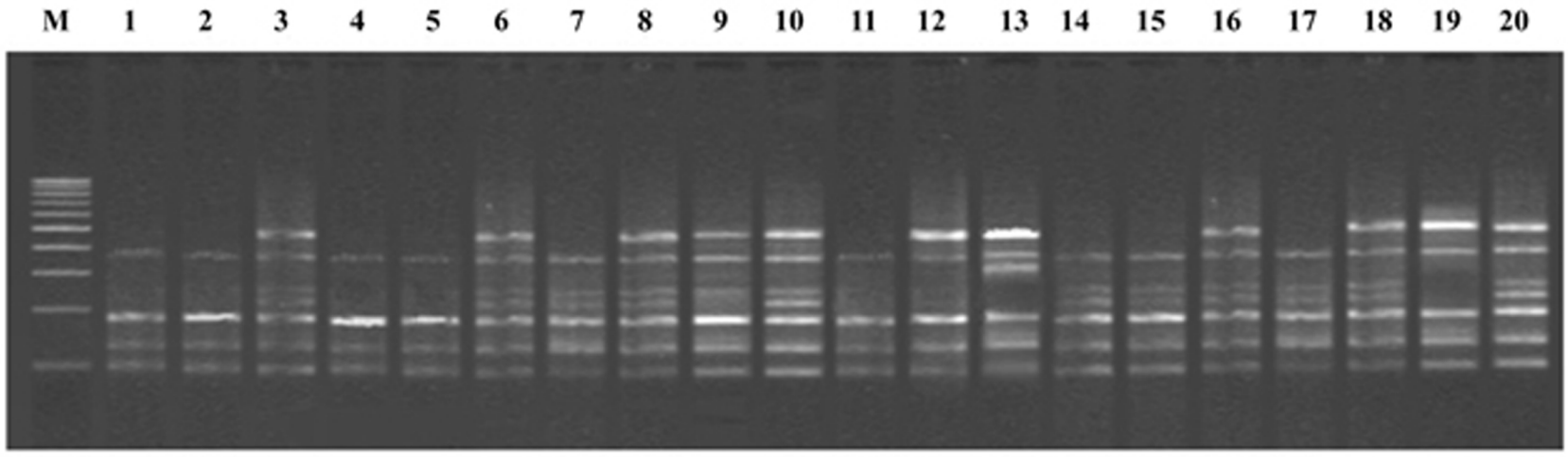
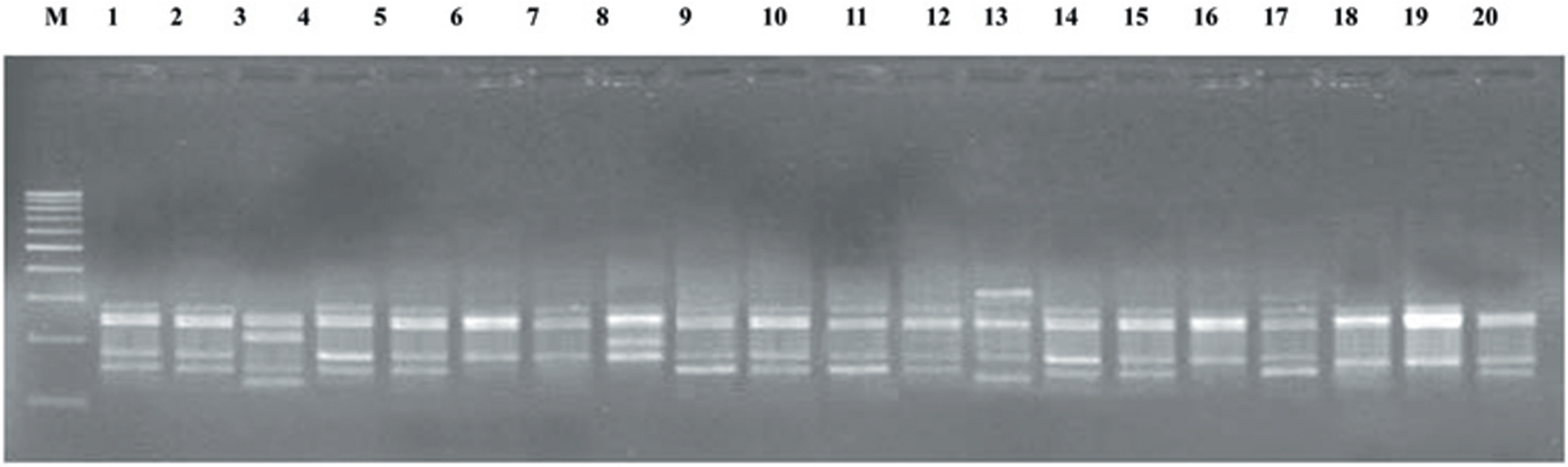


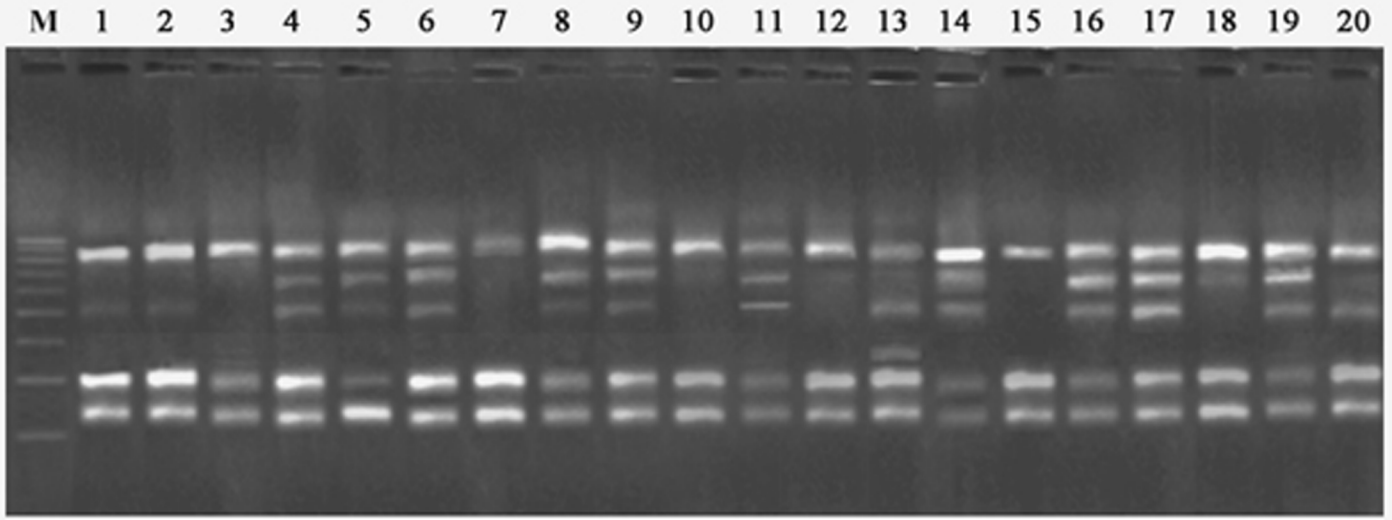
M - Molecular marker

Plate 4 Amplification profiles of the DNA of twenty accessions of *Indigofera tinctoria* using the primer OPA 10



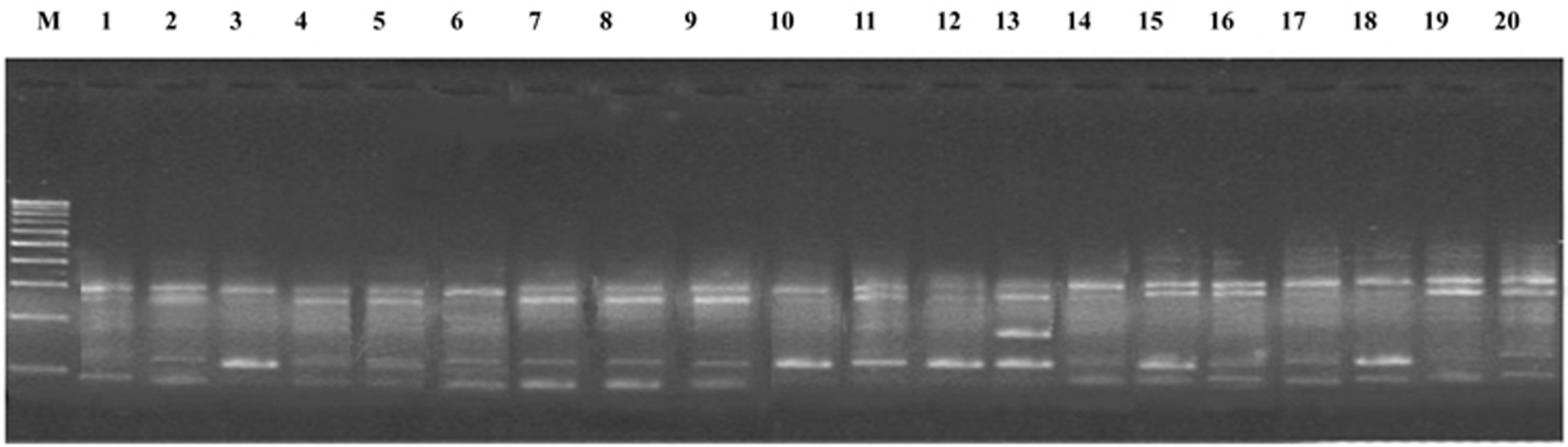
M - Molecular marker

Plate 5 Amplification profiles of the DNA of twenty accessions of *Indigofera tinctoria* using the primer OPB 5



M - Molecular marker

Plate 6 Amplification profiles of the DNA of twenty accessions of *Indigofera tinctoria* using the primer OPB 10



M - Molecular marker

Plate 7 Amplification profiles of the DNA of twenty accessions of *Indigofera tinctoria* using the primer OPB 3

similarity matrix (Table 14) was constructed using the Jaccard's coefficient method (Jaccard, 1908).

The cluster based on RAPD analysis using four primers shows that the similarity indices ranged from 0.46 to 1.00. The twenty accessions that were used for RAPD analysis formed three main clusters in the UPGMA cluster analysis (Fig. 10). At a similarity coefficient of 0.46, the twenty accessions grouped into two clusters, with the accession IT 13 with least similarity coefficients of 0.46 forming a separate cluster, while all the other accessions were grouped into a large cluster. The accessions IT 3, IT 10, IT 12 and IT 15 showed a similarity coefficient of 1.00 with each other. Similarly the accessions IT 6 and IT 17 showed a similarity coefficient of 1.00 with each other. Same is the case with accessions IT1, IT 2, IT 4 and IT 5. At similarity coefficient of 0.784, the dendrogram got divided into five clusters, of which the accessions IT 3, IT 10, IT 12, IT 15, IT 18 and IT 19 formed the first cluster. The second cluster was formed from the accessions IT 17, IT 6, IT 7, IT 16, IT 5, IT 4, IT 2 and IT 1. The three accessions IT 20, IT 8 and IT 14 grouped together to form the third cluster. The fourth cluster had two accessions, namely IT 9 and IT 11. The accession IT 13 formed an individual cluster.

The molecular variability analysis of twenty accessions of *Indigofera tinctoria* revealed that the accession IT 13 was found to be distinct from all the other accessions. The different clusters formed, shows the genetic variability among these accessions.

Table 14. Similarity matrix among twenty accessions of *Indigofera tinctoria* obtained by RAPD analysis using four primer combinations

	IT1	IT2	IT3	IT4	IT5	IT6	IT7	IT8	IT9	IT10	IT11	IT12	IT13	IT14	IT15	IT16	IT17	IT18	IT19	IT20
IT1	1.00																			
IT2	1.00	1.00																		
IT3	0.69	0.69	1.00																	
IT4	1.00	1.00	0.69	1.00																
IT5	1.00	1.00	0.69	1.00	1.00															
IT6	0.85	0.85	0.71	0.85	0.85	1.00														
IT7	0.88	0.88	0.42	0.88	0.88	0.42	1.00													
IT8	0.60	0.60	0.52	0.60	0.60	0.54	0.55	1.00												
IT9	0.65	0.65	0.30	0.65	0.65	0.40	0.40	0.69	1.00											
IT10	0.69	0.69	1.00	0.69	0.69	0.50	0.44	0.52	0.42	1.00										
IT11	0.50	0.50	0.85	0.50	0.50	0.62	0.37	0.69	0.86	0.60	1.00									
IT12	0.69	0.69	1.00	0.69	0.69	0.55	0.33	0.52	0.43	1.00	0.50	1.00								
IT13	0.46	0.46	0.55	0.46	0.46	0.50	0.45	0.39	0.42	0.55	0.42	0.55	1.00							
IT14	0.55	0.55	0.50	0.55	0.55	0.50	0.44	0.90	0.69	0.80	0.69	0.54	0.40	1.00						
IT15	0.69	0.69	1.00	0.69	0.69	0.28	0.16	0.52	0.40	1.00	0.42	1.00	0.55	0.33	1.00					
IT16	0.83	0.83	0.30	0.83	0.83	0.45	0.90	0.57	0.46	0.72	0.54	0.80	0.49	0.58	0.18	1.00				
IT17	0.85	0.85	0.55	0.85	0.85	1.00	0.70	0.54	0.40	0.70	0.50	0.77	0.50	0.70	0.22	0.80	1.00			
IT18	0.66	0.66	0.50	0.66	0.66	0.50	0.44	0.51	0.42	0.80	0.45	0.54	0.43	0.70	0.33	0.58	0.70	1.00		
IT19	0.69	0.69	0.47	0.69	0.69	0.37	0.50	0.53	0.56	0.40	0.50	0.44	0.51	0.55	0.33	0.36	0.44	0.87	1.00	
IT20	0.71	0.71	0.56	0.71	0.71	0.65	0.66	0.82	0.69	0.56	0.69	0.56	0.59	0.82	0.56	0.63	0.65	0.88	0.44	1.00

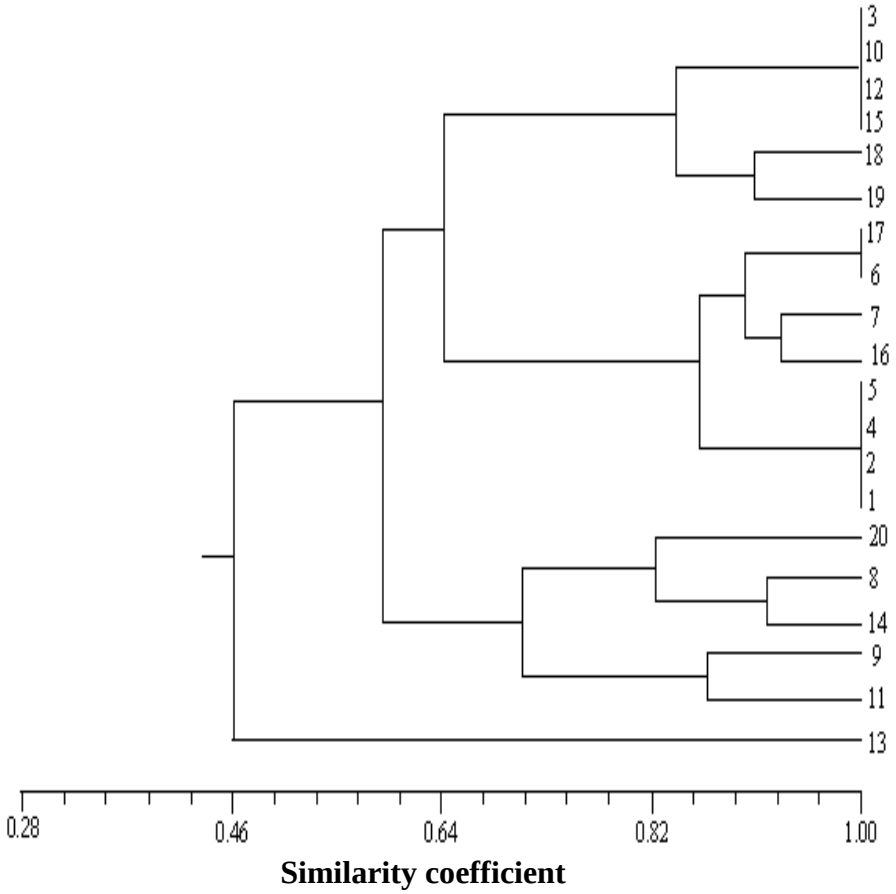


Fig. 10. Dendrogram of RAPD markers for twenty accessions of *Indigofera tinctoria*

DISCUSSION

5. DISCUSSION

Indigofera tinctoria L., commonly known as neelayamari, is an important leguminous medicinal plant in south-eastern Asia. The plant is an important natural source of the blue dye 'indigo'. The leaves form an ingredient in hair tonics, while the roots of the plant are used for the treatment of hepatitis and as an antidote against snake bite poisoning. Despite these useful traits, only very few authentic work is available on the characterization of the accessions at morphological and molecular levels. Molecular characterisation gains importance in the fact that it is not affected by environmental conditions and that it is not time consuming when compared with the former. Therefore the present investigation was carried out with the objective of analyzing the genetic variability of the germplasm of *Indigofera tinctoria* and characterizing them on morphological and molecular basis.

Twenty accessions of *Indigofera tinctoria* collected from different parts of Kerala state were evaluated for morphological and yield parameters. Molecular characterization of these accessions using Random Amplified Polymorphic DNA was also done. The results obtained are discussed in this chapter.

5.1 MEAN PERFORMANCE OF GROWTH PARAMETERS

Analysis of variance showed significant difference among the 20 accessions of *Indigofera tinctoria* for all the characters studied *viz*. plant height, plant spread, height at first branching, number of branches, girth of stem, length and breadth of leaves, number of leaves, leaf area, root length, root girth at collar region, fresh and dry weight of leaves, shoots, roots, pods, number of root nodules, number of effective root nodules, fresh and dry weight of root nodules at all the stages of observation *viz.* pre flowering, flowering and pod maturation stage. This is in accordance with

the observations of Singh *et al*. (2000), Kaushik and Dashora (2001), Kumar and Choudhary (2003) and Solanki *et al*. (2003).

5.1.1 Shoot Characters

Growth parameters namely plant height, plant spread, height at first branching, number of branches, girth of stem, length and breadth of leaves, number of leaves and leaf area showed significant difference among the twenty accessions at all stages of observation namely pre flowering, flowering and pod maturation stages.

The maximum plant length was observed in IT 14 at all stages of observation, but minimum plant height was observed in IT 12 at pre flowering stage and IT 4 at flowering and pod maturation stage. The plant height was found to increase through out the growth stages. Similar results were reported by Kulkarni and Karadge (1991). This indicates that the performance of different accessions differ during various growth stages as reported by Nair (2000).

Maximum plant spread was recorded in IT 1 and IT 16 and IT 6 in the pre flowering and flowering stages, whereas IT 14, IT 4 and IT 2 showed least plant spread during these stages. Variation in plant spread has been attributed to the fact that it is a function of genetic makeup and environmental conditions.

Among the twenty accessions, the lowest height at first branching was shown by IT 12 whereas the first branch at maximum height was observed in IT 15.

In the present study, the maximum branches was found in accession IT 13 during all stages of plant growth, whereas minimum number of branches was produced by IT 15, IT 3 and IT 12 at the three stages of observation. Conspicuous variations were noticed for the number of branches, which is in accordance with the earlier reports of Singh *et al*. (2000).

IT 16, IT 14 and IT 6 recorded maximum stem girth in the three stages of observation while IT 20, IT 9 and IT 6 recorded the lowest stem girth in these stages. This result shows that different accessions exhibited different performance during different stages of growth.

In respect to the length and breadth of leaves, accessions IT 18 and IT 19 produced small leaves, while accessions IT 2, IT 3 and IT 4 produced larger leaves. Number of leaves per plant was highest in IT 13 for all stages of plant growth, while the accession IT 15 showed the lowest number of leaves. An increase in leaf production was found from the pre flowering to the flowering stage, but a decline was found in the seed maturation stage. Nair and Reghunath (2002) and Resmi (2001) observed similar trend in *Clitoria ternatea*. This may be attributed to leaf shedding caused by senescence. In all the accessions the greatest increase in leaf production was between the period of pre flowering and flowering. Variation in number of leaves among the accessions may be attributed to the fact that it is purely a function of genetic make up and environmental conditions.

Leaf area showed significant variation among the accessions at all stages of growth. As in the case of number of leaves, leaf area showed an increasing trend from pre flowering to flowering stage and a declining trend from flowering to seed maturation stage. At pre flowering stage maximum leaf area was recorded for IT 16. At flowering and pod maturation stage, maximum leaf area was recorded for IT 13, where as IT 15 showed minimum leaf area at all the stages. Similar trend was noticed by Nair (2000) and Resmi (2001) in *Clitoria ternatea*.

5.1.2 Root Characters

The accessions IT 12 and IT 2 recorded the longest and shortest root length in pre flowering stage, while in flowering and pod maturation stage, IT 13 produced longest root, IT 1 and IT 2 produced shortest root respectively. The existing variations may be attributed to the genetic make up of the plant. The results revealed that maximum root length was

obtained at the pre flowering stage itself and only very small increase in root length occurs in the flowering and pod maturation stage.

In the case of root girth at collar region, the maximum girth was observed in IT 20, IT 3 and IT 10 at pre flowering, flowering and pod maturation stage respectively, whereas minimum root girth was recorded in IT 19, IT 14 and IT6 for the three stages respectively. The root girth at collar region was found to increase through out the growth period.

5.1.3 Yield Parameters

Maximum fresh and dry weight of leaf was recorded in the accession IT 13 in all the stages, except in pre flowering stage, where the maximum fresh weight was recorded in IT 1. The minimum fresh and dry weight was recorded in the accession IT 15 for all stages of growth. The fresh and dry weight increased during the first two stages, but decreased in the last stage. This can be explained by the production of leaves at all stages, and shedding of leaves at the last stage due to senescence as reported by Samuel (2000).

Considering the fresh and dry weight of shoots, the maximum shoot yield was produced by the accession IT 13 in the pre flowering and flowering stages, but at the pod maturation stage, the accession IT 1 produced maximum shoot. Minimum shoot yield was recorded in the accession IT 19, IT 15 and IT 3 at these stages respectively. The shoot weight was found to increase through out the growth stage. This is in line with the observations of Nair (2000) and Resmi (2001) in *Clitoria ternatea*.

Among the twenty accessions of *Indigofera tinctoria* studied, the accessions IT 13 and IT 12 produced maximum root yield, while IT 1, 2 and 18 were the lowest root yielder. From the data an increasing trend was observed in the fresh and dry weight of roots up to pre flowering and flowering stage and then exhibited a decline towards pod maturation. A

similar rooting pattern was observed in *Clitoria ternatea* by Nair (2000) and Resmi (2001)

5.1.4 Nodule Characters

IT 16 recorded the maximum nodule number at pre flowering and flowering stages. The lowest nodule number was recorded in IT 10 at pre flowering and flowering stages. Nodule number showed an increasing trend up to flowering stage. At pod maturation, nodules were absent. This is due to the degeneration of nodules at this stage.

The fresh and dry weight of nodules was maximum in IT 16 in pre flowering and flowering stages. The least fresh weight was recorded in IT 19 and IT 10 at pre flowering and flowering stages respectively, while least dry weight recorded in IT 5 in pre flowering and IT 10 in flowering stages.

5.1.5 Floral Characters

Days for 50 per cent flowering ranged from 109.63 to 125.27 days. Anitha (1997) reported that *Indigofera tinctoria* needed a mean of 147 days for flowering. The earliness in flowering, when compared can be attributed to the favourable conditions provided.

Time of anthesis and anther dehiscence was in the morning hours (9.10 – 11.10 and 3.10 –5.15 a.m.respectively) in conformity with the studies of Mathew (2002).

Number of pollen grains per anther ranged from 12,000 to 1,96,000.

5.2 GENETIC VARIABILITY

5.2.1 Variability Studies

Attempt has been made to study the genetic variability of different characters such as plant height, plant spread, number of branches, number of leaves, leaf area, root length, weight of leaves, weight of shoots, weight of pods, weight of roots and number of effective root nodules.

Coefficient of variation - PCV and GCV are better indices for comparison of characters with different units of measurement, than estimates of quantitative variations like range and variation around mean.

PCV ranged from 10.414 to 39.453 and GCV ranged from 9.819 to 38.448. The estimates of PCV were higher than that of GCV. This is in conformity with the observations of Singh *et al*. (2000). GCV and PCV were less for plant height, in support to the view of Misra *et al*. (1998). Among the characters studied, leaf yield, number of leaves per plant, number of effective root nodules, weight of pods and weight of shoots and weight of roots had higher PCV and GCV. The observations of Sankaranarayanan *et al*. (1992), Kalamani and Gomez (2001) and Kumar and Choudhary (2003) support this result. Higher GCV and PCV for most of the characters revealed great extent of variability for these characters. Furthermore, the magnitude of genetic variation nearly approaches the phenotypic variations in all the characters, indicating that the selection on phenotypic basis will hold good scope on genotypic upgradation.

5.2.2 Correlation Studies

Correlation coefficient is a statistical measure used to find out the degree and direction of relationship between two or more variables. This helps in understanding the change caused in one character by doing selection based on another character.

In the present study, number of branches, number of leaves, leaf area, weight of shoots, weight of pods and number of effective root nodules showed positive phenotypic and genotypic correlation with leaf yield. Similar reports were published by Natarajaratnam *et al*. (1984), Sankaranarayanan *et al*. (1992), Singh *et al*. (2000) and Misra *et al*. (2000). Among these, number of leaves, leaf area, number of branches and weight of shoots recorded high genotypic correlation. From this it is evident that selection based on these characters will result in higher leaf yield. The

characters plant height and plant spread exhibited negative correlation with leaf yield.

Number of branches, number of leaves, leaf area and leaf weight showed high phenotypic and genotypic correlation with shoot weight. This result showed that selection based on these characters could improve shoot yield, whereas plant height and plant spread recorded negative correlation with shoot weight.

Weight of roots was found highly correlated with root length, so selection based root length is very effective for improving root yield.

Root length and weight of root showed negative correlation with number of effective root nodules, leading to the conclusion that root nodules have no effect on root growth and yield.

Plant height showed positive correlation with pod yield. Similar observation was made by Doss *et al*. (1974) in soybean.

Plant height is positively correlated with plant spread, whereas all other characters studied are negatively correlated with plant height. So selection based on plant height is not effective.

5.2.3 Selection Index

A selection index scores based on plant height, plant spread, number of branches, number of leaves, leaf area, root length, fresh and dry weight of leaves, fresh and dry weight of shoot, fresh and dry weight of pods, fresh and dry weight of roots and number of effective root nodules were used to identify superior genotypes of *Indigofera tinctoria*. An estimation of the discriminant function based on reliable and effective characters is a valuable tool for selection of genotypes. The accession IT 13 showed superior performance followed by IT 11 and IT 9. The accession IT 15 showed the least selection index. Based on selection index the top ranking accessions were identified to be genetically superior from other accessions.

5.3 MOLECULAR CHARACTERISATION

The objective of this study is to estimate the genetic variability among twenty accessions of *Indigofera tinctoria* using Random Amplified Polymorphic DNA analysis.

The use of RAPD markers to genetically finger print plants, which are morphologically similar or indistinguishable, has been established as a reliable, efficient and very informative tool. RAPD analysis has been successfully used to analyse genetic diversity in different medicinal plants as reported by Bradley *et al*. (1996), Farooqui *et al*. (1998), Padmesh *et al*. (1999), Sangwan *et al*. (1999), Khanuja *et al*. (2000), Shasany *et al*. (2000), Zang *et al*. (2001), Darokar *et al*. (2001), Raimondi *et al*.(2001), Sales *et al*. (2001), Vieira *et al*. (2001), Agrimonti *et al*. (2003), Darokar *et al*. (2003), Jain *et al*. (2003), Lal *et al*. (2003a), Shasany *et al*. (2003) and Viera *et al*. (2003). Variability studies in legumes have been made by Yong *et al*. (1997), Mignouna *et al*. (1998), Ahmad (1999), Duarte *et al*. (1999) and Qiang *et al*. (2000).

Tender leaves were used for DNA isolation. Mondal *et al*. (2000) observed that tender leaves contain actively dividing cells with lesser intensity of extra nuclear materials like proteins, carbohydrates and other metabolites that interfere with isolation of nucleic acids which in turn improve the quality of DNA.

The DNA yield of twenty accessions of *Indigofera tinctoria* ranged from 0.51 to 5.85 µg / µl. The purity of DNA A_{260} / A_{280} ratio ranged from 1.53 to 2.01.

Agarose gel electrophoresis was used for analyzing the genomic DNA isolated from different accessions as well as for RAPD products. 0.7 percent agarose gel stained with ethidium bromide was used for visualizing genomic DNA and 1.4 percent was used for RAPD analysis.

Polymerase chain reaction using the method recommended by Williams *et al*. (1990) was done to find the genetic variability among twenty accessions of *Indigofera tinctoria*. The reaction conditions were standardized as an initial denaturation at 94˚C for 4 minutes followed by 40 cycles of denaturation at 94˚C for 1minute, annealing at 35˚C for 1 minute and extended at 72˚C for 1 minute 30 seconds. The synthesis step of the final cycle was extended further by 10 minutes. Forty decamer primers of kit A and B were screened for their efficiency using DNA isolated from IT 15 as the representative sample. Out of this, twenty-two yielded amplification products. The absence of amplification products in the other primers shows that, there is no sequence in them, which are complimentary to the DNA sequence of accession IT 15. According to Prasannalatha *et al*. (1999) the individual primers have the capability to amplify the less conserved and highly repeated regions of genomic DNA.

Based on the reproducibility of the bands, level of polymorphism, number and intensity of bands produced the primers OPA-10, OPB-3, OPB-5 and OPB-10 were selected for further PCR amplification and statistical analysis. The molecular characterisation of seven accessions of *Indigofera tinctoria* done by Jose (2002) revealed that certain distinct and specific bands were produced by the primers OPX-13, OPX-16 and OPX-20. According to him, only those bands which were scorable for their presence and absence in all the seven DNA templates and which were detectable at other template concentration are considered as markers. A total of 26 RAPDs (average 6.5 per primer) were obtained from the four selected primers, of which 17 were polymorphic. The highest number of scorable bands (8) was obtained from the primer OPA-10 of which four bands were monomorphic. The primer OPB-5 produced a total of seven scorable bands, of which one band was monomorphic to all the accessions. Primer OPB-10 could amplify a total of six scorable bands for twenty accessions of *Indigofera tinctoria* among which, three were monomorphic and the rest were polymorphic.

Reproducible bands were scored for their presence (+) or absence (-) for all the accessions studied. The data obtained were subjected to cluster analysis by UPGMA method using NTSYS software to estimate the similarity indices and genetic relatedness among the accessions. Jaccard's similarity coefficient values ranged from 0.46 to 1.00. Cluster analysis revealed that at about 78 per cent genetic similarity the accessions grouped into five clusters. The accessions IT 3, IT 10, IT 12, IT 15, IT 18 and IT 19 formed the first cluster. The accession IT 19 and IT 3 were from Palakkad and Thrissur districts respectively. All the other accessions were from Thiruvananthapuram district. Among these the accessions IT 3, IT 10, IT 2 and IT 15 showed no genetic variability among each other. The second cluster was formed from the accessions IT 17, IT 6, IT 7, IT 16, IT 5, IT 4, IT 2 and IT 1 of which no variability was shown among the accessions IT 5, IT 4, IT 2 and IT 1, collected from Ernakulam, Kottayam and Thiruvananthapuram districts. Cluster analysis revealed that the accessions IT 17 and IT 6 collected from Thiruvananthapuram and Thrissur districts did not show considerable difference. The accession IT 16 (Malappuram) and IT 7 (Thrissur) separated into a different cluster at similarity coefficient of 0.90. The three accessions IT 20, IT 8 and IT 14 grouped together to form the third cluster and all the three accessions were from Thiruvananthapuram district. The fourth cluster had two accessions, namely IT 9 and IT 11 collected from Thiruvananthapuram and Kollam districts. The accession IT 13 from Thiruvananthapuram district formed an individual cluster.

From the above discussion it can be inferred that the cluster formation was not fully in agreement with the geographical locations. It is to be specially noted that the accession IT 13 was distinct from the other accessions both genetically and morphologically. The accessions IT 11 and IT 9 which were noted for its high selection index falls in one cluster. Similarly the lowest yielders, IT 15 and IT 19 also fall in one cluster. Thus, genetic variability analysis in *Indigofera tinctoria* using RAPD analysis revealed that the morphologically distinct and superior lines were genetically differentiable also.

SUMMARY

6. SUMMARY

The study on 'Genetic variability analysis in Indian indigo (*Indigofera tinctoria* L.) using Random Amplified Polymorphic DNA (RAPD) technique' was carried out during 2003-2004 at the Department of Plantation Crops and Spices and at the biotechnology lab of the Department of Plant Biotechnology, College of Agriculture, Vellayani.

The study was conducted in two phases. Collection and raising of twenty accessions of *Indigofera tinctoria* and evaluation of growth, yield, root nodule and floral characteristics were made in the first phase. This phase also included the assessment of genetic variability, correlation among and within components and formulation of a selection index for identifying superior accessions based on yield. The molecular characterization of the accessions using RAPD technique was carried out as the second phase. The salient features of the study are summarized below.

Phase I

Morphological studies were made during this phase. Seeds of twenty accessions of *Indigofera tinctoria* were collected from different parts of Kerala and planted in polythene bags. The experiment was laid out in CRD with three replications.

Evaluation was done for leaf, shoot, root and root nodule characters at three stages of observation, namely pre flowering, flowering and pod maturation stage. Significant differences was observed among the accessions for all the characters studied namely plant height, plant spread, height at first branching, number of branches, girth of stem, length and breadth of leaves, number of leaves, leaf area, root length, root girth at collar region, fresh and dry weight of leaves, shoots, roots, pods, seeds, number of root nodules, number of effective root nodules, fresh and dry weight of root nodules and shell weight.

During pre flowering, flowering and pod maturation stage, maximum plant height was observed in IT 12 (21.87 cm), IT 15 (123.07 cm) and IT 20 (221.4 cm) respectively.

The plant spread was maximum in IT 1 (69.20 cm), in the pre flowering stage. In the flowering and pod maturation stage, the maximum plant spread was observed in IT 16 (113.4 cm) and IT6 (143.1 cm) respectively.

The accession IT 15 produced first branch at a maximum height of 8.67 cm.

The accessions IT 16, IT 14 and IT 6 had the maximum stem girth of 3.37, 4.53 and 7.27 cm respectively at the three observational stages.

The longest leaf (11.47 cm) was seen in IT 18, while the breadth was found maximum in IT 2 and IT 4 (4.53 cm).

The accession IT 13 produced the maximum number of leaves in the three observational stages (141, 579.67 and 499.67 respectively.)

Leaf area was found to be highest in IT 16 (2037.17 cm) during the pre flowering stage. In the flowering and pod maturation stage, the maximum leaf area was found for IT 13 (7769.7 and 6987.1 cm^2 respectively).

The accession IT 12 produced the longest root of 43.73 cm in the pre flowering stage, while in the flowering and pod maturation stage, IT 13 produced the longest root (50.13 and 54.33 cm respectively).

The accessions IT 19 (3.77 cm) IT6 (7.6 cm) and IT 10 (6.07 cm) had the maximum girth at the collar region at the three stages of observation.

The fresh weight of leaves in the pre flowering was found to be highest in IT 13 (46.43 g). In the flowering and pod maturation stages it was for IT 1 (155.13 g) and IT 13 (145.2 g) respectively. The maximum dry

weight was observed for the accession IT 13 at all the three stages (23.6 g, 82.83 g and 74.43 g respectively).

Among the twenty accessions, the fresh weight of shoot was greatest for the accession IT 13 for pre flowering and flowering (133.3 g and 361.93 g) stages and for IT 1 (809.73g) in the pod maturation stage. Similarly the dry weight of shoot was highest for the accession IT 13 in the pre flowering and flowering stages (70.54 g and 177 g) and in IT 1 (408.67 g) in the pod maturation stage.

The accession IT 8 recorded the maximum fresh and dry pod weight of 280.93g and 98.07g respectively. The maximum seed weight of 96.2 g was also recorded in this accession.

The highest root fresh weight (50.03g) was observed in the accession IT 13 in the pre flowering stage. In the flowering and pod maturation stage the maximum root fresh weight was observed in IT 12 (77.03 and 72.5 g respectively).

Similarly maximum root dry weight was observed in the accession IT 13 (25.43 g) in the pre flowering stage and during flowering and pod maturation stage it was noticed in IT 12 (77.03 and 72.5 g respectively).

The accession IT 16 produced the maximum number of root nodule at flowering and pod maturation stage (41 and 117 respectively). At these stages the same accession recorded the highest fresh weight of root nodule *viz*. 0.6 g and 1.75 g respectively. The dry weights of root nodules for the accession during these stages are 0.243 and 0.797 g respectively.

The accession IT 7 was the earliest to flower and the days for 50 % flowering ranged from 109.63 to 125.27 days.

The anthesis and anther dehiscence occurred during the morning hours, anthesis time ranged from 9.10 to 11.10 a.m. and the time for anther dehiscence ranged from 3.10 to 5.15 a.m. The number of pollen grains produced per anther ranged from 12,000 to 1,96,000.

The coefficients of variation, namely PCV and GCV were found to be almost similar. Highest PCV and GCV were obtained for the number of effective root nodule followed by pod yield and shoot yield. The lowest GCV and PCV were observed for the number of branches followed by plant spread.

The correlation analysis was carried out using different characters such as plant height, plant spread, number of branches, number of leaves, leaf area, root length, fresh weight of leaves, shoots, roots and pods and number of effective root nodules. Correlation studies revealed that at both phenotypic and genotypic levels the characters such as number of branches per plant, number of leaves per plant and leaf area were positively correlated with leaf yield, shoot yield, root yield and pod yield. Root length had high positive correlation with root yield. The plant height showed negative correlation with leaf, shoot, root and pod yield. Root length had negative correlation with number of effective root nodules.

Selection index was worked out using the characters plant height, plant spread, number of branches, number of leaves, leaf area, root length, fresh and dry weight of leaves, shoots, roots and pods and number of effective root nodules. Based on the selection index it was found that the accession IT 13 was found to be superior.

From the morphological characterization it was inferred that the accessions IT 13 followed by IT 11 and IT 9 were the superior ones. These accessions had high performance on leaf, shoot and root yield. The selection index worked out also supported the same.

Phase II

Molecular characterization of twenty accessions of *Indigofera tinctoria* using Random Amplified polymorphic DNA technique was conducted in this phase. The DNA isolated from tender leaves was checked for the yield and purity, which ranged from 0.51 to 5.85 µg / µl and 1.53 to 2.01 respectively.

Forty decamer primers of kit A and B were screened for their efficiency using the isolated DNA, of which twenty two yielded amplification products. Based on the reproducibility of the bands, level of polymorphism, number and intensity of bands produced the primers OPA-10, OPB-3, OPB-5 and OPB-10 were selected for further PCR amplification and statistical analysis. A total of 26 RAPDs (average 6.5 per primer) were obtained from the four selected primers, of which 17 were polymorphic. The estimation of Jaccard's similarity coefficients and construction of dendrogram by using UPGMA revealed the presence and extent of genetic similarities among the twenty accessions of *Indigofera tinctoria*. The overall similarity coefficients ranged from 0.28 to 1.0. At a similarity coefficient of 0.46, the twenty accessions grouped into two clusters, with the accession IT13 with least similarity coefficients of 0.46 forming a separate cluster, while all the other accessions were grouped into a large cluster. The accessions IT 3, IT 10, IT12 and IT15 showed a similarity coefficient of 1.0 with each other. Similarly the accessions IT 6 and IT 17 showed a similarity coefficient of 1.0 with each other. Same is the case with accessions IT 1, IT 2, IT 4 and IT 5. At similarity coefficient of 0.784, the dendrogram got divided into five clusters, of which the accessions IT 3, IT 10, IT 12, IT 15, IT 18 and IT 19 formed the first cluster. The second cluster was formed from the accessions IT 17, IT6, IT 7, IT 16, IT 5, IT 4, IT 2 and IT 1. The three accessions IT 20, IT 8 and IT 14 grouped together to form the third cluster. The fourth cluster had two accessions, namely IT 9 and IT 11. The accession IT 13 formed an individual cluster.

The molecular variability analysis of twenty accessions of *Indigofera tinctoria* revealed that the accession IT 13 was found to be distinct from all the other accessions.

It may be, thus concluded that morphologically distinct, superior lines are genetically differentiable also.

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ABSTRACT

GENETIC VARIABILITY ANALYSIS IN INDIAN INDIGO (*Indigofera tinctoria* L.) USING RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) TECHNIQUE

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Abstract of the
Thesis submitted in partial fulfilment of the requirement
for the degree of

Master of Science in Horticulture

Faculty of Agriculture
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2004

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ABSTRACT

The study entitled ‘Genetic variability analysis in Indian indigo (*Indigofera tinctoria* L.) using Random Amplified Polymorphic DNA (RAPD) technique’ was carried out during 2003-2004 at Department of Plantation Crops and Spices and at the biotechnology lab of the Department of Plant Biotechnology, College of Agriculture, Vellayani.

Indigofera tinctoria is a valuable medicinal plant, which is also utilized as a natural source of the blue dye, ‘indigo’. Twenty accessions of *Indigofera tinctoria* collected from different parts of Kerala state were assessed for morphological and molecular variability. The significant difference among the genotypes for different traits such as plant height, plant spread, height at first branching, number of branches, girth of stem, length and breadth of leaves, number of leaves, leaf area, root length, root girth at collar region, fresh and dry weight of leaves, shoots, pods, seeds, number of root nodules, number of effective root nodules, root fresh weight together with fresh and dry weight of root nodules and shell weight revealed considerable amount of genetic variability. The highest phenotypic and genotypic coefficient of variation was recorded for number of effective root nodule followed by pod yield and shoot yield. Correlation studies revealed that at both phenotypic and genotypic levels the characters such as number of branches per plant, number of leaves per plant and leaf area were positively correlated with leaf yield, shoot yield, root yield and pod yield. Root length had high positive correlation with root yield. Selection index was worked out and it was found that the accession IT 13 was found to be superior, followed by the accessions IT 11 and IT 9.

All the twenty accessions of *Indigofera tinctoria* from different parts of Kerala state were used for RAPD profiling. Random Amplified DNA (RAPD) profiling of all accessions was done using polymerase chain reaction

using OPA and OPB primer kits (each primer kit contain different primer sequences). After initial screening, the primers OPA 10, OPB 3, OPB 5 and OPB10 were selected for further analysis. The selection was based on the reproducibility of the bands, level of polymorphism, number and intensity of bands produced. These four primers produced a total of 26 RAPDs (average 6.5 bands per primer) of which 17 were polymorphic (65.38 %) and 7 were monomorphic (34.62 %). Similarity and variation among these accessions were observed by cluster analysis with the help of dendrogram. A similarity matrix was constructed and the similarity coefficients ranged from 0.46 to 1.0. The molecular variability analysis of twenty accessions of *Indigofera tinctoria* revealed that the accession IT 13 was found to be distinct from all the other accessions with respect to the polymorphic banding pattern.

The study, thus indicated morphologically distinct, superior lines were also genetically differentiable.