TAGGING OF *PHYTOPHTHORA* POD ROT DISEASE RESISTANCE GENE IN COCOA (*Theobroma cacao* L.) USING ISSR MARKERS

By

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(2015-11-116)

THESIS

Submitted in partial fulfilment of the requirement for the degree of

Master of Science in Agriculture

(PLANT BIOTECHNOLOGY)

Faculty of Agriculture

Kerala Agricultural University, Thrissur

CENTRE FOR PLANT BIOTECHNOLOGY AND MOLECULAR BIOLOGY
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KERALA, INDIA

2017

DECLARATION

I, hereby declare that the thesis entitled **'Tagging of *Phytophthora* pod rot disease resistance gene in cocoa (*Theobroma cacao* L.) using ISSR markers'** 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.

Vellanikkara,

Date: 13/10/2017

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Certified that the thesis entitled **'Tagging of *Phytophthora* pod rot disease resistance gene in cocoa (*Theobroma cacao* L.) using ISSR markers'** is a record of research work done independently by **Mr. Jeughale Kishor Pundlik** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to him.

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ACKNOWLEDGEMENT

First and foremost I bow my head before the ***Almighty God*** *for enlightening and making me confident and optimistic throughout my life and enabled me to successfully complete the M.Sc. thesis work in time.*

It is with immense pleasure I avail this opportunity to express my deep sense of whole hearted gratitude and indebtedness to my major advisor ***Dr. Minimol J. S.****, Associate Professor, Cocoa Research Centre, Vellanikkara for her expert advice, inspiring guidance, valuable suggestions, constructive criticisms, constant encouragement, affectionate advice, care and above all, understanding and wholehearted co-operation rendered throughout the course of my study. I really consider it my greatest fortune in having her guidance for my research work.*

I consider it as my privilege to express my deep-felt gratitude to ***Dr. M.R. Shylaja****, Professor and Head, CPBMB, College of Horticulture, Vellanikkara and member of my advisory committee for her constant support, valuable suggestions and critical scrutiny of the manuscript.*

I express my gratitude to ***Dr. Sainamole P. Kurian****, Professor, Department of Plant Pathology, CoH, Vellayani and member of my advisory committee for her expert advice and assistance provided for constituting the manuscript.*

I sincerely thanks to ***Dr. Rose Mary Francies****, Professor and Head, Department of Seed Science and Technology, College of Horticulture, Vellanikkara and member of my advisory committee for her support, critical comments and suggestions given during the manuscript writing.*

It is great pleasure to record my sincere thanks to ***Dr. Deepu Mathew****, Assistant Professor, CPBMB, Vellanikkara for his precious advice and generous support during my entire study which helped in successful completion of this work.*

I express my sincere thanks to ***Dr. B. Suma****, Professor and Head, Cocoa Research Centre, Vellanikkara for the avail of facilities at the Centre during my research work. I also express my gratitude towards* ***Dr. Valsala P.****, Professor*

(Retired), ***Dr. P. A. Nazeem****, Professor (Retired), CPBMB, for their support, enthusiasm and relevant suggestions during my course of study.*

I am deeply obliged to ***Dr. Mariet Jose****,* ***Dr. Smini Vargheese*** *and* ***Dr. Binu S****. the Teaching Assistants of CPBMB, Vellanikkara and* ***Mrs. Sheethal Babu****, and* ***Mrs. Asha****, Research Associates for their invaluable help, guidance and critical assessment throughout the period of work. Also teaching and non-teaching staff of College of Horticulture as well as on non-teaching staffs at CPBMB, Vellanikkara, I thank them for all the help and cooperation they had extended to me.*

I wish to express my lovely thanks to all the members and labourers of ***Cocoa Research Centre*** *especially* ***Chithira chechi****,* ***Priya chehci****, and* ***Sudha chechi*** *for their affection, care and help during my two years stay in Kerala for PG study.*

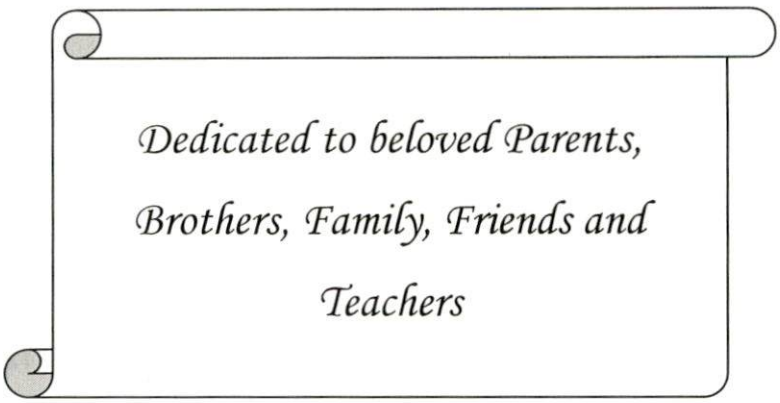
I duly acknowledge the encouragement, moral and unconditional support support, precious suggestions and timely persuasions by my dear seniors especially ***Ajinkya****,* ***Ahamed Mujtaba, Sandesh*** *and* ***Sujith****, not only in my research work but also throughout my PG programme. I express my sincere thanks to my classmates* ***Ashwin, Basil, Debashish, Geethu, Arunima, Giridhari, Pramod, Deepali, Swapnil, Marjan*** *and* ***Priya****, friends* ***Veeresh****,* ***Sunil****,* ***Mahesh****,* ***Nagendra*** *and Juniors whose prayers, love, kind help and affection which gave me enough mental strength and perseverance to get through all odds and tedious circumstances.*

I am in dearth of words to express my love towards ***my beloved Parents*** *and my lovely brothers* ***Sachin*** *and* ***Bhushan*** *and the* ***family*** *for their boundless affection, moral support, eternal love, deep concern, prayers and personal sacrifices which sustains peace in my life.*

I express my deep sense of gratitude to ***Kerala Agricultural University*** *for financial and technical support and facilities provided for completion of my study and research work. I once again express my heartfelt thanks to all those helping hands for the successful completion of this endeavor.*

13/12/17

Jeughale Kishor Pundlik


Dedicated to beloved Parents,
Brothers, Family, Friends and
Teachers

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ABBREVIATIONS

AFLP		Amplified Fragment Length Polymorphism
°C	:	Degree Celsius
cM	:	Centimorgan
cm	:	Centimeter
CPBMB	:	Centre for Plant Biotechnology and Molecular Biology
CTAB	:	Cetyl Trimethyl Ammonium Bromide
dNTP	:	Di-Nucleotide Triphosphate
DNA	:	Deoxyribose Nucleic Acid
EDTA	:	Ethylene Diamine Tetra Acetic acid
g		Gram
ISSR	:	Inter Simple Sequence Repeats
KAU	:	Kerala Agricultural University
kb	:	Kilo base
M	:	Molar
Mb		Mega base pair
mg	:	Milligram
min	:	Minute
ml	:	Mililiter
mm	:	Milimeter
mM		Milimole
nm	:	Nanometer
ng	:	Nano gram
µg	:	Microgram
µl	:	Microliter
µM	:	Micromole
MAS	:	Marker Assisted Selection
min		Minute
OD	:	Optical Density
pH	:	Hydrogen ion concentration
PCR	:	Polymerase Chain Reaction

RFLP		Restriction Fragment Length Polymorphism
rpm	:	Revolution per minute
%	:	Per cent
RNA	:	Ribonucleic Acid
SSR	:	Simple Sequence Repeat
QTL	:	Quantitative Trait Locus
V	:	Volt

Introduction

1. INTRODUCTION

Cocoa, *Theobroma cacao* L., is a small under-story plant native to the lowland tropical rain forest of the Amazon basin (Wood and Lass, 1985; Bartley, 2005). Cocoa was farmed in pre-Columbian times by the Olmec and Mayan cultures. The Mayans used the seeds ('cocoa beans') to prepare beverages for royalty and religious services, and as money (Motamayor *et al.*, 2002). At present, cocoa is cultivated all over the humid tropics, regularly in agro-forest ecosystems with other fruit and commodity crops. It is cultivated extensively as the unique source of cocoa butter and powder for the confectionery industry. Around the world, roughly 5 to 6 million smallholder planters raise 95 per cent of the world's production (Guiltinan *et al.*, 2008).

In the last 100 years, there has been regular growth in demand for cocoa at three per cent each year. The past 10 years had observed an increase in cultivated land area under cocoa, with the West African countries now decisively recognized as the leader in the supply chain. Better demand has been encountered by expansion in production, mainly in the major West African cocoa-producing countries. The demand for cocoa is projected to rise due to cocoa consumption growing in emerging middle-income nations, including India, Brazil, Eastern Europe, China, Russia and Mexico. Income resulting from the sale and export of cocoa offers vital support to livings of planters and landowners all over the tropics. The cocoa yield for 2015 was estimated at a world aggregate of 4,168,000 tons with an inexact value of 12.5 billion USD (ICCO, 2015).

Cocoa production is seriously affected by five major diseases *i.e. Phytophthora* pod rot, witches broom, frosty pod rot, swollen shoot virus and vascular streak die back (Evans, 2002) that annually destroy up to 40 per cent of the total crop. Despite the fact that several key *Phytophthora* diseases of cocoa were recorded from other cocoa growing nations, occurrence of only *Phytophthora palmivora* pod rot has been recorded in India so far (Peter and Chandramohanan, 2011). The genus *Phytophthora* belongs to Oomycete is one of the most damaging plant pathogens, true to its verbal translation in Greek- 'phyto' denoting 'plant' and 'phthora' meaning 'destroyer' (Thines, 2014). Yearly damages from *Phytophthora diseases* were assessed at 30 per cent of the total yield loss (ICCO, 2015) which means a loss of roughly 3.8 billion USD to the cocoa growers around the world.

Disease resistance is governed by number of morphological and physiological characters. Morphological screening is simple to conduct and based on visible traits of the plant. It can help cocoa breeders to handpick the most right accessions for further breeding programs (Engels *et al.*, 1981) and may lead to easier and more cost effective methods of assessment of resistance. But, the morpho-physiological characters greatly depend on environment and ultimately affect the experimental data. During previous two decades, study on the genetics of cocoa had profited tremendously from molecular markers. Huge headway had been completed in the molecular characterization of cocoa germplasm. Molecular markers contrast with morphological markers are superor in genomic richness, the level of polymorphism identified, locus specificity, reproducibility though a little more technically demanding and costly. Hence, they have been used to answer various research problems in cocoa.

Inter simple sequence repeat (ISSR) system is a PCR based technique, which amplify DNA fragment present in between two alike microsatellite repeat regions oriented in opposite direction. The ISSR marker system utilizes microsatellites, usually 16–25 bp long, since primers in a single primer PCR reaction amplifying multiple genomic loci, mainly inter- SSR sequences of dissimilar sizes. This system combines most of the benefits of other marker techniques, like AFLP and microsatellite analysis with the universality of RAPD. ISSRs markers have high reproducibility perhaps due to the usage of longer primers (16–25 mers) as compared to RAPD primers (10- mers) which permit the succeeding use of high annealing temperature (45–60 °C) leading to higher stringency. The ISSR marker has proven to be an efficient tool to tag disease resistance gene in cocoa (Chandrakant, 2014).

In this context, the present study was taken up with the following specific objectives: Tag the gene(s) conferring resistance to *Phytopthora* pod rot disease and thus enable marker assisted selection for developing pod rot resistant varieties.

Review of Literature

2. REVIEW OF LITERATURE

2.1 *THEOBROMA CACAO* – AN INTRODUCTION

2.1.1 Cocoa – Origin and Domestication

Cocoa (*Theobroma cacao* L.) orginated in the tropical rainforest of Central America, is presently cultivated in every tropical swamp of the world (Wood and Lass, 2001). Earlier, cocoa was categorized in the Sterculiaceae family and at present grouped in the family Malvaceae (Bayer and Kubitzki, 2003). The tree is commonly identified as cacao, while the word cocoa is earmarked for the goods prepared from the fermented and dried seeds. Cocoa involves many morphologically flexible populations with a distinct possibility for inter and intra-mattings (Bartley, 2005).

The Europeans was first to begin cultivation of cocoa in Asia and Africa during the sixteenth century (Bartley, 2005) and they were the main force behind the spread of cocoa to other parts of the world. Farming of cocoa began in Central America, where cultural explanation and use of cocoa can be found back more than 3000 years (Henderson *et al.*, 2007; Powis *et al.*, 2011). Amerindian societies including the Mayans (300–900 AD) and the Olmecs (400–1200 BC) deliberately cultivated and used the ancient cultigens (Henderson *et al.*, 2007).

The British brought cocoa to India in 1798, from the island of Amboina (Wood, 1991). From there, it moved consequently to far East Asia. The move in the world's centre of cocoa production from Brazil to West Africa had happened much after establishment of large scale cultivation in Africa (Ruf and Schroth, 2004). In 1822, the Portuguese introduced cocoa to Africa and led the beginning of commercial cultivation (Bartley, 2005) and thereafter, cultivation of cocoa spread to the mainland of West Africa by the 1850s.

2.1.2 Importance and Status of Cocoa

Cocoa is a small understory tree and a standout amongst the most important tropical crops. Today, cocoa is cultivated all through the moist tropics, frequently in agroforestry-ecosystems along with other cash crops. Around the world, roughly 5 to 6 million smallholder planters grow 95 per cent of the world's production (Guiltinan *et*

al., 2008). Cocoa seeds or beans are the basis of chocolate, powder, and cocoa butter and are a noteworthy worldwide exchanged trade item (George, 2013). Exchange of beans was valued at USD nine billion (FAOSTAT, 2014). Cocoa is the chief export commodity of numerous West African nations (70 % of world production), giving key financial assets to Ivory Coast, Cameroon, Nigeria, and Ghana (ICCO, 2015). Presently, the worldwide chocolate commerce is esteemed at 110 billion USD/year (Percival, 2015) and the yearly cocoa production is around 4 million tons (ICCO, 2015).

Since cocoa is a shade loving perennial tree crop with over 50 years cropping cycle, cultivation gives natural advantages by upgrading the biodiversity in avian transient routes, soil and watershed preservation, serving buffer zones close to threatened rainforest territories, *etc.* (Ruf and Zadi, 2003). Cocoa-developing area is to a great extent focused in critical biodiversity hotspots, and in the vicinity to 13 of the world's utmost naturally varied areas (Piasentin and Klare-Repnik, 2004). Both plant and animal variety inside cocoa agro-forests are more prominent than those of other rural land utilizes (Schroth and Harvey, 2007).

2.1.3 Cocoa as an Experimental Plant

Theobroma cacao is a diploid plant with ten sets of chromosomes (2n = 2x = 20) and a lesser genome. The whole genome of cocoa is sequenced and 430-Mb genome was evaluated by flow cytometry (Argout *et al.*, 2011). Examination of the sequence information featured particular extension of some gene families amid evolution. It likewise gives a noteworthy basis of candidate genes for cocoa development.

Cocoa has a few restrictions as a test plant. For instance, its life cycle needs at least 2 to 3 years on or after seed to seed, and it is requisite to maintain progenies of crosses for a long time to completely assess their profitability and disease resistance attributes. Numerous cocoa genotypes are self-incompatible, making breeding methodologies and genetic examination labour intensive (Guiltinan *et al.*, 2008). Moreover, the plants entail substantial zones of acreage and expansive contributions of work to keep up and assess field tests. Furthermore, recalcitrant seeds make it difficult for germplasm conservation and thus must be preserved as alive accumulations in the nurseries or field. The aforementioned and different factors join to make cocoa an actual troublesome and moderate investigational system.

2.2 MORPHOLOGICAL EVALUATION OF COCOA

The morphological descriptors such as pod size, pod and bean weight are considered to be of agro-economic importance (Bekele *et al.*, 1994). Morphological evaluation is a preliminary step to get economic and breeding advances from germplasm pools (Bekele *et al.*, 2006).

Matured fruit is berry but commonly it is called as pod. The fruit varies in shape from spherical to narrow elongated. The immature pod color ranges from green to reddish, whereas matured pods are yellowish to pinkish in color. Usually the pods have 20-40 flat or round beans and these beans may vary in colors like white, pink, brown or purple (Bartley, 2005). Sari and Susilo (2013) observed 14 cocoa accessions for morphological characters viz., number of normal beans per pod, pod girth, pod weight, pod length, wet beans weight per pod, dry bean weight and number of abnormal beans per pod. The outcomes revealed that the trait pod weight had significant part in defining the dry weight of normal bean, and also concluded that wet bean weight per pod character may also be utilized indirectly as a selection standard for dry weight per normal bean. The morpho-agronomic characteristics of leaf, flower, pod and bean can be used for elucidating population structure and to find out genetic distance among the germplasms (Thi *et al.*, 2016).

2.3 COCOA AND DISEASES

In 1900, 80 per cent of the worldwide yield of cocoa originated from the Central and Latin America (Bradeau, 1969), yet, the region had turned into a moderately minor producer by the twenty-first century. Cocoa was the mainstay in "establishment of the Amazon Region's economy up to the late nineteenth century" (Bartley, 2005), and basically all production was coming from this region. Damaging maladies in the Americas that did not happen in the Eastern Hemisphere, particularly witches' broom and frosty pod were major purposes behind such real moves in production (Evans, 2007). In 2012, around 86 per cent of yield originated in the Eastern Hemisphere, besides the main five delivering countries. Altogether of production from the east, delivered around 82 per cent of the aggregate (FAOSTAT, 2014).

In addition to PPR, more noteworthy losses have been anticipated by four other diseases of cocoa (Table 1). Considerably higher damages have been shown for every single of these maladies: witches' broom (100 %), cocoa swollen shoot (100 %), pod rot (90 %), frosty pod (90 %), and vascular streak dieback (VSD) (70 %) (Bowers *et al.*, 2001; Evans, 2002).

Table 1. Major diseases of cocoa

Disease	Region	Estimated world production loss (tons)
Pod rot (*Phytophthora* fungus)	Africa, Brazil, Asia	450,000
Witches broom (fungus)	Latin America	250,000
Frosty pod rot (fungus)	Latin America	30,000
Swollen shoot virus	Africa	50,000
Vascular streak dieback (fungus)	Africa	30,000

(http://www.dropdata.org/, 19/02/2015)

Unmistakably, diseases are prime causes behind yield loss. Cocoa vascular streak dieback and swollen shoot are weakening infections that can destroy trees, though pod rot, witches' broom and frosty pod, those are normally not deadly, and specifically affect harvests by making beans in influenced pods commercially pointless.

2.3.1 *Phytophthora* Pod Rot (PPR) of Cocoa

Despite the fact that several key *Phytophthora* diseases of cocoa were recorded from other cocoa growing nations, in India, only one *Phytophthora* pod rot has been reported so far (Peter and Chandramohanan, 2011). The genus *Phytophthora* of Oomycete is one of the record damaging plant pathogens, and it truly denotes phyto and phthora means plant and destroyer respectively in Greek (Thines, 2014). The cocoa yield for 2015 was estimated at a world aggregate of 4,168,000 tons with an approximate value of 12.5 billion USD (ICCO, 2015). Yearly damage from *Phytophthora* diseases were assessed at 30 per cent of the loss of total product (ICCO, 2015) which means roughly 3.8 billion USD misfortune to the cocoa agriculturists around the world.

2.3.1.1 The Pathogen

A widely accepted cataloguing system to group different species inside the class *Phytophthora* has been looked for extended time. Morphological characters of the different species/isolates are principally utilized to assign *Phytophthora* species. Zoospores produced by *Phytophthora* hung on a sporangial stalk or pedicel like structures called sporangia. The sporangia are hyaline to light yellow in shading and differ in size and shape. Zentamyer (1976) separated *Phytophthora palmivora* isolates into four subgroups as per sporangial stalk qualities and thereby separating them into different strains.

2.3.1.2 Control of Disease

Phytophthora, a novel pathogen, have unique components that should be measured when arranging examinations or control strategies. Lamentably, there is not an even sole remedy or basic solution to control diseases of tropical tree crops caused by *Phytophthora*. The circumstance is composite because of (1) the biology of the pathogen; (2) financial matters; (3) human conduct, and (4) politics (Drenth and Guest, 2013). Conventionally four standards of control strategies have been utilized to oversee plant infections. These standards incorporate prohibition (quarantine), destruction (phyto sanitation/cleanliness), protection (chemical), and immunization (plant resistance). The investigation led by Peter and Chandramohanan (2014) showed that PPR disease can be viably managed in gardens with high disease rate by complementing fungicide application with the cultural practices adopted in the trial. Albeit this, concentrated chemical control will possibly lead to environmental damages. Subsequently, there is solid need to develop resistance genotypes with appropriate breeding programme.

2.3.2 Nature of Disease Resistance

The disease caused by *Phytophthora* can be managed by genetically determined resistance in the host plants. Genetic studies have shown that resistance to PPR infection is polygenic and could be improved by recurrent selection (Iwaro *et al*., 1997; Nyasse *et al*., 2007). Much late research into *Phytophthora* science had used present day molecular biology methods. The sequenced genomes of three *Phytophthora* species

have uncovered remarkable collection of genes coding proteins emitted by *Phytophthora* with the end goal of disabling and disrupting host plant intrinsic and dynamic defence responses (Whisson, 2010).

Rubiyo and Rivaie (2013) proposed that resistance to the disease caused by *P. palmivora* in cocoa was more affected by cumulative gene action. Imperviousness to the different species that cause pod rot infection in cocoa is related with genetic change.

2.4 SCREENING METHODS USED FOR PPR DISEASE RESISTANCE IN COCOA

Concerning field readings of contaminated pods, many specialists battled that the data which can be gathered in the farm was just a rough sign of the genuine susceptibility of those accessions inspected. Moreover, the consequences of leaf inoculation on the field were uncertain and thus testing on leaf plates or detached leaves kept under maintained conditions was recommended (Lawrence, 1978). A typical technique for stem inoculation includes putting an agar plate with mycelium of *Phytophthora* into a vertical opening on the stem and casing the opening with any tape (Lawrence, 1978).

Inoculation tests on injured pods give a appropriate method for checking post-penetration resistance or internal resistance (Prendergast and Spence, 1967). This inoculation procedure was investigated by various workers utilizing distinctive types of inoculum. For example, Bhavani *et al.* (2007) examined 225 different cocoa types against PPR by artificial inoculation of the pathogen on detached cocoa pods and have revealed that cocoa types have shown consistence differences in level of infection. Detached pod method was found to be an appropriate technique for evaluating effect of endophytic isolates in reducing disease on artificially inoculated detached pod (Kurian, 2011). They reported eight potential endophytes for their antagonistic effect on pathogen.

2.5 MORPHOLOGICAL MARKERS IN PLANT DISEASE CHARACTERIZATION

Morphological screening is simple to conduct and based on visible traits of the plant. Morphological characterization may help cocoa breeders to make choice of the most right accessions for further breeding programs (Engels *et al.*, 1981) and may lead to easier and more cost effective methods of assessment of resistance. Disease

resistance is governed by number of morphological and physiological characters. For instance, the joint effect of stomatal frequency and size showed a strong relationship with resistance at penetration stage, suggesting that pathogen entry may be through stomatal opening (Iwaro *et al.*, 1997). Nyadanu *et al.* (2012) reported significant relationship between pod husk thickness and resistance to *P. palmivora* in cocoa.

2.6 MOLECULAR MARKERS FOR GENOMIC MAPPING AND CHARACTERIZATION OF COCOA

2.6.1 The Characterization of Cocoa Germplasm

During previous two decades, the use of molecular markers had helped tremendously in research on cocoa genetics. Huge headway had been completed in the molecular characterization of cocoa germplasm. The main purposes of these studies comprise: dropping mislabeling and repetition in cocoa gene banks, knowing genetic variation in *ex situ* pools and in grower's plantation, confirming pedigree data and describing germplasm for valuable agronomic characters.

The isozymes were the primary molecular markers used in cocoa (Lanaud, 1986). Despite the fact that each isozyme normally produced low number of the accessible loci and the polymorphisms, it enabled appraisal of genetic variability and breeding system, helped genotype identification, and supported to linkage mapping (Sounigo *et al.*, 2005). Nevertheless, the isozyme markers are obsolete on account of their short polymorphism and the ecological impact on the phenotype.

DNA markers generally exploited in cocoa contain Restriction fragment length polymorphism (RFLP), Random amplified polymorphic DNA (RAPD), Amplified fragment length polymorphism (AFLP), Simple sequence repeats (SSRs) and Inter simple sequence repeats (ISSR). Said markers contrast in genomic richness, the level of polymorphism identified, locus specificity, reproducibility, practical necessities and economic charge. Hence, these markers have been used to answer several research problems in cocoa.

2.6.1.1 Restriction Fragment Length Polymorphism

Restriction fragment length polymorphism (RFLP) is a DNA marker free of Polymerase chain reaction (PCR) and it was first practised in cocoa in the mid-1990s

(Laurent *et al.*, 1994). The level of polymorphism of RFLP is reasonably high in cocoa. The properties of this marker like, co-dominant character and great reproducibility make it an appropriate marker for tagging genes and Quantitative trait loci (QTL), development of genetic linkage maps associated to traits of agronomic significance (Risterucci *et al.*, 2000) and evaluation of genetic diversity (Motamayor *et al.*, 2002). Since RFLP markers are always definite to a few number of loci, RFLP is not an extremely viable instrument for cocoa genotype documentation. Moreover, RFLP is not an agreeable to automation and data management is both lengthy and costly.

2.6.1.2 Random Amplified Polymorphic DNA

Random amplified polymorphic DNA (RAPD) was the primary PCR built DNA fingerprinting system to be practised for genetic description of cocoa (Wilde *et al.*, 1992). This marker system is, in fact, easy to execute yet has a few reproducibility amongst laboratories and analyses. This system is not an immediate criteria of heterozygosity which makes it not as much of valuable for authentic genotyping instead of essentially recognizing clones. However, RAPD is an easy to-utilize marker for recording variability among cocoa clones. Russell *et al.* (1993) demonstrated that three RAPD markers could recognize 25 cocoa accessions as indicated by their geographical origin. Different investigations utilized RAPD for examinations of genetic diversity, and identification of accession repetition and mislabeling (Sounigo *et al.*, 2005). Suwastika *et al.* (2017) revealed that the diversity of chloroplast genome within species of local cocoa (*T. cacao*) from Central Sulawesi can be distinguished in view of phenotypic and nuclear genome-based characterization by RAPD marker.

2.6.1.3 Amplified Fragment Length Polymorphism

Amplified Fragment Length Polymorphism (AFLP) is a PCR centered fingerprinting system that joins the quality of RAPD and RFLP (Vos *et al.*, 1995). It is very polymorphic with significant reproducibility inside the laboratory. Like RAPD, AFLP does not need prior information about sequence for primer development. However, as a result of its dominant nature, AFLP is not an immediate criteria of heterozygosity thus it has constrained usage in genotyping. Perry *et al.* (1998) announced characterization of parental plants along with their hybrids of cocoa by AFLP.

Marker AFLP was trustworthy for recognizing firmly linked cocoa varieties (Saunders *et al.*, 2001). Queiroz *et al.* (2003) identified a major QTL associated with resistance to witches' broom disease, by AFLP linkage mapping. Yet, there have been few investigations to date utilizing AFLP marker in cocoa germplasm management. The significant drawbacks of AFLP comprise the strength of alleles and conceivable non-homology of co-migrating parts linking to various loci, which constrain its extensive use in cocoa.

2.6.1.4 Simple Sequence Repeats

In the previous decade, SSRs, otherwise called microsatellites, have developed as a broadly utilized marker in cocoa, empowering extraordinary steps to be prepared in the description of cocoa germplasm (Lanaud *et al.*, 1999). SSRs markers are ordinarily multi-allelic and co-dominant, permitting exact segregation of individual clones in view of the multilocus fingerprints. The information study and understanding of outcomes suited the genetic model of cocoa.

Such as a result of these significant points, SSR has been the technique of choice for cocoa in the previous decade. SSR has been exploited to different parts of cocoa germplasm characterization, containing:

i. Identification of mislabeled and duplicate accessions in germplasm collections

The improper naming of cocoa accession has been a huge issue that has thwarted the effective protection and utilization of cocoa germplasm (Motilal and Butler, 2003). SSR utilized along with more throughput genotyping services, allow expansive scale evaluation of genetic identity in cocoa gene banks. SSR profiles give correct match for multi-locus in identical cocoa genotypes as compare to dominant markers, for example RAPD and AFLP (Zhang *et al.*, 2006). Accordingly, multi-locus genotyping based on SSR has been generally explored in different national and worldwide germplasm assemblies (Johnson *et al.*, 2007). The polymorphic groups produced in SSR study were utilized for creating DNA fingerprints for cocoa varieties of Kerala Agricultural University (KAU) (Sujith, 2016).

ii. Evaluation of on-farm diversity

Aikpokpodion *et al.* (2006) surveyed the cocoa gown in various agro ecosystems in Nigeria for assessing the genetic variability by using SSR markers and observed a small acceptance rate of enhanced germplasm in farmer's fields. Diversity study based on SSR markers was completed in Ivory Coast and Ghana (Opoku *et al.*, 2005). The set of 27 cocoa trees from the significant cocoa growing area of Tamil Nadu were evaluated utilizing 10 SSR markers resulted in identification of five clusters (Thondaiman *et al.*, 2013).

iii. Investigating phylogeograph

Sustainable preservation and powerful utilization of cocoa germplasm need four fundamental key factors *viz.*, progressions of gene flow, understanding the spatial patterns of biodiversity, chronicled consequences and climatic consequences for the sharing of genetic diversity. SSR is an ultimate marker system for evaluating intra-specific phylogeography in cocoa. Motamayor *et al.* (2002) studied the allelic structure in coca varieties from Meso-America. Their outcomes supported the speculation that cocoa evolved in the greater Amazon and propose the possible spreading route from the Amazon to Meso America and Mexico.

Sereno *et al.* (2006) looked at cocoa genotypes of characteristic populaces from the Brazilian Amazon. It was concluded that the Brazilian upper Amazon populace was observed to have the biggest genetic variability and in this way it was proposed to be to part of the centre of diversity for the species. Suwastika *et al.* (2015) reported high evolution rate on cocoa trees considering the variety of recognizable pod morphology and genetic diversity, based on SSR marker from several clones collected from Central Sulawesi ranches.

2.6.1.5 Single Nucleotide Polymorphism (SNP) Marker

Emphasis is currently moving on the way to the improvement of molecular markers that can be recognized by non-gel-based examines. Single nucleotide polymorphism (SNP) is one of the most mainstream of these, which identifies locales in which DNA groupings vary by a single base pair. The SNPs assays do not entail DNA partition by size and, subsequently, can be mechanized in a test plate setup or on

microchips (Rafalski, 2002). SNPs are the utmost widely spread group of polymorphisms in plant genomes (Zhang and Hewitt, 2003).

Improvement of SNP markers is advancing quickly in cocoa. The substantial scale utilization of SNPs in cocoa will essentially expand our capacity to solve more difficult queries in germplasm characterization of cocoa. Genetic fingerprints of West African cocoa were produced from SNPs changed over from microsatellite markers (Dadzie *et al.*, 2013).

2.6.2 Linkage Mapping

In cocoa, the primary linkage map was created for the progenies of 'UPA402' × 'UF676', comprising 193 different marker loci (for the most part RFLPs and RAPDs), covering 759 cM of every 10 linkage clusters, relating to the haploid chromosome number (Lanaud *et al.*, 1995). This map was turned into the consensus linkage map and reference for chromosome numbering for cocoa and linkage group after inundated with extra markers (essentially AFLP and SSRs) to an aggregate of 424 loci (Risterucci *et al.*, 2000; Clement *et al.*, 2001).

The second linkage map for cocoa was developed by Crouzillat *et al.* (1996) from a backcross population of 'Catongo' × 'Pound12', the absolute backcross covered 944 cM, outline with 140 markers (RFLPs and RAPDs) and comprised two morphologic loci (Crouzillat *et al.*, 2000). For the related F_1 population from the same cross an extra genetic map was produced with 162 markers, covering 772 cM (Crouzillat *et al.*, 2000). Consecutively, for identification of genomic regions related with *Phytophthora* resistance, additional eight linkage maps were produced (Risterucci *et al.*, 2003). At the same time, yield traits related QTL and plant life were additionally evaluated in some of these groups.

Faleiro *et al.* (2006) established and utilized an F_2 population of 'ICS1' × 'Scavina 6' cross to recognize genomic areas related to witches' broom disease resistance and this highly dense genetic map presently comprises almost 500 different markers. As of late, extra mapping populations were established in Brazil to recognize QTLs related with resistance to witches' broom disease (Figueira *et al.*, 2006).

2.6.3 Quantitative Trait Loci (QTL) Mapping

Cocoa genetic maps have been utilized to identify QTLs for different argonomically vital attributes, containing resistance to the three major fungal maladies, plant life, quality characteristics and yield components (Risterucci *et al.*, 2003; Brown *et al.*, 2005).

2.6.3.1 Disease Resistance

The *Phytophthora* pod rot is the utmost vital infection of cocoa around the world. *Phytophthora palmivora* occurs all inclusive, whereas *P. megakarya* is limited to West Africa, but in the Americas pathogens *P. capsicii* and *P. citrophthora* occurs. Twelve linkage maps are available to distinguish genomic regions related to *Phytophthora* resistance (Guiltinan *et al.*, 2008).

QTLs for resistance against *P. palmivora* were recognized based on normal disease proportions under field conditions in Ivory Coast. The QTL's were located on chromosome one and nine of the two parents of the 'UPA402' × 'UF676', disclosing 15 to 19 per cent of the variability for the character (Lanaud *et al.*, 2000). One major QTL was recognized on chromosome 10 based on a comparable assessment conducted for a two-year reap period utilizing the 'T60/887' × 'IFC2/IFC5' progeny, clarifying 17 per cent of the phenotypic fluctuation (Flament *et al.*, 2001). After the information gathered between years eight and 13 of planting, Clement *et al.* (2003) identified a critical QTL for resistance against *P. palmivora* for two parents 'DR1' and 'IMC78' on a similar region of chromosome four.

Six QTLs were identified on five linkage groups base on the resistance reaction to *P. palmivora* of the "'Catongo' × 'Pound12' F_1 and the BC_1 offspring, when assessed by detached pod inoculation (Crouzillat *et al.*, 2000). Just a single QTL (on chromosome 9) was normal to the two populations, by a more impact on the F_1, clarifying almost 48 per cent of the variability for the character. Flament *et al.* (2001) additionally utilized simulated inoculation on attached pods of 'T60/887' in the examination for resistance, distinguishing two QTLs on chromosomes two and six.

The QTLs for resistance to *Phytophthora* were distinguished based on inoculation of leaf disks (Lanaud *et al.*, 2000; Flament *et al.*, 2001; Risterucci *et al.*,

2003). In any case, correlations between resistances assessed by leaf disk inoculation with pod rot rate in the field or pod inoculations with *P. palmivora* have been feeble and non-significant, conceivably because leaf disk inoculations are very much impacted by the environment and have low exactness and reproducibility.

Crouzillat *et al.* (2000) detected a QTL on chromosome five for *P. palmivora* resistance based on field pod inoculation of the BC_1 offspring, and in this QTL a group of resistance gene analogue (RGA) has been confined as well (Lanaud *et al.*, 2004). Motilal *et al.* (2001) discovered three significant QTLs on chromosomes one, nine, and three or eight by utilizing leaf inoculation of 'IMC57' × 'Catongo' with *P. palmivora,* and they were co-localized with earlier reported QTLs in other studies. Overall 13 QTLs for *Phytophthora* resistance were identified in six chromosome area, clarifying the phenotypic variability between 7.5 per cent and 12.4 per cent (Risterucci *et al.*, 2003). These QTLs were identified based on the result of leaf disc inoculation in the progeny of ('Scavina6' x Hybrid) × 'IFC1' and using two isolates of each of three *Phytophthora* species (*P. palmivora*, *P. megakarya* and *P. capsicii*).

After dissecting 16 QTL studies, the predicted 76 QTLs were anticipated on a dynamically constructed consensus map (Lanaud *et al.*, 2009). Numerous hot spots with QTLs related with various *Phytophthora* species and different diseases, were recorded. The possible number of 'genuine' QTLs was assessed by using a meta-analysis executed in BioMercator programming. After contrasted with the confidence interval of individual QTLs, a two-fold decrease in normal confidence interval was observed. This tactic affirms the presence of numerous resources of diseases resistance in cocoa which could be cumulated in newly developed varieties to enhance the sustainability of cocoa resistance through marker assisted selection strategies.

The QTLs study for cocoa resistance to *P. palmivora* was carried out in three hybrid progenies in Cote d'Ivoire, which were resistant Forastero clones, moderately susceptible Trinitario clones and a susceptible Forastero clone (Akaza *et al.*, 2016). They followed leaf disc inoculation with zoospores (Folres) and pod rot rate (PRR) in field to evaluate resistance to *P. palmivora.* Eight QTLs of PRR and three QTLs of Folres were detected on different chromosomes in the three progeny. Among these one QTL associated with Folres was identified on chromosome 10, moreover they also

found QTLs for PRR and Folres on chromosome one. Markers associated to QTLs have tremendous potential as candidates for future exploitation of these disease associated QTLs as selection tools.

2.7 ISSR MARKERS FOR GENOMIC STUDIES IN PLANTS

Inter simple sequence repeat (ISSR) system is a PCR based technique, which comprises amplification of DNA fragment present in between two similar microsatellite repeat regions leaning in opposite direction. A single primer PCR reaction utilizes microsatellites as primer normally 16–25 bp long targeting multiple genomic loci to amplify largely the inter-SSR sequences of different sizes. The primers used can be di-nucleotide, trinucleotide, tetranucleotide or pentanucleotide repeat. The primers can be either unanchored (Gupta *et al.*, 1994) or more generally anchored at 3'or 5' end with one to four degenerate bases stretched into the bordering sequences (Zietkiewicz *et al.*, 1994).

The ISSR system combines most of the advantages of AFLP and microsatellite markers with the universality of RAPD. This system has high reproducibility maybe due to the use of longer primers (16–25- mers) as compared to RAPD primers (10- mers) which permit the successive use of increase annealing temperature (45–60 °C) causing higher stringency.

2.7.1 ISSR Marker Analysis in Cocoa

Charters and Wilkinson (2000) used di-nucleotide based ISSR primers anchored at 5' or 3' end for fingerprinting studies with high reproducibility for conservation of cocoa collection. Molecular characterization of a collection of 50 clonal and hybrid cocoa trees (*Theobroma cacao* L.) was done from the UNAS-Tingo Maria by using ISSR markers indicating a spatial distribution between parents and their respective progeny (Wong and Alfonso, 2009).

ISSR markers were used to characterize *Theobroma cacao* L. collections at Tingo Maria region of Peru (Chia *et al.*, 2011). The study was conducted to differentiate 46 cocoa accessions maintained in Tingo Maria - Peru and the similarity relationships between the accessions. In this research, the results showed that ISSR, despite its

dominance nature, could establish the noticeable capacity to group Trinitario accessions into a common cluster.

In KAU, the study conducted by Chandrakant (2014) revealed that ISSR primer UBC 857 was able to differentiate between VSD resistant clones of cocoa from susceptible and partially resistant ones. This finding was further validated by Tulshiram (2016). ISSR analysis was done to check polymorphism in promising cocoa varieties of KAU (Sujith, 2016). By scoring distinguish polymorphic bands, DNA fingerprints were developed to differentiate cocoa varieties of KAU from other.

2.7.2 ISSR Markers for Tagging of Disease Resistance in Other Crops

Many new markers can be recognized in the similar region based on linked ISSR marker to a gene of interest. Ratnaparkhe *et al.* (1998) confirmed that ISSR markers are suitable in gene tagging and also acknowledged a marker, UBC 855_{500}, for *Fusarium* wilt race 4 resistance gene in chickpea. In another study, an F_2 population of chickpea cultivars was used to construct intraspecific linkage map for *Ascochyta rabiei* (Pass.) Lab by ISSR markers (Flandez-Galvez *et al.*, 2003). Study revealed that the Resistance gene analogues (RGA) markers grouped with the ISSR markers on linkage groups showing resistance to disease. In another study, ISSR 8111_{357} marker was identified as tightly associated with the *Mungbean yellow mosaic virus* (MYMV) resistant gene at 6.8 cM (Souframanien and Gopalakrishna, 2006).

Sea buckthorn (*Hippophae* L.), an economically significant plant when screened with ISSR markers, indicated that four ISSR markers were potentially related with resistance to dried-shrink disease (Ruan *et al.*, 2009). These markers provided a possible system for breeding programmes that selected lineages resistant to dried-shrink disease without availability of no other genetic information.

Jogaiah *et al.* (2014) developed SCAR marker from identified ISSR marker tightly linked to downy mildew disease in pearl millet. The F_2 population produced from a cross between a susceptible and a resistant genotypes of Indian cauliflower (*Brassica oleracea* var. *botrytis* L.) was phenotyped by lab inoculation using *Xanthomonas campestris* pv. *campestris* race 1 (Saha *et al.*, 2014). Further Bulk segregant analysis (BSA) analysis in resistant and susceptible bulks of F_2 progeny

revealed two ISSR markers linked to resistant locus Xca1bo from seven differentiating polymorphic markers screened.

In BSA study, marker ISSR 834 related to the powdery mildew resistant gene of resistant parent BPMR-48 was found polymorphic between the parents as well as resistant and susceptible bulk of green gram, suggesting its probable relation in to resistance gene (Bainade *et al.*, 2014). Salah *et al.* (2016) identified QTL linked to maize stalk rot disease resistance by using an ISSR along with other molecular markers. The ISSR primers were exploited to reveal the genetic polymorphisms among 42 mulberry genotypes from seven countries over Asia and South America (Wang *et al.*, 2017). Additionally, a link between mulberry diseases and their genotypes was recorded, which directed possible application for ISSR in studying disease resistance of mulberry.

2.8 POLYMERASE CHAIN REACTION

Polymerase chain reaction (PCR) is a molecular biology technique for enzymatically amplifying DNA without utilizing a living organism, for example, *E. coli* or yeast. The procedure permits a little volume of the DNA to be amplified ordinarily, in an exponential way. With more DNA available, examination is made significantly less demanding. The development of PCR is a technological breakthrough in genome analysis as it allowed the amplification of specific segments of genomic DNA (Saiki *et al.*, 1988).

The introduction of the PCR system has revolutionized standard molecular techniques and enabled new tools deleting DNA polymorphism (Hu and Quiros, 1991). Presence or absence of a precise DNA segment between two individuals serve as standard for polymorphism score. The absence of amplified segment may result from omission of a priming site or addition rendering site too difficult for successful amplification. Addition can change the size of a DNA segment without preventing its amplification (Williams *et al.*, 1990). The PCR system is widely used for detecting disease resistance gene, for developing DNA fingerprints, for phylogenetic analysis, *etc.* For instance, effective PCR based detection assays have been developed for detecting the major strawberry pathogens (Mirmajlessi *et al.*, 2015) with the final aim of optimizing plant disease management approaches.

2.9 CLONING OF PLANT DNA

The polymerase chain reaction (PCR) is perceived as an essential technique in molecular biology and biotechnology. Subsequently, PCR based gene probing, cloning, and sequencing are widely used for genomic study. The vast majority among these methodologies need ligation of the target genes, or DNA fragments, with different specific vectors for ensuing applications. For this reason, it is notable that TA-cloning systems are the best techniques for direct cloning of PCR products, because the strategy can dodge laborious and tedious steps like restriction digestion and ligation (Lim *et al.*, 2010). This approach is based on the principle that non proof reading polymerases, for example, 'Taq' join a nucleotide adenosine into the 3' end out of a template independent manner. Thus, protrusion of a thymine at the terminal ends of the cloning vector can ligate PCR products. This TA-cloning method was acknowledged by a few reports (Mead *et al.*, 1991; Song *et al.*, 1995), and additionally enhanced by the incorporation of the recognition sites into the vector as cloning sites.

The blue white screening method is based on β-galactosidase action for the detection of recombinants. It requires 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) as a substrate and isopropyl-β-D-thiogalactopyranoside (IPTG) as an inducer for increased fidelity; along these lines, it has a moderately high cost. Furthermore, this technique is likewise functional in a lacZ mutation or deficient host. It is easier way for selection of recombinants.

Usually, the PCR product can directly be sequenced but it is better to clone it. Generally, the first 10-50 bp of the Sanger sequence fragments contain a lot of noise and it gives short sequence with accuracy. Hence, for the complete sequence, DNA fragment was cloned on vector and sequenced using specific M13 primers from the vector (Yanisch-Perron *et al.*, 1985). The microsatellite UBC 857 marker was validated by cloning and sequencing (Tulshiram, 2016). The marker was confirmed linked with VSD resistance.

2.10 SEQUENCING OF PLANT DNA

Maxam and Gilbert (1977), and Sanger (Sanger *et al.*, 1977), developed the first practical sequencing methods and were implemented on a large scale. Plant

biotechnology started shortly thereafter with the successful integration of recombinant DNA and sequencing techniques to generate the first transgenic plants using Agrobacterium (Fraley *et al.*, 1983). The first genetic map in plants based on RFLPs (Bernatzky and Tanksley, 1986) enabled the capture of genetic variation and started the era of molecular marker-assisted plant breeding. From that point forward, sequencing methodologies have been essential tools in plant research. They have allowed the characterization and modification of genes and metabolic pathways, as well as the use of genetic variation for studies in species diversity, MAS, germplasm characterization and seed purity.

The determination of the reference genomes in *Arabidopsis thaliana*, rice and maize using Sanger sequencing strategies constituted major milestones that enabled the analysis of genome architecture and gene characterization in plants (Arabidopsis Genome Initiative, 2001; International Rice Genome Sequencing Project, 2005; Schnable *et al.*, 2009). More recently, the development and increasing availability of multiple Next-Generation sequencing (NGS) technologies minimized research limitations and bottlenecks based on sequence information (Glenn, 2011). It is difficult to overstate the influence that these massively parallel systems have had in our understanding of plant genomes and in the expansion, acceleration and diversification of breeding and biotechnology projects.

The new chemistries and platforms, broadly described as NGS technologies, take advantage of diverse chemistries and detection approaches. Currently there are 5 companies commercializing one or more NGS platforms. However, there are only three NGS technologies, Roche 454, Illumina and ABI SOLiD that account for the vast majority of usage in plant research and are widely available in academic institutions, private research centres and service-providing companies.

The draft genome of *T. cacao*, an economically important tropical-fruit tree crop was sequenced and assembled (Argout *et al.*, 2011). This assembly corresponds to 76 per cent of the estimated genome size and contains almost all previously described genes, with 82 per cent of these genes anchored on the 10 cocoa chromosomes. It also provided a major source of candidate genes for cocoa improvement.

Al-Rwahnih *et al.* (2015) compared the bioassay against NGS analysis of the genomic sequences of the viruses and other pathogens extracted as DNA and RNA from infected grapevine material. The BLAST was used to compare contigs to references, using parameters. BLAST study found NGS to be superior to the standard bioassay in detection of viruses of agronomic significance, including virus infections at low titers, in its comprehensiveness, the speed of its analysis, and for the discovery of novel, uncharacterized viruses.

Materials and Methods

3. MATERIALS AND METHODS

The study entitled 'Tagging of *Phytophthora* pod rot disease resistance gene in cocoa (*Theobroma cacao* L.) using ISSR markers,' was carried out with an objective to develop a strategy to detect gene(s) for *Phytophthora* pod rot (PPR) resistance in cocoa. The experiments were conducted at Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture and Cocoa Research Centre (CRC), Vellanikkara during the period 2016-2017. The materials used and methods adopted in this study are described below.

Hybrid progeny of SVI 1.26 × PII 12.11 served as the material for this study (CCRP, 2009). The parents selected with moderate level of resistance for *Phytophthora palmivora* were heterozygous in nature. Therefore, the segregating generation which was obtained in F_1 generation was used for identifying susceptible and resistant genotypes.

3.1 MORPHOLOGICAL ANALYSIS OF HYBRIDS

A total of 28 F_1 hybrids obtained from the cross between SVI 1.26 × PII 12.11 were morphologically characterized for pod and bean characters *viz*., pod weight (g), pod length and breadth (cm), wet bean weight (g) and single dry bean weight (g) recorded. The observations were recorded on five pods from each hybrid. Analysis was done following the completely randomized design (CRD) involving twenty-eight treatments.

3.2 SELECTION OF RESISTANT AND SUSCEPTIBLE HYBRIDS

3.2.1 Isolation of the Pathogen

Infected pods were collected from the field during the peak period of infection. The collected pods were washed thoroughly with detergent and water to avoid surface contamination. Pods were kept for air drying and then surface bits were taken from infected part of the pod. The bits were surface sterilized with 0.1 per cent $HgCl_2$ for two min followed by washing in three changes of sterile water. The surface sterilized bits were placed on potato dextrose agar (PDA) medium and incubated at 22 °C. The plates were observed daily for growth of the pathogen. The cultural and morphological

characters of the isolate *viz*., pattern of fungal growth and shape of sporangiophores were observed. The purified isolate was maintained on PDA plates for further use.

3.2.2 Artificial Inoculation of Pathogen on the Healthy Pods

The procedure suggested by Iwaro *et al*. (2000) was followed for this experiment.

Fresh healthy, immature pods of equal age and size were collected from 28 hybrids of cocoa. Pods from a highly susceptible genotype to PPR served as control. The length and breadth of the pods were recorded. The pods were washed thoroughly with detergent and water, and disinfected with 70 per cent ethanol. The inoculation of the pod with the pathogen was made after giving injury with pin pricks. A disc of about 10 mm diameter of seven days old *Phytophthora* culture grown on PDA was placed over the pin pricks and cotton moistened with sterile water was placed over it. The inoculated pods were incubated in polythene bags with a pad of cotton wetted with sterile water in order to provide humidity. Two replications were maintained for each cocoa hybrid. Observations on the length and breadth of lesion developed were recorded at one day interval for ten days. The per cent infection was calculated by using the formula given below (Bhavani *et al*., 2007) and based on it grouping of genotypes was done (Table 2).

Per cent pod area infection =

$$\frac{\text{Average length of lesion} \times \text{Average breadth of lesion}}{\text{Length} \times \text{breadth of pod}} \times 100$$

Table 2. Grouping of genotypes based on per cent of pod area infection

Sl. No.	Category	Pod area infection (%)
1	Resistant	Less than 25
2	Moderately resistant	>25 to 50
3	Moderately susceptible	>50 to 75
4	Susceptible	More than 75

3.3 MOLECULAR MARKER ANALYSIS

3.3.1 Plant Materials

From hybrid progeny of SVI 1.26 × PII 12.11, three resistant and three susceptible plants were identified after lab screening. These hybrids along with their parents were used for tagging the resistance gene for PPR in cocoa (Table 3).

Table 3. Details of the cocoa hybrids and parents used in the study

Sl. No.	Hybrids and parents	Stand No.	Response to *Phytophthora* pod rot disease
1	H23	7.4	Resistant
2	H25	13.9	Resistant
3	H15	16.8	Resistant
4	H19	9.3	Susceptible
5	H3	13.7	Susceptible
6	H27	15.7	Susceptible
7	P1	SVI 1.26	Moderately resistant
8	P2	PII 12.11	Moderately resistant

3.3.2 Laboratory Chemicals, Glassware and Plastic Wares

The chemicals used in this study were of AR grade procured from SRL, HIMEDIA and Merck India Ltd. The Taq DNA polymerase, Taq buffer and molecular weight marker supplied by GeNei, Invitrogen and ThermoFisher Scientific and RNase A (Sigma, USA) were used. The plastic wares used for the study were purchased from Tarsons, India Ltd.

3.3.3 Equipment and Machinery

The present experiments were carried out using the molecular biology facilities and equipment available at CPBMB, Vellanikkara. Centrifugation was done in KUBOTA 6500 high-speed refrigerated centrifuge. NanoDrop® ND-1000 spectrophotometer was used for the estimation of quality and quantity of DNA. The PCR was done in ProFlex™ PCR Systems from Applied Biosystems®. Horizontal gel

electrophoresis system (BIO-RAD, USA) was used for agarose gel electrophoresis. Gel Doc™ XR+ System (BIO-RAD, USA) was used for imaging and documenting the agarose gel.

3.3.4 DNA Isolation

Tender, brown leaves were selected as the ideal part for extraction of the genomic DNA. It yielded good quality DNA in sufficient quantity. From individual plants, leaves were collected early in the morning. The collected leaves were quickly covered in aluminium foil and kept in the ice box and brought to the laboratory. Modified CTAB method reported by Sujith (2016) with slight alteration was standardized and practised for the extraction of good quality genomic DNA. The concentration of reagents is mentioned below. Quantity and preparation of reagents required for DNA isolation are given in Annexure I.

3.3.4.1 Reagents

I. Extraction Buffer (5X)

- 5.0 per cent CTAB (w/v)
- 100 *mM* Tris base (pH-8.0)
- 20 *mM* EDTA (pH-8.8)
- 1.4 *M* NaCl

II. Polyvinyl pyrrolidone (PVP) – 2.0 per cent

III. β-mercaptoethanol – 0.2 per cent

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

V. Chilled isopropanol

VI. Ethanol – 70 and 100 per cent

VII. RNase A (1.0 %)

VIII. Sterile distilled water

Reagent I and VIII were autoclaved separately and stored at room temperature.

3.3.4.2 Procedure

Autoclaved CTAB extraction buffer (5.0 %) was preheated to 65 °C temperature by keeping it in hot water bath. From collected fresh leaf sample, 0.08-0.1 g of tender leaves were weighed removing midrib region and taken in a pre-cleaned mortar. A pinch of PVP and β-mercaptoethanol (50 µl) was added to the tissue to prevent polyphenol oxidation. Liquid nitrogen was added to the mortar and the tissue was quickly powdered using a pestle. The ground samples were immediately transferred to a sterile 2 ml Eppendorf tube containing 1.25 ml of pre-warmed CTAB buffer. The samples were incubated at 65 °C for 30 min with occasional mixing by gentle inversion. After incubation, the samples were centrifuged (KUBOTA 6500) at 13,000 rpm for 15 min at 4 °C. The top sticky layer containing DNA was transferred to fresh 2 ml Eppendorf tube and to that equal volume of Chloroform: Isoamyl alcohol (24:1) was added. The contents were mixed by slightly inverting the tube and then centrifuged at 13,000 rpm for 15 min at 4 °C. Centrifugation resulted in the separation of tube content into three distinct phases. Top aqueous phase comprised of DNA with a small quantity of RNA and middle phase was formed by protein and other cell debris. Bottom clear phase was formed by Chloroform, isoamyl alcohol and some other leaf pigments.

The top aqueous phase was transferred to a sterile 1.5 ml centrifuge tube and to that, 2 µl of 1 per cent RNase A was added. The content inside the tubes were mixed gently and then kept for incubation at 37 °C for 20 min. An equal volume of Chloroform: Isoamyl alcohol (24:1) was added to the tubes and mixed by gentle inversion of tubes. Then centrifuged at 12,000 rpm for 15 min at 4 °C. After centrifugation, the top clear aqueous phase was transferred to a fresh, sterile 1.5 ml tube. The 0.6 volume of chilled isopropanol was added into the tube. The contents in the tubes were quickly mixed by gentle inversion until DNA got precipitated. For complete precipitation, tubes were immediately transferred for incubation at -20 °C for 2 hours.

Tubes were again centrifuged at 12,000 rpm for 10 min at 4 °C. Supernatant was gently poured off taking care that only pellet was retained in the tube. The pellet was washed by adding 70 per cent ethanol and then spun for five minutes at 8,000 rpm. Ethanol was decanted and the pellets were air dried until ethanol got evaporated

completely. Air dried pellets were dissolved in 70 µl of autoclaved distilled water and stored at -20 °C for daily use and at -80 °C for a long duration storage.

3.3.5 Assessing the Quality of DNA by Gel Electrophoresis

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

3.3.5.1 Reagents and Equipment Used

- Agarose – 0.8 per cent (w/v)
- 50X TAE buffer (pH 8.0)
- Running buffer (1X TAE)
- 6X Gel loading dye
- Ethidium bromide (0.5 µg/ml)
- Gel casting system (BIO-RAD)
- Wide mini-sub electrophoresis unit (BIO-RAD)
- Power pack (BIO-RAD)
- UV transilluminator (Herolab®)
- Gel documentation unit – Gel Doc™ XR+ System (BIO-RAD)

Composition and preparation of reagents are provided in Annexure II.

3.3.5.2 Procedure

Gel casting tray and comb were wiped with 70 per cent ethanol. The tray was kept tight inside the gel casting system with a properly placed comb. Exactly 0.96 g agarose (Merck India) was dissolved in 120 ml 1X TAE buffer to obtain 0.8 per cent agarose. The gel was melted by keeping in a microwave oven for 2 to 2.5 min. The molten gel was then allowed to cool down to about 45 to 60 °C to which 3 µl diluted ethidium bromide was added and mixed gently. The warm gel was poured into the gel casting tray without forming air bubbles and kept undisturbed for 30 to 40 min at room temperature for solidification.

After solidification, the comb was carefully removed from the gel. The gel casting tray along with the gel was placed in the electrophoresis unit. To cover the gel properly, nearly 700 ml of 1X TAE buffer was poured into the electrophoresis tank. A

total of 7 µl samples (5 µl DNA and 2 µl 6X loading dye) were loaded into the wells. An EcoR1-HindIII digested (GeNei) molecular weight ladder was also loaded in to first well. The power supply of about 85 V was provided to run the gel until the dye reaches to 2/3rd of the gel. The gel was removed from the gel apparatus, drained out and carefully transferred to gel documentation unit (Gel Doc™ XR+ System) and observed under UV exposure. QuantityOne software provided by BIO-RAD was used for analysing the electrophoresed agarose gel.

3.3.6 DNA Quantity Assessment by Spectrophotometer

The purity of DNA was further checked using the NanoDrop® ND-1000 spectrophotometer. Nucleic acids show absorption maximum at 260 nm, whereas proteins show peak absorbance at 280 nm. 1 µl of the sample was poured onto the measurement pedestal and absorbance was recorded at both wavelengths and purity was indicated by the ratio OD (A260/280). 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 given as ng/µl.

3.3.7 Normalization of DNA Concentration for PCR

Normalization of DNA was done to bring all DNA concentrations to relatively equal level (20 ng/µl) by appropriate dilutions. Dilutions were done with distilled water.

3.4 SCREENING WITH THE PRIMERS

The study was carried out using two types of markers ISSR (Inter Simple Sequence Repeats) and SSR (Simple Sequence Repeats). Under each marker system, DNA from all eight selected accessions of cocoa were amplified using selected primers and polymorphic bands were identified for further study.

3.4.1 Screening with Inter Simple Sequence Repeats (ISSR) Primers

The concentration of PCR components, as well as the PCR conditions used, was standardized to yield effective amplification using ISSR markers. Master Mix devoid of DNA template was prepared and transferred to 0.2 ml PCR tubes and to this, the template DNA was added. Polymerase chain reaction using standardized PCR conditions was performed using ProFlex™ PCR systems. PCR conditions were

standardized in such a way that, the temperature and time used and cycle numbers were optimum for effective amplification. Blank was prepared by adding all PCR components except DNA.

Good quality diluted DNA (20 ng/µl) from all eight samples were used for ISSR analysis. Initial screening was done using bulked DNA of resistant, susceptible and parental genotypes separately. Primers with good resolving power were used for the further analysis.

Genomic DNA amplification was performed using 20 µl reaction mixture in 0.2 ml thin walled PCR tubes. The PCR reaction mixture consisted of the following components.

- Genomic DNA (20 ng/µl)	:	2.0 µl
- 10X Taq assay buffer	:	2.0 µl
- $MgCl_2$ (25 *mM*)	:	1.5 µl
- dNTP mix (10 *mM* each)	:	1.5 µl
- Taq DNA polymerase (3 U)	:	0.5 µl
- Primer (20 *µM*)	:	1.5 µl
- Autoclaved distilled water	:	11.0 µl
Total volume	:	20.0 µl

The PCR amplification profile was as follows.

1. Initial denaturation at 94 °C temperature for 3 min
2. Denaturation at 94 °C for 45 seconds
3. Primer annealing at 37 to 58 °C for 1 min
4. Primer extension at 72 °C for 2 min
5. Final primer extension at 72 °C for 8 min

Steps two to four were repeated 35 times.

Based on the previous studies in cocoa (Chandrakant, 2014; Sujith, 2016), 50 ISSR primers were selected for the present study (Table 4a).

Table 4a. List of ISSR primers used in the study

Sl. No.	Primer	Nucleotide sequence (5'-3')
1	UBC 807	AGAGAGAGAGAGAGAGT
2	UBC 808	AGAGAGAGAGAGAGAGC
3	UBC 810	GAGAGAGAGAGAGAGAT
4	UBC 811	GAGAGAGAGAGAGAGAC
5	UBC 814	CTCTCTCTCTCTCTCTA
6	UBC 815	CTCTCTCTCTCTCTCTG
7	UBC 818	CACACACACACACACAG
8	UBC 823	TCTCTCTCTCTCTCTCC
9	UBC 825	ACACACACACACACACT
10	UBC 826	ACACACACACACACACC
11	UBC 827	ACACACACACACACACG
12	UBC 834	AGAGAGAGAGAGAGAGYT
13	UBC 835	AGAGAGAGAGAGAGAGYC
14	UBC 836	AGAGAGAGAGAGAGAGYA
15	UBC 841	GAGAGAGAGAGAGAGAYC
16	UBC 843	CTCTCTCTCTCTCTCTRA
17	UBC 844	CTCTCTCTCTCTCTCTRC
18	UBC 846	CACACACACACACACART
19	UBC 847	CACACACACACACACARC
20	UBC 848	CACACACACACACACARG
21	UBC 850	GTGTGTGTGTGTGTGTYC
22	UBC 854	TCTCTCTCTCTCTCTCRG
23	UBC 855	ACACACACACACACACYT
24	UBC 856	ACACACACACACACACYA
25	UBC 857	ACACACACACACACACYG
26	UBC 865	CCGCCGCCGCCGCCGCCG'
27	UBC 866	CTCCTCCTCCTCCTCCTC
28	UBC 873	GACAGACAGACAGACA
29	UBC 880	GGAGAGGAGAGGAGA
30	UBC 889	DBDACACACACACACAC
31	UBC 890	VHVGTGTGTGTGTGTGT
32	UBC 892	TAGATCTGATATCTGAATTCCC'

table 4a continued

33	UBC 895	AGAGTTGGTAGCTCTTGATC
34	UBC 899	CATGGTGTTGGTCATTGTTCC A
35	UBC 900	ACTTCCCCACAGGTTAACACA
36	HB 10	GAGAGAGAGAGACC
37	HB 12	CACCACCACGC
38	Oligo ISSR 04	ACACACACACACACACC
39	Oligo ISSR 05	CTCTCTCTCTCTCTTG
40	Oligo ISSR 06	GAGAGAGAGAGAGAGAC
41	Oligo ISSR 07	CTCTCTCTCTCTCTTG
42	Oligo ISSR 08	GAGAGAGAGAGAGAGA
43	ISSR 2	ATTATTATTATTATTCAT
44	ISSR 3	TTATTATTATTATTACTT
45	ISSR 4	ATTATTATTATTATTGTT
46	ISSR 5	ATTATTGTTGTTGTTTTC
47	ISSR 6	TTATTATTATTATTATAA
48	ISSR 7	ATTATTGTTGTTGTTGTA
49	ISSR 8	ATTATTATTATTATTGTA
50	ISSR 9	TTATTATTATTATTATTACT

3.4.1.1 Separation of Amplified Products by Agarose Gel Electrophoresis

The amplified PCR products were electrophoresed along with 1 kb plus DNA ladder (GeNei) on 1.8 per cent agarose gel stained with ethidium bromide at voltage of 80 V. After 90 to 110 min of the run, the gel was examined under gel doc and the DNA banding pattern was photographed directly in gel documentation unit under UV exposure. The gel pictures were saved in image format for further scoring and detection of polymorphism among the amplicons.

3.4.2 Screening with Simple Sequence Repeats (SSR) Primers

Good quality diluted DNA (20 ng/µl) from all eight samples were used for SSR analysis. Initial screening was done using bulked DNA of resistant, susceptible and parental genotypes separately. Primers with good resolving power were used for further analysis.

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The PCR reaction mixture consisted of the following components.

- Genomic DNA (20 ng/µl)	:	2.0 µl
- 10X Taq assay buffer	:	2.0 µl
- $MgCl_2$ (25 *mM*)	:	1.5 µl
- dNTP mix (10 *mM* each)	:	1.5 µl
- Taq DNA polymerase (3 U)	:	0.3 µl
- Forward primer (20 *µM*)	:	0.75 µl
- Revers primer (20 *µM*)	:	0.75 µl
- Autoclaved distilled water	:	11.2 µl
Total volume	:	20.0 µl

The amplification profile was as follows.

1. Initial denaturation at 94 °C temperature for 3 min
2. Denaturation at 94 °C for 30 seconds
3. Primer annealing at 37 to 58 °C for 45 seconds
4. Primer extension at 72 °C for 1 min
5. Final primer extension at 72 °C for 5 min

Steps two to four were repeated 35 times.

Based on the previous report in cocoa (Lanaud *et al.*, 2009) fifteen SSR primers were selected for the present study (Table 4b).

3.4.2.1 Separation of Amplified Products by Agarose Gel Electrophoresis

The amplified PCR products were electrophoresed along with 100 bp DNA ladder (GeNei) on 2.0 per cent agarose gel stained with ethidium bromide at voltage of 80 V. After 90 to 110 min of the run, the gel was examined under gel doc and the DNA banding pattern was photographed directly in gel documentation unit under UV exposure. The gel pictures were saved in image format.

Table 4b. List of SSR primers used in the study

Sl. No.	Primer		Nucleotide sequence (5'-3')	Primer length (bp)
1	mTcCIR32	F	GACTTACTCCCATCCTAC	18
		R	TGATTGGCACACTTTT	16
2	mTcCIR37	F	CTGGGTGCTGATAGATAA	18
		R	AATACCCTCCACACAAAT	18
3	mTcCIR54	F	AACCTCTTGTCACGTTA	17
		R	GAAGGCATACTTACTACTGT	20
4	mTcCIR100	F	TGATGGAATAAACTAAGAACA	21
		R	TAAGAAGCCAGGTCAGG	17
5	mTcCIR113	F	GGAAAGTTACAGCAAGAGAGA	21
		R	ACAAGCCCGGTGAAGG	16
6	mTcCIR119	F	TGGACTTGTGCTGGAAC	17
		R	GCAAGAAATAAAATAGGAAC	20
7	mTcCIR152	F	CAGTAGTCAAAACATCAAA	19
		R	GTAATCCAAATAATAAGCAT	20
8	mTcCIR156	F	GGCAGGACCAAATGAT	16
		R	AAAACCAGGAACACCAG	17
9	mTcCIR170	F	CTCTTGCACGGCACAGGA	18
		R	TTGCCCCACCCATACG	16
10	mTcCIR199	F	GATTCTTATTTGATTTTCCTTA	22
		R	GCACGGTTACATTTATTACA	20
11	mTcCIR203	F	GTGGATTTGGGTGGGAT	17
		R	ATTGTGTTTTGGCTATGTTC	20
12	mTcCIR215	F	GCTTCAACTCCAAATCAC	18
		R	TAGCATCCCGTATTGTG	17
13	mTcCIR222	F	CTACAGAAAATAGGCAATA	19
		R	TCATTGTATTATCAGGTAGA	20
14	mTcCIR255	F	TTTACCTCCACCATCTT	17
		R	TGGCACTTATCTATTACTGT	20
15	mTcCIR279	F	GTCCATCTACATCATAAGC	19
		R	CAGCAACAGCATCACT	16

3.4.3 Identification of Polymorphism

For every ISSR and SSR primer, the marker profiles were generated through electrophoresis of the PCR products on 1.8 and 2.0 per cent agarose gel respectively. The gel profiles were examined in relation to the reported disease response and any band which is present in resistant genotypes and at least in any one of the parent, or present in susceptible genotypes only considered as polymorphic.

3.4.4 Elution of Polymorphic Band and Cleaning (QIAquick® Gel Extraction Kit by Qiagen©)

The DNA fragments were excised from the agarose gel with a clean, sharp scalpel. The gel slices were weighed in a colourless 1.5 ml microcentrifuge tube. Two volumes of buffer QG was added to 1 volume gel (100 mg gel ~100 µl). The tube was kept for incubation at 50 °C for 10 min (or until the gel slice dissolved completely). The tube was vortexed every 2–3 min to help the gel to dissolve. One gel volume isopropanol was added to the sample and mixed well. A QIAquick spin column was placed in a 2 ml collection tube. The sample was applied to the QIAquick column and centrifuged for one min for DNA binding. Flow-through was discarded and then the QIAquick column was placed back into the same tube. Washing was done by adding 750 µl buffer PE to QIAquick column and the column was kept in the stand for 2–5 min. Then the tube was centrifuged for one min. Flow-through was discarded and the QIAquick column was placed back into the same tube. Again it was centrifuged for one min to remove residual wash buffer and then placed into a clean 1.5 ml microcentrifuge tube. To elute DNA, 30 µl buffer EB was added to the centre of the QIAquick membrane and kept the column stand for four min and then it was centrifuged for one min. The purified DNA was analysed on a 1.2 per cent agarose gel by adding one volume of loading dye to five volumes of purified DNA. The quantity of purified PCR product was checked on a spectrophotometer (NanoDrop® ND 1000).

3.4.5 Transformation of Eluted PCR Products

The purified PCR product was transformed into *E. coli*DH5α cells with Thermo Scientific™ TransformAid™ Bacterial Transformation Kit. The high-quality opened TA cloning vector pTZ57R/T was used for efficient ligation with purified PCR product.

3.4.5.1 Ligation

The optimal insert/vector ratio was 3:1. The amount of purified PCR product was calculated from reference booklet provided in the kit, required for efficient ligation with 0.165 µg (3 µl, 0.172 pmol ends) of the pTZ57R/T vector. The ligation mix was prepared by adding the following components to a 0.2 ml PCR tube.

-	Vector pTZ57R/T (0.165 µg, 0.18 pmol ends)	:	3 µl
-	Purified PCR fragment, (approx. 0.54 pmol ends)	:	4 µl
-	5X Ligation Buffer	:	6 µl
-	Water, nuclease-free	:	up to 29 µl
-	T4 DNA Ligase (5 U)	:	1 µl
	Total volume	:	30 µl

The ligation mixture was incubated at room temperature (22 °C) for 1 hour and at 4 °C for overnight.

3.4.5.2 Transformation

3.4.5.2a. Preparation of Bacteria from Overnight Bacterial Culture

A single colony of one day old *E. coli* DH5α bacterial strain was inoculated in a 2 ml tube containing TransformAid™ C-Medium. The culture tube was incubated overnight at 37 °C in a shaker.

On the next day, culture tube containing the required amount of TransformAid™ C-Medium was pre-warmed at 37 °C. The 1/10th volume of overnight culture was added to the pre-warmed C-Medium and the tube was incubated on a shaker at 37 °C for 20 min.

3.4.5.2b. Preparation of LB-Ampicillin Plates

The 0.5 g agar was added to 100 ml of LB medium (4 %) and kept in the microwave for 1 min. The medium was autoclaved at 121 °C at 15 psi for 15 min. The LB medium was cool down to 55 °C and to this 100 µl of ampicillin (50 µg/ml) was added. The medium was gently mixed and 30-35 ml of LB-agar was poured directly onto each plate. Plates were kept opened for drying at room temperature under UV light

for 30 min. The solution of 40 µl each of X-Gal solution (20 mg/ml) and of IPTG 100 *mM* was spread evenly with a sterile spatula on solidified ampicillin plate. These plates were kept in an incubator for warming for at least 20 min. The composition and preparation of IPTG and X-Gal solution are given in Annexure III.

3.4.5.2c. Transformation Procedure

LB-Ampicillin agar plates were pre-warmed in a 37 °C incubator for at least 20 min. TransformAid™ T-Solution was prepared by mixing equal volumes of T-Solution (A) and T-Solution (B) and immediately kept on the ice. The freshly incubated cultured C-Medium tube was dispensed into a microcentrifuge tube and spined at maximum speed for a minute at room temperature (RT). The supernatant was discarded and the pelleted cells were re-suspended in 300 µl of TransformAid™ T-solution. The pellet was mixed gently and the tube was incubated on ice for 5 min. The tube was centrifuged again for 1 min at RT and then the supernatant was removed. The cells were now re-suspended in 120 µl of TransformAid™ T-solution and incubated on ice for 5min. DNA was prepared for transformation by dispensing 2.5 µl of ligation mixture (10-20 ng of vector DNA) into new microcentrifuge tubes and set them on ice for 2 min. The 50 µl re-suspended cells were added to each tube containing DNA and were incubated on ice for 5 min. The mixture was plated on pre-warmed LB-ampicillin agar plates and the plates were incubated overnight at 37 °C.

3.4.5.3 Analysis of Recombinant Clones by Colony PCR

The putative transformants which appeared to as white colonies along with one blue colony were picked from the plate and streaked on gridded LB-ampicillin agar plates. The plates were incubated overnight at 37 °C. This was done to check whether the white colonies retained their colour and also get more colonies of each transformant. These plates were stored at 4 °C for further use.

3.4.5.3a. Procedure

A single white and blue colony was picked out from the grid plate and mixed separately in a microcentrifuge tube containing 25 µl distilled water. The tubes were then heated at 98 °C for 4 min and centrifuged at 8,000 rpm for 5 min. The supernatant

was taken in a new tube and was used for PCR reaction. The components of the colony PCR were as following.

- Genomic DNA (20 ng/µl)	:	2.0 µl
- 10X Taq assay buffer	:	2.0 µl
- $MgCl_2$ (25 *mM*)	:	0.8 µl
- dNTP mix (10 *mM* each)	:	1.5 µl
- Taq DNA polymerase (3 U)	:	0.3 µl
- M13 forward primer (20 *µM*)	:	0.6 µl
- M13 reverse primer (20 *µM*)	:	0.6 µl
- Autoclaved distilled water	:	12.2 µl
Total volume	:	20.0 µl

The amplification profile of colony PCR was as follows.

1. Initial denaturation at 94 °C temperature for 2 min
2. Denaturation at 94 °C for 30 seconds
3. Primer annealing at 55 °C for 30 seconds
4. Primer extension at 72 °C for 1 min
5. Final primer extension at 72 °C for 5 min

Steps two to four were repeated 30 times.

After the amplification, the PCR product was separated on 1 per cent agarose gel along with 100 bp DNA ladder (GeNei).

3.4.5.4 Maintenance of Transformants

The confirmed white colony was streaked on to LB-ampicillin agar plate and incubated overnight at 37 °C. The culture plate was stored at 4 °C for further use.

3.4.6 Plasmid DNA Isolation and Purification

3.4.6.1 Preparation of Culture

The confirmed single colony was picked from grid plate or maintenance plate and inoculated in a tube containing autoclaved LB broth medium. Inoculated tube was kept for 16 hours incubation at 37 °C in an incubator shaker at 150 rpm, then developed culture was used for plasmid DNA isolation.

3.4.6.1a. Procedure

The 1–5 ml of the overnight LB-culture was centrifuged and the medium was removed completely. 250 µl resuspension buffer (R3) with RNase A was added to the cell pellet and resuspended the pellet until it was homogeneous. 250 µl lysis buffer (L7) was added to the tube and then it was mixed gently by inverting the capped tube until the mixture was homogeneous. The tube was incubated at room temperature for 5 min. Precipitation buffer (N4), 350 µl was added and mixed immediately by inverting the tube, until the mixture was homogeneous. The lysate was centrifuged at 13,000 rpm for 10 min. The supernatant was loaded onto a spin column in a 2 ml wash tube and it was centrifuged at 12,000 rpm for 1 min. The flow-through was discarded and the column was placed back into the wash tube. To this 700 µl wash buffer (W9) with ethanol was added, the column was centrifuged at 12,000 rpm for 1 min. The flow through was discarded and the column was placed back into the wash tube. The column was centrifuged at 12,000 rpm for 1 min and the wash tube was discarded with the flow-through. The spin column was placed in a provided clean 1.5 ml recovery tube. The 75 µl preheated TE buffer (TE) was added to the centre of the column and the column was incubated for 4 min at room temperature. The column was centrifuged at 12,000 rpm for 2 min. The recovery tube contained the purified plasmid DNA. The column was discarded. The plasmid DNA was stored at -20 °C.

3.4.6.2 Assessing the Quality and Quantity of Plasmid DNA

The isolated plasmid DNA was separated on 1 per cent agarose gel along with 1 kb plus DNA ladder (GeNei). The quantity of purified plasmid DNA was checked on a spectrophotometer (NanoDrop® ND 1000).

The isolated plasmid DNA was again confirmed for desired DNA fragment by performing PCR. The PCR was done using the conditions described in section 3.4.5.3a. The purified plasmid DNA was sent for sequencing to the sequencing facility at AgriGenome Lab, Kochi, Kerala, India.

3.5 SEQUENCE ANALYSIS

The sequences were processed and analysed with bioinformatics tools.

3.5.1 VecScreen

The VecScreen system helps in identification and removal of any segment of vector origin from generated nucleotide sequence. Further, nucleotide sequences obtained were processed in the BioEdit tool for removing vector contaminated region. The sequences generated after processing was used for further analysis.

3.5.2 BLASTn and KAAS Analysis

The nucleotide sequences obtained from BioEdit analysis were annotated in BLASTn program for checking of sequence homology.

KEGG Automatic Annotation Server (KAAS) analysis was also done for the sequences. KAAS provides functional annotation of genes and the result contains KO (KEGG Orthology) assignments and automatically generated KEGG pathways. The nucleotide sequences obtained were annotated in KAAS platform to check possible relation of sequence with the metabolic pathway.

3.5.3 Genome Data Viewer Study

The BLASTn annotated sequences were analyzed in NCBI Genome Data Viewer tool to locate the position of markers on chromosome of cocoa.

Results and Discussion

4. RESULTS AND DISCUSSION

The results of the experiments carried out in the study titled 'Tagging of *Phytophthora* pod rot disease resistance gene in cocoa (*Theobroma cacao* L.) using ISSR markers,' during 2016-2017 conducted at Centre for Plant Biotechnology and Molecular Biology, College of Horticulture (CPBMB) and Cocoa Research Centre, Vellanikkara (CRC) are presented and discussed in this chapter.

4.1 MORPHOLOGICAL CHARACTERIZATION OF COCOA POD AND BEAN

Data on distinguishable quantitative characters were recorded on five pods from each Hybrid and further analyzed following a CRD experimental design. The Hybrids differed significantly with respect to all the five characters *viz*., pod weight, pod breadth and length, wet bean weight per pod and single dry bean weight recorded (Table 5).

The highest pod weight was recorded in Hybrid 8 (724 g) which differed significantly from all other hybrids. Pod weight was least in Hybrid 14 and Hybrid 15 (160 g). Pod length was found to be highest in Hybrid 1 (21.17 cm), whereas it was the least (13.18 cm) in Hybrid 5. The highest pod breadth of 8.82 cm was observed in Hybrid 26 and minimum in Hybrid 2 (6.60 cm). Length and breadth of pod were found to be proportional.

Yield expressed as wet or dry bean weight is highly variable (Enriquez and Soria, 1996). Maximum wet bean weight per pod observed in Hybrid 8 (164.32 g) and least in Hybrid 12 (42.40 g) (Table 5). Dry bean weight above the International standard of one gram (Wood and Lass, 1985) was expressed only by four hybrids (Hybrid 2, 7, 14 and 24). Pound (1996) concluded that dry seed weight is the most reliable trait for the description and identification of a cacao genotype. Adewale *et al.* (2014) supported these results by affirming the number of seeds per pod and dry weight are the best descriptors of cacao production.

High variability in quantitative characters like pod size and dry bean weight have been considered as desirable traits for cocoa breeding program (de Schawe *et al.*, 2013). Daniel *et al.* (2014) and Minimol *et al.* (2015) had reported that progeny of same cross vary in characters due to the heterozygous nature of parents and the result was in tune with present study.

SG

Table 5. Pod and bean characters of cocoa hybrid progeny of SIV 1.26 × PII 12.11

Hybrid	Stand No.	Pod weight (g)	Pod length (cm)	Pod breadth (cm)	Wet bean weight/pod (g)	Single dry bean weight (g)
H1	8.50	342.00	21.17	8.82	102.36	0.76
H2	12.60	416.00	14.30	6.68	110.88	1.22
H3	13.70	276.00	13.98	6.72	64.08	0.64
H4	14.80	252.00	15.08	7.36	92.82	0.70
H5	15.60	222.00	13.18	6.86	68.92	0.68
H6	16.50	305.60	16.80	7.86	53.36	0.76
H7	8.11	212.00	17.10	7.18	77.86	1.06
H8	10.60	724.00	15.84	8.24	164.34	0.90
H9	11.50	318.00	20.58	7.88	111.40	0.92
H10	12.40	260.00	14.82	6.90	68.12	0.84
H11	12.50	352.00	17.86	7.68	129.44	0.60
H12	14.40	164.00	17.78	7.64	42.40	0.52
H13	14.60	264.00	14.82	6.90	73.34	0.82
H14	15.40	160.00	14.72	6.82	75.64	1.04
H15	16.80	160.00	17.76	7.64	63.68	0.68
H16	6.50	220.00	20.20	7.74	67.26	0.48
H17	7.80	296.00	20.08	7.68	81.82	0.86
H18	8.90	228.00	17.84	7.64	78.04	0.50
H19	9.30	188.00	15.64	7.94	50.28	0.52
H20	10.40	252.00	17.44	7.42	80.28	0.84
H21	10.70	360.00	18.10	7.84	85.64	0.82
H22	11.90	352.00	17.14	7.32	97.32	0.84
H23	7.40	368.00	15.70	7.92	85.70	0.82
H24	12.10	334.00	18.90	7.98	79.04	0.78
H25	13.90	344.00	18.72	7.74	92.64	1.00
H26	14.50	368.00	21.07	8.82	71.88	0.84
H27	15.70	244.00	17.86	7.74	78.50	0.78
H28	16.70	220.00	17.42	7.52	59.34	0.52
CV (%)		**17.38**	**3.392**	**4.327**	**17.444**	**16.070**
CD (0.05)		**63.106**	**0.740**	**0.407**	**17.872**	**0.150**

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4.2 SCREENING OF HYBRIDS FOR DISEASE RESISTANCE

4.2.1 Isolation and Characterization of Pathogen

The pathogen causing *Phytophthora* pod rot (PPR) of cocoa was isolated from naturally infected cocoa pods (Plate 1). The isolate was purified by hyphal tip method and maintained on PDA plates by periodic sub culturing. The mycelium of the isolate was branched, hyaline and coenocytic. The characteristic papilla shape sporangium development was observed (Plate 1). Sporangia were spherical when young, with less dense protoplasm.

The cultural and morphological characters of the pathogen isolated were studied. The cultural and morphological characters, were typical as observed by earlier workers (Zentamyer, 1988; Bhavani, 2007) and based on the pathogenicty on cocoa, the isolate was identified as *Phytophthora palmivora*.

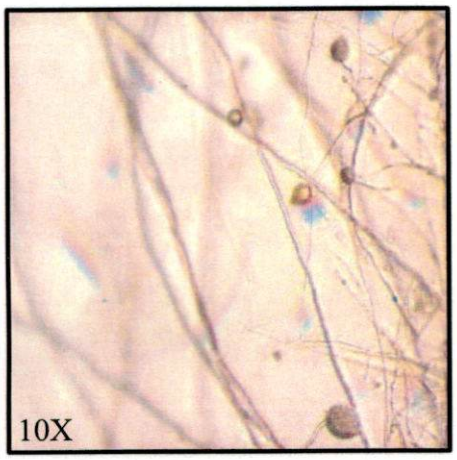
4.2.2 Artificial Inoculation on Detached Pods

Pathogenicity of organism was proved by inoculation on healthy cocoa pods collected from 28 hybrids as mentioned in section 3.2.2.3 (Iwaro *et al.*, 2000). The isolate produced circular water soaked lesions within 48 hours. Later, lesions enlarged and turned into chocolate brown color. Whitish growth of pathogen consisting of mycelia and sporangia was produced over the dark brown area immediately behind the advanced border. The artificially inoculated detached pods were observed up to 10 days and further disease resistance was calculated based on the area of infection by *P. palmivora*. The hybrids screened for detached pod inoculation differ significantly with respect to susceptibility (Table 6).

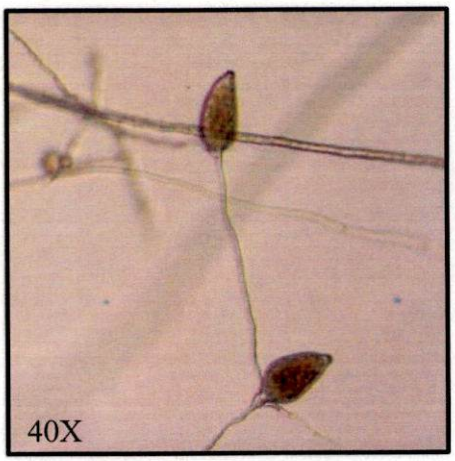
Hybrid 15 (16.53 %), Hybrid 23 (18.11 %) and Hybrid 25 (22.54 %) were found to be the least susceptible to *P. palmivora* (Table 6, Plate 2). However, cent per cent susceptibility was recorded in Hybrid 19 followed by Hybrid 3 (97.45 %) and Hybrid 27 (96.68 %) (Table 6, Plate 3). The aforesaid hybrids were categorized as susceptible and resistance based on the standard scoring table mentioned in section 3.2.2.4 (Table 7). Further these hybrids were selected for molecular analysis and were screened with ISSR and SSR primers.

Several researchers doubted that the data that can be gathered in the plantation was just a rough sign of the genuine susceptibility of those accessions inspected. Inoculation tests on injured pods is a more appropriate method for checking internal resistance or post-penetration resistance (Prendergast and Spence, 1967). Hence, an artificial inoculation method as advocated by Iwaro *et al.* (1997) and Iwaro *et al.* (2000), was used to assess the reaction of fully grown, unripe detached pods to *P. palmivora*.

As recommended by Iwaro *et al.* (2000), two pods were tested per genotype to confirm the reaction of each genotype to *P. palmivora*. The hybrids examined on artificial inoculation showed consistence differences in level of infection. The hybrids were further grouped into resistant and susceptible hybrids based on the score chart given by Bhavani *et al.* (2007). Hybrid 15, 23 and Hybrid 25 were selected as resistant, whereas Hybrid 19, 3 and Hybrid 27 selected as susceptible for gene tagging studies. The progenies of same cross segregated for resistance and susceptibility as reported by Efron *et al.* (2002) and Minimol *et al.* (2016) and same trend was observed in the study.



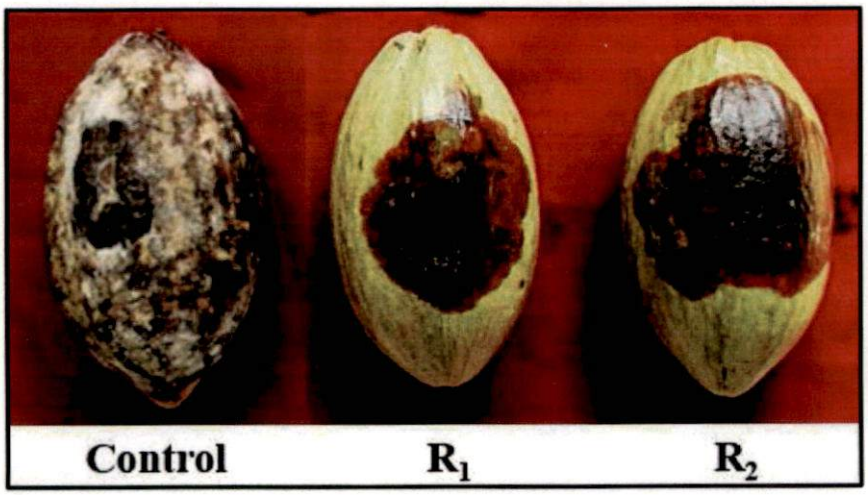
A. Sporangial growth of *P. palmivora*



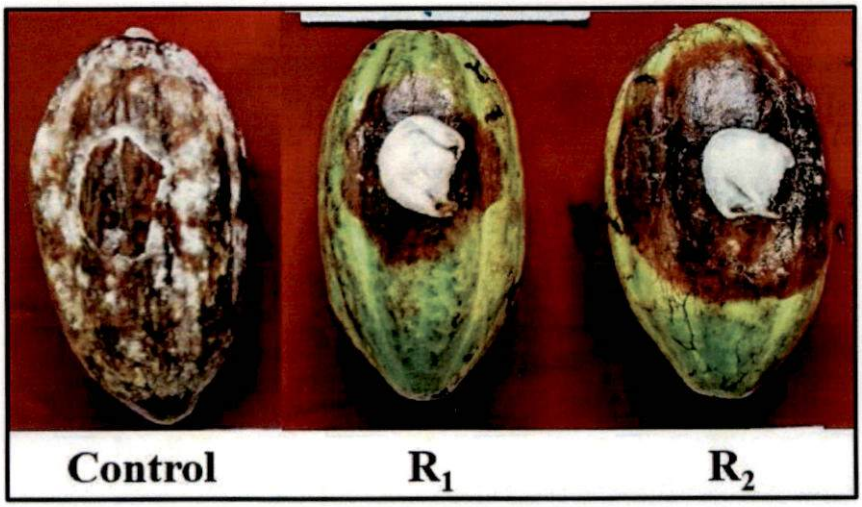
B. A single sporangiophore of *P. palmivora* culture

C. *Phytophthora palmivora* culture plate

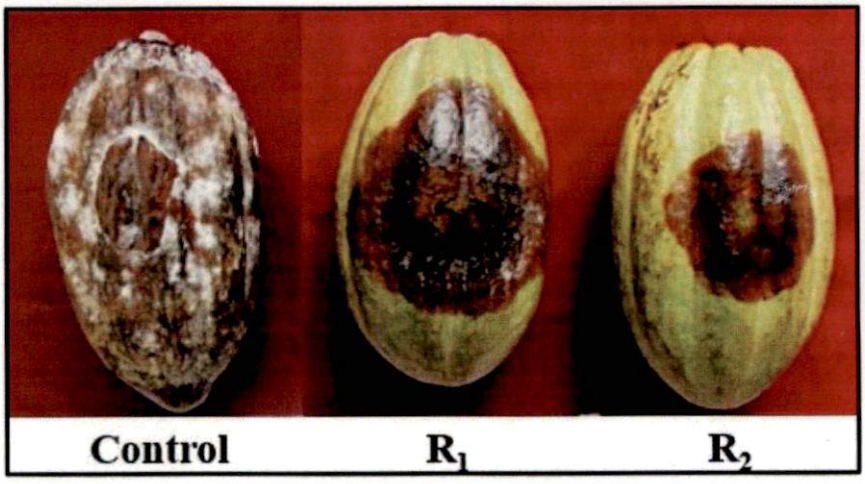
Plate 1. Isolation and confirmation of *Phytophthora palmivora* culture



Hybrid 23

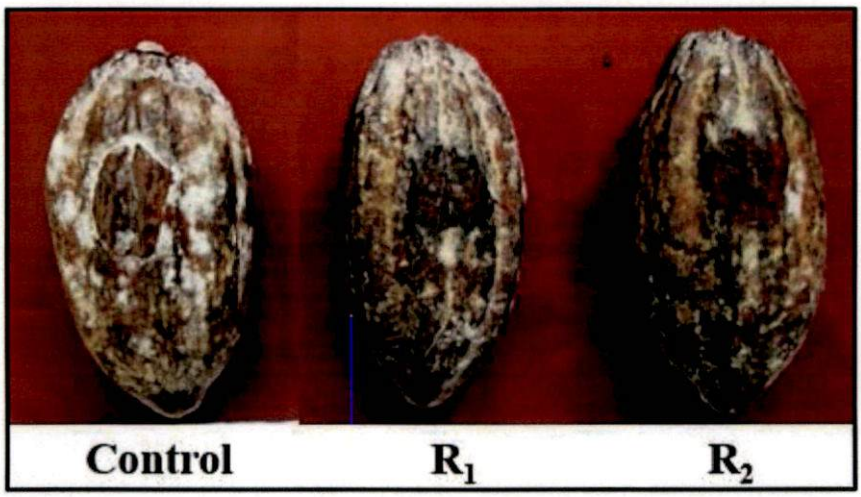


Hybrid 15

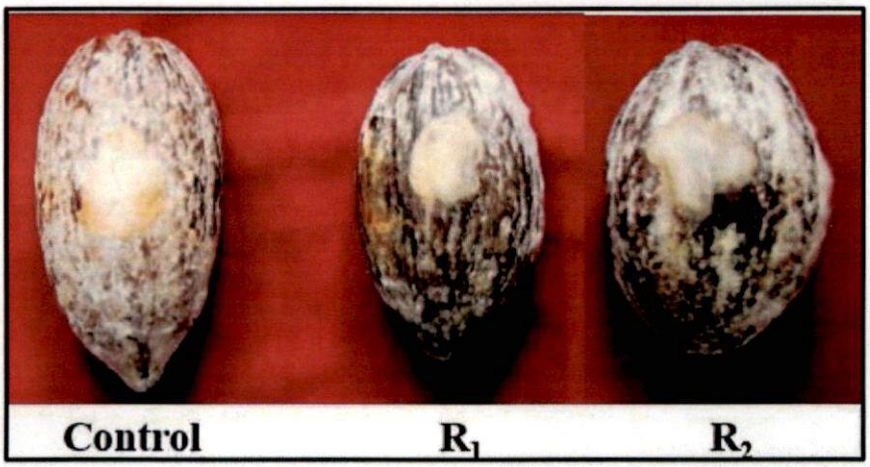


Hybrid 25

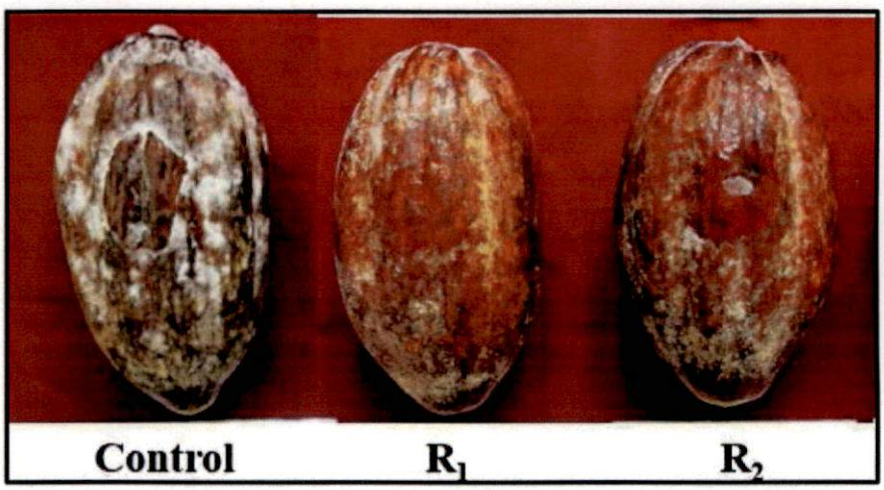
Plate 2. Hybrids resistant to pod rot identified through pod inoculation method



Hybrid 19



Hybrid 3



Hybrid 27

Plate 3. Hybrids susceptible to pod rot identified through pod inoculation method

Table 6. Grouping of hybrids based on pod area infection

Hybrid	Length of pod (cm)	Breadth of pod (cm)	Length of lesion after 10 days (cm)	Breadth of lesion after 10 days (cm)	Pod area infection (%)	Grouping based on disease score
H1	21.75	25.00	20.00	25.00	91.95	S
H2	15.00	26.75	14.50	26.75	96.66	S
H3	14.50	26.16	14.50	25.50	97.45	S
H4	15.50	27.50	14.00	22.00	72.25	MS
H5	13.33	23.83	13.00	21.33	87.27	S
H6	17.00	28.66	16.83	27.33	94.41	S
H7	18.50	25.40	11.50	12.50	30.59	MR
H8	16.25	23.65	11.00	15.25	43.64	MR
H9	20.50	21.25	12.50	16.50	47.34	MR
H10	15.50	26.50	12.75	16.00	49.66	MR
H11	17.83	26.33	13.33	18.33	52.05	MS
H12	17.33	26.83	13.16	17.33	49.06	MR
H13	15.50	21.50	12.23	15.66	57.51	MS
H14	15.00	22.75	13.00	18.75	71.42	MS
H15	17.75	28.90	8.75	9.75	16.63	R
H16	19.50	24.50	18.16	22.66	86.19	S
H17	19.33	26.50	15.83	25.33	78.29	S
H18	17.83	26.66	13.83	17.50	50.90	MS
H19	16.50	21.83	16.50	21.83	100.00	S
H20	17.66	24.33	14.00	20.83	67.84	MS
H21	18.83	25.33	17.16	25.33	91.15	S
H22	17.33	25.66	13.33	18.83	56.44	MS
H23	16.00	23.00	8.00	8.30	18.11	R
H24	19.83	25.33	15.16	21.66	65.40	MS
H25	19.00	27.83	9.66	12.33	22.54	R
H26	21.50	28.50	13.66	20.16	44.97	MR
H27	18.33	27.66	17.83	27.50	96.68	S
H28	17.25	24.25	14.00	19.75	66.09	MS

† R: Resistant, MR: Moderately resistant, MS: Moderately susceptible, S: Susceptible

Table 7. Details of the cocoa hybrids selected for molecular analysis

Sl. No.	Hybrids	Pod area infection (%)	Response to *Phytophthora* pod rot disease
1	H23	18.11	Resistant
2	H25	22.54	Resistant
3	H15	16.53	Resistant
4	H19	100	Susceptible
5	H3	97.45	Susceptible
6	H27	96.68	Susceptible

4.3 MOLECULAR ANALYSIS

4.3.1 Isolation and Quantification of DNA

Regardless of the plant sample and DNA marker system, the DNA isolated should have very good quality and it is the primary need of any molecular analysis. Several DNA extraction protocols were reported for molecular marker analysis in various crops (Dellaporta *et al.*, 1983; Couch and Fritz, 1990; Lanaud *et al.*, 1995; Rogers and Bendich, 1994; Perry *et al.*, 1998). Young leaves (second or third leaf from the shoot tip) were reported to be the desirable part for extraction of good quality DNA (Charters and Wilkinson, 2000). Tender pale yellow or reddish colored leaves were reported to yield good quality DNA in cocoa (Chandrakant, 2014).

In the current study, tender leaves from the actively growing tips of cocoa trees were collected in ice box during early morning for extraction of genomic DNA. The modified Doyle and Doyle (1987) method of extraction protocol was found to yield good quality DNA (Chandrakant, 2014; Sujith, 2016). However, when practiced here, the DNA extracted was found to be dominated by RNA contamination and high amount of mucilage content was observed as the plant belongs to family Malvaceae. Hence, slight alterations were made in the protocol which resulted in good quality DNA (Plate 4).

Sample quantity was reduced to 0.08-0.10 g. Liquid nitrogen was used for homogenization and complete disruption of the tissue which further resulted in isolation of "nick"-free DNA (Blin and Stafford, 1976). Inhibition of polyphenol was done by

addition of anti-oxidant β-mercaptoethanol and PVP in excess quantity. Further, the addition of chloroform: isoamyl alcohol (24:1) during the first centrifugation step was avoided and instead of 2.0 per cent CTAB, 5.0 per cent was used. The protocol used in the study is detailed in 3.2.4.

Malvaceae leaf samples contain high amounts of mucilage (Bayer *et al.*, 1999), and it is very difficult to remove the mucilaginous supernatant after centrifugation without losing most of the DNA. Earlier research works revealed that in cocoa, a plant belonging to Malvaceae family, the leaf tissue used for DNA isolation were characteristically high in polysaccharides, polyphenols, tannins and other secondary metabolites, which interfere with effective DNA extractions and subsequent enzymatic reactions (Echevarría-Machado *et al.*, 2005; Chandrakant, 2014). Polyphenols have viscous, glue-like texture and make the DNA unmanageable in pipetting and unsuitable for PCR by inhibiting Taq polymerase activity (Fang *et al.*, 1992). Upon cell lysis, these compounds bind to DNA and cannot be removed by conventional extraction procedures (John, 1992).

Other techniques for DNA extraction from different highly mucilaginous plants, which included modifications of the CTAB-based protocols, had been reported (Barnwell *et al.*, 1998; Mansoor *et al.*, 1999). Jose and Usha (2000) described a protocol for the extraction of genomic DNA from okra (*Abelmoscus esculentus*), a Malvaceae plant. This method involved a combination of Dellaporta *et al.* (1983), the CTAB method (Doyle and Doyle, 1990), and alkali lysis. Protocols developed for DNA extraction from Malvaceae plant species rich in polysaccharides, polyphenols, and latex were found to be time consuming or expensive (Jeske *et al.*, 2001). Expensive commercial kits are also available and recommended for the extraction of DNA from cocoa (Haymes *et al.*, 2004).

Using NanoDrop ND-10OO®, the ratio of optical density at 260 and 280 nm was worked out to test the quality of isolated DNA. The absorbance ratio was calculated as OD at 260/280, for the various samples. The ratio was observed to vary between 1.89 and 1.98 (Table 8) indicated good quality DNA. An Optical density ratio between 1.8 to 2.0 is considered as good quality DNA (Thakur *et al.*, 2014; Sujith, 2016).

G5

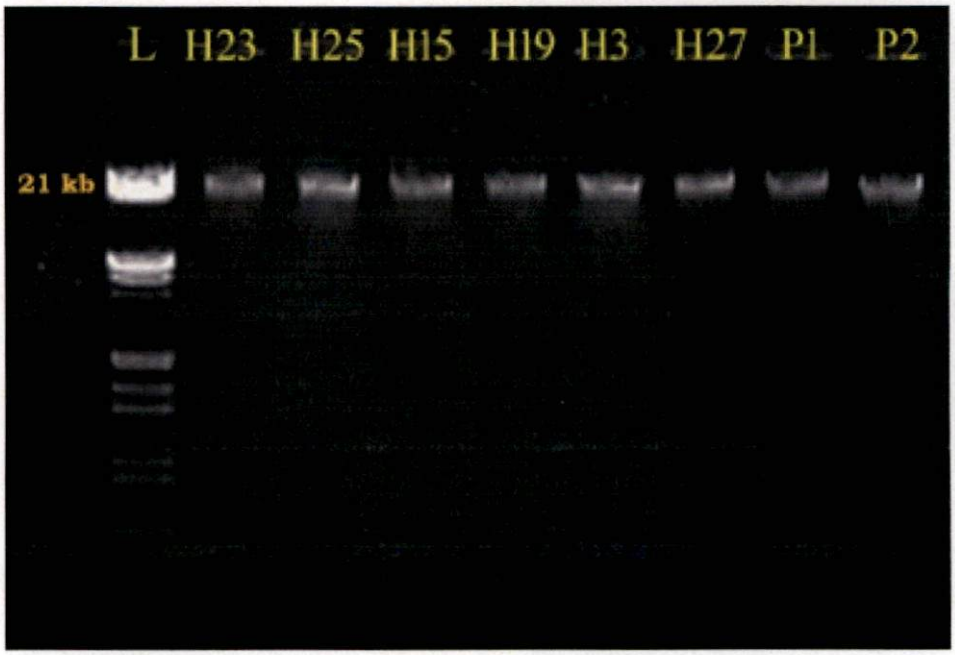


Plate 4. Gel profile of isolated DNA of eight cocoa accessions

The quantified DNA samples were diluted to lower concentration (20 ng/µl) because the presence of polyphenols could interfere with PCR reactions (Sujith, 2016). Moreover, amplification was to be found excellent in both ISSR and SSR analysis at same concentration.

Table 8. DNA quantification result of eight cocoa accessions

Sl. No.	Hybrids	Optical density ($A_{260/280}$)	Quantity of DNA (ng/µl)
1	H23	1.96	429.2
2	H25	1.89	330.1
3	H15	1.98	119.6
4	H19	1.93	214.3
5	H13.7	1.96	318.6
6	H15.7	1.92	105.6
7	P1	1.9	185.3
8	P2	1.95	230.3

4.3.2 Inter Simple Sequence Repeat (ISSR) Analysis

ISSR is a PCR based marker system that assess variation in microsatellite regions distributed in the genome. It is utilized to amplify inter simple sequence repeat regions in the genome (Zietkiewicz *et al.*, 1994). Thus, the designed primers which are successfully annealed to two microsatellite regions within an amplifiable stretch will generate a band of particular molecular weight. The method uses a single 5'- or -3' anchored or unanchored oligonucleotide primer composed of 6 to 18 di or tri nucleotide repeats.

Fifty ISSR primers were used and validated in the initial screening based on their ability to amplify the bulked genomic DNA, with the thermal settings mentioned earlier under section 3.3.1 and they gave varying amplification patterns (Table 9). ISSR is highly reproducible, sensitive and dominant marker system and had been successfully used for evolutionary and genetic diversity study of many crops including finger millet (Salimath *et al.*, 1995), wheat (Nagaoka and Ogihara, 1997), citrus (Fang and Roose, 1997, chickpea (Choudhary *et al.*, 2013), *etc.* The ISSR markers were useful in the

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evaluation of population structure and genetic diversity in natural populations of *Theobroma subincanum* Mart. (Rivas *et al.*, 2013). Giustina *et al.* (2014) also evaluated natural populations of *Theobroma speciosum* with ISSR and detected polymorphism in the populations analyzed and also proved to be reproducible.

ISSR primers are designed from SSR motifs and can be undertaken for any plant species containing a sufficient number and distribution of SSR motifs in the genome (Gupta *et al.*, 1994). Therefore, this technique has been used extensively to evaluate genetic diversity both within and between plant populations in angiosperms and gymnosperms (Osborn *et al.*, 2005) and with this the plant genome fingerprints were developed in many crops *viz.*, *Coffea arabica* (Tesfaye *et al.*, 2014), rice (Shahin Kaleybar *et al.*, 2015) and cocoa (Sujith, 2016).

Based on the quality of amplification patterns, 27 primers were selected for amplification with individual DNA of all eight hybrids. In this screening polymorphism was observed in 11 ISSR primers. Further, their reproducibility was confirmed by repeating PCR amplifications thrice. Details of the selected primers are provided in Table 10. The high level reproducibility and specificity of ISSR markers is already well established and exploited to detect disease resistance in in many crops such as mung bean (Souframanien and Gopalakrishna, 2006), pearl millet (Jogaiah *et al.*, 2014), cocoa (Chandrakant, 2014), maize (Salah *et al.*, 2016), *etc.*

The analysis of result on individual polymorphic primers is detailed here under.

4.3.2.1 UBC 810

The ISSR primer UBC 810 generated on an average 12 clear amplicons on 1.8 per cent agarose gel (Plate 5). The molecular weight of the bands varied from 300 bp to 1.4 kb. Amplicon obtained from this primer was distinctly polymorphic with a band of 850 bp present in all resistant hybrids and both parents, and was absent in two susceptible hybrids. This polymorphism between resistant and susceptible hybrids was repeatable and was useful for identification of a marker associated with resistant trait for PPR.

Table 9. Details of the amplification pattern obtained with 50 ISSR primers

Sl. No.	Primer	Amplification pattern			
		No. of amplicons	Types of amplicons		Remarks
			Distinct	Faint	
1	UBC 807	11	8	3	Not selected
2	UBC 808	12	7	6	Not selected
3	UBC 810	8	6	2	Selected
4	UBC 811	0	0	0	Not selected
5	UBC 814	0	0	0	Not selected
6	UBC 815	1	0	1	Not selected
7	UBC 818	4	1	3	Not selected
8	UBC 823	8	3	5	Not selected
9	UBC 825	6	4	2	Selected
10	UBC 826	7	5	2	Selected
11	UBC 827	9	5	4	Selected
12	UBC 834	10	5	5	Not selected
13	UBC 835	14	7	7	Not selected
14	UBC 836	11	6	5	Selected
15	UBC 841	10	4	6	Not selected
16	UBC 843	1	1	0	Not selected
17	UBC 844	6	1	5	Not selected
18	UBC 846	0	0	0	Not selected
19	UBC 847	0	0	0	Not selected
20	UBC 848	0	0	0	Not selected
21	UBC 850	0	0	0	Not selected
22	UBC 854	2	0	2	Not selected
23	UBC 855	2	1	1	Not selected
24	UBC 856	9	5	4	Selected
25	UBC 857	6	5	1	Selected
26	UBC 865	7	5	2	Not selected
27	UBC 866	5	2	3	Not selected
28	UBC 873	6	2	4	Not selected
29	UBC 880	3	1	2	Not selected
30	UBC 889	6	0	6	Not selected
31	UBC 890	11	4	7	Not selected
32	UBC 892	0	0	0	Not selected

table 9 continued

33	UBC 895	4	0	4	Not selected
34	UBC 899	4	2	2	Not selected
35	UBC 900	3	0	3	Not selected
36	HB 10	12	8	4	Selected
37	HB 12	1	0	1	Not selected
38	Oligo ISSR 04	12	6	6	Selected
39	Oligo ISSR 05	0	0	0	Not selected
40	Oligo ISSR 06	8	6	2	Selected
41	Oligo ISSR 07	3	0	3	Not selected
42	Oligo ISSR 08	14	8	6	Selected
43	ISSR 2	0	0	0	Not selected
44	ISSR 3	0	0	0	Not selected
45	ISSR 4	0	0	0	Not selected
46	ISSR 5	0	0	0	Not selected
47	ISSR 6	0	0	0	Not selected
48	ISSR 7	0	0	0	Not selected
49	ISSR 8	0	0	0	Not selected
50	ISSR 9	0	0	0	Not selected

Table 10. Details of the ISSR primers selected for tagging of resistance gene

Sl. No.	Primer	Annealing temperature (°C)	Nucleotide sequence (5'-3')
1	UBC 810	52	GAGAGAGAGAGAGAGAT
2	UBC 825	52	ACACACACACACACACT
3	UBC 826	53.3	ACACACACACACACACC
4	UBC 827	54	ACACACACACACACACG
5	UBC 836	52	AGAGAGAGAGAGAGAGYA
6	UBC 856	51.6	ACACACACACACACACYA
7	UBC 857	52.7	ACACACACACACACACYG
8	Oligo ISSR 04	48	ACACACACACACACACC
9	Oligo ISSR 06	52	GAGAGAGAGAGAGAGAC
10	Oligo ISSR 08	48	GAGAGAGAGAGAGAGAT
11	HB 10	37	GAGAGAGAGAGACC

4.3.2.2 UBC 825

On an average six amplicons were obtained in each accession, on DNA amplification with the primer UBC 825. The pattern of amplification is shown in Plate 5. The molecular weight of the bands varied from 600 bp to 1.6 kb. Amplicon of size 700 bp obtained with this primer were polymorphic but showed no co-segregation with PPR resistance.

4.3.2.3 UBC 826

Using the primer UBC 826, total of eight amplicons were generated. The pattern of amplification is shown in Plate 6. The molecular weight of the bands varied from 550 bp to >2.0 kb. Amplicons obtained with this primer were distinctly polymorphic and the polymorphic band of 550 bp was present only in susceptible hybrids and one moderately resistant parent, and absent in all the three resistant hybrids. This primer was able to differentiate resistant and susceptible hybrids and could prove useful for identification of marker associated with PPR resistance.

4.3.2.4 UBC 827

ISSR analysis of DNA samples using primer UBC 827 generated eight amplicons (Plate 6). The molecular weight of the amplicons ranged from 400 bp to 2.3 kb. Amplicons obtained with this primer were found polymorphic for resistance to PPR. The polymorphic band of 1200 bp was present in one resistant hybrid, two susceptible hybrids and in one moderately resistant parent.

4.3.2.5 UBC 836

On an averages of 12 amplicons were obtained in each accession, on DNA amplification with the primer UBC 836. The pattern of amplification is shown in Plate 7. The molecular weight of the bands varied from 300 bp to 1.5 kb. Amplicons obtained with this primer were polymorphic. The polymorphic band of 600 bp was present in two susceptible hybrids, one resistant hybrid and in both moderately resistant parents.

4.3.2.6 UBC 856

Averages of nine amplicons were obtained in each hybrid, on DNA amplification with the primer UBC 856 (Plate 7). The molecular weight of the

amplicons varied from 500 bp to 1.1 kb. The polymorphic band of 750 bp was present in one resistant hybrid and in two susceptible hybrids. One another polymorphic band of 800 bp was present only in one moderately resistant parent. The both polymorphic bands obtained had no association with resistance to PPR.

4.3.2.7 UBC 857

When the hybrids along with parents were screened with ISSR primer UBC 857, an averages of six amplicons were obtained. The pattern of amplification is shown in Plate 8. The molecular weight of the bands varied from 600 bp to 1.7 kb. Amplicons obtained with this primer were distinctly polymorphic for resistance to PRR. The polymorphic band of 850 bp was present in two resistant hybrids and in one of susceptible hybrid and in both moderately resistant parents.

4.3.2.8 Oligo ISSR 06

Amplification pattern developed using the primer Oligo ISSR 06 produced an average of eight bands in all samples (Plate 8). Molecular size of the bands developed varied from 400 bp to 1.9 kb. Amplicon generated at 650 bp was found to be polymorphic and the band was present in all samples excluding two resistant hybrids and the marker was particularly polymorphic for the disease

4.3.2.9 Oligo ISSR 04

On an average of 13 amplicons were obtained in each accession, on DNA amplification with the primer Oligo ISSR 04 (Plate 9). The molecular weight of the bands varied from 500 bp to 1.8 kb. Amplicons generated with this primer were clearly polymorphic for resistance to PPR. The polymorphic band of 550 bp was present in all susceptible hybrids and in both moderately resistant parents.

4.3.2.10 Oligo ISSR 08

Oligo ISSR 08 had generated an averages of 14 amplicons in each accession. The pattern of amplification is shown in Plate 9. The molecular size of the bands varied from 300 bp to 1.35 kb. At 850 bp size, a distinct polymorphic amplicon was generated in three resistant hybrids, one susceptible hybrid and in both moderately resistant

parents. Amplicon obtained with this primer was clearly polymorphic for PPR resistance.

4.3.2.11 HB 10

ISSR analysis with primer HB 10 generated 12 amplicons in all accessions (Plate 10). The polymorphic band of 1.4 kb size present in all accessions except one susceptible hybrid. This polymorphic band had no association with resistance to PPR.

To summarise, UBC 826 and Oligo ISSR 04 had yielded distinct polymorphic bands of size 550 bp. In the case of UBC 826, this band was present in all the susceptible hybrids and one moderately resistant parent. Whereas, in case of Oligo ISSR 04 the 550 bp band was present in both moderately resistant parents along with susceptible hybrids. In both cases, these bands were absent in the all resistant hybrids. These ISSR primers may be linked with the PPR disease resistance. According to Iwaro *et al.* (1997) and Nyasse *et al.* (2007), PPR resistance is polygenic and have additive gene effect. The gene linked by this primer may have minor effect or some cumulative epistatic effect is required for gene expression.

Other primers UBC 810 and Oligo ISSR 08 produced the distinct polymorphic amplicon of 850 bp by size in all resistant hybrids, in one susceptible hybrid and in both parents. These markers appear to be a very important and since they were present in all the resistant accessions, it could be observed that these markers are associated with resistance. This gene could be targeted for developing resistant line in marker assisted selection.

UBC 827 and UBC 857 had also given the polymorphism at 1200 bp and 850 bp respectively. The band of 1200 bp size obtained by UBC 827 marker was present in one resistant hybrid, but in two susceptible hybrids and also in one of moderately resistant parents. Whereas, the marker UBC 857 generated band of size 850 bp was present in two resistant hybrids, in one susceptible hybrid and also in both parents. These banding pattern generated by the both primers UBC 827 and UBC 857 showed that these markers could be associated with genes which have low level of contribution to the resistance. This may be the reason why the markers have appeared in the susceptible line also. Obviously the presence of this markers in at least single resistant

hybrid and in moderately resistant parents show that this gene is requisite part of the gene groups which are contributing towards the resistance to PPR in cocoa. It should be concluded that mere presence of these genes alone to which markers are linked will not offer any resistance or the level of resistance offered will be minimal. The polygenic and cumulative epistatic nature of gene action in cocoa are already reported by Iwaro *et al.* (1997), Nyasse *et al.* (2007) and Rubiyo and Rivaie (2013).

In the present study 11 ISSR primers yield polymorphic bands. The parents used in the study were reported to be moderately resistant to PPR. Thus, polymorphics were selected based on: any single band which was presented in resistant or susceptible hybrids and at least in any one of the parent, or present in susceptible genotypes only considered as polymorphic of importance. Based on this criteria, six ISSR markers were selected for cloning sequencing analysis (Table 11).

Table 11. Details of the ISSR markers selected for cloning/sequencing

Sl. No.	Primer	Size of polymorphic amplicon (~bp)
1	Oligo ISSR 04	550
2	Oligo ISSR 08	850
3	UBC 810	850
4	UBC 826	550
5	UBC 857	850
6	UBC 827	1200

4.3.3 Simple Sequence Repeat (SSR) Analysis

Microsatellites, or simple sequence repeats (SSRs), are becoming gradually attractive markers in molecular breeding, diversity evaluation and fingerprinting (Powell *et al.*, 1996). SSRs are short tandemly repeated sequence motifs of nearly 1-8 bp in length, which are distributed throughout the genome and can vary between individuals in repeat length. Primer pairs intended for the flanking sequences can be used in PCR reactions for site-specific amplification of the microsatellite, thereby constructing sequence-tagged microsatellite markers (Powell *et al.*, 1996). A number of SSR markers have been developed for cacao (www.cacaogenomedb.org). SSRs show polymorphism higher than other markers and coupled with other desirable

features, they have been used widely in various genetic studies of cacao, including construction of various genetic and QTL maps (Clement *et al.*, 2003; Brown *et al.*, 2005; Faleiro *et al.*, 2006; Chandrakant, 2014; Sujith, 2016).

In the present study, SSR assay was done with 15 primer sets which are reported to yield good polymorphism and linked with QTL resistant to PPR in cocoa (Lanaud *et al.*, 2009). Details of the SSR amplification profile is given in the table 12. Though the various primer sets screened were found to yield polymorphism among the hybrids and moderately resistant parents at various levels, none of them was successful to give a clear distinction among the resistant and susceptible genotypes. The failure may be due to both experimental and genetic factors. Similar observations have been made in genetic studies using SSR markers in many crop species (Gupta and Varshney, 2000).

Table 12. Details of the amplification pattern obtained with 15 SSR primers

Sl. No.	Primer	Amplification pattern				
		No. of amplicons	Types of amplicons		Polymor -phism	Remarks
			Distinct	Faint		
1	mTcCIR32	2	2	0	Absent	Not selected
2	mTcCIR37	3	2	1	Present	Not selected
3	mTcCIR54	1	1	0	Present	Not selected
4	mTcCIR100	1	1	0	Present	Not selected
5	mTcCIR113	1	1	0	Absent	Not selected
6	mTcCIR119	2	2	0	Present	Not selected
7	mTcCIR152	3	3	0	Present	Not selected
8	mTcCIR156	2	2	0	Present	Not selected
9	mTcCIR170	2	2	0	Present	Not selected
10	mTcCIR199	2	1	1	Absent	Not selected
11	mTcCIR203	3	2	1	Absent	Not selected
12	mTcCIR215	2	2	0	Absent	Not selected
13	mTcCIR222	1	1	0	Absent	Not selected
14	mTcCIR255	3	3	0	Present	Not selected
15	mTcCIR279	1	1	0	Absent	Not selected

The total of 15 SSR primers were primarily screened using the bulks of DNA of resistant and susceptible progenies along with bulk of parent DNA. The gel profile of four SSR primers showed in Plate 11 and 12. Among 15 SSR primers screened, eight primers produced polymorphism but with no clear co-segregation between resistant and susceptible. Other primers either produced monomorphic bands or failed to amplify the expected fragments. Among these, some SSR primers screened were linked with QTLs reported for PPR resistance. The SSR primers used for screening were selected based on the report that they are linked to QTL reported for PPR resistance (Lanaud *et al.*, 2009). However, they did not work on present population indicating that not all SSRs work equally well for all cacao populations.

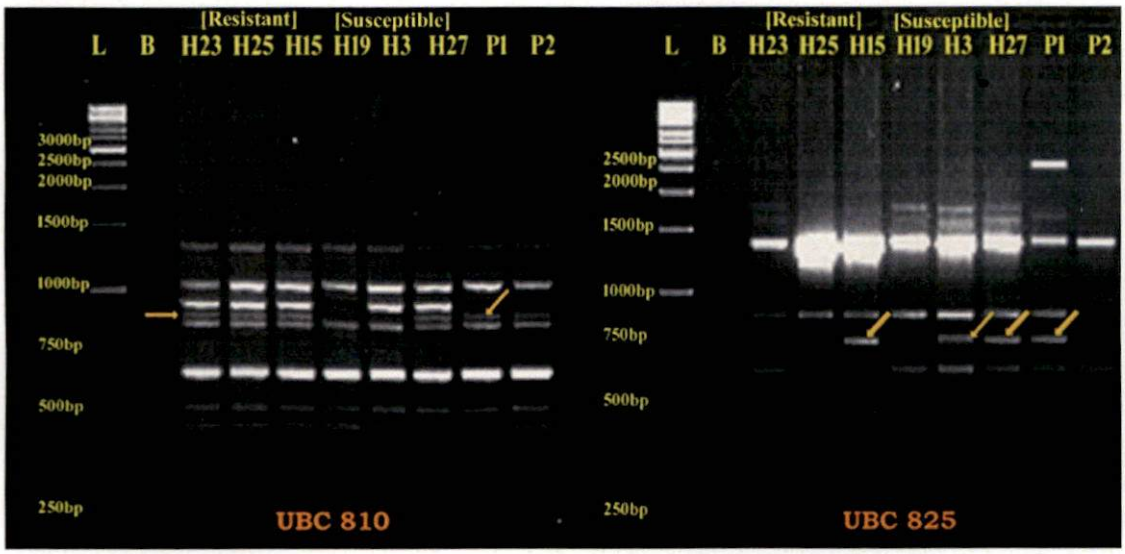

Plate 5. Amplification pattern generated with primer UBC 810 and UBC 825

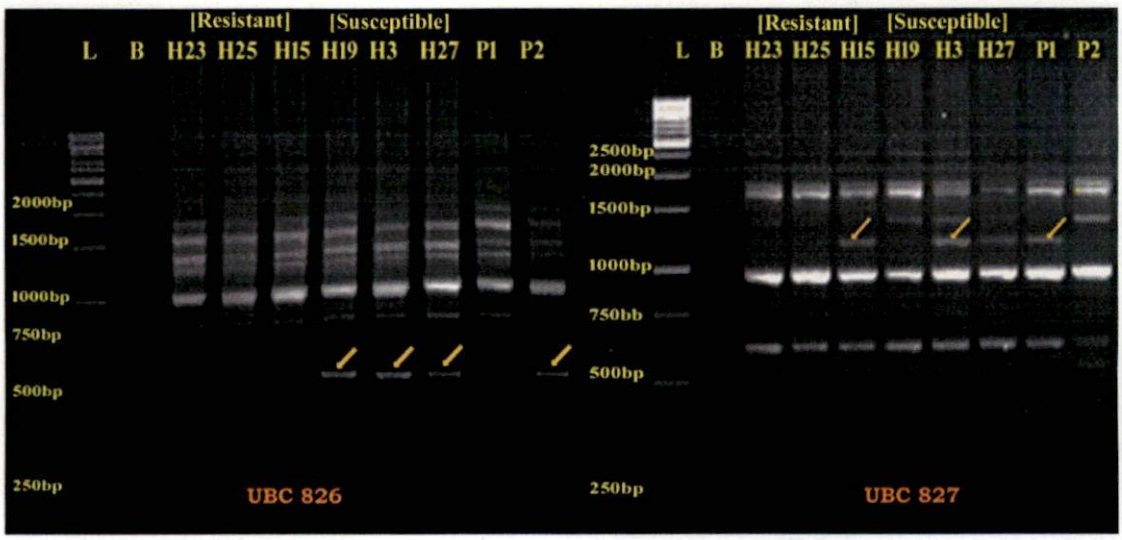

Plate 6. Amplification pattern generated with primer UBC 826 and UBC 827

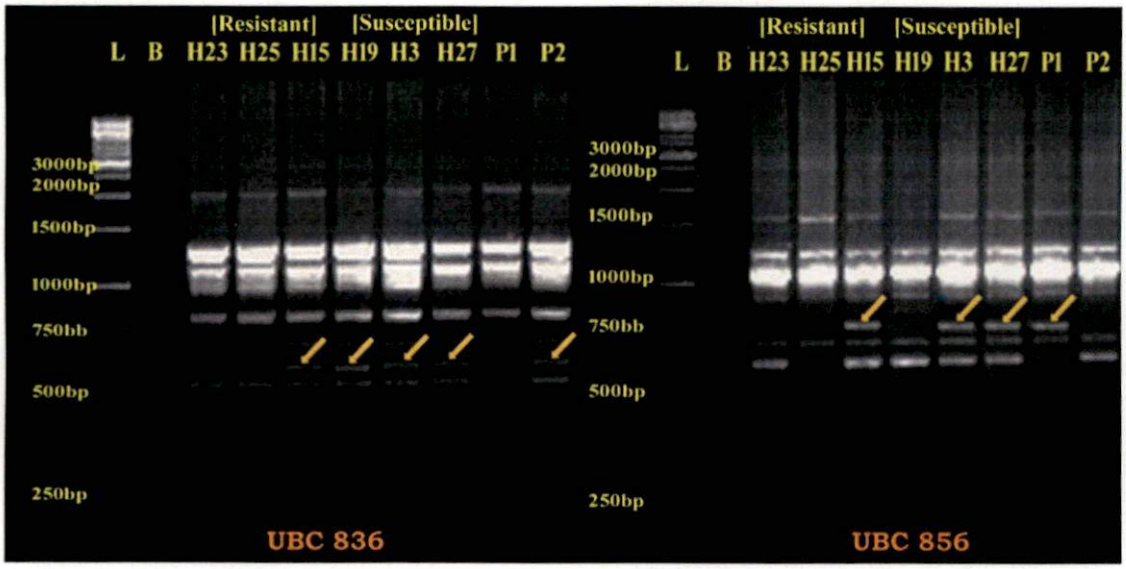

Plate 7. Amplification pattern generated with primer UBC 836 and UBC 856

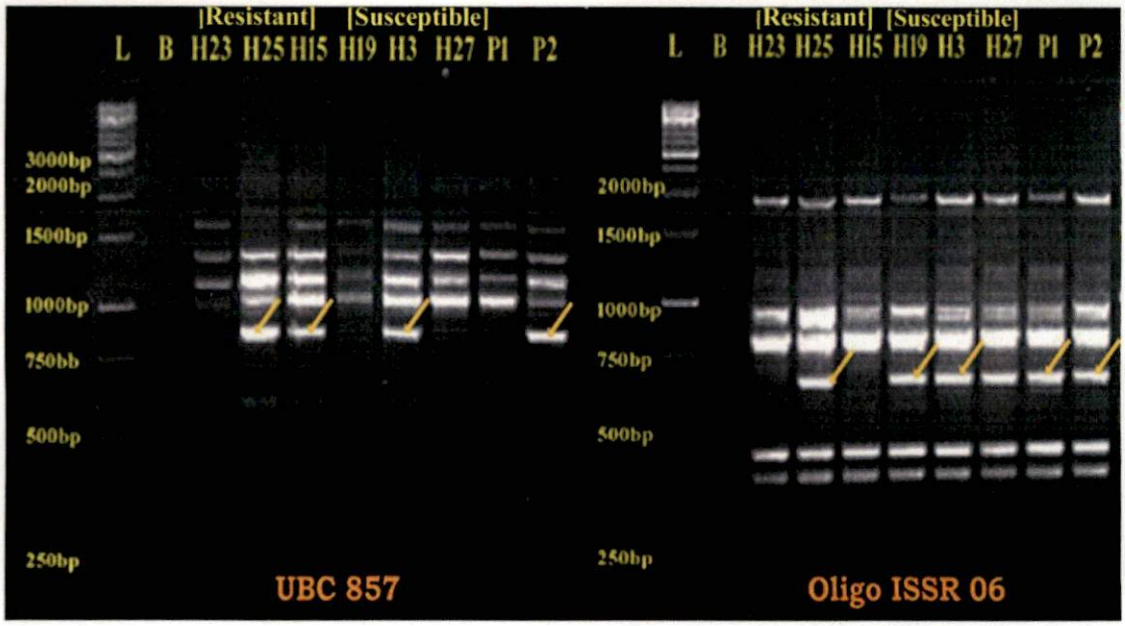

Plate 8. Amplification pattern generated with primer UBC 857 and Oligo ISSR 06

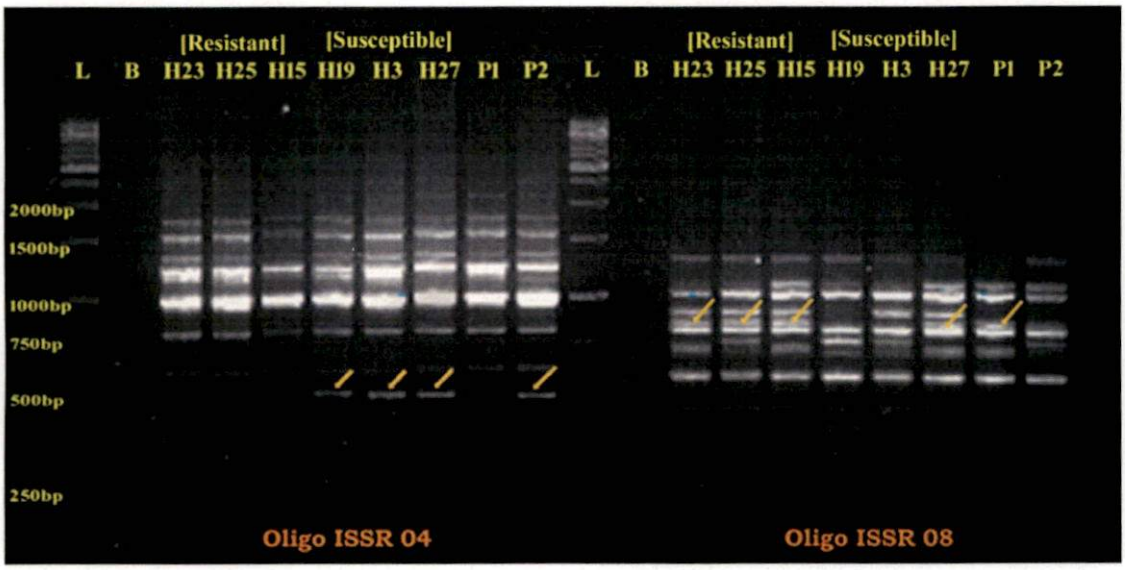

Plate 9. Amplification pattern generated with primer Oligo ISSR 04 and Oligo ISSR 08

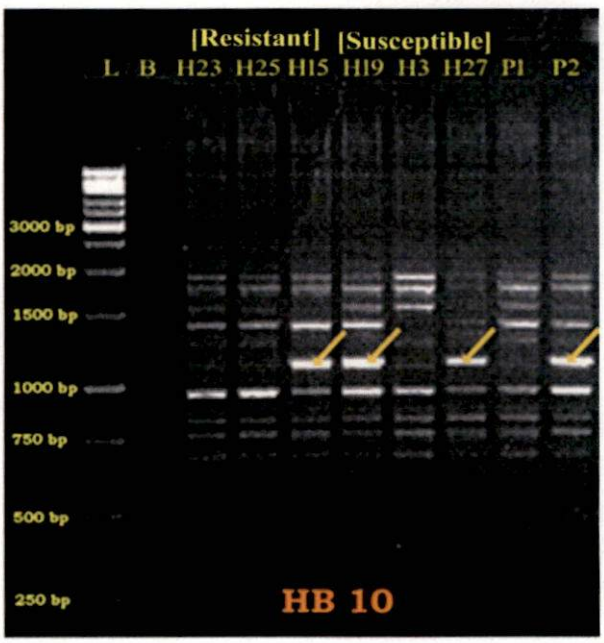

Plate 10. Amplification pattern generated with primer HB 10

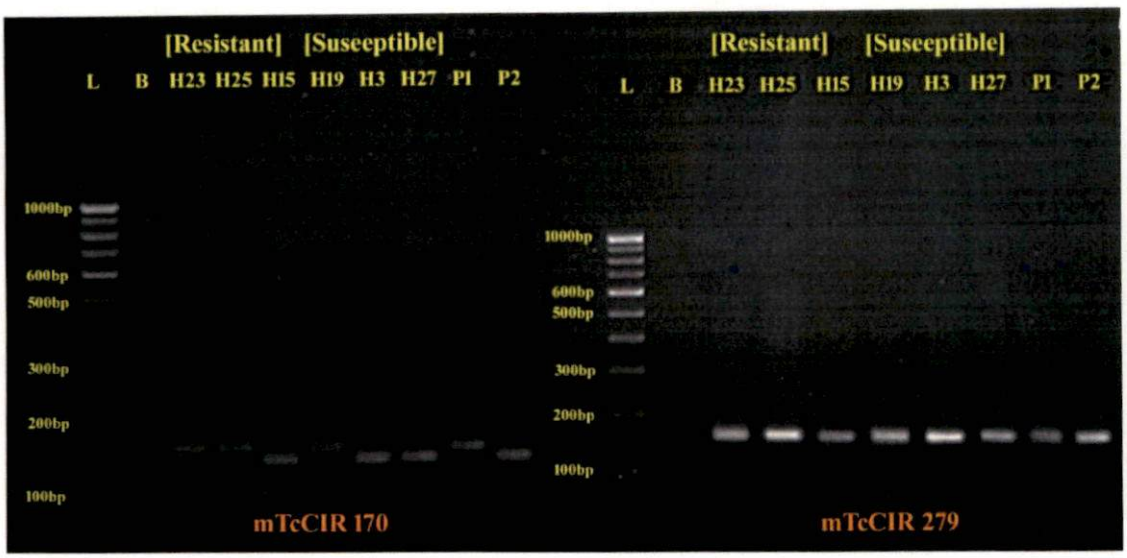

Plate 11. Amplification pattern generated with primer mTcCIR 170 and mTcCIR 279

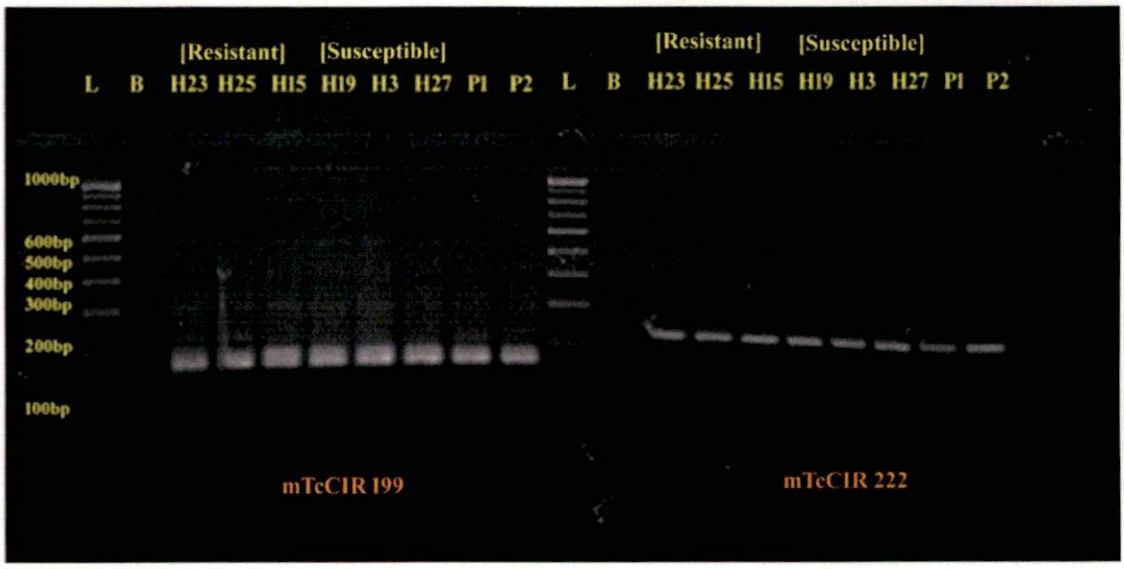

Plate 12. Amplification pattern generated with primer mTcCIR 199 and mTcCIR 222

4.4 CLONING AND SEQUENCING OF ISSR POLYMORPHIC MARKERS

The polymorphic bands generated in relation to the disease response from selected five ISSR markers; UBC 810, UBC 826, UBC 857, Oligo ISSR 04 and Oligo ISSR 08 were eluted (Plate 13). The concentration of eluted DNA was measured. The eluted PCR products, which had adenosine at 3' end, were ligated in to vector pTZ57R and were directly cloned into *E. coli* DH5α (Souii, *et al.*, 2013). A large number of white colonies were developed on LB-ampicillin X-Gal plates which indicated a high degree of transformation (Plate 14) (Kwon *et al.*, 1998). The putative transformants which appeared as white colonies were maintained on LB-ampicillin X-Gal plates (Plate 14). The transformed colonies were selected and confirmed as transformants through colony PCR (Plate 15). Plasmid DNA was isolated from these transformants. The isolated plasmids were confirmed for desired insert through gel electrophoresis and by PCR amplification with specific M13 primer (Yanisch-Perron *et al.*, 1985). The gel profile of isolated plasmid and its PCR product is given in Plate 16.

In the present study, TA cloning system was proved the best technique for direct cloning of PCR products, because the strategy avoided tedious steps like restriction digestion and ligation (Lim *et al.*, 2010). The isolated plasmids were sequenced on Sanger sequencing platform. The DNA sequencing enables construction of genetic map, development of transgenic plants, identification of genes, *etc.* (Fraley *et al.*, 1983; Bernatsky and Tanksley, 1986; Schnable *et al.*, 2009). The microsatellite UBC 857 marker was validated by cloning and sequencing (Tulshiram, 2016). The marker was confirmed linked with VSD resistance.

DNA sequencing also enables development of reference genomes which facilitate identification of candidate genes in crop, Arabidopsis (Arabidopsis Genome Initiative, 2001), rice (International Rice Genome Sequencing Project, 2005), cocoa (Motamayer *et al.*, 2013), *etc.*

The polymorphic ISSR band of 1200 bp size generated from UBC 827 was eluted, reamplified and directly send for sequencing (Plate 17) and this marker could sequence DNA of size 650 bp. ISSR is random primer which will in general produce noise on both ends of reads. This was the clear reason for ultimate reduction in the size of good sequence. The DNA fragments cloned into a plasmid vector and sequenced by

using a flanking M13 primer gave complete sequence read and this result is in tune with Hindley and Phear (1981). The length of remaining five markers UBC 810, UBC 826 UBC 857 Oligo ISSR 04 and Oligo ISSR 08 were 949 bp, 914 bp, 827 bp, 939 bp and 924 bp respectively, and was more than size of actual DNA inserts. According to Tulshiram, (2016), sequencing the cloned plasmid DNA is better way to get complete read of the insert.

Plate 13. Gel profile of eluted DNA samples

Lane **1**: Oligo ISSR 04, **2**: Oligo ISSR 08, **3**: UBC 810, **4**: UBC 826 and **5**: UBC 857

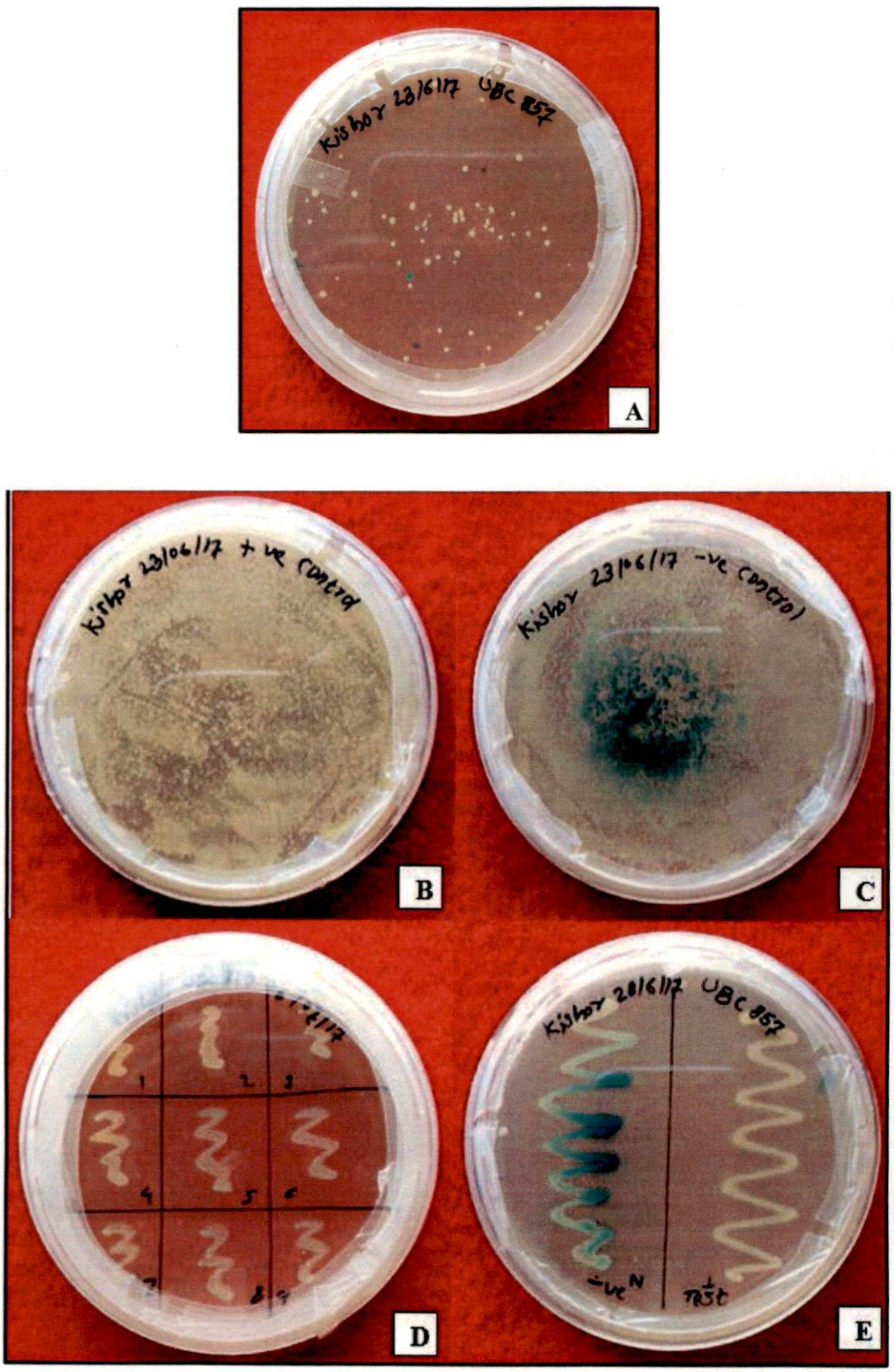

Plate 14. Cloning of eluted PCR products: (A) Transformants (B) Positive control (C) Negative control (D) Grid plate (E) Maintenance of transformants

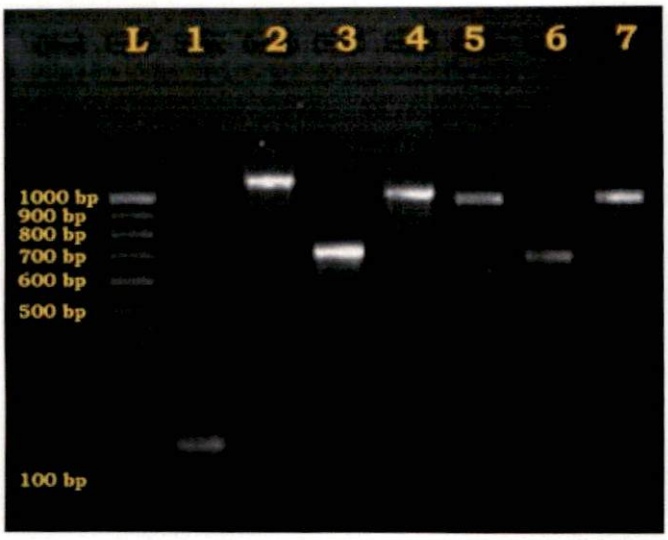

Plate 15. Gel profile of colony PCR products

Lane **1**: Negative control, **2**: Positive control, **3**: Oligo ISSR 04, **4**: Oligo ISSR 08, **5**: UBC 810. **6**: UBC 826 and **7**: UBC 857

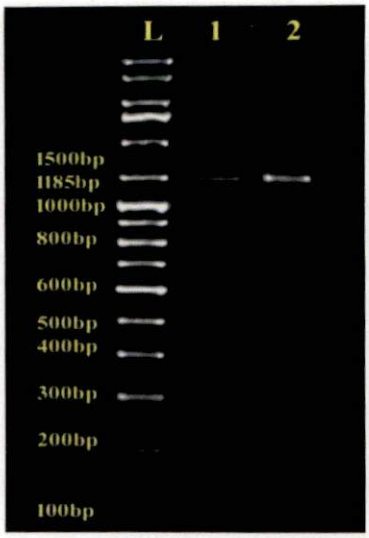

Plate 17. Gel profile of PCR product of primer UBC 827

Lane **1:** Eluted, **2:** Reamplified

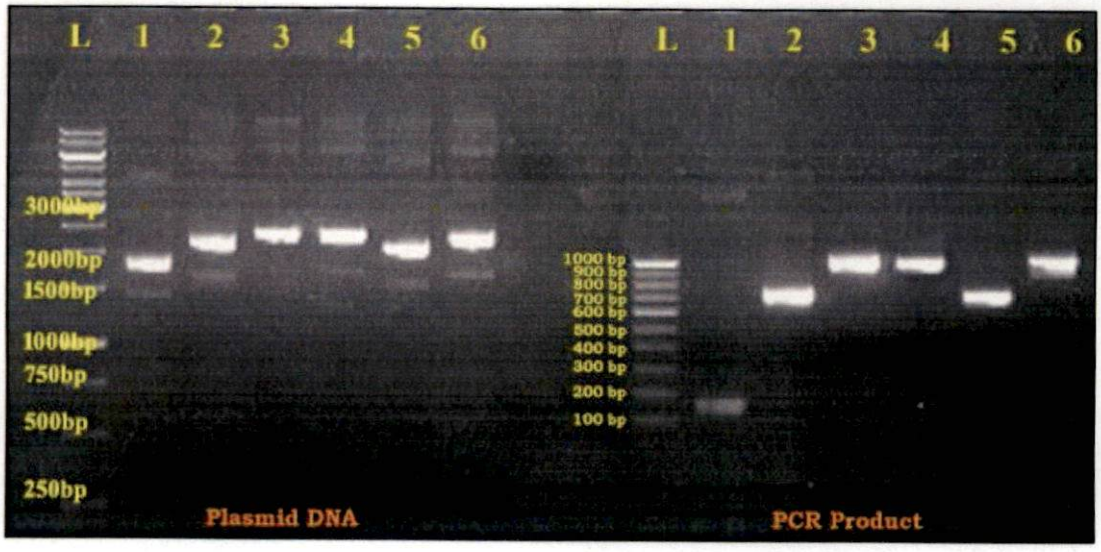

Plate 16. The gel profiles of isolated plasmid DNA of five transformants, and the amplification pattern of five plasmid DNA with M13 primer

Lane **1**: Negative control, **2**: Oligo ISSR 04, **3**: Oligo ISSR 08, **4**: UBC 810, **5**: UBC 826 and **6**: UBC 857

4.5 SEQUENCE ANALYSIS OF ISSR POLYMORPHIC MARKERS

The sequences obtained were processed and analysed with bioinformatics tools.

4.5.1 Sequence Processing

The trimmed sequences generated were processed in VecScreen tool for identification of vector contaminated fragments. The contaminated nucleotide numbers were noted down and were removed from sequences by BioEdit tool. The processed sequences of all markers are given below.

i. Oligo ISSR 04

>Oligo.ISSR.04 (561 bp)

5'CACACACACACCACCAGTCAGAGGGTTGAGGAACTATGTAAGGGTA
TTAAGGATATATATACATGAAGAAAGAGAGAATCATACATGTACAAGCG
TATAGGTGCAGAGACATTTGTGGTGAGAATGCTGAAATCAAAACTGGCA
GCGTCGTCTTGGTTAGGCAGTCATAGCTGCACTACTACTCAGGTCAGTTA
CAAACAAAATGACAGATAAGACAACAAAAAACAATAACCACGCGCCTC
TGTACAGGGGTTATTGGCACCACGCGCCCTTAAAACCCTAGCCTACGCCA
ACGAATACTAGGCCCACAAATAAAACATTCCTAACGACCCTAATCTTAT
AATTTATCCGCCTATTTCTTCTAGGCTATGGGGTAAACGAAGAAGAGGG
AGAGAAAGCACTTGGGGCAATTAACAGAATTACATATGCCATTATTTTCT
AAAGTTAAGTTATAATGAAATTAATTGGTAAATTAACAATTTGACAATGC
ATTTTAGTTGTGTGCATGTCCTGTACCACATTTAACTGTTGGAATAAAAC
AGGTGTGTGTGTGTGTGTA-3'

ii. Oligo ISSR 08

>Oligo.ISSR.08 (871 bp)

5'GATTGAGAGAGAGAGAGAGATAGAGAGAGCTATCTTTTCTAATGCTTT
GGCTTTATGGCTTCAGTTTTACTGAAGTAATGTTGTTTCAATTCTTAAATT
GGTAATGAATCTGTTAGTGAACAAAATAAAATGTTAGATCAATCTGTAG
GAATTGCTAAAATTTGCACCCTTAATCCATTTTGCATCTCTGTTCCTCAAC
CCTTTTTGCCCTTCTTTTACCTTTTTCTTGATAACCACCATTTCATGCAAG
CCTTGACTATAGTCACTTTTGGTTGTATGTTGATATGGTTTTAGTTTCTTC
TTAATTATGTGACTCTCCAAAATGGTGCAGTCACGCTGTCAAGCATGCTT

TTGCACCTTAAACCATATCAACATGTTTGATCTTACTCTTGGCAAACCTA
AAGTAGTTCAGACATGATGCATGTGCATTTCTACGTTGTTTTGGGAGATT
GATGAAACTCTAATTGTTAAACAGGGCTTTCGCACCAGGTTTTCTGGACA
AAGATGTATTTGGTCTTCAAACTTTTCAGCATTTAATGCGAATGACCCGT
ACATGGGATTCCACGATGTCAATGATACCAAAAGGTGGGGTCACTATAT
GGGGTGGGCTTGATTGGTCACCTGAAGGAGGAAGCTTTAACTGTAGTGC
AAAAAAGTTGGAGAACAATGGCACTCGTACAGGCCACAATGCAACCAGC
AACTTGGGTAATATGAAAAGTGTGAATTATGGGAGAATTATTTCATTTGG
GAAAGATGTGGCTGAGGCACATTCCTCCAAGATTGAGAGGGTTGATTTC
AGGGTAATGTCCAAGTTATGATTAAGTTACTTTACATTAGTGAAAGTATA
AGGATGATCTCTCTCTCTCTCTCA-3'

iii. UBC 810

>UBC.810 (877 bp)

5'GCATCTAGATTGAGAGAGAGAGAGAGATAGAGAGAGCTATCTTTTCT
AATGCTTTGGCTTTATGGCTTCAGTTTACTGAAATAATGTTGTTTCAATT
CTTAAATTGGTAATGAATCTGTTAGTGAACAAAATAAAATGTTAGATCA
ATCTGTAGGAATTGCTAAAATTTGCACCCTTAATCCATTTTGCATCTCTG
TTCCTCAACCCTTTTTGCCCTTCTTTTATTTTTTTCTTGATAACCACCATTT
CATGCAAGCCTTTACTATAGTCACTTTTGGTTGTATGTTGATATGGTTTT
AGTTTCTTCTTAATTATGTGACTCTCCAAAATGGTGCAGTCACGCTGTCA
AGCATGCTTTTGCACCTTAAACCATATCAACATGTTTGATCTTACTCTTG
GCAAACCTAAAGTAGTTCAGACATGATGCATGTGCATTTCTACGTTGTTT
TGGGAGATTGATGAAACTCTAATTGTTAAACAGGGCTTTCGCACCAGGT
TTTCTGGACAAAGATGTATTTGGTTTTCAAACTTTTCAGCATTTAATGCG
AATGACCCGTACATGGGATTCCACGATGTCGATGATACCAAAAGGTGGG
GACACTATATGGGGTGGGCTTGATTGGTCACCTGAAGGAGGAAGCTTTA
ACTGTAGTGCAAAAAAGTTGAAGAACAATGGCACTCGTACAGGCCACA
ATGCAAACAGCAACTTGGGTAATATGAAAAGTGTGAATTATGGGAGAA
TTATTTCATTTGGGAAAGATGTGGCTGAGGCACATTCCTCCAAGATTGA
GAGGGTTGATTTCAGGGTAATGTCCAAGTTATGATTAAGTTACTTTACAT
TAGTGAAAGTCTAAGGGTGATCTCTCTCTCTCTCA-3'

iv. UBC 826

>UBC.826 (535 bp)

5'ACACACACACCTATAAACAAAACCTAATATATTTTTTACTATTATATA
TGCATATGAAAAGTAAATAAAAAGAGTTAATAAATGACAATATGTAGG
GGTCACAAAACATAATGAACTTTTTCTTTTTGTTTGATCAAATGGTTATG
TTAAAAATCGAAAGGTAACTAATGAAACTTGAACATCAGATTAATAAA
AACTTAAAAGTTAATTAAAGGTATCATGAAAAAACTTATAAGATGTTGA
AGTAAAATTGAGAAATAAAAAAAAGAACCTCGAAAACATAATTGGGAA
AATACATCTTGGGTAAGAGAAAGAAAGGGAAGACCGTGGAAACAATGG
CCGGTAGTTAAAGAAATTGACAACCTATGCTATGGGGAGGTTAATACTA
TGGCAGACTTCACTAGCTAGGCTTGGTACTGATCGTAATGGTGTGCTTTT
GCTTGGTGATAGATTCTGTTTCCATGGTTTCTTTATCAGTTATGGCCTTTA
TCAAGATTATATATGTGAGTCAGAATCTGGTGTGTGTGTGTGTGTA-3'

v. UBC 857

>UBC.857 (839 bp)

5'TACACACACACACACACTGCTAGCCATATTCTTTTTTAAGGCACTACA
AAACAATAGTTGCTCACATGGCTTAATAAAGTTTCCGATTAAATTTGGG
ACAGCTCACTGAAAATCCAACCAGTTGAGGACACAACACCAAAAGACT
ATATATAATTGCAAGAAAGGAGATAACCTTTTTAAGCACATATATAGGA
AAGGAGATCACCAAACAGCTCACTGTTCTTTCAATAAACTCCCAAAGAT
CACCTTTCTAAGCACATACAAATGAAATATTAAAGAAAAAAATAGTTTC
AAATGCAAATGCCAAGAAAAAATATGCTTCAAAGTTTTTTGTGGCAAAC
TCGGATCATAATAATATATCAATAGTCATAGTTTAAAAACCAGCACACC
GATGGTATTATAAACTGCTTGGCTATTTAATTTGGTAAAAGTTGTAGTTT
AATCCGACATATTTTAGATGAAAGTGGAGTGGCCTTGTCTCCATAGAAG
TCTCAAATTAGGTTTTACAAAAGCAAGAAATTCTGGAATTGCGAAAAAA
TTAAGGGAGAGTTAATGATGAATTGCAAATTTGTTGCCAAGCTCACTCT
CTTGTAAATGGAAAATTAAGGGTAATGGAGTCCACCTTAGCTACTACAA
AGTGCTAATTCTGGCAAGATCTTCTCACATCCCACAATTCCAACTTTCTC
TTTCCAAAATCAAATGTTAATTAATGGTGCATGTGGTTTTACGTGGAGG
AGACTCCTTCGTAATTTAAGAAGAGATTTTTGAGGTGCAGATGACTGAT

AAGATTGTTGTTGGATGTTGTACTCTTTGTTCCCTTCGGTGTGTGTGTGT
GTGTA-3'

Sequence of marker 827 was not processed since it was sequenced directly by avoiding cloning. The sequence of marker UBC 827 is given below.

vi. UBC 827

>UBC.827 (571 bp)
5'CATTATTCATCACATTATACATTTTAAAATGTAAAAGTATCACTTGTGA
AAAGCTTGTTCTCGTGCATAAATTTGAAAAGAGTCAACCAAGGATATTC
TAGCATTTCATTCAACACATATGCATTTGTTCAAAACCTTTTAAATGTAA
GTTCACTCACCTTACAATAACAAAATACTACATCGCTATTAGTTCGGAA
GCGTATCTAGCTATTTCTACTTGCCGGTCACTCGTTATTTCCTCCGGCAC
GCCACCACAGTACACTATCTTTACTTTTGCACTTCTTAGCAACAATCTAA
TTTCAATATCTTTACTGTATATCATTTCTACTTCTCTTTTTCATTCAATCG
TGAATCGTTATTCAACCAACTTATATCTTGACACATTTTTACCAAAGTGG
CATTTATCCTTTGCTTGCATAATTCTTTAGATTATAAATATCGGTAACTC
ATTTATGACTCTAGCACATATATTAACCTTTTCTAAGGATATTTATCAAG
TTCAACCATCATTTCCACACCAATAACCTAGCATATGGCAGCAACTATA
AATTTACTTCCATCAAGCATTTTC-3'

4.5.2 Sequence Analysis

BLASTn analysis of the sequence Oligo ISSR 04 had shown 96 per cent identity with *Theobroma cacao* genome assembly, chromosome: IX (LT594796.1), 92 per cent with Predicted: *Herrania umbratica* histidine-containing phosphotransfer protein 1-like (LOC110412650), transcript variant X2 and 98 per cent with Predicted: *Theobroma cacao* histidine-containing phosphotransfer protein 1 (LOC18590364) mRNA (XM_007015823.2) (Plate 18).

Histidine-containing phosphotransfer proteins (HPts) take part in hormone signal transduction in higher plants (Ruszkowski *et al.*, 2014). They function as two-component phosphorelay mediators between cytokinin sensor histidine kinases and response regulators (B-type ARRs). It also plays an important role in propagating cytokinin signal transduction through the multistep His-to-Asp phosphorelay (Miyata *et*

al., 1998; Tanaka *et al.*, 2006). Cytokinin is found to be associated in many aspects of plant growth and development, including cell division, shoot initiation and development, vascular development, leaf senescence, deetiolation, chloroplast differentiation and in stress response (Sakakibara, 2006; Nishiyama *et al.*, 2013) and more recently its implication with disease resistance has been revealed (De Vleesschauwer *et al.*, 2013).

The fungal pathogen *Plasmodiophora brassicae*, the causal agent of the Brassicaceae clubroot disease, had shown to down regulate the cytokinin degradation pathway during infection of Arabidopsis, and transgenic overexpression of cytokinin oxidases/ dehydrogenases suppressed club root development, indicating the importance of cytokinin in the pathogenicity of *P. brassicae* (Siemens *et al.*, 2006). Transgenic plants with increased cytokinin levels exhibited delayed leaf senescence and enhanced resistance to *B. cinerea* infection in tomato (Swartzberg *et al.*, 2008) and enhanced resistance to *A. brassicicola* KACC40036 in Arabidopsis (Choi *et al.*, 2010).

BLASTn analysis of marker Oligo ISSR 08 resulted in 99 per cent of identity with *Theobroma cacao* genome assembly, chromosome: IX (LT594796.1) (Plate 19). The BLASTn analysis of marker UBC 810 was also showed 99 per cent identity with *Theobroma cacao* genome assembly, chromosome: IX (LT594796.1) (Plate 20). It also resulted in 99 per cent sequence identity with Predicted: *Theobroma cacao* phospholipid: diacylglycerol acyltransferase 1 mRNA (XM_018128234.1) and showed high sequence identity with Predicted: *Gossypium raimondii* phospholipid: diacylglycerol acyltransferase 1-like and Predicted: *Glycine max* phospholipid: diacylglycerol acyltransferase 1-like, transcript variant X3, mRNA (XM_006593880.2). KASS analysis of UBC 810 marker had given hit for enzyme phospholipid: diacylglycerol acyltransferase (PDAT, EC 2.3.1.158) (Plate 21) involved in glycolipid pathway. Function of this enzyme is characterized by transferring the fatty acyl moiety from the sn-2 position of a phosphatidylcholine (PC) to the sn-3 position of sn-1, 2-DAG to form triacylglycerols (TAG) (Stahl *et al.*, 2004).

According to Yuan *et al.* (2017), five different PDAT encoding genes (CsPDATs) are involved in different types of stress responses in *Camelina sativa*

seedlings and they may have practical applications in increasing oil accumulation and enhancing stress tolerance in other plants as well.

When the marker UBC 826 sequence was analyzed on BLASTn, showed 97 per cent identity with *Theobroma cacao* genome assembly, chromosome: VI (LT594793.1) (Plate 22). The marker sequence UBC 857 resulted in 99 per cent identity with *Theobroma cacao* genome assembly, chromosome: IV (LT594791.1) (Plate 23). The marker UBC 827 had shown 98 per cent identity with *Theobroma cacao* genome assembly, chromosome (LT594792.1) and 73 per cent with *Theobroma cacao* clone TCC_BA049P20, complete sequence (JN127765.1) (Plate 24). TCC_BA049P20 BAC is reported to be QTL rich region associated with different traits of *T. cacao* (Feltus *et al.*, 2011).

From the results of BLASTn analysis, polymorphic ISSR primers UBC 810, UBC 826, UBC 857 and Oligo ISSR 04 are identified as putative markers linked to PPR. Hereafter, these are referred to as UBC 810_{877}, UBC 826_{535}, UBC 857_{839} and Oligo ISSR 04_{561} where, numbers in subscript denote the sequence length (bp) of the polymorphic amplicon.

Table 13. Results of BLASTn analysis

Sl. No.	Query sequence	BLASTn analysis results		
		Subject sequence title	**Identity (%)**	**Function**
1	Oligo ISSR 04	1. *Theobroma cacao* genome assembly, chromosome: IX (LT594796.1)	96	-
		2. Predicted *Herrania umbratica* histidine-containing phosphotransfer protein 1-like (LOC110412650), transcript variant X2	92	Histidine-containing phosphotransfer proteins (HPts) take part in cytokinin signal transduction. Cytokinin is associated with disease resistance.
		3. Predicted *T. cacao* histidine-containing phosphotransfer protein 1 (LOC18590364), mRNA	98	

table 13 continued

2	Oligo ISSR 08	*Theobroma cacao* genome assembly, chromosome: IX	99	-
3	UBC 810	1. *Theobroma cacao* genome assembly, chromosome: IX (LT594796.1) 2. *T. cacao* phospholipid: diacylglycerol acyltransferase 1 mRNA (XM_018128234.1) 3. *Gossypium raimondii* phospholipid: diacylglycerol acyltransferase 1-like	99 99 97	- Phospholipid: diacylglycerol acyltransferase (PDAT) is involved in different types of stress responses
4	UBC 826	*Theobroma cacao* genome assembly, chromosome: VI (LT594793.1)	97	-
5	UBC 827	1. *Theobroma cacao* genome assembly, chromosome: V (LT594792.1) 2. *T. cacao* clone TCC_BA049P20, complete sequence (JN127765.1)	98 73	- QTL rich region associated with different traits
6	UBC 857	*Theobroma cacao* genome assembly, chromosome: IV (LT594791.1)	99	-

4.5.3 Location of Markers on Chromosome

ISSR markers UBC 810_{877}, UBC 826_{535} and UBC 857_{839} are located on chromosome nine, six and four respectively as inferred from NCBI Genome Data Viewer tool through BLASTn annotations. These marker locations were compared with SSR markers in meta-QTL map for PPR resistance in cocoa (Lanaud *et al.*, 2009).

Marker UBC 810_{877} is found to be located on the chromosome nine in between the reported SSR marker mTcCIR30 and mTcCIR251 on right side while markers mTcCIR166 and mTcCIR172 on the left side (Plate 25). This marker is positioned in the already reported PPR resistance associated gene rich region (Lanaud *et al.*, 2009).

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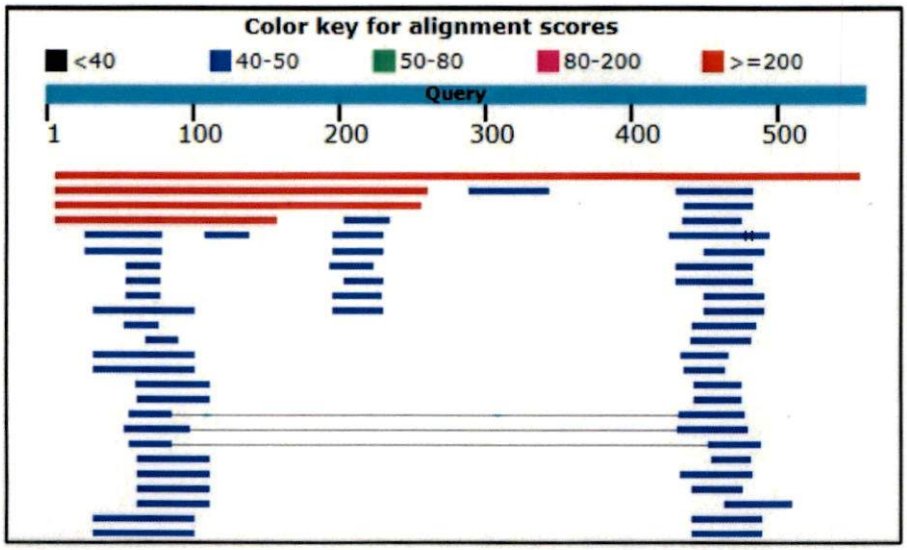
These genes include *viz.*, Regulatory protein NPR1, Probable disease resistance protein At1g12280, Mitogen-activated protein kinase (MAPK) 2, Phosphatase 2C 77, trans-membrane signaling protein genes, *etc.*, which is reported to be associated with disease resistance (Argout *et al.*, 2011; Belchí-Navarro *et al.*, 2013; Durian *et al.*, 2016). NPR1 is an Arabidopsis BTB/POZ domain protein that acts as a central mediator of the plant defense signal transduction pathway (Shi *et al.*, 2010). Arabidopsis MAPK3/MAPK6 signaling cascade had shown to regulate WRKY33 (Pecher *et al.*, 2014). The protein Phosphatase 2A (PP2A) has been identified as a crucial component that controls pathogenesis responses in various plant species by involving or regulating various immune pathways in plants (Durian *et al.*, 2016).

The marker UBC 826_{535} was bracketed by SSR markers mTcCIR136 and mTcCIR182 on chromosome six (Plate 26). This marker was linked with probable disease resistance protein At1g12290, Reticuline oxidase-like protein, Flavin-dependent oxidoreductase FOX1/2/5, *etc.* genes and their role in disease resistance was already described (Lanen and Zeier 2009; Daniel *et al.*, 2016). Reticuline oxidase (berberine bridge enzyme) catalyzes the formation of the berberine bridgehead carbon in the production of benzophenanthridine alkaloids which will accumulate in certain species of the Papaveraceae and Fumaraceae in response to pathogen attack (Dittrich and Kutchan, 1991). Belchí-Navarro *et al.*, (2013) examined expression of reticuline oxidase-like protein in *Vitis vinifera* involved in the modification of the cell wall architecture during cell culture growth and in the prevention of pathogen attack.

Location of marker 857_{839} on chromosome four was sandwiched by SSR marker mTcCIR183 and mTcCIR107 on the left while marker mTcCIR221 on the right side (Plate 27). This region is rich in disease associated genes like, Transcription factor MYB75, GDP-L-galactose phosphorylase 1, Serine/threonine-protein kinase CDL1, *etc.* Plant receptor like serine/threonine-protein kinase CDL1 plays an important role in plant signaling and defense (Afzal *et al.*, 2008). GDP-L-galactose phosphorylase 1 is a key regulator in ascorbic acid synthesis, which is acting as an antioxidant and provides plants with protection against a wide variety of biotic and abiotic environmental stresses (Conklin *et al.*, 2013). Expression of a GDP-L-galactose phosphorylase-like gene in a Chinese wild *Vitis* species induced responses to defense signaling molecules as reported by Hou *et al.*, (2013). Transcription factor MYB75 and MYB family regulates jasmonic

acid/salicylic acid immune pathway in plants and expression of defense related WRKY transcription factors (Schluttenhofer *et al.*, 2014).

To conclude, association of ISSR marker Oligo ISSR 04_{561} with cytokinin regulator Histidine-containing phosphotransfer proteins (HPts) need to be validated in relation to PPR resistance. ISSR markers UBC 810_{877}, UBC 826_{535} and UBC 857_{839} are found to be located in PPR resistance linked region of chromosome 9, 6 and 4 respectively. Moreover, these markers are surrounded by many major defense associated genes. Hence if validated, these markers will prove to be a strong tool to enable marker assisted selection for developing pod rot resistant varieties in cocoa.



A. Distribution of hits on query sequence

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Theobroma cacao genome assembly, chromosome: IX	895	895	98%	0.0	96%	LT594796.1
PREDICTED: Herrania umbratica histidine-containing phosphotransfer protein 1-like (LOC110412650), transcript variant X2, mRNA	365	365	45%	8e-98	92%	XM_021423242.1
PREDICTED: Herrania umbratica histidine-containing phosphotransfer protein 1-like (LOC110412650), transcript variant X1, mRNA	361	361	44%	1e-96	92%	XM_021423241.1
PREDICTED: Theobroma cacao histidine-containing phosphotransfer protein 1 (LOC18590364), mRNA	259	259	26%	9e-66	98%	XM_007015823.2
Cucumis melo genomic chromosome, chr_5	50.0	50.0	9%	0.008	81%	LN713256.1
Cucumis melo genomic scaffold, anchoredscaffold00003	50.0	50.0	9%	0.008	81%	LN681847.1
Solanum lycopersicum chromosome ch10, complete genome	46.4	90.9	9%	0.10	83%	HG975522.1
Solanum lycopersicum strain Heinz 1706 chromosome 10 clone hba-6c1 map 10, complete sequence	46.4	46.4	8%	0.10	83%	AC254783.3
PREDICTED: Sorghum bicolor zinc finger BED domain-containing protein RICESLEEPER 2 (LOC8082778), mRNA	44.6	44.6	9%	0.36	81%	XM_002442903.2
Arabidopsis thaliana Thioredoxin superfamily protein (GRX480), mRNA	44.6	44.6	4%	0.36	100%	NM_102616.2
Theobroma cacao genome assembly, chromosome: I	44.6	44.6	7%	0.36	85%	LT594788.1
Vigna angularis var. angularis DNA, chromosome 1, almost complete sequence, cultivar: Shumari	44.6	130	12%	0.36	81%	AP015034.1
Phanerophlebia nobilis voucher G. Yatskievych et al. 85_211 rps4-trnS intergenic spacer, partial sequence; chloroplast	44.6	44.6	7%	0.36	86%	KF020384.1

B. Sequence description

PREDICTED: Theobroma cacao histidine-containing phosphotransfer protein 1 (LOC18590364), mRNA

Sequence ID: XM_007015823.2 Length: 982 Number of Matches: 1

Range 1: 1 to 151 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
259 bits(286)	1e-65	148/151(98%)	0/151(0%)	Plus/Minus

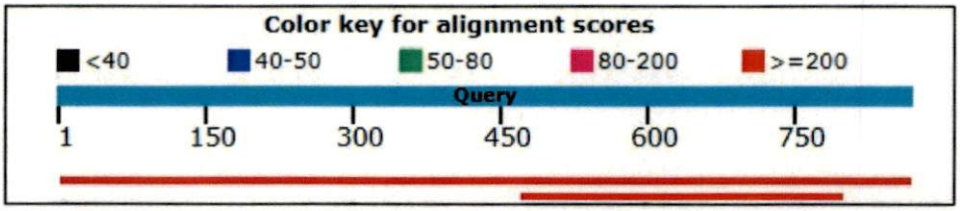
```
Query  8    acacacCACCAGTCAGAGGGTTGAGGAACTATGTAAGGGTATTAAGGATATATATACATG  67
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  151  ACACACCACCAGTCCGAGGGTTGAGGAACTATGTGAGGGTATTAAGGATAAATATACATG  92

Query  68   AAGAAAGAGAGAATCATACATGTACAAGCGTATAGGTGCAGAGACATTTGTGGTGAGAAT  127
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  91   AAGAAAGAGAGAATCATACATGTACAAGCGTATAGGTGCAGAGACATTTGTGGTGAGAAT  32

Query  128  GCTGAAATCAAAACTGGCAGCGTCGTCTTGG  158
            |||||||||||||||||||||||||||||||
Sbjct  31   GCTGAAATCAAAACTGGCAGCGTCGTCTTGG  1
```

C. Sequence alignment

Plate 18. BLASTn output of marker Oligo ISSR 04



A. Distribution of hits on query sequence

Sequences producing significant alignments:

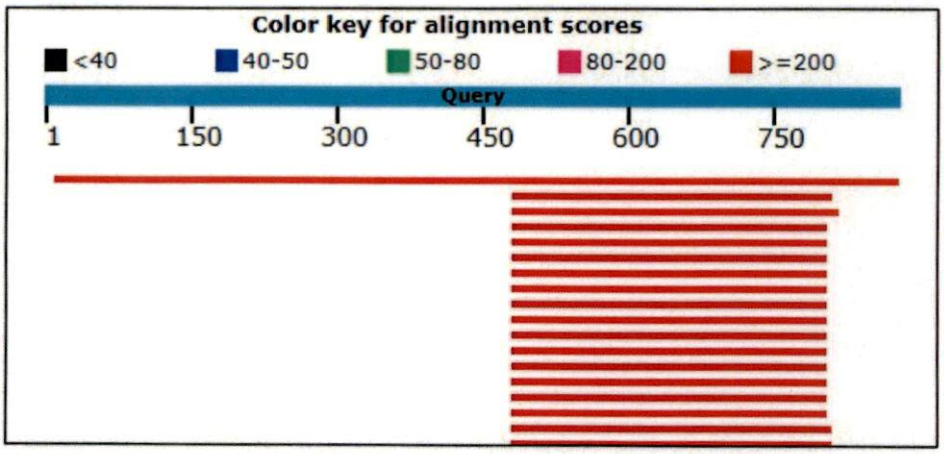
Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Theobroma cacao genome assembly, chromosome: IX	1519	1519	99%	0.0	99%	LT594796.1
PREDICTED: Theobroma cacao phospholipid:diacylglycerol acyltransferase 1 (LOC18588322), mRNA	574	574	37%	1e-160	99%	XM_018128234.1

B. Sequence alignment

Plate 19. BLASTn output of marker Oligo ISSR 08



A. Distribution of hits on query sequence

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Theobroma cacao genome assembly, chromosome: IX	1534	1534	98%	0.0	99%	LT594796.1
PREDICTED: Theobroma cacao phospholipid:diacylglycerol acyltransferase 1 (LOC18588322), mRNA	587	587	37%	2e-164	99%	XM_018128234.1
PREDICTED: Herrania umbratica phospholipid:diacylglycerol acyltransferase 1-like (LOC110410575), mRNA	535	535	38%	1e-148	95%	XM_021420341.1
PREDICTED: Gossypium raimondii phospholipid:diacylglycerol acyltransferase 1-like (LOC105772831), transcript variant X4, mRNA	417	417	36%	3e-113	88%	XM_012594301.1
PREDICTED: Gossypium raimondii phospholipid:diacylglycerol acyltransferase 1-like (LOC105772831), transcript variant X3, mRNA	417	417	36%	3e-113	88%	XM_012594300.1
PREDICTED: Gossypium raimondii phospholipid:diacylglycerol acyltransferase 1-like (LOC105772831), transcript variant X2, misc_RNA	417	417	36%	3e-113	88%	XR_001126893.1
PREDICTED: Gossypium raimondii phospholipid:diacylglycerol acyltransferase 1-like (LOC105772831), transcript variant X1, mRNA	417	417	36%	3e-113	88%	XM_012594299.1
PREDICTED: Gossypium hirsutum phospholipid:diacylglycerol acyltransferase 1-like (LOC107901080), transcript variant X4, mRNA	412	412	36%	1e-111	88%	XM_016826938.1
PREDICTED: Gossypium hirsutum phospholipid:diacylglycerol acyltransferase 1-like (LOC107901080), transcript variant X3, mRNA	412	412	36%	1e-111	88%	XM_016826937.1
PREDICTED: Gossypium hirsutum phospholipid:diacylglycerol acyltransferase 1-like (LOC107901080), transcript variant X2, mRNA	412	412	36%	1e-111	88%	XM_016826936.1
PREDICTED: Gossypium hirsutum phospholipid:diacylglycerol acyltransferase 1-like (LOC107901080), transcript variant X1, mRNA	412	412	36%	1e-111	88%	XM_016826935.1
PREDICTED: Gossypium arboreum phospholipid:diacylglycerol acyltransferase 1-like (LOC108474099), transcript variant X2, mRNA	399	399	36%	7e-108	87%	XM_017775869.1
PREDICTED: Gossypium arboreum phospholipid:diacylglycerol acyltransferase 1-like (LOC108474099), transcript variant X1, mRNA	399	399	36%	7e-108	87%	XM_017775868.1
PREDICTED: Gossypium hirsutum phospholipid:diacylglycerol acyltransferase 1-like (LOC107897707), transcript variant X3, mRNA	399	399	36%	7e-108	87%	XM_016823248.1

B. Sequence alignment

Plate 20. BLASTn output of marker UBC 810

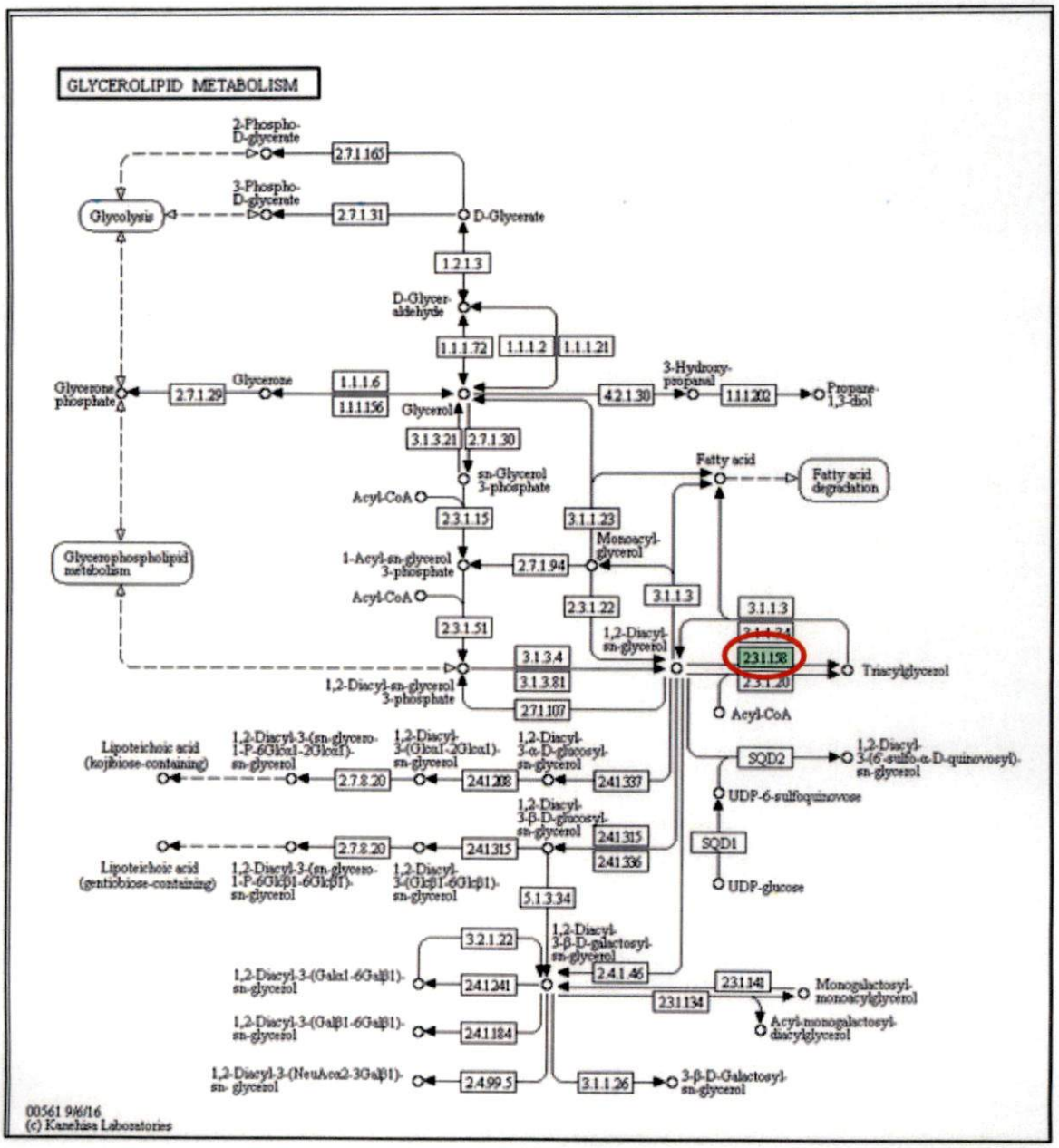
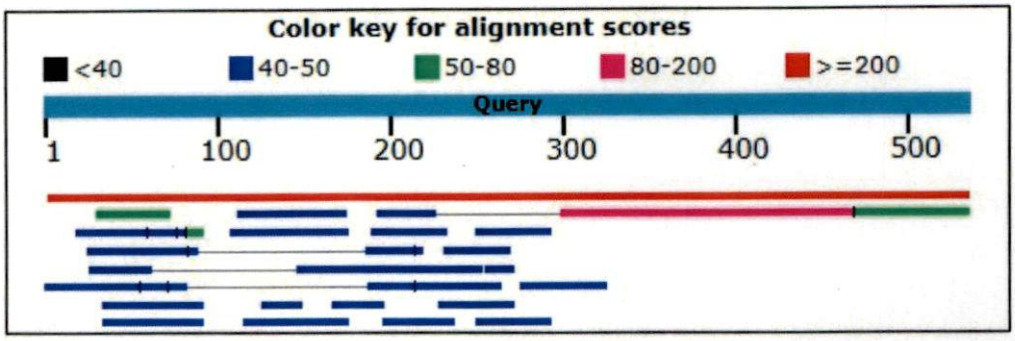


Plate 21. KASS analysis output of marker UBC 810



A. Distribution of hits on query sequence

Sequences producing significant alignments:

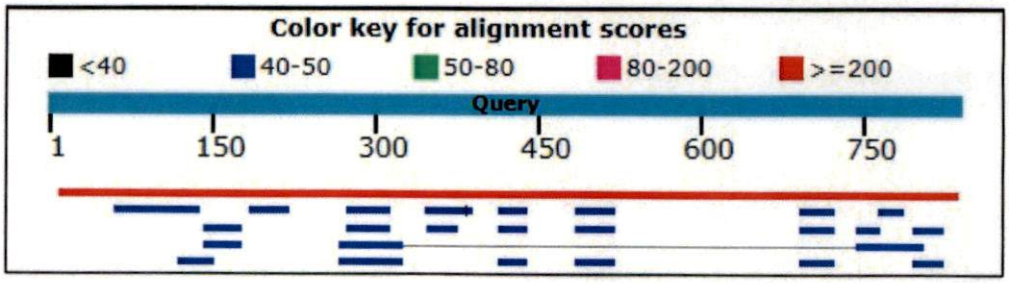
Select: All None Selected 0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Theobroma cacao genome assembly, chromosome: VI	901	984	99%	0.0	97%	LT594793.1
Theobroma cacao genome assembly, chromosome: IV	116	238	50%	7e-23	70%	LT594791.1
Lotus japonicus genomic DNA, chromosome 4, clone: LjT11L10: TM1734, complete sequence	60.8	60.8	8%	4e-06	91%	AP010347.1
Vigna angularis var. angularis DNA, chromosome 1, almost complete sequence, cultivar: Shumari	53.6	304	13%	7e-04	81%	AP015034.1
Vigna angularis var. angularis DNA, chromosome 4, almost complete sequence, cultivar: Shumari	50.0	215	18%	0.008	79%	AP015037.1
Vigna angularis var. angularis DNA, chromosome 9, almost complete sequence, cultivar: Shumari	50.0	133	26%	0.008	89%	AP015042.1
Vigna angularis var. angularis DNA, chromosome 5, almost complete sequence, cultivar: Shumari	50.0	301	29%	0.008	82%	AP015038.1
PREDICTED: Populus euphratica uncharacterized LOC105141971 (LOC105141971), transcript variant X4, mRNA	50.0	50.0	11%	0.008	81%	XM_011049399.1

B. Sequence alignment

Plate 22. BLASTn output of marker UBC 826



A. Distribution of hits on query sequence

Sequences producing significant alignments:

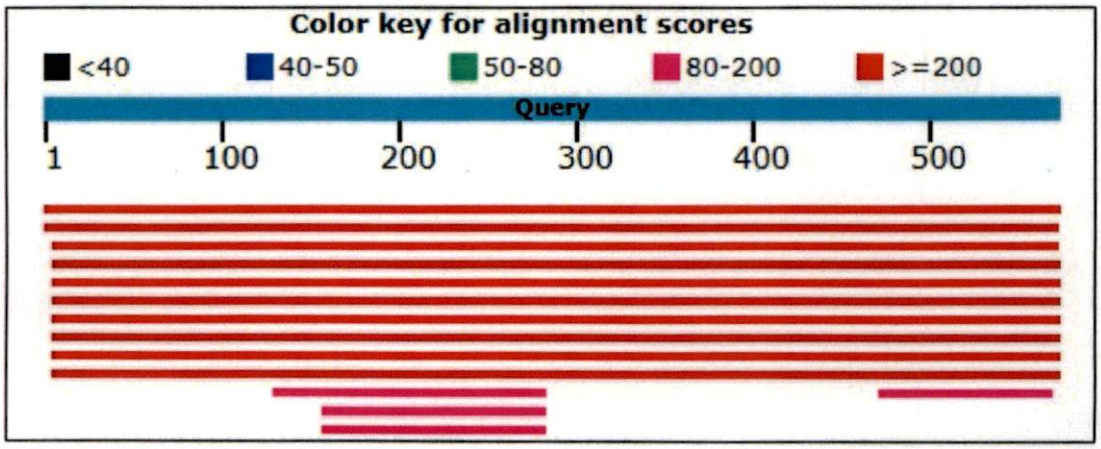
Select: All None Selected 0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Theobroma cacao genome assembly, chromosome: IV	1433	1540	98%	0.0	99%	LT594791.1
PREDICTED: Theobroma cacao WEB family protein At2g40480 (LOC18587409), transcript variant X2, mRNA	48.2	48.2	3%	0.045	94%	XM_018129618.1
PREDICTED: Theobroma cacao WEB family protein At2g40480 (LOC18587409), transcript variant X1, mRNA	48.2	48.2	3%	0.045	94%	XM_018129617.1
Theobroma cacao genome assembly, chromosome: X	48.2	48.2	3%	0.045	94%	LT594797.1
Theobroma cacao genome assembly, chromosome: VII	48.2	48.2	9%	0.045	73%	LT594794.1

B. Sequence alignment

Plate 23. BLASTn output of marker UBC 857



A. Distribution of hits on query sequence

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Theobroma cacao genome assembly, chromosome: V	989	69005	100%	0.0	98%	LT594792.1
Theobroma cacao genome assembly, chromosome: II	834	66340	100%	0.0	92%	LT594789.1
Theobroma cacao genome assembly, chromosome: X	832	40294	100%	0.0	93%	LT594797.1
Theobroma cacao genome assembly, chromosome: III	825	61933	100%	0.0	92%	LT594790.1
Theobroma cacao genome assembly, chromosome: I	825	46337	100%	0.0	92%	LT594788.1
Theobroma cacao genome assembly, chromosome: IX	816	29684	100%	0.0	92%	LT594796.1
Theobroma cacao genome assembly, chromosome: VI	816	29014	100%	0.0	92%	LT594793.1
Theobroma cacao genome assembly, chromosome: IV	807	56651	100%	0.0	92%	LT594791.1
Theobroma cacao genome assembly, chromosome: VII	785	28530	99%	0.0	91%	LT594794.1
Theobroma cacao genome assembly, chromosome: VIII	751	12695	99%	0.0	90%	LT594795.1
PREDICTED: Theobroma cacao uncharacterized LOC108661735 (LOC108661735), ncRNA	178	178	26%	3e-41	86%	XR_001927512.1

A. Sequence alignment

Plate 24. BLASTn output of marker UBC 827

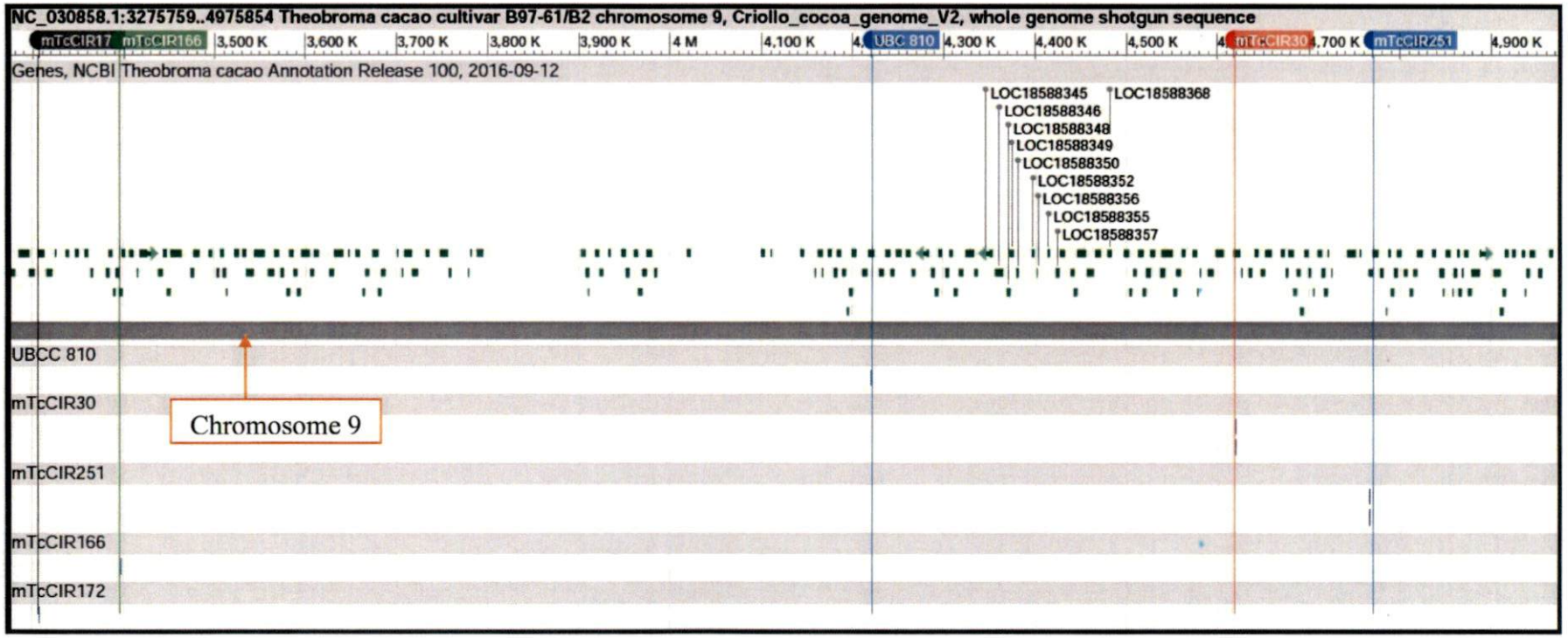


Plate 25. Location of marker UBC 810$_{877}$ on chromosome nine

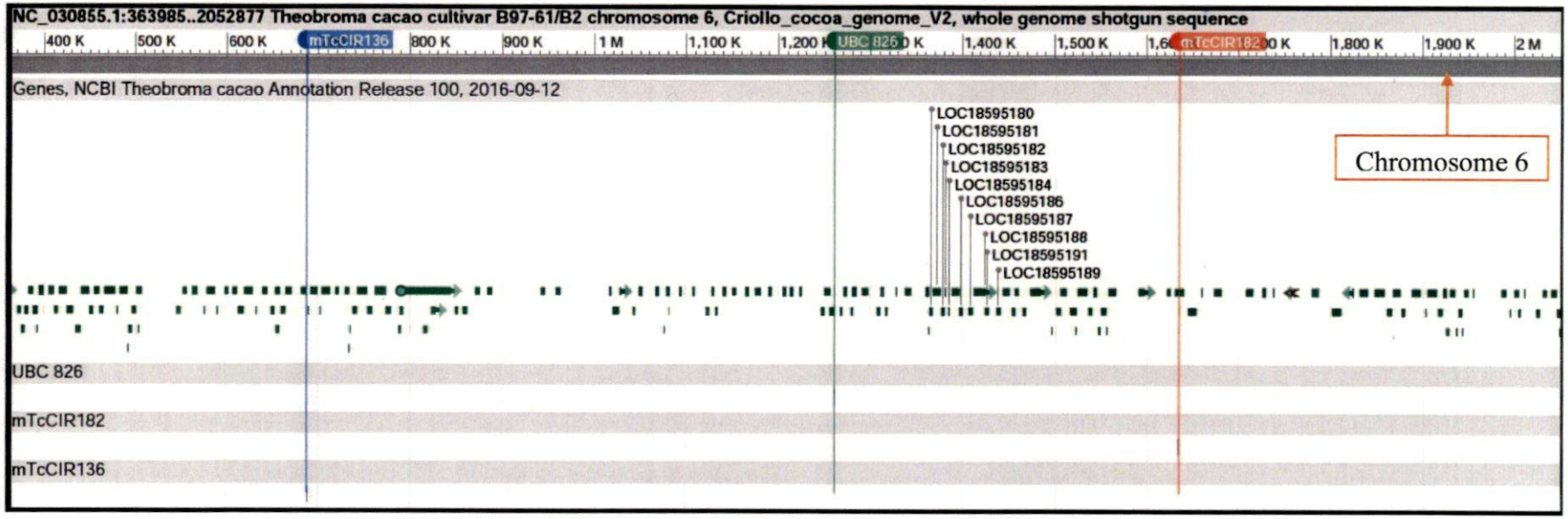


Plate 26. Location of marker UBC 826$_{535}$ on chromosome six

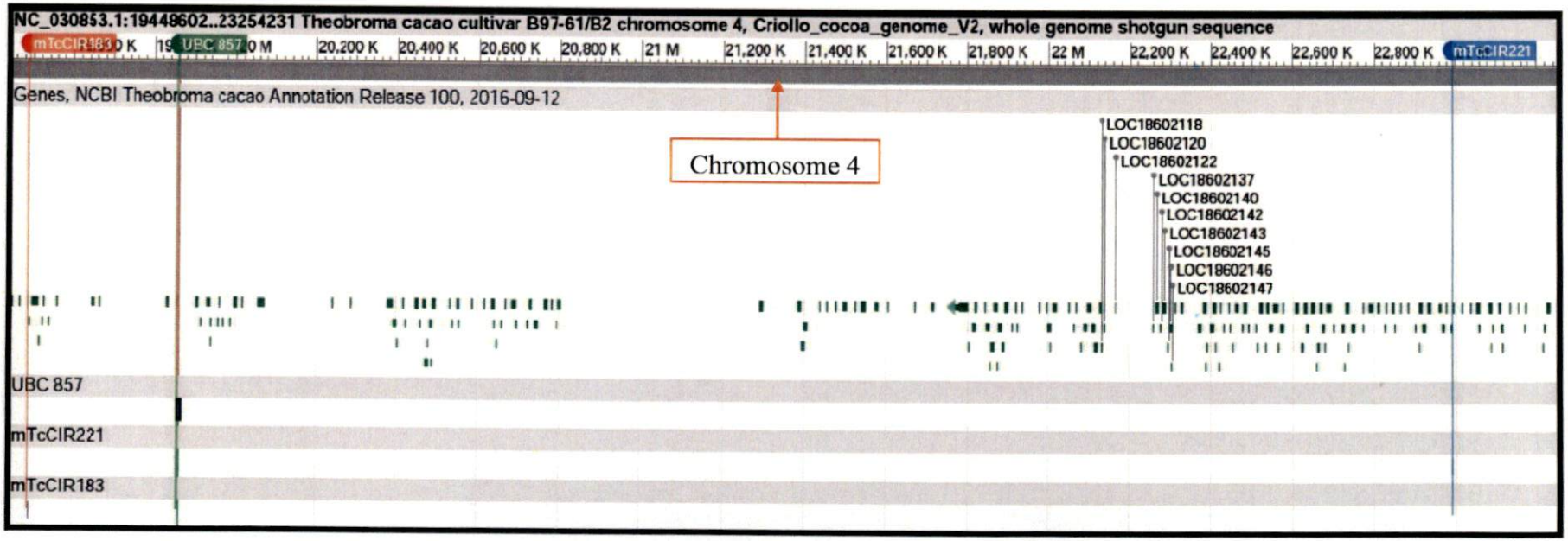


Plate 27. Location of marker UBC 857$_{839}$ on chromosome four

Summary

5. SUMMARY

The study entitled "Tagging of *Phytophthora* pod rot disease resistance gene in cocoa (*Theobroma cacao* L.) using ISSR markers" was carried out with an objective to develop a strategy to detect gene(s) for *Phytophthora* pod rot (PPR) resistance in cocoa. Hybrid progeny of SVI 1.26 × PII 12.11 along with their parents maintained at Cocoa Research Centre (CRC), Vellanikkara served as the material for this study. The experiments were conducted at Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture and CRC, Vellanikkara during the period 2016-2017.

The salient features of the study were as follows

1. Morphological characterization of 28 hybrids obtained from SVI 1.26 × PII 12.11 were carried out by recording five characters *viz*., pod weight, length and breadth of pod, wet bean weight per pod and single dry bean weight. High variability was observed for the characters among the progeny of same cross.

2. Pathogen was isolated from infected pod and used for screening resistance by detached pod inoculation technique. Based on the result, each of three resistance and susceptible hybrids were selected.

3. Modified CTAB method reported by Sujith (2016) with slight modification was standardized and practised for the extraction of good quality genomic DNA.

4. The quality of isolated DNA was evaluated by agarose gel electrophoresis. Quantification of isolated DNA was further done using the NanoDrop® ND 1000 spectrophotometer. The absorbance ratio was recorded in between 1.89 to 1.98.

5. The study was carried out using two types of markers ISSR (Inter Simple Sequence Repeats) and SSR (Simple Sequence Repeats). Under each marker system, DNA from all six selected hybrids and their parents was amplified using selected primers and polymorphic bands were identified.

6. Optimum PCR conditions and master mix for ISSR analysis was standardized.

7. Fifty ISSR primers were used in the initial screening to amplify the bulked genomic DNA. Based on the quality of amplification patterns, 27 primers were

selected for amplification with individual DNA of all eight accessions. In this screening polymorphism was observed in 11 ISSR primers. Further, their reproducibility was confirmed by repeating PCR amplifications for three time.

8. In the present study SSR assay was done with 15 primer sets. Though the various primer sets screened were found to yield polymorphism among the hybrids and moderately resistant parents at various levels, none of them was successful to give a clear distinction among the resistant and susceptible hybrids.

9. Six ISSR polymorphics *viz.*, UBC 810, UBC 826, UBC 827, UBC 857, Oligo ISSR 04 and Oligo ISSR 08 were eluted, cloned and recombinant plasmids were isolated. The nucleotide sequences obtained after sequencing were processed in VecScreen and BioEdit tool, and were analyzed using BLASTn.

10. BLASTn analysis of the sequence Oligo ISSR 04 had shown 96 per cent identity with *Theobroma cacao* genome assembly, chromosome: IX (LT594796.1), and 98 per cent with predicted: *T. cacao* histidine-containing phosphotransfer protein 1 (HPt). HPts also play an important role in propagating cytokinin signal transduction and so in disease resistance.

11. Marker Oligo ISSR 08 resulted in 99 per cent identity with *Theobroma cacao* genome assembly, chromosome: IX (LT594796.1).

12. The BLASTn analysis of marker UBC 810 also showed 99 per cent identity with *Theobroma cacao* genome assembly, chromosome: IX (LT594796.1). It also resulted in 99 per cent sequence identity with predicted *T. cacao* phospholipid: diacylglycerol acyltransferase 1 mRNA (XM_018128234.1).

13. When the marker UBC 826 sequence was analyzed on BLASTn, it showed 97 per cent identity with *Theobroma cacao* genome assembly, chromosome: VI (LT594793.1). Whereas, the marker sequence UBC 857 resulted in 99 per cent identity with *T. cacao* genome assembly, chromosome: IV (LT594791.1).

14. UBC 827 had shown 98 per cent identity with *Theobroma cacao* genome assembly, chromosome (LT594792.1) and 73 per cent with *T. cacao* clone TCC_BA049P20, complete sequence (JN127765.1).

15. ISSR markers UBC 810_{877}, UBC 826_{535} and UBC 857_{839} are located on chromosome nine, six and four respectively as inferred from NCBI Genome Data Viewer tool through BLASTn annotations. These marker locations were compared with already reported SSR markers in meta-QTL map for PPR resistance in cocoa.

16. Marker UBC 810_{877} is found to be located on the chromosome nine with SSR marker mTcCIR30 and mTcCIR166 present either side. Genes associated with this region are Regulatory protein NPR1, probable disease resistance protein At1g12280, Mitogen-activated protein kinase (MAPK) 2, Phosphatase 2C 77, trans-membrane signaling protein genes, *etc.*

17. The marker UBC 826_{535} was bracketed by SSR markers mTcCIR136 and mTcCIR182 on chromosome six. This marker was linked with probable disease resistance protein At1g12290, Reticuline oxidase-like protein, Flavin-dependent oxidoreductase FOX1/2/5, *etc.*

18. Location of marker 857_{839} on chromosome four was sandwiched by SSR marker mTcCIR183 and mTcCIR107 on the left while marker mTcCIR221 on the right side. This region is rich in disease associated genes like, Transcription factor MYB75, GDP-L-galactose phosphorylase 1, Serine/threonine-protein kinase CDL1, *etc.*

The future line of work includes validation, and exploitation of polymorphic amplicons or markers in response to PPR resistance. The linkage of Oligo ISSR 04_{561} with HPts has to be validated and understood the association and role of cytokinine with/in *Phytophthora* pod rot disease resistance. ISSR markers UBC 810_{877}, UBC 826_{535} and UBC 857_{839} are found to be located in PPR resistance region rich in many major defense associated genes. Hence if validated, these markers will prove to be a strong tool to enable marker assisted selection for developing pod rot resistant varieties in cocoa. In addition more ISSR and SSR primers have to be screened to identify suitable ones that tag PPR resistance gene/s.

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Annexures

ANNEXURE I

1. 5X extraction buffer (100 ml)

CTAB	:	5 g
Tris base	:	1.21 g
EDTA	:	0.745 g
NaCl	:	8.18 g
PVP	:	1.0 g

Adjusted the pH to 8 and made final volume up to 100 ml.

2. Chloroform- Isoamyl alcohol (24:1 v/v)

To chloroform (24 parts), isoamyl alcohol (1 part) was added and mixed properly.

3. Chilled isopropanol

Isopropanol was stored in refrigerator at 0 °C and was used for the study.

4. Ethanol (70 %)

Ethyl alcohol 70 parts of absolute ethanol (100 %), 30 parts of sterile distilled water was added to make 70 per cent ethanol.

ANNEXURE II

Composition of buffers and dyes used for gel electrophoresis

1. TAE buffer (50X)

Tris base	:	242 g
0.5 *M* EDTA (pH 8.0)	:	100 ml

2. Loading dye (6X)

0.25 per cent bromophenol blue

0.25 per cent xylene cyanol

30 per cent glycerol in water

3. Ethidium bromide

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

ANNEXURE III

1. X-Gal stock solution (20 mg/mL)

200 mg X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was dissolved in 10 ml N, N-dimethylformamide, stored at -20 °C in the dark.

2. IPTG stock solution (100 *mM*)

1.2 g IPTG (isopropyl-β-D-thiogalactopyranoside) was dissolve in 50 ml deionized water then filter-sterilized, aliquot and stored at 4 °C.

TAGGING OF *PHYTOPHTHORA* POD ROT DISEASE RESISTANCE GENE IN COCOA (*Theobroma cacao* L.) USING ISSR MARKERS

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(2015-11-116)

ABSTRACT OF THE THESIS

Submitted in partial fulfilment of the requirement for the degree of

Master of Science in Agriculture

(PLANT BIOTECHNOLOGY)

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2017

ABSTRACT

Cocoa (*Theobroma cacao* L.) known as 'Chocolate tree', is a major cash crop in tropical countries. Cocoa production is seriously affected by pod rot diseases caused by many *Phytophthora* species. Among these, the pod rot caused by *Phytophthora palmivora* has been reported in India. Yearly losses to the cocoa growers around the world from *Phytophthora* diseases were assessed at 30 per cent of the total yield loss. Disease resistance can be scored using a number of morphological and physiological characters. However, the morpho-physiological characters greatly depend upon the environment which ultimately affect the experimental data. Hence, confirmation of transfer of genes by tagging with the help of a strong tool is of utmost importance in crop breeding. Molecular markers such as Inter simple sequence repeats (ISSRs) have already proven to be a good tool to detect and tag the genes of interest and will help to reduce the breeding cycle. In this context, the present study was taken up with an objective to develop a strategy to tag gene(s) for *Phytophthora* pod rot (PPR) resistance in cocoa using ISSR markers.

Morphological characterization of 28 hybrid progenies of SVI 1.26 × PII 12.11 was carried out by recording five pod and bean characters. High variability was observed for characters *viz*., pod weight, pod length and breadth, wet bean weight per pod and single dry bean weight among the progeny of the same cross. Detached pod inoculation technique was adopted to classify the hybrids into resistant and susceptible ones. The wide variability was also recorded for disease reaction among the progenies. Based on the resistance score, three resistant and three susceptible hybrids were selected from the segregating progeny.

The eight accessions were screened with fifty ISSR and 15 SSR primers to observe polymorphism between resistance and susceptible genotypes. Polymorphism was observed in 11 ISSR primers and from these, six primers *viz*., UBC 810, UBC 826, UBC 827, UBC 857, Oligo ISSR 04 and Oligo ISSR 08 were eluted and cloned. Plasmid DNA was isolated from clones and sequenced. Though various SSR primer sets screened were found to yield polymorphism, none of them was successful to give a clear distinction among the resistant and susceptible hybrids. This may be due to the fact that,

Quantitative trait loci (QTLs) associated with these reported SSR primers may be absent in the genotypes considered for the study.

BLASTn analysis specific to plants was done for all six sequences. Upon analysis, Oligo ISSR 04_{561} had shown 98 per cent identity with Predicted: *T. cacao* histidine-containing phosphotransfer protein 1 (HPt). HPts play an important role in propagating cytokinin signal transduction. Cytokinins are instrumental in mediating disease resistance by generating a green island around the infection zones, exhibiting delayed leaf senescence and upregulating the expression of the pathogenesis related (PR) gene/s. In addition to this, the auxin-cytokinin antagonism that occurs as part of a complex hormonal interplay, exerts a critical influence on the core SA-JA/ET plant immunity pathways. The BLASTn analysis of marker UBC 810_{877} resulted in 99 per cent sequence identity with Predicted: *T. cacao* phospholipid: diacylglycerol acyltransferase (PDAT) 1 mRNA. This protein regulates the synthesis of triacylglycerol, which is a building component of oils in the plant. Accumulation of oil content in plant cells could impart resistance against the pathogen. UBC 827_{571} had shown 73 per cent sequence identity with *T. cacao* clone TCC_BA049P20 complete sequence and it is reported to be QTL rich region associated with different traits of *T. cacao.*

Moreover, ISSR markers UBC 810_{877}, UBC 826_{535} and UBC 857_{839} are located on chromosome nine, six and four respectively as inferred from NCBI Genome Data Viewer tool through BLASTn annotations. These markers are found to be located in PPR resistance regions rich in defense associated genes.

Further validation and exploitation of polymorphic amplicons or markers in response to PPR would be required. The linkage of Oligo ISSR 04_{561} and UBC 810_{877} with HPts and PDAT correspondingly have to be validated to elucidate the association and role of cytokinin and triacylglycerol with PPR disease resistance. If validated, UBC 810_{877}, UBC 826_{535} and UBC 857_{839} and Oligo ISSR 04_{561} could be employed as a marker in PPR resistance breeding programmes in cocoa.