GENETIC VARIABILITY FOR YIELD AND ITS ATTRIBUTING CHARACTERES IN F_5 GENERATION RECOMBINANT INBRED LINES AND STUDY OF PARENTAL POLYMORPHISM FOR STERILITY MOSAIC DISEASE IN PIGEONPEA [*Cajanus cajan* (L.) Millsp.]

SATHEESH NAIK, S. J.

PAK 9217

DEPARTMENT OF GENETICS AND PLANT BREEDING

UNIVERSITY OF AGRICULTURAL SCIENCES

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SATHEESH NAIK, S. J.

PAK 9217

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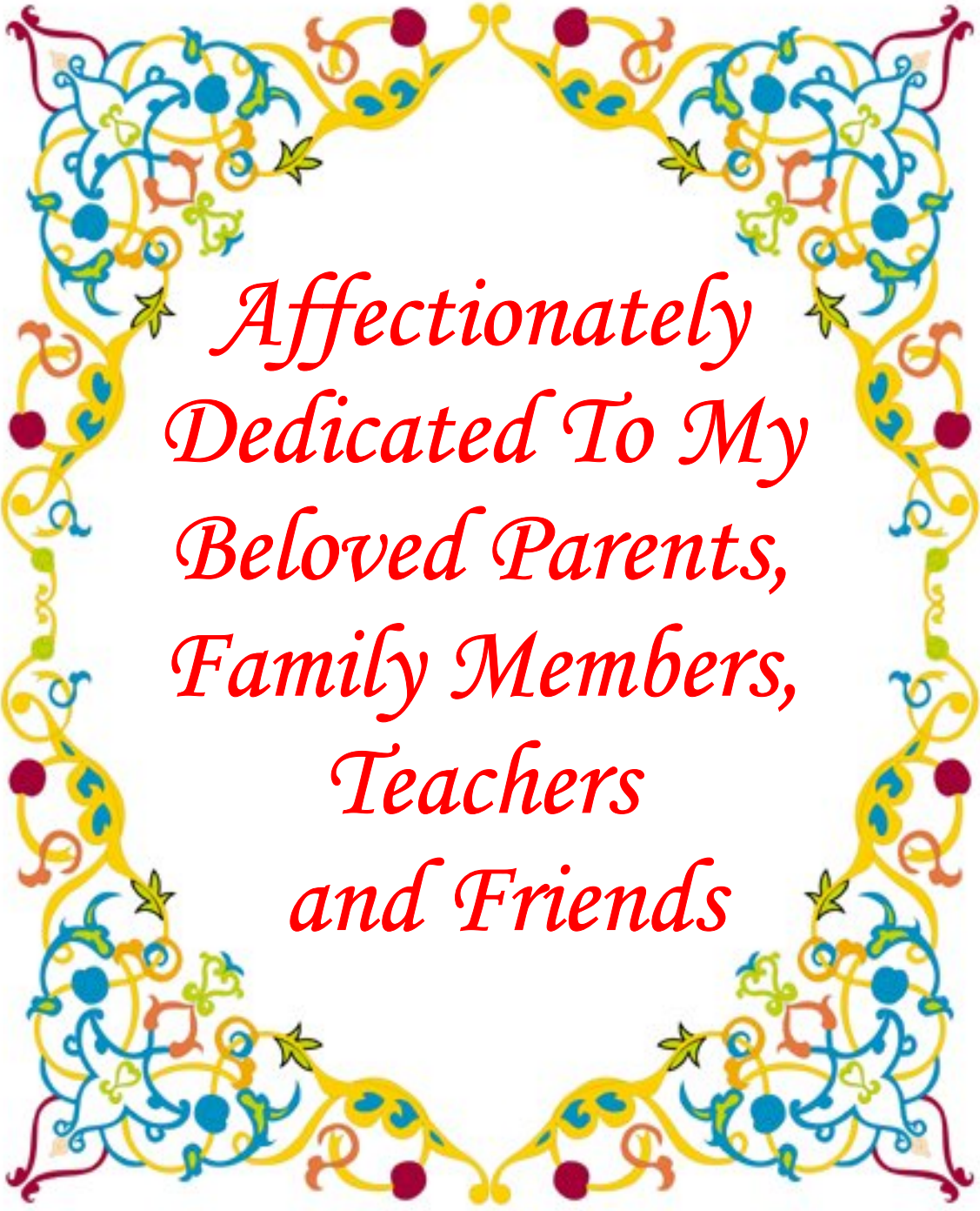
Affectionately
Dedicated To My
Beloved Parents,
Family Members,
Teachers
and Friends

DEPARTMENT OF GENETICS AND PLANT BREEDING
UNIVERSITY OF AGRICULTURAL SCIENCES
GKVK, BENGALURU –560 065

CERTIFICATE

This is to certify that the thesis entitled **"Genetic variability for yield and its attributing characters in F_5 generation Recombinant Inbred Lines and study of parental polymorphism for sterility mosaic disease in pigeonpea [*Cajanus cajan* (L.) Millsp.]"** submitted by **Mr. Satheesh Naik S. J.,** ID NO. **PAK 9217** in partial fulfillment of the requirements for the award of degree of **MASTER OF SCIENCE (Agriculture)** in **GENETICS AND PLANT BREEDING** to the University of Agricultural Sciences, Bengaluru, is a record of bonafide research work done by him during the period of his study in this university under my guidance and supervision and that no part of the thesis has been submitted for the award of any degree, diploma, associateship, fellowship or any other similar titles.

Bengaluru
July, 2011

(**M. Byre Gowda)**
Major advisor

APPROVED BY :

Chairman : _____
(**M. BYRE GOWDA)**

Members : 1. _____
(**SHAILAJA HITTALMANI)**

2. _____
(**D.L. SAVITHRAMMA)**

3. _____
(**M. SAIFULLA**)

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Bangaluru

21st July 2011 ***(SATHEESH NAIK, S. J.)***

Genetic variability for yield and its attributing characters in F_5 generation recombinant inbred lines and study of parental polymorphisms for sterility mosaic disease in pigeonpea [*Cajanus cajan* (L.) Millsp.]

SATHEESH NAIK, S. J.

ABSTRACT

The present study was carried out at AICRP on pigeonpea, UAS, Bangaluru, to study the extent of genetic variability for yield & its attributing characters and screening for sterility mosaic disease in F_5 generation RIL progeny lines of cross TTB 7 x ICP 7035 and to estimate the extent of molecular diversity existing between susceptible and resistant genotypes of pigeonpea against SMD. In the present study PCV and GCV estimates were high for secondary branches per plant, pods per plant, seed yield per plant, primary branches per plant, length of inflorescence. Moderate PCV and GCV values were recorded for 100 seed weight and plant height, while low values were recorded for days to maturity and days to fifty per cent flowering. High heritability with high genetic advance as per cent of mean were observed for secondary branches per plant, pods per plant, seed yield per plant, shelling percentage and plant height, while, Days to fifty percent flowering and days to maturity recorded moderate heritability with low genetic advance as per cent of mean.

One hundredane twenty F_5 generation RILs of cross TTB 7 x ICP 7035 were screened for SMD resistance. Out of which, none of them were immune, three showed resistance, 25 moderately resistance and 92 susceptible reaction. Among seven genotypes screened for SMD, BRG1 and ICP 7035 recorded resistant reaction, Hy3C and Bahar were moderately resistant and BRG 2, TTB 7 and ICP 8863 were exhibited susceptible reaction.

Out of 61 SSR primers screened for molecular diversity in seven parental genotypes, 13 exhibited polymorphism between the genotypes, with 28.23 per cent polymorphism. The PIC value ranged between 0.23 – 0.60, with an average of 0.41. Highest (0.60) PIC value was observed for the primer CCttc008 and lowest for CCtc012. Highest (0.796) similarity was observed between Hy3c and BRG 1 and lowest similarity (0.593) was between ICP 8863 and BRG 1. The dendrogram grouped SMD resistant varieties ICP 7035 and BRG 1 and moderately resistant varieties Bahar and HY 3c in major cluster I. These genotypes were differing at their genotypic level from the susceptible genotypes BRG 2, TTB 7 and ICP 8863 which formed a separate and distinct cluster (cluster II).

Department of Genetics and Plat Breeding
UAS, GKVK, Bangaluru
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M. BYRE GOWDA
Major Advisor

CONTENTS

CHAPTERS	TITLE	PAGE NO.
I	INTRODUCTION	1-4
II	REVIEW OF LITERATURE	5-16
III	MATERIAL AND METHODS	17-35
IV	EXPERIMENTAL RESULTS	36-50
V	DISCUSSION	51-60
VI	SUMMARY	61-63
VII	REFERENCES	64-75
	APPENDIX	

LIST OF TABLES

TABLE No.	TITLE	PAGE No.
1	List of SSR primers used to study parental polymorphism	25
2	Important morphological characters of parental genotypes	27
3	Analysis of Variance (Mean sum of squares) for 11 different yield and yield attributing characters in the F_5 generation RILs of the cross TTB 7 × ICP 7035	37
4	Estimates of genetic parameters for yield and its attributing characters in F_5 generation RILs of cross TTB 7 × ICP 7035	40
5	Mean performance of parents and F_5 generation RILs for yield and its attributing characters	42
6	Transgressive recombinants in F_5 generation RILs of cross TTB7 x ICP 735	44
7	Reaction of parents and other genotypes to sterility mosaic disease	45
8	Reaction of F_5 generation RILs of cross TTB 7 × ICP 7035 to sterility mosaic disease	45
9	Details of 61 SSR primers tested for parental polymorphism	47
10	Details of polymorphic SSR primers obtained in the study on parental polymorphism	49
11	Jaccard's similarity coefficients for seven parental genotypes of pigeonpea	50

LIST OF FIGURES

FIG. No.	PARTICULARS	BETWEEN PAGES
1	Frequency distribution of RILs to pigeonpea sterility mosaic disease incidence	46-47
2	Dendrogram showing grouping of pigeonpea genotypes based on SSR primers	48-49

LIST OF PLATES

PLATE No.	PARTICULARS	BETWEEN PAGES
1	SMD nursery for screening of RILs, parents and other pigeonpea genotypes.	24-25
2	General view of leaf stapling technique	24-25
3	Quantification of DNA samples on 0.8% agarose gel	29-30
4	Polymorphic SSR primers of pigeonpea genotypes on 3 % agarose gel	47-48
5	Polymorphic SSR primers of pigeonpea genotypes on 4.5% acrylamide gel	47-48

Introduction

I. INTRODUCTION

Pigeonpea (*Cajanus cajan* (L). Millsp.) is an important grain legume crop of rainfed agriculture in the semi-arid tropics. It is the only cultivated food crop of the *Cajaninae* sub-tribe and has a diploid genome with 11 pairs of chromosomes (2n = 2x = 22) and a genome size estimated to be 858 Mbp (Greilhuber and Obermayer, 1998). The genus *Cajanus* comprises 32 species, most of which are found in India, Australia and one species is native to West Africa. Pigeonpea is cultivated in more than 25 tropical and sub-tropical countries, either as the sole crop or a inter crop with finger millet, sorghum, pearl millet, maize or with short duration legumes. It plays an important role in food security, balanced diet and alleviation of poverty because of its diverse usages as a food, fodder and fuel (Rao *et al*. 2002).

In India, pigeonpea is grown in an area of 3.53 m. ha with a production of 2.46 m. tonnes and productivity of 723 kg/ha (Anon, 2010). In Karnataka, it is grown in an area of 5.97 lakh ha with an annual production of 3.15 lakh tones and productivity of 467 kg/ha (Anon, 2010). The Indian sub continent alone contributes nearly 92 per cent of the total world production. Major pigeonpea growing states are Maharashtra, Karnataka, Madhya Pradesh, Andhra Pradesh, Uttar Pradesh and Gujarat together contributes about 90 per cent of area and 93 per cent of production.

Yield being a complex trait is collectively influenced by various component characters, which are polygenically inherited and highly influenced by environmental variations. So, the observed variability for these characters is the sum total of hereditary effects and the influence of environment. Hence, it becomes necessary to partition the observed variability into heritable and non-heritable components measured as

genotypic and phenotypic coefficient of variations, heritability and genetic advance. Higher genotypic coefficient of variance is more important than the phenotypic coefficient of variance because the heritable portion of the character depends upon the proportion of genotypic variance present in the total phenotypic variance.

Although, India leads the world both in area and production of pigeonpea, its productivity is lower than the world average. This is attributed to factors such as biotic (*Fusarium* wilt, sterility mosaic and pod borers) and abiotic (e.g., drought, salinity and water-logging) stresses. Among the biotic stresses, sterility mosaic is considered to be the most important disease of pigeonpea in India and can cause yield loss upto 95 per cent under favourable condition (Kannaiyan *et al.* 1984). The disease is caused by pigeonpea sterility mosaic virus (PPSMV) (Kumar *et al.* 2003) and transmitted by eriophyid mite (*Aceria cajani* Channabasavanna). The disease is characterized by the symptoms like bushy and pale green appearance of plants followed by reduction in leaf size and mosaic mottling of leaves and finally partial or complete cessation of reproductive structures.

Control of sterility mosaic disease (SMD) by chemical method though effective but economically not feasible and non eco-friendly (Nene *et al.* 1977). Breeding for resistant varieties is considered to be one of the most effective and economic methods of reducing crop losses and has received top priority. Due to outcrossing nature and long life cycle of the crop, there is a problem to screen varieties and breeding population for SMD resistance. The task of developing resistant varieties is further complicated in view of the genetic plasticity of the pathogen which has warranted the use of strain specific sources of resistance in crop improvement. Though conventional plant breeding in pigeonpea has been facilitated greatly to develop improved varieties with enhanced resistance

to biotic/abiotic stresses (Varshney *et al.* 2005, 2006), selection process is tedious and time consuming.

Therefore, it is necessary to tackle these problems at molecular level by developing cultivars which are resistant to biotic stresses and have greater recovery from the damage. Genomic tools especially molecular markers have facilitated breeding in many crops leading to development of several improved cultivars/varieties with enhanced resistance/tolerance to biotic stresses (Varshney *et al.* 2006). Among different kinds of molecular markers available at present, simple sequence repeat (SSR) markers have proven the markers of choice in practical breeding (Gupta and Varshney, 2000; Varshney *et al.* 2005) because they are more reliable and reproducible as compared to RAPD markers and less cumbersome and time consuming than RFLPs and AFLP techniques (Vos *et al.* 1995).

In pigeonpea, availability of number of molecular markers are meagre and also the level of polymorphism is low (Yang *et al.* 2006, Odeny *et al.* 2007). However, recent reports indicated many SSR markers are available in pigeonpea. Recently, Ganapathy *et al.* (2010) and Gnanesh *et al.* (2011) have identified few linked markers for SMD resistance in pigeonpea. Thus availability of more number of SSRs in pigeonpea would enable the breeders to know the location of specific genes and QTLs making it possible to improve the efficiency of breeding through marker assisted selection (MAS). Use of molecular markers in precise mapping of genes in the genome will be pivotal for MAS in breeding programmes. Hence there is a need to integrate SSR markers to genetic maps and to identify the markers linked to SMD resistance and to enhance the marker density on genetic maps. For integrating the SSR markers into genetic maps, the markers should detect polymorphism between parental genotypes and mapping populations. Hence,

information on marker polymorphism in parental genotypes and mapping populations is essential.

With this background the present study was undertaken with the following objectives

1. To estimate the genetic variability parameters for yield and its attributing characters in F_5 generation RILs of cross TTB 7 x ICP 7035.

2. Screening of parents and F_5 generation RILs for sterility mosaic disease (SMD).

3. To study parental polymorphism using SSR markers for SMD resistance.

Review of Literature

II. REVIEW OF LITERATURE

In the present investigation the literature pertaining to genetic variability studies, screening for SMD and molecular diversity studies has been reviewed from the year 1980 onwards and presented under the following headings.

2.1 Genetic variability parameters for yield and its attributing traits in pigeonpea

The progress in the improvement of plant type by the plant breeders will be determined by the variability existing in the populations. Thus, for effective selection and utilization of genotypes in breeding programme a thorough study on genetic variability, heritability and genetic advance is essential. Genotypic coefficient of variation, which indicates the relative magnitude of genetic diversity present in the material, will help to study the genetic variability present for different characters.

Bashiruddin and Sreeramulu (1981) observed high genotypic coefficient of variation for 100 seed weight, cluster per plant, pods per plant and branches per plant.

Jagashoram (1983) studied 100 genotypes of pigeonpea in two years and reported high magnitude of phenotypic variability for all the characters except for seeds per pod. High estimates of genotypic coefficient of variation and heritability accompanied by moderate to high genetic advance for pods per plant, days to maturity, plant height and days to flowering were observed.

Premsagar and Jatasra (1984) reported high GCV for branches per plant, followed by pods per plant. High heritability was observed for days to maturity, seed yield, pods per plant, days to flowering and plant

height. The genetic advance was high for pods per plant followed by seed yield and branches per plant.

In a study of traits in 12 genotypes, Balyan and Sudhakar (1985) reported high estimates of phenotypic and genotypic coefficient of variation, heritability and expected genotypic advance for primary and secondary branches, pods per plant, 100 seed weight and seed yield.

Sindhu *et al.* (1985) found high variability for pods per plant. Heritability estimates were high for all the traits studied except seed yield and seeds per pod. Genetic advance was high for pods per plant while, higher values of heritability and genetic advance for days to maturity, days to flowering and plant height were reported by Kanwan and Hazarika (1988).

Tikka (1986) reported high estimates of heritability for days to flowering, branches per plant, plant height, pods per plant, pod length, pod weight and 100 seed weight.

High genotypic coefficient of variation was observed for pod number followed by cluster number and seed yield while, it was lowest for seeds per pod. High heritability and high genetic advance were observed for pod number, cluster number and seed yield (Natarajan *et al.* 1990).

Holkar *et al.* (1991) evaluated 27 pigeonpea genotypes and reported high GCV and PCV estimates for pods per plant and seed yield and high heritability and genetic advance for days to flowering, maturity and pods per plant.

Khapre and Nerker (1992) reported high genetic variability and high genetic advance for seed yield per plant and high heritability for all important yield attributes studied except primary branches per plant.

Ghodke *et al.* (1994) reported high estimates of heritability for number of pods, days to flowering and days to maturity.

Gamber *et al.* (1996) reported moderate PCV and GCV estimates for days to 50% flowering, pod length and 100 seed weight at both genotypic and phenotypic level.

Aher *et al.* (1998) observed wide genetic variability for plant height, number of secondary branches per plant and days to flowering. High heritability accompanied by high genetic advance was observed for primary and secondary branches per plant, followed by seed yield per plant, days to flowering and plant height.

Pansuriya *et al.* (1998) reported information on variability, heritability and correlation coefficients from the 20 early maturing pigeonpea genotypes. Plant height and pods per plant showed a wide range of phenotypic variation. The genotypic and phenotypic coefficients of variation were the highest for dry matter per plant, harvest index, pods per plant and seed yield per plant. Heritability estimates were high for all the characters studied. However, high genetic advance was obtained only for dry matter per plant followed by pods per plant and plant height.

Takalkar *et al.* (1998) reported the highest level of variability for pods per plant followed by straw yield per plant and plant height. The high heritability estimates were observed for all the characters under study except, straw yield per plant. The expected genetic advance was high for pods per plant, plant height, straw yield per plant and days to maturity. Low genetic advance was observed for branches per plant, seeds per pod and 100 seed weight.

Vikas and Singh (1998) reported high estimates of GCV for number of pods per plant, seed yield and plant height. High heritability estimates

were observed for plant height and 100 seed weight. The genetic advance was high for plant height and seed yield.

Jagdish Singh (1999) reported that genotypic and phenotypic coefficients of variations were high for seed yield per plant, pods per plant and branches per plant. These characters also exhibited high heritability coupled with high genetic advance.

Srinivas *et al.* (1999) reported higher values of genetic variability for number of pods and lower values for seeds per pod. Heritability estimates were high for all the traits, except seeds per pod.

Basavarajaiah *et al.* (2000) reported high phenotypic and genotypic coefficient of variation values for days to flowering, pods per plant, seed yield per plant and length of pod bearing branches. High heritability coupled with high genetic advance was observed for days to 50 per cent flowering, yield per plant, length of pod bearing branches and 100 seed weight.

Deshmukh *et al.* (2000) reported that secondary branches per plant recorded the highest genetic variation. Heritability estimates were high for days to 50 per cent flowering, days to maturity, 100 grain weight and primary and secondary branches per plant.

Venkateswarlu (2001) reported maximum variability for number of pods per plant and plant height. High heritability estimates were observed for number of secondary branches per plant, seed yield per plant, days to maturity and number of primary branches per plant. The expected genetic advance was high for plant height, number of pods per plant, grain yield per plant and days to maturity. Ahmad Neyaz and Bajpai (2002) reported high heritability and genetic advance values for 100 seed weight.

Firoz Mahmad (2003) reported high estimates of genotypic and phenotypic coefficient of variation values for branches for plant, pods per plant and low values for days to flowering, days to maturity, pod length and pod width.

Singh *et al.* (2003) reported that heritability and genetic advance was high for seed yield per plant, seeds per pod, 100 seed weight and length of pod bearing branch.

Ram Dhari *et al.* (2004) reported that branches and pods per plant exhibited high genotypic and phenotypic coefficient of variation.

Chattopadyaya and Dhiman (2005) reported high genotypic and phenotypic variation for seeds per pod and days to maturity and high heritability and genetic advance for seed yield, plant height, branches per plant, days to maturity and seeds per pod.

Sathish Kumar *et al.* (2006) reported high PCV and GCV values for pods per plant, primary and secondary branches per plant and low for days to maturity. High heritability in broad sense coupled with high genetic advance as per cent mean were recorded for pods per plant, seed yield per plant and plant height. Moderate heritability and low genetic advance for days to maturity, days to fifty per cent flowering and seeds per pod.

Anantharaju and Muthiah (2008) reported low PCV and GCV for traits like days to maturity, days to first flowering and 100 seed weight and moderate to high PCV and GCV for plant height and 100 seed weight in F_2 populations. High heritability estimates were recorded for traits *viz.*, days to first flowering, days to maturity, plant height and seeds per pod while, moderate to high heritability estimates for primary branches , pods per plant, 100 seed weight and seeds per pod. Genetic advance as per cent of mean was high for traits *viz.*, seeds per pod, seed yield per

plant and moderate to high for days to first flowering, plant height, primary branches per plant, pods per plant and 100 seed weight.

Ganapathy (2009) reported high PVC and GCV values for pods per plant, secondary branches, length of the pod bearing inflorescence and seed yield per plant. High heritability coupled with high predictable genetic advance for secondary branches per plant, plant height and pods per plant this indicates that these characters are under additive gene action.

Vange and Egbe (2009) reported high PCV and GCV values for pods per plant, pod weight, grain yield and numbers of primary branches: moderate for days to 50% flowering, maturity and plant height. Heritability (broad sense) was high for all the traits studied with exception of seeds per pod. High expected genetic advance was obtained for number of branches per plant (58.26%), number of pods per plants (161.84%), pod weight (68.48%) and grain yield (82.65%). High heritability and expected genetic advance in these traits indicated the presence of additive gene effects.

Ajay (2010) reported high PCV and GCV values for secondary branches per plant, Primary branches per plant, pods per plant, pod yield and seed yield. Whereas moderate to low values for plant height, shelling per cent, 100 seed weight, Seeds per pod had moderate PCV and low GCV. High heritability with high genetic advance for primary branches, secondary branches, pods per plant and plant height.

Bhadru (2010) reported moderate to high PCV and GCV for number of pods, seed yield per plant, plant height and plant spread. High heritability with high genetic advance for number of pods, primary and secondary branches per plant, test weight, plant height and plant spread. Days to 50% flowering, plant spread, primary and secondary

branches per plant, number of pods and raceme length had moderate to low direct effect and its true relationship of these traits with grain yield. Hence, direct selection for these traits would be rewarding for yield improvement in pigeonpea.

Singh *et al.* (2010) studied seven genotypes of pigeonpea, comprising three cultivars, four wild relatives and 10 interspecific crosses for genetic variability and character association. The high estimates of PCV and GCV were exhibited for all the characters except days to 50% flowering and days to maturity. High heritability accompanied with high genetic advance was observed for primary branches/plant, pod length, number of chambers/pod, 100-seed weight and seed yield/plant, whereas days to 50% flowering showed high heritability and low genetic advance as per cent of mean. Seed yield/plant had significant and positive correlation coefficient with plant height. Similar correlation was noticed between numbers of chambers/pod with number of seeds/pod

Hamid *et al.* (2011) reported high PCV and GCV for primary branches, plant height, shelling percentage and seed yield per plant. In general heritability (broad sense) was high for all the traits except days to maturity and days to fifty percent flowering.

2.2 Screening parents and F_5 generation RILs for SMD in pigeonpea

Three methods are being used for evaluating resistance to SMD. An effective technique called “Leaf stapling technique” for screening pigeonpea germplasm and breeding material for resistance to sterility mosaic virus was developed by Nene and Reddy (1977). It is the most commonly used method under field and glass house conditions. This technique involves stapling of a portion of SMD infected pigeonpea leaves on the healthy seedlings. Mites infected with virus from the stapled leaf migrate and transmit the virus to the test plants. This technique was

shown to facilitate inoculation at primary leaf stage and to express disease symptoms rapidly.

Saifulla and Byregowda (2003) reported BRG 3, ICPL 99-06 and ICP 99055 as resistant genotypes to SMD with 0, 1.7 and 2.4 per cent disease incidence respectively. Among the three resistant genotypes BRG 3 was completely free from disease coupled with high seed yield.

Three genotypes *viz.*, BRG 3, ICP 7035, Hy 3c showed moderately resistant reaction to SMD with 11- 30 per cent disease incidence while, the susceptible check ICP 8863 recorded more than 50 per cent disease incidence (Saifulla *et al.*2005a).

Among the seventy nine genotypes screened against SMD, seven genotypes *viz.,* ICP 7035, BAD 2001-6, NDA 98-8, Hy 3c, MAL 24, MAL 23 and BRG 3 showed moderately resistant reaction with 11- 30 per cent SMD incidence while, rest 72 genotypes including the susceptible check TTB 7 were found to be susceptible with disease incidence more than 50 per cent (Saifulla *et al.*2005b)

Several pigeonpea genotypes were screened against SMD for 3 consecutive years from 2002-03 to 2005-06. BRG 3 and ICP 7035 recorded resistant reaction while, the genotypes HY3c and Bahar recorded moderate resistant reaction to SMD. The susceptible checks ICP 8863 and TTB 7 recorded 100 per cent disease incidence (Saifulla *et al.* 2006).

2.3 Molecular diversity studies using SSR markers

Varietal identification is important for the documentation of genetic resources. Traditional techniques like morphometric traits observation and biochemical techniques based on protein and isozyme polymorphism have been used. But for differentiation and characterization of varieties

at molecular level, fingerprinting of crop varieties using DNA markers are very useful and this is found to be more reliable than traditional markers (Vasconcelos *et al.* 1996). The relative advantages of molecular markers over morphological markers for most genetic and breeding applications were discussed by Tanksley *et al.* (1989).

Microsatellite or simple sequence repeat markers are short tandem repetitive DNA sequences with a repeat length of a few (1-5) base pairs (Litt and Luty, 1989). Microsatellite markers have been increasingly used to assess the genetic diversity and population structure among plants (Li *et al.* 2000; Pillen *et al.*2000). The high variability of repeat numbers among individuals has led to the use of microsatellite markers for the development of genome specific DNA fingerprints (Weising *et al.* 1992; Zavodra *et al.*2000).

Burns *et al.* (2001) reported a set of 10 simple sequence repeat (SSR) markers in pigeonpea. Screening for polymorphic SSRs was conducted on a set of 12 diverse pigeonpea accessions using 20 primer pairs. Of the 20 primer pairs, 10 loci exhibited polymorphism when applied to the set of 12 diverse pigeonpea accessions.

Dendrogram constructed by combined RAPD and SSR data depicted that the SMD susceptible genotypes TTB 7 and ICP 8863 clustered together while, the resistant genotype Hy 3c and BRG 3 subclustered with ICP 7035 indicating ICP 7035, BRG 3, HY 3C are differing at molecular level from susceptible genotypes TTB 7 and ICP 8863 (Gangadhara, 2006).

Diversity array technology (DArT) markers revealed low level of genetic diversity in cultivated pigeonpea as compared to wild relatives. Most of the diversity was among the wild relatives of pigeonpea or between the wild and the cultivated species (Yang *et al.,* 2006).

Odeny *et al.* (2007) identified 19 SSR primers to be polymorphic among 15 cultivated and nine wild pigeonpea accessions providing evidence for cross species transferability within the genus Cajanus. A total of 98 alleles were detected at the 19 polymorphic loci with an average of 4.9 alleles per locus. Less alleleic variation (31 alleles) was observed within the cultivated species than across the wild species (92 alleles) and suggested for development of more microsatellite markers for future genomic studies in pigeonpea.

Singh *et al.* (2008) used 21 SSR markers obtained from different crop species to assess polymorphism in 16 cultivated pigeonpea genotypes. Based on SSR fingerprinting, 16 genotypes were grouped into two groups as early and late duration genotypes indicating that SSR markers could be used as a good choice to classify the pigeonpea genotypes.

Ganapathy *et al.* (2010) identified SSR and AFLP markers associated with the sterility mosaic disease in the F_2 population of the cross TTB 7 (susceptible) and BRG 3 (resistance). A total of 156 SSRs and 16 AFLP primer pairs were surveyed for identification of polymorphic markers between the parents and DNA bulks of susceptible and resistance F2 individuals. Out of 10 polymorphic SSRs identified between the parents, none of them were polymorphic between the DNA bulks. From 13 polymorphic AFLP primer combinations between the parents, two AFLP primer pairs generated 4 markers which were polymorphic between the resistant and susceptible bulks indicating that these markers are linked to SMD.

Saxena *et al.* (2010a) reported 36 microsatellite loci from a SSR-enriched genomic library. Primer pairs were designed for 23 SSR loci, of which 16 yielded amplicons of expected size. Thirteen SSR markers were polymorphic amongst 32 cultivated and eight wild pigeonpea genotypes

representing six *Cajanus* species. These markers amplified a total of 72 alleles ranging from two to eight alleles with an average of 5.5 alleles per locus. The polymorphism information content for these markers ranged from 0.05 to 0.55 with an average of 0.32 per marker.

In order to maximize polymorphism in the mapping populations for mapping loci for *Fusarium* wilt (FW) and sterility mosaic disease (SMD) resistance in pigeonpea, a set of 32 pigeonpea lines were screened for polymorphism with 30 microsatellite markers. A total of 23 marker loci showed polymorphism with 2-4 alleles and the polymorphism information content for these markers ranged from 0.12 to 0.65 with an average of 0.43 per marker. Based on the genetic diversity five parental combinations were identified and populations were developed. Of these crosses, one cross segregate for *Fusarium* wilt resistance, two for SMD resistance and the remaining two crosses segregate for resistance to both. (Saxena *et al.* 2010b).

Datta *et al* (2010) revealed a significant transferability (46%) of chickpea microsatellites to *Cajanus*. In cultivated pigeonpea, chickpea-specific SSRs showed 38–39% transferability, while among wild *Cajanus* species, it ranged from 26% in *Cajanus sericeus* ICP 15760 to 40% in *C. sericeus* ICP 15761. The transferable primers exhibited extensive polymorphism in *Cajanus* with an average number of 4.11 alleles per marker. High level of polymorphism exhibited by chickpea microsatellite markers in the present study indicates their usefulness in diversity analysis, mapping agronomically important traits and marker-assisted breeding in pigeonpea.

Metkar *et al.* (2010) reported the SSR based molecular characterization of CMS lines in twenty five pigeonpea genotypes. Totally 16 SSR primers were used for detection of polymorphism, out of which four primers *viz.*, CCB-5, CCB-8, CCB-9, CCB-10, were found to be

polymorphic. A total of 13 alleles were detected on an average 3.25 alleles.

Raju *et al.* (2010) generated 908 ESTs which are available in public domain. At the time of analysis, a set of 5,085 unigenes were used for identification of molecular markers in pigeonpea. For instance, 3,583 simple sequence repeat (SSR) motifs were identified in 1,365 unigenes and 383 primer pairs were designed. Assessment of a set of 84 primer pairs on 40 elite pigeonpea lines showed polymorphism (28.8%) with an average of four alleles per marker with an average polymorphic information content (PIC) value of 0.40.

Datta *et al.* (2011) reported that 550 validated genic- SSR markers in pigeonpea using deep transcriptome sequencing. From these, 20 highly polymorphic markers were used to evaluate the genetic relationship among species of the genus *Cajanus.* A comprehensive set of genic- SSR markers were developed as an important genomic resource for diversity analysis and genetic mapping in pigeonpea.

Gnanesh *et al.* (2011) used microsatellite markers to screen F_2 population of cross ICP 8863×ICPL 20097 (segregating for Patancheru SMD isolate) and TTB7× ICP7035 (segregating for both Patancheru and Bangalore SMD isolates) and identified QTLs linked to SMD. After screening over 3000 SSR markers on parental genotypes of each mapping population, Intra-specific genetic maps comprising of 11 linkage groups and 120 and 78 SSR loci were developed for ICP 8863 × ICPL 20097 and TTB 7 × ICP7035 populations, respectively. Composite interval mapping (CIM) based QTL analysis by using genetic mapping and phenotyping data provided four QTLs for Patancheru SMD isolate and two QTLs for Bang lore SMD isolate. One QTL namely qSMD4 identified within an interval of 2.8 cM on LG7 explaining 24.72% of phenotypic variance.

Material and Methods

III. MATERIAL AND METHODS

The details of materials and methods adopted in the present investigation are described in this chapter under the following headings

3.1. Genetic variability parameters for yield and its attributing characters in F_5 generation RILs of cross TTB 7x ICP 7035

3.2. Screening of F_5 generation RILs, parents and other genotypes for sterility mosaic disease (SMD).

3.3. Genetic diversity studies using SSR markers.

The present investigation was carried out in two broad experiments at the experimental plots of All India Co-ordinated Research Project on Pigeonpea, University of Agricultural Sciences, Gandhi Krishi Vignana Kendra, Bangalore. The experimental material consisted of 120 F_5 generation RILs and seven pigeonpea genotypes. Each of the RILs was divided in to two parts; one part was utilized for genetic variability studies and the other part was grown for SMD screening. Seven pigeonpea genotypes differing for their resistance/susceptibility to SMD (Saifulla *et al.*, 2006) were used for parental polymorphism studies using SSR markers. All the above material was procured from AICRP on pigeonpea UAS, GKVK, Bangalore.

3.1 Genetic variability parameters for yield and its attributing characters

3.1.1 Experimental material

First part of 120 F_5 generation RILs of the cross TTB 7 x ICP 7035, its two parents TTB7, ICP7035 and susceptible variety ICP 8863 as checks were grown during *kharif* 2010 following augmented experimental design. The design has been divided into 30 blocks of 4.2 m^2 each, where all the lines and checks were raised with spacing of 60 x 30 cm wherein,

checks were replicated in each block and F_5 generation RILs were unreplicated. Standard package of practices was followed for raising a good and healthy crop.

3.1.2 Observations recorded

In each lines and checks, five plants were selected randomly and tagged for recording observations. Each character was observed for eliciting the information and described hereunder.

3.1.2.1 Days to 50 per cent flowering

Total number of days taken from the day of sowing to the day on which fifty per cent of the plants showed anthesis was recorded.

3.1.2.2 Days to maturity

The number of days taken from the day of sowing to physiological maturity was recorded.

3.1.2.3 Primary branches per plant

The number of primary branches per plant were counted and recorded at the time of harvest and mean was calculated.

3.1.2.4 Secondary branches per plant

The number of secondary branches per plant were counted and recorded at the time of harvest and mean was calculated.

3.1.2.5 Plant height (cm)

The plant height was measured at maturity from the base of the plant to the tip of main branch and expressed in centimeters.

3.1.2.6 Length of the inflorescence (cm)

The length of inflorescence was measured from the base of the inflorescence to the tip and mean was calculated and expressed in centimeters.

3.1.2.7 Pods per plant

The total number of filled pods per plant was counted at the time of harvest and mean was calculated.

3.1.2.8 Seeds per pod

The number of seeds per pod was counted; mean was calculated and expressed in grams.

3.1.2.9 100 seed weight (g)

The weight of hundred seed weight was recorded in grams and mean was computed.

3.1.2.10 Shelling percentage (%)

The ratio of seed yield per plant to pod yield per plant was worked out and expressed in percentage.

$$\text{Shelling per cent} = \frac{\text{Seed weight (g)}}{\text{Pod weight (g)}} \times 100$$

3.1.2.11 Seed yield per plant (g)

Total seed weight per plant was recorded after threshing, cleaning and drying and the mean was calculated and expressed in grams.

3.1.3 Statistical analysis

The data was analyzed by using statistical software packages of AUGMENT, GENERES and SAS. The details of the statistical methods followed are furnished below.

The class of augmented experiment design (AED) contains two kinds of treatments, checks/standard (replicated) and entries/new genotype (unreplicated). The letters used are usually considered being random and user fixed. The entries/ genotypes are usually unreplicated while the checks are replicated to obtain an estimate of the error and to obtain estimates of blocking effects. An experiment design is selected for the check treatments and then the blocks, rows, and or columns are enlarged to accommodate the new treatments and this forms the augmented design. AED was introduced by Federer (1961) as an alternative to the systematically arranged check and new treatments. AED have several advantages over the systematic arrangement. They are useful for screening new treatments such as genotypes, insecticides, herbicides, drugs, etc.

ANOVA for augmented design

Source of variation	Degrees of freedom	Sum of squares	Mean sum of squares	F- ratio
Blocks	(B-1)	BSS	BMSS	BMSS / eMS
Genotypes	(G-1)	TSS	TMSS	TMSS / eMS
Checks	(C-1)	CSS	CMSS	CMSS / eMS
URG	(U-1)	USS	UMSS	UMSS / eMS
Check vs. URG	1	C vs USS	C vs UMSS	C vs UMSS / eMS
Error	(B-1) (C-1)	eSS	eMSS	

Where,

B = Number of blocks

G = Number entries (G = C + U)

C = Number of checks as repeater

URG = Number of unreplicated entries/genotypes

The significance was tested by referring to the table given by Fisher (1936).

3.1.3.1 Components of variance

The genotypic variance was computed by using mean sum of squares of unreplicated genotypes and replicated entries (checks).

$$1)\ Vg = \text{MSS of URG} - \frac{\text{MSS of Checks}}{\text{No. of Blocks}}$$

Where,

Vg is the genotypic variance

MSS of URG is the mean sum of square of unreplicated genotypes

MSS of checks is the mean sum of square of checks

2) Ve = Vg + MSS of Error

Where,

Vg is the genotypic variance

MSS of error is the mean sum of square of error

3) Vp = Vg+Ve

Where,

Vp= Phenotypic variance

3.1.3.2 Coefficients of Variation

The co-efficient of variability both at phenotypic and genotypic levels for all the characters were computed by applying the formula as suggested by Burton and De Vane (1953).

Genotypic coefficient of variation (GCV)

$$GCV\ (\%) = \frac{\sigma_g}{\overline{X}} \times 100$$

Phenotypic coefficient of variation (PCV)

$$PCV\ (\%) = \frac{\sigma_p}{\overline{X}} \times 100$$

Where,

$\overline{X}$ = General mean of the character

σ_p = Phenotypic standard deviation

σ_g = Genotypic standard deviation

The GCV and PCV were classified as suggested by Robinson *et al.* (1949) into low (0 - 10%), moderate (10.1% - 20%) and high (>20%).

3.1.3.3 Heritability (h^2)

Heritability in broad sense for all the characters was computed by the formula given by Lush (1945).

$$h^2 = \frac{\sigma^2_g}{\sigma^2_p} \times 100$$

Where,

h^2 = Heritability per cent (Broad sense)

σ^2_g = Genotypic variance

σ^2_p = Phenotypic variance

Heritability was classified as per Robinson *et al.* (1949) into low (0 - 30%), Moderate (30.1% - 60%) and High (>60%).

3.1.3.4 Predicted genetic advance

The predicted genetic advance was estimated according to the formula given by Johnson *et al.* (1955).

$$\text{Genetic advance (GA)} = K.\ H.\ \sqrt{Vp}$$

Where,

K= Selection differential at given intensity

H = Broad sense heritability

$\sqrt{V_p}$ = Phenotypic standard deviation

3.1.3.5 Genetic advance as per cent mean (GAM)

The expected GA as per cent of mean (GAM) was estimated as given below

$$\text{Expected GAM} = \frac{GA}{\overline{X}} \times 100$$

Where,

GA = Genetic advance

$\overline{X}$ = General mean

The genetic advance as per cent of mean was categorized as suggested by Johnson *et al.* (1955) and the same is given below.

Low (0 - 10%), Moderate (10.1% - 20%) and High (20% and above)

3.1.3.6 Identification of transgressive recombinants/progenies

The mean performance of the individual F_5 generation RILs was compared with the better parental mean and the transgressive progenies were identified.

3.2 Experiment: Screening of genotypes and F_5 generation RILs for SMD

The second part F_5 generation RILs and seven genotypes *viz.,* BRG 1, BRG 2, TTB 7, ICP 7035, ICP 8863, Hy3C and Bahar were used for SMD screening. The seeds were raised in polythene bags using compost and soil as a filler material.

Screening of SMD was done by following the standard method "Leaf stapling technique" (Nene and Reddy 1977) during *kharif* 2010 (Plate 1 and 2). For infection of SMD from diseased plants to the test seedlings, mite infected leaves were collected from the SMD infected plants and stapled at 2-3 leaf stage of plants to be screened. As the stapled leaflets from the infected plants gets dried, mites from the infected leaves migrate to healthy leaf and inoculates the virus. After transmission of virus from the infected plants to healthy seedlings, seedlings were scored for SMD incidence at 15 days interval upto 75 days. The plant will be considered as 'susceptible' if it shows mosaic symptoms while the other will be considered as 'resistant'. The mean data on SMD screening is given in Appendix 1.

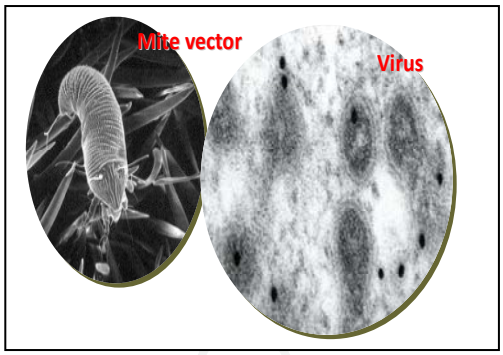
3.3 Genetic diversity studies using SSR markers

The laboratory work on molecular diversity of pigeonpea was carried out at the Research laboratory, AICRP on Pigeonpea, UAS, GKVK, Bangalore. In the present study 61 SSR primers (Table 1) available in public domain (Saxena *et al.*, 2010a; Saxena *et al.*, 2010b and Metkar *et al.*, 2010) were used to screen parents and other pigeonpea genotypes.

3.3.1 Plant material

The material for the study comprised of seven pigeonpea genotypes including parents of F_5 generation RILs of the cross TTB 7 x ICP 7035, *viz.*, BRG 1, BRG 2, TTB 7, ICP 7035, ICP 8863, HY 3c and Bahar.

Plate 1: SMD nursery for screening of RILs, parents and other pigeonpea genotypes.



A

B

C_1

C

C_2

C_4

C_3

Plate 2: General view of leaf stapling technique

A: SMD vector eriophyid mite and pigeonpea sterility mosaic virus (PPSMV)
B: Infected SMD source plants ICP 8863
C: Leaf stapling technique
C_1 - Collection of infected leaf samples
C_2 - Folding the infected leaf with the healthy leaf
C_3 - Stapling
C_4 - Infected leaf stapled with healthy leaf

Table 1: List of SSR primers used to study parental polymorphism in pigeonpea

SL. No.	Primer name	Tm (^{0}C)	SL. No.	Primer name	Tm (^{0}C)
1	ICPM1A08	58	33	CCtta015	56
2	ICPM1B04_a	57	34	CCttc003	56
3	ICPM1B04_b	57	35	CCttc006	56
4	ICPM1C02	60	36	CCttc007	48
5	ICPM1C11	58	37	CCttc008	56
6	ICPM1D01	53	38	CCttc018	48
7	ICPM1D10	59	39	CCttc025	48
8	ICPM1E04	55	40	CCttc033	56
9	ICPM1E10	57	41	CCB1	57
10	ICPM1F11	57	42	CCB2	57
11	ICPM1G01	55	43	CCB3	52
12	ICPM1G04	53	44	CCB4	57
13	ICPM1G11	59	45	CCB5	57
14	ICPM1H01	53	46	CCB6	57
15	ICPMCT20	57	47	CCB7	55
16	ICPM2B8	52	48	CCB8	52
17	CCac003	56	49	CCB9	55
18	CCac010	56	50	CCB10	50
19	CCac012	59	51	PB1	55
20	CCac013	56	52	PB3	55
21	CCac020	48	53	PB7	48
22	CCac036	56	54	PB8	55
23	CCat011	59	55	PB9	53
24	CCcct004	48	56	PB10	53
25	CCcta001	56	57	PB11	55
26	CCgaaa002	56	58	PB12	55
27	CCtc004	48	59	PB13	53
28	CCtc012	48	60	PKM5	56
29	CCtc013	48	61	PKS31	56
30	CCtc014	48			
31	CCtc020	56			
32	CCtta011	48			

Seeds were raised in polythene bags and first trifoliate leaves from three seedlings were taken for DNA isolation. Brief descriptions of morphological characters of parental genotypes used in the study are given in Table 2.

3.3.2 Buffers and solutions used for DNA extraction

1. 1M TRIS HCL pH 8.0

1. 121.1 g of TRIS [Tries-(Hydroxymethyl) Aminomethane]
2. Dissolve in about 650 ml of millipore water
3. Bring pH down to 8.0 by adding concentrated HCL
4. Bring total volume to 1 liter with millipore water
5. Sterilize/autoclave the stock solution

II. 0.5M EDTA

1. 186.12 g of EDTA [Ethylene Diamine Tetra Acetic acid]
2. Add about 650 ml of millipore water
3. Adjust the pH to 8.0 by adding 16-18 g of NaOH pellet. (EDTA will not be dissolve until the pH is near 8.0)
4. Bring total volume to 1 liter with millipore water
5. Sterilize/autoclave the stock solution

III. 5M NaCl

1. 292.2 g of NaCl (Sodium Chloride)
2. Dissolve in about 650 ml of millipore water
   (Note: do not add NaCl all at once because it will never go into solution)
3. Bring the total volume to 1 liter with millipore water
4. Sterilize/autoclave the stock solution

Table 2: Important morphological characters of parental genotypes

Line	Origin	Pedigree	Characteristics
ICP 7035	India (Madhya Pradesh)	Germplasm line	Mid late, indeterminate with semi spreading growth habit. Flowers are red colour with denser purple streaks. Purple colour pods, with reddish brown and bold pea shaped seeds. Resistant to SMD.
BRG 1	India (Karnataka)	Pedigree selection (Hy3C X Hoskote local)	Mid late, semi determinate and semi spreading growth habit, sparsely arranged pink flowers, green pods with few black streaks, dull white and mottled seed colour and moderately resistant to SMD
Hy3C	India (Andhra Pradesh)	Germplasm line	Mid late, purple stem colour, red colourd flowers arranged in clusters, light green with block streaks on pods, white seed colour. Resistant to SMD
TTB 7	India (Karnataka)	Selection from tuth togari	Mid late, indeterminate with spreading growth habit, Yellow flowers with red streaks. Green colour pods with purple streaks. Produces light brown oval seeds. Susceptible to SMD.
BRG 2	India (Karnataka)	Pureline selection from Nelamangala local	Mid late, semi determinate with spreading growth habit, yellow flowers, green colour pods, produces white seeds. Susceptible to SMD
ICP8863	India (Andhra Pradesh)	Gernplasmline	Mid late, spreading growth habit, yellow colour flowers and green colour pods with streaks. It produces brown colored seeds. Susceptible to SMD
Bahar	India (Bihar)	-	Mid late, indeterminate with spreading growth habit, yellow flowers with red streaks. Green colour pods with purple streaks. Produces light brown seeds. Susceptible to SMD

IV. TE buffer

1. 10 ml of 1M TRIS HCL pH 8.0
2. 2 ml of 0.5M EDTA
3. Bring the total volume to 1 liter with millipore water

V. DNA Extraction CTAB working buffer

1. 100 ml of 1M TRIS HCL pH 8.0
2. 40 ml of 0.5M EDTA
3. 280 ml of 5M NaCl
4. 20-25 g of CTAB (N, N, N, N-Cetyl Trimethyl Ammonium Bromide)
5. Make up the volume to 1 liter with sterilized millipore water

3.3.3 DNA extraction protocol

Total genomic DNA was extracted from fully expanded first trifoliate leaves by following CTAB method as per Murray and Thompson (1980) with slight modifications.

1. About 2 gram of leaves were taken and ground with liquid nitrogen to fine powder.
2. Then add 5ml pre-heated extraction buffer and 50µl of β-mercaptaethanol.
3. Then contents were shaken briefly and the tubes were placed on hot water bath at 65 ^{0}C for 30 minutes.
4. Then the contents of the tube were shaken gently and equal volume of 24:1 chloroform/isoamyl alcohol was added and centrifuged at 8000 rpm for 20 minutes.

5. The upper aqueous phase (aliquot) was taken into fresh labeled tube and equal volume of 24:1 chloroform : isoamyl alcohol was added and centrifuged at 6000 rpm for 20 minutes.
6. The upper aqueous phase was taken into fresh labeled tube and add 1/10th of 5M NaCl + equal volume of 100% Isopropanol and kept at -20^{0}c for 2 h, then spin at 12,000 rpm for 20 minutes.
7. Decant the supernatant and add 500 µl of 70% chilled ethanol and spin at 6000 rpm for 20 minutes.
8. Discard the supernatant and air dry the pellets.
9. Finally 50-100 µl of T_{10} $E_{0.1}$ buffer was added to dissolve DNA pellets.

3.3.4 Quantification of genomic DNA

The DNA was quantified by electrophoresis on 0.8 % Agarose gel using 1X TBE electrode buffer and Ethdium Bromide (5 µl /100 ml of Agarose) as staining chemical. Then 2 µl of DNA sample from each genotype was mixed with 2 µl of loading dye (50 % sucrose + 5 µl of bromophenol blue and xylene cyanone) and loaded in to the gel. Known quantity of 'λ' uncut DNA (50 ng/µl) was also loaded as reference to quantify the DNA. The electrophoresis was carried out at 70 Volts for one hour. The gel was viewed under UV trans-illuminator (BIO-RAD gel documentation system, DOL-008.XD, England) to ascertain the quantity of DNA (Plate 3).

3.3.5 Simple Sequence Repeats (SSR) analysis

SSR analysis was carried out for the assessment of genetic diversity among pigeonpea genotypes and for the assignment of genotypes to heterotic groups based on the molecular diversity.

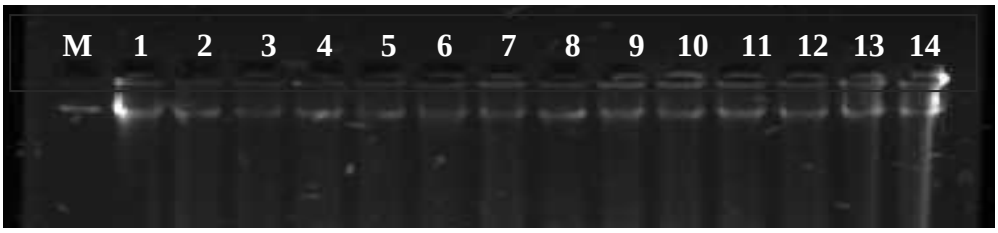


Plate 3: Quantification of DNA samples on 0.8% agarose gel

Note: M1: standard marker of known concentration 50ng/µl (lambda uncut DNA)
Lane 1 to 7 represents seven genotypes
Lane 8 to 14 represents the replica of same seven genotypes

The genomic DNA sample obtained from seven genotypes was diluted using $T_{10}E_{0.1}$ buffer for the required concentration of 15 ng/ µl and used to screen 61 SSR primers.

3.3.5.1 SSR reaction mixture

Polymerase chain reactions (PCR) were performed by using Touch - Down PCR. Then DNA was amplified in 10 µl reaction mixture using mastercycler (model eppendorf gradient PCR system 9700). The cocktail of reaction mixture is given below

SSR reaction mixture	1 X (µl)
Template DNA (5- 10 ng)	1.0 µl
Primer mix (forward and reverse)	2.0µl
dNTP mix (1mM)	2.0 µl
Taq buffer (10X)	1.0 µl
Taq polymerase (5U/µl)	0.3 µl
Sterile water	3.7 µl
Total	10.0 µl

3.3.5.2Amplification programme

A Touch - Down PCR programme was used to amplify the DNA fragments. The primers used in the study were varied with the annealing temperature. Hence, three different touch-down annealing temperatures *viz.,* 55-45 ^{0}C, 60-55 ^{0}C and 65-60 ^{0}C were used for PCR amplification. The details of the programme followed are given below.

Steps	Reactions	Temp (^{0} C)	Duration	
1 :	Initial denaturation	95	3 min	
2 :	Denaturation	94	20 sec	
3 :	Primer annealing	55	20 sec	(-1.0^0 C per cycle- touch down)
4 :	Extension	72	30 sec	(Go to 2 repeat 9 times)
5 :	Denaturation	94	20 sec	
6 :	Primer annealing	48	20 sec	(Go to 6, repeat 32 times)
7 :	Extension	72	30 sec	
8 :	Extension	72	20 min	
9 :	Storage	4	30min	

3.3.5.3 Gel electrophoresis

The amplified PCR products were fractioned on both agarose and polyacrylamide gel to detect polymorphism among the seven genotypes.

Agarose gel electrophoresis

The PCR amplified products were fractionated on 3.0 per cent agarose horizontal gel electrophoresis. The gel was prepared in 1 x TBE buffer. The solution was added with Ethidium bromide (5 µl/100 ml). Then the gel was poured into the gel casting platform after placing the comb in the trough. While pouring, sufficient care was taken to ensure that there is no air bubbles trapped inside the gel.

The comb was removed after the gel solidification. Then the solidified gel was transferred into the electrophoresis unit containing sufficient buffer so as to cover the wells completely. The amplified PCR

products of 2 µl volume with 2 µl of loading dye were carefully transferred into the wells. The electrophoresis conditions were held at 100 V for 4 h at room temperature. After the completion of electrophoresis, the DNA profile was documented using UV trans-illuminator (BIO-RAD gel documentation system, DOL-008.XD, England).

Polyacrylamide gel electrophoresis (PAGE)

The PCR amplified products were also resolved on 4.5% polyacrylamide gel.

Solutions used for plate preparation

1. **Bind silane stock solution:** 100 % ethanol 40 ml + 150µl of bind silane
2. **Bind silane working solution:** 4 ml of stock bind solution + 1ml 10 % acetic acid
3. **Repelcote working solution:** Repelcote 3 ml + 95 % Aceto ethanol (1ml).

Plate preparation

1. Both the plates were cleaned using detergent solution and rinsed. The plates were then rinsed with de -ionized water and dried with paper towels.
2. The larger plate surface was treated with repelcote silane by spreading repelcote with paper towel. An ethanol wipe was given over the surface to evenly spread the silane and the final polish was given with second ethanol wipe.
3. The small plate was treated with bind silane by spreading evenly over the whole surface with paper towel.

4. The spacers were aligned on the edges of large plate. The smaller plate was placed on top and tape was put on both sides to secure both plates together.

Pouring of gel

1. Hundred ml of 4.5% polyacrylamide gel was taken in a pouring bottle and 50µl of chilled TEMED (Tetramethyl ethylenediamine) and 500µl of chilled 10% Ammonium persulphate were added.
2. The polyacrylamide was allowed to flow slowly and gently between the plates. The comb was placed in reverse direction between plates.
3. The gel was allowed to polymerize for 3h.
4. The comb was removed by sliding it out horizontally.
5. The plates were mounted onto the electrophoresis unit, with the longer plate in the outer position.
6. Five hundred ml of 1 x TBE was poured into the top reservoir and also into bottom reservoir.
7. The comb was put into position so that it just touched the gel.
8. Three µl of each DNA sample was loaded.
9. Electrophoresis was carried out at 1200 V for 2 h until the dark blue dye ran off.
10. The gel was unmounted and the two plates were separated using plastic wedge. The gel remained in complete contact with the smaller plate.

Vertical gel electrophoresis

After polymerization of gel, the PCR products were added with loading dye and fractioned on 4.5% polyacrylamide gel on Genei ™,

Vertical –Gel Apparatus. Gels were made of 11.5 ml of acrylamide solution (40% acrylamide and bisacrylamide solution), urea (8 M) and 10 ml of 10xTBE buffer and the total volume was 100 ml per gel. Electrophoresis was carried out by loading 3µl of each DNA sample. Then gels were run for 90 min. at 1200 V. The gels were then separated and developed by silver staining (Bassam and Caetano-Anolles 1993) Stained gels were air dried and scored under white light trans-illuminator.

3.3.5.4 Silver staining of the gel (NaOH staining)

Solutions prepared for staining of the gel

1. **Fixer** -200 ml ethanol +10 ml acetic acid then make up the volume to 2*l* with millipore water and store at 4^{o} c
2. **Silver stain** – 3g of silver nitrate in 2*l* of millipore water and to that add 2 ml of farmaldehyde.
3. **Developer** – 1.5% sodium hydroxide was prepared (30 g of sodium hydroxide in 2*l* of millipore water and add 3ml of formaldehyde.) and stored at 4^{o} C

Silver staining procedure

1. The gel was placed in trays and fixer was poured, under shaking condition gel was kept for 5min
2. Then under shaking condition, gel was placed in silver stain solution for 5 min
3. The gel was rinsed in 3 liter of distilled water for 10 sec
4. Gel was immediately placed in developing solution and immersed up and down holding the top end of the plate
5. The reaction was stopped by adding 2*l* fixer when bands near the bottom of the gel started to develop

6. The gel was rinsed in distilled water for 10 min and left to dry over night in vertical position

3.3.5.5 SSR data scoring and data analysis

The profile produced by different **SSR** primers were scored, each allele was scored as present (1) or absent (0) for each of the SSR loci. Then 0–1 matrix was subjected to similarity analysis based on Jaccards index (Jaccard, 1908) to derive a matrix of similarity coefficient. Pairwise comparisons from the similarity matrix were used to generate a dendrogram of genetic relatedness using NTSYS pc version 2.0 program (Rolf 1998). In addition, PIC values were also computed using the formula given by Anderson *et al*. (1993). It refers to the value of a marker for detecting polymorphism within the genotypes, depending on the number of detectable alleles and the distribution of their frequency. The PIC value of a marker was calculated as follows

$$PIC = 1 - \sum_{I=/}^{k} Pi^2$$

Where k is the total number of alleles detected for a given marker locus and Pi is the frequency of the i^{th} allele in the set of genotypes investigated.

Experimental Results

IV. EXPERIMENTAL RESULTS

The data on genetic variability studies in F_5 generation RILs, screening for SMD and parental polymorphism studies was subjected to statistical analysis and the results obtained are presented in this chapter under the following headings.

4.1 Estimation of genetic variability parameters for yield and its attributing characters in F_5 generation RILs

The variability parameters *viz.,* mean, genotypic coefficient of variation (GCV), phenotypic coefficient of variation (PCV), heritability in broad sense and genetic advance as per cent of mean (GAM) with respect to days to fifty per cent flowering, days to maturity, primary and secondary branches per plant, plant height (cm), length of the inflorescence, pods per plant, seeds per pod, 100 seed weight (g), shelling per cent and seed yield per plant (g) are presented below.

Data on analysis of variance (ANOVA) for all the above parameters is presented in Table 3. The data indicated that, significant difference was noticed among all the characters studied.

The mean, range, phenotypic coefficient of variation (PCV), genotypic coefficient of variation (GCV), broad sense heritability (h^2) and genetic advance as per cent mean (GA%) for all the characters are furnished in Table 4.The data indicated that F_5 generation RILs exhibited wide variation for all the parameters studied.

Days to 50 per cent flowering ranged from 95 (RIL No.17) to 126 (RIL No.94) days with a mean of 107.47 days and days to maturity ranged from 137 (RIL No.16) to 193 (RIL No.96) days with an average of 172.89 days.

Table 3: Analysis of Variance (mean sum of squares) for 11 different yield and yield attributing characters in the F_5 generation RILs of cross TTB 7 × ICP 7035

SOURCE	d. f.	DFF	PB/P	SB/P	LOI	PHT	PPP	SPP	100TW	DTM	SH.P	YLD
Blocks	29	27.26	4.53	54.88	93.57	531.51	2778.30	0.15	2.88	54.92	54.81	852.26
Genotype	122	75.96	6.67	94.64	125.03	425.07	4260.98	0.15	3.21	125.16	99.85	1830.37
Checks	2	380.22	3.71	33.32	7.02	6.43	1995.89	0.03	2.96	727.17	16.14	1167.13
URG	119	69.33*	6.76*	94.91**	128.02*	369.69**	4324.13**	0.15*	3.23*	112.64*	92.43**	1850.03**
Checks / URG	1	257.14	0.78	185.03	4.90	7851.84	1276.19	0.70	1.83	412.10	1150.07	816.98
ERROR	58	56.54	7.19	52.62	88.10	221.92	2364.56	0.10	1.98	97.95	55.74	983.16

Note: * and ** indicates significant at 5 % and 1 % level respectively

URG- Unreplicated genotypes

DFF = Days to fifty % flowering

PB/P = Primary branches per plant

SB/P = Secondary branches per plant

LOI = Length of the inflorescence (cm)

PHT = Plant height (cm)

PPP = Pods per plant

SPP = Seeds per pod

100SW = 100 seed weight (g)

DTM = Days to maturity

SH.P = Shelling per cent

YLD = Seed yield per plant (g)

Primary branches per plant ranged from 2 (RIL No.118) to 13 (RIL No.53) with an average of 6.88 while the secondary branches per plant ranged from 2 (RIL No.39) to 75 (RIL No.78) with an average 15.86.

Plant height varied from 112.50 (RIL No.120) to 223.60 cm (RIL No.41) with an average of 157.38 cm and the length of the inflorescence was ranged from 15.00 (RIL No.34) to 76.00 cm (RIL No.71) with an average of 37.82 cm.

Pods per plant ranged from 40.75 (RIL No.100) to 329.00 (RIL No.78) with an average of 132.96 and seeds per pod ranged from 3 (RIL No.58) to 5 (RIL No.37) with an average 4.15.

Hundred seed weight and shelling per cent ranged from 8.68 (RIL No.50) to 16.87 g (RIL No.69) and 35.20 (RIL No.99) to 75.36 (RIL No.84), respectively, while the mean values were 51.56, and 13.03g respectively.

Seed yield per plant ranged from 20.30 (RIL No.60) to 221.80g (RIL No.48) with an average of 88.41 g.

High phenotypic coefficient of variance (PCV) and genotypic coefficient of variance (GCV) values were estimated for secondary branches per plant (76.29 and 61.06 %), pods per plant (61.20 and 49.07 %), seed yield per plant (58.98 and 47.49%), primary branches per plant (53.99 and 37.42 %) and length of inflorescence (38.84 and 29.88 %).

100 seed weight (16.79 and 13.03%) and plant height (15.45 and 12.21%) exhibited moderate PCV and GCV values.

Low PCV and GCV values were recorded for days to maturity (7.89 and 5.44 %) and days to fifty per cent flowering (9.89 and 7.00 %).

Moderate PCV and low GCV values were recorded for seeds per pod (12.5 and 9.32 %), whereas, shelling per cent recorded high PCV (23.80) and moderate GCV (18.89).

Heritability in broad sense was computed by taking ratios of genotypic variance to phenotypic variance and converted to per cent heritability (Table 4).

Heritability values were high for plant height (71.13%), seed yield per plant (64.81%), pods per plant (64.29%), secondary branches per plant (64.06%), shelling per cent (63.00%) and 100 seed weight (61.26%).

Whereas, moderate heritability values were recorded for seeds per pod (59.83%), length of the inflorescence (59.19%), days to fifty per cent flowering (50.05%), primary branches per plant (51.77%) and days to maturity(47.43%).

Genetic advance as per cent of mean was calculated and presented in Table 4.

Secondary branches per plant exhibited the highest genetic advance as per cent of mean (99.7%) followed by pods per plant (80.91%), seed yield per plant (78.75%), length of the inflorescence (47.36%), primary branches per plant (45.87%), shelling per cent (30.88%), 100 seed weight (23.73%) and plant height (22.63%).

Genetic advance as per cent of mean was moderate for seeds per pod (14.85 %) and days to fifty per cent flowering (10.20%) whereas, days to maturity (7.71%) recorded low genetic advance.

From the studies on the genetic parameters on yield and its attributing characters, it is evident that, seed yield per plant, pods per plant and secondary branches per plant exhibited high estimates of PCV

Table 4: Estimates of genetic parameters for yield and its attributing characters in F_5 generation RILs of cross TTB 7 × ICP 7035

Sl. No.	Characters	Range		Mean ± S.E.m	PCV (%)	GCV (%)	h^2_{bs} (%)	GA as % mean
		Lowest	Highest					
1.	Days to 50% flowering	95.00	126.00	107.47 ± 0.757	9.89	7.00	50.05	10.20
2.	Primary branches per plant	2.00	13.00	6.88 ± 0.237	53.99	37.42	48.27	45.87
3.	Secondary branches per plant	2.00	75.00	15.86 ± 0.889	76.29	61.06	64.06	99.70
4.	Length of the inflorescence (cm)	15.00	76.50	37.82 ± 1.033	38.84	29.88	59.19	47.36
5.	Plant height (cm)	112.50	223.60	157.38 ± 1.756	15.45	12.21	71.13	22.63
6.	Pods per plant	40.75	329.00	132.96 ± 1.003	61.20	49.07	64.29	80.91
7.	Seeds per pod	3.00	5.00	4.15 ± 0.035	12.05	9.32	59.83	14.85
8.	100 seed weight (g)	8.68	16.87	13.03 ± 0.164	18.80	14.70	61.26	23.73
9.	Days to maturity	147.00	193.00	172.89 ± 0.969	7.82	5.44	47.43	7.71
10.	Shelling per cent	35.20	75.36	51.56 ± 0.878	23.80	18.89	63.10	30.88
11.	Seed yield per plant (g)	20.30	221.80	88.41 ± 3.926	58.98	47.49	64.81	78.75

and GCV, heritability in broad sense and genetic advance as per cent mean.

4.1.1 Mean performance of parents for yield and its attributing traits

The mean performance of parents TTB 7 and ICP 7035 for yield and its attributing characters is given below (Table 5).

a) TTB 7

The mean values for various yield attributing traits in TTB 7 variety are as follows. Days to fifty per cent flowering (111.80); primary branches per plant (9.50); secondary branches per plant (29.83); length of the inflorescence (25.89 cm); plant height (153.8 cm); pods per plant (182.00); seeds per pod (3.87); 100 seed weight (9.46 g); days to maturity (185); shelling per cent (64.41) and seed yield per plant (121.2g).

b) ICP 7035

The mean values for various yield attributing traits in ICP 7035 are as follows. Days to fifty per cent flowering (105.35); primary branches per plant (5.67); secondary branches per (7.50); length of the inflorescence (42.45 cm); plant height (127.7 cm); pods per plant (117.5); seeds per pod (4.65); 100 seed weight (14.63 g); days to maturity (176); shelling per cent (63.34) and seed yield per plant (46.32 g).

4.1.2 Identification of transgressive recombinants

All the F_5 generation RIL progeny lines which exceeded their parental limit for seed yield, primary branches, secondary branches and pods per plant were identified. Details of the positive transgressive recombinants are given in Table 6 and briefly described hereunder.

Table 5: Mean performance of parents and F_5 generation RILs for yield and its attributing characters

SI. No.	Material	DFF	PBP	SBP	LOI	PHT	PPP	SPP	100 SW	DTM	SHP	SYLD
1	TTB7	111.80	9.50	29.83	25.89	153.8	182.0	3.87	9.46	185	64.41	121.2
2.	ICP7035	105.35	5.67	7.50	42.45	127.7	117.5	4.65	14.63	176	63.34	46.32
3.	Mean of RILs	107.47	6.88	15.86	37.56	157.38	132.96	4.14	12.02	172.89	51.56	89.61
4.	Range of RILs	95-126	2-13	2-75	15-76	112.5-223.6	40.75-329	3-5	8.68-16.87	137-193	35.2-75.36	20.3-221

DFF = Days to first flowering

PBP = Primary branches per plant

SBP = Secondary branches per plant

LOI= length of inflorescence (cm)

PHT = Plant height (cm)

PPP = Pods per plant

SPP = Seeds per pod

100 SW = 100 seed weight (g)

DTM= days to maturity

SHP = Shelling per cent

SYLD = seed yield per plant (g)

For the seed yield per plant, a total of 26 positive transgressive recombinants were observed of which RIL 48 recorded the highest seed yield per plant (221.80g) followed by RIL 78 (210.40 g) and RIL 41 (204.10 g).

Twenty positive transgressive segregants were identified for pods per plant. The RIL 78 exhibited the highest pods per plant (329.00) followed by RIL 48 (320.00) and RIL 41 (300.00).

While, for secondary branches per plant a total of 8 positive transgressive recombinants were noticed of which RIL 78 recorded the highest secondary branches per plant (75.00) followed by RIL 108 (45.00) and RIL 13 (40.80).

4.2 Screening of pigeonpea genotypes and F_5 generation RILs for SMD

4.2.1 Reaction of parental genotypes to SMD

The disease incidence in parental genotypes exhibited wide range of variability and it varied from 6.7 to 96.0 per cent. Genotypes ICP 7035 and BRG 1 showed resistance reaction with the SMD score of 6.7% and 9.7% respectively. While, Bahar (23.0%) and HY 3c (25.0%) recorded moderately resistant reaction. Genotypes BRG 2 (64.3%), TTB 7 (94.1%) and ICP 8863 (96.0%) exhibited susceptibility reaction to SMD (Table 7).

4.2.2 Reaction of F_5 generation RILs to SMD

In F_5 generation RILs also, SMD incidence exhibited wide variability and it ranged from 5.0 to 100 per cent. Out of 120 RILs studied, none of the families were immune, only 3 lines (RIL numbers 9, 23 & 33) were resistant, 25 were moderately resistant and the remaining 92 RILs showed susceptible reaction (Table 8). The mean disease reactions of individual RILs are presented in Appendix I.

Table 6: Transgressive recombinants in F_5 generation RILs of cross TTB7 x ICP 7035

SL. No.	RIL No.	Secondary branches/ plant	RIL No.	Pods / plant	RIL No.	Seed yield/ plant
1	78	55.00	78	329.00	48	221.80
2	108	45.00	48	320.00	78	210.40
3	13	40.80	41	300.00	41	204.10
4	99	32.60	82	311.00	26	171.62
5	98	32.20	108	280.00	82	170.28
6	41	32.00	49	243.80	75	168.20
7	47	31.60	39	243.40	106	160.24
8	118	30.00	26	226.25	40	157.22
9			75	215.00	37	153.21
10			40	202.00	42	148.21
11			85	200.10	49	148.20
12			1	200.00	85	142.78
13			42	200.00	65	141.22
14			65	198.50	23	138.02
15			37	192.00	108	137.82
16			17	189.32	36	132.11
17			23	187.40	45	131.30
18			36	186.20	1	128.31
19			61	182.46	89	128.21
20			50	182.42	27	128.00
21					7	123.86
22					17	123.62
23					72	123.42
24					114	123.37
25					9	121.60
26					99	121.31

SL. No.	Parents	Secondary branches/ plant		Pods /plant		Seed yield/ plant
1	TTB 7	29.83		182.00		121.20
2	ICP 7035	7.50		117.50		46.32

Table 7: Reaction of parents and other genotypes to SMD

Disease incidence scale	Genotypes	Total no. of plants	Infected plants	Per cent disease incidence	Reaction to SMD
0-10 % of plants infected	BRG 1	32	03	09.7	Resistant
	ICP 7035	30	02	06.7	Resistant
10.1 – 30 % of plants infected	HY 3C	25	06	25.0	Mod. resistant
	Bahar	26	06	23.0	Mod. resistant
30.1 – 100 % of plants infected	BRG 2	28	18	64.3	Susceptible
	ICP 8863	25	24	96.0	Susceptible
	TTB 7	34	32	94.1	Susceptible

Table 8: Reaction of F_5 generation RILs of cross TTB 7 × ICP 7035 to SMD

Disease incidence scale	No. of RILs	SMD reaction
0-10 % of plants infected	3	Resistant
10.1 -30 % plants infected	25	Mod. resistant
30.1 - 100 % plants infected	92	Susceptible
Total	**120**	

4.2.3 Frequency distribution of F_5 generation RILs to SMD

The SMD incidence in RILs ranged between 5.0 to 100.0 % with a mean of 78.94 %. The variation existed for SMD incidence is represented graphically using frequency distribution of means (Fig 1.). The disease scores were plotted on X-axis against genotypic frequency on Y-axis with equal class intervals. The resulting histogram showed near normal curves with skewed towards susceptibility for SMD. In general the distribution of RILs for SMD incidence was within the parental limits.

4.3 Parental polymorphism studies using SSR markers for SMD resistance

Sixty one SSR primers available in public domain were selected to screen the parental polymorphism among seven pigeonpea genotypes. Amplicons of all the 61 primers were electrophoreses on agarose (3%). Out of 61 primers, 46 primers showed amplification of which three showed polymorphism (ICPM2B08, CCat011 and CCttc033) and the remaining 34 primers showed monomorphism and nine primers showed non-specific amplification with 6.5 per cent polymorphism (Table 9 and plate 4).

In order to have the higher resolution, all the PCR products were also electrophoresed on 4.5 per cent polyacrylamide gel. Out of 46 primers amplified, nine were non-specific, 24 were monomorphic (Table 9). The 13 primers exhibited polymorphism on PAGE in pigeonpea genotypes are CCttc003, CCttc006, CCttc008, CCat011, CCtc012, CCttc033, CCac036, ICPM1D10, ICPM1E04, ICPM1H01, ICPM2B08, PB1 and PB12 (Plate 4 and 5). The polymorphic information content (PIC) ranged between 0.23 – 0.60, with an average of 0.41. The highest (0.60) PIC value was observed for the primer CCttc008 and lowest for CCtc012 (Table 10).

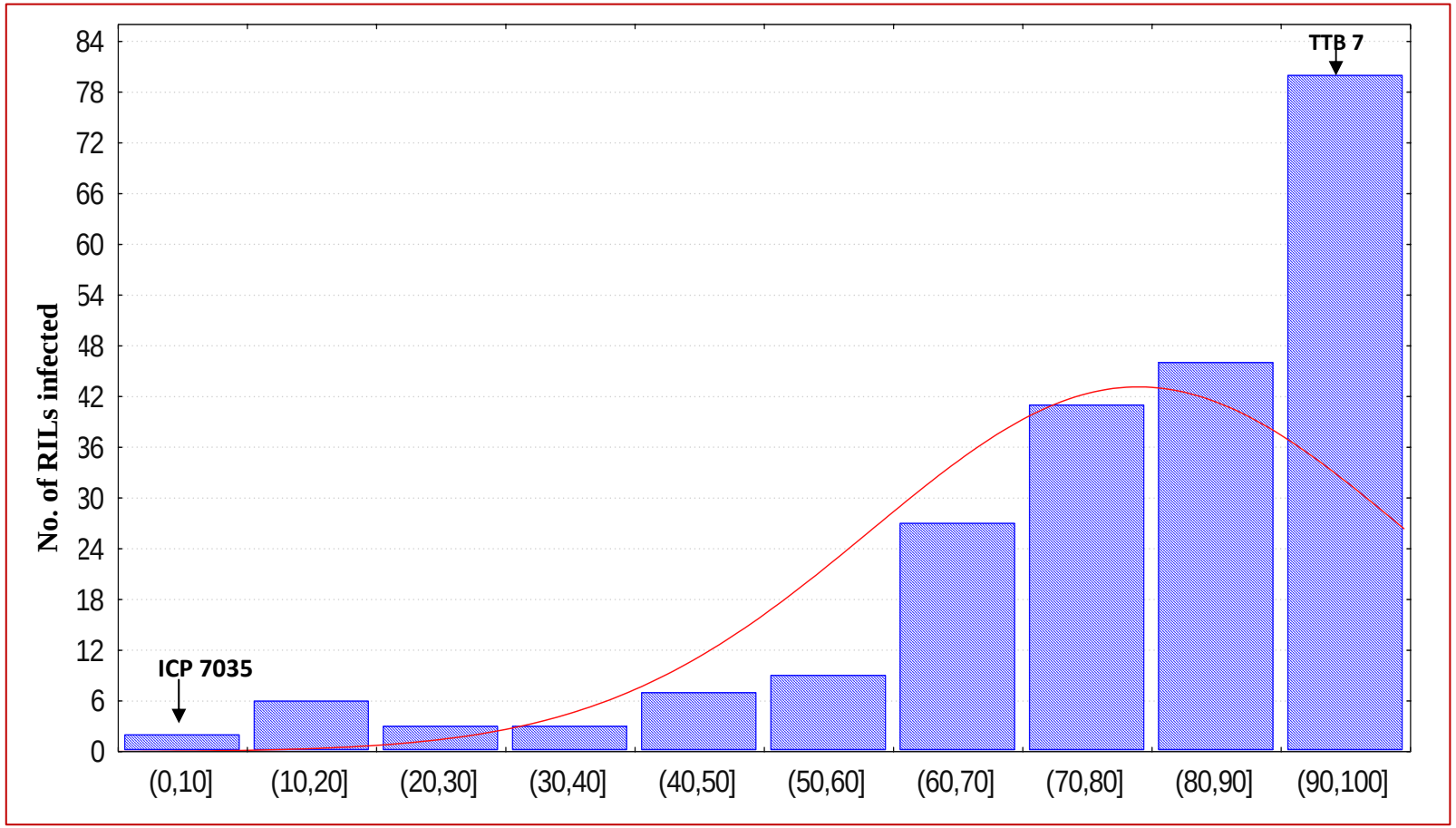


Fig 1: Frequency distribution of F_5 generation RILs to pigeonpea sterility mosaic disease incidence

Table 9: Details of 61 SSR primers tested for parental polymorphism

Sl. No.	Details of SSR primers	Agarose (3%)	PAGE (4.5 %)
1	Primers not amplified	15	15
2	Monomorphic primers	34	24
3	Polymorphic primers	03	13
4	Non-specific amplification	09	09
5	Total primers amplified	46	46
6	**Per cent polymorphism**	**6.5**	**28.3**

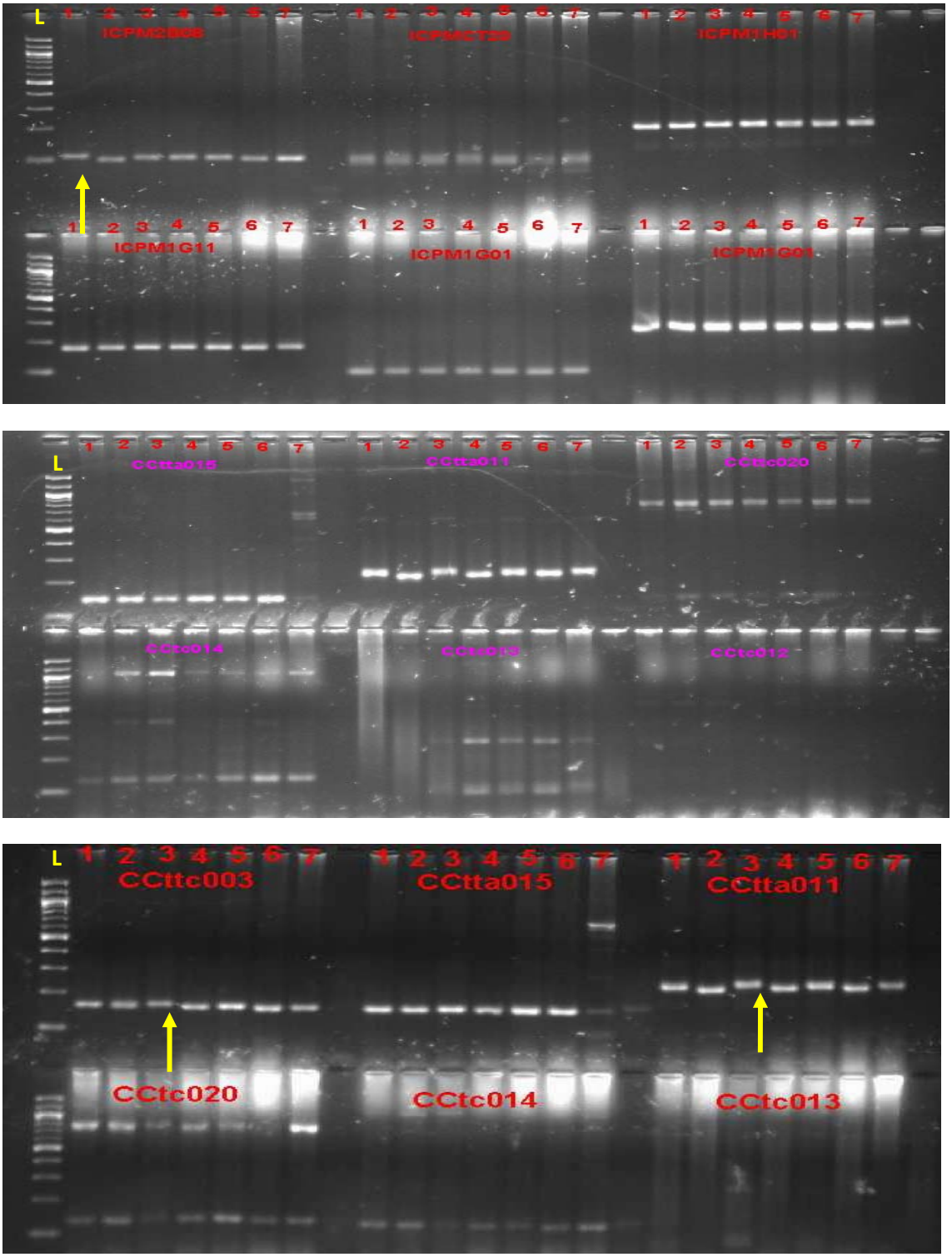


Plate 4: Polymorphic SSR primers of pigeonpea genotypes on 3 % agarose gel

L-100bp, 1-BRG 1, 2-BRG 2, 3-TTB 7, 4-ICP 7035, 5-ICP 8863, 6-Hy 3C, 7-Bahar

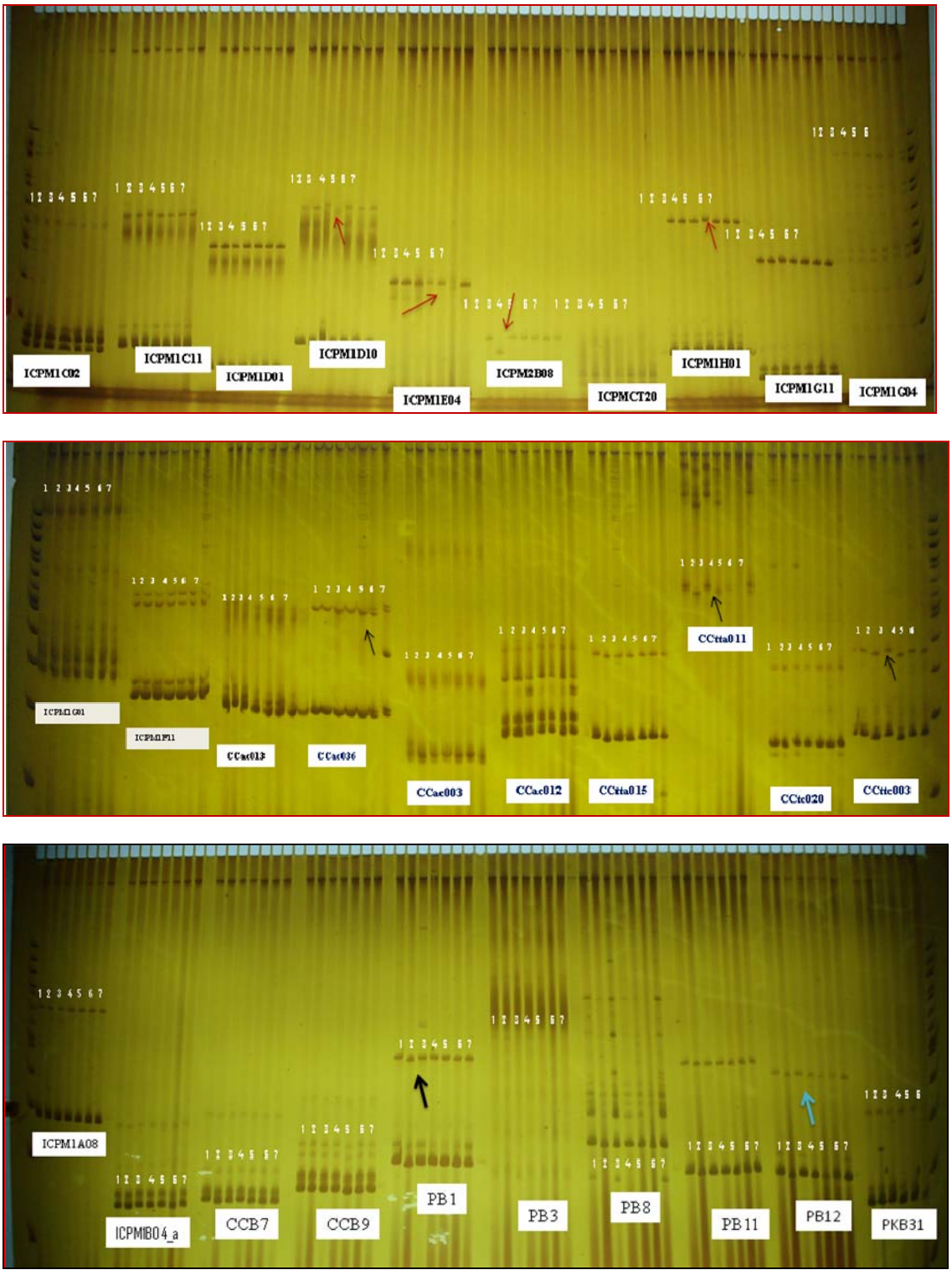


Plate 5: Polymorphic SSR primers of pigeonpea genotypes on 4.5% acrylamide gel

L-100bp, 1-BRG 1, 2-BRG 2, 3-TTB 7, 4-ICP 7035, 5-ICP 8863, 6-Hy 3c, 7-Bahar

Between susceptible variety TTB 7 and resistant variety ICP 7035, eight SSR primers (CCttc003, CCttc006, CCttc008, CCat011, ICPM1D10, ICPM1E04, ICPM1H01, and PB12) showed polymorphism (Plate 5).

Genetic similarity and clustering of varieties

The SSR data on 58 polymorphic bands was subjected to genetic similarity analysis based on cluster analysis by UPGMA method using NYSYS pc programme. The similarity coefficient was ranged from 0.62 to 0.80. The highest (0.796) similarity was observed between Hy3c and BRG 1 and lowest (0.539) was between ICP 8863 and BRG 1 (Table 11).

Dendrogram constructed by the SSR data depicted that, the SMD resistant and moderately resistant genotypes viz., ICP 7035, BRG 1, Bahar and HY 3c clustered together in major cluster I. Genotypes BRG 1 and HY 3c formed a separate sub cluster within the major cluster and very close to Bahar. These genotypes are differing at genotypic level from SMD susceptible genotypes *viz.*, BRG 2, TTB 7 and ICP 8863 which formed a separate and distinct major cluster II. The genotypes TTB 7 and ICP 8863 formed a separate sub cluster within the major cluster II which is very close to BRG 2 (Fig. 2).

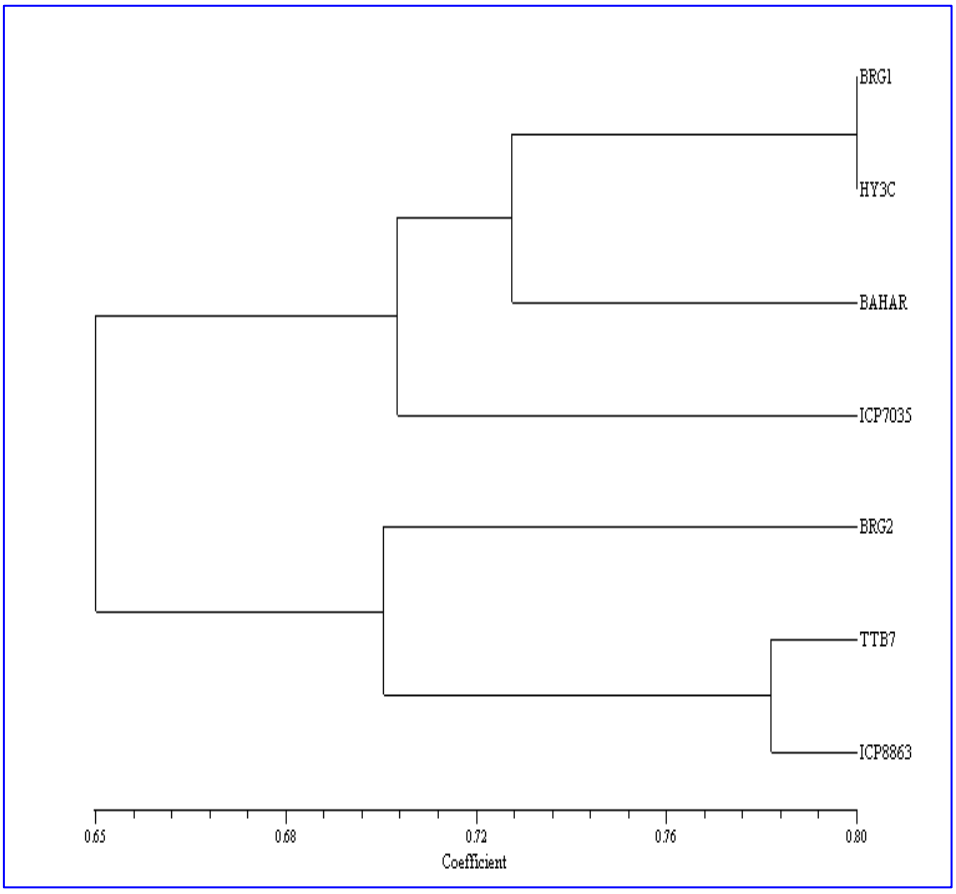


Fig 2: Dendrogram showing grouping of pigeonpea genotypes based on SSR primers

Table 10: Details of polymorphic SSR primers obtained in the study on parental polymorphism

SI. No.	Primer name	Forward primer sequence (5'-----3')	Reverse primer sequence (5'-----3')	Tm (^{0}C)	PIC value
1.	CCttc003	ACACCACCATCGTAAAGAACAAG	CCAAGCAAGACACGAGTAATCATA	56	0.49
2.	CCttc006	CTAGAGGAGGTTCCAAATGACATA	ATCTGTCTGGTGTTTTAGTGTGCT	56	0.57
3.	CCttc008	TCACAGAGGACCACACGAAG	TGGACTAGACATTGCGTGAAG	56	0.60
4.	CCat011	TGCTCTAATGCTAGTTCATCC	AAACACTCATGGGTTAGATTCTCC	59	0.28
5.	CCtc012	GAGGATTGCACCAAGCAACT	GCACTGCTGGCCTTACCATA	48	0.23
6.	CCttc033	ATTCCCTCTCTATCTCAGACTTTT	TCGTGATGGAACTCAAGATACACT	56	0.60
7.	CCac036	ATCGGCTTTTGTCTTGATGA	AAGCTACAAGGGATACACATGC	56	0.54
8.	ICPM1D10	GGATTAACCAATTGTGAGTGAACC	TGCACTTTATAAGCATTTACCAACA	59	0.33
9.	ICPM1E04	TTTTTATGGAATATTTATGAGTTGGC	AAGAGTTTCCCAACCCTGCT	55	0.36
10.	ICPM1H01	CCCGAACTGCATTCAAAAAT	GCGTAGGTGGAAGAAGATCG	53	0.49
11.	ICPM2B08	AGTTTGAAATTGCTTTTGGCT	GAATTGGGAGAGACCGCATA	52	0.49
12.	PB1	GGGCTTCCTTTTCTTCTC	GTCTTCGTGTAAGTCATACT	55	0.49
13.	PB12	GTCTTCGTGTAAGTCATACT	CCGAGGTGCTCCAAGTGAC	55	0.42

Note: Tm- annealing temperature, PIC- polymorphic information content

Table 11: Jaccard's similarity coefficients for seven parental genotypes of pigeonpea

Parents	BRG 1	HY 3c	Bahar	ICP 7035	BRG 2	TTB 7	ICP 8863
BRG 1	1.000						
HY 3c	0. 796	1.000					
Bahar	0.610	0.728	1.000				
ICP 7035	0.728	0.677	0.610	1.000			
BRG 2	0.627	0.677	0.779	0. 762	1.000		
TTB 7	0.644	0.644	0.610	0.628	0.627	1.000	
ICP 8863	0. 593	0.711	0.711	0.661	0.694	0.694	1.000

Discussion

V. DISCUSSION

The results obtained from the present investigation are discussed under the following headings to arrive at valid conclusions.

5.1 Genetic variability parameters for yield and its attributing characters in F_5 generation RILs

The improvement of character in a population is a function of variability existing in the population. Hence, formulation of objectives in breeding programme should be essentially accompanied with assessment of existing variability. The variability quantified by range includes influence of environment and genotype × environmental interactions. Since, all these variations are not heritable therefore, it is appropriate to partition the phenotypic variation into heritable (genetic) and non-heritable (environmental) components and thus, breeding value of the genotype can be precisely estimated by separating genetic variance from environmental variance. In this direction the components of variance such as phenotypic coefficient of variance (PCV), genotypic coefficient of variance (GCV), heritability in broad sense (h^2b.s) and predicted genetic advance as per cent mean were computed for all the characters studied and discussed below.

In the present investigation, significant variation was observed for seed yield and its yield attributing characters studied. This variation indicated substantial scope for selection of these characters for further breeding work to improve yield in pigeonpea. Similar significant genetic variations for the yield and yield attributing characters were reported by Deshmukh, *et al.* (2000); Venkateswarulu, (2001); Anantharaju and Muthiah, (2008); Bhadru (2010) and Hamid *et al.* (2011) in pigeonpea.

High PCV and GCV values were recorded for secondary branches per plant, pods per plant, seed yield per plant, primary branches per plant and length of the inflorescence. This indicates that the variance present in these characters is due to the superiority of the RIL genotypes and the present results are in conformity with the findings of earlier workers (Jagashoram, 1983; Balyan and Sudhakar, 1985; Aher *et al.*1998; Vikas and Singh 1998; Venkateshwaralu 2001; Satish Kumar *et al.* 2006; Anantharaju and Muthiah 2008 and Ganapathy 2009).

Moderate values of PCV and GCV were recorded for 100 seed weight and plant height; the results are in accordance with the findings of Premsagar and Jatasra (1984), Sindhu et al. (1985), Holkar *et al.* (1991) and Gamber *et al.* (1996). On the contrary, high PCV and GCV estimate for these traits were reported by Vikas and Singh (1998); Singh *et al.* (2003), Chattopadyaya and Dhimam (2005) and Satish Kumar *et al.* (2006).

Low PCV and GCV estimates exhibited by days to 50 per cent flowering and days to maturity indicating limited scope for selection. This is due to the lesser variability for these characters in the population studied. Similar observations were reported by Firoz mahamad (2003) and Anantharaju and Muthiah (2008). On contrary, high PCV and GCV values for these traits were reported by Singh *et al.* (2003) and Chattopadhyaya and Dhiman (2005).

High PCV and moderate GCV were recorded for shelling percentage. Similar reports were made by Ganapathy (2009) and Ajay (2010). Moderate PCV and low GCV were recorded for seeds per pod and these results are in accordance with the results of Ganapathy (2009), Vange and Egbe (2009), Bhadru (2010) and Ajay (2010).

Genotypic coefficient of variation (GCV) would be more useful for the assessment of variability than the phenotypic coefficient of variation (PCV) since, it depends upon the heritable portion of the total variability (Allard, 1970). Higher the proportion of GCV more will be the chance for exploitation of that particular character. Many practical decisions in breeding programs are based on the magnitude of heritable variation. By comparing PCV and GCV estimates, the influence of environment on the expression of the characters can be assessed. In the present study, the differences between PCV and GCV was low for length of the inflorescence, secondary branches, pods per plant and primary branches indicating the greater role of genetic factors causing variability in these characters. These observations indicated ample scope for improvement of the above traits by undergoing selection form their phenotypic values.

Knowledge of genetic advance and heritability is useful in designing effective breeding programme. Evaluation of the recombinant inbreds lines helps in estimation of various genetic and non-genetic components of variance. Heritability is a fraction of variance in phenotypic expression that arises from genetic effects. The nature of selection units and sampling errors also influences greatly the magnitude of heritability estimates. The heritability estimates in segregating generation helps to know genetic variance, genotype environment interaction and progress to be expected from selection. The estimate of genetic advance may be biased upward if phenotypic variance contains a fraction of whatever genetic variance due to non additive effects (dominance or epistasis) if present (Hanson *et al.*, 1956). Hence, heritability and predicted genetic advance as per cent mean (GAM) were also computed.

Heritability value alone provides no information of the amount of genetic progress that would result from selecting the best individuals

since their scope is restricted by their interaction with environment. Johnson *et al.* (1955) reported that heritability estimates along with genetic gain would be more useful than the former alone, in predicting the effectiveness of selecting the best individuals. Therefore, it is essential to consider the predicted genetic advance along with heritability estimates as a tool in the selection programme for better efficiency in the selection.

In the present study, heritability in broad sense was high for plant height, seed yield per plant, pods per plant, secondary branches per plant, shelling per cent and 100 seed weight. This is due to higher genotypic variance than the phenotypic variance for these characters. These results are in conformity with the results of Pansuriya (1998); Vikas and Singh (1998), Srinivas *et al.* (1999), Basavarajaiah *et al.* (2000), Venkateswarlu (2001); Singh *et al.* (2003); Chattopadyaya and Dhiman (2005), Anantharaju and Muthaiah (2008), Ganapathy (2009) and Ajay (2010).

Moderate heritability was recorded for seeds per pod, length of the inflorescence, days to fifty per cent flowering, primary branches per plant and days to maturity. This is due to higher phenotypic variance than the genotypic variance. These results are in accordance with Anantharaju and Muthiah (2008). On contrary Vange and Egbe (2009) recorded the highest heritability for these traits.

High heritability accompanied with high genetic advance indicates that most likely the heritability is due to additive effects and selection may be effective. High heritability accompanied with low genetic advance indicates non-additive gene action and selection for such trait may not be rewarding (Johnson *et al.*, 1955).

High heritability with high genetic advance was observed for secondary branches, pods per plant, seed yield per plant, shelling per cent, 100 seed weight and plant height. Similarly, Balyan and Sudhkar (1985), Aher *et al.* (1998), Chattopadyaya and Dhiman (2005), Ganapathy (2009), Ajay (2010) and Singh *et al.* (2010) also reported high heritability coupled with high genetic advance as per cent of mean for these characters.

Primary branches per plant and length of the inflorescence recorded moderate heritability with high genetic advance. These results are in accordance with results of Jagdish Singh (1999), Basavarajaiah *et al.* (2000) and Singh *et al.* (2003)

Seeds per pod recorded moderate genetic advance with moderate heritability; the results are in accordance with the findings of Jagashoram (1983) and Sindhu *et al.* (1985). On contrary Holkar *et al.* (1991) and Deshmukh *et al.*(2000) recorded high genetic advance as per cent mean and high heritability for seeds per pod.

Characters like days to 50 per cent flowering and days to maturity recorded moderate heritability with low genetic advance as per cent of mean. Similar results were also reported by Satish Kumar *et al. (*2006) in pigeonpea. The straight selection on the basis of phenotypic performance alone would not be rewarding in the improvement of these traits.

High estimates of PCV, GCV, heritability and genetic advance as per cent of mean were observed for secondary branches per plant, pods per plant and seed yield per plant. The results indicated that these traits are under the control of additive gene action and directional phenotypic selection for these traits could be effective for desired genetic improvement.

5.2 Identification of desirable transgressive recombinants

Recombination breeding is one of the important breeding methods to incorporate and get the favourable quantitative and qualitative traits of both the parents in its progenies. Selection in F_5 for transgressive recombinants is likely could help the breeder to pick out the favourable recombinant inbreed lines (RILs).

In the present investigation, for the seed yield per plant, a total of 26 positive transgressive recombinants were observed, of which RIL 48 recorded the highest seed yield per plant. It might be due to its higher 100 seed weight (16.87g). Twenty positive transgressive segregants were identified for pods per plant. The RIL 78 exhibited the highest pods per plant (329.0) may be due to higher number of secondary branches. While, for secondary branches per plant, a total of 8 positive transgressive recombinants were noticed of which RIL 78 recorded the highest secondary branches per plant. Ganapathy (2009) and Ajay (2010) also reported higher frequencies of positive transgressive segregants for seed yield per plant, pods per plant and secondary branches per plant in pigeonpea advanced breeding population.

5.3 Screening of F_5 generation RILs and parental genotypes for SMD resistance

A basic knowledge of number of genes governing the traits is essential for efficient selection. There are conflicting reports about the genetics of resistance to sterility mosaic disease claiming both susceptibility and resistance to be dominant. However in most cases, susceptibility was shown to be dominant and resistance to be under the control of recessive genes (Singh *et al.* 1983). The task of developing resistant varieties has been complicated in view of the reported genetic variability of the pathogen. This dynamic nature of the SMD has

warranted the identification and use of isolate specific sources of resistance in the crop improvement programmes.

In the RIL population, SMD incidence varied from 5.0 to 100 per cent with wide range of variability. Out of the 120 RILs, none of were immune, only three were resistant, 25 were moderately resistant and 92 were susceptible. Absence of immune plants and rare occurrence of extreme phenotypes indicates polygenic control of SMD inheritance and higher level of virulence to Bangalore isolate of SMD. Similar results were reported by Kulkarni *et al.* (2003) and Gnanesh (2010). Inheritance of SMD seems to be complex and does not fit a simple gene- for-gene interaction. In the present study, number of plants with high level of resistance to SMD is fewer in number. In general, resistance to SMD in pigeonpea is controlled by recessive genes and the causal organism has higher level of virulence, hence it is very difficult to realize plants with resistance to SMD. Hence, there is a need to search sources with high level resistance from either primary or tertiary gene pools.

The pattern of frequency distribution of SMD incidence in the RILs was found to be continuous depicting quantitative nature of SMD resistance. However, large number of plants could be classified into moderately resistant and susceptible class. Only few plants were classified in to resistant group. Genetics of SMD has been studied earlier and depending on the resistance source, SMD isolate and scoring method, resistance to SMD in pigeonpea appears to be complex and showing continuous variation (Saxena, 2008; Gnanesh, 2010). On contrast, resistance to SMD has been reported to be controlled by single gene (Murugesan *et al.* 1997 and Srinivas *et al.* 1997), oligogenic (Singh *et al.* 1983; Sharma *et al.* 1984; Amala Balu and Rathnasamy, 2003; Nagaraj *et al.* 2004; and Ganapathy *et al.* 2010).

5.4 Parental polymorphism studies

The most important step in a breeding programme is the choice of parents with good performance and wide genetic base. Thus, measures of the genetic divergence, ahead of making any cross, may help breeders to concentrate their efforts only on most promising combinations. However, most of the times, many breeders develop the mapping populations just based on phenotypic data without caring of the adequate amount of genetic diversity between the parental genotypes. Indeed, there have been several cases where screening of parental genotypes of the mapping populations provided no/ very low polymorphism and populations were already advanced to recombinant inbred line stage (Chandra *et al.* 2004 and Odeny 2007).

Conventional breeding of pigeonpea has continued entirely without the aid of molecular methods and made limited use of germplasm resources, resulting in a very narrow genetic base in the domesticated species. As a consequence, pigeonpea genetic improvement programme have made relatively little progress in addressing the primary constraints to crop production, which includes a range of biotic (SMD, *Fussarium* wilt and pod boring insects) and abiotic stresses(drought, salinity, and water logging). With the advent of next generation sequencing technologies several crop legumes have recently been subjected to intensive analysis, making marker assisted breeding a reality (Varshney *et al.*, 2006).

To develop diverse mapping populations, in addition to contrasting phenotype, it is important to select the diverse genotypes as well. Therefore, efforts have been made to estimate genetic diversity among seven genotypes using 61 SSR primers, out of 61 primers only 13 primers showed polymorphism. The low polymorphism might be due non availability of SSRs in pigeonpea genome compared to other legume

genomes or the SSR isolation protocols deployed so far are less effective. The polymorphism observed in the present study (28.3%) is Low. Low level of polymorphism in pigeonpea was also reported by Panguluri *et al.* (2005), Odeny *et al.* (2007) and Yang *et al.* (2006), Ganapathy *et al.* (2010) and Gnanesh *et al.* (2010). On contrary Saxena *et al.* (2010a) reported higher level of polymorphism (69.56 %) and it can be attributed to the use of higher number of genotypes representing more wild species and tertiary gene pool as compared with the present study. Similar was the case for PIC value and average number of alleles detected by the marker. The average PIC value and number of alleles were 0.41 and 4.4, respectively, for the polymorphic primers identified in the present study. The similarity coefficient was ranged from 0.593 to 0.796, the highest (0.796) similarity was observed between Hy3c and BRG 1 due to the common utilization of gene pool, as HY 3c is one of the parent for BRG 1. While, the lowest similarity (0.593) was observed between ICP 8863 and BRG 1.

Between TTB 7 (S) and ICP 7035 (R), the genotypes used for developing RIL population, eight SSR primers showed polymorphism (CCttc003, CCttc006, CCttc008, CCat011, ICPM1D10, ICPM1E04, ICPM1H01, and PB12). These polymorphic SSR primers could be employed for genetic mapping of population TTB 7 x ICP 7035.

Dendrogram constructed by the SSR data depicted that, the SMD resistant and moderately resistant genotypes ICP 7035, BRG 1, Bahar and HY 3c clustered together in major cluster I. The genotypes BRG 1 and Hy 3c formed a separate sub cluster within the resistance cluster which is very close to Bahar due to common utilization of gene pool, as HY 3c is one of the parent for BRG 1. These are differing at genotypic level from SMD susceptible genotypes *viz.*, BRG 2, TTB 7 and ICP 8863 which formed a separate and distinct major cluster II. The genotypes TTB

7 and ICP 8863 formed a separate sub cluster within the susceptible cluster II which is very close to BRG 2 Gangadhara (2006) also studied the parental polymorphism in pigeonpea using RAPD and SSR markers and reported grouping of susceptible and resistant genotypes in to separate clusters. However, Saxena *et al.* (2010b) indicated that large numbers of markers are needed to understand the amount of genetic similarity among genotypes with higher resolution.

Future line of work

- Secondary branches per plant, pods per plant, length of the inflorescence and pod yield exhibited high GCV, PCV, heritability and genetic advance as per cent mean. Therefore, more weightage has to be given for these characters while selecting the genotypes for higher yield.

- Transgressive recombinants/progenies identified in the present study needs to be further evaluated.

- Number of SSR primers used in the study is very few. Hence, more number of polymorphic SSR primers need to be identified for genetic mapping and marker assisted selection.

Summary

VI. SUMMARY

The present study was carried out at AICRP on pigeonpea, UAS, Bangaluru, to study the extent of genetic variability for yield and its attributing characters, screening for sterility mosaic disease in F_5 generation RIL progeny lines of cross TTB 7 x ICP 7035 and parental genotypes, and to estimate the extent of molecular diversity existing between susceptible and resistant genotypes of pigeonpea. The research findings of the study are briefly summarized here under.

PCV and GCV estimates were high for secondary branches per plant (76.29 and 61.6%), pods per plant (61.20 and 49.7%), seed yield per plant (58.98 and 47.49%), primary branches per plant(53.99 and 37.42%), length of inflorescence (38.84 and 29.88%).Moderate for 100 seed weight (18.8 and 14.70%) and plant height (15.45 and 12.21%), while low values were recorded for days to maturity (7.82 and 5.44%) and days to fifty per cent flowering (9.89 and 7.0%). Moderate PCV and low GCV values were recorded for seeds per pod (12.5 and 9.32%). High PCV and moderate GCV estimates were recorded for shelling percentage (23.80 and 18.89%).

High heritability with high genetic advance as per cent of mean were observed for secondary branches per plant (64.6 and 99.70%), pods per plant (64.29 and 80.91%), seed yield per plant (64.81 and 78.75%), shelling percentage (63.1 and 38.8%) and plant height (71.13 and 22.63%), indicating that these traits are under the control of additive gene action. Days to first flowering (50.05 and 10.20%) and days to maturity (47.43 and 7.71%) recorded moderate heritability with low genetic advance as per cent of mean indicating these traits are controlled by non-additive genes.

Totally, 26 positive transgressive recombinants were observed for seed yield per plant of which RIL 48 recorded the highest (221.80g) seed

yield per plant followed by RIL 78 (210.40g). For pods per plant, 20 positive transgressive recombinants were identified. The RIL 78 exhibited the highest (329.00) pods per plant followed RIL 48 (320.00). The RIL 78 recorded the highest secondary branches per plant (55.00) followed by RIL 108 (45.00) out of 8 positive transgressive recombinants. This indicated that, RILs 48 and 78 exhibited high number of secondary branches, pod number and seed yield.

F_5 generation RILs of cross TTB 7 x ICP 7035 and seven parental genotypes *viz.*, BRG1, BRG2, TTB 7, ICP 7035, ICP 8863, Hy3C and Bahar were screened for SMD resistance. Out of 120 RIL screened, none of them were immune, three showed resistant reaction, 25 exhibited moderately resistant reactions and 92 susceptible reaction. Screening of seven pigeonpea genotypes for SMD revealed that, BRG 1 and ICP 7035 recorded resistant reaction, Hy3C and Bahar were moderately resistant and BRG 2, TTB 7 and ICP 8863 were susceptible to SMD.

Amplicons of 61 primers electrophoresed on agarose (3%) resulted in 46 amplified primers from which three showed polymorphism (ICPM2B08, CCat011 and CCttc033) and the remaining 34 primers showed monomorphism and nine primers showed non-specific amplification with 6.5 per cent polymorphism.

All the PCR products were also electrophoresed on 4.5 per cent polyacrylamide gel. Out of 46 primers amplified, nine were non-specific, 24 were monomorphic. Thirteen primers exhibited polymorphism on PAGE in pigeonpea genotypes are CCttc003, CCttc006, CCttc008, CCat011, CCtc012, CCttc033, CCac036, ICPM1D10, ICPM1E04, ICPM1H01, ICPM2B08, PB1 and PB12. Between susceptible variety TTB 7 and resistant variety ICP 7035, eight SSR primers (CCttc003, CCttc006, CCttc008, CCat011, ICPM1D10, ICPM1E04, ICPM1H01, and PB12) showed polymorphism.

The PIC value ranged between 0.23 – 0.60, with an average of 0.41. The highest (0.60) PIC value was observed for the primer CCttc008 and the lowest for CCtc012.The similarity coefficient was ranged from 0.593 to 0.796. The highest (0.796) similarity was observed between Hy3c and BRG 1 and the lowest similarity (0.593) was exhibited between ICP 8863 and BRG 1.

Dendrogram constructed by the SSR data showed the grouping of SMD resistant and moderately resistant genotypes *viz.*, ICP 7035, BRG 1, Bahar and HY 3c in major cluster I. Genotypes BRG 1 and HY 3c formed a separate sub cluster within the major cluster and very close to Bahar. These genotypes are differing at genotypic level from SMD susceptible genotypes *viz.*, BRG 2, TTB 7 and ICP 8863 which formed a separate and distinct major cluster II. The genotypes TTB 7 and ICP 8863 formed a separate sub cluster within the major cluster II which is very close to BRG 2.

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Appendix

APPENDIX

Mean SMD disease reaction in F_5 generation RILs of cross TTB 7 × ICP 7035

RIL No	Reaction	RIL No	Reaction	RIL No	Reaction
1	100.0	26	87.5	51	100.0
2	100.0	27	78.6	52	60.7
3	40.20	28	82.5	53	70.7
4	77.50	29	15.5	54	95.0
5	13.82	30	79.5	55	89.4
6	87.51	31	90.5	56	91.7
7	66.3	32	24.5	57	31.4
8	100.0	33	10.0	58	61.1
9	10.0	34	85.5	59	95.5
10	70.2	35	80.0	60	100.0
11	95.0	36	73.9	61	58.3
12	21.0	37	13.6	62	100.0
13	13.3	38	63.5	63	15.2
14	15.9	39	90.9	64	63.1
15	88.9	40	100.0	65	23.8
16	77.9	41	93.8	66	87.5
17	17.5	42	68.9	67	77.9
18	75.0	43	77.3	68	91.2
19	93.8	44	74.1	69	71.4
20	93.8	45	85.7	70	47.3
21	87.5	46	94.4	71	20.9
22	81.8	47	90.9	72	49.4
23	5.0	48	87.3	73	53.2
24	86.7	49	100.0	74	20.0
25	23.0	50	95.8	75	100.0

Contd...

RIL No	Reaction	RIL No.	Reaction	RIL No.	Reaction
76	44.3	91	30.0	106	32.5
77	96.4	92	17.4	107	65.5
78	20.0	93	12.5	108	81.3
79	15.0	94	27.1	109	20.0
80	25.9	95	16.3	110	75.9
81	27.3	96	55.0	111	41.1
82	11.7	97	92.9	112	22.5
83	76.4	98	20.0	113	100.0
84	26.2	99	100.0	114	68.2
85	100.0	100	75.0	115	100.0
86	100.0	101	66.0	116	100.0
87	80.6	102	92.9	117	30.0
88	95.8	103	68.1	118	67.8
89	91.7	104	100.0	119	100.0
90	45.8	105	94.4	120	100.0