DEVELOPMENT OF MOLECULAR MARKERS FOR ANTHRACNOSE RESISTANCE IN VEGETABLE COWPEA [*Vigna unguiculata* (L.) Walp.]

By

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(2013-11-109)

THESIS

Submitted in partial fulfillment of the
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COLLEGE OF HORTICULTURE

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KERALA, INDIA

2015

DECLARATION

I, hereby declare that the thesis entitled "Development of molecular markers for anthracnose resistance in vegetable cowpea [*Vigna unguiculata* (L.) Walp.]" is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.
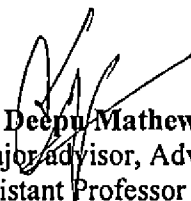
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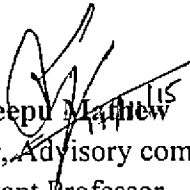
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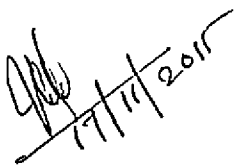
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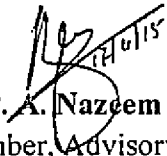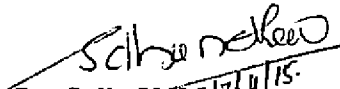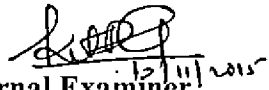
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And so comes the time to look back on the path traversed during the endeavor and to remember the faces and spirits behind the action with a sense of gratitude. Nothing of significance can be accomplished without the acts of assistance, words of encouragement and gestures of help from the other members of the society.

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Dolagobinda Pradhan

Dedicated to my Parents, Brothers and Friends

TABLE OF CONTENTS

LIST OF TABLES

LIST OF PLATES

LIST OF ANNEXTURES

ANNEXTURE NO.	TITLE
I	List of laboratory equipments used for the study
II	Composition of reagents used for DNA isolation and purification
III	Composition of media for culturing of pathogen

ABBREVIATIONS

°C	:	Degree Celsius
AFLP	:	Amplified Fragment Length Polymorphism
cM	:	Centimorgan
cm	:	Centimeter
m	:	Meter
CPBMB	:	Centre for Plant Biotechnology and Molecular Biology
CDA	:	Carrot Dextrose Agar
CTAB	:	Cetyltrimethyl Ammonium Bromide
dNTP	:	Di-Nucleotide Triphosphate
DNA	:	Deoxyribose Nucleic Acid
EDTA	:	Ethylene Diamine Tetra Acetic acid
EST	:	Expressed Sequence Tag
FAO	:	Food and Agriculture Organization
GD	:	Genetic Distance
Ha	:	Hectare
ISSR	:	Inter Simple Sequence Repeat
Ds	:	Double stranded
g	:	Gram
KAU	:	Kerala Agricultural University
kb	:	Kilo base
M	:	Molar
mg	:	Milligram
min.	:	Minute
mm	:	Milimeter
ml	:	Milliliter
nm	:	Nanometer

ng	:	Nano gram
μg	:	Microgram
μl	:	Microliter
μm	:	Micromole
pM	:	Pico molar
MAS	:	Marker Assisted Selection
MAB	:	Marker Assisted Breeding
NGA	:	Neo-peptone Glucose Agar
NIL	:	Near Isogenic Line
OD	:	Optical Density
p^H	:	Hydrogen ion concentration
PCR	:	Polymerase Chain Reaction
PDA	:	Potato Dextrose Agar
PCA	:	Potato Carrot Agar
ppm	:	Parts per million
rpm	:	Revolution per minute
%	:	Per cent
RAPD	:	Random Amplified Polymorphic DNA
RIL	:	Recombinant Inbred Lines
RNA	:	Ribonucleic Acid
SSR	:	Simple Sequence Repeat
SCAR	:	Sequence Characterized Amplified region
Sec.	:	Second
QTL	:	Quantitative Trait Locus
V	:	Volt

Introduction

1. INTRODUCTION

Cowpea [*Vigna unguiculata* (L.) Walp.] is one of the most important food legume crops in the semiarid tropics covering Asia, Africa, Southern Europe, and Central and South America. Since it is well known as a drought-tolerant and warm-weather crop, cowpeas are well-adapted to the drier regions of the tropics, where other food legumes do not perform well. It also has the useful ability to fix atmospheric nitrogen through its root nodules. The cowpea is cultivated in an area of around 12.5 million hectares with a production of 6.5 tonnes per hectares (FAO, 2013).

In India, it is exclusively known as a kharif season pulse and the demand of cowpea as vegetable is increasing because of consumption habit of people. All pole type [*Vigna unguiculata* (L.) ssp. *sesquipedalis*] and some semi-trailing type are consumed as vegetable [*Vigna unguiculata* (L.) Walp. ssp. *cylindrica*] and rest are of grain type. These are cheap sources of dietary protein and Lysine (Bresami *et al.*, 1985). Hundred grams of green tender pods contain 4.3 g protein, 2.0 g fiber, 8.0 g carbohydrates, 74 mg phosphorus, 2.5 mg iron, 13.0 mg vitamin-C, 0.9 mg minerals. Thus, it is an important source of proteins for vegetarians as a substituent of meat. In India, cowpea is grown in about 0.5 million ha with an average productivity of 600 to 750 kg grains ha^{-1} (Mandal *et al.*, 2009). Using cowpea in crop rotation and as cover crop has special advantages for enrichment of soil fertility, preventation of soil and water loss due to wind erosion and evapo-transpiration. In Kerala, it is grown as a floor crop in coconut gardens, as an inter-crop in tapioca, fringe crop in rice fields and in garden lands. It is grown throughout India for its long, green vegetable pods, seeds, and foliage for fodder.

The major constraints of farming the pole type vegetable is its susceptibility to various pests and diseases (William, 1975). Anthracnose is one of the most destructive fungal disease caused by *Colletotrichum lindemuthianum* (Sacc. & Magn.). Prassana (1985) has first reported the cowpea anthracnose incidence in India. Losses can be accounted up to 74 per cent (Masangwa *et al.*, 2013), depending on the susceptibility of cultivar especially in the humid climate of tropic and subtropics regions. Most of the vegetable cowpea cultivars having
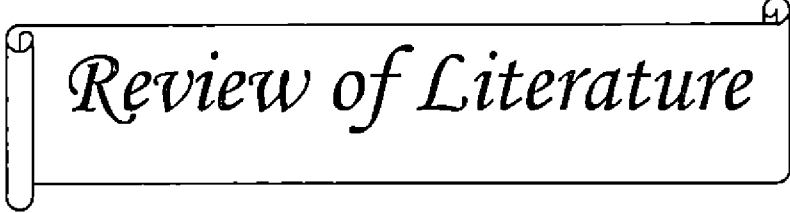
trailing habit are susceptible to anthracnose but contributes higher yield as compared to the bushy or semi-trailing types which offer resistance to this disease. Inefficiency of existing management measures coupled with the multiple races, make this disease highly problematic throughout the world. Since fungicides and chemicals are not fully effective, the transfer of resistance from the bushy type cultivars to trailing types can only be a durable solution. Conventional breeding takes almost 16 years to complete the breeding cycle and to reach farmers. Apart from this, the major problem in conventional breeding is the inability to confirm the presence of resistance gene, since the healthy plant status at field level may be due to escape or unfavorable environmental conditions for the fungus or due to the presence of antagonistic microorganisms in the soil.

Biotechnology offers tool for tagging, characterization and isolation of resistance genes. This can be made possible by suitable combination of molecular markers and breeding methods. One such combination is marker assisted selection (MAS) which is identified as a dependable, reproducible and time saving strategy to confirm the presence of desirable gene and to quicken the breeding cycle. Neither the any gene for cowpea anthracnose resistance is characterized nor its genetics is known, so use of random marker systems is recommended and hence the RAPD and ISSR systems were adopted in the present study.

The well-known strategy followed for the identification of potential markers linked to disease resistance genes is bulked segregant analysis (BSA). BSA is a breeding method reported by Michelmore *et al.*, (1991) which involves comparing two pooled DNA samples of individuals from a segregating population originating from a cross between resistant and susceptible parents, which can reduce the time period as it removes the need for screening of entire mapping population with every primer.

Tagging of anthracnose resistance gene in cowpea through molecular markers will help in marker assisted selection while transferring the same to the genetic background of other susceptible but high yielding varieties.

The objective of the present study was to develop molecular markers for anthracnose disease resistance through bulked segregant analysis method by using RAPD and ISSR markers. Resistant variety Kanakamony and susceptible variety Sharika and segregating F_2 population of Sharika × Kanakamony and Kanakamony × Sharika served as the source of DNA for molecular marker analysis.

Review of Literature

2. REVIEW OF LITERATURE

Anthracnose incited by *Colletotrichum* spp. occurs commonly in a wide variety of legumes including cowpea. Anthracnose which is derived from Greek word meaning 'coal', is characterized by very dark, sunken lesions, containing spores in infected plant (Ogu and Owoege, 2013), causing significant yield losses (Masangwa *et al.*, 2013). Report from Nigeria, which is considered as the place of origin of cowpea, revealed the association of *Colletotrichum* spp. in two different diseases in this crop namely anthracnose and brown blotch (Adegbite and Amusa, 2008). According to Kumar (1999), anthracnose in vegetable cowpea is caused by *C. lindemuthianum* and the infestation is more severe for pole types, mainly affecting the stem of the plant. This disease is reported to cause 35-50 per cent crop loss and if occurs at seedling stage, the loss will be complete (Varma and Langerak, 1988). In India, the incidence of the anthracnose disease was first reported from Maharashtra in 1966 (Rao, 1966). Kumar (1999) has reported that, in Kerala the losses due to this disease may be accounted up to 58.8 per cent. If a susceptible cultivar is growing in a cool, moderate temperature climate with abundant moisture, the loss can be incurred up to 100 per cent (Shao and Teri, 1985). Hence effort was made by Kumar (1999) to tackle the problem, where he screened many cowpea genotypes for resistance to this disease.

2.1 General background

2.1.1 Salient features of cowpea varieties used in this study

2.1.1.1 Kanakamony

Kanakamony is a high yielding dual purpose variety developed by RARS, Pattambi, Kerala Agricultural University, by the pure line selection of Kunnamkulam Local in 1977. The plant is semi-trailing with medium long and dark green pods with an average length of 15-18 cm and yield 2.8 tonnes. It is best suited for kharif and summer seasons and its life cycle is completed almost within 70-80 days. Kumar (1999) reported that this cultivar is immune to anthracnose disease.

2.1.1.2 Sharika

Sharika is also high yielding vegetable type cultivar developed by KAU. The plant is trailing with long and white colour pod with an average length of 40 cm. It is best suited to both kharif as well as rabi season and its lifecycle is completed almost within 80-90 days. It is widely accepted because of its long pods which attract the farmers and also its suitability during harvesting. One of the major constraint in production is high amount of susceptibility towards anthracnose.

2.1.2 Anthracnose symptomology and spread

Anthracnose disease of cowpea was reported for the first time in Islamabad by Quereshi *et al.* (1985).

According to Onesirosan and Barker (1971) and Williams (1975) the lesions become lenticular to circular, brown to tannish pink and sunken with dark red margins on the stem. Later, the lesions develop into large, spreading, dark lesions which griddle stem, branches and petioles. Coalescence of lesions lead to chlorosis and death of the leaves. Brown sunken lesions also appear on pods.

Kumar (1999) studied the detailed symptomatology of the anthracnose disease and has observed that in wet conditions, reddish brown streaks appear on stem. In seedlings, the streaks coalesce, spreading over stem, resulting in wilting of the plant. In mature plants, individual lenticular to oval light brown lesions with dark reddish brown margins appear. Reddish brown streaks appear initially on lower surface of the leaves and later on extend to upper surface also. In few plants, numerous minute black dots appear on leaf lamina. As the disease advance, these lesions coalesce and extend all over the stem and vines, leading to drying up of the stem and veins. Finally leaves become chlorotic and defoliates. The whole plant appears chlorotic and stunted. Reddish brown streaks on the pods results in rotting of the pods and the seeds become shriveled and discoloured. Black fruiting bodies of the fungus could be observed on the dried pods.

The fungus is both soil and seed transmitted. The fungus survives for 2 years in infected thrash or seeds and up to 5 years at controlled temperature of 4°C as mycelia or spores (Enyiukwu *et al.*, 2014). *C. lindemuthianum* survives as

dormant mycelium within the seed coat, sometimes even within cells of cotyledons, as spores between cotyledons or elsewhere in the seed. The fungus survives in the seed as long as the seed remains viable. It also survives in infected crop residues (Zaumeyer and Meiners, 1975).

There are two different reports which support the growth of the fungus. According to Ferrante and Bisiach (1976) the infection is favored by moderate temperatures between 13 and 26°C while Aylesworth and Tu (1980) reported that infection is favored by an optimum temperature of 17-24°C.

Zaumeyer and Thomas (1957) reported that the dissemination, spread and · the development of the conidia and the development of severe anthracnose epidemics is favored by wind or rain. *C. lindemuthianum* required about 10 mm of rain to establish the infection (Tu, 1981). Conidia spread may be dispersed within the crop by insects, animals and man, especially when foliage is moist.

2.1.3 Morpho-cultural characteristics of *C. lindemuthianum*

Anthracnose disease of cowpea is caused by the fungus *C. lindemuthianum*, belonging to Melanoconiales of class Coelomytes under Deuteromycotina. Its perfect stage is *Glomerella lindemuthianum* (Sacc. and Magn.) in family Polystigmataceae, order Sphaeriales and class pyrenomycetes of Ascomycotina (Agrios, 2005).

2.1.4 Anthracnose in major pulses

C. lindemuthianum is hemibiotrophic pathogen that means it produces cell wall degrading enzymes and low molecular weight phytotoxins which participate in killing host cells. Advance invasion of hyphae contribute to the necrotrophic growth of the fungus (Enyiukwu *et al.*, 2014).

In cowpea, this fungus is noticed to be severe under hot and humid climate. It affects all the above ground parts of the plants. The way of infection is hemibiotrophy especially when the seedlings become susceptible (Enyiukwu *et al.*, 2014). After 6-9 hrs. of spore germination, the fungus penetrates through the anticlinal wall of the epidermis by the help of appressoria and infection peg. At 48 hr., infection vesicles form, which becomes multilobed and gives rise to secondary hyphae, then penetrate the host cell wall to initiate necrotrophic phase

exactly at 60 hr. acervuli with a single melanize seta forms at the plant surface after 96 hr. (Shen *et al.*, 2001).

Table 2.1 *Colletotrichum* anthracnose reported from different crops in India

Host	Species identified	References
Vitis vinifera	*C. capsici*	Sawant *et al.* (2012)
Saccharum officinarum	*C. falcatum*	Bukhari *et al.* (2012)
Chlorophytum borivilianum	*C. capsici*	Sattar *et al.* (2005)
Capsicum sp.	*C. capsici*	Paul and Behl (1990)
Boehravia diffusa	*C. gloeosporioides*	Gautam and Bhadauria (2012)
Persea bombycina	*C. gloeosporioides*	Handique *et al.* (2008)
Clusterbean	*C. truncatum*	Kumar and Henry (2005)
Azadirachta indica	*C. truncatum*	Bhanumathi and Rai (2007)
French basil	*C. capsici*	Alam *et al.* (1981)
Sansevieria trifasciata	*C. sansevieriae*	Gautam *et al.* (2012a)
Solanum melongena	*Colletotrichum* sp. and *Fusarium* sp.	Kumar (2011)
Pisonia alba	*C. gloeosporioides*	Vidyalakshmi and Divya (2013)
Murraya koenigii	*C. gloeosporioides*	Basnayake *et al.* (2000)
Momordica charantia	*Colletotrichum* sp.	Ekka and Dubey (2003)
Curcuma longa	*C. gloeosporioides*	Patel *et al.* (2005)
Carica papaya	*C. gloeosporioides*	Bag (2004)
Aloe vera	*C. gloeosporioides*	Avasthi *et al.* (2011)
Capsicum sp. (bell pepper)	*C. gloeosporioides*	Gupta *et al.* (2009)
Morinda citrifolia	*C. gloeosporioides*	Hubballi *et al.* (2012)
Geranium sp.	*C. acutatum*	Sattar *et al.* (2002)
Tuberose	*C. capsici*	Dubey *et al.* (2006)
Capsicum sp. (bird pepper)	*C. coccodes*	Roat *et al.* (2009); Sharma *et al.* (2011)
Pedilanthus tithymaloides	*C. gloeosporioides*	Gautam *et al.* (2012b)
Persea bombycina	*C. gloeosporioides*	Das *et al.* (2005)

Susceptibility of soybean (*Glycine max* L.) to anthracnose was first reported in Korea in 1917 (Akem, 1994). Symptoms usually appear in the early reproductive stages on stems, pods and petioles as irregular brown lesions, which later turn black from presence of acervuli that produce minute black spines (setae) visible to the naked eye. Causal organisms are *C. truncatum* and *C. glycines* leading to necrosis of lamina veins, premature defoliation, pod blanking and shriveled seeds; resulting in 16-26 per cent yield reduction or total crop failure in severe instances. Recently involvement of *C. gloeosporioides* was reported in Malaysia as another causal organism for this disease. (Mahmodi *et al.*, 2013).

Bean (*Phaseolus vulgaris* L.) anthracnose is caused by the fungus *C. lindemuthianum* (Sacc. and Magn.). This pathogen has been reported to be associated with anthracnose of mung bean (*Vigna radiata* L.), cowpea (*Vigna unguiculata*) and broad bean (*Vicia faba*) (Mohammed, 2013). Under favorable climatic condition, field losses are up to 90 per cent due to seedling, leaf, stem and pod infections. Dark brown necrotic lesions on leaves decrease photosynthetic area, early leaf senescence and plant death, ultimately reducing the yield (Mohammed, 2013).

Pigeon pea (*Cajanus cajan* L.) anthracnose is caused by *Cajanus cajani*. During rainy season, the disease becomes more destructive, affecting leaves, pods and seeds. Symptoms include spots on pods and leaves, blackening and shriveling of veins of infected leaves, defoliation and seed discoloration. Generally affected plants, show up to 86 per cent pods infection and 36 per cent unmarketable seeds (Thakur *et al.*, 2011).

2.2 Mechanism of host plant resistance to *Colletotrichum*

Plants have developed sophisticated and efficient mechanism to prevent the invasion of their tissues by pathogen, and disease rarely occurs. In a study by Torregrossa *et al.* (2004) after symptom analysis and cytological studies, it was shown that the resistance of cv. Jemalong was associated with a hypersensitive response of the plant which is a common feature of the of disease resistance achieved through the death of immediately surrounding cells at the infected sites. Hypersensitive response of resistant bean cultivars was the appearance of red-

brownish wounds of different sizes, to check the spread of the pathogenic fungus. (Elliston *et al.*, 1976).

The study of Chakraborty and Datta (2003) revealed the loss of aggressiveness of the fungal anthracnose pathogen *C. gloeosporioides* due to increase in elevated CO_2 level. The experimental analysis was on inoculated cultivars Fitzroy (susceptible) and Seca (resistant) of *Stylosanthes scabra* by subjecting them to CO_2 level of 350 and 700 ppm.

Inducible defense mechanism such as accumulation of host-synthesized phytoalexin antibiotics, deposition of lignin-like material, accumulation of hydroxyl proline like glycoprotein (HRGPs) and proteinase inhibitors, and increases in the activity of certain hydrolytic enzymes such as chitinase are the different features of resistant plants (Sequeira, 1983).

2.3 Different media favorable for the growth of the fungi

According to Kumar (1999) isolates obtained from different places in Kerala are having almost similar colony and spore characters of *C. lindemuthianum* as per the study of Mordue (1971), except from Kanakkari area. The pathogen has shown some of the common characters in different media as described below.

a) Potato dextrose agar medium

Kumar (1999) described the slow growth of colonies on PDA medium. First, they became grey color and later, rapidly turned darker with compact aerial mycelium. Pink pigmentation, appressoria and chlamydospore were failed to develop but setae were properly developed. Less sporulation i.e. 5.5×10^2 spores ml^{-1} were measured.

b) Potato carrot agar medium

Colony growth on PCA medium was faster as compared to PDA. Whitish grey colonies with compact fluffy mycelium, dark pink pigmentation were well noticed, and then the whole colony turned darker with well developed setae and dark brown appressoria. Good amount of sporulation, nearly 3.9×10^4 was observed.

c) Neo-peptone glucose agar medium (selective medium)

Using the selective medium faster growth of colonies in comparison with the above two media was achieved within 7 days, at room temperature. Colonies with sparse whitish mycelium, light pink pigmentation, abundant setae, presence of appressoria and intercalary chlamydospore, with no black discoloration were observed.

Watnabe (2010) has reported that *C. lindemuthianum* produce acervuli only on natural media containing bean straws and agar. The acervuli in culture was pale brown, hemi-spherical composed of conidiophores, conidia and indistinct setae. Conidiophores were hyaline, simple or branched, erect, bearing 2-3 conidia at phialicles. The conidia were phialosporous, hyaline, single celled and cylindrical. Setae were brown, thick walled and sharp at the apex having 2-3 setae. The dimensions of the conidia were 13-17.5×4.7-5.3 μm in diameter and the setae was 65-107.5×3.7-6 μm.

2.4 Breeding of legume cultivars for anthracnose resistance

2.4.1 Conventional breeding for anthracnose resistance in legumes

Pathania *et al.* (2006) reported the inheritance of genes conferring resistance to different Indian races of anthracnose pathogen from crosses involving cvs. of kidney bean Jawala × KRC-5, Kanchan × KRC-5, Jawala × G 2333, Kanchan × G 2333, Kanchan × G 2333, Jawala × G 2333 and Kanchan × G 2333 revealed different ratio of 3:1, 3:1, 15:1, 15:1, 15:1 and 15:1, which involve single dominant and two independent dominant genes.

Goncalves-Vidigal *et al.* (2012) reported the presence of a single dominant gene which is conferring resistance to anthracnose in dry bean cultivar Pitanga by crossing the cultivars independently with susceptible cultivars. Subsequently F_1 and F_2 populations were screened and *Co-14* had different genes and alleles than those were already characterized in other common bean cultivars.

Resistance was identified in Ethiopian white lupin germplasm which were extensively used in the crosses with the superior exiting lines. Selection was done in the F_4/F_5 generations by SSD method. The resistant lines developed were Tanjil, P27174, Andromeda and Amira (Adhikari *et al.*, 2013).

A study conducted by Torregrossa *et al.* (2014) to establish a relationship between *Medicago truncatula* and *C. trifolii* by screening of a few *M. truncatula* lines, has identified the two cultivars Jemalong and F83005.5 as resistant and susceptible respectively to *C. trifolii* race 1.

Yang *et al.* (2012) reported presence of *RCT1* gene in *Medicago truncatula* which encodes a TIR-NBSLRR type R protein that confers broad-spectrum anthracnose resistance when transferred into the susceptible alfalfa plants. This gene is analogous to *An1* which is an independent dominant gene conferring resistance to *C. trifoli* race 1 (Elgin and Ostazeski 1985; Mackie *et al.*, 2003).

Chi-square analysis on $F_{7:8}$ recombinant inbred lines of *Lens ervoides* obtained from the cross between susceptible *L. culinaris* cultivar, Eston, and a resistant accession of *L. ervoides* cv., L-01-827A has shown that the resistance to two races of anthracnose is governed by two recessive genes *ct1* and *ct2* (Fiala *et al.*, 2009).

In India, work was conducted by taking 16 genotypes which are widely grown in different places of the country. Genotypes such as JS-71-0S, JS-72-280, PK472, JS-335 were found to be immune, and cvs. JS-72-44, JS-7S-46, PK-262, JS-33S. MACS-S8,VLS-2, Panjab-1, Bragg, Monetta, KHSb-2, NRC-7, VLS-S8, Shivalik were highly resistant, according to the scale given by Mayee and Datar (1986) and Shrirao *et al.* 2009.

2.4.2 Molecular breeding for anthracnose resistance

Goncalves-Vidigal *et al.* (2011) performed linkage mapping to establish the inheritance pattern of the *Co-1* gene with the *Phg-1*, which were found linked with two molecular markers, CV542014[450] and TGA1.1[570] and reported to be the resistance genes to anthracnose and angular leaf spot diseases in common bean.

Genetic mapping of the resistance allele *Co-5²* to *C. lindemuthianum* in the common bean MSU 7-1 line has established the linkage of *g1233* at a distance of 1.2 cM (Sousa *et al.*, 2014). Schwartz *et al.* (1982) described another locus on Pv07, named *Co-6* in the cv. AB136, and linked with the molecular marker OPZ04. This marker was converted to SCAR Z04 (Mendez-Vigo *et al.*, 2002;

Kelly *et al.*, 2003; Queiroz *et al.*, 2004). SCAR markers SB12^{350} linked to resisitance loci *Co-3/9* and SQ4^{1440} and SCH20^{1000} to *Co-2* respectively were developed in common bean by Ferreira *et al.* (2012).

The linkage of three SCAR markers SCARY20, SCARZ20, SCARF10 with *Co-4, Co-6, Co-10* were reported by Rocha *et al.* (2012) who screened five populations of carioca common bean families after crossing with resistant line Ruda-R.

RAD (restriction-site associated DNA) sequencing method for development of cost effective molecular marker was successfully done in lupin cv. Tanjil (*Lupinus angustifolius* L.) by Yang *et al.* (2012). They identified a single dominant gene *Lanr1* conferring resistance to anthracnose through NGS technology. Five putative markers AnSeq1, AnSeq2, AnSeq3, AnSeq4 and AnSeq5, closely linked at a distance of 0.9 cM were identified.

Tullu *et al.* (2003) performed BSA on RIL population derived from cross between PI 320937 and Eston lentil where they analyzed RAPD, ISSR and AFLP molecular markers linked with the single dominant gene locus *Lct-2. A*. Three AFLPs EMCTTACA and EMCTTAGG and EMCTAAAG flanking markers, RAPD OPE06 (6.4 cM) and ISSR UBC 704 (10.5 cM) were found to be linked with the locus.

2.5 Molecular Markers for tagging disease resistant genes

The process of locating genes of interest via linkage of markers is referred as "gene tagging" (Chawla, 2002). RAPD, RFLP, SSR, AFLP and ISSR markers are suitable for tagging major genes for the character of interest.

2.5.1 RAPD (Random Amplified Polymorphic DNA) for tagging the disease resistance gene

Winter *et al.* (2000) found 17 RAPDs markers linked to Fusarium wilt resistance of chick pea from 130 recombinant inbred lines derived from a *Cicer arietinum × Cicer reticulatum*.

Reddy *et al.* (2009) performed bulk segregant analysis on a segregating population of ICPL 7035 × ICPL 8863 for identification of RAPD markers associated with pigeon pea sterility mosaic disease (PPSMD) resistance. The

primer OPA 18 revealed polymorphism between the parents and the resistant and the susceptible bulks, which was converted into SCAR marker for identification of PPSMD resistant plants in the segregating population.

Kotresh *et al.* (2006) used bulk segregant analysis with 39 RAPD primers which led to identification of two markers (OPM03 and OPAC11) which were associated with *Fusarium* wilt susceptibility allele in a pigeonpea F_2 population derived from cvs. GS1 × ICPL87119.

Arruda *et al.* (2000) have identified two putative markers OPY20 and OPAS 13 tightly linked with anthracnose resistance genes *Co-4* and *Co-4²* in cultivars G2333 and Selection 1308, respectively. Based on these studies Silva *et al.* (2007) identified a putative markers OPAS 13 tightly linked with the cultivar PI 207262 by using a F_2 derived F_3 population ($F_{2:3}$) obtained from a cross between PI 207262 and Ruda.

Katoch *et al.* (2010) identified a powdery mildew resistance gene *er2* in pea that was associated with a RAPD marker OPX 17. For further enhancing the reproducibility, it was converted in to SCAR marker. They had used F_2 population derived from Lincoln × JI2480.

Table 2.2. List of the genes/loci which are reported to offer resistance to anthracnose disease and their respective markers

Gene/ Allele	Linked Marker	Crop	Reference	Sequence (5'-3')
Co-1	CV542014[450]	Common bean	Goncalves-Vidigal *et al.* (2011)	F:CACTTTCCACTGACGGTTT GAACC R:GCACAAGGACAAGTGGT CTGG
Co-2	SCH20[1000] and SQ4[1440]	Common bean	Ferreira *et al.* (2012)	SCH20 F:GGGAGACATCCATCAGACA ACTCC R:GGGAGACATCTTCATTTGA TATGC SQ4 F:CCTTAGGTATGGTGGGAAA CGA R:TGAGGGCGAGGATTTCAGC AAGTT
Co-3/9	SB12[350]	Common bean	Ferreira *et al.* (2012)	F:CCTTGACGCACCTCCATG R:TTGACGCATGGGTTGGCC

Co-4	SCARY20[830]	Common bean	Rocha et al. (2012)	F:AGCCGTGGAAGGTTGTCAT R:CCGTGGAAACAACACACA AT
Co-6	SCARAZ20[940]	Common bean	Rocha et al. (2012)	F:ACCCCTCATGCAGGTTTTA R:CATAATGCATTCATGCTCA CC
Co-6	SCAR Z04[567]	Common bean	Schwartz et al. (1982); Freyre et al. (1998)	F:GGCTGTGCTGATTAATTCT GG R:TGCTCATTTTATAATGGAG AAAAA
Co-5, Co4, Co-7	g1233	Common bean	Sousa et al. (2014)	F:GGCAAAAGAGGAAAAGAA GGAAGGAC R:CTCTTCCACACTTACGGCA CTG
Co-10	SCARF10[1050]	Common bean	Rocha et al. (2012)	F:GGAAGCTTGGTGAGCAAG GA R:GGAAGCTTGGCTATGATGG T
Co-14		Pitanga dry bean	Goncalves-Vidigal et al. (2012)	Sequence are not reported
R	AnSeq3 and AnSeq4	Lupin	Yang et al. (2012)	Sequence are not reported

2.5.2 ISSR marker for tagging disease resistance genes

Ratnaparkhe et al. (1998) were the first to demonstrate that ISSR markers are useful in gene tagging and can be used for finding markers linked to the gene of interest.

Bainade et al. (2014) had undertaken a study for tagging gene for resistance to powdery mildew in green gram cultivar BPMR-48 through a crossing progarmme arranged with the cultivar Kopargaon which reported as susceptible. Out of 75 ISSR markers 54 were polymorphic in both the accessions and only one marker UBC 834 was found polymorphic between the parents as well as resistant and susceptible bulk, indicating its possible linkage in BSA of F3 population.

Souframanien and Gopalakrishna, (2006) found the linkage of ISSR 811 marker with the gene governing mung bean Yellow Mosaic Virus (MYMV) in vigna, at a distance of 6.8 cM. They developed a RIL population of F8 generation for screening mung bean Yellow Mosaic Virus obtained from the cross between

Vigna mungo (cv. TU 94-2) with *Vigna mungo* var. *silvestris*. Later the ISSR811 marker was sequenced and SCAR primers were designed (YMV1-F and YMV1-R) to amplify the marker for screening the RIL population to distinguish resistant and susceptible genotypes differing in their MYMV response.

2.5.3 Bulked Segregant Analysis to tag the genes of interest

BSA is a method to identify molecular markers linked to gene of interest without having to construct a map of the genome (Michelmore *et al.,* 1991). Instead of screening dozens of individuals to test for linkage, DNA bulks with fewer than 10 individuals are normally bulked with selected polymorphic primers saving time and resources. Another advantage of BSA is that only polymorphic bands potentially linked to the gene of interest are scored. Two bulk DNA sample are generated form a segregating population usually from F_2 population. Each pool or bulk contains individuals that are identical for a particular trait or genomic region but arbitrary at all unlinked regions. The bulked are screened for differences using selected polymorphic primers obtained after the screening of parental population. BSA removes the need for screening the entire mapping population with every primer. It has several advantages over the use of near-isogenic lines to identify markers in specific region of the genome.

Avila *et al.* (2003) used BSA to identify the first molecular markers $OPI20^{900}/OPL18^{1032}$ associated with the gene *Uvf-1*, controlling a race-specific resistance to rust in faba bean.
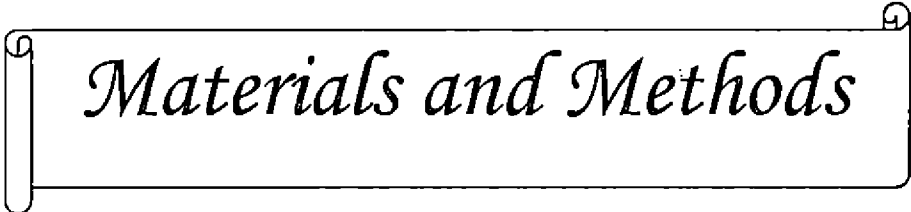
Roman *et al.* (2003) utilized the technique of BSA and found out the markers linked with *Ascochyta* blight in faba beans. F_2 from two susceptible and resistant varieties Vf6 and Vf136 revealed the linkage of $OPA11^{1045}/OPAB07^{1026}$ OPE17/OPJ18 RAPD markers with the genes *Af1* and *Af2*. The same work was done by Avila *et al.* (2003) after screening the F_2 obtained from the cross between 29H and Vf136 resistant and susceptible varieties respectively to find the markers OPD16, OPG04, OPJ18 and OPG11.

Gutierrez *et al.* (2006, 2007 and 2008) found out the SCAR markers linked with the seed quality traits of faba bean in terms of zero tannins and low vicine-

convicine where the genes involved were *zt1*, *zt2* and *vc*, linked with the markers SCC5/SCG11 SCAD16-B/SCAD16-H and SCH01/SCAB12.

Janila and Sharma (2004) have shown the linkage between the SCAR marker ScOPD-10 and the powdery mildew resistance gene at 3.4 cM in field pea.

Through MAS, Ek *et al.* (2005) identified five linked SSR markers namely PSMPSAD60, PSMPA5, PSMPSAA369 and PSMPSAD51 with powdery mildew resistance in field pea. They screened a total of 192 F_2 progenies obtained after crossing of one resistant cultivar 955180 and susceptible Majoret.

Materials and Methods

3. MATERIALS AND METHODS

The research work on development of molecular markers for anthracnose resistance in vegetable cowpea was carried out at Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Kerala Agriculture University, Vellanikkara, Thrissur. The methodology and materials used are described in this chapter.

3.1 Materials

3.1.1 Plant materials

The two cowpea varieties Kanakamony and Sharika were selected for the study (Plate 3.1). Of these, Kankamony is semi-trailing type [*Vigna unguiculata* (L.) Walp. ssp. *cylindrica*] and reported to be immune whereas Sharika is a pole type [*Vigna unguiculata* (L.) Walp. ssp. *sesquipedalis*] and considered as susceptible to anthracnose disease.

These varieties were used in controlled breeding to develop the mapping population to identify the markers for the gene governing resistance. F_1 population was selfed to obtain the F_2 seeds. The F_2 population acted as the segregating population. From this candidate plants were identified and used in BSA.

3.1.2 Laboratory chemicals, glasswares and equipment items

The chemicals used in the study were of good quality (AR grade) procured from Merck India Ltd., HIMEDIA and SISCO Research Laboratories. The *Taq* DNA polymerase, dNTPs, Taq buffer and primers used in this study were supplied by Bangalore Genei Ltd. or Sigma. All the plasticwares were obtained from Axygen and Tarson India Ltd. ISSR and RAPD primers were obtained from Sigma Aldrich Chemicals Pvt. Ltd.

Centrifugation was done in High speed refrigerated centrifuge (KUBOTA 6500). NanoDrop® ND-1000 spectrophotometer was used for the estimation of quality and quantity of DNA. The DNA amplification was carried out in Proflex (Applied Biosystem) and Agilent themal cycler. Agarose gel electrophoresis was performed in horizontal gel electrophoresis unit (BIO-RAD, USA).

3.2 Methods

3.1.2 Raising the parental population for the development of mapping population

The variety Kanakmony was raised on the sides of the ridges. The ridges and furrows were taken at a spacing of 60 cm. On one sides of the ridges two seeds each were placed at 60 cm spacing. The basal fertilizers as per the package of practices recommendations and FYM were added to the soil at the time of field preparation.

Both the varieties were raised in an area of 5 cents each. The semi trailing Kanakamony was permitted to trail on the ground where as pandal was raised to support the trailing Sharika variety. Standard crop production techniques were followed as per the Package of Practices recommendation of KAU.

3.2.2 Hybridization and development of F_1 seeds

F_1 population was developed by crossing the parents. A reciprocal cross was made between the anthracnose resistant genotype Kanakamony and susceptible genotype Sharika to develop two lines of F_1. For crossing, flowers of the female parents were emasculated at 5:00-6:00 pm on the day prior to anthesis and covered with butter paper cover. Such flowers were pollinated on the next day morning 6:30- 7:30 am, using the pollen from fresh opened flower from the male plant. The pollinated flowers were covered with butter paper cover and tagged, indicating the cross and date of pollination. A total of six cross pods were obtained.

After 4 to 6 days, the butter paper covers were removed. These pods were harvested at maturity and pods and seeds were collected.

3.2.3 Production of F_2 seeds by selfing

The F_1 plants were raised in open field during January-April 2014 at a spacing of 2 m × 2 m by following the standard package of practices. Seeds from 4 pods of Sharika × Kanakamony and 2 pods of Kanakamony × Sharika were used to raise the F_1 crop. Seeds from different pods were sown in 6 different rows. Since it is a self-pollinated crop, F_1 plants were left as such to get seeds by self-pollination. Subsequently the F_1 were selfed to obtain the seeds for F_2 generation.

a. Kanakamony (Bushy type – Immune to anthracnose)

b. Sharika (Pole type – Susceptible to anthracnose)

Plate 3.1. Cowpea varieties selected for the study

3.2.4 Morphological characteristics of parents and F_1 progenies

Morphological characters of F_1 progenies obtained from Sharika × Kanakamony and reciprocal crosses in comparison with parents were observed. The morphological parameters were recorded as per the minimal description for cowpea hybrid. The characters were recorded.

1. Days to flowering

The number of days from date of planting to the appearance of first flower was recorded.

2. Growth habit

Plants were classified in two groups such as trailing and semi-trailing according to the growth pattern.

3. Plant height

The plant height has been measured from ground level to tip of the plant at the time of crop senescence and plant height was expressed in centimeters.

4. Leaf area

The middle leaflet of each trifoliate leaf was selected and at least 5 leaves from each plant were measured. The leaf area was calculated (cm^2) by using graph paper method.

5. Pod length

Pod length (cm) was recorded for the parents and the F_1s by measuring the length of at least 10 pods of individual plant.

6. Pod weight

The weight (g) of ten freshly harvested mature pods was taken each parent and F_1s and the mean pods weight was identified.

7. Number of pods per plant

Pods were harvested periodically from each plant and recorded on the number of pods from each plant was maintained.

8. Number of seeds per pod

Ten pods from each parent and F_1s were harvested at random and the mean number seeds per pod were recorded.

9. **Seed colour**

Seed colour at maturity was recorded in both the parents in F_1 lines.

10. **Yield per plant**

The total vegetative pod yield (gm) was recorded from each parent from F_1 lines. The yield of all the harvests was added.

3.2.5 Raising of F_2 segregating population

The crops were raised under greenhouse condition in pots during March 2015. The green house at Department of Plant Pathology, College of Horticulture, Kerala Agricultural University, was used for this purpose. Seventy pots were filled with well sterilized soils and decomposed cow dung. To achieve maximum segregation in F_2, eight pods from individual F_1 were selected and from each pod a single seed was sown for F_2. A total of four seeds were sown in each pot. So for single plant of F_1 total two pots were sown. Out of seventy pots four pots were sown with parent seeds. Remaining sixty six pots were used for sowing F_2 plants. Totally 264 seeds were sown, out of which 163 viable seedlings were maintained properly for artificial screening of anthracnose resistance/ susceptibility.

3.2.6 Isolation, identification and maintenance of the pathogen

Anthracnose infected samples of cowpea were collected from Central Nursery field and brought to the Plant Pathology laboratory, washed with tap water and dried with blotting paper. Isolation of the pathogen was carried out using following standard protocol.

The infected stem portions were cut into small bits of 5 mm size and then surface sterilized with one per cent sodium hypochlorite for one minute and rinsed with three changes of sterile water. The bits were then transferred aseptically to sterile Petriplates containing potato dextrose agar, carrot dextrose agar (CDA) and neopeptone glucose agar media were amended with streptomycin sulphate separately. Petriplates were incubated at room temperature ($26\pm2^\circ C$) and examined daily for the growth of the pathogen for 7 days. The fungus developed on NGA and CDA were purified and maintained in the refrigerator for further

studies. The cultured and morphological characteristics of the fungus were observed.

3.2.7 Inoculum preparation

Secondary culture of 7 days old pathogen which was grown on carrot agar medium in Petriplates was used for the preparation of inoculum. The inoculums were prepared by scrapping off the mycelium and spores from the medium using a sterile glass slide and suspending in 100 ml sterile distilled water. The concentration of the inoculum was then adjusted to 10^6 spores ml^{-1} by following serial dilution method.

3.2.8 Artificial inoculation on plants

Artificial inoculation was carried out in the morning before 8:30 AM in the greenhouse on 15 days old plants. Each plant was sprayed with 20 ml of spore suspension. Then the plants were covered with moistened polythene covers and the pots were kept in basins containing water to maintain high humidity (Plate 3.2). Plants sprayed with sterile distilled water served as control.

Observations on disease incidence were initially assessed at 10 days after inoculation and finally the plant were left for 2 weeks for the symptoms to be well expressed.

3.2.9 Molecular analysis to identify the marker

Molecular analysis of the parent genotypes, F_1 and F_2 plants carried out with two different marker systems - Inter Simple Sequence Repeats (ISSR), Random Amplified Polymorphic DNA (RAPD).

3.2.9.1 Genomic DNA extraction

Young tender, pale green leaves were collected in the morning hours on ice from each of the populations. The surface was cleaned by washing with sterile water and wiping with 70 per cent alcohol. The fresh leaves were ground into a fine powder in liquid nitrogen along with β-mercaptoethanol and PVP using sterile ice-cold mortar and pestle, in order to prevent browning due to phenol oxidase activity.

Plate 3.2. Artificially inoculated F_2 plants covered with moistened polyethylene cover inside the green house

Among the most commonly used protocols, CTAB method developed by Doyle and Doyle (1990) was used for the extraction of genomic DNA. Details are as follows.

3.2.9.1.1 Reagents

I. CTAB buffer (2X):

- 2 per cent CTAB (w/v)
- 100mM Tris (pH 8.0)
- 20mM EDTA (pH 8.0)
- 1.4M NaCl
- 1 per cent PVP

II. 10 per cent CTAB solution:

- 10 per cent CTAB (w/v)
- 0.7M NaCl

III. TE buffer:

- 10mM Tris (pH8)
- 1mM EDTA

IV. Chloroform: Isoamyl alcohol (24:1 v/v)

V. Isopropanol (100 %)

VI. Ethanol 70 per cent and 100 per cent

VII. Sterile distilled water

3.2.9.1.2 Procedure

➤ 0.2 gram of clean leaf tissues was ground in presence of liquid nitrogen in pre-chilled mortar and pestle in 10µl of β-mercaptoethanol (100%) and a pinch of Poly Vinyl Pyrrolidone (PVP).

➤ The homogenized sample was transferred into an autoclaved 2ml centrifuge tube and 1ml of pre-warmed extraction buffer was added (total 1ml).

➤ The contents were mixed well and incubated at 65°C for 20 to 30 min. with occasional mixing by gentle inversion.

➤ After the completion of incubation, the sample was taken out and equal volume of (1ml) of chloroform: Isoamyl alcohol (24:1) was added and mixed by gentle inversions to emulsify. Spun at 12,000 rpm for 15 minutes at 4°C

> After centrifugation, the contents got separated into three distinct phases.
  - Aqueous topmost layer   - DNA and RNA
  - Interphase   - Fine particles and proteins
  - Lower layer   - Chloroform, pigments

> The tubes were carefully taken out from the centrifuge without disturbing the different layers. Transferred the top aqueous layer to a clean centrifuge tube and added 1/10th volume of 10 per cent CTAB solution and equal volume of Chloroform:Isoamyl alcohol (24:1) and gently mixed by inversions.

> The contents were centrifuged at 12,000 rpm for 15 minutes at 4°C.

> The aqueous phase was transferred into a clean centrifuge tube and 0.6 volume of chilled isopropanol was added and mixed by quick gentle inversions till the DNA got precipitated. The tubes were kept at -20°C for half an hour for complete precipitation of DNA.

> After the expiry of time the tubes were taken out and centrifuged at 10,000 rpm for 05 minutes at 4°C. The pellet at the bottom of the centrifuged tube was observed and the supernatant was gently poured off.

> The DNA pellet was washed with 70 per cent ethanol followed by 100 per cent ethanol. Spun for 5 min at 10,000 rpm and decanted the ethanol.

> The pellet was left for air drying for 10 to 15 minutes under room conditions with the open tube kept upside down.

> The pellet was dissolved in70μl of sterilized water and stored at -20°C.

3.2.9.1.3 Quality and quantity checking of extracted DNA

(I) Agarose gel electrophoresis

The quality of isolated DNA was evaluated through agarose gel electrophoresis and spectrophotometer.

Reagents and equipments

1. Agarose - 0.8 per cent (for genomic DNA)
2. ·50X TAE buffer (pH 8.0)
   - 10 ml Tris buffer (1 M)
   - 45 ml glacial Acetic acid
   -· 2 ml M EDTA (0.5 M)

3. Tracking/loading dye (6X)

4. Ethidium bromide (stock 10 mg/ml; working concentration 0.5 µg/ml): 5 µl/100 ml of agarose gel composition of reagents is provided in Annexure II

Procedure

➢ The gel tray was prepared by sealing the ends with tape. Comb was placed in gel tray about 1 inch from one end of the tray and positioned the comb vertically such that the teeth are about 1 to 2 mm above the surface of the tray.

➢ Agarose (0.8 per cent, 0.8 g in 100 ml) was prepared in a glass beaker or conical flask with 100 ml 1X TAE buffer. Microwaved for 45 to 60 seconds until agarose was dissolved and solution was clear.

➢ Solution was allowed to cool to about 42 to 45°C and 4 µl Ethidium bromide was added at this point and mixed well.

➢ This warm gel solution was poured into the gel tray to a depth of about 5 mm. and allowed to solidify for about 30 minutes at room temperature.

➢ The comb was subsequently removed and the tape used for sealing removed, the tray was placed in electrophoresis chamber, and covered (just until wells are submerged) with TAE buffer (the same buffer used to prepare the agarose).

➢ To prepare samples for electrophoresis, 1 µl of 6X gel loading dye was added for every 5 µl of DNA solution. Mixed well and loaded 6 µl DNA sample per well.

➢ Electrophoresis was carried out at 70 volts until the dye has migrated two third the length of the gel. On completion of the electrophoresis, the gel was documented in a gel doc machine.

➢ Intact DNA appeared as orange fluorescent bands. The degraded was appeared as smear. The gel profile was examined for intactness, clarity of DNA band, presence of RNA and protein.

(II) Gel documentation

Gel documentation was done with BioRad gel documentation system using PDQuest™ software. PDQuest is a software package for imaging, analyzing

and data basing the gels. An image of a gel was captured using the controls in the imaging device window and displayed on computer screen. .

(III) Assessing the quality and quantity of DNA by spectrophotometer

The purity of DNA was further checked using The NanoDrop ND-1000 spectrophometer. Nucleic acids show absorption maxima at 260 nm whereas proteins show peak absorbance at 280 nm. Absorbance was recorded at both wavelengths and purity was indicated by the ratio OD_{260}/OD_{280}. The values between 1.8 and 2.0 indicated that the DNA is pure and free from proteins. The quantity of DNA in the pure sample was calculated using the relation 1 OD_{260} equivalent to 50 ng double stranded DNA/ml sample.

1 OD at 260 nm = 50 ng DNA/ml

Therefore OD at 260 × 50 gives the quantity of DNA in ng/ml.

Procedure for quantity detection using NanoDrop

➢ NanoDrop spectrophotometer was connected to the System and the software ND-1000 was opened.

➢ The option Nucleic acid was selected.

➢ With the sampling arm open, 1 μl distilled water was pipetted onto the lower measurement pedestal.

➢ The sampling arm was closed and a spectral measurement was started using the operating software. The sample column was automatically drawn between the upper and lower measurement pedestals and the spectral measurement was made.

➢ The reading was set to zero with blank sample.

➢ 1 μl of the sample was pipetted onto measurement pedestal and measure was selected.

➢ When the measurement was complete, the sampling arm was opened and the sample was wiped from both the upper and lower pedestals using a soft laboratory wipe. Simple wiping was sufficient to sample carryover in successive measurements for samples varying by more than 1000 fold in concentration.

3.2.9.1.4 Purification of DNA

The DNA which had RNA contaminant was purified by RNase treatment, further precipitated, washed and air dried.

Reagents

- ➤ Phenol: Chloroform mixture (24:1, v/v)
- ➤ Chilled isopropanol (100 %)
- ➤ 70 per cent ethanol
- ➤ TE buffer
- ➤ Chloroform: Isoamyl alcohol (24:1, v/v)
- ➤ 1 per cent RNase

One per cent solution was prepared by dissolving RNase (Sigma, USA) in TE buffer at 100°C for 15 minutes. The solution was cooled to room temperature, dispensed into aliquots and stored at -20°C.

Procedure

- ➤ To 100 µl DNA sample, 1 per cent RNase solution (2 µl) was added and incubated at 37°C in dry bath for 1 hr.
- ➤ The volume was made up to 250 µl with distilled water.
- ➤ Equal volume of chloroform: isoamyl alcohol (24:1) mixture was added and mixed gently.
- ➤ Centrifuged at 12,000 rpm for 15 minutes at 4°C.
- ➤ The aqueous phase was transferred into a fresh micro centrifuge tube and equal volume of chloroform: isoamyl alcohol (24:1) was added.
- ➤ Centrifuged at 12,000 rpm for 15 minutes at 4°C.
- ➤ The aqueous phase was transferred into a clean centrifuge tube and 0.6 volume of chilled isopropanol was added and mixed by quick gentle inversions till the DNA gets precipitated. The tubes were Kept at -20°C for half an hour for complete precipitation.
- ➤ The mixture at -20°C for 30 minutes and centrifuged at 10,000 rpm for 15 minutes at 4°C.
- ➤ The DNA pellet was washed with 70 per cent ethanol.

> The pellet was air dried and dissolved in 50 to 100 µl sterilized water.

> The samples were loaded on 0.8 per cent agarose gel at constant voltage of 100 V and the quality of DNA tested. The shearing during RNase treatment was also checked.

3.2.10 Molecular markers for identification of marker

RAPD and ISSR marker system were used in this study to identify the markers linked with genes governing resistance to anthracnose disease in vegetable cowpea.

3.2.10.1 Reaction mixture for thermal cycling

The PCR condition required for effective amplification in RAPD and ISSR analysis included appropriate proportions of the component of the reaction mixture. The reaction mixture comprised of template DNA, assay buffer A or B, $MgCl_2$, Taq DNA polymerase, dNTPs and primers. The aliquot of this master mix were dispensed into 0.2 ml PCR tubes. The PCR was carried out in Thermal Cycler (Proflex from Applied Biosystems and Surecycler from Agilent).

The thermocycler was programmed for desired number of cycles and temperatures for denaturation, annealing and polymerization, based on the number of nucleotides in the primer and method of assay.

3.2.10.1.1 Initial screening of RAPD primers

Genomic DNA at the concentration of 30-35ng was amplified using selected random primers as per the protocol given by Zannou et al. (2008). Initially, 47 random decamer primers were screened and out of them 33 were selected.

The PCR amplified products were electrophoresed on 1.2 per cent agarose gel stained with ethidium bromide using 1X TAE buffer along with marker (100-bp DNA ladder). The profile was visualized under gel documentation unit and docked for further analysis. The documented RAPD profiles were carefully examined for amplification.

PCR amplification was performed in a 20 µl reaction mixture and the composition of the reaction mixture consisted of

a)	Genomic DNA (30 ng)	:	2.0 µl
b)	10X Taq assay buffer B	:	2.0 µl
c)	dNTP mix (10 mM each)	:	1.0 µl
d)	Taq DNA polymerase (3U)	:	0.4 µl
e)	Primer (10 pM)	:	1.5 µl
f)	Autoclaved distilled water	:	13.1 µl
	Total volume	:	20.0 µl

The amplification was carried out with the following programme.

a)	94°C for 4 minutes	:	Initial denaturation
b)	94°C for 45 seconds	:	Denaturation
c)	38°C to 45°C for 1 minutes	:	Primer annealing
d)	72°C for 2 minutes	:	Primer extension
e)	72°C for 8 minutes	:	Final extension
f)	4°C for infinity to hold the sample		

35 cycles (Denaturation, Primer annealing, Primer extension)

Table 3.1. List of RAPD decamers primers used for screening of parents

Sl. No.	Name of primers	Sequence of primers (5'-3')	Sl. No.	Name of primers	Sequence of primers (5'-3')
1	OPA 01	CAGGCCCTTC	25	OPC-01	TTCCCAGCAG
2	OPA-02	TGCCGAGCTG	26	OPC-02	GTGAGGCGTC
3	OPA-03	AGTCAGCCAC	27	OPC-05	GATGACCGCC
4	OPA 04	AATCGGGCTG	28	OPC-07	GTCCCGACGA
5	OPA 05	AGGGGTCTTG	29	OPC-12	TGTCATCCCC
6	OPA-07	GAAACGGGTG	30	OPC-13	AAGCCTCGTC
7	OPA-08	GTGACGTAGG	31	OPC-15	GACGGATCAG
8	OPA-09	GGGTAACGCC	32	OPC-19	GTTGCCAGCC
9	OPA-10	GTGATCGCAG	33	OPC-20	ACTTCGCCAG
10	OPA-12	TCGGCGATAG	34	OPD-07	TTGGCACGGG

11	OPA-14	TCTGTGCTGG	35	OPD-08	GTGTGCCCCA
12	OPA-27	GAAACGGGTG	36	OPD-10	GGTCTACACC
13	OPAH-1	TCCGCAACCA	37	OPD-14	CTTCCCCAGG
14	OPAH-3	GGTTACTGCC	38	OPD-18	GAGAGCCAAC
15	OPAH-5	TTGCAGGCAG	39	OPF-09	CCAAGCTTCC
16	OPAH-12	CTGCTGGGAC	40	OPK-01	CATTCGAGCC
17	OPB-1	GTTTCGCTCC	41	OPK-07	AGCGAGCAAG
18	OPB-2	TGATCCCTGG	42	OPL-12	GGGCGGTACT
19	OPB-06	TGCTCTGCCC	43	OPL-18	ACCACCACCC
20	OPB-07	GGTGACGCAG	44	OPP-19	GGGAAGGACA
21	OPB-09	TGGGGGACTC	45	OPO-04	AAGTCCGCTC
22	OPB-10	CTGCTGGGAC	46	OPX-17	GACACGGACC
23	OPB-11	GTAGACCCGT	47	OPY-02	CATCGCCGCA
24	OPC-05	GATGACCGCC			

3.2.10.2 Initial screening of ISSR primers

Genomic DNA at the concentration of 30-35ng was amplified using selected ISSR primers as per the protocol given by Anatala *et al.* 2014. For initial screening 43 ISSR Primers were used out of them 19 primers were selected for marker analysis. The amplified products were run on 1.5 per cent agarose gel stained with ethidium bromide using 1X TAE buffer along with marker (100-bp DNA ladder). The profile was visualized under gel documentation unit and docked for further analysis. The documented ISSR profiles were carefully examined for amplification of bands.

PCR amplification was performed in a 20 µl reaction mixture and the composition of the reaction mixture consisted of

g)	Genomic DNA (30 ng)	:	2.0 µl
h)	10X Taq assay buffer B	:	2.0 µl
i)	MgCl₂	:	2.0 µl
j)	dNTP mix (10 mM each)	:	1.5 µl

k)	Taq DNA polymerase (3U)	:	0.4 µl
l)	Primer (10 pM)	:	1.5 µl
m)	Autoclaved distilled water	:	10.6 µl
	Total volume	:	20.0 µl

The amplification was carried out with the following programme.

g)	94°C for 4 minutes	:	Initial denaturation
h)	94°C for 45 seconds	:	Denaturation
i)	42°C to 56°C for 1 minutes	:	Primer annealing
j)	72°C for 2 minutes	:	Primer extension
k)	72°C for 10 minutes	:	Final extension
l)	4°C for infinity to hold the sample		

35 cycles

Table 3.2. List of ISSR primers used for screening the parents DNA

Sl. No.	Primers	Sequence of primers (5'-3')	Number of bases
1	UBC 807	AGAGAGAGAGAGAGAGT	17
2	UBC 808	AGAGAGAGAGAGAGAGC	17
3	UBC 809	AGAGAGAGAGAGAGAGG	17
4	UBC 810	GAGAGAGAGAGAGAGAT	17
5	UBC 811	GAGAGAGAGAGAGAGAC	17
6	UBC 812	GAGAGAGAGAGAGAGAA	17
7	UBC 813	CTCTCTCTCTCTCTCTT	17
8	UBC 814	CTCTCTCTCTCTCTCTA	17
9	UBC 815	CTCTCTCTCTCTCTCTG	17
10	UBC 816	CACACACACACACACAA	17
11	UBC 817	CACACACACACACACAA	17
12	UBC 818	CACACACACACACACAG	17
13	UBC 820	GTGTGTGTGTGTGTGTC	17
14	UBC 825	ACACACACACACACACT	17
15	UBC 826	ACACACACACACACACC	17

16	UBC 834	AGAGAGAGAGAGAGAGYT	18
17	UBC 835	AGAGAGAGAGAGAGAGYC	18
18	UBC 840	CTCTCTCTCTCTCTCTRC	18
19	UBC 844	CTCTCTCTCTCTCTCTRG	18
20	UBC 845	CACACACACACACACART	19
21	UBC 846	CACACACACACACACARG	19
22	UBC 848.	TCTCTCTCTCTCTCTCRG	20
23	UBC 855	ACACACACACACACACYT	18
24	UBC 856	ACACACACACACACACYA	18
25	UBC 857	ACACACACACACACACYG	18
26	UBC 864	ATGATGATGATGATGATG	18
27	UBC 866	CTCCTCCTCCTCCTCCTC	18
28	UBC 868	GAAGAAGAAGAAGAAGA	17
29	UBC 880	GGAGAGGAGAGGAGA	15
30	UBC 890	VHVGTGTGTGTGTGT	21
31	UBC 892	TAGATCTGATATCTGAATTCCC	22
32	UBC 895	AGAGTTGGTAGCTCTTG	17
33	UBC 899	CATGGTGTTGGTCATTGTTCCA	22
34	UBC 900	ACTTCCCCACAGGTTAACACA	21
35	ISSR 1	GTTGTTGTTGTTGTT	15
36	ISSR 2	ATTATTATTATTATTCAT	18
37	ISSR 3	TTATTATTATTATTACTT	18
38	ISSR 4	ATTATTATTATTATTGTT	18
39	ISSR 5	GTCGTCGTCGTCGTCA	19
40	ISSR 6	TTATTATTATTATTATAA	18
41	ISSR 7	ATTATTGTTGTTGTTGTA	18
42	ISSR 8	CCATTATTATTATTATAG	18
43	ISSR 9	TTATTATTATTATTATTACT	20

3.2.10.3 Bulked segregant analysis and identification of polymorphic markers

The bulked segregant analysis proposed by Michelmore *et al.* (1991) details the methodology to tag the genes of interest, using a structurally developed population segregating for the gene of interest. To perform this assay, DNA was isolated from 10 highly resistant and 10 highly susceptible F_2 plants. BSA was performed for each polymorphic primer using the DNA samples from susceptible parent, resistant parent, susceptible F_2 bulk and resistant F_2 bulk. Initial BSA with susceptible and resistant bulks of 10 plants each has not yielded any marker. Subsequently, the number of plants for bulking was reduced to 7, 5 and 3. BSA with the bulked DNA from 3 F_2 plants each in susceptible and resistant bulks was found to yield distinct markers in both RAPD and ISSR assays.

Key to symbol: R=A+G, Y=C+T, H= A+T+C, V=G+A+C
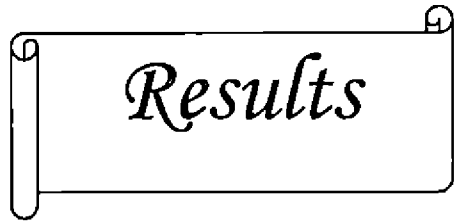
Table 3.2. List of RAPD primers used for BSA

Sl. No.	Name of primers	Sequence of primers (5'-3')
1	OPA 02	TGCCGAGCTG
2	OPA 14	TCTGTGCTGG
3	OPA 27	GAAACGGGTG
4	OPAH 3	GGTTACTGCC
5	OPAH 5	TTGCAGGCAG
6	OPC 05	GATGACCGCC
7	OPC 08	TGGACCGGTG
8	OPD 18	GAGAGCCAAC
9	OPF 09	CCAAGCTTCC
10	OPK 07	AGCGAGCAAG
11	OPL 18	ACCACCACCC
12	OPX 17	GACACGGACC

Table 3.4. List of ISSR primers used for BSA

Sl. No.	Name of primers	Sequence of primers (5'-3')
1	UBC 807	AGAGAGAGAGAGAGAGT
2	UBC 809	AGAGAGAGAGAGAGAGG
3	UBC 810	GAGAGAGAGAGAGAGAT
4	UBC 811	GAGAGAGAGAGAGAGAC
5	UBC 816	CACACACACACACACAA
6	UBC 834	AGAGAGAGAGAGAGAGYT
7	UBC 840	CTCTCTCTCTCTCTCTRC
8	UBC 846	CACACACACACACACARG
9	UBC 855	ACACACACACACACACYT
10	UBC 864	ATGATGATGATGATGATG

Key to symbol: R=A+G, Y=C+T, H= A+T+C, V=G+A+C

Results

4. RESULTS

The results of the study on "Development of molecular markers for anthracnose disease resistance in vegetable cowpea [*Vigna unguiculata* (L.) Walp.]" undertaken during the period 2013 to 2015 was carried out at the Centre of Plant Biotechnology and Molecular Biology, College of Horticulture, Kerala Agricultural University, are described in this chapter.

4.1 Evaluation of parents with F_1

The two selected varieties Sharika and Kanakamony were observed for important morphological and yield characters which were compared with the F_1 progenies obtained from the cross between Sharika × Kanakamony and Kanakamony × Sharika. The results are presented in Table. 4.1.

The characters were evaluated from randomly selected 10 plants of each of the 4 genotypes under study.

1. Plant height

The average plant height recorded from the F_1s of cross Sharika × Kanakamony and Kanakamony × Sharika were 201.01 and 199.57 cm whereas the values for the parents Sharika and Kanakamony were 210.14 cm and 128.57 cm respectively.

2. Number of branches

The average number of branches of F_1s of the cross Sharika × Kanakmony and Kanakamony × Sharika were 7.95 and 7.77 whereas for the parents Sharika and Kanakamony the values were recorded 7.89 and 6.20 respectively.

3. Leaf area

The average leaf area of F_1s obtained from the cross Sharika × Kanakamony and Kanakamony × Sharika were recorded 77.79 and 76.78 cm^2 whereas for the parents Sharika and Kanakamony the value were recorded 79.40 and 60.67 cm^2, respectively.

4. Number of days for first flowering

The average number of days to flowering was recorded 50.60 and 50.75 days for the F_1s obtained from the cross Sharika × Kanakmony and Kanakamony

a) Susceptible parent Sharika
raised in open field

b) Resistant parent Kanakam
raised in open field

c) F_1 hybrids raised in open field

Plate 4.1. Field raised parents and F_1 plants

× Sharika, whereas for the parents Sharika and Kanakamony the values were recorded 48.45 and 41.19 days, respectively.

5. Number of pods per plant

The average number of pods harvested from F_1 plants of cross Sharika × Kanakamony and Kanakamony × Sharika were 48.03 and 49.97 whereas for parents Sharika and Kanakamony the values were 20.71 and 19.26 respectively.

Table 4.1. Morphological observation of parents and F_1 for different characters

Characters	Plant Heig ht (cm)	Numb er of branc hes	Leaf area (cm²)	Days to 1st flower ing	Numb er of pods per plant	Leng th of pods (cm)	Pod weig ht (g)	Seed colour	Numb er of seeds per pod	Seed weight (g) (100 seeds)	Seed Yiel d per plant (g)
Sharika	210.14	7.89	79.40	48.45	20.71	40.24	18.40	Black	18.65	19.24	110.68
Kanakamo ny	128.57	6.20	60.67	41.19	19.26	17.67	9.79	Red	12.45	12.22	102.91
Sharika × Kanakamo ny	201.01	7.95	77.79	51.68	48.03	18.26	10.83	Black	16.59	14.49	150.73
Kanakamo ny × Sharika	199.57	7.77	76.78	50.75	49.97	18.17	10.48	Black	15.92	14.12	158.02

6. Length of pod

The average length of pods for the F_1s from the cross Sharika × Kanakamony and Kanakamony × Sharika were recorded 18.26 and 18.17 cm whereas for parents Sharika and Kanakamony the values were 40.24 cm and 17.67 cm respectively.

7. Pod weight

The average fresh pod weight of cross Sharika × Kanakamony and Kanakamony × Sharika were 18.40 g and 9.79 g whereas for the parents the values were recorded 10.83 g and 10.48 g, respectively.

8. Number of seeds per pod

The number of seeds per pod for the F_1s obtained from the cross Sharika × Kanakamony and Kanakamony × Sharika were 16.59 and 15.92 whereas for the parents the values were 18.65 and 12.45, respectively.

9. Seed weight (100 seeds)

The average seed weight of 100 seeds of parent Sharika were recorded 19.24 g and Kanakamony parent 12.22 g whereas the average seed weight for the F_1s obtained from the cross Sharika × Kanakamony and Kanakamony × Sharika were recorded 14.49 g and 14.12 g, respectively.

10. Seed yield per plant

The average seed yield per plant of parent Sharika and Kanakamony were recorded 110.68 g and 102.91 g whereas for F_1s the values were recorded 150.73 g and 158.02 g, respectively.

In F_1s of the Sharika × Kanakamony and reciprocal cross, pole type and black seed colour characters of Sharika were dominant over semi-trialing and red seed colour of Kanakamony.

4.2 Development and raising of F_1 generation

Reciprocal crosses were made using the immune cultivar Kanakamony and susceptible cultivar Sharika. The pod setting in the cross was 17 per cent. Thirty F_1 plants were raised from the six pods obtained from the crosses (2 pods from Sharika× Kanakamony and 4 from Kanakamony × Sharika).

4.3 Development and raising of F_2 population

Eight F_2 plants were raised form a single F_1 plant by selfing and they were used as segregating population for tagging the anthracnose resistance gene. A total 264 plants were sown for F_2. Due to poor germination, only 163 plants were artificially screened.

4.4 Isolation and identification of pathogen

The pathogen associated with the anthracnose disease of cowpea was isolated initially on 3 different medium viz. potato dextrose agar (PDA), neopeptone glucose agar (NGA) and carrot dextrose agar (CDA) medium. The fungus grown on PDA medium failed to produce fruiting bodies for which it was

a. Culture of *C. lindemuthianum* on
neopeptone glucose agar medium
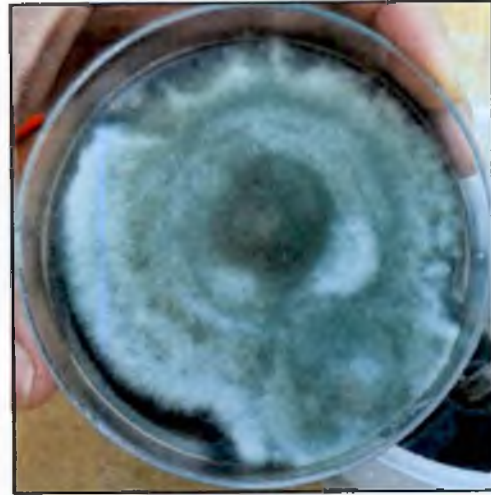
b. Culture of the pathogen on
Carrot dextrose agar medium

Plate 4.2. Mycelial development in *C. lindemuthianum*, 7 days after inoculation on different media
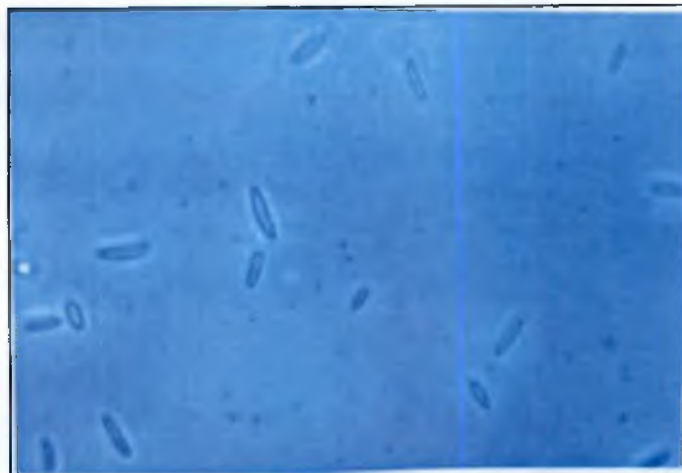
Plate 4.3. Micrograph of *C. lindemuthianum* conidiospore

discarded and finally the other two media were selected for the isolation of fungus. The growth of pathogen on the two media and spore characteristics were shown in the Plate 4.2.

On NGA medium, colonies were growing faster, sparsh whitish mycelium, sporulation observed not to the satisfied level, light pink pigmentation and very few sclerotia like bodies (Plate 4.2a).

On CDA medium, colonies were fast growing, whitish mycelium, high sporulation, no pink pigmentation were observed (Plate 4.2b). Conidia were hyaline, cylindrical with both ends obtuse, aseptate and uninucleate. Based on cultural and morphological characteristics the pathogen was identified as *C. lindemuthianum* (Plate 4.3).

4.4.1 Artificial inoculation

Artificial inoculation was carried out during May 2015. The symptoms appeared late as the condition for the disease development was not met due to absence of rain and less humidity.

After the first inoculation, symptoms have appeared on green leaves as small pin head black colour spots, surrounded by yellow hallow (Plate 4.4). These enlarged to 3.4 mm diameter size and covered the leaf surface. Finally, leaves became yellow and detached from the plants. In few plants, the breakage of vines also noticed. Symptoms have appeared on the stem fifteen days after artificial inoculation as typical vine blackening (Plate 4.5a and 4.5b).

4.5 Assessment of disease severity and disease incidence

One hundred and sixty-three plants of F_2 progenies were observed for the development of symptoms which are categorized in to highly susceptible and highly resistant plants (Table 4.2).

Table 4.2. Plant response to anthracnose disease in F_2

Disease response	Number of plants
Highly Susceptible	149
Moderately susceptible	0
Moderately resistant	0
Highly resistant	14

Development of early stage leaf symptoms after the artificial inoculation

Plate 4.4. Symptoms development in the susceptible Sharika following artificial inoculation under controlled condition.

a) Resistance and Susceptibilty responses in plants in the segregating F_2 population

b) Typical Vine blackening in F_2 generation, leading to complete plant mortality

Plate 4.5. Development of typical symptoms in F_2 segregating population, following artificial inoculation under controlled condition

4.6 Molecular marker analysis to identify the marker for resistance
4.6.1 Isolation, purification and quantification of DNA

Two cowpea genotypes, Kanakamony which is reported to have anthracnose immunity and Sharika which is susceptible were selected as parents in crossing programme. The genomic DNA isolated through the CTAB method reported by Doyle and Doyle (1990). The isolation of DNA samples had RNA contamination which was completely removed by RNase treatment (Plates 4.6a, b).

4.6.1.1 Assessing the quality of DNA by agarose gel electrophoresis

The quality and quantity of DNA were assessed using agarose gel electrophoresis. The DNA isolated by this method appeared as clear and single intact band of high intensity. This indicated non-degraded good quality DNA free from RNA and protein contamination.

4.6.2 Assessing the quality and quantity of DNA using NanoDrop method

The result of the NanoDrop® ND-1000 spectrophotometer analysis is presented in Table 4.3 and Table 4.4. The ratio of UV absorbance ($A_{260/280}$) ranged between 1.83-2.09, based on these values DNA were rated as good and standard. The quantity of DNA in the isolated samples ranged from 1349.68 to 2853.82.

4.7 RAPD (Random Amplified Polymorphic DNA) analysis

After isolation of good quality genomic DNA the cowpea genotypes were subjected RAPD based BSA analysis. The different experiments carried out under this include screening of random primers, bulked segeregant analysis and co-segregation of cowpea genotypes using selected primers.

4.7.1 Screening of primers for RAPD analysis

Forty seven primers were belonging to 13 Operon series *viz.*, OPA, OPAH, OPB, OPC, OPD, OPK, OPL, OPM, OPF, OPO, OPP, OPX, and OPY subjected for initial screening of parental DNA (Table 3.1). From that thirty three were finally selected for screening of parents to test their ability to produce polymorphism for resistance. The amplification pattern is described below.
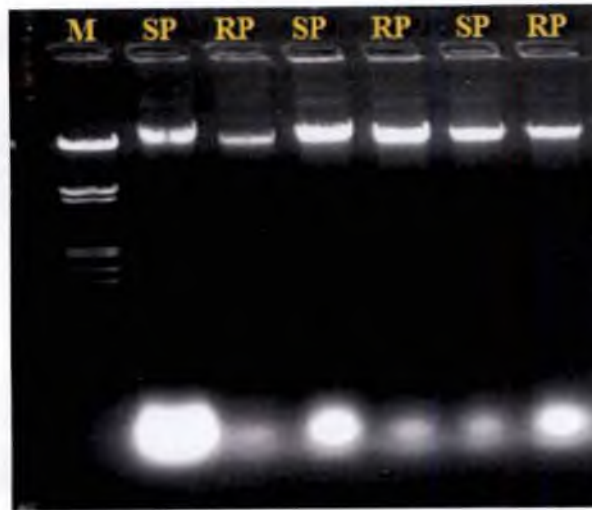
a) DNA isolated by 2x CTAB method
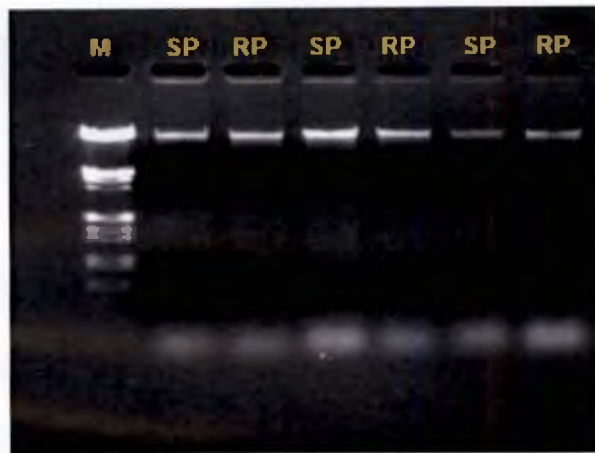
b) DNA isolated by 2x CTAB method after
RNase treatment

Plate 4.6. Genomic DNA isolation and purification from the
parental lines used in the study

a) DNA isolated from five susceptible F_2 progenies

b) DNA isolated from five resistant F_2 progenies

Plate 4.7. Genomic DNA isolated from susceptible and resistant F_2 plants

Seven primers from A series were screened in this series. The efficiency of amplification is given in Table 4.5 and Plate 4.8a, 4.8b, and 4.8c, respectively. Mean number amplicons ranged between 0 to 10.5. In this series primer OPA-02 has given polymorphism and polymorphic bands were of size 0.65, 0.7, 0.9, 1, 1.1 and 0.4, respectively.

Table 4.3. Quality and quantity of DNA isolated from cowpea genotypes assessed by NanoDrop method

Genotypes	A $_{260/280}$	Quantity (ng/µl)	Quality
RP	1.87	1562.72	Good
SP	1.97	2754.7	Good

Table 4.4. Quality and quantity of DNA isolated from parents and F$_2$ progenies

Genotypes	A $_{260/280}$	Quantity (ng/µl)	Quality
RP	1.84	1426.28	Good
SP	1.98	2853.82	Good
SF$_2$ 1	1.83	1352.23	Good
SF$_2$ 2	1.95	2380.78	Good
SF$_2$ 3	1.86	1698.37	Good
SF$_2$ 4	1.84	1855.10	Good
SF$_2$5	2.06	2635.18	Good
RF$_2$ 1	2.09	1349.68	Good
RF$_2$ 2	1.92	1754.32	Good
RF$_2$ 3	2.06	1968.74	Good
RF$_2$ 4	1.94	2265.12	Good
RF$_2$ 6	1.89	2605.24	Good

RP: Resistant parent, SP: Susceptible Parent

SF$_2$: Susceptible F$_2$, RF$_2$: Resistant F$_2$

The primer OPA-02, OPA-14 and OPA-27 were selected for BSA based on polymorphism and good amplification. Six primers from B series were screened. Efficiency of amplification is given in Table 4.5 and Plate 4.8b and 4.9a. Mean number of amplicons range between 3 to 8 kb. In this series no

a)

b)

c)

d)
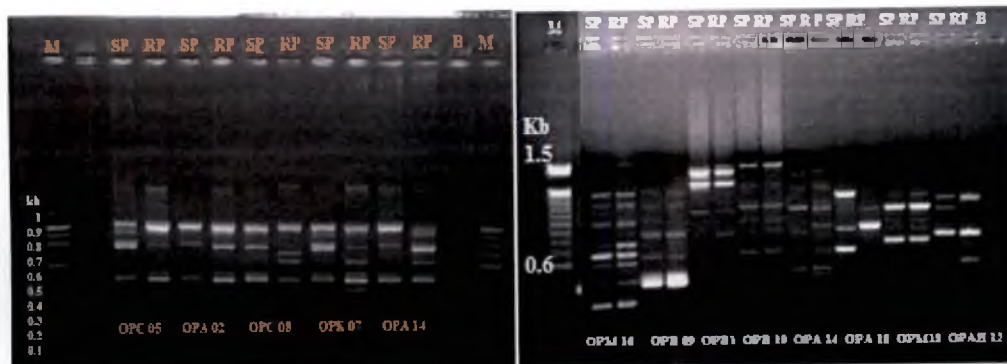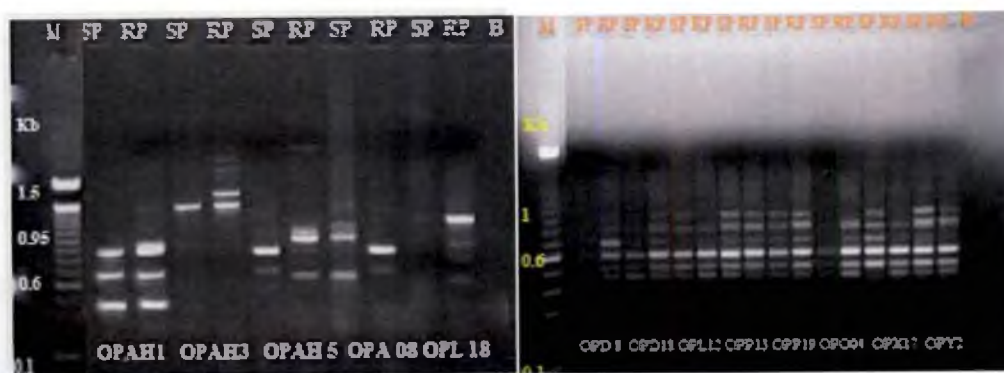
M: Marker (100 bps)

SP: Susceptible parent   RP: Resistant parent

a) OPC 05, OPA 02, OPC 08, OPK 07 and OPA 14

b) OPM 16, OPB 09, OPB 1, OPB 10, OPA 14, OPA 10, OPM 18, OPAH 12

c) OPAH 1 OPAH 3 OPAH 5, OPA 08, OPA 02

d) OPD 8, OPD 18, OPL 12, OPP 13, OPP 19, OPO 04, OPX 17 and OPY 2

Plate 4.8. Screening primers of OPC, OPK, OPA, OPB, OPD, OPAH,
OPP, OPL, OPM, OPO, OPX and OPY series with
susceptible and resistant parents

primers have given amplification. Five primers from C series screened. The efficiency of amplification observed for different primers OPC series is given in Table 4.8 and Plate 4.8a, 4.9b. The mean number of bands in this series varied from 2 to 6 kb. The primers OPC-05 and OPC-08 polymorphic with bands size 0.55, 0.8 and 0.65, 0.7, 0.75 Kb, respectively.

Two primers from OPD series were screened. The amplification pattern observed for these two primers is given in Table 4.7 and Plate 4.8d. The mean number of bands generated from these primers were varied from 5.5 to 6. The primers OPD 18 gave a polymorphic band of size more than 1 kb was chosen for BSA. In L series two primers were screened. OPL 12 primer was given a polymorphic band of size. The amplification pattern is given in Table 4.7 and Plate 4.8c and 4.8d, respectively. The mean numbers of ampilcon were varied from 1.5 to 4.5. Two primers from M series were screened. The amplification pattern of these two primers is given in Table 4.7 and plate 4.8b. The mean numbers of ampilcon were varied from 7 to 10.5.

Two primers from P series were screened. The amplification pattern is given in the Table 4.7 and Plate 4.10b. OPF 09 primer was screened with both the parents and the amplification pattern is given in the Table 4.11 and Plate 4.8a. The polymorphic bands were observed at 0.4 kb and less than 0.5 kb sizes. OPO 04 primer was screened with the parents and the amplification pattern is given in the Table 4.11 and Plate 4.7d. The mean number of amplicons obtained was 6.5. OPX 17 primer was screened to obtain polymorphism in parents. The amplification pattern was given in the Table 4.11 and Plate 4.7d. The mean number of amplicons obtained was 5.5. A polymorphic band of size 0.6 Kb obtained and the primer was selected for BSA. The OPY 02 primer was screened to obtain polymorphism in both the parents. The amplification pattern is given in the Table 4.11 and Plate 4.7d. The mean number of amplicons was 6.

Table 4.5. Amplification pattern of cowpea genotypes with A series primers

Sl. No.	Name of primers	No. of bands		Mean	No. and Molecular weight of polymorphic bands		Amplification pattern
		SP	RP			kb	
1	OPA 02	4	5	4.5	1	0.65 in RP	Medium
2	OPA 03	0	0	0	0	0	Poor
3	OPA 08	3	2	2.5	0	0	Good
4	OPA 10	6	3	4.5	0	0	Medium
5	OPA 12	4	1	2.5	0	0	Poor
6	OPA 14	7(9)	8(12)	7.5 and 10.5	2	0.7 and 0.9 in RP	Good
7	OPA 27	8	10	9	3	1 in SP 1.1 and 0.4 in RP	Good
8	OPB 1	6	5	5.5	0		Medium
9	OPB 06	2	4	3	0		Poor
10	OPB 7	8	8	8	0		Good
11	OPB 9	9	7	8	0		Good
12	OPB 10	7	8	7.5	0		Good
13	OPB 11	4	6	5	0		Medium

Table 4.6. Amplification pattern of cowpea genotypes with AH series primers

Sl. No.	Name of the primers	No. of bands		Mean	No. of and molecular weight of polymorphic bands		Amplification pattern
		SP	RP			kb	
1	OPAH 1	1	2	1.5	1	0.95 in RP	Poor
2	OPAH 3	1	4	2.5	1	1.5 in RP	Poor
3	OPAH 5	2	3	2.5	2	0.6 in SP 0.55 in RP	Poor
4	OPAH 12	3	3	3	1		

SP: Susceptible parent   RP: Resistant parent
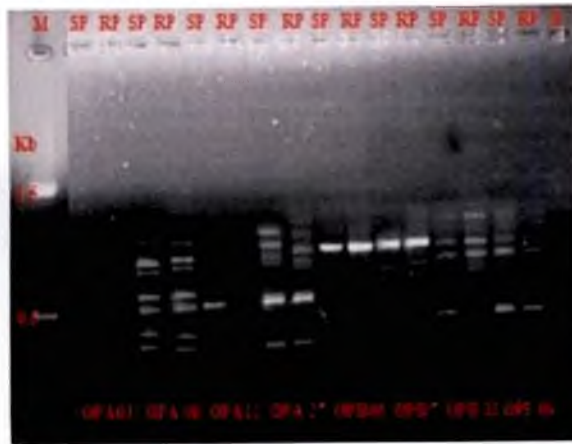
a)

b)

M: Markers (100 bps)

SP: Susceptible parent   RP: Resistance parent

a) OPA 03, OPA 08, OPA 12, OPA 27,OPB 06, OPB 07, OPB 11 and
    OPF 09

b) OPC 11. OPC 12, OPC 15 and OPC 20

**Plate 4.9. Screening primers of OPC, OPA, OPB, OPD, OPP, OPL,
          OPO series with susceptible and resistant parents**

Table 4.7. Amplification pattern of cowpea genotypes with D, L, M and P
series primers

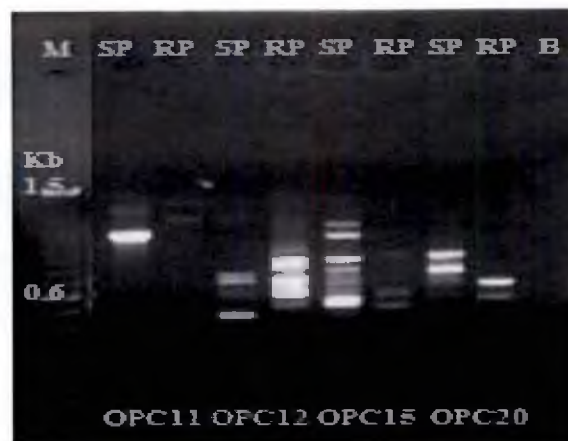| No. of primers	Name of primers	No. of bands		Mean	No. of and molecular weight of polymorphic bands		Selection for BSA
		SP	RP			kb	
1	OPD 08	6	6	6	0		Not selected
2	OPD 18	5	6	5.5	1	> 1 in RP	Selected
3	OPK 07	6	7	6.5	1	0.7	
4	OPL 12	5	4	4.5	1	>1 in SP	Selected
5	OPL 18	0	3	1.5	0		Not selected
6	OPM 16	10	11	10.5	0		Not selected
7	OPM 18	6	8	7	0		Not selected
8	OPP 13	5	5	5	0		Not selected
9	OPP 19	6	6	6	0		Not selected

Table 4.8. Amplification pattern of cowpea genotypes with C series primers

Sl. No.	Name of primers	No. of bands		Mean	No. of and molecular weight of polymorphic bands		Selection for BSA
		SP	RP			kb	
1	OPC 05	8	6	6	2	0.55 in SP 0.8 in SP	Selected
2	OPC 08	6	6	6	3	0.65 in SP 0.7 in RP 0.75 in RP	Selected
3	OPC 11	2	2	2	0		Not selected
4	OPC 12	3	6	4.5	0		Not selected

Table 4.9. Amplification pattern of cowpea genotypes with OPF 09, OPO 04,
OPX 17 and OPY 02 primers

No. of primers	Name of primers	No. of bands		Mean	No. of and molecular weight of polymorphic bands		Selection for BSA
		SP	RP			kb	
1	OPF 09	10	7	8.5	1	SP- 0.5	Selected
2	OPO 04	4	9	6.5	0		Not selected
3	OPX 17	5	6	5.5	1	0.6 in RP	Selected
4	OPY 02	6	6	6	0		Not selected
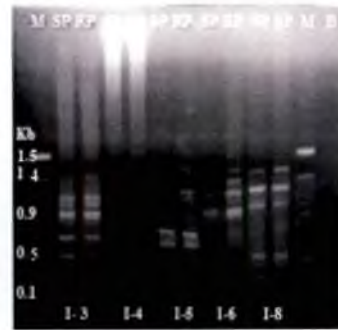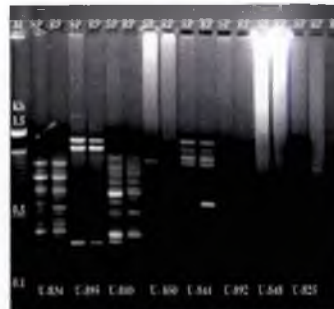
SP: Susceptible parent   RP: Resistant Parent
a) UBC 807, UBC 808, UBC 809 and UBC 810
b) ISSR 3, ISSR 4, ISSR 5, ISSR 6 and ISSR 8
c) UBC 934, UBC 895, UBC 840, UBC 850, UBC 844, UBC 892, UBC 848
   and UBC 825
d) UBC 846, UBC 857, UBC 864, UBC 868 and UBC 855
e) UBC 811 and UBC 816

Plate 4.10. Screening of susceptible and resistant parents with ISSR primers

4.7.2 ISSR analysis

The different steps carried out under this included the screening of random primers, bulked segeregant analysis cowpea genotypes using selected primers.

The gradient PCR revealed amplification of 19 primers out of 43 which were subsequently subjected for the screening of parents. The amplification patterns are described below.

4.7.2.1 Screening of parents with ISSR primers

Out of 43 ISSR primers 19 primers that gave amplification were subsequently studied for screening of parental DNA as described in Table 4.12. The mean number of amplicons varied from 0 to 13.5. Eleven primers *viz.,* UBC 807, UBC 809, UBC 810, UBC 811, UBC 816, UBC 934, UBC 840, UBC 844, UBC 846, UBC 855 and UBC 864 were polymorphic.

The amplification pattern of primers UBC 807 is given in Table 4.12 and Plate 4.9a which mean number of amplicons generated was 11. A polymorphic band of 0.7 Kb was obtained in susceptible parent. Screening of UBC 809 primers revealed the presence of 2 polymorphic bands of size >0.9 kb and 1 kb in resistant parent. The details of the primer are given in Table 4.12 and Plate 4.9a. The mean number of amplicons obtained was 7. The primer UBC 810 screening revealed the presence of 2 polymorphic bands of size 0.9 and 1.3 kb in resistant parent. The amplification pattern is given in the Table 4.10 and Plate 4.10a. The mean number of amplicon was 5. The primer UBC 811 gave 3 polymorphic bands of size 0.7 and 1 kb in susceptible parent and 1.45 kb in resistant parent. The details of the primer are given in the Table 4.10 and Plate 4.10e. The mean number of amplicons was 6.

Screening with the UBC 816 has given a single polymorphic band of size 1.3 kb. The amplification pattern of the primer is given in the Table 4.10 and Plate 4.10e. The mean number of amplicon obtained was 4.5. UBC 834 primer has given 1 polymorphic band of size 0.6 kb in susceptible parent. The amplification pattern is given in Table 4.10 and Plate 4.10c. The mean number amplicon generated by this primer is 13.5 which are highest amongst all the primers selected for the study.

UBC 840 primer screening revealed the presence of a single polymorphic band of size 0.55 kb. The details of the primer are given in the Table. 4.10 and Plate 4.10c. The mean number of amplicons was 11.5. The primer UBC 844 has given two polymorphic bands of size 0.6 and 1.1 kb in susceptible parent. The amplification pattern is given in the Table. 4.10 and Plate 4.10c. The mean number of amplicons obtained was 9.

The primer UBC 846 screening result has yielded a polymorphic band of size 1.4 kb in resistant parent. The amplification pattern is given in the Table 4.10 and Plate 4.10d. The mean number of amplicon generated was 4.5. Screening of UBC 855 primers revealed the presence of a single polymorphic band of size 0.7 Kb in resistant parent. The amplification pattern is given in the Table 4.10 and Plate 4.10d. The mean numbers of amplicon was 12.5 which is the second highest amongst the primers under study. The amplification pattern of the primer UBC 864 is given in the Table 4.10 and Plate 4.10d. A single polymorphic band was of size 0.4 Kb in susceptible parent. The mean number of amplicon was 9.5.

The parental DNA screening with RAPD and ISSR primers concluded with the results that, 12 RAPD and 10 ISSR primers were found to be polymorphic and subjected to bulked segregant analysis for the discrimination of resistant and susceptible F_2 progenies.

Table 4.10. Amplification pattern of cowpea genotypes with ISSR primers

Sl. No.	Name of primers	No. of bands		Mean	No. and Molecular weight of polymorphic bands		Selection for BSA
		SP	RP			kb	
1	ISSR 3	8	8	8	0	NA	Not selected
2	ISSR 4	0	0	0	0	NA	Not selected
3	ISSR 5	4	5	4.5	0.	NA	Not selected
4	ISSR 6	2	6	4	0	NA	Not selected
5	ISSR 8	9	9	9	0	NA	Not selected
1	UBC 807	11	11	11	1	SP-0.7	Selected
2	UBC 808	12	12	12	0		Not selected
3	UBC 809	6	8	7	2	RP-> 0.9 and 1	Selected
4	UBC 810	4	6	5	2	RP-0.9 and1.3	Selected
5	UBC 811	7	5	6	3	SP- 0.7 and 1	Selected

						RP- 1.45	
6	UBC 816	4	5	4.5	1	RP- 1.3	Selected
7	UBC 825	1	3	2	0		Not selected
8	UBC 834	14	13	13.5	1	SP-0.6	Selected
9	UBC 840	12	11	11.5	1	RP- 0.55	Selected
10	UBC 844	9	9	9	2	SP-0.6 and 1.1	Selected
11	UBC 846	4	5	4.5	1	RP-1.4	Selected
12	UBC 848	0	0	0	0		Not selected
13	UBC 850	2	2	2	0		Not selected
14	UBC 855	12	13	12.5	1	RP-0.7	Selected
15	UBC 857	9	9	9	0		Not selected
16	UBC 864	10	9	9.5	1	SP-0.4	Selected
17	UBC 868	6	6	6	0		Not selected
18	UBC 892	0	0	0	0		Not selected
19	UBC 895	5	5	5	0		Not selected

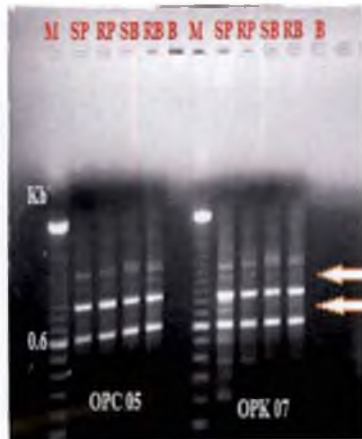SP: Susceptible parent RP: Resistant parent

4.8 BSA using selected RAPD and ISSR primers

BSA was done with twelve RAPD and ten ISSR primers. The list of primers used for BSA is given in Table 4.11 and 4.12, respectively. The sample for BSA consisted of DNA of susceptible parent, resistant parent, bulked DNA from three susceptible F_2 and bulked DNA from three resistant F_2 progenies, negative control without DNA and marker. Amplification profile created by each primer was examined for polymorphism between resistant and susceptible genotypes. The results obtained are furnished below.

4.8.1   BSA using selected 12 RAPD primers

4.8.1.1 OPA 02

Screening with primer generated nine and six amplicons in resistant and susceptible parent respectively. The molecular weight of amplicons ranged between 0.3 to 1.5 kb. The amplification pattern observed is given in Table 4.11 and Plate 4.11d. The polymorphic 0.85 kb band was absent in both the resistant parent and resistant bulk but present in susceptible parent and susceptible bulk.
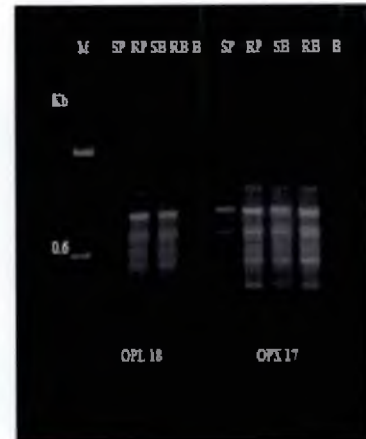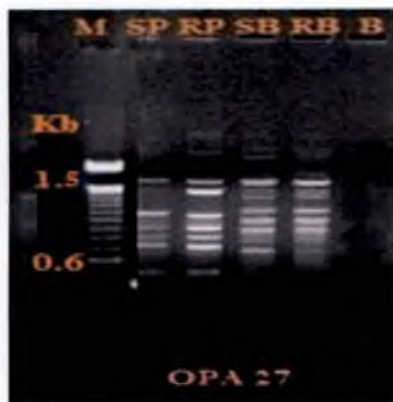
M: Marker (100 bps), SP: Susceptible parent, RP: Resistant parent

SB: Susceptible bulk, RB: Resistant bulk

Plate 4.11. BSA with OPC 05, OPK 07, OPL 18, OPX 17, OPA 27 and OPA 02 primers

a)

b)

c)

M: Marker (100 bps), SP: Susceptible parent, RP: Resistant parent
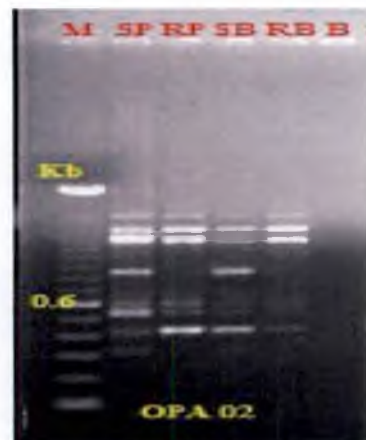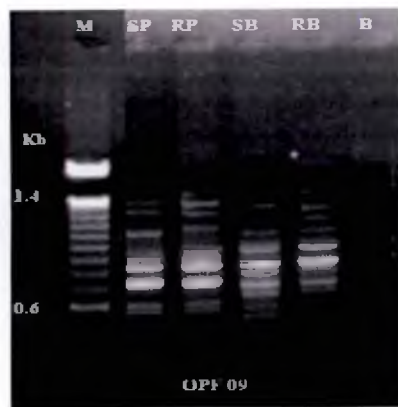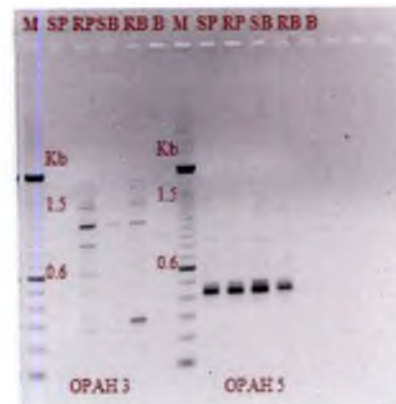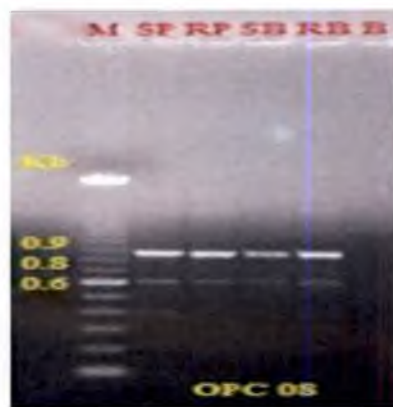
SB: Susceptible bulk, RB: Resistant bulk

Plate 4.12. BSA with OPF 09, OPAH 3, OPAH 5 and OPC 08 primers

4.8.1.2 OPA 14

Two and three amplicons were observed in susceptible parent and resistant parent, respectively. The amplification pattern observed is given in Table 4.11 and Plate 4.13b. Two polymorphic bands of 1.1 kb and 1.5 kb were observed in susceptible parent but absent in other genotypes. The molecular weight of the products was ranged between 0.4 to 1.4 kb. This primer was able to differentiate susceptible parent from other genotypes.

4.8.1.3 OPA 27

Six and nine amplicons were observed in susceptible parent and resistant parent, respectively, on the agarose gel for the DNA amplified with the primer OPA 27. The amplification pattern observed is given in Table 4.11 and Plate 4.11c. All bands were monomorphic among the genotypes. The molecular weight of the products was ranged between 0.5 to 1.5 kb. This primer was unable to differentiate between the resistant and susceptible genotypes.

4.8.1.4 OPC 05

Amplification with this primer generated three amplicons in resistant as well as susceptible parent. The molecular weight of amplicons ranged between 0.6 to 1.4 kb. The amplification pattern observed is given in Table 4.11 and Plate 4.11a. The primer was unable to differentiate between the resistant and susceptible genotype.

4.8.1.5 OPC 08

Three and two amplicons were observed in susceptible parent and resistant parent, respectively on the agarose gel for the DNA amplified with the primer OPC 08. The amplification pattern observed is given in Table 4.11 and Plate 4.12c. One polymorphic band of 0.3 kb was observed in susceptible genotypes. The molecular weight of the products was ranged between 0.6 to 0.85. This primer was unable to differentiate susceptible and resistant genotypes.

4.8.1.6 OPD 18

Four amplicons were observed in susceptible parent and resistant parent, respectively, on the agarose gel for the DNA amplified with the primer OPD 18.

a)

b)

M: Marker (100 bps), SP: Susceptible parent, RP: Resistant parent

SB: Susceptible bulk, RB: Resistant bulk

Plate 4.13. BSA with OPD 18 and OPA 14 primers

The amplification pattern observed is given in Table 4.11 and Plate 4.13a. A polymorphic band of size 0.65 kb was observed in the susceptible parent. The molecular weight of the products was ranged between 0.45 to 1.5 kb. This primer was able to differentiate susceptible parent from other genotypes.

4.8.1.7 OPF 09

Eight and nine amplicons were observed in susceptible parent and resistant parent, respectively. The amplification pattern observed is given in Table 4.11 and Plate 4.12a. All bands were monomorphic among the genotypes. The molecular weight of the products was ranged between 0.55 to 1.5 kb. This primer was unable to differentiate between the resistant and susceptible genotypes.
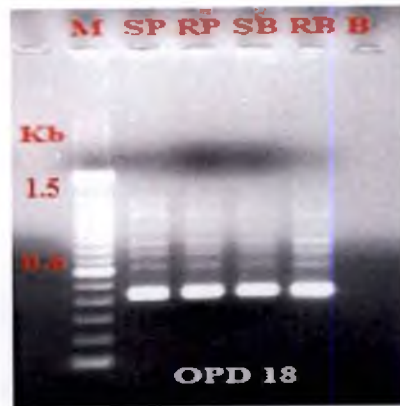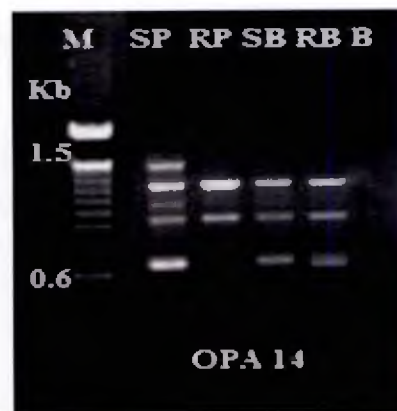
4.8.1.8 OPAH 3

Ten amplicons were observed in resistant parent. The amplification pattern observed is given in Table 4.11 and Plate 4.12b. All bands were monomorphic among the genotypes. The molecular weight of the products was ranged between 0.3 to 1.5 kb. This primer was unable to differentiate between the resistant and susceptible genotypes.

4.8.1.9 OPAH 5

Three amplicons were observed both in susceptible and resistant parent. The amplification pattern observed is given in Table 4.11 and Plate 4.12b. All bands were monomorphic among the genotypes. The molecular weight of the products was ranged between 0.4 to 0.85 kb. This primer was unable to differentiate between the resistance and susceptible genotypes.

4.8.1.10 OPX 17

Six and ten amplicons were observed in susceptible and resistant parent, respectively. The amplification pattern observed is given in Table 4.11 and Plate 4.11b. All bands were monomorphic among the genotypes. The molecular weight of the products was ranged between 0.4 to 1.3 kb. This primer was unable to differentiate between the resistance and susceptible genotypes.

In BSA with 12 RAPD primers only the primer OPA 02 has produced polymorphism among susceptible and resistant genotypes. The primer OPA 02

has produced a polymorphic band of 0.85 kb in susceptible parent and susceptible bulk which can be eluted and sequenced for further information.

Table 4.11. Amplification pattern in genotypes of BSA with selected RAPD primers

Sl. No.	Name of primers	No. of bands				No. of polymorphic bands
		SP	RP	SB	RB	
1	OPA 02	9	6	7	6	1
2	OPA 14	5	2	3	3	0
3	OPA 27	6	9	9	7	0
4	OPAH 3	0	10	1	7	0
5	OPAH 5	3	3	3	3	0
6	OPC 05	3	3	3	3	0
7	OPC 08	3	2	2	2	0
8	OPD 18	4	4	4	4	0
9	OPF 09	8	9	10	9	0
10	OPK 07	9	4	4	4	0
11	OPL 18	0	7	7	0	0
12	OPX 17	6	10	10	10	0

SP: Susceptible Parent, RP: Resistant Parent, SB: Susceptible Bulk, RB: Resistant Bulk

4.8.1.11 BSA using selected ISSR primers

4.8.1.12 UBC 864

Six and five amplicons were observed in susceptible parent and resistant parent, respectively. The amplification pattern observed is given in Table 4.12 and Plate 4.14d. A polymorphic band of 0.5 kb was present in susceptible parent. The molecular weight of the products was ranged between 0.6 to 1.2 kb. This primer was able to differentiate the susceptible parent amongst others.

4.8.1.13 UBC 809

Seven and nine amplicons were observed in susceptible parent and resistant parent, respectively. The amplification pattern observed is given in Table 4.12 and Plate 4.14a. All bands were monomorphic among the genotypes. The molecular weight of the products was ranged between 0.7 to 1.5 kb. This primer was unable to differentiate between the resistant and susceptible genotypes.

a)

b)

c)

d)

M: Marker (100 bps), SP: Susceptible parent, RP: Resistant parent

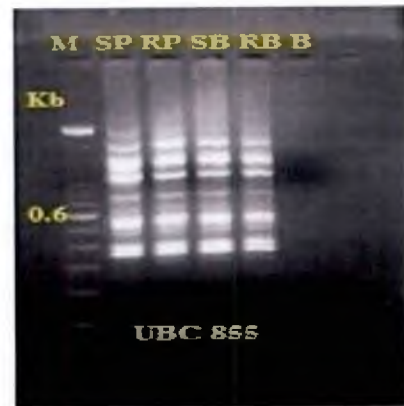Plate 4.14. BSA with UBC 809, UBC 855, UBC 810 and UBC 864 primers

4.8.1.14 UBC 810

Eleven and six amplicons were observed in susceptible parent and resistant parent, respectively. The amplification pattern observed is given in Table 4.12 and Plate 4.14c. Here a polymorphic band of 1.4 kb size was observed in resistant parent as well as resistant bulk. The molecular weights of the products were ranged between 0.55 to 1.5 kb. This primer was able to differentiate between the resistant and susceptible genotypes.

4.8.1.15 UBC 855

Amplification with this primer generated eleven numbers amplicon in both resistant and susceptible parent. The molecular weight of amplicons ranged between 0.35 to 1.5 kb. The amplification pattern observed is given in Table 4.12 and Plate 4.14b. All bands were monomorphic.

4.8.1.16 UBC 811

Amplification with this primer generated six and seven amplicons in resistant and susceptible parent respectively. The molecular weight of amplicons was ranged between 0.45 to 1.5 kb. The amplification pattern observed is given in Table 4.12 and Plate 4.15c.The polymorphic band of 1.5 kb was present in both the resistant parent and resistant bulk but absent in susceptible parent and susceptible bulk.
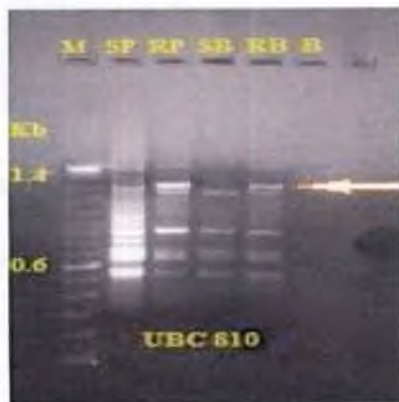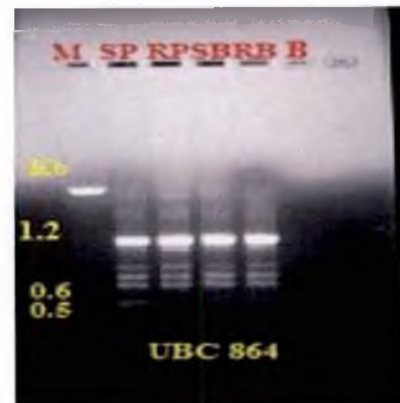
4.8.1.17 UBC 816

Eight and six amplicons were observed in susceptible parent and resistant parent, respectively. The amplification pattern observed is given in Table 4.12 and Plate 4.15a. A band of 0.9 kb was present in resistant parent, susceptible bulk and resistant bulk but absent in susceptible parent. The molecular weight of the products ranged between 0.35 to 1.6 kb.

4.8.1.18 UBC 834

Twelve amplicons were observed in susceptible parent as well as resistant parent. The amplification pattern observed is given in Table 4.12 and Plate 4.15d. All bands were monomorphic among the genotypes. The molecular weights of the
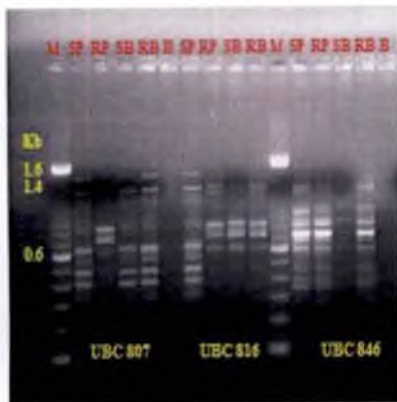
a)

b)

c)
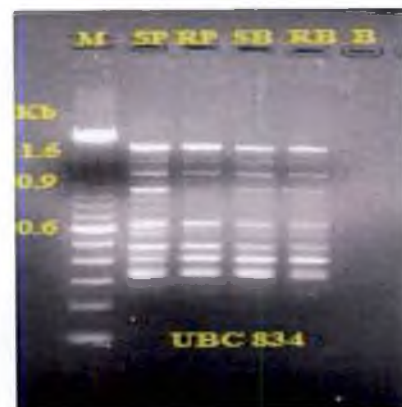
d)

M: Marker (100 bps), SP: Susceptible parent, RP: Resistant parent

SB: Susceptible bulk, RB: Resistant bulk

Plate 4.15. BSA with UBC 807, UBC 816, UBC 846, UBC 840, UBC 811 and UBC 834 primers

products were ranged between 0.3 to 1.5 kb. This primer was unable to differentiate between the resistant and susceptible genotypes.

4.8.1.19 UBC 840

BSA of UBC 840 revealed four amplicons which were observed in susceptible parent and a single band was present in resistant parent. The amplification pattern observed is given in Table 4.12 and Plate 4.15b. All bands were monomorphic among the genotypes. The molecular weight of the products was ranged between 0.4 to 1.1 kb. This primer was unable to differentiate between the resistant and susceptible genotypes.
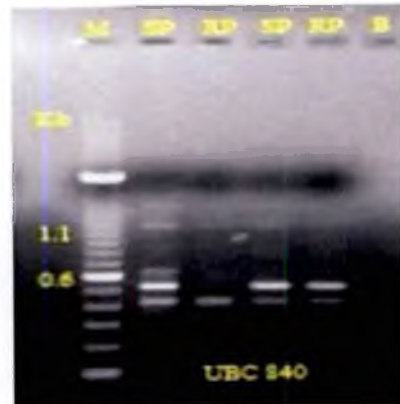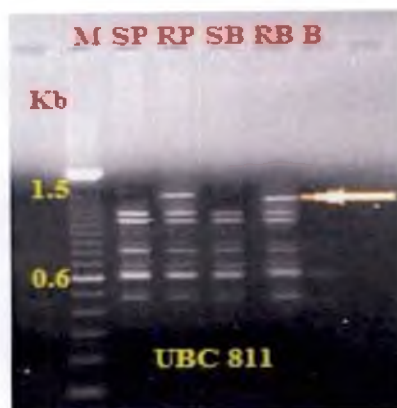
4.8.1.20 UBC 846

Nine and eight amplicons were observed in susceptible parent and resistant parent, respectively. The amplification pattern observed is given in Table 4.12 and Plate 4.15a. All bands were monomorphic among the genotypes. The molecular weights of the products were ranged between 0.35 to 1.4 kb. This primer was unable to differentiate between the resistant and susceptible genotypes.

4.8.1.21 UBC 807

Seven and three amplicons were observed in susceptible parent and resistant parent, respectively, on the agarose gel for the DNA amplified with the primer UBC 807. The amplification pattern observed is given in Table 4.12 and Plate 4.15a. A polymorphic band of 0.7 kb was present in resistant parent which enable this genotype to be different from others. The molecular weights of the products were ranged between 0.7 to 1.6 kb.
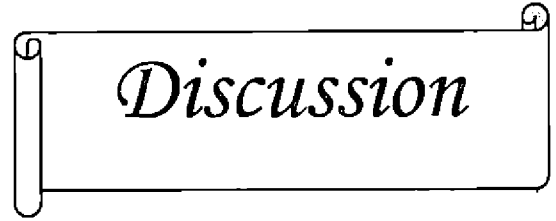
In BSA with 10 selected ISSR primers only UBC 810 and UBC 811 has produced polymorphism among susceptible and resistant genotypes. The primer UBC 810 has produced a polymorphic band of 1.4 kb in resistant parent and resistant bulk. The primer UBC 811 has produced a polymorphic band of 1.5 kb in resistant parent and resistant bulk. The polymorphic products can be eluted and sequenced.

Table 4.12. Amplification pattern in genotypes of BSA with selected ISSR primers

Sl. No.	Name of primers	No. of bands				No. of polymorphic bands
		SP	RP	SB	RB	
1	UBC 807	7	2	6	8	0
2	UBC 809	7	9	6	6	0
3	UBC 810	11	6	5	6	1
4	UBC 811	6	7	6	7	1
5	UBC 816	8	6	6	6	0
6	UBC 834	12	12	12	12	0
7	UBC 840	4	1	2	2	0
8	UBC 846	9	8	3	9	0
9	UBC 855	11	11	11	11	0
10	UBC 864	6	5	5	5	0

SP: Susceptible Parent, RP: Resistant Parent, SB: Susceptible Bulk, RB: Resistant Bulk

Discussion

5. DISCUSSION

Cowpea [*Vigna unguiculata* (L.) Walp.] is one of the important kharif pulses grown in India. It is a warm season crop, well adapted to many areas of the humid tropics and subtropical zones. Anthracnose of cowpea was first reported in India by Prassana (1985). A significant amount of yield losses up to 74 per cent (Masangwa *et al.*, 2013) depending on the susceptibility of cultivar. This disease stands as the most destructive disease of cowpea in humid areas of India and South Africa (Adegbite and Amusa, 2008).

Breeding resistant variety is the most effective means for control of anthracnose. Conventional breeding involving germplasm collection, selection and heterosis breeding has resulted in the release of resistant varieties with desirable agronomic traits. But in this crop no such varieties has been developed that can really tackle the problem. The problem for breeding a durable cultivar resistant to this disease is due to the existence of many races and multiple hosts of the fungi (Sharma *et al.*, 2007).

Knowledge of the inheritance of resistance is important in breeding. Study of the nature of inheritance showed that the gene controlling the resistance to such a wide range of races of the pathogen are polygenic in nature (Goncalves-Vidigal *et al.*, 2011; Ferreira *et al.*, 2012; Rocha *et al.*, 2012 Schwartz *et al.*, 1982; Freyre *et al.*, 1998; Sousa *et al.*, 2014) except in lupin where resistance is governed by a single dominant gene (Yang *et al.*, 2012).

Molecular markers offer a great potential in breeding for disease and pest resistance as they can be employed in selection of genotypes with specific character. Molecular markers such as RFLP, RAPD, SSR, ISSR, AFLP, SCAR and STS using F_2 and back cross population NILs, RILs and double haploid are used for this.

Mapping and tagging agriculturally important genes is an important area of molecular breeding where molecular markers form the foundation for the marker assisted selection. The advantage of using molecular markers is due to its accurate information about the susceptbility or resistant genotypes to a particular disease at an early stage of plant growth compared to field screening with artificial

inoculation. It also eliminates environmental influence in resistant or susceptibility reaction of genotype of field screening.

Availability of molecular markers linked to genes will help in identifying plants, carrying these genes in various generations without subjecting them to pathogen infection. When reliable markers are identified and gene is tagged with them they would prove to be a powerful tool especially in screening and selection for qualitative and quantitative characters there by reducing burden of plant breeders.

Sharika, a pole type variety reported as susceptible to anthracnose, but the long pods and firmness of the variety looks attractive and draws an attention towards farmers for a wide range of cultivation. Continuous losses due to anthracnose have trouble for the acceptance of this variety. On the other way a semi-trailing dual purpose type, variety Kanakamony developed by KAU is reported as immune to the disease under study. This could be used as a source of resistance; hence, the effort was made by KAU in the department of CPBMB, Vellanikkara, Thrissur for identification of a potential reliable marker linked to the gene governing resistance to anthracnose. Hence the present study based on "Development of molecular markers for anthracnose resistance in vegetable cowpea [*Vigna unguiculata* (L.) Walp.]". Cultivars Sharika and Kanakamony was taken up for the programme. The methodology involved was bulked segregant analysis. The technical programme involved the following aspects.

1. Development of mapping population.

2. Screening of ISSR and RAPD primers for getting polymorphism between resistant and susceptible genotypes.

· The results obtained on various aspects are discussed here ·

5.1 Development of F_2 segregating generation for trait under consideration

Controlled crosses were made by using the resistant variety Kanakamony as pollen parent and susceptible variety Sharika as ovule and vice versa, and F_1 plants were generated (Plate 4.3c). Evaluation of parents with F_1 of Sharika × Kanakamony and reciprocal cross for morphological characters revealed that pole

type and black seed colour characters of Sharika were dominant over semi-trailing and red seed colour of Kanakamony.

A total of 30 plants of F_1 were raised to obtain seeds for F_2 (Plate 4.3c). Eight F_2 plants were raised from a single F_1 plants in a total of 70 pots kept inside the greenhouse, this manner a total of 163 plants were raised along with the resistant and susceptible parents. Segregating population for the character under consideration is necessary to know the gene expression under homozygous dominant, homozygous recessive and heterozygous condition. It will reveal monogenic or polygenic nature of the character. F_2 plants have certain advantages over other mapping populations (DHlines, NILs) because of fixed segregation (Burr, 1999).

The usual method to locate and compare loci regulating quantitative traits (QTLs) requires segregating populations of plants are grouped according to phenotyping expression of trait and bulked them as resistant or susceptible to perform bulked segregant analysis (Michelmore et al., 1991).

5.2 Symptomatology of the disease under artificial condition

The artificial inoculation was carried out inside the greenhouse to get maximum humidity as shown in the Plate 3.2. This particular fungus needs a special condition for the infection to be prevailed as reported by Zaumeyer and Thomas (1957). Only 20 ml of spore suspension for a single dose (Kumar, 1999) was not sufficient enough to establish a good amount of infection. The inoculation at twice after two weeks of first inoculation was found to be more effective (Plate 4.5).

The infection was first noticed in the leaves Initially after 10 days of first inoculation, symptoms appear on green leaves as small pin head black colour spots, surrounded by yellow hallow (Plate 4.4). Then enlarged to 3.4 mm size and covered totally the leaf surface. Finally, leaves became yellow and detached from the plants. Some plants the breakage of vines also noticed. The same observation was noticed by Kumar (1999) and Shiny (2013). Seedlings of Kanakamony have remained symptomless, following the report on its immune nature (Kumar, 1999).

The typical symptoms were noticed fifteen days after the second artificial inoculation which is commonly known as vine blackening (Plate 4.5b). The same symptoms was observed by (Onesirosan and Barker, 1971; Williams, 1975; Thakur et al., 2011) in pigeon pea anthracnose.

Out of 163 F₂ plants inoculated twice, 14 plants were found resistant which could be utilized in marker assisted breeding programme. Many races causing the anthracnose disease have been reported and gene/alleles conferring resistance to such a wide range of races are present in different cultivars of a particular genus. One cultivar have resistance at a particular period of time may become susceptible at another time. For example, break down of resistance has been reported in cultivar G2333 of kidney bean against the races exiting in CoastaRica, Mexico and Argentina reported from CIAT (1995) and Mahuku et al. (2002). This happens due to absence of particular locus/ loci which is specific for the new race. In such cases breeding of cultivar which can offers durable resistance becomes a great challenge for the breeders to incorporate all the genes especially through conventional breeding. This problem can be solved by identification of suitable markers against the race and its utilization in Marker Assisted Breeding (MAB) programmes.

5.3 Screening of parents with the RAPD and ISSR primers

Random amplified polymorphic DNA and inter simple sequence repeat are important tools for tagging the gene/ genes conferring resistance. Advantages of these primers over the other primers like RFLP, AFLP and SSR which need a primer sequence information and good quality genomic DNA.

5.3.1 Genomic DNA extraction from the parents

The genomic DNA isolated through the CTAB method reported by Doyle and Doyle (1990) was initially contaminated with RNA but after following three time wash with Chloroform and Isoamyl alcohol-24:1 (v/v) pure with less RNA contamination was obtained Plate 4.6b, 4.7a and 4.7b. Both NanoDrop and Gel electrophoresis results showed the DNA isolated by this method appeared as clear and single intact band of high intensity.

5.3.2 Isolation DNA for RAPD analysis

The genomic DNA isolated as per standardized procedure from resistant parent, susceptible parent and 20 F_2 progenies were carried out. The DNA was isolated immediately after development of symptoms in plants and was dissolved in TE buffer and maintained in the refrigerator at -80°C for long term use. This technique relies on the differential enzymatic amplification of small DNA fragments using PCR with arbitrary oligonucleotide primers. In RAPD markers the polymorphism results from changes in either of the sequence of the primer binding site due to insertion deletion or inversion etc. This procedure requires only a small amount of DNA which needs has to be free from RNA contamination.

RAPD markers are dominant in nature (Waugh and Powell, 1992). The marker OPZ04 was used for mapping of *Co-6* gene in the F_2 progenies, developed form the cross of two bean lines Mexico 222 × MSU-7 by Rocha *et al.* 2012 was subsequently converted in to SCAR Z04. They have selected RAPD markers for tagging of anthracnose resistance gene as the procedure is rapid and requires only a small amount of DNA.

5.3.2.1 Screening of primers for RAPD analysis

Initially, 47 primers belonging to 13 Operon series *viz.*, OPA, OPAH, OPB, OPC, OPD, OPK, OPL,OPM, OPF, OPO, OPP, OPX, and OPY were screened, which includes seventeen primers used for the genetic diversity study of cowpea by Patil *et al.* (2013). When these primers were used for screening of parents, only five *viz.* OPA 02, OPC 05, OPC 08, OPA 14 and OPD 18 have yielded polymorphism. The template DNA of immune cultivar Kanakamony and susceptible Sharika were used.

Twelve RAPD primers were selected for tagging of genes from the selected genotypes through bulked segregant analysis based on presence of one or more polymorphic bands. Among them OPA 02, OPA 14, OPA 27, OPAH 3, OPAH 5, OPC 05, OPC 08, OPD 18, OPF 09, OPK 07, OPL 18 and OPX 17, have produced at least one polymorphic band and were used for bulked segregant analysis.

5.3.2.2 Isolation of DNA for ISSR analysis

The same protocol of DNA extraction (modified Doyle and Doyle, 1990) for RAPD analysis was followed in ISSR analysis. Like RAPD, ISSR also is a dominant marker.

Forty three ISSR primers, including 18 belonging to UBC series as reported by Anatala *et al.* (2014), were used. Based on the amplification pattern, 19 primers were selected for screening of resistant and susceptible parents. Nine primers ISSR1, ISSR2, ISSR3, ISSR4, ISSR5, ISSR6, ISSR7, ISSR8 and ISSR9 were selected for screening were monomorphic (Table 4.10 and Plate 4.10b). Ten primers belongs to UBC series UBC 807, UBC 809, UBC 810, UBC 811, UBC 816, UBC 834, UBC 840, UBC 846, UBC 855 and UBC 864 were used for Bulked segregant analysis.

5.4 Bulked segregant analysis

The usual method to locate and compare loci regulating quantitative traits (QTLs) requires segregating population of plants with each one genotyped with molecular markers. However, plants from such segregating populations can also be grouped in to resistant and susceptible bulk according to phenotypic expression of a trait which reduce the time for genotyping each and every individual of the segregating population (Michelmore *et al.*, 1991).

The BSA when performed with the DNA bulked from 10, 7 and 5 F_2 plants have failed to yield the markers. In the legumes such as common bean (Sousa *et al.*, 2014; Rocha *et al.*, 2012; Ferreira *et al.*, 2012; Gonçalves-Vidigal *et al.*, 2011), the anthracnose resistance is reported to be polygenic. This particular observation from BSA also points that the anthracnose resistance in cowpea is also governed by more than one gene, which have the capability to offer complete resistance. Identification of marker through BSA is impossible when the bulking of DNA happens from resistant plants whose resistance is governed by different genes. In this case, when more than 3 F_2 plants were used in DNA bulking, at least one plant whose resistance is governed by another gene might have been included.

Since the segregation for anthracnose resistance in F_2 plants was in 15:1 ratio (149 susceptible plants out of 163), it is most likely that the resistance is governed by polygenes, except when a single gene under recessive condition is coding for resistance. If the recessive alleles are governing the resistance, all the F_1s are supposed to be susceptible. In the present study, the F_1 plants were raised under open field conditions with high atmospheric humidity, which is highly favourable for the development of symptoms. Even then, none of the F_1s have developed the symptoms, suggesting that gene action for resistance is polygenic. For a polygenically inherited character, $1/(4^n)$ = ratio of F_2 individuals expressing either of the two most extreme phenotypes, where 'n' is the number of gene pairs involved (Klug and Cummings, 2010). For the present F_2 ratio of 1/16 for resistance, two genes may be contributing for the resistance. Twelve primers from RAPD and 10 primers from ISSR belonging to OPA, OPAH, OPC, OPD, OPF, and OPK, OPX and OPY series and UBC series respectively were used for bulk segregant analysis.

Out of 12 selected primers of RAPD, OPA 02 primers produced a single polymorphic band of 0.85 kb in susceptible parent and susceptible bulk. But it was absent in resistant parent band resistant bulk.

Out of 10 selected ISSR primers of UBC group, two primers UBC 810 and UBC 811 have yielded polymorphic bands of size 1.4 kb and 1.5 kb which were present in resistant parent and resistant bulk but absent in susceptible parent and susceptible bulk.

Since bulked segregant analysis of 12 RAPD primers didn't give polymorphic band which would present in resistant parent and resistant bulk, no RAPD primers linked with anthracnose resistant was identified. Since two primers UBC 810 and UBC 811 give polymorphic band between resistant and susceptible genotypes the two primers could be considered to be linked with anthracnose resistant trait which further will be confirmed after co-segregant analysis of individual F_2 resistant plants used for resistant bulk.

Summary

6. SUMMARY

The study on "Development of molecular markers for anthracnose disease resistance in vegetable cowpea [*Vigna unguiculata* (L.) Walp.]" was carried out at the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara during the period from 2013 to 2015. The main objective of the study was to identify an ISSR or RAPD molecular marker linked with anthracnose resistance in vegetable cowpea, using bulked segregant analysis to enable marker assisted selection. The genotypes used for the study were semi-trailing Kanakamony [*Vigna unguiculata* (L.) ssp. *cylindrica*] which is reported to be immune and pole type Sharika [*Vigna unguiculata* (L.) ssp. *sesquipedalis*] which is susceptible to anthracnose disease. These parents and F_2 progenies of Sharika × Kanakamony and Kanakamony × Sharika were used in the analysis.

The works of study are summarized below:
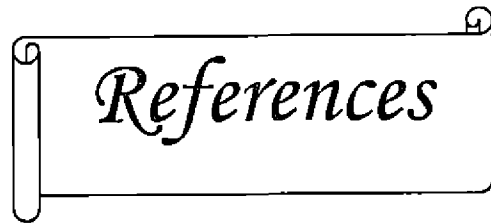
I.   Thirty F_1 plant were raised by crossing immune cultivar Kanakmony as the male parent and susceptible cultivar Sharika as female parent and vice versa. Out of 63 crosses, a total of 6 fruits (4 pods from the cross Sharika × Kanakmony and 2 pods from Kanakamony × Sharika) were obtained. The average fruit set was found to be 9.5 per cent. The F_1 was sown in six different lines and allowed for selfing develop the F_2 seeds.

II.   Evaluation of parents with F_1 of Sharika x Kanakamony and reciprocal cross for morphological characters revealed that pole type and black seed colour characters of Sharika were dominant over semi-trailing and red seed colour of Kanakamony.

III. F_2 segregating population was pot-raised in May-June 2015, inside the greenhouse. Totally, 66 pots filled with soil and FYM 1:1 were prepared. To achieve maximum segregation, one seed from each pod was sown from each individual F_1 plants. A total of 8 seeds were sown from individual plants (4 seeds per each pot). Out of the 264 seeds sown to raise F_2 progenies, 163 plants were raised.

IV.   The parents and F_2 progenies were screened by artificial inoculation of *Colletotrichum lindemuthianum*. Artificial inoculation was done by

spraying the inoculum on 15 days old seedlings and was repeated after 15 days. Humid environment was provided for the seedlings to facilitate infection. Kanakamony was resistant with 85 per cent survival. Sharika was highly susceptible as survival percentage was zero. Out of 163 plants inoculated, 14 were disease resistant with the survival percentage of 8.5.

V.   The CTAB method for DNA isolation, using 2X extraction buffer and washing solution of 70 per cent ethanol has yielded good quantity DNA from the tender leaves. RNA contamination was removed by RNase treatment. The ratio of UV absorbance ($A_{260/280}$) ranged between 1.83-2.09. The quantity of DNA in the isolated samples has ranged between 1352.23-2853.82 ng/µl.

VI.   Primer screening was done with ninty primers (43 ISSR and 47 RAPD). Among them 10 ISSR primers and 12 RAPD primers were finally subjected to BSA based on their capability to discriminate the resistant and susceptible genotypes.

VII. Two ISSR primers, UBC 810 and UBC 811 have given polymorphism with respect to the anthracnose resistance in F_2 segergating population. The polymorphic bands of 1.4 and 1.5 kb, respectively, were yielded by the primers. RAPD marker assay has yielded a marker linked with anthracnose susceptibility, using the primer OPA 02 at 0.85 kb.

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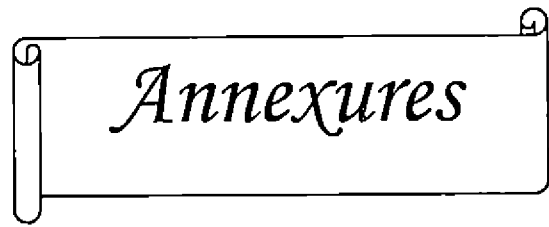
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Annexures

APPENDIX-1

List of Laboratory Equipments used for the study

Nano Drop ND-1000 spectrophotometer - Thermo Scientific, USA

Cooling Centrifuge — - KUBOTA model No. 65000, Japan

Minispin — - Eppendorf, Germany

Horizontal electrophoresis system - BIO-RAD, Italy and Ge Nei, India

Agilent thermal Cycler, Veriti - Agilent and Applied Biosystem

Gel documentation system - BIO- RAD, Italy

AccuBlock ™ Digital Dry bath - model D110, Labnet International, Inc

Laminar air flow - THERMODYNE, Faridabad

Composition of reagents used for DNA isolation and purification

Doyle and Doyle (CTAB) Method

I. **2X CTAB extraction buffer**

        a. CTAB (2% (V/V)

        b. 1 M Tris buffer (p^H 8)

        c. 0.5 mM EDTA (p^H 8)

        d. 1.4 M NaCl

II. **TE Buffer**

        a. 10 mM Tris Buffer (p^H 8)

        b. 1mM EDTA

        c. Distilled water

III. **70% washing solution**

        Ethanol 70%

IV. **Chilled Isopropanol**

V. **RNase stock**

        RNase A   -100 mg

        Autoclaved distilled water1 ml   -1 ml

Stocks was prepared by dissolving 10 mg RNase A in 1 ml water and was stored under refrigerated conditions at -20°C. The RNase A was used to prepare RNase. Ten per cent RNA solution was prepared by dissolving the same in water at 1:10 ratio. The solution was stored at -20°C and use for RNase treatment.

VI. **TAE Buffer (1X)**

        40mM Tris

        1mM EDTA

        20mM Acetic acid

VII. **Agarose gel composition**

        Agarose

        TAE buffer – 2 ml per 100 ml of autoclaved distilled water

APPENDIX- III

Composition of media for culturing of pathogen
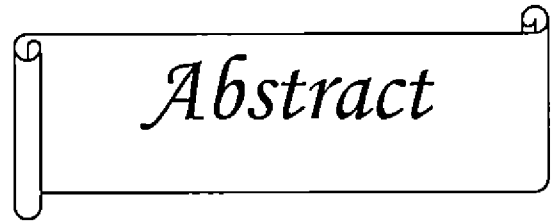
Potato Dextrose Agar (PDA)

Potato	-	200 g
Dextrose	-	20 g
Agar		20 g
Distilled water		1 L

Neopeptone Glucose Agar

Neopeptone	-	20 g
Glucose	-	28 g
Mg SO$_4$	-	12.3 g
KH2PO4	-	27.2 g
Agar	-	20 g
Distilled water	-	1L

Carrot Dextrose Agar (CDA)

Carrot	-	200 g
Dextrose	-	20 g
Agar	-	20 g
Distilled water	-	1 L

Abstract

DEVELOPMENT OF MOLECULAR MARKERS FOR ANTHRACNOSE

RESISTANCE IN VEGETABLE COWPEA [*Vigna unguiculta* (L.) Walp.]

By

DOLAGOBINDA PRADHAN

(2013-11-109)

ABSTRACT OF THE THESIS

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MASTER OF SCIENCE IN AGRICULTURE

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Faculty of Agriculture

Kerala Agricultural University, Thrissur

CENTRE FOR PLANT BIOTECHNOLOGY AND MOLECULAR BIOLOGY

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2015

ABSTRACT

Cowpea is one of the most important food legume crops in the semi-arid tropics, covering Asia, Africa, Southern Europe and Central and South America. Nigeria is the centre of origin for cowpea and in India, it is exclusively known as a kharif season pulse but the demand of cowpea as vegetable is increasing. In India, cowpea is grown in about 0.5 million ha but productivity is as low as 600-750 kg grains ha^{-1}. All the pole type vegetable cultivars are highly susceptible to anthracnose caused by *Colletotrichum lindemuthianum*, which is the most destructive disease, not only for cowpea but also in other legumes. Since no effective measures are capable to manage this disease, the losses can be up to 74-100 per cent and hence breeding the resistant cultivars is suggested as the only promising strategy. Molecular marker assisted selection offers precision in the breeding process but so far no markers are identified in this crop for the anthracnose resistance gene.

With this background, the study on "Development of molecular markers for anthracnose resistance in vegetable cowpea [*Vignaunguiculata* (L.) Walp.]" was carried out at Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, during August 2013 to June 2015. The objective of the study was to identify an ISSR or RAPD molecular marker linked with anthracnose disease resistance in vegetable cowpea, using bulked segregant analysis to enable marker assisted selection.

The dual purpose semi-trailing cultivar Kanakamony, which is reported as immune to anthracnose and the high yielding trailing vegetable type cultivar Sharika, which is susceptible, were used for the development of the mapping population for BSA to identify the marker. The parental populations were grown in separate fields in the month of October 2014 as per the package and practices recommended from KAU. Controlled crosses were made to develop F_1 seeds during November and December months. Thirty F_1 plants were grown during January - March 2015 and the F_2 seeds were developed through selfing. The F_2 segregating progenies were raised during May-June, in the pots and maintained in greenhouse.

Evaluation of parents with F_1 of Sharika × Kanakamony and reciprocal cross for morphological characters revealed that pole type and black seed colour characters of Sharika were dominant over semi-trialing and red colour seed of Kanakamony.

To isolate the fungal spores for artificial inoculation on the F_2 plants, infected plant parts were collected and initially cultured in 3 different media *viz.* PDA, NGA and CDA. NGA and PDA medium were selected for artificial inoculation based on the characteristics of fungal spores. Artificial inoculation was initially done on 15 days old seedlings and was repeated after 15 days with an approximate concentration of 10^6 spores/ml and the highly resistant and susceptible F_2 plants were identified for BSA.

Good quality DNA was isolated from the parents and 47 RAPD and 43 ISSR primers were screened for their capability to generate polymorphic bands. Subsequently, based on the initial screening, 12 RAPD and 10 ISSR primers were selected for bulk segregant analysis. The bulked segregant analysis was done using the DNA from the resistant parent, susceptible parent, resistant F_2 bulk and susceptible F_2 bulk. BSA with the RAPD primer OPA 02 revealed a polymorphic band at 850 bp which was present in the susceptible parent and susceptible bulk and absent in the resistant parent and bulk. BSA with ISSR primers UBC 810 and UBC 811 produced polymorphic bands at 1.4 kb and 1.5 kb, respectively, which were present in resistant parent and resistant bulks. The co-segregation analysis has to be performed, the marker has to be characterized and validated for the use in breeding programmes.