MOLECULAR CHARACTERIZATION AND DEVELOPMENT OF TRAIT RELATED MARKERS FOR APHID RESISTANCE IN COWPEA (*Vigna unguiculata* (L) Walp.)

By

RAMYA HARIDAS

THESIS

Submitted in partial fulfilment of the requirement for the degree of

Master of Science in Agriculture

(PLANT BIOTECHNOLOGY)

Faculty of Agriculture
Kerala Agricultural University

Centre for Plant Biotechnology and Molecular Biology

COLLEGE OF HORTICULTURE
VELLANIKKARA, THRISSUR - 680 656
KERALA, INDIA

2007

DECLARATION

I, hereby declare that this thesis entitled **"Molecular characterization and development of trait related markers for aphid resistance in cowpea (*Vigna unguiculata* (L) Walp.)"** is a bonafide record of research work done by me during the course of research and that it has not been previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title, of any other University or Society.

Vellanikkara
14-02-08

Ramya Haridas

CERTIFICATE

Certified that this thesis entitled **"Molecular characterization and development of trait related markers for aphid resistance in cowpea (*Vigna unguiculata* (L) Walp.)"** is a bonafide record of research work done independently by **Ms. Ramya Haridass** under my guidance and supervision and that it has not formed the basis for the award of any degree, diploma, fellowship or associateship to her.

R. Keshavachandran

Vellanikkara
14-02-08

Dr. R. Keshavachandran

Major Advisor, Advisory Committee
Professor
Centre for Plant Biotechnology
and Molecular Biology
College of Horticulture
Vellanikkara, Thrissur.

CERTIFICATE

We, the undersigned members of the Advisory Committee of **Ms. Ramya Haridas,** a candidate for the degree of **Master of Science in Agriculture**, with major field in **Plant Biotechnology**, agree that the thesis entitled **"Molecular characterization and development of trait related markers for aphid resistance in cowpea (*Vigna unguiculata* (L) Walp.)"** may be submitted by Ms. Ramya Haridas in partial fulfillment of the requirements for the degree.

Dr. R. Keshavachandran
(Major Advisor, Advisory Committee)
Professor
Centre For Plant Biotechnology and Molecular Biology
College of Horticulture
Vellanikkara, Thrissur

Dr. P.A. Nazeem
(Member, Advisory Committee)
Professor and Head
Centre for Plant Biotechnology and
Molecular Biology
College of Horticulture
Vellanikkara, Thrissur.

Dr. D. Girija
(Member, Advisory Committee)
Associate Professor
Centre for Plant Biotechnology
and Molecular Biology
College of Horticulture
Vellanikkara, Thrissur.

Dr. Salikutty. Joseph
(Member, Advisory Committee)
Professor
Department of Olericulture
College of Horticulture
Vellanikkara, Thrissur.

(External Examiner)

ACKNOWLEDGEMENT

I humbly bow my head before the God Almighty who blessed me with will power and courage to complete this endeavour successfully.

I am ineffable in expressing deep sense of gratitude to Dr. R. Keshavachandran, Professor, CPBMB and chairman of my advisory committee, for his kind concern, expert advice, constant support and encouragement throughout the course of study and preparation of thesis. I am obliged for the wholehearted co-operation rendered to me during his endeavour.

I wish to place on record my deep sense of gratitude to Dr. P.A. Nazeem, Professor and Head, CPBMB, member of my advisory committee for her constant inspiration, expert guidance, critical suggestions and invariable help rendered for the completion of my research work.

Iam deeply obliged to Dr. D. Girija, Associate Professor, CPBMB, member of my advisory committee for her meticulous support, sustained encouragement and sincere help during the entire course of study.

I express my heartfelt gratitude to Dr. Salikutty Joseph, Professor, Department of Olericulture, member of my advisory committee for her valuable suggestions, wholehearted co-operation and support given to me during the conduct of the research work.

My sincere thanks to all the staff members of CPBMB, especially Dr. A.Augustine, Dr.P.A.Valsala, Dr.P.C.Rajendran, Dr.R.Sujatha and Dr.Lisamma Joseph for their frequent enquiries and moral support during the period of study.

I owe special thanks to Dr.P.Suresh Kumar, Professor, Radio Tracer Laboratory, for providing the necessary facilities for carrying oyt the radioactivity work. His unfailing patience and ever willingness to help blessed me to complete my research work.

I extend my profound thanks to Dr.P.K.Rajeevan, Associate Dean, College of Horticulture for his unlenching support during the fledging stages of my M.Sc. course.

I express my sincere gratitude to all he RAs of CPBMB especially Sheeba chechi, Firoz chetan, Beena, Soumya, Sherin, Shruti, Rekha, Praveena, Rajasekharan, Megha, Vipin, Jithesh, Dileep and Aleesha for all their help rendered to me during the period of investigation.

I have no words to express my deep sense of gratitude o my dearest and beloved friends Sheetal, Neema, Nidhi, Seena chechi, Nisha, Shifa and Shareesh who were always with me and provided all the encouragement and mental strength during the entire course of study.

On a personal note, I would like to pour out my heartfelt thanks to my dear friends Sameera, Anjali Divakaran, Anjali Nambiar, Geena, Likhitha, Fayas, Liffey, Saisree, Hima, Divya and Shajna for their unbrided help and spendous support.

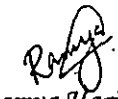
I thank all my seniors and juniors who have helped me in one way or the other during the period of study.

I thankfully acknowledge Shylaja chechi, Simy chechi and Sreekumar chettan or their assistance and support for the successful completion of my thesis work.

I sincerely thank all the labourers of CPBMB for their help and co-operation during the course of study. The award of KAU scholarship is duly acknowledged.

My profound thanks to Santhosh chettan, Computer club for the assistance rendered during the preparation of thesis.

I am in dearth of words to express my love towards my beloved parents, sisters, and Mahesh for their boundless affection, moral support, eternal love, deep concern, prayers, personal sacrifices which sustains peace in my life.



Ramya Haridas

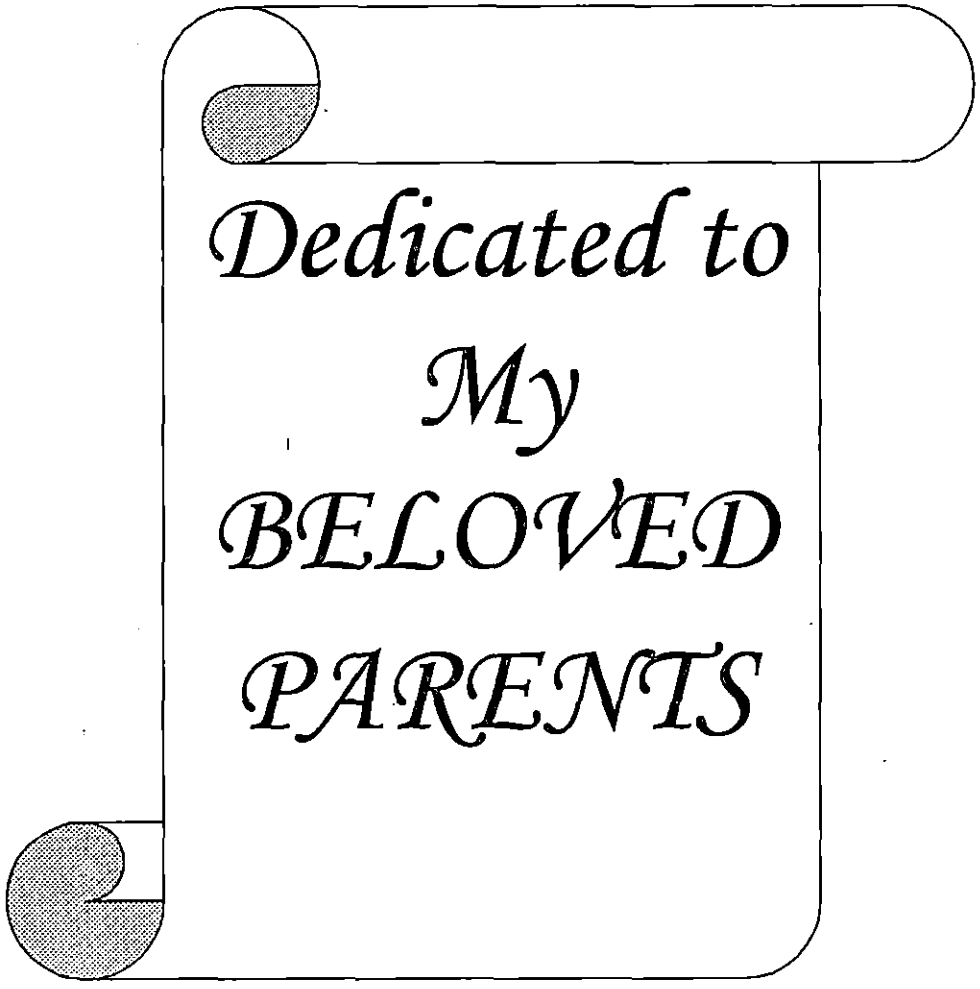

Dedicated to
My
BELOVED
PARENTS

TABLE OF CONTENTS

LIST OF TABLES

LIST OF PLATES

LIST OF FIGURES

LIST OF APPENDICES

ABBREVIATIONS

AFLP	- Amplified Fragment Length Polymorphism
ATP	- Adenosine triphosphate
bp	- base pair
CPBMB	- Centre for Plant Biotechnology and Molecular Biology
DNA	- Deoxyribonucleic acid
dNTPs	- Deoxy ribonucleoside triphospate
DTT	- Dihiotriol
^0C	- degee celsius
EDTA	- Ethylene diamine tetra acetic acid
g	- gram
hr	- hours
IITA	- International Institute of Tropical Agriculture
kb	- kilo base
µg	- micro gram
µl	- micro litre
ml	- milli litre
%	- percentage
PCR	- Polymerase Chain Reaction
PAGE	- Polyacrylamide gel electrophoresis
RAPD	- Random Amplified Polymorphic DNA
RFLP	- Restriction Fragment Length Polymorphism
rpm	- revolutions per minute
RTL	- Radio Tracer Laboratory
Rp	- Resolving power
SCAR	- Sequence Characterized Amplified Region
SDS	- Sodium dodecyl sulphate

TAE	- Tris acetate EDTA buffer
TBE	- Tris borate EDTA buffer
TE	- Tris EDTA buffer
UV	- Ultra violet
v/v	- volume/volume
QTL	- Quantitative trait loci

Introduction

1. INTRODUCTION

Cowpea (*Vigna unguiculata* (L) Walp) is a food legume of significant economic importance world wide. It is grown in North and South America, Africa, Europe and Asia, predominantly in the semiarid and humid tropical regions lying between 35^0 N and 30^0S of the equator. Cowpea is cultivated on at least 12.5 million hectares with an annual production of over 3 million tones of grain world wide (Singh *et al.*, 1997). Currently the Central and West Africa account for more than 64 percent of the total area under cowpea cultivation, followed by South America, Asia, East and South Africa.

Part of the popularity of cowpea as a food staple for people in the semiarid and humid tropical regions of Africa is that it is relatively drought tolerant and performs well under conditions whereas most other food legumes do not. Its unique ability to fix nitrogen even in very poor soils with low organic matter also contributes to its widespread use among farmers (Singh *et al.*, 1997).

Like most crop plants, cowpea production is also limited by numerous biotic and abiotic factors. Both severe heat and drought limit cowpea productivity (Marfo and Hull, 1992). It is also attacked by a wide range of insect pests, microbial and fungal diseases, nematodes and two different parasitic angiosperms (Basher and Hapton, 1996, Ehlers and Hall, 1997, Fery and Singh, 1997).

Aphids are one among the most important pests of cowpea causing considerable damage especially during dry seasons. Cowpea aphid dwells on the crop in the period between germination and flower initiation. They attack all the vegetative parts and cause leaf curling, loss of vitality and reduction in growth. In severe cases, plants fail to bear flowers and pods resulting in 20-40 percent loss in yield (Allen and Singh, 1980). The use of insecticides has been the recommended cultural practice but is expensive in a low value crop, destroys natural enemies and causes toxic hazards in the environment. Therefore use of resistant varieties

was thought to be an alternative method in the control of aphids. Several aphid resistant lines have been identified, but conventional breeding alone may not provide an ultimate solution.

The advent of biotechnology paved the way towards improving many of the agronomic traits and also in developing varieties that are resistant to pests and diseases. Development of resistant specific markers will help in the marker assisted selection of resistant genotypes. Identification of genes contributing resistance to varieties is possible by introduction of these genes to cultivated varieties of cowpea. Molecular markers such as RAPD, AFLP, RFLP etc can be used to identify markers linked to aphid resistance in cowpea. Development of molecular markers to aphid resistance will help in molecular based screening of aphid resistant lines. This method could be exploited for effective control of aphids other than the use of costlier insecticides.

So far there is no published information on the use of molecular markers in the characterization of aphid resistance in cowpea. In this context, the following study has been conducted.

1) To identify the sources of resistance to aphid by screening and scoring of cowpea accessions.

2) To characterize and develop trait related markers for aphid resistance in cowpea.

Review of Literature

2. REVIEW OF LITERATURE

2.1. Origin, domestication and distribution

Cowpea (*Vigna unguiculata*) is one of the most ancient of human food sources and has probably been used as a crop since Neolithic times (Summerfield *et al.*, 1974). A lack of archaeological evidence has resulted in contradicting views supporting Africa, Asia, and South America as origin (Johnson, 1970., Summerfield *et al*, 1974., Coetzee, 1995). One view is that cowpea was introduced from Africa to the Indian subcontinent approximately 2000-3500 years ago (Allen, 1983). Before 300BC, cowpea had reached Europe ad possibly North Africa from Asia. In the 17th century AD, the Spanish took the crop to West India. Another view was that the Transvaal region of the Republic of South Africa was the centre of speciation of *Vigna unguiculata* due to the presence of most primitive wild varieties (Padulosi and Ng, 1997). Presently cowpea is grown throughout the tropic and sub tropic areas around the whole world.

Ng (1995) postulated that during the process of evolution of *Vigna unguiculata* there was a change of growth habit from perennial to annual breeding and from predominantly outbreeding to inbreeding, while cultivated cowpea (sub sp *unguiculata*) evolved through domestication and selection of annual wild cowpea (var *dekinotiana*). According to Prabhakara *et al* (2001), there are five distinct subspecies of cowpea, out of which two are wild, viz. *dekinotiana* and *mensenis* and three are cultivated. They are *Vigna unguiculata* sub sp *unguiculata*, sub sp *cylindrica* and sub sp *sesquipedalis*.

The wide geographical distribution of var *dekinotiana* throughout sub Sahara Africa suggests that the species could have been brought under cultivation in any part of the region. However the centre of maximum diversity of cultivated cowpea is found in West Africa, in an area encompassing the Savannah region of Nigeria, Southern Niger, Northern Benin, Togo and North Western part of Cameroon (Ng and Marechal, 1985). Carbon dating of cowpea has been carried

out by (Flight, 1976) and is the oldest archaeological evidence of cowpea found in Africa. This shows the existence of gathering of cowpea by African hunters or food gatherers as early as 1500 BC.

2.2. Morphology and Biology

Summerfield *et al* (1974), Kay (1979) and Fox and Young (1982) described cowpea as an annual herb reaching a height of up to 80cm with a strong taproot and many spreading lateral roots in the surface soil. Growth forms may vary and many are erect, trailing, climbing or bushy, usually intermediate growth under favourable condition.

Leaves are alternate and trifoliate. The first pair of leaves is simple and opposite. Leaves exhibit considerable variation in size and shape and they are usually dark green. The stems are striate, smooth or slightly hairy and sometimes tinged with purple colour.

Flowers are arranged in racemose or intermediate inflorescence at the distal ends of 5-60 cm long peduncle. Flowers are borne in alternate pairs usually two flowers per inflorescence. They are conspicuous, self pollinating, borne on short pedicels and corollas may be white, dirty yellow, pink or pale blue in colour.

Fruits are pods that vary in size, shape, colour and texture. They may be erect, crescent shaped or coiled. These are usually 8-20 seeds per pods. Seeds vary considerably in size, shape and colour.

2.3. Cowpea taxonomy

Verdcourt (1970) and Marechal *et al* (1978) classified cowpea as follows

Order- Fabales

Family – Fabaceae

Subfamily- Faboideae

Tribe - Phaseoleae

Sub tribe - Phaseolinae

Genus – Vigna

Vigna has several species, but the exact number varies according to different authors. All cultivated cowpeas are grouped under *Vigna unguiculata* which are subdivided into four semigroups- *unguiculata, biflora, sesquipedalis* and *textilis* (Westphall, 1974., Marechal *et al*, 1978, Ng and Marechal 1985).

A few studies have reported a chromosome number of 2n=22 for *Vigna unguiculata* (Barone and Saccado., 1990). They also described the karyotype of cowpea as being composed of one very long chromosome and one very short chromosome, with the remaining nine groups being allocated to three groups of intermediate size. On the other hand, Pignone *et al* (1990) described 11 chromosome pairs falling into three size groups: five long, five medium and one short.

The size of cowpea genome has been estimated at $613x10^6$ bp (Armuganathan and Earle, 1991).

2.4. Cowpea-uses

Cowpeas are grown both for its tender pods and also for its dry seeds and are used as pulse for culinary purpose. According to Fery (2002), subsistence farmers in the semi arid and sub humid regions of Africa are the major producers and consumers of cowpea. These farmers not only grow cowpeas for dry seeds for human consumption and fodder for animal feed, but also utilize the leaves and fruits for vegetables. Cowpeas are widely grown in Eastern Africa and South East Asia primarily as a leafy vegetable. The pods are rich in proteins, vitamin B and minerals. Steele *et al* (1985) noted that the protein content of leafy cowpea parts consumed annually in Africa and Asia is equivalent to 5 million tones of dry cowpea seeds and that this represents as much as 30 percent of the total food legume production in the low land tropics. It is also used as a green manure crop, a nitrogen fixing crop or for erosion control (Davis *et e*l, 1991).

2.5. Production status

Based on the information available from Food and Agricultural Organization (FAO), cowpea researchers at IITA estimated that cowpea is now cultivated on at least 12.5million hectares with an annual production of over 3 million tonnes world wide. Cowpea is widely distributed through out the tropics but Central and West Africa account for over 64 percent of the area with about 8 million hectares, followed by about 2.4 million hectares in Central and South America, 1.3 million hectares in Asia and about 0.8 million hectares in East and South Africa. Some cowpea is also cultivated in Middle East and Southern Europe. However substantial part of cowpea production comes from the drier regions of Northern Nigeria (about 4 million hectares with 1.7 million tones), Southern Niger Republic (about 3 million hectares with 1 million tonne) and Brazil (about 1.9 million hectares with 0.7 million tones). (Singh *et al.*, 1997)

2.6. Cowpea aphid

Cowpea aphid (*Aphis craccivora*) belongs to the family Aphididae and order Hemiptera.

It is a soft bodied, pear shaped insect having shiny black body with white appendages. The nymphs are pale green to grey with powdery coating.

Cowpea aphid has a wide distribution across many parts of Europe, Asia, Africa, Australia, Pacific Islands and West Indies (Singh and Van Emden, 1979). In India the pest was reported in Tamil Nadu, Punjab, Maharashtra, Bihar, West Bengal, Orissa, Andhra Pradesh, Karnataka and Kerala. The host plants of cowpea aphid include lima bean, chick pea, lentil, red gram, lablab bean, alfalfa, groundnut, broad bean and peas (David, 1957).

Aphis craccivora dwells on the cowpea crop in the period between germination and flower initiation. Aphids congregate on the lower leaf surfaces and on the terminal buds from which they extract the plant sap. The plant tissues

dry and yellowing and wilting symptoms are seen. Heavily infested leaf curls. They excrete honey dew which causes sticky, shiny leaves. A dark sooty mould grows on the honey dew coated surfaces of aphid infested leaves.

Multiplication of aphids was favoured by moist and cloudy weather. Consequently with the occurrence of favourable weather conditions for a longer period of time, a severe outbreak of aphids could be apprehended. Higher temperature and radiation increased the aphid (*Aphis fabae*) population on field bean in late June and mid July or in early August (Way, 1967).

Mathew *et al* (1972) studied the population fluctuation of *Aphis craccivora* on cowpea and reported that high and low population occurred from September to April and from May to August.

Saleh *et al* (1972) revealed that the population density of *Aphis caccivora* reached the maximum on the broad bean during March and during august on cowpea.

Pal *et al* (1978) delineated the ideal condition for the outbreak of *Aphis craccivora* as about 80percent relative humidity, 27.5 to 28.5^{0}C air temperature and a low number of sunshine hours.

Sulochana (1984) reported that aphid population got reduced considerably during rainy days. Frequent rains during the population rise phase adversely affected the population build up.

2.7. Reaction of cowpea genotypes to aphids

Singh (1977) at IITA found cowpea lines Tvu408, Tvu416, Tvu2740, Tvu3417, and Tvu3509 resistant to aphids.

Bell (1980) evaluated 29 cowpea lines for resistance to aphids and reported P476, EC4276, V1 and T422/2 as resistant

Dhanorkar and Davare (1980) found that out of 14 lines evaluated for the incidence of aphids, lines P1 473, and P1 476 were completely free from aphid infestation.

Karel and Malinga (1980) evaluated 11 cowpea accessions and found that the lines Tvu 408 P2, Tvu410 and lfe brown were resistant to aphid *Acyrthosiphon gossypii.*

Trials conducted at IITA, Nigeria proved Tvu36 as an aphid resistant source. The lines Tvu9836, Tvu9914, Tvu9929, Tvu9930 and Tvu9944 were resistant to cowpea aphid borne viruses and cowpea aphids. Of several cowpea accessions evaluated for their reaction to aphids, Tvu18, Tvu36, Tvu42, Tvu109, Tvu310, Tvu801,Tvu1037, Tvu2755 were resistant (IITA, 1982).

Pathak (1983) reported high levels of resistance in two cowpea cultivars ICV11 and ICV12 obtained by induced mutations.

Mcfoy and Dabrowski (1984) found that the cultivars Tvu310 and 408 P2 were relatively resistant as compared to variety Vita1. The line ITB10-1020 was resistant to aphids as reported at IITA (IITA, 1985).

Messina *et al* (1985) evaluated nearly 200 accessions of cowpea for resistance to cowpea aphids. The varieties reported resistant in West Africa were highly susceptible to aphids from Southern United States.

Pathak (1988) reported four cultivars ICV10, ICV11, ICV12 and Tvu310 as resistant to aphids.

The repeated resistant behaviour of three cowpea accessions VS 350, VS 438 and VS 452 confirmed their resistance to aphids (Joseph, 1990).

IITA (1995) reported that the excellent sources of resistance to aphids were found in wild species, namely *Vigna vexillata* and *Vigna oblongitelia*.

Ampily (2005) reported that there was no aphid incidence in accessions VS 1282, VS 1294, VS 1230, VS 1277, VS 1075, VS 1053, VS 1286, VS 1111, VS 1133, Kanakamony, VS 1034, VS 1032, VS 1177, VS 1231 and VS 1248 during kharif. During summer the accessions VS 1282, VS1230, VS 1047, VS 1151, VS 1230, Kanakamony and VS 1231 showed no incidence of aphids. In rabi also the accessions VS 1140, VS 1230, VS 1047, VS 1151, VS 1168, VS 1156, VS 1160, VS 1263 and VS 1231 showed no aphid infestation. The accessions VS 1230 and VS 1231 were free from aphid infestation during all the seasons. In general the incidence of aphids was lowest during kharif (32.5%) followed by summer (34.99%) and rabi (35.64%).

2.8. Genes associated with resistance to aphids

A resistant gene may produce an enzyme, which makes some chemical toxic to aphid or may produce a protein that activates some other forms of defense.

Pathak (1988) reported two different genes controlling aphid resistance in different cultivars of cowpea. He assigned the gene symbols Rac1 for the genes in cultivars ICV10 and Tvu310 and Rac2 for the gene in the cultivars ICV11 and ICV12.

In wheat, a gene Dn5 was found which showed resistance to Russian wheat aphid (*Diuraphis noxia*). This gene was located on the chromosome 7D of wheat. The study was carried out which identifies the gene on the chromosome arm (Toit *et al.*, 1995).

Resistance against the aphid *Macrosiphum euphorbiae* previously was observed in tomato and attributed to a novel gene, designated Meu1, tightly linked to the nematode resistant gene Mi which confers resistance to *Meloidogyne incognita*. Mi has recently cloned and studies were conducted to determine whether Meu1 and Mi are the same gene. It was shown that Mi is expressed in the leaves that aphid resistance is isolate specific and that susceptible tomato transformed with Mi is resistant to the same aphid isolates as the original resistant lines (Rossie *et al.*, 1998).

Marais *et al.* (1998) associated a stem rust resistant gene Sr45 and two Russian wheat aphid resistance genes (Dn5 and Dn7) with mapped structural loci in common wheat.

Klinger *et al.* (2002) studied that the resistance to *Aphis gosypii* (cotton melon aphid) in melons is conferred by a single dominant gene that causes an inhibition of feeding from the phloem sieve element. Aphid resistance in melon is genetically linked to the resistance to virus transmission and resistance to powdery mildew fungus, raising the possibility that *Aphis gosypii* resistance locus Agr resides in a cluster of resistant genes.

Anderson *et al.* (2003) identified a gene in rye, Dn7, located on chromosome IRS which confers resistance to Russian wheat aphid in wheat.

Birch *et al.* (2003) identified a strongest resistant gene A10 which confers resistance to large raspberry aphid (*Amphorophora idaei*) in red raspberry.

Ml-1.2 , a member of the intracellular nucleotide binding site-leucine rich repeat family of resistance genes, confers resistance in tomato against both root feeding nematode (*Meloidogyne* sp) and leaf feeding aphids (*Macrosiphum euphorbiae*). Ml mediated aphid resistance is developmentally regulated and

protects the mature plants but not seedlings against aphid infestation (Goggin *et al.*, 2004).

Aphids including the blue green aphid (*Acyrthosiphon kondoi*) are important pests of Australian pasture legumes. Improved aphid resistance is one of the priorities of the breeding programme for *Medicago truncatula* and other forage legumes. Aphid resistance has been introgressed into two popular *Medicago truncatula* varieties from resistant stock to create new aphid resistant cultivars (Edwards *et al.*, 2004). This study also paved the path in the identification of plant defense genes induced by aphid feeding.

The alkaloids were found to be more active against cowpea aphid than against green peach aphid. Lupanine was the only compound with greater activity and was the only alkaloid highly active against both species (Ridsdill *et al.*, 2004)

Klinger *et al* (2005) identified aphid resistant genes in *Medicago truncatula* as a single dominant gene and also found that plant showed phloem specific defense.

Flavanoids are reported to have a direct correlation with aphid resistance in cowpea. Identification of genes related to flavonoid production will help in enhancing the production of these resistance factors.

Lectins are the proteins lethal to sucking pests like aphids. Genes encoding snowdrop lectin (*Galanthus nivalis* L.agglutinin, GNA) under the control of a phloem specific promoter was transfered to maize (Wang *et al.*, 2005). The transgenic plants synthesized GNA and showed resistance to aphids.

2.9. Genetics of resistance to aphids

IITA (1982) reported resistance as dominant to susceptibility and F_2 population segregated in a ratio of 3 resistant: 1 susceptible. The F_1, F_2, F_3 and backcross population involving aphid resistant and susceptible parents were evaluated for their reaction to aphids (IITA, 1985).

Resistance to aphids is a dominant trait and monogenically inherited as evident by 3 resistant to 1 susceptible in F_2 population and 1 resistant and 1 susceptible ratio in backcross population. Similar results were reported at ICIPE (Pathak, 1984).

Pathak (1988) studied the inheritance of resistance to aphid in four resistant cultivars of cowpea and determined whether the genes for resistance were independent. The parental, F_1, F_2 and BC_1 populations involving resistant x susceptible and resistant x resistant cultivars were evaluated in the screen house. The reactions of F_1, F_2 and BC_1 populations arising from the crosses of four resistant cultivars ICV 10, ICV 11, ICV 12 and TVu310 with susceptible cultivar ICV1 revealed that resistance in each of these cultivars was governed by a single dominant gene. Tests for allelism in the F_1, and F_2 populations from crosses between resistant cultivars indicated that the resistance of ICV10 and Tvu310 is controlled by a common locus as are those of ICV11 and ICV 12. The locus of the resistance gene in ICV10 and Tvu310 is not linked to the locus of resistant gene in ICV11 and ICV12. Thus the resistant gene common to ICV 10 and TVu310 was non allelic to and independent of the resistance gene of cultivars ICV11 and ICV12.

Bata *et al.* (1987) undertook a study to elucidate the inheritance of resistance to aphids in cowpea. Three resistant lines were used for the study. Parental, F_1, F_2, F_3 and backcross population involving resistant x susceptible and resistant x resistant lines were evaluated in the green house using artificial infestation with aphids. The F_1 plants in all crosses were resistant and F_2

populations derived from all the crosses involving resistant and susceptible parents segregated into 3 resistant: 1 susceptible ratio. The back cross population involving susceptible parents segregated into 1 resistant: 1 susceptible ratio whereas backcross population involving resistant parents were completely resistant. The F_3 progenies derived from resistant F_2 plants showed a ratio of 1/3 line breeding resistant: 2/3 segregating like F_2's. These data indicated monogenic dominant inheritance of aphid resistance.

Inheritance of resistance to cowpea aphid *Aphis craccivora* in three resistant cultivars of cowpea *Vigna unguiculata* was studied by Ombhakho *et al.* (1987). The parents F_1 and F_2 population were grown in an insect proof screen house. Each of three day old seedlings was infested with 10 apterous adult aphids. Seedling reaction was recorded when susceptible check was killed. The segregation data revealed that the resistance of ICV11 and Tvu310 was governed by a single dominant gene. All the F2 seedlings of the cross ICV10 and Tvu310 were resistant indicating that they have the same gene for resistance. However the F_2 populations from the crosses ICV10 x ICV11and ICV11 x Tvu310 segregated in the ratio of 15:1 indicating that dominant genes in ICV11 and Tvu310 are non allelic and independent of each other.

Nieto and Blake (1994) studied the inheritance of Russian Wheat Aphid resistance in barley and molecular markers linked to it.

Githri *et al.* (1997) studied the genetic linkage of the aphid resistance gene, Rac with various polymorphic loci controlling morphological traits and aspartate amino transferase (AAT) isozyme in cowpea. The objective was to identify simply inherited and easily identifiable markers for aphid resistance and to distinguish between Rac1 and Rac2 reported by Pathak (1988). It was found that there was no genetic linkage between aphid resistance genes and the genes controlling other polymorphic morphological traits or AAT isozyme.

Two sources of resistance to the corn leaf aphid (*Rhopalosiphum maidis*) were verified in Hawaii. Ratings were performed on the extent of infestation of whorls during tassel development. Generation mean analyses were conducted on parents, F_1, F_2, BC_1 to determine the gene action involved. Resistance is shown to be monogenic and recessive in Hi38-71 (Lu and brewbaker, 1999)

The genetics of resistance to the aphid *Aphis craccivora* of cowpea lines Vs 350, Vs 438 and Vs 452 was investigated in Kerala, India. The resistant lines were crossed with two susceptible lines Kanakamony and Pusa Komal at all possible combinations with the susceptible lines as males. F_1's were crossed with resistant and susceptible lines to produce BC_1 and BC_2 generations. The parental generation as well as the F_1, F_2 and backcross generation was used to evaluate the inheritance of pest resistance. The monogenic dominant inheritance of aphid resistance was confirmed in the backcross generation. The ratio of resistant: susceptible plants closely fitted 3:1 ratio. Results indicate that the resistance to aphid was governed by a single dominant gene (Joseph and Peter, 2003).

Another study was conducted by Joseph and Peter (2003) regarding the management of aphids (*Aphis craccivora*) through physical mixtures in cowpea. In cowpea, the physical mixtures generated by mixing of seeds from three resistant (Vs 452, Vs 350) and two susceptible lines (Kanakamony and Pusa komal) were evaluated in two , three, and four way combinations for three seasons in Kerala. During all the seasons all the physical mixtures expressed compensatory effect for the level of aphid resistance. There was a positive correlation between the level of aphid resistance expressed and the percentage obstruction created through physical blending. Both the susceptible lines had a general coexistence ability index of more than one for the level of aphid resistance showing improvement in the level of aphid resistance over purestand of susceptible lines.

Kiplagat (2005) studied the effect of the Russian wheat aphid infestation on seedlings and adult plants of eight Kenyan wheat cultivars. Segregation in the F2 populations indicated that resistance in two accessions of PI 294994, a resistant cultivar was controlled by two genes (one dominant and the other recessive).

2.10. Molecular markers in crop improvement

Genetic improvement of crop plants through conventional plant breeding has tremendous contribution to the breakthrough in the global agricultural production. Recently an array of tools and techniques in the field of molecular biology have become available and found to be supplementing the conventional approaches.

Molecular markers are heritable differences in the nucleotide sequences of DNA at corresponding position on homologous chromosomes of two different individuals which follow Mendelian pattern of inheritance. They offer several advantages which include abundance, co-dominance, absence of epistasis and developmental stage, tissue and environment independent expression (Mohapatra, 2005).

Molecular markers have been used to develop linkage maps for many important crop species. These genetic linkage maps show the location of markers and genes and show the distance from other genes. Polygene characters which are difficult to analyze using traditional plant breeding methods can now be easily tagged using molecular markers (Anand, 2005).The important DNA based molecular markers include RAPD, RFLP, AFLP, SSR etc.

Molecular markers are finding widespread use in enhancing traditional breeding programmes. When tightly linked to a gene of interest, selecting for the marker can replace direct selection for the trait. This is particularly useful if the direct screening is difficult or inconvenient. Conversely, markers can aid the

identification of rare recombinants permitting selection against unwanted chromosome segments linked to a desirable gene. Thus markers form a tool kit available to improve the precision and speed of breeding programme (Chapman, 1996).

Molecular markers have helped to elucidate the numbers, effects and interactions of insect resistance QTL's in Japanese plant introductions and markers are now being used in breeding programs to facilitate the transfer of resistant alleles while minimizing linkage drag (Boerma and Walker, 2005). Molecular markers also make it possible to evaluate QTL's independently and together in different genetic backgrounds. Recently markers have also been identified or the identification of aphid (*Rhopalosiphum padi*) genotypes and evaluation of barley genotypes for aphid resistance.

2.10.1. Random Amplified Polymorphic DNA

RAPD is one of the important molecular markers which are used widely for map construction and linkage analysis (Williams *et al.*, 1990). RAPD markers are generated by PCR amplification of random genomic DNA segments with single primers, usually ten nucleotides long. The primer target complexes are used as the substrates for DNA polymerase to copy the genomic sequences 3^1 to the primers (Staub *et al.*, 1996). Repetition of this process yield a discrete set of amplified DNA products that represent the target sequences flanked by opposite oriented primer annealing sites. The amplified products are separated by electrophoresis either on agarose gels and detected with ethidium bromide. RAPD's are usually dominant markers with polymorphism between individuals defined as the presence or absence of a particular band.

2.10.2. Amplified Fragment Length Polymorphism

AFLP is based on PCR amplification of a subset of restriction fragments (Vos *et al.*, 1995). This method surveys a large number of restriction fragments simultaneously, facilitating the detection of polymorphism. AFLP markers are

codominant which is important for the study of population genetics of diploid organisms and highly reproducible. Greater genome coverage, high reproducibility, rapid generation and high frequency of identifiable polymorphisms make AFLP analysis an attractive method for germplasm characterization and in the construction of genetic map (Mohan *et al,* 1997; Savelkoul *et al.,* 1999).

There are several reports of studies using AFLP in evaluating genetic relationships (Roa *et al.*, 1997, Loh *et al.*2000, Zhao *et al.*, 2005), germplasm characterization (Larson *et al.*,2001, Tang *et al.*,2003), and varietal identification (Clerc *et al.*, 2002, Diaz *et al.*, 2003). AFLP has also been used in studying the relationship between cultivars and wild relatives (Aggarwal *et al.*, 1999, Xu *et al.*, 2000).

2.10.3. Molecular markers for insect resistance

Keim *et al.* (1997) constructed a high density genetic linkage map in soyabean using 300 recombinant inbred lines. The resulting map is comprised of 840 markers including 165 RFLP, 25 RAPD and 650 AFLP markers.

Roche *et al.* (1997) identified four RAPD markers and three RFLP markers linked to sd1 in a cross between *Dysaphis devecta* susceptible variety prima and the resistant variety Fiesta. This is the first report for an aphid resistance gene in fruit tree crops.

Myburg *et al.* (1998) developed RAPD and SCAR markers linked to the Russian wheat aphid resistance gene Dn2 in wheat. Four polymorphic RAPD fragment were identified as putative RAPD markers for the Dn2 gene.

Lawlor *et al.* (1998) used AFLP for the identification of molecular genetic markers against resistance to native bud worm (*Helicoverpa* sp) in chickpea.

Skov and Wellendorf (2000) identified RAPD makers linked to major genes behind field resistance against the green spruce aphid *Elatobium abietinum* in *Picea sitchensis*. Three RAPD markers have been identified each of which is linked to segregating resistance gene loci coding for aphid resistance.

Silva *et al.* (2000) used RAPD markers for the selection of resistant and susceptible progenies to *Helicoverpa zeae*. 86 primers were tested for PCR reactions with resistant and susceptible bulks for the detection of RAPD polymorphism. The results identified the primer OPC-02 as a possible marker for the identification of resistant progenies.

Hayashi *et al.* (2001) constructed a linkage map of Japanese black pine based on AFLP and RAPD markers including markers linked to resistance against pine needle gall midge.

Klinger *et al.* (2001) used RAPD markers in the mapping of cotton melon aphid resistance in melon.

Ouedraogo *et al.* (2001) identified AFLP markers linked to the resistance of cowpea to parasitism to *Striga gesnerioides*. The identification of AFLP markers linked to striga resistance provides a stepping stone for marker assisted selection programme and the eventual cloning and characterization of the genes encoding resistance to his noxious parasitic weed.

Selvi *et al.* (2002) used Random Amplified Polymorphic DNA (RAPD) analysis in conjunction with bulked segregant analysis to identify four phenotype specific RAPD markers for yellow stem borer (*Scirpophaga incertulus*) in rice.

The green bug, *Schizaphis graminum* is the most economically damaging aphid pest of wheat in the Southern Great Plains of USA. In this study the single dominant green bug resistance gene, Gb3 was molecularly tagged and genetically

mapped using AFLP and SSR markers. The two AFLP markers cosegregating with Gb3 are valuable tools in developing molecular markers for marker assisted selection of green bug resistance in wheat breeding (Weng and Hazar, 2002).

Najini *et al.* (2002) analysed markers associated with H5 and H22 Hessian fly resistance genes in bread wheat using AFLP. The technique was used in conjunction with near isogenic lines and bulked segregant analysis to identify molecular markers linked to Hessian fly resistance genes in bread wheat.

Wiliams *et al.* (2002) used bulked segregant analysis to identify AFLP markers linked to *Pratylenchus neglectus,* root lesion nematode resistance in the wheat cultivar Excalibur.

Hueyjiuan *et al.* (2002) screened RAPD markers linked with the gees resistant to rice brown plant hopper. A chromosome region linked to the marker OPL 9, which is significantly related to BPH resistance was detected. OPK 16 and OPL 9 markers are useful tools to be applied to marker assisted selection for cultivars resistant to BPH.

Cevik and King (2002) analysed high resolution genetic analysis of the sd-1 locus in *Malus* sp using AFLP markers.

Ouedraogo *et al.* (2002) used AFLP analysis in combination with bulked segregant analysis to identify molecular markers linked to two cowpea genes conferring resistance to *Striga gesnerioides* race 1.

Cerenak and Javornik (2002) conducted a study to analyze the association of RAPD and AFLP markers of hop resistance to damson hop aphid (*Phorodon humuli*). Seven specific AFLP markers and three specific RAPD markers were detected among the polymorphic fragment in resistant genotypes.

The determined specific fragments represent potential markers for hop resistance to damson hop aphid.

Yueying *et al.* (2003) detected RAPD markers linked to the single dominant gene in the F2 bulked segregant populations of sorghum. 500 random primers were used to screen the markers linked to the resistant gene and 1614 DAN bands were amplified by using the primers OPA-01, OPP-09, OPP-14, OPN-07, OPN-08, OPN 20, OPY 14, OPS20 and OPJ-06.

Sharma *et al.* (2003) constructed a linkage map of rice brown plant hopper resistance gene, Bph1. RFLP and AFLP markers were selected by the bulked segregant analysis and used in the mapping study.

Bose *et al.* (2004) identified RAPD markers for the detection of a gall midge resistance gene, Gm4t in rice. The markers amplified a 583bp fragment in all the susceptible lines and a 570bp fragment in all the resistant lines. The results demonstrate that the markers are highly specific for the Gm4t gene.

Sauge *et al.* (2004) identified RAPD marker Qo6-350 and RFLP marker AG 50A which were strongly associated with aphid resistance (*Myzus persicae*) in the wild peach.

Moreira et al. (1999) used bulked segregant analysis to find Random amplified polymorphic DNA markers linked to the resistance of *Liriomyza trifolii* in tomato.

Herselman *et al.* (2004) identified AFLP markers linked to peanut (*Arachis hypogaea* L) resistance to the aphid vector of groundnut rosette disease. Bulked segregant analysis and AFLP analysis were employed to identify DNA markers linked to aphid resistance for the development of a partial genetic linkage map. A total of 308 AFLP primer combinations were used to identify markers

associated with the aphid resistance. This study represents the first report on the identification of molecular markers closely linked to the aphid resistance to groundnut rosette disease and the construction of the first partial genetic linkage map for cultivated peanut.

Braendle *et al.* (2005) generated AFLP markers for the genetic mapping of *Aphicarus*- a sex linked locus controlling a wing polymorphism in the pea aphid (*Acyrthosiphum pisum*). Seven AFLP markers were identified.

Sargent *et al.* (2007) used AFLP and microsatellite markers for mapping of A1 confering resistance to the aphid *Amphorophora idaei* and dw (dwarfing habit) in red raspberry.

2.10.4. Molecular markers in genetic variation studies

Cervera *et al* (2000) studied the genetic relationships among biotypes of *Bemisia tabaci* based on AFLP analysis. The AFLP assay allowed the scoring of a total of 354 polymorphic bands with the use of two primer combinations.

Metais *et al* (2001) assessed common bean genetic diversity using RFLP, RAPD, AFLP and ISSR markers. Reproducible RAPD profiles were obtained to differentiate all the genotypes tested with only seven primers.

Junghyun *et al.* (2001) constructed molecular genetic linkage map of cowpea using RAPD markers. Five hundred and twenty primers were screened for parental polymorphism. Seventy RAPD markers were found to be genetically linked and formed eleven linkage groups.

Genetic diversity of cowpea (*Vigna unguiculata* (L)Walp) genotypes were analyzed by Cidrack *et al.*2001. Nearly eight genotypes were identified by means of chromosome number identification and amplification of RAPD molecular markers. A somatic chromosome number of 2n=22 was found in all the

genotypes. On the other hand RAPD markers were shown to be polymorphic in these cowpea genotypes with a total of 68 bands amplified by nine primers used.

Belaj *et al.* (2002) used RAPD markers for analyzing the genetic diversity of Albanian olive germplasm and its relationship with other Mediteranean countries. A total of 76 polymorphic bands out of 107 reproducible bands were obtained using 16 primers.

Coulibaly *et al.* (2002) used amplified fragment polymorphisms to evaluate the genetic relationships within cowpea and to assess the organization of its genetic diversity. AFLP variation was also used to study the genetic variation among and within domesticated and wild accessions based on their geographical origin. Wild cowpea was more diverse than domesticated ones.

Fall *et al.* (2003) studied genetic diversity in cowpea (Vigna unguiculata L Walp) varieties which was determined by ARA and RAPD techniques.

Nazeem *et al.* (2003) used RAPD and AFLP techniques to assess the genetic variability in 49 black pepper varieties. They observed an average similarity of 63 percent among the accessions.

Herselman (2003) used AFLP technique employing two different rare cutters, *Eco*R1 and *Mlu*1 in combination with frequent cutter, was used to assess the genetic diversity and relationships among 21 closely related cultivated Southern African peanut genotypes.

Molecular profiles of 24 selections and 11 hybrids of cashew were developed using a combination of five RAPD and four ISSR primers for maximum discrimination and repeatability (Archak, 2003)

Ntundu *et al.* (2004) used Amplified Fragment Length Polymorphism to assess the genetic diversity among 100 selected bambara groundnut landraces from a diverse geographic area of Tanzania. Eleven informative AFLP primer combinations generated 49 scorable polymorphic amplification fragments across the bambara groundnut accessions.

Keshavachandran *et al.* (2005) reported the genetic finger printing of *Piper nigrum* and *Piper longum* cultivars using RAPD markers. Fourteen land races and three advanced cultivar of *Piper longum* were amplified using 10 sets of random primers to give 119 amplification products. The analysis indicated that the accessions could be differentiated based on heir RAPD profiles.

Roychoudhury *et al.* (2005) examined the diversity among nineteen wild and cultivated germplasms of the genus *Vigna* using RAPD marker. Ten decamer primers were used in the amplification reactions and five of them produce reproducible bands.

Kavitha *et al.* (2005) evaluated he genetic diversity of *Zingiber* sp and their response to *Pythium aphanidermatum*, the causal agent of soft rot. Diversity of 21 cultivars and three wild accessions of *Zingiber officinale*, 84 accessions of five wild species were examined using AFLP markers. Analysis revealed high variability in *Zingiber cernuum* and relatively low variation in other species.

2.11. Ongoing Works

To obtain a better understanding of the genetic make up of cowpea and its wild relatives, IITA and collaborators are constructing genetic map using RFLP. This will help scientists to identify the approximate location of specific genes for desired traits within the pant genome.

CSIRO (Commomwealth Scientific and Industrial Research Organization) Entomology will study the genetic mechanism involved in the natural host plant resistance in *Medicago truncatula* to aphid.

At the University of California, aphid resistant lines of cowpea have been identified. This will further be evaluated to find out the most effective source of resisance. Germplasm will be characterized as to the mode of resistance based on the relative ability of the lines to avoid, tolerate or develop induced resistance to aphid attack.

Swarna Bharat Biotechnics Pvt. (SBBPL), Hyderabad, is set to license a lectin gene (Lec GNA 2) that produces a protein lethal to sucking pests like aphids.

Materials and Methods

3. MATERIALS AND METHODS

The study on "Molecular characterization and development of trait related markers for aphid resistance in cowpea (*Vigna unguiculata* (L) Walp.) was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB) and Radio Tracer Laboratory (RTL), College of Horticulture, Vellanikkara during the period 2005-2007.

3.1. Materials

3.1.1. Plant Materials

The seeds collected from different districts of Kerala and maintained in the Department of Olericulture, College of Horticulture were used for the present study. The list of accessions used are given in the Table 1.

3.1.2. Laboratory chemicals, glassware and equipments

The chemicals used in the present study were of good quality (AR grade) procured from Merck India Ltd. and SISCO Research Laboratories. The Taq DNA polymerase, dNTPs, taq buffer and molecular weight marker (λDNA/*Hind* III+ *Eco* RI) were supplied by Bangalore Genei Ltd. The random primers were obtained from Operon Technologies. The reagents and primers for AFLP analysis were supplied by Invitrogen, USA. The plastic wares used for the study were purchased from Tarson. Radioactivity was obtained from Board of Radiation Isotope and Technology, Mumbai.

The equipments available at the Centre for Plant Biotechnology and Molecular Biology, college of Horticulture were used for the present study.

3.2. Methods

3.2.1. Screening for aphid resistance

Sixty accessions of cowpea were sown in the field during the months April-May 2006 and September-October 2006. They were raised in the field

according to the recommendations from KAU package of practices (Plate 1). Ten plants of each accession were grown in a channel. These accessions were screened and scored for aphid resistance. The screening was done by taking the average count of aphids (Plate 2). The count was taken from the terminal shoots, flower buds and pods of three plants from each accession. Five each of susceptible and resistant plants were selected for doing further assays.

Development of aphids in plants was monitored at all the stages of plant growth ie during 20, 30, and 40 days after sowing and the intensity of infestation was measured as a 0-2 scale (Joseph, 1990) as detailed below.

Aphid count	Class of infestation	Grade	Resistance rating
<100 aphids	Low	0	Resistant
100-200 aphids	Medium	1	Moderately resistant
>200 aphids	High	2	Susceptible

3.2.2. Molecular characterization

3.2.2.1. Standardisation of genomic DNA isolation

Isolation of good quality genomic DNA is one of the most important prerequisites for doing RAPD and AFLP analysis. The procedures reported by Doyle and Doyle (1987) and Rogers and Bendich (1994) for the extraction of nucleic acids were tried for the isolation of genomic DNA from cowpea. Tender leaves from the selected plants were collected early in the morning and used for the genomic DNA isolation.

3.2.2.1.1. *Procedure reported by Doyle and Doyle (1987)*

Reagents

Extraction buffer

Lysis buffer

TE buffer

Plate 1. Cowpea accessions in the field

Plate 2. Aphid infestation on the plant

Isopropanol

Chloroform: Isoamyl alcohol (24:1)

5 % sarcosin

Ethanol 70%

Procedure

Leaf sample (1g) was ground to a fine powder in excess of liquid nitrogen using a mortar and pestle. A pinch of sodium metabisulphite and 50µl of β-mercaptoethanol was added. Later 3ml extraction buffer was also added. The homogenate was transferred to a 50 ml oakridge tube containing 4 ml prewarmed lysis buffer and 1 ml sarcosin. The contents were mixed well and maintained at 65^0C for 15 minutes. Equal volume of chloroform:isoamyl alcohol (24:1) mixture was added to the tube, mixed gently by inversion and centrifuged at 10000 rpm for 15 minutes at 4^0C. The contents get separated into three distinct phases. The upper aqueous phase containing DNA was pipeted out into a fresh 50ml oakridge tube. Then 0.6 volume of ice cold chilled isopropanol was added and the contents were mixed gently. The sample was then incubated at -20^0C for 30 minutes or overnight to precipitate the DNA completely. The DNA was then pelleted by centrifuging at 10000 rpm for 15 minutes at 4^0C. The isopropanol was poured out and the pellet was washed with 70 percent alcohol. The pellet was air dried to remove the alcohol and finally dissolved in 250 µl TE buffer.

3.2.2.1.2. Procedure reported by Rogers and Bendich (1994)

Reagents

2 x CTAB extraction buffer

10% CTAB solution

TE Buffer

Isopropanol

Chloroform:isopropanol (24:1)

Ethanol 70% and 100%

Procedure

Leaf sample (1g) was weighed accurately and ground using liquid nitrogen. The ground tissue was transferred to a 50ml oakridge tube containing 5ml prewarmed 2x CTAB extraction buffer and 60µl β- mercaptoethanol. The contents were mixed and incubated at 65^{0}C for 15-20 minutes. Then equal volume of chloroform:isoamyl alcohol mixture was added, mixed gently by inversion and centrifuged at 10000rpm for 10 minutes at 4^{0}C. The supernatant was transferred to a fresh tube and 1/10 volume of 10% CTAB solution was added. Equal volume of chloroform: isoamyl alcohol was added again, mixed gently and centrifuged at 10000 rpm for 15 minutes at 4^{0}C. The aqueous phase was transferred to another fresh tube and 1/6 volume of isopropanol was added and kept at -20^{0}C for 30 minutes for complete precipitation of DNA. The DNA was pelleted by centrifuging at 10000 rpm for 15 minutes at 4^{0}C. The supernatant was discarded and the pellet was washed with 70 percent ethanol. The pellet was air dried and dissolved in 250µl TE buffer.

3.2.3. Purification of DNA

The DNA isolated contains RNA as contaminant and was purified by phenol precipitation and RNAse treatment (Sambrook *et al.*, 1989).

Reagents

1. Phenol: Chloroform mixture (1:1,v/v)
2. Chilled isopropanol
3. 70 per cent ethanol
4. TE buffer
5. Chloroform:Isoamyl alcohol (24:1;v/v)

The RNAse A from Sigma, USA was used to prepare RNAse. One per cent solution was prepared by dissolving RNAse A in TE buffer at 100^{0}C for 15minutes.The solution was cooled to room temperature, dispensed into aliquots and stored at -20^{0}C.

The procedure followed for DNA purification is as follows

RNAse solution (2µl) was added to 100µl DNA sample and incubated at 37^{0}C in dry bath (Genei, Thermocon) for 1hour. The volume was made up to 250µl with distilled water and equal volume of Phenol: Chloroform mixture was added. This was then centrifuged at 12,000 xg for 10minutes at 4^{0}C. The aqueous phase was collected in a fresh micro centrifuge tube and added equal volume of Chloroform: Isoamyl alcohol (24:1). Again hey were centrifuged at 12,000 xg for 10minutes at 4^{0}C. The above two steps were repeated and finally precipitated DNA from the aqueous phase with 0.6volume of chilled isopropanol. The mixture was then incubated at -20^{0}C for 30minutes and centrifuged at 10,000rpm for 15minutes at 4^{0}C. The pellet of DNA obtained was washed with 70 per cent ethanol. The pellets were air dried and dissolved in 250µl TE buffer.

3.2.4. Assessing the quality of DNA

The quality of isolated DNA was evaluated through agarose gel electrophoresis (Sambrook *et al.*, 1989).

Reagents

Agarose

50X TAE buffer

Tracking dye (6X)

Ethidium bromide

The procedure followed for agarose gel electrophoresis is as follows

1X TAE buffer was prepared from the 50 X TAE stock solutions. Agarose (1%) was weighed and dissolved in TAE buffer by boiling. Then ethidium bromide was added at a concentration of 0.5 µg ml^{-1} and mixed well. The open end of gel casting tray was sealed with a cellotape and kept on a horizontal surface. The comb was placed desirably and the dissolved agarose was poured on to the tray. The gel was allowed to set for 30 minutes after which the comb was

removed carefully. The tray was kept in the electrophoresis unit with well side directed towards the cathode. 1X TAE buffer was added to the tank. Then DNA sample (5 µl) along with the tracking dye (1 µl) was loaded into the wells using a micropipette carefully. λDNA/EcoRI + HindIII double digest was used as a molecular marker. After closing the tank, the anode and cathode ends were connected to the power pack and the gel was run at a constant voltage (100V) and current (50A). The power was turned off when the tracking dye reaches 2/3rd length of the gel.

3.2.4.1. Gel documentation

The gel was taken from the electrophoresis unit and viewed under UV light in a transilluminator. The DNA fluoresces under UV light due to ethidium bromide dye. The image was documented and saved in gel documentation system (Alpha Imager 1200).

3.2.5. Purity of DNA

The purity of DNA was further analysed by using NanoDrop® ND-1000 spectrophotometer. This is a full spectrum (220-750mm) spectrophotometer that measures 1µl samples with high accuracy and reproducibility. It utilizes a patented sample retention technology that employs surface tension alone to hold the sample in place. The small sample requirement and ease of use to make the NanoDrop® ND-1000 spectrophotometer is ideally suited for measuring nucleic acid concentration and purity of nucleic acid samples up to 3700ng/µl (ds DNA) without dilution.

A 1µl sample is pipetted on to the end of a fibre optic cable (the receiving cable). A second fiber optic cable (the source fibre) was then brought into contact with the liquid sample causing the liquid to bridge the gap between the fibre optic ends. The gap was controlled to both 1mm and 0.2mm paths. A pulsed xenon flash lamp provides the light source and a spectrometer utilizing a linear CCD array was used to analyze the light after passing through the sample. The

instrument was controlled by special software run from a PC and the data is logged in an archive file on the PC.

The absorbance of nucleic acid samples were measured at a wavelength of 260nm and 280nm.

A260 – absorbance of sample at 260nm represented if measured with a conventional 10mm path.

A280 – sample absorbance at 280nm represented if measured with conventional 10mm path.

260/280 – The ratio is used to assess the purity of nucleic acids. A ratio of approximately 1.8 is generally accepted as pure for DNA. If the ratio is lower, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280nm.

3.2.6. RAPD assay

After the isolation of good quality genomic DNA the cowpea accessions were subjected to RAPD. It is a PCR based molecular marker technique. Here single short oligonucleotide primers usually a 10 base primer are arbitrarily selected to amplify a set of DNA segments distributed randomly throughout the genome (Williams et al., 1990). The number of amplified products in RAPD depends on the length of the primer and size of the target genome. The reaction products are conveniently analyzed on agarose gels, stained with ethidium bromide and seen under UV light.

An RAPD reaction mixture contains different constituents like template DNA, random primers, enzyme, dNTPs,$Mgcl_2$ and assay buffer which are subjected to repeated cycles of denaturation, primer annealing and elongation in a thermal cycler.

The procedure reported by (Roychoudhary et al., 2005) was modified for the amplification of cowpea DNA. A total of 20µl reaction mixture was prepared.

10x PCR buffer	-2 µl
dNTPs (10mM)	-1 µl
$Mgcl_2$ (25mM)	-1 µl
Primer (10pM)	-1.5 µl
Template DNA	- 2 µl
Taq DNA polymerase	- 0.3 µl
Sterile double distilled water	- 12.2 µl

3.2.6.1. Screening of primers

Primer screening was carried out to identify the best primers for RAPD analysis. Random decamer primer kits obtained from Operon Technologies, USA were used. 10 primers each under OPA, OPS and OPY were tried. The template DNA was kept the same throughout the screening procedures. A master mix was prepared for the required number of reactions by adding all the constituents except the primer. Aliquots of the master mix were pipetted out into each of the 0.2ml PCR tubes and then the primer was added into each tube separately. The primers that gave good amplification with five or more distinct and reproducible bands were selected and used for the characterization of 10 accessions.

3.2.6.2. Molecular characterization of cowpea accessions

Of the total 30 primers screened 10 primes that gave good reproducible bands were used for the characterization of the cowpea accessions. A 20 µl reaction mixture containing all the above constituents were prepared in a 0.2ml thin walled PCR tubes. A control tube containing all the components but no genomic DNA was also run with each primer to check for the contamination. Polymerase chain reaction was carried out in the thermal cycler programmed for an initial denaturation of 94^0C for 5 minutes followed by 40 cycles of 1 minute denaturation at 94^0 C, 1minute primer annealing at 37^0 C and 2 minutes polymerization at 72^0 C. There is a final extension for 5 minutes at $72\ ^0$C. After completion of amplification, the reaction was held at 4^0 C for 5 minutes.

The amplified products were resolved through 1.2 percent agarose gel electrophoresis. The gel was visualized under UV light on a transilluminator and documented. The reliability of polymorphic bands was tested by repeating the assay.

3.2.6.3. Analysis of amplification profiles

Amplification profiles of 10 accessions were compared with each other and bands of DNA fragments scored manually as (1) or (0) depending on the presence or absence of particular band respectively. The data was analysed using Numerical Taxonomy System of Multivariate Statistical Programme (NTSYS) software package (Rohlf,1990). The SIMQUAL programme was used to calculate Jaccard's coefficient, a common estimator of genetic identity. Clustering was done using Sequential Agglomerative Hierarchial Nested Clustering (SAHN) routine and a dendrogram was constructed using Unweighted Pair Group Method of Arithmetic Averages (UPGMA) by Sneath and Sokal (1973) using NTSYS package.

Resolving power (Prevost and Wilkinson, 1999) was used to identify the primers that would distinguish the accessions most efficiently. Resolving power (Rp) of a primer was calculated as the sum of 'band informativeness' of all the bands produced by the primer. Band informativeness (Ib) is = 1-(2x [0.5-p]) where p is the proportion of accessions containing the band. Resolving power of the primer is represented as: $Rp= \sum Ib$. Finally the data obtained was pooled together to generate a combined dendrogram

3.2.7. AFLP analysis

AFLP is a highly sensitive method for detecting polymorphism throughout the genome. It is based on PCR amplification of genomic restriction fragments generated by specific restriction enzymes and oligonucleotide adapters of a few nucleotide bases (Vos et al., 1995).

The AFLP analysis has been designed for use with plants ranging in size from $5x10^8$ to $6x10^9$bp. This technique involves mainly 3 steps.

1. Restriction endonuclease digestion of DNA and ligation of adapters.
2. Amplification of restriction fragments.
3. Gel analysis of amplified fragments

3.2.7.1. Procedure

3.2.7.1.1. Restriction digestion of genomic DNA

To prepare an AFLP template, genomic DNA was isolated and digested with two restriction endonucleases simultaneously. This step generates the requisite substrate for ligation and subsequent amplification. The two restriction endonucleases used were *Eco*RI, which is a rare cutter with a 6 base pair recognition site, and *Mse*I, which is a frequent cutter with a 4 base pair recognition site. When used together these enzymes generate small DNA fragments that will amplify well and are in the optimal size range (<1kb) for separation on denaturing polyacrylamide gels.

The digestion reaction was set up as given below. The following components were added to a 1.5ml microcentrifuge tube.

5x reaction buffer	- 5µl
Sample DNA	- 2µl
*Eco*RI / *Mse*I	- 2µl
Distilled water	- 16µl
Total volume	25µl

A 25µl reaction was prepared. The reaction was mixed gent and collected by brief centrifugation. The mixture was then incubated at 37^0C for 2 hours. After 2 hours the restriction endonucleases was incubated by inactivation at 70^0C for 15 minutes and immediately placed on ice. The reaction contents were collected by brief centrifugation.

3.2.7.1.2. Ligation of adapters

Following heat inactivation of the restriction endonucleases, EcoRI and MseI adapters were ligated to the restricted fragments to generate template for amplification. The adapter sequences will serve as the primer binding sites for the amplification of restricted fragments.

The reaction for adapter ligation was set up as given below. The following components were added to the digested DNA samples.

Adapter ligation solution - 24µl

T_4 DNA ligase - 1µl

The contents were mixed gently, centrifuged briefly and incubated at 20^0C for 2 hours. Following incubation, a 1:10 dilution of the ligated product was prepared as follows:

10µl of the ligated product was transferred to a 1.5ml microcentrifuge tube containing 90µl of TE buffer. The contents were mixed properly. The unused portion of the ligated product was stored at -20^0C for future use.

3.2.7.1.3. Amplification reactions

A) Preamplification reaction

PCR amplification was carried out in two consecutive steps. In the first reaction, called preamplification, the ligated product was amplified with an *Mse*I primer containing one selective nucleotide (N+1) and an *Eco*RI primer containing no selective nucleotide(N+0). The reaction was set up as given below. The following components were added to a 0.5ml thin walled microcentrifuge tube

Diluted DNA (1:10 ligated product)	- 5µl
Pre-amp primer mix	- 40µl
10x PCR buffer	- 5µl
Taq DNA polymerase	- 1µl
Total volume	51µl

A 51µl reaction volume was prepared, mixed gently and centrifuged briefly to collect the contents. PCR was performed in a thermalcycler for 20 cycles at 94^0 C for 30 seconds, 56^0C for 60 seconds and 72^0C for 60 seconds. Following amplification, a1:50 dilution of the PCR product was done as follows: 3 µl of the amplified product was transferred to a fresh 1.ml microcentrifuge tube containing 147µl of TE buffer. Both the dilution as well as undiluted reaction products were stored at -20^0C.

B) *Primer labeling*

The selective N+2 *Eco*RI primer was end labeled by phosphorylating the 5'end with γ^{32}P ATP using T_4 kinase. ^{32}P labeled primers were preferred as they gave a better resolution of the PCR products on the gels and the reaction products were less prone to degradation due to autoradiolysis. The labeling reactions for 10 samples were set up as given below. The following components were added to a 0.2ml microcentrifuge tube.

*Eco*RI primer	- 1.8 µl
5x kinase buffer	- 1.0 µl
γ32P ATP	- 2 .0µl
T4 kinase	- 0.2 µl
Total volume:	5 µl

A 50 µl reaction volume was prepared which was sufficient for 100 selective amplifications. The contents were mixed gently and centrifuged briefly. The reaction was then incubated at 37^0C for one hour. Following incubation, the enzyme was heat inactivated at 70^0C for 10 minutes. The tube was centrifuged briefly to collect the reaction contents.

Alongside, the 30-330bp AFLP ladder (Invitrogen) was also labeled using γ32P ATP. The labeling reaction was set up as given below.

30-330 bp AFLP ladder - 2 µl

5X exchange reaction buffer - 1 µl

γ^{32}P ATP - 1 µl

T_4 polynucleotide kinase - 1 µl

The components were mixed thoroughly collected by brief centrifugation and incubated for 10 minutes at 37^{0}C. Then the reaction was inactivated at 65^{0}C for 15 minutes. An equal volume (5 µl) of TE buffer was added to the reaction mixture followed by 20 µl of denaturing solution. The solution was incubated at 70^{0}C for 5 minutes and stored at -20^{0}C.

C) Selective amplification reaction

The 1:50 dilution of the amplified product was used as template for the second amplification reaction called selective amplification using *Mse*I primer containing 3 selective nucleotides (N+3) and an *Eco*RI primer containing two selective nucleotides (N+2). The reaction for selective amplification was set up as given below.

A) For each primer pair, the following components were added to a 0.2ml microcentrifuge tube and it was marked as Mix 1.

Labelled *Eco*RI primer	- 5 µl
*Mse*I primer	- 45 µl
Total volume:	50 µl

B) The following components were added to another 1.5ml microcentrifuge tube which was marked as Mix 2.

Distilled water	- 79 µl
10x PCR buffer	- 20 µl
Taq DNA polymerase	- 1 µl
Total volume	100 µl

C) The final selective amplification reaction was assembled by combining the following in a 0.5ml microcentrifuge tube

Diluted template DNA (1:50 dilution of preamplified product) - 5 µl

Mix 1 -5 µl

Mix 2 -10 µl

A 20 µl reaction volume was prepared and amplified on a thermal cycler with the following conditions: one cycle at 94^{0}C for 1 minute, 65^{0}C for 1 minute and 72^{0}C for 1hour 30 minutes. Next the annealing temperature was lowered in each cycle by 0.7^{0}C during 12 cycles. This was followed by 23 cycles at 94^{0}C for 30 seconds, 56^{0}C for 30 seconds, and 72^{0}C for 1 minute. After completion of amplification, the reaction was held at 4^{0}C.

3.2.7.1.4. Gel analysis of the amplified fragments

After selective amplification an equal volume (20 µl) of formamide dye was added to each reaction. The samples were denatured by incubation at 94^{0}C for 5 minutes and immediately placed on ice. The amplified products were resolved in a 4% denaturing polyacrylamide gel. The composition of the gel mix was as follows:

Acrylamide	- 11.5ml
TBE	- 15ml
Urea	- 31.5g
APS	- 0.1g in 1ml distilled water
TEMED	- 50 µl

The gel was casted on a BIORAD gel sequencer apparatus using 0.2mm spacers and 23 well sharp tooth comb following manufacturer's instruction. The gel was given a prerun at constant power (45W) for 30 minutes and 8 µl of the samples were loaded on to the gel after flushing the wells carefully. Following loading, the gel was electrophoresed at constant power for 2 hours until the xylene cyanol was two-thirds down the length of the gel. After the run, the gel was cooled to room temperature. The glass plates were separated very carefully and the gel was transferred on to the filter paper, wrapped carefully with cling film and dried at 80^{0}C for two hours under vaccum in a Gel Dryer (BIORAD). The dried gel was exposed to an Imaging Plate (IP) and

kept in a BAS cassette (FUJIFILM) for about 20 – 45minutes.The IP plate accumulates and stores radiation energy while it is exposed. This plate has an image recording layer consisting of polyester base material densely coated with accelerated phosphorescent fluorescent material of fine crystals. The recording surface of the IP was scanned with a laser beam inside the Fluorescent Image Analyzing System FLA-5100 (FUJI PHOTO FILM Co., Ltd.) and the digital image was recorded in the analyzer unit. The dried gel can also be exposed to an X-ray film for two days and then developed by washing it in developer, sterile water and fixer. The exposed screen was scanned and the image was used for further analysis.

3.2.7.2. Data Analysis

Each AFLP band was treated as a unit character and was scored manually as independent binary codes (1 for presence and 0 for absence). Only distinct and well resolved fragments were scored. The resulting data were used to construct an UPGMA (Unweighted Pair Group Method with Arithmetic means) dendrogram using the software package NTSYS (Rohlf, 1990). Resolving power (Rp) of the primer combination was also calculated as the sum of 'band informativeness' of all the bands produced by the primer. Band informativeness (Ib) is = 1-(2x [0.5-p]) where p is the proportion of accessions containing the band. Resolving power of the primer is represented as: $Rp= \sum Ib$ (Prevost and Wilkinson, 1999).

Table 1 List of accessions used for the present study

Sl No	Accessions	District
1	VS 1025	Wayanad
2	VS 1028	Kottayam
3	VS 1030	Kottayam
4	VS 1032	Alappuzha
5	VS 1034	Kasargod
6	VS 1035	Kasargod
7	VS 1042	Thrissur
8	VS 1053	Thrissur
9	VS 1058	Kannur
10	VS 1075	Alappuzha
11	VS 1086	Pathanamthitta
12	VS 1087	Thrissur
13	VS1088	Thrissur
14	VS 1105	Malappuram
15	VS 1111	Malappuram
16	VS 1127	Malappuram
17	VS 1128	Malappuram
18	VS 1131	Malappuram
19	VS 1133	Malappuram
20	VS 1134	Malappuram
21	VS 1135	Malappuram
22	VS 1138	Malappuram
23	VS 1139	Kasargod
24	VS 1145	Malappuram
25	VS 1147	Malappuram
26	VS 1153	Kozhikode
27	VS 1160	Kottayam
28	VS 1166	Kottayam
29	VS 1168	Wayanad
30	VS 1170	Wayanad

31	VS 1171	Wayanad
32	VS 1173	Wayanad
33	VS 1174	Wayanad
34	VS 1177	Wayanad
35	VS 1179	Wayanad
36	VS 1180	Wayanad
37	VS 1185	Wayanad
38	VS 1195	Malappuram
39	VS 1201	KAU, Thrissur
40	VS 1213	Kannur
41	VS 1220	Kannur
42	VS 1221	Kannur
43	VS 1225	KAU, Thrissur
44	VS 1230	Wayanad
45	VS 1231	Trivandrum
46	VS 1248	Trivandrum
47	VS 1263	Kannur
48	VS 1275	KAU, Thrissur
49	VS 1277	Idukki
50	VS 1296	Malappuram
51	Anaswara	KAU, Thrissur
52	Aryavaibhavalakshmi	KAU, Thrissur
53	Lola	KAU, Thrissur
54	Pusa komal	KAU, Thrissur
55	Vanitha	KAU, Thrissur
56	Kanakamony	KAU, Thrissur
57	Bagyalakshmi	KAU, Thrissur
58	Varun	KAU, Thrissur
59	IVRCP- 3	KAU, Thrissur
60	IVRCP- 5	KAU, Thrissur

Results

4. RESULTS

The study on 'Molecular characterization and development of trait related markers for aphid resistance in cowpea (*Vigna unguiculata* (L) Walp.)' was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB) and Radio Tracer Laboratory (RTL), College of Horticulture, Vellanikkara during the period 2005-2007. The results of different experiments are described in this chapter.

4.1 Screening of cowpea accessions for the incidence of aphids

Sixty accessions of cowpea collected from different parts of Kerala and maintained in the Department of Olericulture, College of Horticulture were used for the present study. The data on the average count of aphids recorded is presented in the Table 2.

There was no aphid incidence for the accessions sown during April. Aphids were seen on the accessions which were sown in September after 40 days of sowing. The average aphid count ranged from 0 to 494.67. The highest average aphid count was observed for the accession VS 1034 (494.67).

After screening these accessions were given the score 0-2 based on the average count of aphid population (Table 3). Out of sixty accessions, fifty were severely infected with aphids and they were given the score 2. Four accessions were moderately resistant to aphids and were given the score 1 (VS 1145, VS 1032, VS 1296, and VS 1171). Six accessions viz. VS 1230, VS 1231, VS 1248, VS 1263, VS 1201 and VS 1213 were not found affected by aphids during the entire crop season and they were graded as scale 0 and grouped as resistant.

Based on the average aphid count, five susceptible (VS 1177, VS 1179, VS 1173, VS 1208, VS 1034) and five resistant (VS 1230, VS 1231, VS 1201, VS 1248, VS 1263) accessions were used for the further study (Plate 3 and Plate 4).

Table 2 Screening of cowpea accessions for aphid infestation

Accession No:	Aphid count									Total (from 3 parts of plants)			Average count
	Plant 1			Plant 2			Plant 3			Plant 1	Plant 2	Plant 3	
	* T.S	Leaves	Pods	* T.S	Leaves	Pods	* T.S	Leaves	Pods				
VS 1025	200	50	100	150	80	100	200	70	200	350	330	470	383.33
VS 1030	85	75	85	80	75	80	70	50	60	245	235	180	220
VS 1032	75	40	70	70	50	70	75	40	70	185	190	185	186.67
VS 1034	195	60	200	215	100	164	250	100	200	455	479	550	494.67
VS 1035	70	55	75	100	50	60	98	63	75	200	230	236	222
VS 1042	150	52	110	155	55	115	157	60	55	312	325	322	319.67
VS 1053	146	70	125	130	70	110	135	70	115	341	310	320	323.67
VS 1058	150	100	90	150	75	200	175	120	100	340	425	395	386.67
VS 1075	90	75	90	130	100	100	97	70	100	250	330	267	282.33
VS 1086	100	100	100	90	80	90	100	50	90	300	260	240	270
VS 1087	120	90	100	115	90	100	125	93	100	310	305	318	311
VS1088	100	100	100	100	85	100	100	85	100	300	285	285	290
VS 1105	100	75	200	95	80	110	200	75	200	375	255	475	378.33
VS 1111	75	60	58	70	75	80	65	65	55	193	195	225	226.67
VS 1127	100	50	100	100	75	100	85	70	80	250	275	235	253.33

VS 1128	95	90	95	100	100	100	90	80	100	280	300	270	283.33
VS 1131	70	40	70	75	75	75	70	40	70	180	225	180	241.67
VS 1133	180	100	175	155	90	190	85	80	120	455	435	235	391.67
VS 1134	100	70	200	100	50	100	200	70	200	370	250	470	363.3
VS 1135	110	80	110	100	70	110	105	60	100	300	235	265	266.67
VS 1138	100	85	100	150	90	100	110	90	95	285	340	295	306.67
VS 1139	125	80	120	130	90	150	135	95	155	325	370	385	360
VS 1145	70	50	70	60	65	50	70	50	55	190	175	175	180
VS 1147	100	100	100	150	70	95	100	70	100	300	315	270	266.67
VS 1153	100	50	80	100	50	80	85	55	80	230	230	220	226.67
VS 1160	85	60	85	87	65	68	80	60	70	230	220	210	220
VS 1166	154	95	170	120	100	100	95	90	100	419	320	285	341.33
VS 1168	128	90	100	130	90	120	110	85	115	318	348	310	322.67
VS 1170	200	100	120	150	100	100	100	100	100	420	350	300	356.67
VS 1171	70	60	70	70	55	60	70	60	70	200	185	200	195
VS 1173	275	200	150	200	95	200	200	98	200	625	495	498	539.33
VS 1174	125	90	120	90	80	200	150	50	130	335	370	330	345
VS 1177	170	85	150	100	100	155	200	70	200	405	355	470	410
VS 1179	250	155	175	200	50	200	185	75	185	550	450	445	465
VS 1180	100	100	90	90	80	100	100	50	100	290	270	250	270
VS 1185	140	70	120	130	60	110	125	60	100	330	300	255	305
VS 1195	100	100	100	150	100	100	140	80	90	300	350	310	320
VS 1201	–	–	–	–	–	–	–	–	–	–	–	–	0

VS 1208	200	100	180	190	115	190	170	90	185	480	495	44 5	473.33
VS 1213	–	–	–	–	–	–	–	–	–	–	–	–	0
VS 1220	100	75	100	100	100	100	100	50	95	275	300	245	273.33
VS 1221	150	50	150	100	50	100	90	60	90	350	250	240	280
VS 1225	175	80	150	125	100	150	90	75	120	405	375	285	355
VS 1230	–	–	–	–	–	–	–	–	–	–	–	–	0
VS 1231	–	–	–	–	–	–	–	–	–	–	–	–	0
VS 1248	–	–	–	–	–	–	–	–	–	–	–	–	0
VS 1263	–	–	–	–	–	–	–	–	–	–	–	–	0
VS 1277	100	96	100	80	80	80	85	50	70	296	240	205	247
VS 1296	80	45	75	70	60	75	70	35	70	200	205	175	193.33
Anaswara	100	100	100	100	96	100	100	100	100	300	296	300	298.67
Aryavaibhavalakshmi	100	50	100	100	75	100	100	60	100	250	275	260	261.67
Lola	110	75	100	110	75	100	100	60	100	285	285	260	276.67
Pusa komal	100	100	100	100	50	100	100	90	100	300	250	290	280
Vanitha	90	65	120	100	50	100	120	70	125	275	250	315	280
Kanakamony	95	60	95	75	65	150	150	70	100	250	290	320	286.67
Bagyalakshmi	90	60	90	100	70	100	95	90	95	240	270	280	263.3
Varun	100	75	100	100	50	100	95	95	95	275	250	285	270
IRCP- 3	100	50	100	85	60	80	100	65	100	250	225	265	246.67
IVRCP- 5	90	90	90	150	80	150	85	60	80	270	380	225	291.67

*T.S- Terminal shoots

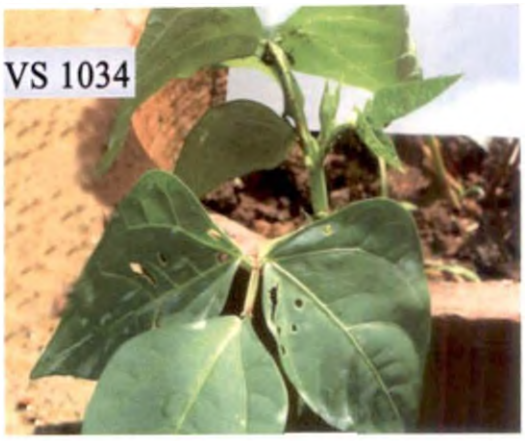
Table 3. Score chart for reaction of cowpea accessions for aphid resistance

Accession no:	Scale (0-2)	Resistance rating
VS 1025	2	Susceptible
VS 1030	2	Susceptible
VS 1032	1	Moderately resistant
VS 1034	2	Susceptible
VS 1035	2	Susceptible
VS 1042	2	Susceptible
VS 1053	2	Susceptible
VS 1058	2	Susceptible
VS 1075	2	Susceptible
VS 1086	2	Susceptible
VS 1087	2	Susceptible
VS1088	2	Susceptible
VS 1105	2	Susceptible
VS 1111	2	Susceptible
VS 1127	2	Susceptible
VS 1128	2	Susceptible
VS 1131	2	Susceptible
VS 1133	2	Susceptible
VS 1134	2	Susceptible
VS 1135	2	Susceptible
VS 1138	2	Susceptible
VS 1139	2	Susceptible
VS 1145	1	Moderately resistant
VS 1147	2	Susceptible
VS 1153	2	Susceptible
VS 1160	2	Susceptible
VS 1166	2	Susceptible
VS 1168	2	Susceptible
VS 1170	2	Susceptible
VS 1171	1	Moderately resistant
VS 1173	2	Susceptible
VS 1174	2	Susceptible
VS 1177	2	Susceptible
VS 1179	2	Susceptible

VS 1180	2	Susceptible
VS 1185	2	Susceptible
VS 1195	2	Susceptible
VS 1201	0	Resistant
VS 1208	2	Susceptible
VS 1213	0	Resistant
VS 1220	2	Susceptible
VS 1221	2	Susceptible
VS 1225	2	Susceptible
VS 1230	0	Resistant
VS 1231	0	Resistant
VS 1248	0	Resistant
VS 1263	0	Resistant
VS 1277	2	Susceptible
VS 1296	1	Moderately resistant
Anaswara	2	Susceptible
Aryavaibhavalakshmi	2	Susceptible
Lola	2	Susceptible
Pusa komal	2	Susceptible
Vanitha	2	Susceptible
Kanakamony	2	Susceptible
Bagyalakshmi	2	Susceptible
Varun	2	Susceptible
IVRCP- 3	2	Susceptible
IVRCP- 5	2	Susceptible

Plate 3. List of susceptible accessions

a



b

c

d

e

Plate 4. List of Resistant Accessions

a

b

c

d

e

4.2 Standardization of genomic DNA isolation

The protocols suggested by Doyle and Doyle (1987) and Rogers and Bendich (1994) were tried for the extraction of genomic DNA from cowpea.

The quality of DNA isolated using the protocols were assessed using agarose gel electrophoresis. Good quality genomic DNA was obtained using Doyle and Doyle (1987) protocol which showed clear distinct bands. The DNA samples isolated using Rogers and Bendich (1994) were partially degraded which showed bands with smear (Plate 5).

The quantity of DNA in selected samples was analysed using NanoDrop® ND-1000 spectrophotometer. The recovery of genomic DNA was highest for the accession VS 1034 (4363.2 µg/ml). The ratio of absorbance at 260nm and 280nm was also obtained for every sample. The absorbance ratio ranges between 1.78 - 1.88 which indicates the good quality of DNA. The quantity and quality of genomic DNA isolated from the selected cowpea accessions using the standardized protocol are presented in Table 4 and Plate 6. The RNA contamination in the samples was completely removed after RNase treatment. The electrophoretic profile showed clear narrow bands (Plate 7).

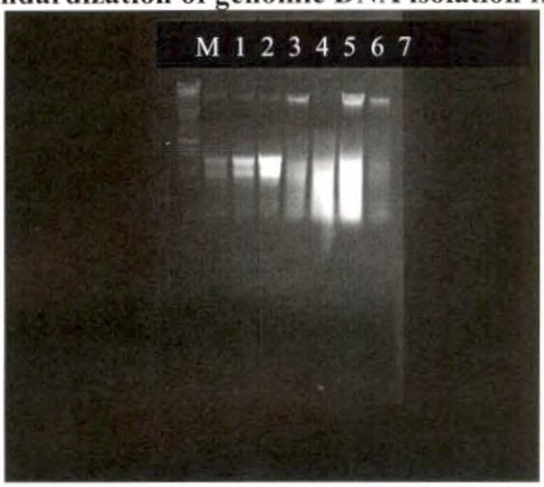
4.3 RAPD assay

The different experiments carried out under this include screening of random primers, screening of cowpea accessions using selected primers and finally the analysis of results using NTSYS pc. (ver 2.1).

4.3.1 Primer screening

Thirty random primers from three different Operon kits (10 from OPA, 10 from OPS and 10 from OPY) wee screened using the genomic DNA from cowpea.

Plate 5. Standardization of genomic DNA isolation from cowpea



Lane 1: Molecular marker, Lane 2, 3, 4: DNA isolated by Doyle and Doyle method, Lane 5, 6, 7: DNA isolated by Rogers and Bendich method

Plate 6. DNA isolated from 10 cowpea accessions using Doyle and Doyle protocol

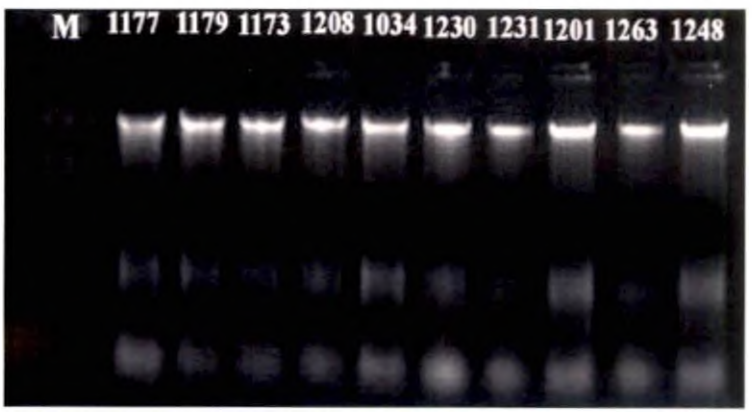
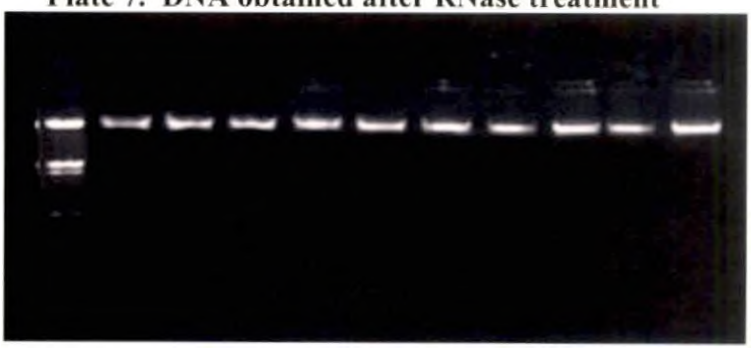


Plate 7. DNA obtained after RNase treatment



4.3.1.1OPA series

Ten primers in this series were screened. The amplification pattern obtained for different primers in OPA series are given in the Table 5 and Plate 8. The number of bands ranged between zero and fifteen. Eight primers OPA 1, 2, 3, 4, 6, 7, 9, and 10 gave good amplification with more number of discrete bands (above9) and were selected for further analysis. No amplification was obtained for the primer OPA 5. OPA 8 gave average banding pattern (less than 5).

4.3.1.2 OPS series

Results of screening with ten primers in OPS series were given in the Table 6 and Plate 9. Number of bands produced by the primers varied from zero and nine. All the primers gave good amplification pattern, the band numbers ranging from 5 and 9. For further study, OPS 4 and OPS 5 were selected due to distinct banding pattern with good quality amplification and reproducibility.

4.3.1.3 OPY series

Ten primers under this series were also screened. None of the primers produced any conspicous repeatable banding pattern and hence were not selected for further amplification.

4.3.2 Screening of cowpea accessions with selected primers

Ten accessions of cowpea showing susceptibility and resistance to aphids were screened using ten selected random primers (Table 7) belonging to two different Operon primer kits (OPA and OPS). The selected primers used for the amplification gave 74 scorable amplification products of which 26 bands (36percent) were monomorphic and 48 bands (64 percent) were polymorphic (Table 8).

4.3.2.1 Primers from OPA series

Eight primers of this series were selected after initial screening for the characterization of cowpea accessions.

The primer OPA 1 generated a total of 8 amplified products, out of which 5 bands were polymorphic and exhibited about 62.5 percent polymorphism. The accession VS 1248 produced the maximum number of bands and least number of bands by VS 1034. The RAPD profiles generated by OPA 1 for the accessions are given in the Table 9 and Plate 10.

OPA 2 primer exhibited 57.1 percent polymorphism. Seven amplified products were produced out of which 4 bands were polymorphic. No polymorphic bands were shown by VS 1179. The number of bands ranged from 4 and 6. Two bands were shown by the resistant accessions VS1230 and VS 1201 of molecular weight 0.44kb. The RAPD profiles generated by OPA 2 are shown in the Table 10 and Plate 11.

Eleven amplified products were obtained with the primer OPA 3.The maximum number of 11 bands was observed with the accession VS 1263 (Table 11 and Plate 12). The polymorphism exhibited by this primer was 75 percent. Two distinct bands of molecular weight 0.2 kb were seen for the aphid resistant accessions VS 1263 and VS 1248. Another two distinct bands of 0.4kb size were also observed for these accessions. Three bands of size 5.2kb, 3.6 kb and 1.9kb were unique to the accession VS 1248 which was rated as resistant to the aphids.

The amplification pattern produced by the primer OPA 4 for 10 cowpea accessions were given in the Table 12 and Plate 13. The maximum number of bands per sample ranged from 6 to 9. The primer generated 9 amplified products out of which 4 bands were polymorphic (44.4 percent). The maximum number of nine bands was observed for the accession VS 1179.

The RAPD profile generated by OPA 6 showed the maximum number of monomorphic bands with a total of 9 amplified products (Table 13 and Plate 14). Least polymorphism (22.2 percent) was exhibited by this primer. Only two bands were polymorphic. VS 1177, VS 1173 and VS 1179 gave maximum number of nine bands.

The primer OPA 7 generated a total of 7 amplified products with 5 polymorphic bands. The maximum number of bands ranged between 0 and 6 (Table 14 and Plate 15). No amplification was obtained for VS 1248. This primer exhibited 71.4 percent polymorphism.

OPA 9 primer produced 80 percent polymorphism. Five bands were generated by 10 accessions out of which 4 bands were polymorphic The maximum number of bands produced by 10 accessions was 1 to 5 (Table 15 and Plate 16).

The amplification pattern obtained with the primer OPA 10 is given in the Table 16 and Plate 17. This primer generated 6 amplified products. The number of bands generated ranged from 3 and 6. The polymorphism exhibited by this primer was 83.3 percent. A unique band was seen for the resistant accession VS 1248 of molecular weight 0.82 kb.

4.3.2.2 Primers from OPS series

Two primers of this series were selected for the characterization of cowpea accessions (OPS 4 and OPS 5).

The primer OPS 4 shows 80 percent polymorphism. The maximum number of bands produced were 1 to 5. Five amplified products were obtained and 4 of them were polymorphic. The RAPD profile for the primer OPS 4 is given in the Table 17 and Plate 18.

The primer OPS 5 produced 7 amplified products. Maximum number of seven bands was obtained for VS 1179 (Table 18 and Plate 19). Six bands were polymorphic.

In general, least polymorphism was observed with the OPA 6 (Table 19). The specific bands generated by random primers for cowpea accessions are given in the Table 20. The accessions VS 1230, VS 1201, VS 1263 and VS 1248, which produced specific bands, were resistant to the aphids. The resolving power obtained for different primers are presented in the Table 21. The results show that maximum resolving power was given by the primer OPA 4 followed by OPA 6, OPA 1, OPA 3, OPS 5, OPA 2, OPA 7, OPS 4, OPA 10 and OPA 9. A diagrammatic representation of RAPD based markers in the selected cowpea accessions generated by the primer which produced accession specific bands in cowpea were depicted in the Fig 1. The specific bands produced by the primers OPA 2, OPA 3 and OPA 10 were observed. These bands could be treated as trait related markers for aphid resistance.

4.3.3 Genetic analysis

The RAPD data was used to generate a similarity matrix using the SIMQUAL programme. Based on the estimated genetic similarity matrix (Table 22) the highest and lowest genetic similarities were noticed between the accessions VS 1231 and VS 1230 (8.97), and VS 1179 and VS 1248 (5.31) respectively.

The phenetic representation of similarity coefficients among 10 accessions are presented in Fig 2. In the dendrogram, all the 10 accessions were divided into two major clusters, 1 and 2 at 62 percent similarity. The first cluster of 8 accessions was again divided into three main sub clusters 1A, 1B and 1C. The sub cluster 1A had two susceptible accessions VS 1177 and VS 1173. Four accessions were grouped in the sub cluster 1B. Out of the four accessions, VS 1230, VS 1231 and VS 1201 were resistant to the aphids. VS 1230 and VS 1231

Table 4. Quality and Quantity of genomic DNA isolated from cowpea

Accession No:	Absorbance		Absorbance 260/280	Quantity (µg/ml)	Quality
	260nm	280nm			
VS 1177	1.284	0.709	1.81	64.2	Good
VS 1173	6.12	3.31	1.84	306	Good
VS 1179	108.5	60.9	1.78	3407.9	Good
VS 1208	85.21	45.9	1.85	4260.5	Good
VS 1034	87.26	47.12	1.85	4363.2	Good
VS 1230	14.25	7.85	1.81	712.5	Good
VS 1231	14.63	7.96	1.83	2643.2	Good
VS 1201	59.28	32.5	1.82	2964.4	Good
VS 1263	102.12	65.8	1.83	3312.9	Good
VS 1248	65.25	34.68	1.88	1164.2	Good

Table 5. Amplification pattern obtained by the primer OPA series

Primer code	Number of bands	Primer sequence	Amplification pattern
OPA 1	15	CAGGCCCTTC	Very good
OPA 2	13	TGCCGAGCTG	Very good
OPA 3	9	AGTCAGCCAC	Good
OPA 4	10	AATCGGGCTG	Very good
OPA 5	_	AGGGGTCTTG	No amplification
OPA 6	11	GGTCCCTGAC	Very good
OPA 7	9	GAAACGGGTG	Good
OPA 8	4	GTGACGTAGG	Average
OPA 9	11	GGGTAACGCC	Very good
OPA 10	12	GTGATCGCAG	Very good

Table 6. Amplification pattern obtained with the primer OPS series

Primer code	Number of bands	Primer sequence	Amplification pattern
OPS 1	6	GTTTCGCTC C	Good
OPS 2	5	TGATCCCTGG	Good
OPS 3	5	CATCCCCCTG	Good
OPS 4	8	GGACTGGAGT	Good
OPS 5	7	TCGGCCCTTC	Good
OPS 6	9	TGCTCTGCCC	Good
OPS 7	6	GGTGACGCAG	Good
OPS 8	8	GTCCACACGG	Good
OPS 9	_	TGGGGGACTC	No amplification
OPS 10	8	CTGCTGGGAC	Good

Table 7. List of selected decamer primers used for RAPD analysis

Sl No:	Primer code
1	OPA 1
2	OPA 2
3	OPA 3
4	OPA 4
5	OPA 6
6	OPA 7
7	OPA 9
8	OPA 10
9	OPS 4
10	OPS 5

Plate 8. Amplification pattern obtained by the primer OPA series

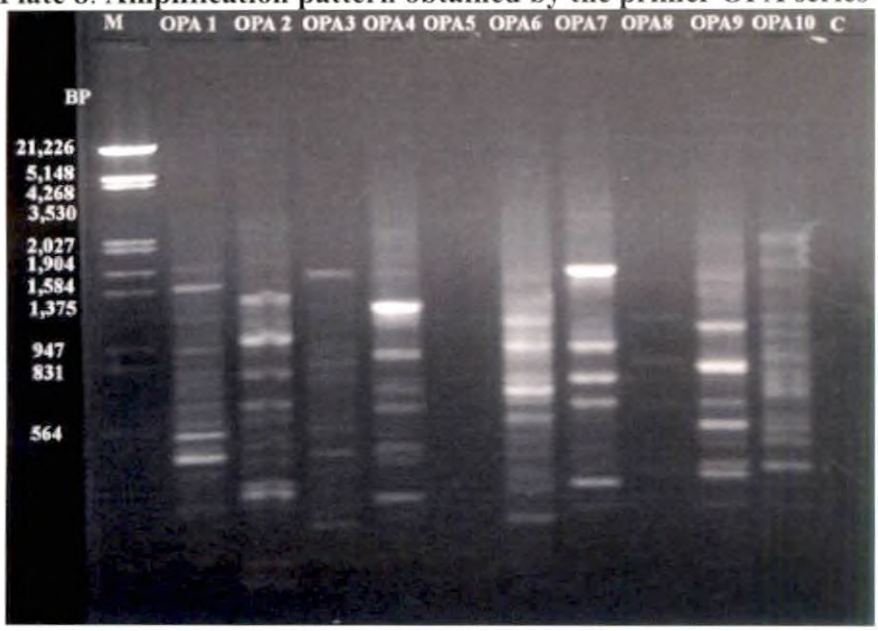
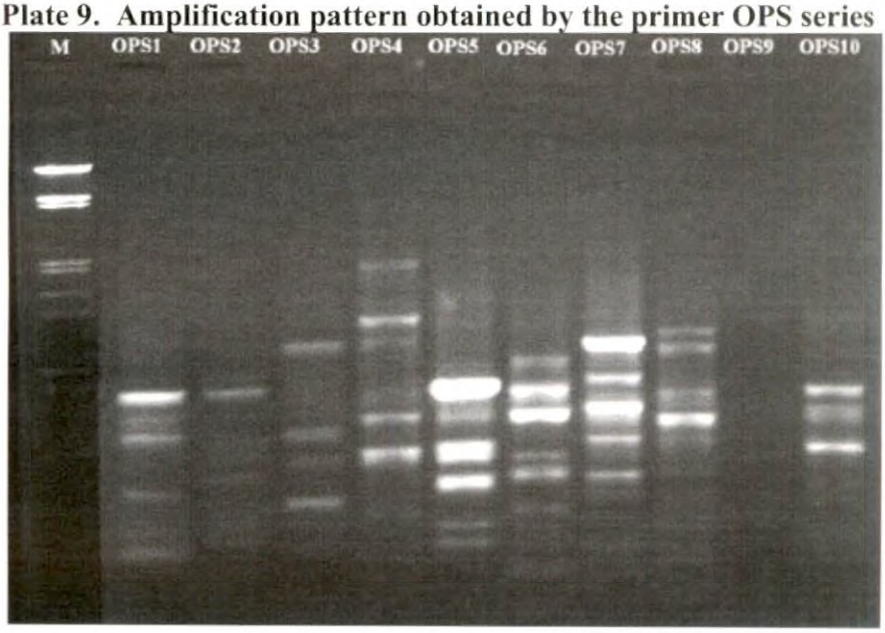


Plate 9. Amplification pattern obtained by the primer OPS series



able 8. Total number of amplicons produced by the random primers in RAPD

Primer	Total number of amplicons	Number of polymorphic bands
OPA 1	8	5
OPA 2	7	4
OPA 3	11	9
OPA 4	9	4
OPA 6	9	2
OPA 7	7	5
OPA 9	5	4
OPA 10	6	5
OPS 4	5	4
OPS 5	7	6
Total	74	48

Table 9. RAPD profile of cowpea accessions with primer OPA 1

Accession	Maximum number of bands/sample	Number of polymorphic bands
VS 1177	6	3
VS 1179	5	2
VS 1173	6	3
VS 1208	6	3
VS 1034	4	1
VS 1230	7	4
VS 1231	7	4
VS 1201	6	3
VS 1263	7	4
VS 1248	8	5
Total	62	32

Table 10. RAPD profile of cowpea accessions with primer OPA 2

Accession	Maximum number of bands/sample	Number of polymorphic bands
VS 1177	5	2
VS 1179	3	0
VS 1173	4	1
VS 1208	4	1
VS 1034	6	3
VS 1230	6	3
VS 1231	5	2
VS 1201	6	3
VS 1263	5	2
VS 1248	5	2
Total	49	19

Table 11. RAPD profile of cowpea accessions with primer OPA 3

Accession	Maximum number of bands/sample	Number of polymorphic bands
VS 1177	4	2
VS 1179	4	2
VS 1173	3	1
VS 1208	6	4
VS 1034	3	0
VS 1230	3	0
VS 1231	3	0
VS 1201	3	0
VS 1263	8	6
VS 1248	11	9
Total	50	24

Table 12. RAPD profile of cowpea accessions with primer OPA 4

Accession	Maximum number of bands/sample	Number of polymorphic bands
VS 1177	7	2
VS 1179	9	4
VS 1173	6	1
VS 1208	7	2
VS 1034	6	1
VS 1230	8	3
VS 1231	7	2
VS 1201	7	2
VS 1263	8	3
VS 1248	7	2
Total	71	21

Table 13. RAPD profile of cowpea accessions with primer OPA 6

Accession	Maximum number of bands/sample	Number of polymorphic bands
VS 1177	9	2
VS 1179	9	2
VS 1173	9	2
VS 1208	7	0
VS 1034	7	0
VS 1230	8	1
VS 1231	7	0
VS 1201	8	1
VS 1263	8	1
VS 1248	8	1
Total	80	10

Table 14. RAPD profile of cowpea accessions with primer OPA 7

Accession	Maximum number of bands/sample	Number of polymorphic bands
VS 1177	4	2
VS 1179	7	5
VS 1173	4	2
VS 1208	4	2
VS 1034	4	2
VS 1230	6	4
VS 1231	6	4
VS 1201	6	4
VS 1263	6	4
VS 1248	0	0
Total	47	29

Table 15. RAPD profile of cowpea accessions with primer OPA 9

Accession	Maximum number of bands/sample	Number of polymorphic bands
VS 1177	5	4
VS 1179	2	1
VS 1173	4	3
VS 1208	1	0
VS 1034	2	1
VS 1230	3	2
VS 1231	3	2
VS 1201	3	2
VS 1263	2	1
VS 1248	3	2
Total	28	18

Table 16. RAPD profile of cowpea accessions with primer OPA 10

Accession	Maximum number of bands/sample	Number of polymorphic bands
VS 1177	5	4
VS 1179	6	5
VS 1173	5	4
VS 1208	5	3
VS 1034	4	4
VS 1230	5	3
VS 1231	5	4
VS 1201	3	3
VS 1263	3	2
VS 1248	5	4
Total	46	36

Table 17. RAPD profile of cowpea accessions with primer OPS 4

Accession	Maximum number of bands/sample	Number of polymorphic bands
VS 1177	5	4
VS 1179	3	2
VS 1173	5	4
VS 1208	5	4
VS 1034	4	3
VS 1230	3	2
VS 1231	4	3
VS 1201	4	3
VS 1263	1	0
VS 1248	5	4
Total	39	29

Table 18. RAPD profile of cowpea accessions with primer OPS 5

Accession	Maximum number of bands/sample	Number of polymorphic bands
VS 1177	2	1
VS 1179	7	6
VS 1173	4	3
VS 1208	5	4
VS 1034	5	4
VS 1230	5	4
VS 1231	5	4
VS 1201	5	4
VS 1263	6	5
VS 1248	1	0
Total	45	35

Table 19. Percentage polymorphism exhibited by the selected primers

Selected primers	Percentage polymorphism
OPA1	62.5
OPA 2	57.1
OPA 3	81
OPA 4	44.4
OPA 6	22.2
OPA 7	71.4
OPA 9	80
OPA 10	83.3
OPS 4	80
OPS 5	85.7

Table 20. Accession specific bands generated by random primers in cowpea

Sl.No.	Accessions	Primer	Band *size(Kbp)*	Resistance to *aphids*
1	VS 1230	OPA 2	0.44	Resistant
2	VS 1201	OPA 2	0.44	Resistant
3	VS 1263	OPA 3	0.4, 0.2	Resistant
4	VS 1248	OPA 3	0.4,0.2	Resistant
5	VS 1248	OPA 3	5.2, 3.6, 1.9	Resistant
6	VS 1248	OPA 10	0.82	Resistant

Table 21. Resolving power of the selected primers in RAPD

Sl .No	Primer	Resolving power (Rp)
1	OPA 1	12.6
2	OPA 2	9.6
3	OPA 3	11
4	OPA 4	15.4
5	OPA 6	14.2
6	OPA 7	9.2
7	OPA 9	5.6
8	OPA 10	8.4
9	OPS 4	8.8
10	OPS 5	10.4

Table 22. Similarity values based on the RAPD profiling of cowpea accessions

Rows\Cols	VS1177	VS1179	VS1173	VS1208	VS1034	VS1230	VS1231	VS1201	VS1263	VS1248
VS1177	1.0000000									
VS1179	7.0454545	1.0000000								
VS1173	8.1818181	7.0454545	1.0000000							
VS1208	7.3863636	7.1590909	7.6136363	1.0000000						
VS1034	7.2727272	7.0454545	7.5000000	7.8409090	1.0000000					
VS1230	7.7272727	7.2727272	6.8181818	6.9318181	7.7272727	1.0000000				
VS1231	8.0681818	8.0681818	7.6136363	7.7272727	8.2954545	8.9772727	1.0000000			
VS1201	7.8409090	7.3863636	7.1590909	7.0454545	7.6136363	8.9772727	8.6363636	1.0000000		
VS1263	5.9090909	5.9090909	5.4545454	6.2500000	5.6818181	7.0454545	6.4772727	7.1590909	1.0000000	
VS1248	6.7088607	5.3164556	5.9493670	6.3291139	5.4430379	6.2025316	6.3291139	6.8354430	7.3417721	1.0000000

Primer	Marker	Accessions									
	Size (bp)	1	2	3	4	5	6	7	8	9	10
OPA 2	1741										
OPA 2	1232										
OPA 2	1011										
OPA 2	770										
OPA 2	600										
OPA 2	447										
OPA 2	405										
OPA 3	5275.76										
OPA 3	3601										
OPA 3	1983										
OPA 3	1760										
OPA 3	902										
OPA 3	801										
OPA 3	728.35										
OPA 3	534.14										
OPA 3	497										
OPA 3	401.16										
OPA 3	169.96										
OPA 10	2458.4										
OPA 10	1631										
OPA 10	1344.63										
OPA 10	959.08										
OPA 10	820.05										
OPA 10	700.79										
OPA 10	621.12										
OPA 10	524.57										
OPA 10	300										

Fig 1 Diagrammatic representation of RAPD based markers of selected cowpea accessions

Presence of band

Absence of band

Susceptible accessions

1- VS 1177
2- VS 1179
3- VS 1173
4- VS 1208
5- VS 1034

Resistant accessions

1- VS 1230
2- VS 1231
3- VS 1201
4- VS 1263
5- VS 1248

Plate 10. RAPD profile of cowpea accessions with primer OPA 1

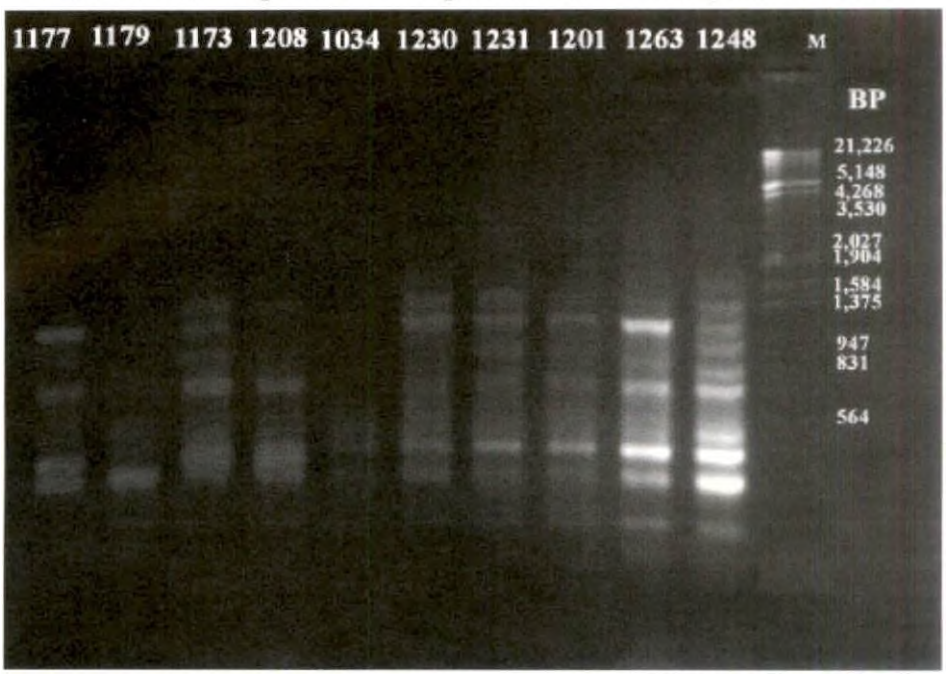


Plate 11. RAPD profile of cowpea accessions with primer OPA 2

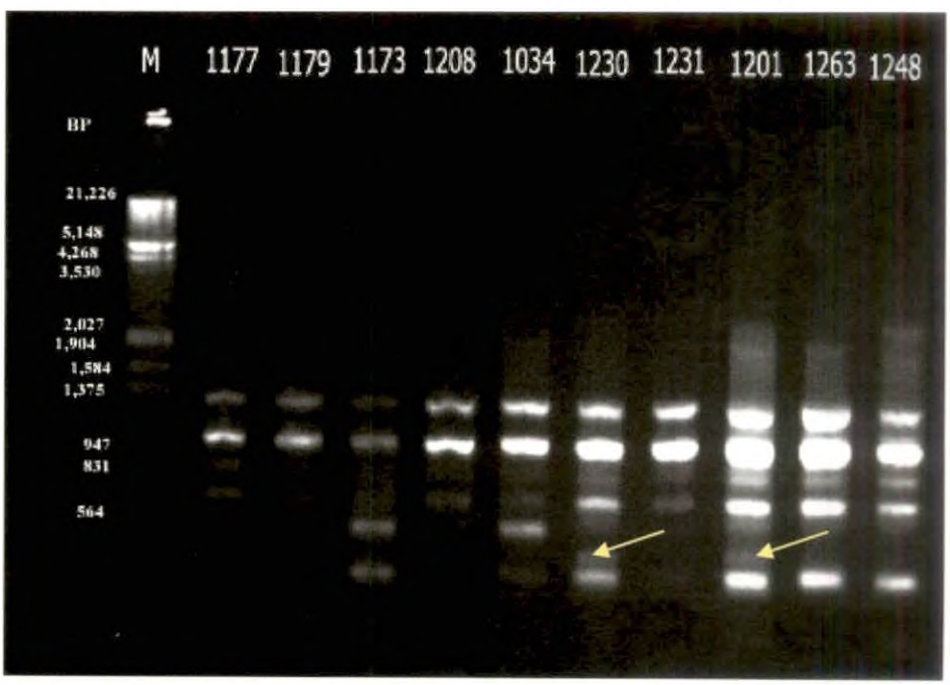


Plate 12. RAPD profile of cowpea accessions with primer OPA 3

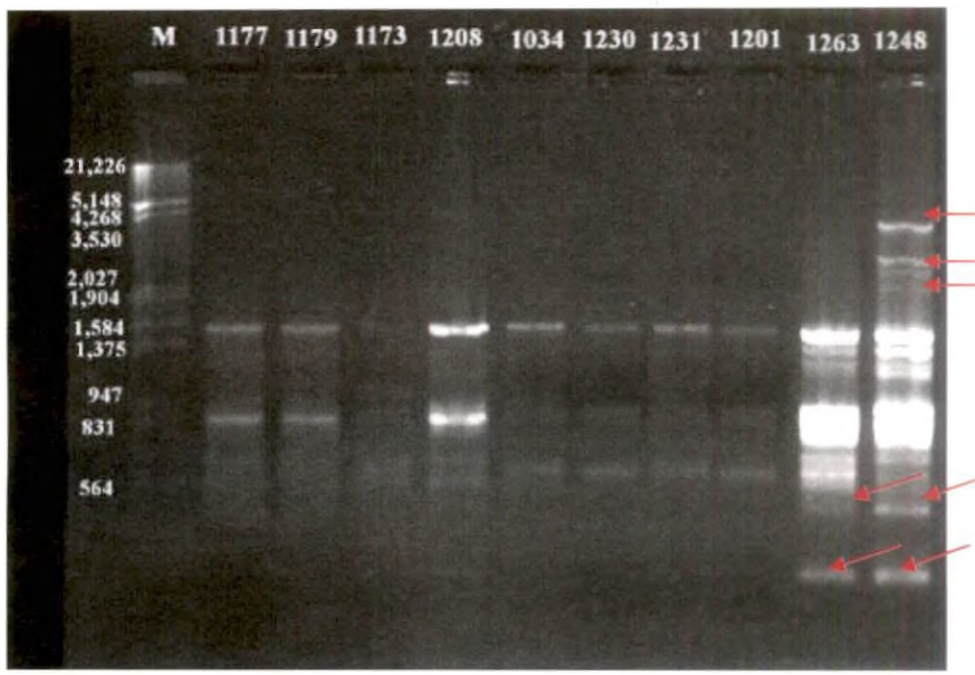


Plate 13. RAPD profile of cowpea accessions with primer OPA 4

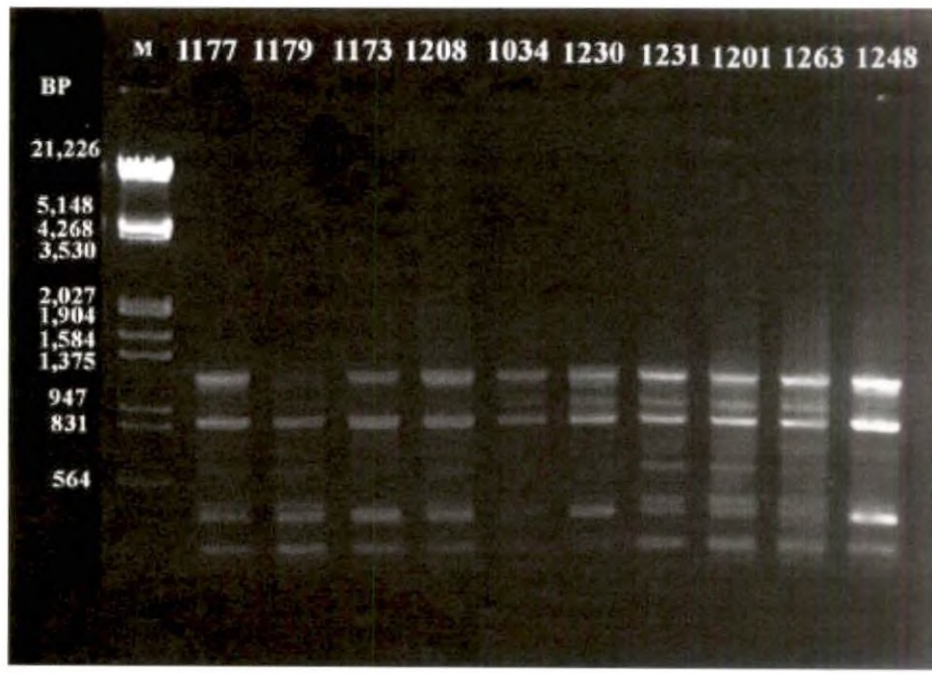


Plate 14. RAPD profile of cowpea accessions with primer OPA 6

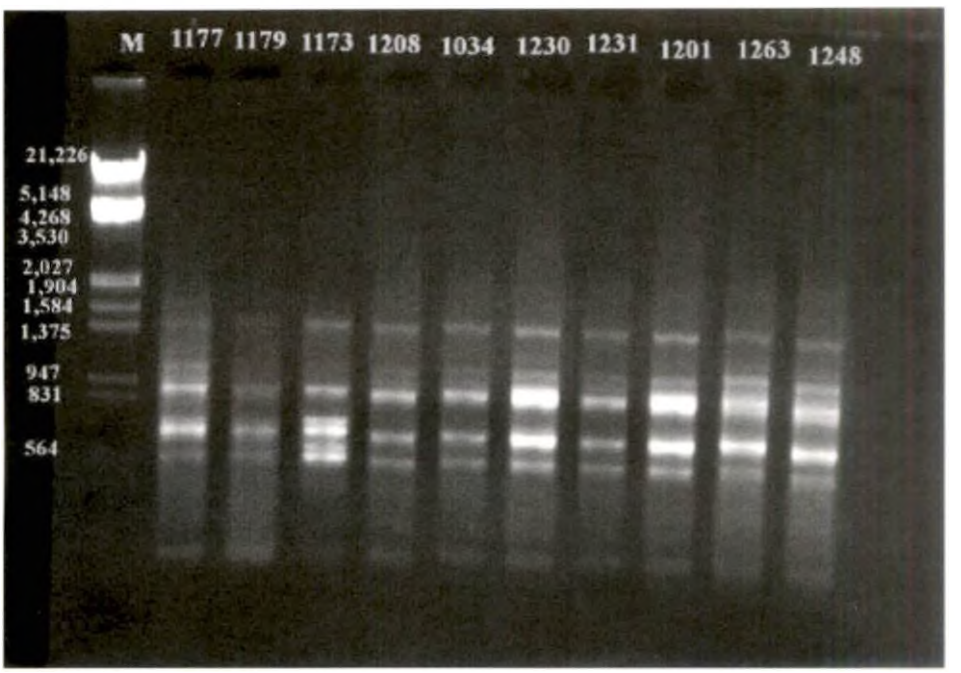


Plate 15. RAPD profile of cowpea accessions with primer OPA 7

Plate 16. RAPD profile of cowpea accessions with primer OPA 9

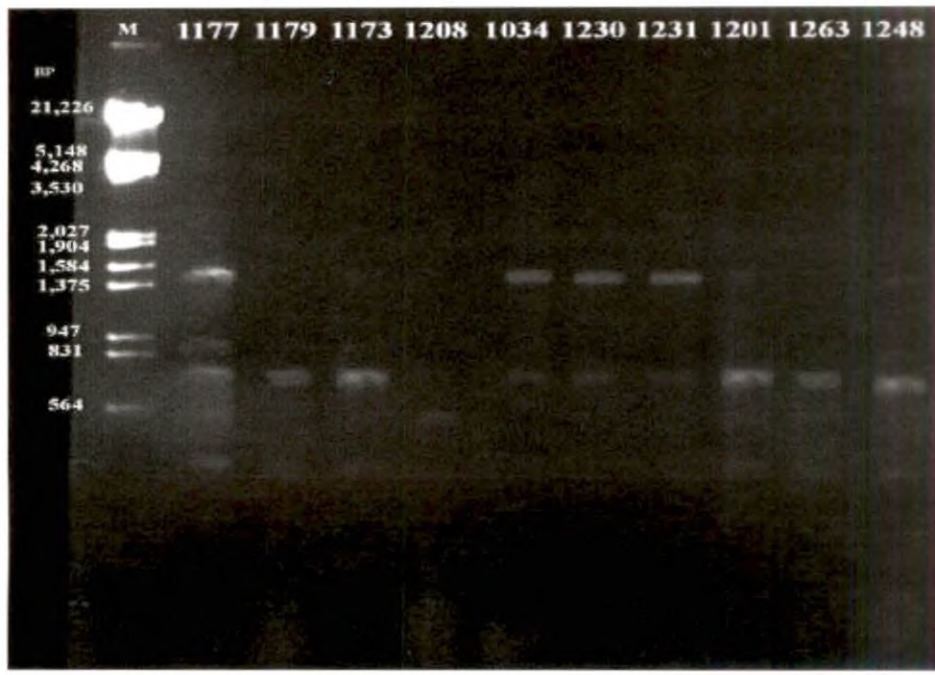


Plate 17. RAPD profile of cowpea accessions with primer OPA 10

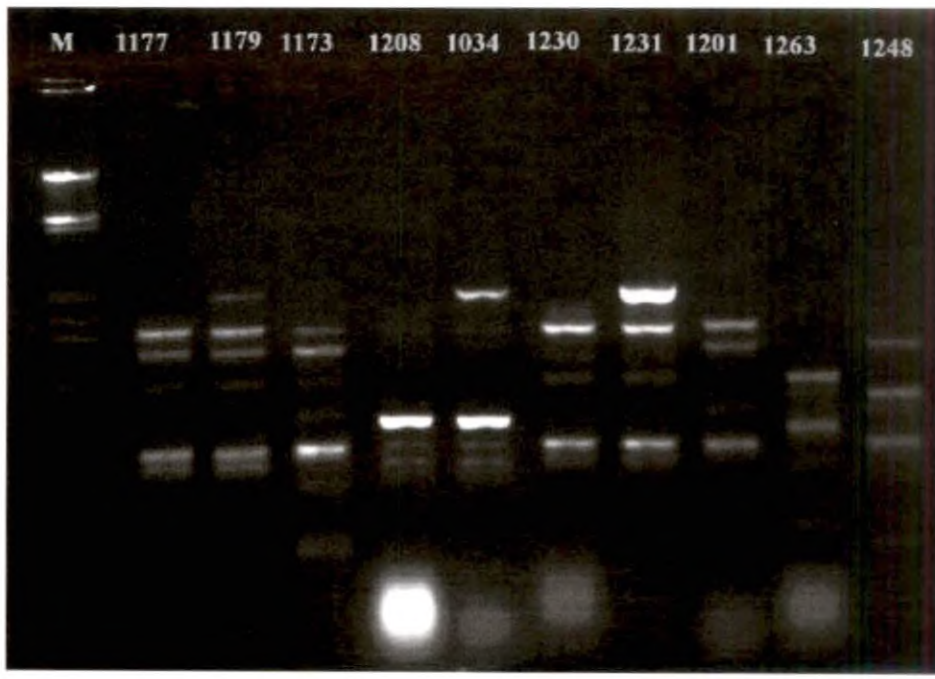


Plate 18. RAPD profile of cowpea accessions with primer OPS 4

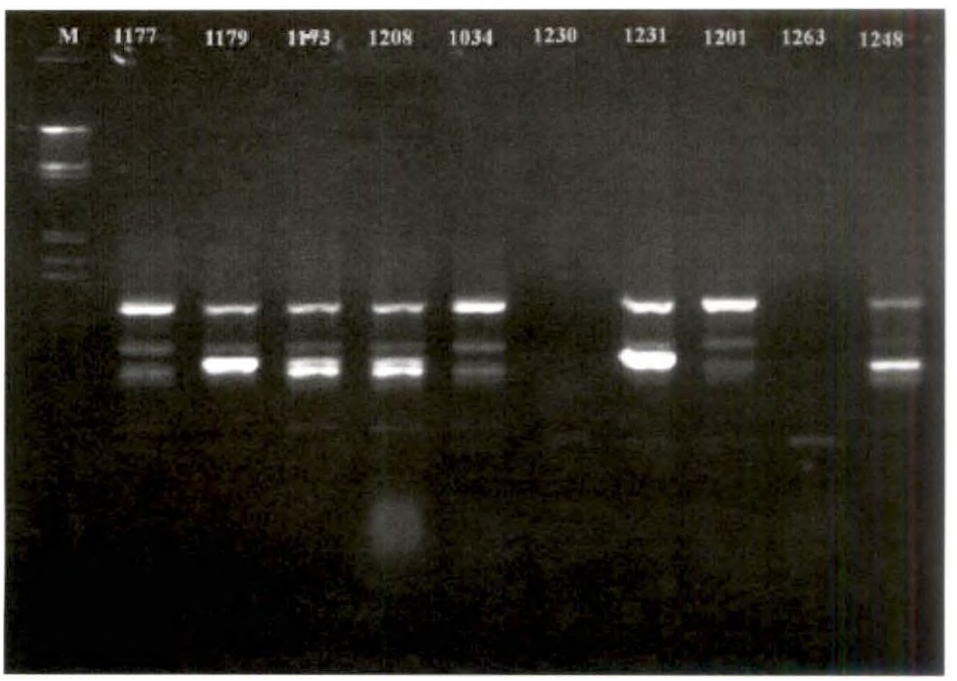
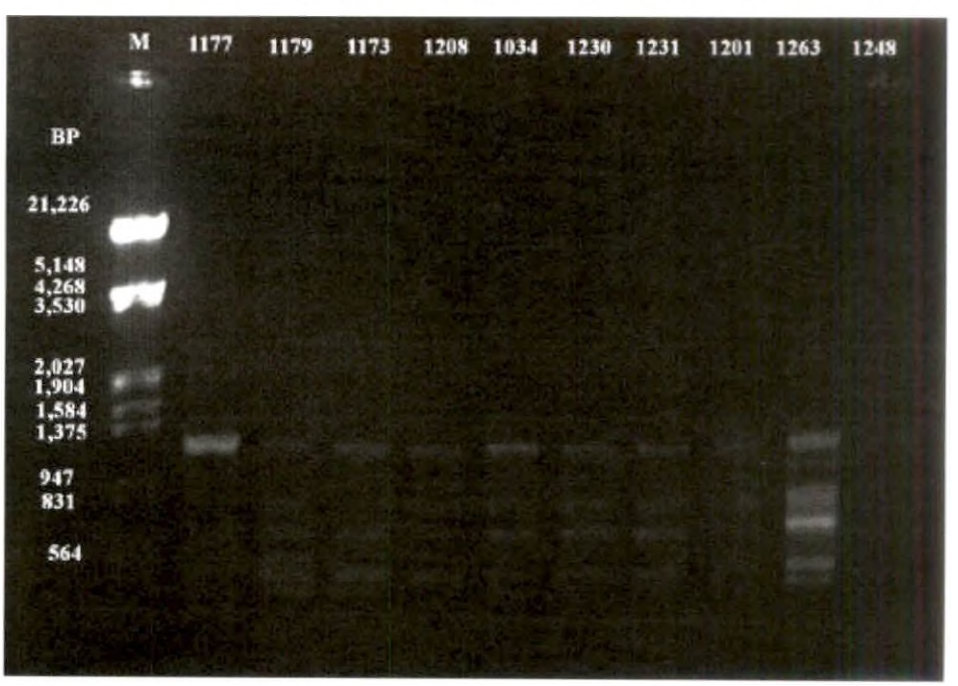


Plate 19. RAPD profile of cowpea accessions with primer OPS 5



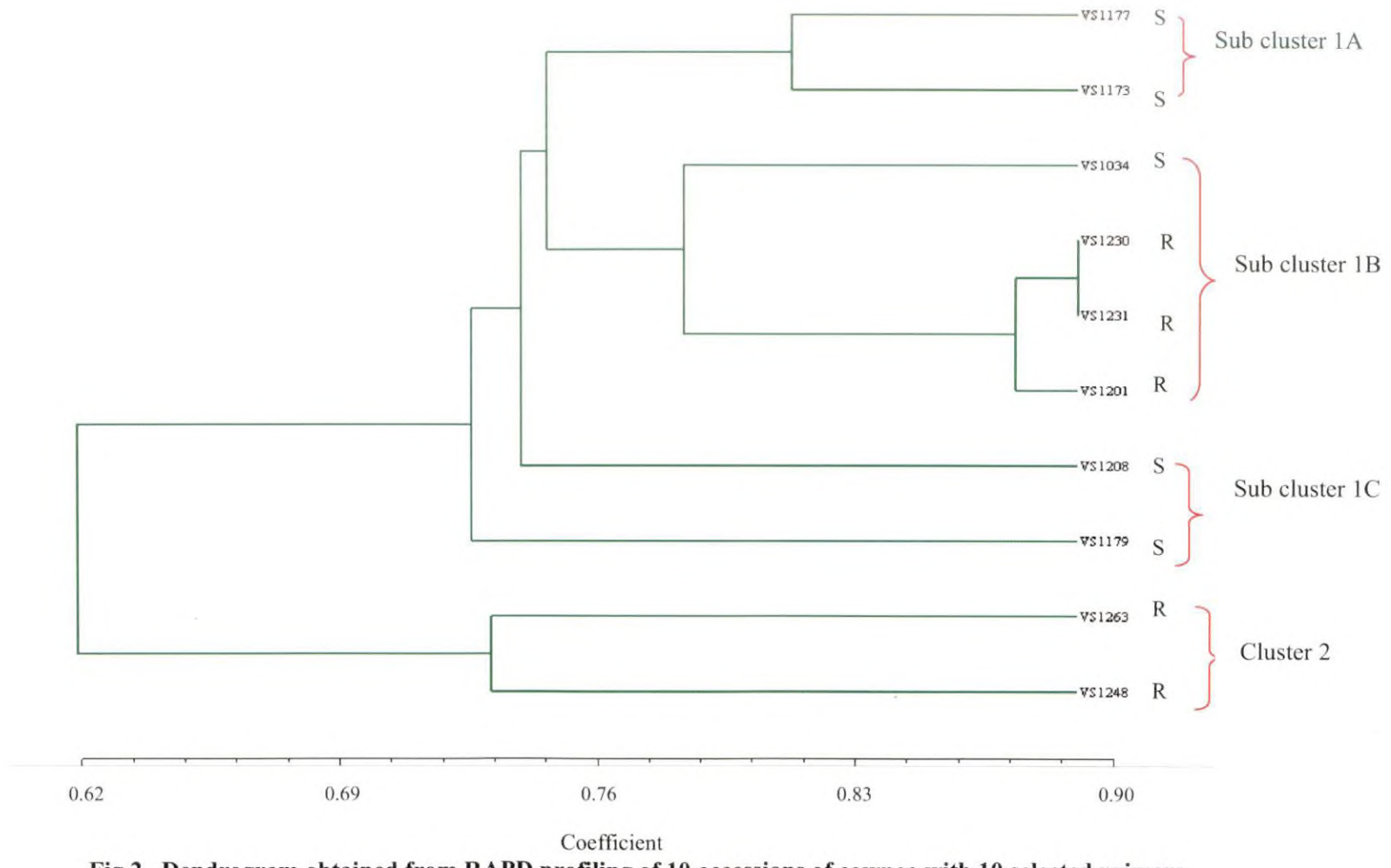


Fig 2 Dendrogram obtained from RAPD profiling of 10 accessions of cowpea with 10 selected primers

S- Susceptible, R-Resistant

were grouped together and showed a close similarity of 90 percent. In the sub cluster 1C, the susceptible accessions VS 1208 and VS 1179 formed different groups. The second cluster had two accessions VS 1263 and VS 1248 and they differed from the first cluster at 73 percent similarity. These accessions were also resistant to the aphids.

4.4 AFLP assay

4.4.1. Screening of cowpea accessions with AFLP primers

Five susceptible and five resistant cowpea accessions were subjected to AFLP assay with different combinations of *Eco*R1 and *Mse*1 primers. The primers used for the present study is given in the Table 23.

The cowpea accessions gave a total of 237 scorable amplification products with the five primer pairs of which 144 (60.7 percent) bands were monomorphic and 93 (39.2 percent) were polymorphic band (Table 24). The amplification pattern obtained by each primer pair is detailed below.

4.4.1.1. EAAG+MCAA

Forty six amplified products were generated by this primer combination. No amplification was obtained for the accession VS 1208. The AFLP profile generated by this combination is given in the Table 25 and Plate 20. Three unique bands of molecular weight approximately between 150 and 100bp were seen for the accessions VS 1230, VS 1263 and VS 1248 which were resistant to aphids. Another two bands of approximately 150bp were seen for the accession VS 1263. The percentage level of polymorphism exhibited by his primer was 54.3 percent. The number of bands produced by this primer combination was 0 and 46. The bands unique to these accessions could be identified as trait related markers for resistance to aphids.

4.4.1.2. EACG+MCAC

The amplification pattern obtained by this primer is given in the Table 26 and Plate 21. A total of 47 scorable amplification products were generated by this primer pair. The highest number of 47 bands was produced by VS 1201 and the percentage polymorphism exhibited by this primer pair was 2 percent.

4.4.1.3. EACG+ MCAA

Ten cowpea accessions gave 45 scorable amplification products. This primer pair yielded 28 polymorphic bands. The maximum number of bands ranged between 22 and 45. The polymorphism obtained by this primer pair was 62.2 percent. The amplification pattern produced by this primer pair is given in the Table 27.

4.4.1.4. EACT+MCAA

The primer combination generated a total of 49 amplification products of which 17 bands were polymorphic. The AFLP pattern of this primer pair is given in the Table 28 and Plate 22. The maximum number of bands were produced by he accession VS 1231 followed by VS 1248. The percentage polymorphism exhibited by this primer pair was 34.6 percent.

4.4.1.5. EAGG+MCAA

The AFLP profile generated by this primer pair generated a total of 50 amplification products The percentage polymorphism obtained by this primer pair was 44. The maximum number of bands was observed with the accession VS 1231 and the number of bands ranged between 39 and 50 (Table 29 and Plate 23). The percentage polymorphism exhibited by different primer combination was given in the Table 31.

The resolving power of the primer combinations used in AFLP are presented in the Table 30. The results show that EACT+MCAA exhibits

Table 23. List of primer pairs used for AFLP

Sl no:	Primer combination (EcoR1/Mse1)
1	ACG+CAA
2	AGG+CAA
3	ACG+CAC
4	AAG+CAA
5	ACT+CAA

Table 24. Total number of amplicons produced by the primer combinations in AFLP

Primer combination(EcoR1/Mse1)	Total number of amplicons	Number of polymorphic bands
EAAG+MCAA	46	25
EAGG+MCAA	50	22
EACT+MCAA	49	17
EACG+MCAA	45	28
EACG+MCAC	47	1
Total	237	93

Table 25. AFLP profile of cowpea accessions with EAAG+MCAA

Accession	Maximum number of bands/primer	Number of polymorphic bands
VS 1177	46	25
VS 1179	46	25
VS1173	45	24
VS 1208	0	0
VS 1034	34	13
VS 1230	32	11
VS 1231	39	18
VS 1201	30	19
VS 1263	31	10
VS 1248	38	17
Total	341	162

Table 26. AFLP profile of cowpea accessions with EACG+MCAC

Accession	Maximum number of bands/primer	Number of polymorphic bands
VS 1177	46	17
VS 1179	45	16
VS1173	31	2
VS 1208	44	15
VS 1034	35	6
VS 1230	44	15
VS 1231	35	6
VS 1201	47	1
VS 1263	35	6
VS 1248	42	13
Total	404	114

Table 27. AFLP profile of cowpea accessions with EACG+MCAA

Accession	Maximum number of bands/primer	Number of polymorphic bands
VS 1177	41	24
VS 1179	27	10
VS1173	38	21
VS 1208	22	5
VS 1034	44	27
VS 1230	26	9
VS 1231	45	28
VS 1201	28	11
VS 1263	43	26
VS 1248	42	25
Total	356	186

Table 28. AFLP profile of cowpea accessions with EACT+MCAA

Accession	Maximum number of bands/primer	Number of polymorphic bands
VS 1177	42	10
VS 1179	40	8
VS1173	40	8
VS 1208	37	5
VS 1034	44	12
VS 1230	38	6
VS 1231	52	20
VS 1201	44	12
VS 1263	47	15
VS 1248	49	17
Total	433	113

Table 29. AFLP profile of cowpea accessions with EAGG+MCAA

Accession	Maximum number of bands/primer	Number of polymorphic bands
VS 1177	45	17
VS 1179	44	16
VS1173	43	15
VS 1208	33	5
VS 1034	40	12
VS 1230	39	11
VS 1231	50	22
VS 1201	39	11
VS 1263	47	19
VS 1248	48	20
Total	428	148

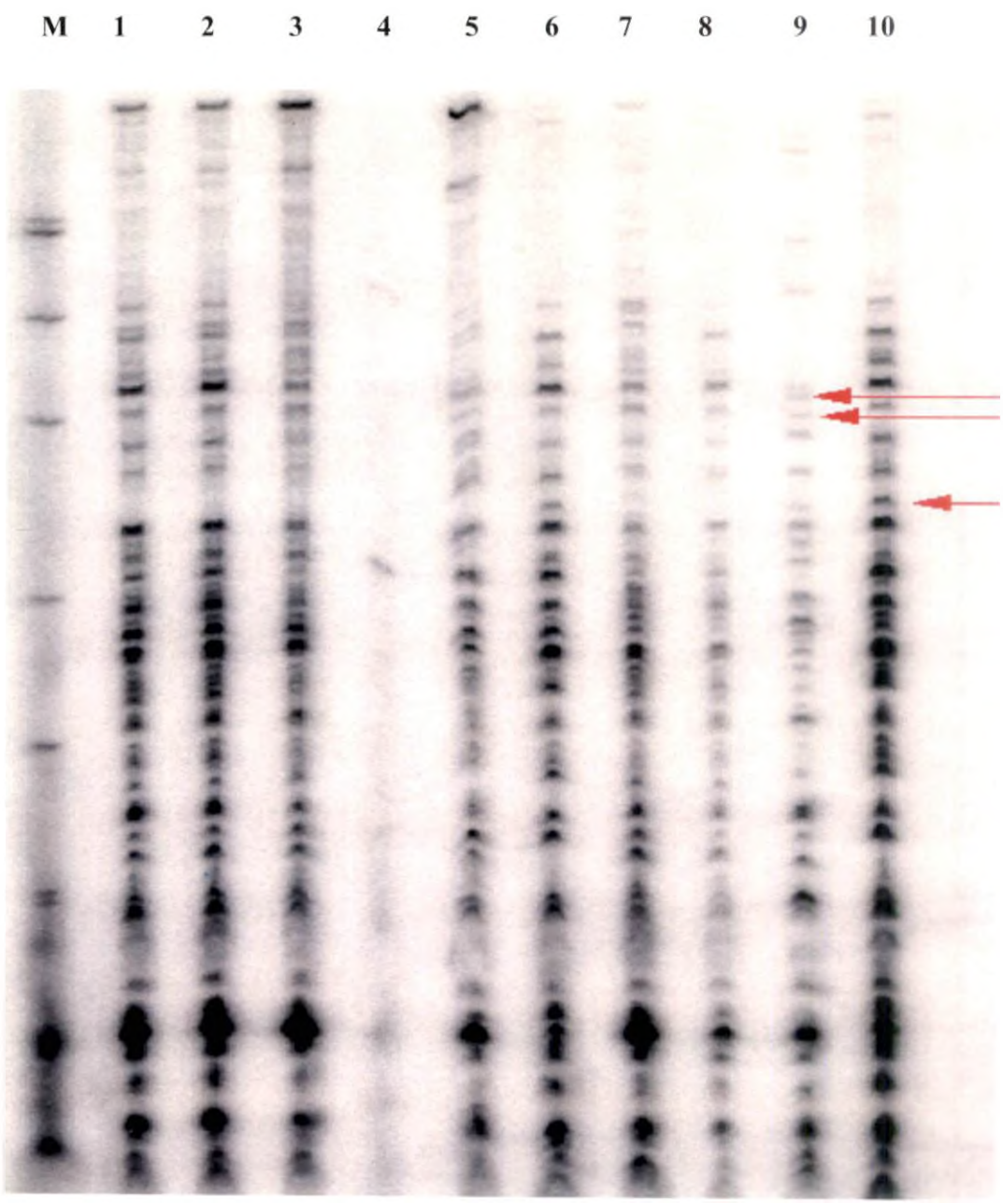
Table 30. Resolving power of the primer combination in AFLP

Sl.No.	Primer combination (EcoR1/Mse1)	Resolving power (Rp)
1	EACG+MCAC	77.4
2	EAAG+MCAA	63.6
3	EACG+MCAA	78.2
4	EACT+MCAA	86
5	EAGG+MCAA	84.2

Table 31. Percentage polymorphism exhibited by different primer combinations

Primer combination	Percentage polymorphism
EAAG+MCAA	54.3
EAGG+MCAA	44
EACT+MCAA	34.6
EACG+MCAA	62.2
EACG+MCAC	2

Plate 20. AFLP profile of cowpea accessions with EAAG+MCAA



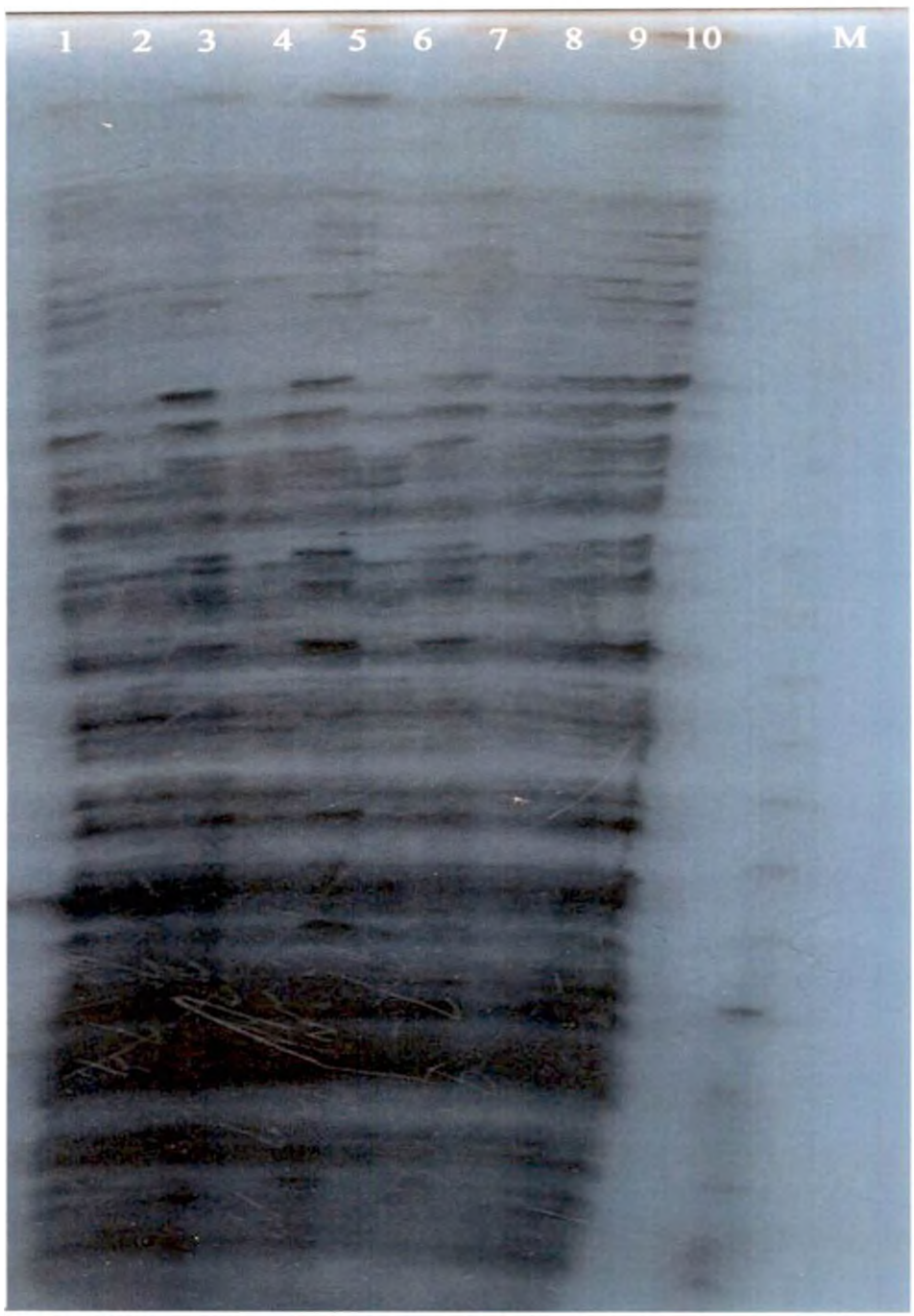
Susceptible accessions

1-VS 1177
2-VS 1179
3-VS 1173
4-VS 1208
5-VS 1034

Resistant accessions

6-VS 1230
7-VS 1231
8-VS 1201
9-VS 1263
10-VS 1248

Plate 21. AFLP profile of cowpea accessions with EACG+MCAC



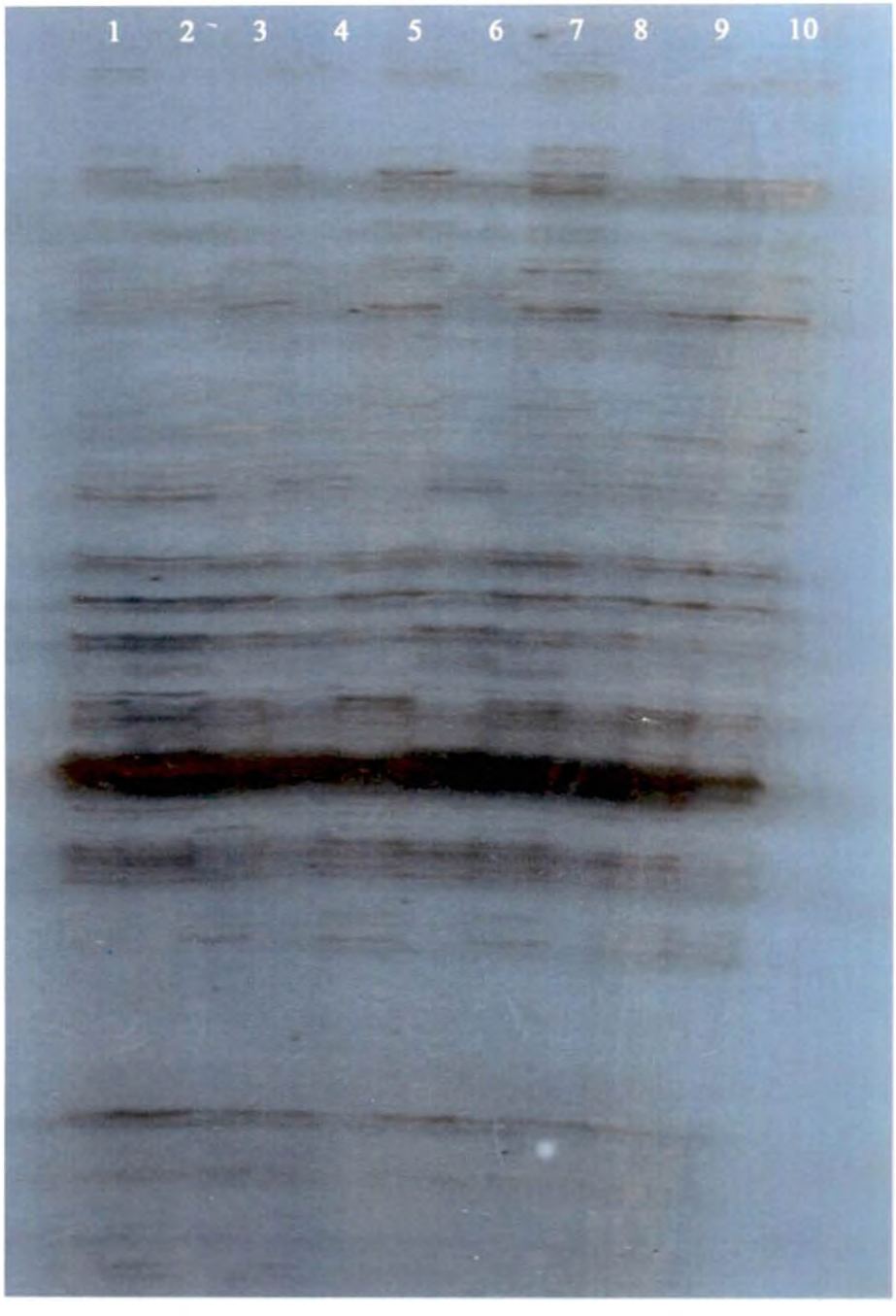
Susceptible accessions

1- VS 1177
2- VS 1179
3- VS 1173
4- VS 1208
5- VS 1034

Resistant accessions

6- VS 1230
7- VS 1231
8- VS 1201
9- VS 1263
10- VS 1248

Plate 22. AFLP profile of cowpea accessions with EACT+MCAA



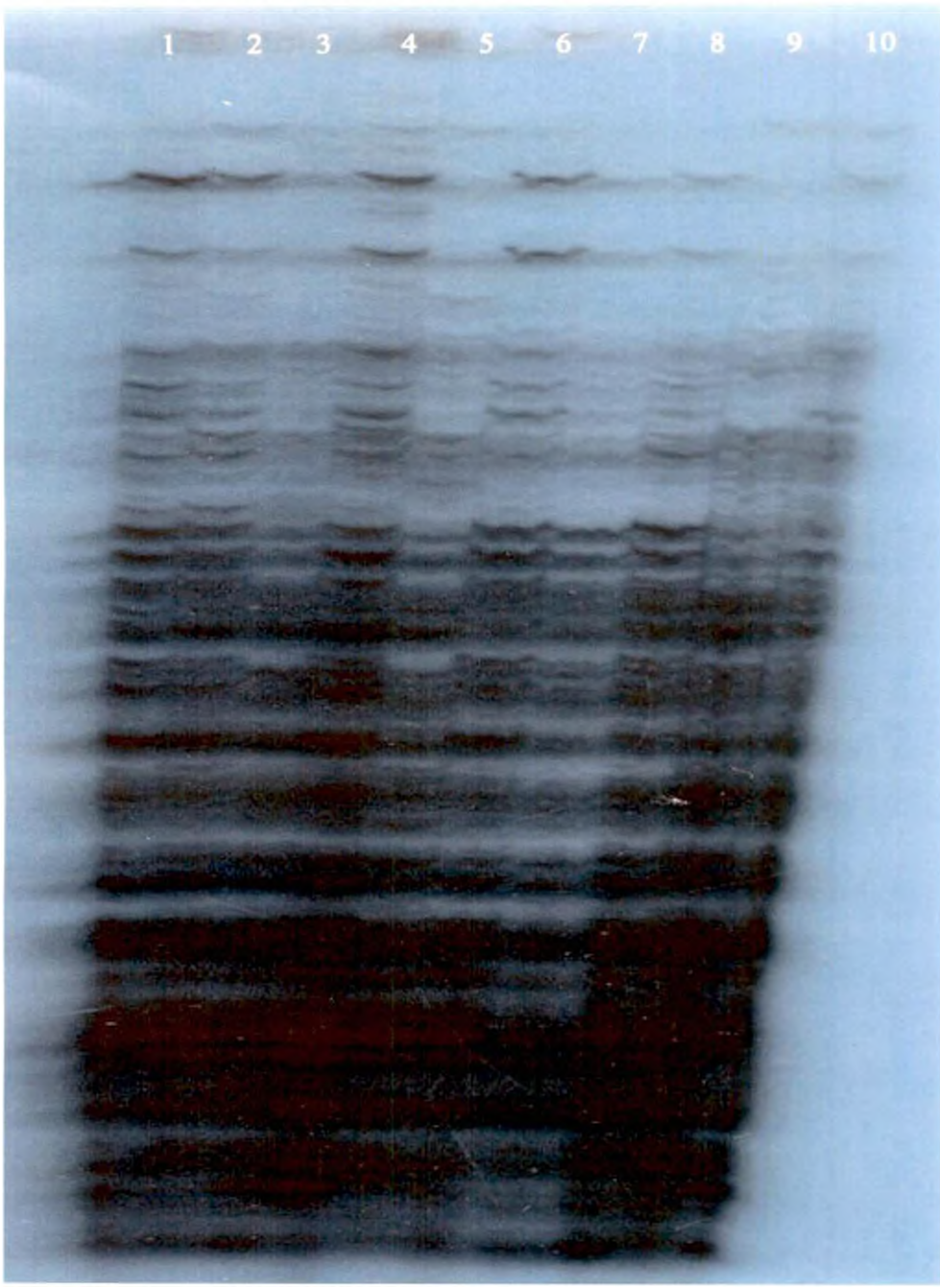
Susceptible accessions

1-VS 1177
2-VS 1179
3-VS 1173
4-VS 1208
5-VS 1034

Resistant accessions

6-VS 1230
7-VS 1231
8-VS 1201
9-VS 1263
10-VS 1248

Plate 23. AFLP profile of cowpea accessions with EAGG+MCAA



Susceptible accessions

1-VS 1177
2-VS 1179
3-VS 1173
4-VS 1208
5-VS 1034

Resistant accessions

6-VS 1230
7-VS 1231
8-VS 1201
9-VS 1263
10-VS 1248

maximum resolving power. This was followed by EAGG+MCAA, EACG+MCAA, EACG+MCAC, EAAG+MCAA.

4.4.2 Analysis of AFLP dendrogram

AFLP data was used to generate a phenetic representation of similarity coefficients among 10 accessions of cowpea as shown in the Figure 3. In the dendrogram, there were two clusters 1 and 2. The cluster 1 was again divided in to several sub clusters with different groups. The cluster 2 had only one accession and it differed from the cluster 1 at 62 percent similarity. In sub cluster A, there were three accessions VS 1177, VS 1179 and VS 1173, which were susceptible to the aphid infestation. The accessions VS 1177 and VS 1179 showed 91 percent similarity. The sub cluster B had four accessions out of which the resistant accessions were grouped together at 82 percent similarity. The sub cluster C comprised of two resistant accessions VS 1230 and VS 1201 and they differed from sub clusters A and B at 77 percent similarity.

Based on the genetic similarity matrix table (Table 32) the highest similarity value was obtained for the accessions VS 1177 and VS 1179 (9.10). The accessions VS 1231 and VS 1208 showed the lowest similarity value (5.44).

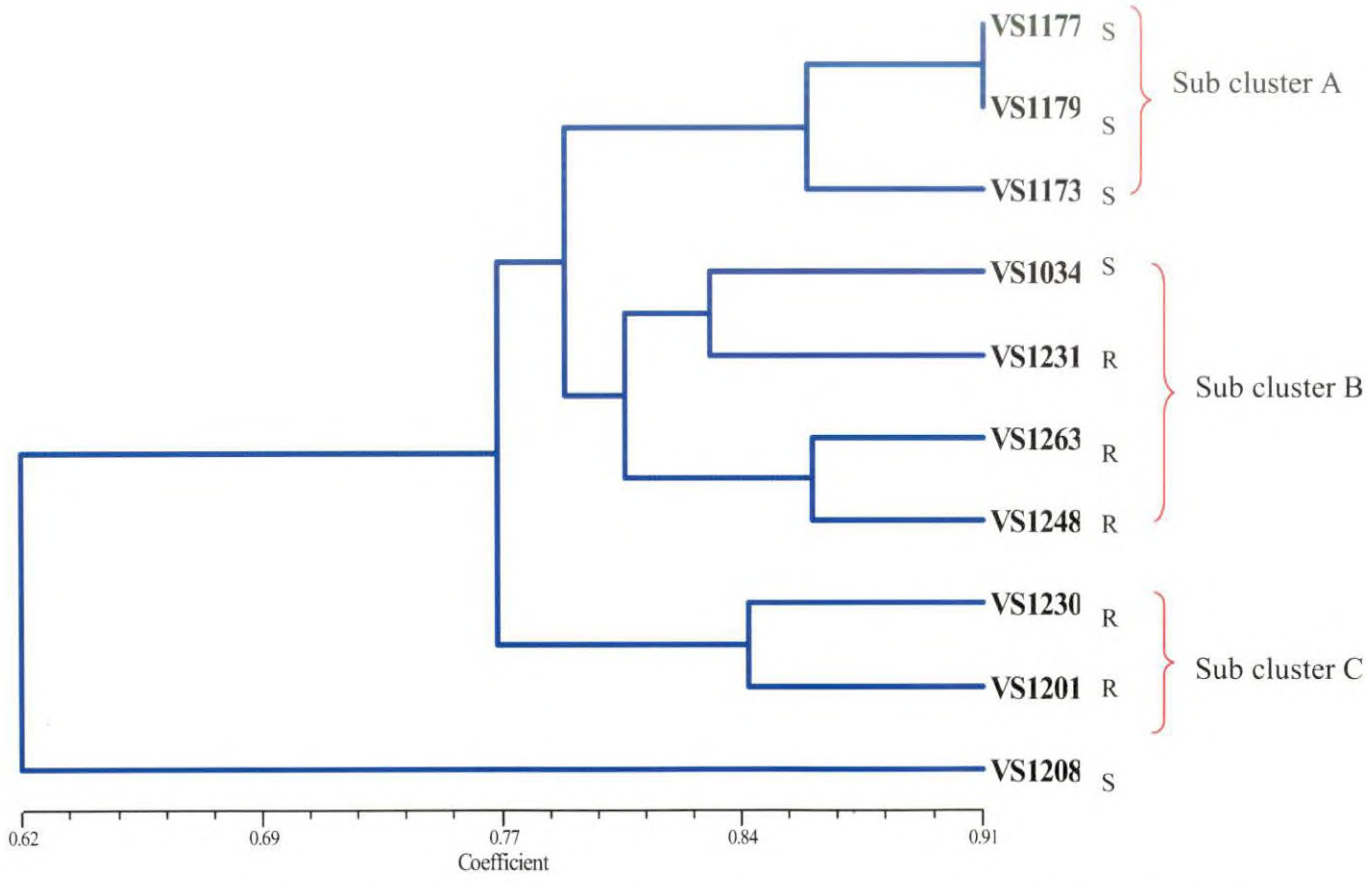
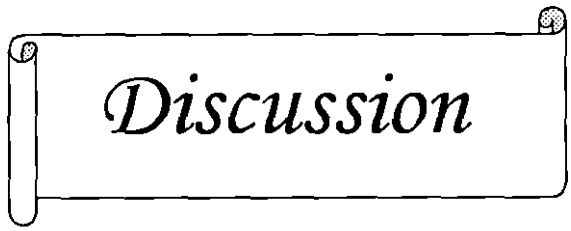


Fig 3 Dendrogram obtained from the AFLP profiling of 10 cowpea accessions with 5 primer pairs

S- Susceptible
R- Resistant

Table 32. Similarity values based on the AFLP profiling of cowpea accessions

Rows\Cols	VS1177	VS1179	VS1173	VS1208	VS1034	VS1230	VS1231	VS1201	VS1263	VS1248
VS1177	1.0000000									
VS1179	9.1050583	1.0000000								
VS1173	8.9105058	8.2490272	1.0000000							
VS1208	6.3424124	6.6147859	6.2256809	1.0000000						
VS1034	7.9377431	7.2762645	8.3268482	5.8754863	1.0000000					
VS1230	7.9766536	8.4046692	7.5097276	7.0038910	7.3151750	1.0000000				
VS1231	8.0155642	7.5097276	7.9377431	5.4474708	8.2879377	7.2373540	1.0000000			
VS1201	7.8599221	8.0544747	7.0817120	6.6536964	7.3540856	8.4046692	7.5875486	1.0000000		
VS1263	7.9377431	7.3540856	7.9377431	5.9143968	7.8988326	7.3929961	7.9766536	7.5097276	1.0000000	
VS1248	8.3268482	7.7431906	7.9377431	5.9533073	7.8210116	7.7042801	8.4435797	8.1322957	8.5992217	1.0000000


Discussion

5. DISCUSSION

Grain legumes form an important component in the tropical cropping system. Several species are cultivated either as monocrops or in mixed cropping with other crops, particularly cereals. Several legumes are also grown as backyard crops near small farm houses. In general, grain legumes are grown in a wide range of environment often on poor soils with marginal moisture and with no fertilization. In subsistence agriculture, on small farms the nitrogen fixing ability of grain legumes is of special advantage. Grain legumes are also important sources of proteins, energy, minerals, vitamins and roughage, in addition to their miscellaneous uses in animal feed. (VanEmden and Singh, 1979).

Cowpea, also known as the black eyed bean is grown throughout the land of tropics in Africa. The main area of production is in West Africa particularly Nigeria, Niger, Sierra Leone and Senegal. In West Africa, the dry grains are eaten, but both the green leaves and dry grains are eaten in East Africa. Cowpea is also extensively cultivated in India and South East Asia and is harvested as dry grains in dry areas and as green pods in more humid condition.

Insect pests are probably the main factor limiting grain legume yields in the tropics. Cowpea is also affected by a wide range of insects and aphids are one among them. Small populations have an insignificant effect on the plant, but large numbers can cause damage, distortion of leaves and stunted plant growth with small, poorly nodulated root system as well. Yield is reduced and in extreme cases the plant is killed. An indirect and generally more harmful effect even of small populations is the transmission and spread of legume viruses which severely reduced the yield. Advances in DNA sequencing, data analysis and PCR have resulted in powerful techniques, which can be used for the characterization and evaluation of germplasm and genetic resources and for the identification of markers for use in the breeding programmes.

In this context, it was considered of interest to study on the characterization of cowpea using molecular markers such as RAPD and AFLP with special reference to aphids. Sixty accessions of cowpea which were collected from different parts of Kerala and maintained in the Department of Olericulture, College of Horticulture were used for the present study. The main objective of the study was to identify the sources of resistance and to develop trait related markers for resistance to aphids in cowpea.

5.1. Screening of cowpea accessions for resistance to aphids

Attempts on exploration of sources of resistance to aphids in cowpea germplasm are very much limited in India. So there is a necessity to strengthen the line of research on this aspect. An extensive screening of germplasm is very essential criterion for any breeding programme which involves host resistance to the pathogens, pests or vectors. Success in identifying resistant source is directly related to the diversity of germplasm available and probability of resistance occurring in the host population.

Sixty accessions of cowpea were evaluated for resistance to aphids under field conditions. The population build up of aphids in different cowpea accessions was considered as the criterion to assess the resistance. Infestation of aphids were recorded in all the accessions and the average count of aphids were made and rated on a 0-2 scale. (0-resistant, 1-moderately resistant and 2-susceptible). The count was taken from the terminal shoots, leaves and pods. The aphid population was comparatively less in leaves and they were mainly concentrated on the terminal shoots. The average aphid count for three plants from each accession ranged between 0 to 494.67 (VS 1034). The count was above 200 for all the susceptible accessions.

There were no aphids on six accessions VS 1230, VS 1231, VS 1201, VS 1248, VS 1263, and VS 1213 which were rated as resistant. This was in line with the report of Ampily (2005) that there was no aphid incidence for the accessions

VS 1230, VS 1231 and VS1263. These accessions were free from the aphids during the entire crop season. Similar studies were conducted by Joseph (1990) in which 204 lines of cowpea were screened and they were rated according to this scale.

Haneefa *et al.* (1973) considered field bean cultivars as resistant if they had less than 104 aphids per 2.5 cm length of terminal shoots, while other cultivars were taken as susceptible. Counts of aphid population build up on terminal shoots, terminal leaves, flowers and pods were taken. Since the aphids mostly colonize on the terminal shoots, the population was recorded from the 2.5 cm length of the terminal shoots.

Similarly Singh (1977) found TVU-408P2, TVU-416, TVU-240, TVU-3417 and TVU- 3509 to be resistant on the basis of the average aphid population per 2.5 cm length of terminal shoots. Bell (1980) rated P-1476, EC-4276, T-4222 as being resistant among 259 cowpea germplasm screened. Dhanorkar and Daware (1980) screened 14 cultivars of cowpea by counting the number of aphids per leaf and per 2.5cm length of stem and pods. Karel and Malinga (1980) evaluated 11 cowpea cultivars for resistance to *Acrythosiphon gossypii* based on the foliar damage scale of 0-5.

Jayappa and Lingappa (1988) screened 408 accessions of cowpea for aphid resistance in field under natural infestation. The intensity of infestation on five plants per cultivar were placed on a 0-4 scale (0- free from aphids, 1- less than 26 percent, 2- between 26 and 50 percent, 3- between 51 and 75 percent and 4- between 76 and 100 percent of the plant area covered by the aphids). According to their findings, the moderately resistant entries are able to withstand the damage by a higher aphid population than resistant ones. Difference in the performance of specific entries could be due to the variation in the species involved, the evaluation standards or the population level in the field.

Another study on the screening for resistance to the aphids was done by Sulochana (1984). Based on the count of aphids in the leaves, internodes and pods, the lines were classified as immune (0), resistant (less than 100), moderately susceptible (above 100 and less than 250), susceptible (above 250 and less than 1000), and highly susceptibe (above 1000). Out of 83 lines tested, nearly all the 70 of them were highly susceptible.

In the present study, aphids were not found on the accessions sown during April-May. But they were heavily infested during September. Sulochana (1984) observed that the aphid population showed a diminishing trend during the rainy days. Aphid population as influenced by the changes in the meteorological parameters is an important information in the evaluation of cowpea lines for resistance to the insects.

Due to variation in the infestation level of the pest, field screening is often inconsistent. So repeated trials are necessary before making conclusions about the performance of the variety and a common evaluation standard should be formulated.

5.2. Isolation of genomic DNA from cowpea

Two protocols were tried for the isolation of genomic DNA from cowpea Doyle and Doyle (1987) and Rogers and Bendich (1994). The method suggested by Doyle and Doyle (1987) was the best for the isolation of the genomic DNA from cowpea since distinct bands were observed.

Good quality DNA was obtained when the tissues were frozen with liquid nitrogen and using beta mercaptoethanol while grinding. Liquid nitrogen was used to powder the leaves so that the entire tissues got disrupted. Low temperature provided by liquid nitrogen could reduce the DNase activity (He *et al.*, 1992). When the leaf tissues are frozen with liquid nitrogen, the chances of degradation of nucleic acids is very low.

Tender leaves were used for the DNA extraction because of their better performance in yielding DNA with good quantity and quality. Babu (2000) reported that the quality and quantity of DNA was the best with tender leaves as compared to mature and half mature leaf samples. Tender leaves contain actively dividing cells with lesser concentration of extra nuclear materials like protein, oil, carbohydrates, fats, and other metabolites that interfere with the isolation of nucleic acids. Another finding by Babu (2000) is that the time between the thawing of the frozen pulverized tissues and its exposure to the extraction buffer should be minimized to avoid the nucleolytic degradation of DNA.

Beta mercaptoethanol used during the grinding of leaves disrupts the protein disulfide bonds and was thus capable of initiating protein degradation. The chemical components in the extraction mixture could have contributed greatly to the isolation of nucleic acids. The cell membrane must be disrupted to release the nucleic acids into the buffer. This is achieved by the detergents like sarcosin and CTAB. The extraction buffer contained EDTA which could effectively chelate Mg^{2+} ions and mediate the aggregation of nucleic acids.

Rogers and Bendich (1994) observed that there is a shearing of the DNA molecules during the chloroform: isoamylalcohol extraction done twice, as vigorous mixing is necessary for the effective removal of proteins. This might have caused the shearing of DNA in this sample.

Proteins were removed by chloroform:isoamylalcohol treatment. Chloroform will remove the pigments, denature and separate the proteins from DNA. In addition to denaturing proteins, chloroform will also remove lipids.

Isopropanol (0.6 volume) was used for the initial precipitation of DNA at low temperature (-20^0 C). Washing the pellets in 70 percent ethanol were also used for the final precipitation. Finally the pellet was dissolved in TE buffer for long term storage. EDTA present in TE buffer could chelate and remove Mg^{2+}

ions, which were required for the nuclease activity. Tris HCl present in TE buffer and sorbitol present in extraction buffer maintains the osmotic pressure.

The DNA isolated using the standardized protocol was found to be contaminated with RNA. Since RNA often influences the reproducibility of the RAPD and amplification pattern of AFLP, an attempt was made to remove the contaminants by treatment with ribonuclease A. Intact DNA bands were obtained after the treatment.

The quality of DNA was based on the electrophoretic pattern of DNA bands on agarose gel after ethidium bromide staining and based on the absorbance at 260 and 280nm. The absorbance ratio ranged between 1.78 and 1.88 which indicated the good quality of DNA.

5.3. RAPD assay

RAPD analysis is a PCR based molecular marker technique in which single short oligonucleotide primers are arbitrarily selected to amplify a set of DNA segments distributed randomly throughout the genome. Welsh and McClelland (1990) showed hat the pattern of amplified bands so obtained could be used for genomic fingerprinting. RAPD is a simple technique since there is no need of any sequence information or developing probes which is a costly and time consuming process. This marker system has been used in many different applications involving the detection of DNA, sequence polymorphism, isolation of markers linked to various traits, varietal identification and parentage analysis.

5.3.1. Primer screening

Random decamer primer kits obtained from Operon Technologies USA were used for the present study. Operon primers were popular among the researchers mainly because of the ease of availability and better results. Tosti and Negri (2002, Ba *et al.* (2004), Archak *et al.* (2002), Belaj *et al.* (2002) and Neeraja *et al.* (2002) have used the random primers from different Operon series for RAPD studies in different crops.

Thirty random primers from different Operon primer kits were used for the present study. The number of amplification products ranged from zero and fifteen. The primers for further analysis were selected based on the number of bands, quality of amplification and stability of expression. Twelve primers showed no amplification while 18 gave good amplification. Those primers which gave good and reproducible amplification pattern were selected for the RAPD assay. Difference in the intensity of RAPD products is due to the primers annealing to genomic loci which are mismatched resulting in reduced priming and extension efficiencies.

According to Williams *et al.* (1990), a single base change in the primer sequence could cause a complete change in the set of amplified DNA segments. They also found that GC content in the 10 mer primer influenced the amplification and a GC content of 40 percent or more in the primer sequence was needed to generate detectable levels of amplified products.

In the present study, 10 primers that yielded consistent and clear banding pattern were selected for the final analysis of 10 accessions of cowpea. Tosti and Negri (2002) used 54 primers that showed clear reproducible banding pattern for the characterization of cowpea land races. Ba *et al.* (2004) used 28 random primers to reveal the genetic diversity in cowpea by RAPD markers. Twenty random primers were used by Neereja *et al.* (2002) to identify the genetic diversity in Indian land races of rice.

5.3.2. RAPD analysis of cowpea accessions

The arbitrary primers which gave good reproducible bands were selected for amplifying the genomic DNA isolated from 10 accessions of cowpea. A control reaction was also included along with each set of PCR reactions. Genomic DNA was omitted from the control so as to confirm that the observed bands were amplified DNA and not the primer artifacts. The selected primers used for the amplification gave 75 scorable amplification products of which 27 bands (36percent) were monomorphic and 48 bands (64 percent) were polymorphic. The total number of amplicons ranged from 5 (OPA 9 and OPS 4) to 12 (OPA 3).

The range of polymorphic markers per primer were 2 (OPA 6), 4 (OPA 2, OPA 4, OPA 9 and OPS 4), 5 (OPA 1, OPA 7 and OPA 10) 6 (OPS 5) and 9 (OPA 3). The total frequency of polymorphic markers obtained was 77.8 percent. Two bands of molecular weight 0.44kb were shown by two aphid resistant accessions VS 1230 and VS 1201 with primer OPA 2. The accessions VS 1263 and VS 1248 showed conspicuous bands of molecular weight 0.2 kb with OPA 3. Another two distinct bands of 0.4kb size were also observed for these accessions. Three bands of size 5.2kb, 3.6 kb and 1.9kb were unique to the accession VS 1248. The primer OPA 10 produced a single specific band (0.8 kb) for the accession VS 1248. The accessions VS 1248 and VS 1263 were also resistant to aphids. These accessions could have shared some common genes which contribute resistance to the aphids. So these bands obtained could be identified as trait related markers contributing to the aphid resistance.

RAPD markers have been developed for rosy leaf curling aphid resistance in apple by Roche *et al.* (1997) and aphid resistance in wheat by Myburg *et al.* (1998).

RAPD has been used in the genetic diversity studies of cowpea. Tosti and Negri (2002) analysed 13 cowpea accessions to assess the genetic variation using 54 selected primers and 36 primers generated polymorphism. This could clearly

discriminate the commercial varieties from other land races. Ba *et al.* (2004) observed a good polymorphism in RAPD banding pattern of 56 accessions of cowpea which differentiated wild and domesticated types.

5.3.3. Genetic analysis

The scored data of all 10 primers was used for the similarity based analysis using NTSYS pc (ver.2.1). The SIMQUAL programme was used to calculate the Jaccard's coefficient, a common estimator of genetic diversity. Based on the estimated genetic similarity, the highest genetic similarity was noticed between the accessions VS 1231 and VS 1230 (8.97) and he lowest between VS 1179 and VS 1248 (5.31).

The dendrogram constructed from the pooled data of RAPD profile from 10 accessions showed that there were two main clusters. The cluster 1 was divided in to several sub clusters with different groups. Out of four accessions grouped together, three accessions VS 1230, VS 1231 and VS 1201 were resistant to the aphids. The accessions VS 1230 and VS 1231 showed the greatest similarity of 90 percent. These accessions were more related to VS 1201 at 88 percent similarity. VS 1034 which was a susceptible accession also was grouped along with these resistant accessions. This may be due to the peculiar banding pattern observed with the primer OPA 4. The sub cluster 1A had two susceptible accessions VS 1173 and VS 1177. These accessions were collected from the same place which accounts for their similarity. The cluster 2 had two resistant accessions VS 1263 and VS 1248 and they differed from the first cluster at 73 percent similarity. Two distinct bands were seen for these accessions with the primer OPA 3. This could be the reason for the clustering of these accessions together. These four accessions were rated as resistant to aphids which were obtained after screening. Ampily (2005) also supported the accessions VS 1230, VS 1231 and VS 1263 to be resistant to aphids. However the other four accessions VS1230, VS 1231, VS1263 and VS 1248 which were collected from different places also showed similarity with respect to the resistance with aphids.

Similar results were obtained by Neeraja *et al.* (2002) who used 22 land races of rice to identify the genetic diversity using RAPD. Here the most identical land races of rice were grouped together at a similarity coefficient of 0.9.

RAPD being a multilocus random marker, may be amplifying more number of bands in the varieties with the availability of more priming sites (Neeraja *et al.*,2002). The use of RAPD as a marker has been done in other crops such as cotton (Rubeena *et al.*, 2002), blackpepper (Nazeem *et al.*, 2005), onion (Ganesh and Veeragowda, 2005), and rubber (Sobhana *et al.*, 2005).

Considering the origin of polymorphic fragments in the RAPD technique, arbitrary oligomers anneal to random homologous target sites, the polymorphism is based on the disruption or the displacement of these sites or on small deletions or insertions between two priming sites. Mismatched annealing of a RAPD primer can occur (Neale and Hary, 1994) and leads to the lack of a detection of single point mutation in the target site. According to Tosti and Negri (2002), when the objective is to discriminate among genetically distinct accessions of cowpea, the RAPD technique appears to be the best alternative because it provides good discrimination in a short time and at low cost.

5.4. AFLP assay

AFLP is a novel technique for fingerprinting genomic DNA which is highly sensitive method for detecting polymorphism throughout the genome. The technique is based on the selective PCR amplification of restriction fragments from a total digest of genomic DNA. The three main steps involved are restriction of DNA and ligation of oligonucleotide adapters, selective amplification of sets of restriction fragments and gel analysis of amplified fragments

AFLP technique provides a novel and very powerful DNA fingerprinting technique for DNAs of any origin or complexity (Vos *et al.*, 1995).

5.4.1. Screening of cowpea accessions with AFLP primer combinations

The cowpea accessions were subjected to AFLP assay with different combinations of *Eco*R1 and *Mse*1 primers. In the present study, cowpea accessions gave a total of 237 scorable amplification products with the five primer pairs of which 144 (60.7 percent) bands were monomorphic and 93 (39.2 percent) were polymorphic bands.

The primer pairs yielded different levels of polymorphism as recorded in the Table 23. Three unique bands of molecular weight approximately between 150 and 100 bp were seen on the accessions VS 1230, VS 1263 and VS 1248 which were rated as resistant to aphids with the primer pair EAAG+MCAA. This can be identified as a putative trait related marker for resistance to aphids. Another two bands of approximately 150bp were seen for the resistant accession VS 1263 for the same primer pair.

The same combination of enzymes *Eco*R1 and *Mse*1 were used by James *et al.* (2003), Herselman *et al.* (2004), Coulibaly *et al.* (2002), Sharma *et al.* (2003) and Ouedraogo *et al.* (2002). Other combinations of enzymes such as *Mlu*1/*Mse*1 and *Pst*1/*Mse*1 were tried by Herselman *et al.* (2004). James *et al.* (2003) also tried with the primer pair *Pst*1/*Kpn*1.

Depending on the size of the genome to be analyzed, different sets of primers will have to be used. The determining factor in the efficiency of AFLP technique as applied to cowpea is the selection of the primer combination (Ouedraogo *et al.*, 2002). AFLP analysis can be effective depending on the nature of the selective primer combinations used in the analysis.

The first step of AFLP involves the restriction endonuclease digestion of genomic DNA. The enzymes used for the present study was *Eco*R1 and *Mse*1. *Eco*R1 has a 6bp recognition site and *Mse*1 has a 4bp recognition site. When these

enzymes were used together they generate small DNA fragments that will amplify well in the optimal size range (<1kb).

According to Herselman (2003), *Eco*R1/*Mse*1 enzyme combination detected greater number of fragments per primer combination in peanut than *Mlu*1/*Mse*1. The result indicated that *Eco*R1 recognition sequences (GAATTC) are more in peanut genome than *Mlu*1 recognition sequences (ACGCGT) and this is supported by the notion that DNA of most eukaryotes is AT rich (Blears *et al.*, 1998).

James *et al.* (2003) reported that very few bands were generated with the enzyme combination *Pst*1/*Kpn*1.

Following the heat inactivation of restriction endonucleases, the genomic DNA fragments were ligated to *Eco*R1 and *Mse*1 adapters to generate a template DNA for amplification. These common adapter sequences flanking variable genomic DNA sequences serve as primer binding sites on these restriction fragments. Using this strategy it is possible to amplify many DNA fragments without having prior sequence knowledge.

PCR is performed in two consecutive reactions. In the first reaction called preamplification, genomic DNAs were amplified with AFLP primers each having one selective nucleotide. The PCR products of the preamplification reaction were diluted and used as template for the selective amplification using two AFLP primers each containing 3 selective nucleotides. The selective amplification was achieved by the use of the primers that extend into the restriction fragments amplifying only those fragments in which the primer extensions match the nucleotides flanking the restriction sites. The method allows specific coamplfication of higher number of restriction fragments. This technique is therefore powerful for the identification of DNA polymorphisms.

The most important factor in determining the number of restriction fragments amplified in the number of selective nucleotides is the selective primer. Another factor is the C and G composition of the selective nucleotides. In general, the more Cs and Gs used as selective nucleotide in the amplification primers, the fewer are the DNA fragments amplified.

The number of fragments that can be analyzed simultaneously is dependent on the resolution of the detection system. Typically 50-100 restriction fragments were amplified and detected in denaturing polyacrylamide gel electrophoresis (PAGE). Polyacrylamide is a cross linked polymer of acrylamide. The length of polymer chains is detected by the concentration of acrylamide used. In case of DNA, polyacrylamide is used for separating fragments of less than 500bp. However under appropriate conditions, fragments of DNA differing in length by a single base pair are easily resolved.

A study was conducted by Herselman *et al.* (2002) to identify and map AFLP markers linked to peanut resistance to the aphid vector of groundnut rosette disease. They could detect a high level of polymorphism in peanut and identified one putative QTL for aphid resistance in the linkage group.

AFLP makers were used to identify molecular markers linked to cowpea genes conferring resistance to *Striga gesnerioides*. Seven AFLP markers were identified and linked to resistance with respect to particular trait (Ouedraogo *et al.* (2002).

AFLP technique has been used to identify markers linked to disease and insect resistant genes (Harlt *et al.*, 1999). There are reports for the use of AFLP markers to detect resistance against aphids in other crops (Cerenak and Javornik, 2002 in plum; Breandle *et al*, 2005 in pea; and Sargent *et al*, 2007 in raspberry).

RAPD and AFLP have been used to detect resistance to various insects in many crops (Sauge *et al.*, 2004, Sharma *et al.*, 2003, Najini *et al.*, 2002 and Weng and Hazar, 2002). This marker is used in genetic variation studies n different crops such as peanut (Herselman, 2003, Ntundu *et al.*, 2004) and common bean (Metais *et al.*, 2001).

The effectiveness of AFLP technique compared to other molecular techniques (isozymes, RFLP and RAPD) may be due to a more efficient detection of single nucleotide changes (at sites of restriction and selective amplification) (He and Prakash, 1997). AFLP has the advantage of being reproducible and having a high multiplex ratio (Herslman *et al.*, 2002). Since relatively small amounts of DNA are digested and detection of AFLP fragments does not depend on hybridization, partial digestion and faint patterns which are the sources of irreproducibility with RFLP is avoided (Vaneechoutte, 1996). Furthermore, the possibility of using stringent PCR annealing temperatures renders the AFLP analysis method more reproducible and robust than RAPD (Blears *et al.*, 1998).

Polymorphisms detected in the DNA fingerprint may be due to mutations in the restriction site, mutations in the sequences adjacent to the restriction sites and complementary to the selective primer extensions and insertions or deletions within the amplified fragments (Savelkoul *et al.*, 1999).

Vos *et al* (1995) observed that species having a large genome yielded more AFLP fragments than those with a small one. According to them, AFLP is insensitive to the template concentration. Differences may occur when templates are extensively diluted. Since AFLPs are based on the restriction digestion of DNA, the quality of the extracted DNA and the method of extraction could affect the profiles found.

5.4.2. Analysis of AFLP dendrogram

A dendrogram was constructed for the cowpea accessions using the AFLP data. There were two clusters which were divided in to several sub clusters with different groups. The resistant accessions VS 1248 and VS 1263 were grouped together as in RAPD. These accessions could have shared some genes which confered resistance to aphids. On screening they were graded as resistant since there was no aphid incidence during the entire crop season. Three susceptible accessions came in a single cluster (VS 1177, VS 1179 and VS 1173) which were collected from Wayanad. This could have accounted for the similarity between the accessions. Another two resistant accessions VS 1230 and VS 1201 were also grouped together at 84 percent similarity. The accessions VS 1230 and VS 1263 were reported to be resistant by Ampily (2005). VS 1248, VS 1263, VS 1230 and VS 1201 inspite of their different sources showed similarity with respect to resistance to aphids.

AFLP fragments correspond to unique positions on the genome and hence can be exploited as land marks in genetic and physical maps. The linked markers can be used for the indirect selection as they allow fast screening of large number of plants without subjecting them to insects in early stages of development (Liu et al., 2000). Molecular markers linked to resistance genes allow simultaneous screening of multiple markers without the limitation of environmental factors. Thus markers were identified in the resistant accessions VS 1230, VS 1201, VS 1263 and VS 1248 using RAPD and in VS 1230, VS 1248 and VS 1263 on AFLP analysis. The confirmation of resistance to aphids is of great importance. So the utility of RAPD and AFLP can be increased by converting these markers to a more specific amplification, a technique called Sequence Characterized Amplified Regions (SCAR).

Summary

6. SUMMARY

The study on 'Molecular characterization and development of trait related markers for aphid resistance in cowpea (*Vigna unguiculata* (L) Walp.)' was conducted at Centre for Plant Biotechnology and Molecular Biology and Radio Tracer Laboratory, College of Horticulture, Vellanikkara during the period 2005-2007. The main objective of the study was to identify the sources of resistance to aphids and to develop markers for aphid resistance in cowpea. The salient findings of the study are summarized below:

1. Sixty accessions of cowpea collected from different parts of Kerala and maintained in the Department of Olericulture, College of Horticulture were used for the present study

2. Cowpea was sown during two seasons April-May and September-October of 2006.

3. There was no aphid incidence for the accessions sown during April. But aphids were seen on the accessions sown during September after 40 days of sowing.

4. The count was taken from the terminal shoots, leaves and pods.

5. The average aphid count ranges from 0-494.67. The highest average aphid count was observed for the accession VS 1034 (494.67)

6. The accessions wee given the score 0-2 based on the average count of aphid population (0-resistant, 1- moderately resistant, 2- susceptible).

7. Out of sixty accessions, fifty were severely infested with aphids and were given the score 2. Four accessions were moderately resistant to aphids and were scored as 1. Six accessions were not at all affected by aphids during the entire crop season which were given the score 0.

8. Based on the average aphid count, five susceptible (VS 1177, VS 1179, VS 1173, VS 1208 and VS 1034) and five resistant (VS 1230, VS 1231, VS 1201, VS 1248 and VS 1263) accessions were used for the present study.

9. The protocol for the isolation of genomic DNA from cowpea has been standardized. The protocol suggested by Doyle and Doyle (1987) was found to be the best for the isolation of genomic DNA from cowpea.

10. The quantity and quality of DNA was analyzed by spectrophotometer. The absorbance ratio ranges from 1.78-1.88 which indicates the good quality of DNA.

11. The RNA contamination was completely removed through ribonuclease A treatment.

12. Thirty random decamer primers from Operon series were used for screening the cowpea accessions.

13. The primers which showed good amplification were selected. The primers used for the amplification gave 75 scorable amplification products of which 27 bands (36percent) were monomorphic and 48 bands (64 percent) were polymorphic.

14. OPA 2 produced two bands of size 0.44kb for VS 1230 and VS 1201. Two unique bands (0.2kb) were produced by VS 1248 and VS 1263 with OPA 3. Another two distinct bands of 0.4kb size were also observed for these accessions. Three bands of size 5.2kb, 3.6 kb and 1.9kb were unique to the accession VS

1248. Finally the primer OPA 10 generated a unique band for the accession VS 1248 (0.82 kb). These accessions having specific bands were resistant to the aphids.

15. The scored data based on RAPD banding pattern was used to construct a dendrogram using the NTSYS pc. (ver 2.1) software. In the dendrogram, all the 10 accessions were split into two main clusters at 62 percent similarity. The accessions VS 1230 and VS 1231 which were resistant to aphids were grouped together and show a similarity coefficient of 0.9. The cluster 2 had only two accessions VS 1248 and VS 1263 which also showed resistance to the aphids.

16. The cowpea accessions were subjected to AFLP assay with 5 primer combinations (*Eco*R1/*Mse*1).

17. The accessions generated a total of 1962 scorable amplification products of which 723 (36.8 percent) were polymorphic bands.

18. Three unique bands of approximately between 150 and 100 bp were observed for the accessions VS 1230, VS 1263 and VS 1248 which were resistant to aphids. Another two bands approximately 150bp were seen for VS 1263 with the primer combination EAAG+MCAA.

19. AFLP data was used to construct a dendrogram using the NTSYS pc. (ver 2.1) software. The resistant accessions VS 1230 and VS 1201, VS 1248 and VS 1263 were grouped together in dendrogram. While comparing the dendrogram obtained from RAPD and AFLP, the resistant accessions VS 1263 and VS 1248 were grouped together.

20. The unique bands produced by the resistant accessions could be identified as trait related markers for aphid resistance.

21. The resolving power of the primers used in RAPD and AFLP were calculated. The maximum resolving power was given by the primer OPA 4 followed by OPA 6, OPA1, OPA 3, OPS 5, OPA 2,OPA 7,OPS 4, OPA 10,OPA 9.In AFLP, EACT+MCAA exhibits maximum resolving power. This was followed by EAGG+MCAA, EACG+MCAA, EACG+MCAC, EAAG+MCAA.

22. The future studies should be focused on converting these RAPD and AFLP markers linked to aphid resistance to a more specific amplification, a technique called Sequence Characterized Amplified regions (SCAR).

References

REFERENCES

Allen, D.J. 1983. *The Pathology Of Tropical Legumes.* John Wiley and Sons. Chichester. 200p.

Allen, D.T. and Singh, S.R. 1980. Pests, disease resistance and protection in cowpea. In: Summerfield, R.J. and Bunting, A.H. (eds.). *Advances in Legume Science.* University of Reading, England. pp150-180.

Aggarwal, R.K., Brar, D.S., Nandi, S., Huang, N. and Khush, G.S.1999. Phylogenetic relationships among *Oryza* species revealed by AFLP markers. *Theor.Appl.Genet.* 98: 1320-1328.

Ampily, M. 2005. GxE interaction of semi erect cowpea genotypes. MSc (Ag) thesis. Kerala Agricultural University. 113p.

Anand, L. 2005. DNA marker technology in the improvement of horticultural crops. In:Rao,G.S.L.H.V.P., Nazeem,P.A., Girija,D., Keshavachandran,R., Joseph,L. and John,P.S. (eds.). *Proceedings of the ICAR National Symposium on Biotechnological Interventions for the Improvement of Horticultural Crops*; 10-12 January; College of Horticulture, Kerala Agricultural University, Thrissur, pp 8-17.

Anderson, G.R., Papa, D., Peng, J., Tahir, M. and Lapitan, N. L.2003. Genetic mapping of Dn7, a rye gene confering resistance to the Russian wheat aphid. *Theor.Appl.Genet.* 107 (7): 1297-303.

Archak, S., Gaikwad, A.B., Gautam, D., Rao, E.V.V.B., Swamy, K.R.M. and Karihaloo, J.L. 2003. DNA figerprinting of Indian cashew (*Anacardium occidentale* L) varieties using RAPD and ISSR techniques. *Euphytica.* 230: 397-404.

Armuganathan, K. and Earle, E. 1991. Nuclear DNA content of some important plant species. *Plant Mol.Biol.Rep.* 9: 208-218.

Ba, S.F., Pasquet, R.S. and Gepts, P. 2004. Genetic diversity cowpea (*Vigna unguiculata* (L) Walp.) as revealed by RAPD markers. *Genet.Resou.Crop.Evolution.* 51: 539-550.

Babu, T.P.H. 2000. RAPD analysis to assess the genetic stability in tissue culture derived black pepper (*Piper nigrum* L.) plants. MSc (Ag.) thesis, Kerala Agricultural University, Thrissur, 83p.

Barone, A. and Saccado, F. 1990. Pachytene morphology of cowpea chromosomes. In: Ng, N. Q. and Monti, L. M (eds.), *Cowpea Genetic Resources.* IITA, Ibadan, Nigeria, pp 137-143.

Basher,M. and Hapton, R.O.1996. Sources of genetic resistance in cowpea (*Vigna unguiculata* (L) Walp) to cowpea aphid borne mosaic virus. *European.J.Plant.Path.* 102: 411-419.

Bata, H.D., Singh, B.B., Singh, S.R. and Ladeinda, T.A.O.1987. Inheritance of resistance to cowpea aphid. *Crop.Sci.* 27 (5): 892-894.

Belaj, A., Satovic, J., Ismail, H., Panajoti, D., Rallo, L. and Trujillo, I. 2003. RAPD genetic diversity of Albanian olive germplasm and its relationships with other Mediteranean counries. *Euphytica.* 130: 387-395.

Bell, C.R.1980. Studies on resistance in cowpea, *Vigna unguiculata* (L)Walp to the aphid *Aphis craccivora* Koch. MSc (Ag) thesis, TamilNadu Agricultural University, Coimbatore, 79p.

*Birch, A.N.E., Jones, A.T., Kenton, B., Malloch, G. and Geoghegan, I. 2003. Resistance breaking raspberry aphid biotypes: a challenge for plant breeding. *Bulletin-01LB/SROP.* 26 (2): 51-54.

Blears, M.J., Grandis, D.S.A., Lee, H. and Trevors, J.T. 1998. Amplifed fragment length polymorphisms (AFLP): a review of the procedure and its applications. *J.Ind.Microbiol.Biotech.* 21: 99-114.

*Boerma, H.R. and Walker, D.R. 2005. Discovery and utilization of QTLs for insect resistance in soyabean. *Genetica.* 123 (3): 181-189.

Bose, L.K., Sahu, S.C., Behera, L., Nair, S. and Mohan, M. 2004. Application of molecular markers for the detection of a gall midge resistance gene, Gm4t in a rice breeding programme. *J.Appl.Zool.Res.* 15 (1): 9-13.

Breandle, C., Caillaud, M.C. and Stern, D.L. 2005. Genetic mapping of aphicarus- a sex linked locus controlling a wing polymorphism in the pea aphid (*Acyrthosiphon pisum*). *Heredity*. 94 (4): 435-42.

*Cerenak, A. and Javornik, B. 2002. Analysis of DNA markers for damson hop aphid resistance in hop. *Zbornik-Biotechniske-fakultete-Univerze-v-Lijublgani-Kmetijstvo*. 79 (2): 361-369.

Cervera, M.T., Cabezas, J.A., Simon, B., Zapater, M.J.M., Beitia, F. and Cens, J.L. 2000. Genetic relationships among bioypes of *Bemisia tabaci* (Hemiptera:Aleyrodidae) based on AFLP analysis. *Bull.Ent.Res.*90 (5): 391-396.

Cevik, V. and King, G.J. 2002. High resolution genetic analysis of the sd-1 aphid resistance locus in *Malus* sp. *Theor. Appl.Genet.* 105 (2): 346-354.

Chapman, C.G.D. 1996. Using molecular markers to breed for pest and disease resistance. *Proceedings of an International conference on crop protection: pests and diseases*; 18-21 November, 1996; Brighton, United Kingdom, pp783-788.

*Cidrack, D.S., Alves, M.A.O., Filho, F.F.R., Friere, E.A., Cavada, B.S. and Grangeiro, T.B. 2001. Analysis of geneic diversity of cowpea (*Vigna unguiculata* (L) Walp) genotypes. *Avancos tecnoloicos no fejao caupi. V Reuniao Nacional de Pesquisa de Caupi*. 4-7 Dezembro, 2001; pp140-144.

Clerc, L.V., Briard, M. and Revellon, P. 2002. Influence of number and map distribution of AFLP markers on similarity estimates in carrot. *Theor.Appl.Genet.* 106: 157-162.

*Coetzee, J.J. 1995. *Cowpea: A traditional crop in Africa*. Africa crop info leaflet. Vegetable and Ornamental Plant Institute, Agricultural Research Council, Pretoria. 10p.

Coulibaly, S., Pasquet, R.S., Papa, R. and Gepts, P. 2002. AFLP analysis of the phenetic organization and genetic diversity of *Vigna unguiculata* (L)Walp reveals the extensive gene flow between wild and domesticated types. *Theor.Appl.Genet.*104: 38-366.

David, K.S.1957. Notes on South Indian aphids III. Lachininse to Aphidinae. *Indian.J.Ent.* 19:171-180.

Davis, D.W., Delke, E.A., Ophnger, E.S., Doll, J.D., Hanson, C.V. and Putnam, D.H.1991. Alternative plant and animal products: programs in information exchange and research. In: Janick, J. and Simon, J.E (eds.), *Alternative Field Crops Manual*. JohnWiley and Sons, New York, pp133-143.

Dhanorkar, B.K., and Daware, D.G.1980. Differences in the number of aphids found on lines of cowpea in replicated trial. *Trop. Grain Legume Bull.*19: pp 3-4.

Diaz, S., Pire, C., Ferrer, J. and Bonete, M.J. 2003. Identification of *Phoenix dactylifera* L. varieties based on amplified fragment length polymorphism (AFLP) markers. *Cell. Mol. Biol. letters*. 8: 891-899.

Doyle, J.J. and Doyle, J.L.1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem. Bull.* 19:11-15.

Edwards, O., Ridsdill, S.J. and Horbury, R. 2004. Blue green aphid resistance in breeding lines of model legume *Medicago truncatula*. Aphids in a New Millenium. *Proceedings of the sixth International Symposium on Aphids*, Rennes, France, pp 435-440.

Ehlers, J.D. and Hall, A.E. 1997. Cowpea (Vigna unguiculata (L) Walp). *Field Crops Research*. 53: 187-204.

Fall, L., Diouf, D., Ndiaye, F.M.A., Badiana, F.A. and Guee, M. 2003. Genetic diversity in cowpea (*Vigna unguiculata* (L) Walp) varieties determined by ARA and RAPD techniques. *African.J.Biotech.*2 (2):48-50.

Fery, L.R.2002. New opportunities in Vigna. In: Janick, J.and Whipkey, A (eds.),*Trends in new crops and new uses.* ASHS Press, Alexandria, pp 424-428.

Fery, R.L. and Singh, B.B. 1997. Cowpea genetics: a review of the recent literature. In: Singh, B.B., Raj, M.D.R., Dashiell, K.E. and Jackai, L.E.N. (eds.). Advances in cowpea research. Copublication of the International Institute of Tropical Agriculture (IITA) and Japan International Research Centre for Agricultural Sciences (JIRCAS), IITA, Ibadan, Nigeria, pp 13-29.

Flight, C.1976.The Kintampo culture and its place in the economic prehistory of West Africa. In: Harlan, J.R., Dewed, J.M.J. and Stemler, A.B.L (eds.), *Origins of African plant domestication.* Mouton, The Hague, Netherlands, pp212-217.

Fox, F.W. and Young, M.E.N. 1982. *Food from the Veld.* Delta books. Johannesburg. 256p.

Ganesh, C.T. and Veergowda, R. 2005. Fingerprinting and genetic diversity of purple blotch disease resistant and susceptible genotypes of onion (*Allium cepa*) using RAPD marker. In: Rao, G.S.L.H.V.P., Nazeem, P.A., Girija, D., Keshavachandran, R., Joseph, L. and John, P.S. (eds.). *Proceedings of the ICAR National Symposium on Biotechnological Interventions for the Improvement of Horticultural Crops*; 10-12 January; College of Horticulture, Kerala Agricultural University, Thrissur, pp 248-251.

Githri, S.M., Kimani, P.M. and Pathak, R.S. 1997. Genetic linkage of the aphid resistance gene, *Rac* in cowpea. *African Crop.Sci.J.* 5 (2):145-150.

Goggin, F.L., Shah, G., Williamson. and Ullman, D.E. 2004. Developmental regulation of Ml mediated aphid resistance is independent of Ml-1.2 transcript levels. *Mol. Plant Microbe Interactions*. 17 (5): 532-536.

Haneefa, A.M., Balasubramanian, G., David, L. and Subramanian, T.R. 1973. Screening of lablab varieties for resistance to black bean aphid, *Aphis craccivora* Koch. *South Indian Hort.* 2:131-133.

Harlt, L., Moler, Y., Zeller, F.J., Hsans, S.L.K. and Schweizer, G.1999. Identification of AFLP markers closely linked to powdery mildew resistance genes pm1c and pm2a in common wheat (*Triticum aestivum*). *Genome*. 42:322-329.

Hayashi, E., Kondo, T., Terada, K., Kuramoto, N., Goto, Y., Okamura, M. and Kawasaki, L. 2001. Linkage map of Japanese black pine based on AFLP and RAPD markers including the markers linked to resistance against the pine needle gall midge. *Theor.Appl. Genet.*102 (6): 871-875.

He, G., Prakash, C.S. 1997. Identification of polymorphic DNA markers in cultivated peanut (*Arachis hypogeae* L). *Euphytica*. 97: 143-149.

He, S., Oba, H. and Mackenzic, S. 1992. Detection of DNA sequence polymorphisms among wheat varieties. *Theor.Appl.Genet.* 84: 573-578.

Herselman, L. 2003.Genetic variation among South African cultivated peanut (*Arachis hypogea* L) genotypes as revealed by AFLP analysis. *Euphytica*.133: 319-327.

Herselman, L., Thwaies, R., Kimmins, F.M., Courtois, B., Vandermerve, P.J.A.and Seal, S.E. 2004. Identification and mapping of AFLP markers linked to peanut (*Arachis hypogea* L) resistance to the aphid vector of groundnut rosette disease. *Theor.Appl.Genet.*109:1426-1433.

Hueyjiuan, H., Changpei, L., Chyrguan, C., Chinghuan, C., Jawshu, H. and Shuntu, L. 2002. Screening molecular markers linked with genes resistant in brown plant hopper. *J.agric.Res.* 51(4):19-27.

IITA.1985. Research highlights for 1984. IITA, Ibadan,Nigeria. pp79-80.

IITA.1982. Annual report for 1981. IITA, Ibadan, Nigeria. pp140-142.

IITA.1995. Annual report and research highlights. IITA,Ibadan, Nigeria.

James, M.C., Lesemann, S.S. and Down, J.G. 2003. Modifed AFLP analysis method for species with small genomes. *Plant.Mol.Biol.Rep.* 21: 303-307.

Jayappa and Lingappa. 1988. Screening of cowpea germplasm for resistance to *Aphis craccivora*. Trop.Pest. Mgmt. 34 (1): 62-64.

Joseph, S.1990. Genetics of resistance to aphids (*Aphis caccivora* koch) and utility of line mixtures in cowpea (*Vigna unguiculata* (L) Walp). Ph.D thesis. Kerala Agricultural University. 194p.

Joseph,S. and Peter,K.V. 2003. Inheritance of aphid resistance in cowpea. *Legume Res.*. 20 (1): 57-59.

JungHyun, S., Jongll, C., Misuk, G., Shim, J.H., Chung.J.I. and Go, M.S. 2001. Construction of molecular genetic linkage map using RAPD markers in cowpea. *Korean.J.Crop.Sci.* 46 (4): 341-343 .

Karel, A.K. and Malinga, Y. 1980. Leaf hopper and aphid resistance in cowpea varieties. *Trop. Grain Legume Bull.* 20: 10-11.

Kavitha, P.G., Nair, P., Nair, R.A., Jayachandran, B.K., Sabu, M. and Thomas, G. 2005. AFLP polymorphism and *Pythium* response in *Zingiber* sp. In:Rao, G.S.L.H.V.P., Nazeem, P.A., Girija, D., Keshavachandran, R., Joseph, L. and John, P.S. (eds.). *Proceedings of the ICAR National Symposium on Biotechnological Interventions for the Improvement of Horticultural Crops*; 10-12 January; College of Horticulture, Kerala Agricultural University, Thrissur, pp 232-234.

Kay, D.E.1979. *Food Legumes*. Tropical Development and Research Institute, London. 270p.

Keim, P., Schupp, J.M., Travis, S.E., Dayton, K., Tong, Z., Liang, S., Ferriera, A., Webb, D.M., Zhu, T. and Shi, L.A. 1997. A high density soyabean genetic linkage map based on AFLP markers. *Crop.Sci.* 37 (2): 537-543.

Keshavachandran, R., Nazeem, P.A. and Karihaloo, J.L.2005. Genetic fingerprinting of *Piper nigrum* L. and *Piper longum* L. cultivars using RAPD markers. In: Rao, G.S.L.H.V.P., Nazeem, P.A., Girija, D., Keshavachandran, R., Joseph, L. and John, P.S. (eds.). *Proceedings of the ICAR National Symposium on Biotechnological Interventions for the Improvement of Horticultural Crops*; 10-12 January; College of Horticulture, Kerala Agricultural University, Thrissur, pp 226-229.

Kiplagat, O.K.2005. The Russian wheat aphid (*Diuraphis noxia* Mord): Damage on Kenyan wheat (*Triticum aestivum* L.) varieties and possible control through resistance breeding. Wageningen University, Netherlands, 141p.

Klinger, J., Creasy, R., Gao, L., Nair, R.M., Calix, A.S., Jacob, H.S., Edwards, R. and Singh, B. 2005. Aphid resistance in *Medicago truncatula* involves antixenosis and phloem specific, inducible antibiosis, and maps to a single locus flanked by NBS-LRR resistance gene analogues. *Plant.Pysiol.* 137(4):1445-55.

Klinger, J., Kovalski, I., Siberstein, L., Trever, P. R., Thompson, G.A. and Katzir. 2002. *Aphis gosypii* resistance in *Cucumis melo*: the genetic base of a phloem specific defense. *Proceedings of 7th Eucarpia meeting on cucurbit breeding and genetics, Maale Ha Hamisha*, Israel, pp313-320.

Klinger, J., Kovalski, I., Silberstein, L., Thompson, G.A. and Treves, P.2001. Mapping of cotton melon aphid resistance in melon. *J.Am.Soc.hort.Sci.*126 (1): 56-63.

Larson, S.R., Cartier, E., Cracken, M.C.L., Dyer, D. 2001. Mode of reproduction and amplified fragment length polymorphism variation in purple needle grass: Utilization of natural germplasm sources. *Mol. Ecology*. 10: 1165-1177.

Lawlor,H.J., Siddique, K.H.M., Sedgley, R.H., Thuling, N. and Drew, R.A. 1998. Improving cold resistance in chick pea and the use of AFLP for the

identification of the molecular markers for these traits. *Acta-Horticulturae.* 46: 185-192.

Liu, J., Liu, J.D., Li, W.W., Wang, S., Chen, P., Cheng, S. and Gao, D. 2000. Molecular marker facilitated pyramiding of different genes for powdery mildew resistance in wheat. *Plant Breeding.* 119: 21-24.

Loh, J.P., Riew, R., Set, O., Gan, I.H. and Gan, Y.Y. 2000. Amplified fragment length polymorphism fingerprinting of 16 banana cultivars (*Musa* cvs). *Molecular Phylogenetics and Evolution.* 17: 360-366.

*Lu, X.W. and Brewbake, J.L.1999. Genetics of resistance to the corn leaf aphid (Homoptera:Aphididae). *Maize Genetics Cooperation Newsletter*, 73: 36-37.

Marais, G.F., Wessels, W.G., Hom, M., Toit, F., Dutoit. 1998. Association of a stem rust resistant gene (sr45) and two Russian wheat aphid resistance genes (Dn5 and Dn7) with mapped structural loci in common wheat. *South African J. plant and soil.* 15:67-71.

*Marechal, R., Mascherpa, J.M. and Stainer, F. 1978. Etude taxonomiqco groupe d'especes des genres phaseolus et vigna (Papilionaceae) Sur la base des donnees morphoogiques et polliques,traitees pour analyse informatique. *Biossiera.*28 :1-273.

Mathew, K.P., Thomas, M.J. and Nair, M.R.G.K. 1972. Population fluctauations of pea aphid in relation to climate and predators. *Agric.Res.J.Kerala.* 9 (1): 23-28.

Mcfoy,C.C.A. and Dabrowski,Z.I.1984. Preliminary studies on cowpea resistance to *Aphis craccivora* Koch. *Zeitschript for Angewandle Entomologie.* 97 (20): 202-209.

Messina, F.J., Renwick, J.A.A. and Bormore. 1985. Resistance to *Aphis craccivora* (Homoptera:Aphididae) in selected varieties of cowpea. *J.Ent.Sci.* 20 (2): 263-269.

Metais, I., Aubury, C., Hamo, B., Jalonzot, R. and Peltier, D. 2001. Assessing common bean genetic diversity using RFLP, ISSR, RAPD and AFLP markers. In:Dore, C. and Dosba, F.(eds.). *Proceedings of the International Symposium on Molecular Markers for Characterizing Genotypes and Identifying Cultivars in Horticulture*. 6-8 March, 2000; France. pp459-461.

Mohan, M., Nair, S., Bhagwat, A., Krishna, T.G., Yano, M., Bhatia, C.R. and Sasaki, T. 1997. Genome mapping, molecular markers and marker assisted selection in crop plants. *Mol.Breeding*.3:87-163.

Mohapatra, T. 2005. Molecular markers in crop improvement: current applications and future prospects. In: Rao, G.S.L.H.V.P., Nazeem, P.A., Girija, D., Keshavachandran, R., Joseph, L. and John, P.S. (eds.). *Proceedings of the ICAR National Symposium on Biotechnological Interventions for the Improvement of Horticultural Crops*; 10-12 January; College of Horticulture, Kerala Agricultural University, Thrissur, pp 3-7.

Moreira, L.A., Mollema, C. and Van, H.S. 1999. Search for molecular markers linked to *Liriomyza trifoli* resistance in tomato. *Euphytica.* 109(3): 149-156.

Myburg, A.A., Cawood, M., Wingfield, B.D. and Botha, A.M. 1998. Development of RAPD and SCAR markers linked to the Russian wheat aphid resistance gene Dn2 in wheat. Theor.Appl.Genet. 96 (8): 1162-1169.

Najini, B., Boukhaten, N., Jaafari, S.E., Jlibene, M., Paul, R. and Jacquemin, J.M. 2002. Amplified fragment length polymorphism (AFLP) analysis of markers associated with H5 and H22 Hessian fly resistance genes in bread wheat. *Biotechnol. Agron. Soc. Environ.* 6 (2): 79-85.

Nazeem, P.A., Keshavachandran, R., Babu, T.D., Achutan, C.R., Girija, D. and Peter, K.V. 2005. Assessment of genetic variability in black pepper (*Piper nigrum*) varieties through RAPD and AFLP analysis. In: Rao, G.S.L.H.V.P., Nazeem, P.A., Girija, D., Keshavachandran, R., Joseph, L. and John, P.S. (eds.). *Proceedings of the ICAR National Symposium on*

Biotechnological Interventions for the Improvement of Horticultural Crops; 10-12 January; College of Horticulture, Kerala Agricultural University, Thrissur, pp 226-229.

Neale, D.B. and Hary, D.E. 1994. Genetic mapping in forest trees: RFLPs, RAPDs and beyond. In: *Agbiotech news and information.* Vol 6. Commonwealth Agricultural Bureaux, Wallingford, United Kingdom. pp 107-114.

Neeraja, C.N., Sarla, N. and Siddiq, E.A. 2002. RAPD analysis of genetic diversity in Indian land races of rice (*Oryza sativa* L). *J.Plant.Biochem.Biotech.* 11: 93-97.

Neito, L. and Blake, T.K. 1994. Russian wheat aphid resistance in barley: Inheritance and linked molecular markers. *Crop.Sci.* 34(3): 655-659.

*Ng, N.Q. 1995. Cowpea-*Vigna unguiculata* (Leguminosae-Papilionidae).In: *Evolution of crop plants.* pp326-332.

Ng, N.Q. and Marechal, R. 1985. Cowpea taxonomy, origin and germplasm. In: Singh, S.R. and Rachie, K.O (eds.), *Cowpea research, production and utilization.* John Wiley and Sons, Chichester, pp 11-12.

Ntundu, W.H., Bach, I.C., Christiansen, J.L. and Anderson.S.B. 2004. Analysis of genetic diversity in bambara groundut (*Vigna suberrana* (L) Verdc) landraces using amplified fragment length polymorpism (AFLP) markers. *African.J.Biotech.* 3(4): 220-225

Ombakho, G.A., Tyagi, A.P. and Pathak, R.S.1987. Inheritance of resistance to the cowpea aphid. *Crop.Sci.* 27 (5): 890-892.

Ouedraogo, J.T., Gowda, B.S., Jean, M.,Close, T.J., Ehlers, J.D., Hall, A.E., Robers, P.A., Ismail, A.M., Timko, M.P. and Gepts, P. 2002. An improved genetic linkage map for cowpea (*Vigna unguiculata* (L) Walp) combining AFLP, RAPD, biochemical markers and biological resistance traits. *Genome.* 45 (1): 175-188.

Ouedraogo, J.T., Maheswari, V., Berner, D.K., Piere, C.A., Belzile, F. and Timko, M.P. 2001. Identification of AFLP markers linked to resistance of cowpea (*Vigna unguiculata* L) to parasitism by *Striga gesnerioides*. *Theor.Appl.Genet.* 102 (6): 1029-1036 .

Padulosi, S. and Ng, N.Q. 1997. Origin, taxonomy and morphology of *Vigna unguiculata* L Walp. In: Singh, B.B., Mohanraj, D.R., Dashiell, K.E., and Jackai, L.E.N. (eds.), *Advances in cowpea research*. IITA, Ibadan, Nigeria, pp1-11.

Pal, S.K., Rao, G.G.S.N. and Kushawaha, K.S. 1978. A note on the pest fluctuations in relation to weather elements in eight vaieties of Moong (*Vigna radiata*) under arid conditions. *Indian.J.Ent.* 39: 375-376.

Pathak, R.S.1983. Induction of aphid resistance in cowpea, 2nd FAO Research coordination. *Proceedings: Improvement of Leguminous and Oil Seed Crops in Latin America through Induced Mutations*, 7-11 Nov.1983, Maracaibo, Venezuela.

Pathak, R.S. 1984. Genetics of plant resistance to insects: Cowpea resistance to aphids. *12th Annual Report. International Centre for Insect Physiology and Ecology.* pp 15-16.

Pathak, R.S.1988. Genetics of resistance to aphid in cowpea. *Crop.Sci.* 28: 474-476.

Pignone, D., Cifareli, S. and Perrino, P. 1990. Chromosome identification in Vigna unguiculata (L) Walp. In: Ng, N.Q. and Monti, L.M (eds.), *Cowpea genetic resources*. IITA, Ibadan, Nigeria, pp 144-150.

Prabhakara, B.S., Naik, L.B., Mohan and Varalakshmi, B. 2001. Cowpea (*Vigna unguiculata* (L) Walp). In: Thamburaj, S. and Singh, N.(eds.), *Textbook of vegetables, tuber crops and spices.* Directorate of Information and Publication of Agriculture, Indian Council of Agricultural Research, New Delhi, pp 206-208.

Prevost, A. and Wilkinson, M.J. 1999. A new system for comparing PCR primers applied to ISSR fingerprinting of potato cultivars. *Theor. Appl.Genet.* 98: 107-112.

Ridsdill, S.J., Edwards, O.,Wangshao, F., Wang, S.F. and Reidycrofts, J. 2004. Aphid response to plant defensive compounds in lupines. *Aphids in a New Millenium. Proceedings of the sixth International Symposium on Aphids*, Rennes, France, pp 491-497.

Roa, A.S., Maya, M.M., Duque, M.M., Tohme, J., Allem, A.C. and Bonierbale, M.W. 1997. AFLP analysis of relationships among cassava and other *Mannihot* species. *Theor.Appl.Genet.* 95: 741-750.

Roche, P., Alston, F.H., Maliepaard, C., Evans, K.M., Vrielink, R., Dunemann, F., Markussen, T., Tartarini, S., Brown, L.M., Ryder, C. and King, G.J. 1997. RFLP and RAPD markers linked to rosy leaf curling aphid resistance gene (Sd1) in apple. *Theor.Appl.Genet.* 94 (3): 528-533.

Rogers, S.O. and Bendich, A.J. 1994. Extraction of total cellular DNA from plants, algae and fungi. *Plant.Mol.Biol.Manual.* 91: 1-18.

Rohlf, F.J. 1990. NTSYS-PC. Numerical taxonomy and multivariate analysis system exeter software. *Euphytica.* 68: 69-76.

Rossi, M., Goggin, F.L., Milligan, S.B., Kaloshian,I., Ullman, D.E., Williamson, V.M. 1998. The nematode resistance gene Mi of tomato confers resistance against potato aphid. *Proceedings of the National Academy of Sciences of United States of America*, 95: 9750-9754.

Roychoudhury,B., Ghosh,S., Mandel,N. and Dasgupta,T. 2005. Genetic diversity in some wild and cultivated germplasm in *Vigna* by RAPD marker. In: Rao, G.S.L.H.V.P., Nazeem, P.A., Girija, D., Keshavachandran, R., Joseph, L. and John, P.S. (eds.). *Proceedings of the ICAR National Symposium on Biotechnological Interventions for the Improvement of Horticultural Crops*;

10-12 January; College of Horticulture, Kerala Agricultural University, Thrissur, pp 239-242.

Rubeena, L.S., Randhawa and Singh, H. 2000. Varietal identification in cotton by RAPD markers. *Crop.Improv.*27 (2): 145-151.

Saleh, M.R.A., Hassanein, M.H. and Elsebae, A.M.1972. Population dynamics of *Aphis craccivora* Koch on broad bean and cowpea under Egypt (Homoptera). Annamalai University. *Res.Ann.* 1:110-112.

Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989. *Molecular Cloning: A Laboratory manual.* Second edition. Cold Spring Harbor Laboratory Press, New York, USA, 1322p.

Sargent, D.J., Fernandez, F., Rys, A., Knigt, V.H., Simpson, P.W. and Obtt, K.R. 2007. Mapping of A1 conferring resistance to the aphid *Amphorophora idaei* and dw (dwarfing habit) in red raspberry (*Rubus idaeus* L) using AFLP and microsatellie markers. *Plant Biol.*20 (7):15.

Sauge, M.H., Pascal, T., Lacroze, J.P., Pteiffer, F. and Kuvella, J. 2004. Mapping of a genetic factor of partial resistance to *Myzus persicae* in the wild peach *Prunus davidiana*, that impedes phloem sap ingestion by the aphid. *Proceedings of the Sixth International Symposium on Aphids;* September, 2001; France. pp 499-505.

Savelkoul, P.H.M., Aarts, H.J.M., Haas, J., Dijkshoorn, L., Duim, B., Rademaker, J.L.W., Otson, M., Schouls, L. and Leustra, J.A. 1999. Amplified fragment length polymorphism analysis: the state of an art. *J.Clin.Microbiol.* 37 (10): 3083-3091.

Selvi, A., Shanmughasundaram, P., Kumar, S.M. and Raju, J.A. 2002. Molecular markers for yellow stem borer *Scirpophaga incertulus* resistance in rice. *Euphytica.*124 (3): 371-177.

Sharma, P.N., Ketipeatachchi, Y., Murata, K., Torii, A., Takumi, S., Mori, N. and Nakamura, C. 2003. RFLP/AFLP mapping of a brown plant hopper

(*Nilaparvata lugens* Stal) resistance gene Bph1 in rice. *Euphytica*.129:109-117.

Silva, S.D., Lemos, M.V.F. and Osuna, A.J.T. 2000. RAPD marker for improving resistance to *Helicoverpa zea* in corn. *Maydica*.45 (4): 289-294.

Singh, S.R.1977. Cowpea cultivars resistant to insect pests in world germplasm collection. *Trop. Grain Legume Bull.* 9: 3-7.

Singh, B.B., Chambliss, O.L. and Sharma, B. 1993. Recent advances in cowpea breeding. In: Singh, B.B., Mohanraj, D.R., Dashiell, K.E. and Jackai, L.E.N (eds.), *Advances in cowpea research*. Copublication of the International Institute of Tropical Agriculture (IITA) and Japan International Research Center for Agricultural Sciences (JIRCAS), IITA. Ibadan, Nigeria, pp 30-49.

Singh, S.R. and VanEmden, H.F.1979. Insect pests of grain legumes. *Ann.Rev.Ent.* 24: 255-78.

Skov, E. and Wellendorf, H. 2000. RAPD markers linked to major genes behind field resistance against the green spruce aphid *Elatobium abietinum* (Walker) in *Picea sitchensis. Forest.Genet*.7 (3): 233-246.

Sneath, P.H.A. and Sokal, R.R. 1973.*Principles of numerical taxonomy*. W.H.Freeman and Co., San Fransisco, 359p.

Sobhana, P., Thomas, M. and Jacob, J. 2005. RAPD analysis of monoclonal and polyclonal seedlings of *Hevea brasiliensis*. In: Rao, G.S.L.H.V.P., Nazeem, P.A., Girija, D., Keshavachandran, R., Joseph, L. and John, P.S. (eds.). *Proceedings of the ICAR National Symposium on Biotechnological Interventions for the Improvement of Horticultural Crops*; 10-12 January; College of Horticulture, Kerala Agricultural University, Thrissur, pp 246-247.

Steele, W.M., Allen, D.T. and Summerfield, R.J. 1985. Cowpea (*Vigna unguiculata* (L)Walp).In: Summerfield, R.J. and Roberts, E.H (eds.),*Grain legume crops*. William Collins, Sons and Co. Ltd, London, pp 520-583.

Staub, J.E., Bergers, F.C. and Gupta, M. 1996. Genetic markers, map construction and their application in plant breeding. *Hort.Sci.* 31(5):729-741.

Sulochana, K.A.1984. Screening of resistance to *Aphis craccivora* in vegetable cowpea (*Vigna unguiculata* var *sesquipedalis* and var *cylindrica*) and evaluation of early types. MSc thesis. Kerala Agriculture University, Vellanikkara. 45p.

Summerfield, R.J., Huxley, P.A., and Steel, W. 1974. Cowpea (*Vigna unguiculata* (L)Walp.).*Field Crop Abstracts*. 27:301-312.

Tang, T., Zhong, Y., Jian, S. and Shi, S. 2003. Genetic diversity of *Hibiscus tiliaceus* (Malvaceae) in China assessed using AFLP markers. *Annals of Botany*. 92: 409-414.

Toit, F., Wessels, W.G., Marais, G.F.1995. The chromosome location of the Russian wheat aphid resistance gene, Dn5. *Cereal Research Communication*. 23:1-17.

Tosti, N. and Negri, V. 2002. Efficiency of three PCR based markers in assessing genetic variation among cowpea (*Vigna unguiculata* subsp *unguiculata*) land races. *Genome*. 45: 268-275.

Vaneechoutte, M. 1996. DNA fingerprinting techniques for microorganisms. A proposal for classification and nomenclature. *Mol. Biotech*. 6:115-142.

*Verdcourt, B.1970. Studies in Leguminosae-Papilionoidaea for the flora of tropical East Africa. *Kew Bull*. 24 (4): 507-569.

Vos, P., Hogers, R., Blecker, M., Reijans, M., Lee, J.V., Hornes, M., Frijters, A., Pot, J., Peeman, J., Kuiper, M. and Zabeau, M.*1995*. AFLP: a new technique for DNA fingerprinting. *Nucleic acids Res.* 23: 4407-4414.

Wang, Z., Zhang, K., Sun, X., Tang, K. and Zhang, J. 2005. Enhancement of resistance to aphids by introducing snowdrop lectin gene, gna into maize plants. *J.Biosci.* 30 (5): 627-38.

Way, M.J.1967. Nature and causes of annual fluctuations in the number of *Aphis fabae* scop on field beans *Vicia fabae*. *Ann.Appl.Biol.* 59:175-188.

Welsh, J. and McClelland, M.1990. Fingerprinting genomes using PCR with abitrary primers. *Nucleic acids Res.* 18: 7213-7218.

Weng, Y. and Lazar, M.D. Amplified fragment length polymorphism and simple sequence repeat based molecular tagging and mapping of green bug resistance gene Gb3 in wheat. *Plant Breeding*. 121 (3): 218-223.

Westphall, E.1974. Pulses in Ethiopia: their taxonomy and agricultural significance. *Field Crop Abstracts*. 24: 213-232.

Williams, J.G., Kubelk, A.R., Livak, K.J., Rafalski, J.A. and Tingey, S.V. 1990. DNA polymorphism amplified by arbitrary primers are useful as genetic markers. *Nucleic acids Res.* 18: 6531-6535.

Williams, K.J., Taylor, S.P., Bogacki, P., Pallotta, M., Bariana, H.S. and Wallwork, H. 2002. Mapping of root lesion nematode (*Pratylenchus neglectus*) resistance gene R1nn1 in wheat. *Theor.Appl.Genet.*104 (5): 874-879.

Xu, R.Q., Tomooka, N. and Vaughan, D.A. 2000. AFLP markers for characterizing the azuki bean complex. *Crop.Sci.* 40: 808-815.

*Yueying, L., Shubha, Z., Liguo,Y. and Shiqiang,L. 2003. Study on molecular markers linked o aphid resistance gene of sorghum. *Acta-Agronomica-Sinica*.29 (4):534-540.

Zhao, J., Wang, X., Deng, B., Lou, P., Wu, J., Sun ,R., Xu, Z., Vromans, J. Koornnef, M. and Bonnemma, G. 2005. Genetic relationships within *Brassica rapa* as infered from AFLP fingerprints. *Theor.Appl.Genet.* 110: 1301-1314.

* Originals not seen

Appendices

APPENDIX-I

Laboratory equipments used for the study

Spectrophotometer	Spectronic Genesys-5, Spectronic Instrument, USA
Refrigerated centrifuge	Kubota, Japan
Horizontal electrophoresis system	Biorad
Vertical electrophoresis system cell	Biorad Sequi-Gen® GT sequencing
Thermal cycler	1. Eppendorf 2. MJ Research PTC-200 Peltier Thermal Cycler
Gel dryer	Biorad, Model 583
Gel documentation system	1. Alpha Imager 2. Phosphor Imager FLA-5100 Fuji

APPENDIX-II

Composition of reagents used for DNA isolation

1. **Doyle and Doyle method**

4x Extraction Buffer

Sorbitol – 2.5g

Tris HCl – 4.8g

EDTA – 0.74g

The chemicals were dissolved in about 80ml of distilled water, adjusted the pH to 7.5 and made up to 100ml with distilled water and then autoclaved.

Lysis buffer

Tris HCl (1M, pH 8) – 20ml (15.76g per 100ml)

EDTA (0.2ml) – 20ml (9.305g per 100ml)

NaCl (5M) – 40ml (29.22g per 100ml)

Distilled water – 20ml

CTAB – 2g (Dissolved in 20ml distilled water and then added to the remaining components).

5% Sarcosin

Sarcosin – 5g

Distilled water – 100ml

TE Buffer

(Tris HCl – 10mM ; EDTA – 1mM)

10mM Tris (pH 8) – 1 ml

0.25 M EDTA (pH 8) – 0.4 ml

Distilled water - 98.6 ml

2. **Rogers and Bendich (CTAB) method**

2x CTAB Extraction Buffer

CTAB (2%, v/v)

100mM Tris buffer (pH 8)

20mM EDTA (pH 8)

1.4M NaCl

10% CTAB Solution

10% CTAB (w/v)

0.7M NaCl

TE Buffer

10mM Tris (pH 8)

10mM EDTA (pH 8)

APPENDIX-III

Composition of buffers and dyes

1. **TAE Buffer 50x (for 1l)**

   242g Tris base

   57.1ml glacial acetic acid

   100ml 0.5M EDTA (pH 8.0)

2. **TBE Buffer 10x (for 1l)**

   54g Tris base

   27.5g Boric acid

   20ml 0.5M EDTA (pH 8.0)

3. **Loading Dye (6X)**

   0.25% bromophenol blue

   0.25% xylene cyanol

   30% glycerol in water

4. **Formamide Dye**

   Formamide – 10ml

   Xylene cyanol – 10mg

   Bromophenol blue – 10mg

   0.5M EDTA (pH 8.0) - 200µl

APPENDIX- IV

Reagents for agarose gel electrophoresis

I. **Agarose**

II. **50X TAE** (1L)

Tris base-242 g

0.5M EDTA (pH 8)-100ml

Glacial acetic acid- 57.1ml

III. **Tracking dye** (6X)

Bromophenol blue 0.25%

Xylene cyanol FF 0.25%

Glycerol in water 30%

IV. **Ethidium bromide** (Intercalating dye)

The dye was prepared as a stock solution of 10mg/ml in water and was stored at room temperature in a dark bottle

APPENDIX- V

Reagents used for AFLP

1. ***EcoRI/Mse*1** (1.25 units/µl)

   10mM Tris HCl (pH 7.5)

   50mM NaCl

   0.1mM EDTA

   1mM DTT

   0.1mg/ml BSA

   50% (v/v) glycerol

   0.1% Triton

2. **5X reaction buffer**

   50mM Tris HCl (pH 7.5)

   50mM Mg-acetate

   250mM K-acetate

3. **Distilled water**

4. **Adapter ligation solution**

   *Eco*R1/*Mse*1 adapters

   0.4mM ATP

   10mM Mg-acetate

   10mM Tris HCl (pH7.5)

   50mM K-acetate

5. **T_4 DNA ligase** (1 unit/µl)

   10mM Tris HCl (pH 7.5)

   1mM DTT

   50mM KCl

50% glycerol (v/v)

6. **TE buffer**

10mM Tris HCl (pH 8)

0.1mM EDTA

7. **T_4 kinase** (10 units/μl)

50mM Tris HCl (pH 7.6)

25mM KCl

1mM 2-mercaptoethanol

0.1μM ATP

50% glycerol (v/v)

8. **5X kinase buffer**

350mM Tris HCl (pH 7.6)

50mM $MgCl_2$

500mM KCl

5mM 2-mercaptoethanol

9. **10X PCR buffer plus Mg**

200mM Tris HCl (pH 8.4)

15mM $MgCl_2$

500mM KCl

APPENDIX – VI

Metereological observations from April 2006 to March 2007

Month	Mean Maximum temperature(^{0}C)		Mean minimum temperature(^{0}C)		Number of rainy days		Rainfall (mm)		Relative humidity(%)		Mean sunshine (hrs)	
	2006	2007	2006	2007	2006	2007	2006	2007	2006	2007	2006	2007
April	33.4	-	24.7	-	3	-	86.2	-	75	-	7.0	-
May	31.8	-	24.3	-	14	-	675.5	-	79	-	5.8	-
June	29.9	-	23.6	-	17	-	608.8	-	84	-	3.8	-
July	29.5	-	23.3	-	29	-	519.0	-	85	-	2.2	-
August	29.9	-	23.1	-	15	-	550.6	-	83	-	4.2	-
September	29.4	-	23.0	-	17	-	522.2	-	84	-	3.9	-
October	31	-	23.0	-	11	-	323.7	-	79	-	4.8	-
November	30.7	-	23.7	-	5	-	79.5	-	72	-	6.5	-
December	31.5	-	23.7	-	0	-	0	-	57	-	7.8	-
January	-	32.5	-	22.0	-	0	-	0	-	54	-	8.7
February	-	34.3	-	22.2	-	0	-	0	-	55	-	9.8
March	-	34.8	-	24.4	-	0	-	0	-	63	-	8.2

MOLECULAR CHARACTERIZATION AND DEVELOPMENT OF TRAIT RELATED MARKERS FOR APHID RESISTANCE IN COWPEA (*Vigna unguiculata* (L) Walp.)

By

RAMYA HARIDAS

ABSTRACT OF THE THESIS

Submitted in partial fulfilment of the requirement for the degree of

Master of Science in Agriculture

(PLANT BIOTECHNOLOGY)

Faculty of Agriculture
Kerala Agricultural University

Centre for Plant Biotechnology and Molecular Biology

COLLEGE OF HORTICULTURE
VELLANIKKARA, THRISSUR - 680 656
KERALA, INDIA

2007

ABSTRACT

Cowpea is one of the most important grain legumes that has been grown throughout the tropics. It is a rich source of proteins (25 percent). Their ability to fix nitrogen is of great advantage on small farms. However their production is greatly limited by many pests, especially aphids. Keeping in view of the importance of this crop, a study was conducted on 'Molecular characterization and development of trait related markers of aphid resistance in cowpea (*Vigna unguiculata* (L) Walp.)'.

The present study was undertaken at the Centre for Plant Biotechnology and Molecular Biology and Radio Tracer Laboratory, College of Horticulture during the period 2005-2007. The objectives of the study were to identify the sources of resistance to aphids and to develop markers for aphid resistance in cowpea. The cowpea accessions which were susceptible, moderately resistant and resistant were identified based on the average count of aphids. Five susceptible (VS 1177, VS 1179, VS 1173, VS 1208, VS 1034) and five resistant (VS 1230, VS 1231, VS 1201, VS 1248, VS 1263) accessions were used for the present study. These 10 accessions were subjected to molecular characterization using RAPD and AFLP markers.

For RAPD and AFLP analysis, the protocol for genomic DNA isolation from cowpea was standardized. The protocol suggested by Doyle and Doyle (1987) was found to be the most appropriate.

Thirty random primers were screened and ten were selected for RAPD profiling of cowpea accessions. The primer OPA 4 was found to have the highest resolving power. A total of 75 scorable amplification products were generated by 10 random primers of which 48 bands were polymorphic. Specific bands were generated for the resistant accessions VS 1230 and VS 1201 with OPA 2, VS 1248 and VS 1263 using OPA 3 and VS 1248 alone with OPA 10. In the

dendrogram, the 10 accessions were grouped into two major clusters of 8 and 2 accessions each. The resistant accessions VS 1230 and VS 1231 were the most closely related with 90 percent similarity. Another two resistant accessions VS 1248 and VS 1263 were also grouped together in the dendrogram.

The cowpea accessions were subjected to AFLP analysis with 5 primer combinations of EcoR1 and Mse1. A total of 237 scorable amplification products were produced by the primer pairs of which 93 bands were polymorphic. The highest resolving power was obtained for EACT+MCAA. Specific bands were produced for EAAG+MCAA in resistant accessions VS 1230, VS 1263 and VS 1248. The dendrogram obtained for AFLP showed that VS 1177 and VS 1179 were most closely related at 91 percent similarity. Similarly the resistant accessions VS 1263 and VS 1248, VS 1230 and VS 1201 were grouped in different sub clusters.

Thus RAPD and AFLP markers were utilized to characterize cowpea accessions for aphid resistance. The specific bands identified in the resistant accessions could be treated as trait related markers for aphid resistance. Further studies should be conducted on screening the cowpea accessions with more number of primers to develop more specific markers with reference to the aphid resistance. The studies should also be focused on converting these RAPD and AFLP markers linked to aphid resistance to a more specific amplification, a technique called Sequence Characterized Amplified Regions (SCAR).