# INHERITANCE OF MOLECULAR MARKERS LINKED TO VASCULAR STREAK DIEBACK DISEASE RESISTANCE IN HYBRID PROGENIES OF COCOA (*Theobroma cacao* L.)

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**KERALA, INDIA**
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**KERALA, INDIA**
**2019**

## DECLARATION

I, hereby declare that the thesis entitled **"Inheritance of molecular markers linked to vascular streak dieback disease resistance in hybrid progenies of cocoa (*Theobroma cacao* L.)"** is a bonafide record of research work done by me during the course of research and that it has not been previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title, of any other University or Society.

Vellanikkara **Midhuna M. R.**

Date: __ /__ /2019 (2016-11-106)

## CERTIFICATE

Certified that the thesis entitled **"Inheritance of molecular markers linked to vascular streak dieback disease resistance in hybrid progenies of cocoa (*Theobroma cacao* L.)"** is a bonafide record of research work done independently by **Mrs. Midhuna M. R. (2016-11-106)** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, associateship or fellowship to her.

Vellanikkara

Date: __ /__ /2019

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## CERTIFICATE

We, the undersigned members of the advisory committee of **Mrs. Midhuna M. R. (2016-11-106)**, a candidate for the degree of **Master of Science in Agriculture** with major field in **Plant Biotechnology**, agree that the thesis entitled **"Inheritance of molecular markers linked to vascular streak dieback disease resistance in hybrid progenies of cocoa (*Theobroma cacao* L.)"** may be submitted by **Mrs. Midhuna M. R.,** in partial fulfillment of the requirement for the degree.

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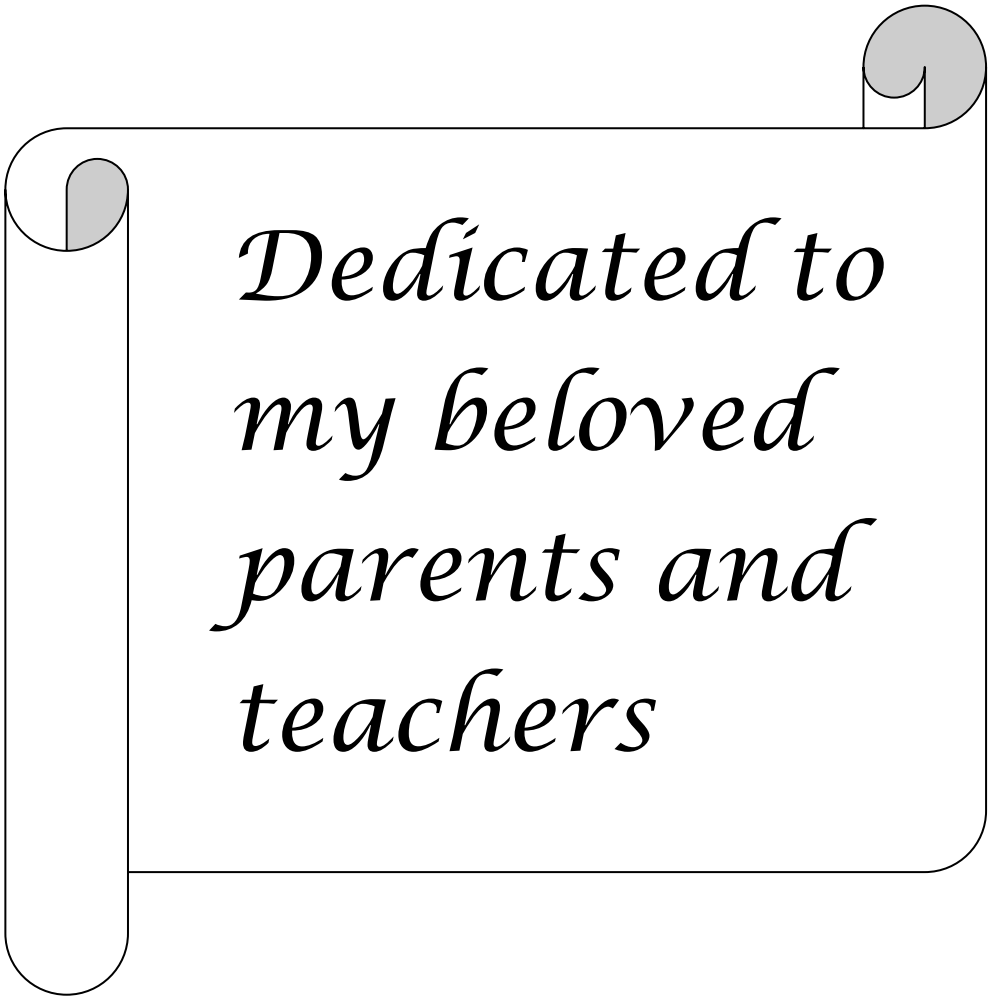
Dedicated to
my beloved
parents and
teachers

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# ABBREVIATIONS

| | |
|---|---|
| % | Percentage |
| = | Equal to |
| µg | Microgram |
| µL | Microlitre |
| AFLP | Amplified Fragment Length Polymorphism |
| PCR | Polymerase Chain Reaction |
| AP-PCR | Arbitrarily Primed Polymerase Chain Reaction |
| BLAST | Basic Local Alignment Search Tool |
| bp | Base pair |
| cm | Centimetre |
| h | Hours |
| CTAB | Cetyl Trimethyl Ammonium Bromide |
| NaCl | Sodium Chloride |
| SDS | Sodium Dodecyl Sulphate |
| $MgCl_2$ | Magnesium Chloride |
| NaOAC | Sodium Acetate |
| KOAC | Pottassium Acetate |
| DNA | Deoxyribonucleic Acid |
| DNase | Deoxyribonuclease |
| dNTPs | Deoxyribo Nucleoside Triphosphate |
| EDTA | Ethylene Diamine Tetra Acetic acid |
| EST | Expressed sequence tags |
| g | Gram |
| ha | Hectare |
| Kb | Kilo base pairs |
| L | Litre |
| M | Molar |

| | |
|---|---|
| MAS | Marker Assisted Selection |
| mg | Milligram |
| mL | Millilitre |
| mM | Milli mole |
| ng | Nanogram |
| $^{o}$C | Degree Celsius |
| OD | Optical Density |
| pH | Hydrogen ion concentration |
| pM | Pico Mole |
| PVP | Poly vinyl pyrolidone |
| QTL | Quantitative Trait loci |
| RNA | Ribonucleic acid |
| RNase | Ribonuclease |
| rpm | Revolutions per minute |
| SCAR | Sequence Characterized Amplified Region |
| TAE | Tris Acetate EDTA |
| TE | Tris EDTA |
| U | Unit |
| UBC | University of British Columbia |
| UV | Ultra violet |
| V | Volts |
| β | Beta |
| VSD | Vascular streak dieback |

# INTRODUCTION

# 1. INTRODUCTION

*Theobroma cacao* L. (also known as the chocolate tree) is the costliest beverage crop. It is native to the Amazon region of South America and northern parts of Central America (Wood and Lass, 1985). Cocoa is a diploid fruit tree species (2n= 20), belonging to the family *Malvaceae* (Alverson *et al*., 1999), having a small genome with the size of 390 Mb (Couch *et al*, 1993).

Cocoa was introduced to India during 20$^{th}$ century (Ratnam, 1961) and now it is widely cultivated as an intercrop/mixed crop under coconut, arecanut and oil palm in Kerala, Karnataka, Andhra Pradesh and Tamil Nadu. Humid climatic conditions of Kerala favours increased occurrence of various diseases and pests which act as a serious limitation to the commercial cocoa cultivation in Kerala. Vascular Streak Dieback (VSD) caused by the fungus *Ceratobasidium theobromae* (Samuels *et al*., 2012) is a serious disease in cocoa. Occurrence of this disease was first reported by Keane (1972) and Prior (1979). Since then, this disease has been reported from many South East Asian countries. VSD was first noticed in India in the districts of Pathanamthitta, Kottayam, Idukki and Kozhikode in Kerala (Abraham, 1981; Chandramohan and Kaveriappa, 1982). After a meticulous survey, Abraham and Ravi (1991) observed that this disease was spreading very fast in all the cocoa cultivated areas of Kerala. However the disease is now under control due to systemic resistance breeding programme under taken by Kerala Agricultural University.

Cocoa is the main host of the VSD pathogen and the initial symptoms of infection include green spotted chlorosis and fall of leaves beginning on the second or third flush behind the stem apex. However, the pathogen has not been reported to sporulate in axenic culture. Hence, screening for resistance has to be done by exposing the test plants to natural inoculum. The management strategies advocated are cutting and removing all infected twigs and pruning off the affected branches 30 cm below the last point of visible symptom of the disease.

Screening for the disease in the field by using conventional techniques is highly influenced by environmental factors. Moreover, cocoa being a perennial tree, has a long breeding cycle, molecular marker techniques play a significant role in accelerating the breeding programmes (Figueira, 2004). Molecular markers are specific regions on the chromosomes near the target gene and they help in the early identification of the desirable genotypes. Therefore, marker assisted selection for the confirmation of transfer of desired gene in the offspring is most reliable (Salimath *et al*., 1995).

Simple Sequence Repeats (SSR) markers are co-dominant, reproducible and are highly polymorphic and have been used widely in many genetic studies of cocoa including identification of Quantitative Trait Loci (QTL) and genetic mapping (Queiroz *et al*., 2003; Brown *et al*., 2005). Inter Simple Sequence Repeats (ISSR) markers have been used widely to analyze the diversity within and between plant populations in gymnosperms and angiosperms (Osborn *et al*., 2005).

Cocoa Research Centre (CRC), Kerala Agricultural University (KAU), India, had developed about five thousand nine hundred and twenty one hybrids exhibiting various levels of resistance for vascular streak dieback disease and their field performance has been evaluated for the past one and a half decade. The clones GVI 55, GVI 18.5 and GVI 126 were the best source of resistance for VSD with maximum recovery of good hybrids among the parents used in the crossing programme (Minimol and Amma, 2013). A total of two hundred and sixty seven hybrids were found free from VSD and out of that, fifty one hybrids were found to have satisfactory yield levels. In view of all the parameters included for evaluation of performance of the hybrids, VSD I 31.8 (CCRP 15) was ranked as a novel hybrid released first in the world with VSD resistance and considerable yield (Minimol *et al*., 2018).

Five ISSR markers *viz*., UBC 811, UBC 815, UBC 826, UBC 857 and UBC 866 and one SSR marker mTcCIR42, were identified to be linked with VSD

resistance (Chandrakant, 2014) in a study conducted at Kerala Agricultural University. These identified ISSR and SSR markers were validated and it was found that the ISSR markers UBC 811, UBC 815 and UBC 857 and one SSR marker mTcCIR42 were linked with VSD resistance (Tulshiram, 2016).

In line with the above, the current research work aimed to study the inheritance of identified ISSR and SSR markers linked to vascular streak dieback resistance in hybrid progeny of cocoa.

# REVIEW OF LITERATURE

# 2. REVIEW OF LITERATURE

The present study on 'Inheritance of molecular markers linked to vascular streak dieback disease resistance in hybrid progenies of cocoa (*Theobroma cacao* L.)' had been carried out to validate ISSR and SSR markers linked to VSD tolerance. In this chapter, the relevant literatures obtainable on distinct aspects of this study were compiled and reviewed under different headings.

## 2.1 Introduction

### 2.1.1 Origin of cocoa

Cocoa (*Theobroma cacao* L.), also known as the chocolate tree, is an important tropical tree species native to the Amazon region of South America and northern parts of Central America (Wood and Lass, 1985). Previously, cocoa was classified under the family *Sterculiaceae.* Alverson *et al*. (1999) congregated cocoa in the family *Malvaceae.* During the sixteenth century, Europeans were the first to embrace cocoa cultivation in Asia and Africa. Evidences of cocoa plantations in Central America dated back to 2000- 4000 years before Spanish contact has been unveiled in the archaeological records (Bergman, 1969; Henderson *et al*., 2007; Powis *et al*., 2011). These plantations were then spread to other localities in South America and the Caribbean from Mexico (Wood and Lass, 1985). Worldwide, cocoa is cultivated on more than five million hectares of tropical land (Krauss and Soberanis, 2001). First commercial cultivation of cocoa began after the Portuguese introduced cocoa to Africa in 1822 (Bartley, 2005).

Cocoa was introduced to India in the $20^{th}$ century by the British, from the island of Amboina (Ratnam, 1961). Cocoa cultivation is done as a part of agro-ecosystems expansion and thus making it available to the farmers benefits in the form of economic and ecological terms (Wood and Lass, 1985). Currently, in India, it is extensively cultivated as an intercrop/mixed crop under coconut, arecanut and oil palm in the states of Kerala, Andhra Pradesh, Karnataka and

Tamil Nadu with the total area of 88,515 ha and an average productivity of 580 kg/ha (DCCD, 2017).

### 2.1.2 Importance of cocoa

Cocoa is one of the most important tropical crop and has been described as a 'virtuous' crop. There is an increasing appreciation of its value for provision of sustainable incomes in less developed regions. According to Ruf and Zadi (2003), the ecological importance of cocoa includes conservation of soil and watershed, boosting up of biodiversity, owing to its perennial cropping cycle of more than fifty years. Other than the normal land conservation, varieties of flora and fauna are also observed in cocoa agroforest system (Schroth and Harvey, 2007).

According to Guiltinan *et al*. (2008), around ninety five per cent of the world's cocoa production is done by five to six million small holder farmers. Beans or seeds of cocoa is an important trade commodity worldwide, serving as the raw material for chocolate production (George, 2013). Now a days, cocoa cultivation around the tropics is common in agroforestry systems co-jointly with other cash crops.

## 2.2 Botany of cocoa

Cocoa is a diploid fruit tree species (2n=20) with ten sets of chromosomes and small genome size ranging from 390 Mb to 415 Mb (Figueira *et al*., 1992). Cocoa is cultivated under shade canopies. In his first edition of the book *Species Plantarum*, Carolus Linnaeus assigned botanical nomenclature *Theobroma cacao* to cocoa.

Cocoa exhibits cauliflory, *ie*., the flowers are produced on the trunk and on the branches. Flowers are produced under favourable conditions, on wood usually of two to three years old. Flowers are composed of five sepals, five petals, ten

stamens (five sterile staminodes and five fertile stamens) and an ovary of five united carpels. Androecium are arranged in two whorls, an outer whorl and an inner whorl, with an outer whorl consists of five long sterile staminodes and inner whorl consists of five fertile stamens. Petals have pouches in which anthers of the corresponding stamen lies. The ovary contains many ovules arranged around a central axis. There is a considerable variation in the size and colour of the flowers between different cultivars (Wood and Lass, 1985). Usually, the flowers are cream with darker tissue on staminodes and petals.

Matured fruit of cocoa, generally called as pod, differs in shape from narrow and elongated to spherical. The seeds or beans in the pods also differs in colour, varying from purple, brown, pink or white (Bartley, 2005). The colour of the pods also varies in both immature stage and mature ripened stage. It is green to reddish colour, when immature and yellow to pinkish in colour, when ripened. According to Thi *et al*. (2016), leaf, flower, pod and bean characters which are the agro-morphological characters can be utilized in analyzing the genetic distance and in demonstrating the population structure in cocoa.

Depending upon the genotype and on the natural conditions, cocoa trees produce large number of flowers at certain times of the year, however only 1-5 per cent of these flowers will be successfully pollinated to form cocoa pods. Higher proportions of pod formations are recorded for Amelonado type (Posnette and Entwistle, 1958). Pollination is carried out by many small insects, the most important among them being the midges which belongs to the family *Ceratopogonidae* (Wood and Lass, 1985). Like self incompatibility, cross incompatibility has also been reported in cocoa and the reason for this is the identical genetic constitution among the genotypes (Mallika *et al*., 2002) According to Guiltinan *et al*. (2008), the methodologies of breeding and examination of genetic makeup is difficult because most of the genotypes of cocoa are self incompatible. More than 90 per cent germination has been reported in cocoa under normal conditions (Amma *et al*., 2009). Since, the seeds of cocoa

are recalcitrant, conservation of germplasm is difficult and conservation must be done by planting in field.

### 2.3 Diseases of cocoa

Unlike many exotic crops, cocoa proves to be highly susceptible to major diseases as well as pests. Hence, sustainability of cocoa is under serious threat from both coevolved and newly encountered diseases (Evans, 2007). Global crop loss due to various diseases annually is almost 30 per cent, with some farms experiencing almost cent per cent loss of crop (Keane, 1992; Bowers *et al*., 2001). Major diseases of cocoa include *Phytophthora* pod rot, Witches' broom disease, Frosty pod rot, Swollen shoot virus, Vascular streak dieback etc. Details of major diseases of cocoa and the global crop loss caused by them are furnished in the Table 1.

**Table 1. Major diseases of cocoa and global crop losses**

| Sl No. | Disease | Causal organism | Region | Potential crop loss (tonnes) |
|---|---|---|---|---|
| 1 | Pod rot | *Phytophthora spp.* (ex. *P. palmivora*) | Ubiquitous | 4,50,000 |
| 2 | Witches' broom | *Crinipelli sperniciosa* | Latin America | 2,50,000 |
| 3 | Frosty pod rot | *Moniliophthora roreri* | Latin America | 30,000 |
| 4 | Swollen shoot virus | *Cocoa Swollen Shoot Virus* (CSSV) | West Africa | 50,000 |
| 5 | Vascular streak dieback | *Ceratobasidium theobromae* | South East Asia | 30,000 |

(http://www.dropdata.org/cocoa/cocoa_prob.htm#severity, 12/03/2019)

## 2.4 Cocoa dieback

According to Lass (1985), dieback refers to the general condition where the cocoa tree suffers from progressive desiccation of the branches from the growing tip towards downside. Dieback can be caused by either physiological or pathological factors or even both. There are several causes of cocoa dieback, including water stress or drought, lack of shade, strong or dry winds, and nutritional problems, particularly toxicity or deficiency of micronutrients.

Drover (1970) found that a nutrient imbalance was involved in cocoa dieback in Papua New Guinea. Severe attacks by insects could also cause dieback (Lass, 1985). *Verticillium* wilt and *Oncobasidium theobromae* pathogens have also been reported to cause dieback symptoms in Uganda and South East Asia (Lass, 1985).

## 2.5 Vascular Streak Dieback (VSD) disease of cocoa

Vascular streak dieback is a major devastating disease of cocoa and the global crop loss caused by this disease is inevitable. Widespread incidence of vascular streak dieback disease in 37 cocoa growing countries in Asia, Africa, Central America and South America was reported by Turner (1967).

The devastating dieback disease was first recorded in Papua New Guinea in the 1960s when many trees were killed due to the infection and it was not possible to replant the fields since the outbreak also killed the young cocoa plants (Shaw, 1963; Bridgland *et al*.,1966). This disease was alluded as vascular streak dieback by Keane (1972). The disease has caused major economic losses to cocoa productivity in Melanesia and some part of Asia, including New Britain, China, Thailand, India, Malaysia, Vietnam, Burma, Philippines, and Indonesia (Prior 1980; Efron *et al*., 2002; Guest and Keane, 2007). In Indonesia, VSD has been

reported in Kalimantan, Java, Maluku, Sulawesi, Papua, and Sumatra Islands since the early 1980s. However, the severity of the disease varied between locations, with cocoa in some areas remaining healthy, while plants in other areas were highly infected (Pawirosoemardjo *et al*., 1990).

In India, initially this disease was reported in the Kottayam district of Kerala by Abraham (1981) and Chandramohan and Kaveriappa (1982). Further spread of this disease was reported from Kottayam to the other districts of Kerala like Thiruvananthapuram, Kozhikode, Pathanamthitta, Ernakulam and Idukki (Abraham and Ravi, 1991). A survey conducted in 1993 had reported the occurrence of this disease in Thrissur district of Kerala as well (KAU,1995).

### 2.5.1 The pathogen

Fungus causing VSD of cocoa had been first described as a new genus and species of *Basidiomycotina* by Talbot and Keane (1971). They morphologically characterized it as white and membraneous fruiting bodies occurring as small adherent patches on leaf scars with mycelia emerging from xylem vessels. Hyphae are thin walled, firm and smooth without clamp connections. Basidiospores are smooth, hyaline, thin walled and broad ellipsoid with one side flattened. Conidial and sclerotial states are not known to occur. This fungus was named as *Oncobasidium theobromae*.

Samuels *et al*. (2012) studied the DNA structure of the pathogen and identified the fungus as *Ceratobasidium theobromae*. The only known host for *C. theobromae* is *Theobroma cacao*, although avocado was reported as a host and presented similar disease symptoms to those found on cocoa (Anderson, 1989; Dennis, 1991).

Many studies have been conducted to analyse the morphological characters of the causal organism of VSD occurring in Indonesia and Malaysia (Ahmad,

1982; Lam *et al*., 1988 and Pawirosoemardjo *et al*., 1990). Only few basidia will be visible as it collapses immediately after spore shedding (Keane and Prior, 1992).

### 2.5.2 Disease symptoms

Symptamology for vascular streak dieback was first described by Shaw (1963). Later, detailed description of the symptoms were given by Keane *et al.* (1972), Keane and Prior (1991) and Abraham and Ravi (1991). The first sign of VSD is chlorosis in the second or third leaf from the growing tip which appear as green spotted islets with a yellow background. A different pattern of leaf symptom is observed in some regions where it appears as necrosis on the edge of the leaf. A few days after the chlorosis appear, the leaf falls off and the symptoms then appear on other nearby leaves on the same branch. Leaves in the latest flush of the seedlings affected by the disease often show interveinal necrosis. The leaf fall occurs right up to the growing tip and will eventually result in the death of the whole plant.

Symptoms showing three dots on the leaf scars are observed when the leaf has fallen. When most of the leaves have fallen, the lenticels expand which result in a coarser surface on the bark. Necrosis along the xylem is seen when the branch is cut vertically. The other branches that develop from the axil of this infected twig are also affected by the disease.

After a five month period from the first sign of chlorosis, the disease spreads to the twigs and causes the death of the branches. When the disease develops on the trunk, death of the tree is inevitable. According to Keane (1981), severe damage is caused when the pathogen infects young cocoa trees up to 10 months old. In this situation, fungal infection will reach the main stem and cause mortality of the young trees. White, effused fruiting bodies of the fungus will cover the leaf scars generated by the abscised leaves. These fruiting bodies will be

seen only on the leaf scar and the adjacent bark. Xylem discolouration is observed in the form of a brown streak, when the bark of the diseased stem is split in to two.

Occurrence of the symptoms of the disease from Papua New Guinea, India, Malaysia and Indonesia has been detailed by many scientists (Prior, 1980; Abraham, 1981, Ahmad, 1982 and Abraham and Ravi, 1991). In Malaysia, occurrence of interveinal necrosis was more common compared to the yellow leaves with green islets as that in Papua New Guinea (Wood and Lass, 1985).

### 2.5.3 Disease spread and infection

When an infected leaf falls during wet weather, hyphae may emerge from the leaf scar and develop into a basidiocarp. This structure is visible as a white, flat, velvety coating over the leaf scar and adjacent bark (Keane *et al*., 1972). Keane (1981) studied the epidemiology of VSD disease and found a correlation between basidiospore production and rainfall. Basidiospores produced by the diseased plants increased with increasing monthly rainfall. Basidia or sporulation occurs on the basidiocarp after evening rainfall. Formation and forcible discharge of basidiospores occurs mainly after midnight into the early morning.

Basidiospores germinate and hyphae penetrate tender leaves at branch termini by growing directly through the cuticle, above leaf veins (Prior, 1979). It is believed that the germination requires free water and high humidity (95% or more); unless these conditions prevail, successful infection is relatively low. Infected leaves do not show symptoms for three to five months, by which time the pathogen has ramified through the xylem in to the adjacent stem.

Basidiospores only remain viable for a few hours in the morning. Direct or indirect exposure to sunlight eventually kills the spores, thus infection must start during the night when the spores are released from the basidia. Extended periods

of wetness are required for basidiocarp formation, basidiospore release, and infection of leaves. Thus, there is a critical link between rainfall peaks and infection periods (Keane, 1981).

Dennis and Keane (1992) revealed that the release of spores is affected by a period of wetness rather than the amount of rainfall. In their study, it was observed that heavy dew deposition in the absence of rainfall could maximize sporulation when compared with late afternoon rainfall.

### 2.5.4 Management of VSD disease

The following practices may be undertaken for the management of VSD in cocoa.

#### 2.5.4.1 Chemical control

A wide range of fungicides have been evaluated in Malaysia and Papua New Guinea. Prior (1980) reported that protective fungicides were unlikely to be effective in controlling VSD of cocoa in Papua New Guinea. Ooi and Chew (1985) reported that Bitertanol at 1500 ppm sprayed at 14 days interval gave complete protection to nursery seedlings. Varghese *et al.* (1985) reported that the chemicals Triadimenol, Paropiconazole and PP969 are promising in *in vitro* screening against *Ceratobasidium.*

Musa and Tey (1986) reported that Benomyl (Benlate), Pyracarbolid and Triforine, totally inhibited the growth of VSD mycelium *in vitro*. About 90 per cent control for VSD was observed with the soil drenching of triadimefon in seedlings at monthly intervals (Gurmit, 1986). Sidhu (1987) reported that triadimenol and PP969 when applied as foliar spray at weekly and fortnightly intervals provided protection against VSD in the nursery. Systemic fungicides *viz*. tebuconazole, hexaconazole and triademenol, when given as foliar spray at

monthly intervals, gave good protection. However, they resulted in the stunting of seedlings (Holderness, 1990).

In older trees, foliar spraying of bordeaux mixture and kitazin were effective in checking the severity of the disease to a certain extent (Abraham and Ravi, 1991). Flutriafol at 200 ppm and triadimefon at 500 ppm, when applied as foliar spray, gave good disease control (Hee *et al*., 1992). Since the fungus responsible for causing VSD remains active within the vascular tissue of the host, chemical control of VSD has been difficult, and it has been found to be almost ineffective in long term controlling of the disease (Prior, 1980; Ajayakumar, 1996).

#### 2.5.4.2 Cultural practices

Pruning to control VSD is a much debated subject. By cutting and removing all infected twigs and pruning off the affected branches 30 cm below the last point of visible symptom of the disease, *ie*., below the darkened xylem, inoculum levels will be highly reduced and thus prevents the further spread of infection (Keane and Turner, 1971; Prior, 1980). Incidence of infection was kept below one per cent, when the infected branches were pruned every two weeks for nearly two years in a mature plantation in Java (Keane, 1981). However, in an unpruned plantation, spreading of the infection and disease incidence increased from about 30 to 90 per cent in 10 months. Since the spreading of the infection requires moist conditions, management of shade, to increase the aeration and incidence of sunlight on the foliage is of much importance (Dennis and Keane, 1992).

#### 2.5.4.3 Isolation/ barrier

Soekirman and Agus (1992) reported that very high levels of infection had been observed in unprotected plants raised near or under diseased plants. Under normal conditions, the VSD fungal spores do not travel for more than 200 m. An

isolation belt of more than 200 m would normally reduce the inoculum potential sufficiently and thus reducing the chances of infection.

Isolation is most useful for nursery. Isolated nurseries generally have less VSD problem. In very high inoculum areas, covering the nurseries with ultra-violet light resistant polythene sheets has been reported to confer protection in Papua New Guinea. The main effect is to keep the leaves dry and hence environment conducive for fungal spore germination is prevented (Dennis and Keane, 1992).

#### 2.5.4.4 Disease avoidance

It is advocated to avoid the planting of cocoa during maximum risk period *i.e.*, when the weather is unusually wet for a prolonged period and when the inoculum potential is very high (Ooi and Chew, 1985). However the following factors taken together may be considered to constitute a high risk period:-

- In very wet years when rainfall is more than 2000 mm per year.
- When there is continuous wet weather for four or more days.
- When very high incidence of VSD is noted and hence high disease pressure

#### 2.5.4.5 Quarantine

Byrne (1976) reported that in Malaysia, movement of cocoa vegetative planting material from VSD infected areas to non infected areas is prohibited. In Papua New Guinea, although the incidence and distribution of VSD is common in some regions of the country and islands, it is not recorded in some of the other islands (Prior, 1980). Hence, as a method of controlling the disease, a restriction in the movement of materials from disease infected and prone areas to disease free areas had been recommended. Also, screening of the seeds collected from the cocoa pods of infected trees failed to show that the transmission of the disease is

through seeds (Keane and Prior, 1992). Hence, transfer of planting materials, seeds from the VSD infected and prone areas to uninfected areas is not restricted.

#### 2.5.4.6 Varietal component in disease management

Studies mainly focus on the management measures, cultural practices and chemical control, used to combat disease occurrence. Breeding and selecting seedlings with disease resistance is probably the best long term solution for VSD control (Prior, 1978). Host resistance against VSD, was reported primarily from Papua New Guinea. According to Van der Vossen (1997), the usual resistance to VSD is found to be partial, and hence prevents the fungus from killing trees. As per the study conducted by Tan and Tan (1988), cocoa in Sulawesi islands were highly resistant to VSD, the reason was due to the natural selection from diverse cocoa germplasm.

VSD, which had occurred in Papua New Guinea in an epidemic amplitude, has diminished in its severity and to a certain degree, due to the distribution of resistant clonal materials. Attempt of screening in seedlings and clonal cuttings had been done by inoculating the spores of the fungus on to the upper surface of young leaf and on to the stipules of the apical bud. Apart from the considerable variation in resistance and susceptibility observed, no cultivars were found to be completely resistant to VSD (Prior, 1978).

Evolving resistant varieties was very effective in controlling the disease in Papua New Guinea (Tan and Tan, 1988). Less susceptible genotypes will still become infected by the disease, but symptoms are less severe and the pathogen grows more slowly and only rarely sporulates; invasion into larger branches and the main trunk also appears to be restricted in these genotypes. Kerala Agricultural University, after 15 years of screening has released VSD I 31.8 (CCRP 15), which is a novel hybrid released in the world with VSD resistance and considerable yield (Minimol *et al*., 2018).

## 2.6 Cocoa yield losses due to VSD

In cocoa, due to VSD alone, the overall fall in yield was assessed to be around 25 to 40 per cent of the total production in Papua New Guinea (Byrne, 1976). Significant crop losses has been reported in Malaysia, where VSD is reported to be the main disease of cocoa (Guest and Keane, 2007).

There happened to be a considerable reduction in the yield in cocoa plantations in the severe VSD affected areas (Shepherd *et al*., 1976). Keane (1981) reported that in Papua New Guinea, under pruned condition, only the most susceptible trees were killed, while in unpruned trees, there were yield losses in 12 year old trees, due to VSD infection. However, this disease was found to be detrimental in seedlings upto 18 months old. Ahmad (1982), reported that the death of the cocoa seedlings in the nursery and in the field due to VSD is often witnessed in Malaysia.

## 2.7 *In vitro* culturing of the pathogen

*In vitro* culturing of the pathogen has not been successful so far. The fungus failed to produce spores in the culture. Many studies were done by inoculating the spores obtained from the infected stems in the field (Keane, 1972; Prior, 1978). However, these attempts to establish the fungus with in cocoa callus tissue to produce spores were not successful (Prior, 1978).

## 2.8 Gene action in VSD resistance

Horizontal resistance attributes to the resistance to VSD. For most of the characters of cocoa, the gene effects were found to be predominantly additive (Tan and Tan, 1988). Additive gene effect is observed for VSD resistance in cocoa *ie*., when each additional gene increases, the expression of the trait also increases.

## 2.9 Breeding for VSD resistance in cocoa

During the period of 1995 to 1998, an extensive breeding programme was conducted at Cocoa Research Centre, Kerala Agricultural University, for the production of disease resistant planting materials. A total of 298 parents showing resistance to VSD were selected and an extensive hybridization programme was carried out in which 1012 crosses were done which resulted in 45,000 hybrid seedlings.

The seedlings exhibiting high levels of resistance (2546 in number) were selected. Among them, 2435 vigorous seedlings were field established since 1998 in three separate plots (Mallika *et al*., 2002). These hybrid populations are segregating since the parents used were heterozygous in nature (Minimol *et al*., 2016). After fifteen years of field screening, 51 hybrids, identified to exhibit field resistance were selected.

When yield contributing characters were evaluated, VSD I 31.8 (CCRP 15) was found to be superior and it is considered as a novel hybrid released in the world with considerable yield and resistance to VSD. Resistance to the vascular streak dieback disease had been further checked by screening with previously reported ISSR marker UBC 857 and SSR marker mTcCIR42 (Minimol *et al*. 2018).

## 2.10 Molecular markers

Molecular markers were introduced in the 1990s. They are DNA sequences, which are fragments of nuclear, mitochondrial or chloroplast DNA, which are tightly linked with the gene of interest and is inherited in similar pattern as the gene does and hence acting as representative of the gene. There is no influence of environmental factors on molecular markers and thus the process of evaluation for

the identification of genotypes is made possible in a shorter time span (Kumar *et al*., 2009).

Molecular markers are more stable than morphological and biochemical markers (Cohen *et al*., 1991). According to Karp *et al*. (1997), types of markers varies, and each type will be having its own advantages and disadvantages, and also their properties also differs. Weising *et al.* (2005) reported that, inorder to satisfy the objective of the research, one can select any type of molecular marker system.

Yoshima *et al*. (1995) evaluated that the marker assisted gene pyramiding in rice by the use of molecular markers that are tightly linked to the resistance genes reduced the duration of the breeding programme. However, finding a molecular marker with all the ideal properties which could meet the need adequately is difficult (Lowe *et al*., 2004). In crops, in order to tag resistant genes, a variety of molecular marker systems such as RFLP, RAPD, ISSR, AFLP and microsatellites (SSRs) are being used.

### 2.10.1 Markers in cocoa

Application of molecular markers on the studies of genetics of cocoa has provided large insights. Lanaud (1986) used isozymes in genetic diversity and population structure studies in cocoa. Figueira (2004) pointed out that, even though they have a potential use in germplasm characterization, population studies, supported linkage mapping and cultivar identification, there is only a limited number of polymorphic enzymatic systems in cocoa, and hence their use in other genetic studies is restricted.

First DNA based marker that was applied to cocoa was Restriction Fragment Length Polymorphisms (RFLPs) (Lanaud *et al*., 1995; Crouzillat *et al*., 1996). However, their use is highly restricted to advanced laboratories, as they are

technically demanding, time-consuming, labour intensive and are not amenable to automation.

Based on polymerase chain reaction (PCR), in cocoa, the next groups of DNA markers were developed. These include Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), microsatellites or Simple Sequence Repeats (SSRs) and Inter Simple Sequence Repeats (ISSRs). These molecular markers aided in providing several solutions to problems in cocoa research. N'goran *et al*. (1994) used RAPDs for cocoa genetic diversity studies. In cocoa, AFLPs are mostly used for genetic mapping (Clement *et al*., 2003; Queiroz *et al*., 2003; Risterucci *et al*., 2003).

Lanaud *et al.* (1999) first described SSRs in cocoa. Since they are uniformly distributed throughout the genome, highly polymorphic, co-dominant and reproducible, SSRs proved to be the best markers. SSRs have been widely used in various genetic studies of cocoa. About 300 SSRs which covers over 10 cocoa linkage groups from various genomic DNA and EST (Expressed Sequence Tag) libraries have been developed (Kuhn *et al*., 2003; Pugh *et al*., 2004; Lanaud *et al*., 2004; Borrone *et al*., 2007; Schnell *et al*., 2007).

#### 2.10.1.1 Random Amplified Polymorphic DNA (RAPD) markers in cocoa

Williams *et al*. (1990) developed an effective and simple marker system called Random Amplified Polymorphic DNA (RAPD). For the genetic characterization of cocoa, Polymerase Chain Reaction (PCR) based first multi locus protocol was done by RAPD analysis (Wilde *et al*., 1992). Russell *et al*. (1993); Laurent *et al*. (1993); Lerceteau *et al*. (1997); Whitkus *et al*. (1998) studied the genetic relationships among cocoa plantations using RAPD markers.

Sane *et al*. (2002) carried out RAPD analysis using polymorphic primers to distinguish between the cocoa accessions and found that the polymorphism was

highest in KAU collections and lowest in Nigerian accessions. According to the geographical origin, three RAPD markers distinguished twenty five cocoa accessions (Russell *et al*., 1993). In order to recognize whether there are any mislabelling or repetitions in accessions, RAPD markers can be analysed for genetic diversity evaluation (Sounigo *et al*., 2005). By using the RAPD and microsatellite markers among the 19 clonal accessions of cocoa from Brazilian, Ecuadorian and Peruvian regions, Faleiro *et al*. (2004) evaluated the genetic variability.

For the evaluation of genetic diversity among 30 clonal cocoa accessions which were resistant to witches broom disease, RAPD marker system was used (Santos *et al*., 2005). Dias *et al*. (2005) showed that, in relation with yield components also, RAPD marker system had proven to be efficient. Within the local cocoa species of Central Sulawesi, variegation in the chloroplast genome has been analysed by using genome characterization by RAPD marker (Suwastika *et al*., 2017).

#### 2.10.1.2 Restriction Fragment Length Polymorphism (RFLP) markers in cocoa

RFLP is a non PCR based molecular marker. Polymorphism level of RFLP is immense in cocoa. It is a co-dominant marker with great reproducibility. However, automation is not possible in case of RFLP markers. In order to evaluate the genetic diversity among 203 genotypes of cocoa, Laurent *et al*. (1993) used RFLP markers. Risterucci *et al*. (2000) studied the linkage analysis among the cocoa seedlings from a cross between Forastero and Trinitario. Motamayor *et al*. (2002) evaluated the genetic diversity among cocoa, using RFLP markers.

#### 2.10.1.3 Amplified Fragment Length Polymorphism (AFLP) markers in cocoa

AFLP is a PCR based molecular marker system that coalesce the advantages of RAPD and RFLP markers. It has high reproducibility and is polymorphic in nature. Sequence information is not needed for the development of primers. Characterization of parental plants along with their progeny has been analysed by Perry *et al*. (1998) in cocoa. A major QTL associated with witches' broom disease resistance in cocoa has been analysed by linkage mapping by using AFLP markers (Queiroz *et al*., 2003). Araujo *et al*. (2009) developed a linkage map from the $F_2$ population resulting from the cross between Scavina-6 and ICS-1 based on AFLP, RAPD and SSR markers.

#### 2.10.1.4 Inter Simple Sequence Repeats (ISSR) in cocoa

The ISSR system of molecular markers coalesces the advantages of AFLP and RAPD markers. Charters and Wilkinson (2000) had done the genome analysis of cocoa and characterized fifty clonal and hybrid cocoa trees by using ISSR markers. Also, the ISSR markers were able to differentiate clearly between 46 cocoa accessions which were maintained at Peru (Chia *et al*., 2011).

To study and evaluate the genetic diversity between three natural populations of *Theobroma subincanum* which are geographically separated, Rivas *et al.* (2013) used ISSR markers. In promising cocoa varieties of KAU, Sujith (2016) had done ISSR analysis to analyse the polymorphism among them and to distinguish KAU cocoa varieties from others, DNA fingerprints were developed.

#### 2.10.1.5 Simple Sequence Repeats (SSR) in cocoa

Lanaud *et al*. (1999) first developed SSR markers in cocoa. SSR markers are multi-allelic, co-dominant markers. Motamayor *et al*. (2002) analysed the allelic structure of varieties of cocoa from Central America by using SSR markers. Using 170 SSR markers, gene mapping of witches' broom resistance in

cocoa was constructed and two QTLs for witches' broom disease resistance in cocoa was found (Brown *et al*., 2005). Genetic diversity in cocoa from different geographical areas were studied by Efombagn *et al*. (2006) by using 13 SSR markers.

For evaluating the genetic variability of cocoa grown in different ecosystems of Nigeria, SSR markers were used by Aikpokpodion *et al*. (2006). According to Johnson *et al*. (2007), many national and worldwide germplasms of cocoa had been exploited by SSR markers. Nagai *et al*. (2009) had done the DNA fingerprinting of 100 cocoa trees, which included Criollo, Trinitario, Forastero and their hybrid types, by using eleven SSR markers.

Lima *et al*. (2010) had identified EST SSR markers linked to witches' broom disease resistance in cocoa. SSR markers had been reported for differentiating the genetic variations among Trinitario cultivars (Yang *et al.*, 2011). Genetic diversity in resistance to witches broom disease resistance in cocoa clones were evaluated (Lima *et al*., 2013). In the major cocoa cultivating regions of Tamil Nadu, Thondaiman *et al*. (2013) had detected polymorphism between 27 cocoa trees by using 10 SSR markers. In the different clones of cocoa collected from Central Sulawesi farms, Suwastika *et al*. (2017) had studied the genetical and morphological variation.

#### 2.10.1.6 Single Nucleotide Polymorphism (SNP) markers in cocoa

SNPs are an upgraded system of molecular markers in which difference in a single base pair will analyse the polymorphism between the samples and they can be identified by non-gel based techniques. According to Zhang and Hewitt (2003), SNPs are the most widely spread polymorphisms in the genome of plant.

## 2.11 Molecular markers associated with VSD disease resistance in cocoa

For the selection of cocoa clones resistant to vascular streak dieback disease, the possibility of developing morphological, biochemical and DNA markers were evaluated by Rosmin (1999) and found that a cloned unique fragment of resistant cocoa clone obtained from an AFLP analysis was able to differentiate between resistant and susceptible cocoa clones. This DNA marker was named VSDr1.

In relation to VSD resistance, ISSR and SSR primers were identified by Chandrakant (2014). A detailed analysis of twelve cocoa clones using 71 primers had shown that the primers UBC 811, UBC 815, UBC 826, UBC 857 and UBC 866 were capable to yield polymorphic bands, thereby differentiating resistant and susceptible cocoa clones, whereas, SSR assay with 46 primer sets had failed to generate any marker for the same. Hence, the most distinct polymorphic marker generated in the resistant lines by the ISSR primer UBC 857 was eluted. The direct sequencing of UBC 857 yielded 246 nucleotides which on BLASTn had shown 94 per cent identity with the *Theobroma cacao* microsatellite DNA clone of mTcCIR42 SSR (NCBI number AJ271944). When the clones were subjected to SSR assay using the primer mTcCIR42, distinct polymorphic banding pattern was obtained which differentiated resistant clones from partially resistant and susceptible cocoa clones.

Tulshiram (2016) validated these primers by using twenty resistant and four susceptible genotypes and found that ISSR markers UBC 811, UBC 815 and UBC 857 and SSR marker mTcCIR42 were successful in differentiating resistant and susceptible genotypes of cocoa. When the genomic DNA of all the genotypes were amplified using the primer UBC 811, a clear polymorphic band of 950 bp was detected in all the resistant genotypes screened, while it was absent in the susceptible progeny analysed. When the marker UBC 815 was evaluated, a clear amplicon of size 750 bp was present in all the resistant genotypes screened, while

in the susceptible progeny, this band was absent. In the case of the marker UBC 857, when it was screened in both the resistant and susceptible genotypes, a distinct polymorphic band of 450 bp was detected in all the resistant genotypes and it was absent in the screened susceptible genotypes. A distinct band of 200 bp was identified to be linked with the gene providing resistance to VSD, when screened with the SSR marker mTcCIR42. Minimol *et al*. (2018) used the ISSR marker UBC 857 and SSR marker mTcCIR42 to tag resistant gene in VSD I 31.8 (CCRP 15), which is considered as a novel hybrid released in the world with VSD resistance and considerable yield. These markers were expressed in the hybrid along with resistant genotype used in the study but was absent in susceptible genotype.

**2.12 Proteins involved in disease resistance**

The inhibitory action of proteins against aflatoxins were analysed by Hang *et al*. (1997). In solanaceous plants, hydroxyproline-rich glycopeptide systemins were found to be involved in defense mechanism (Pearce *et al*., 2001). According to Huffaker *et al*. (2006), *At*Pep1 peptide was found to be linked with the disease defense mechanism of *Arabidopsis* plant.

**2.13 Cytoskeletal elements in disease resistance**

According to Kobayashi *et al*. (1992), immunocytochemical localization of cytoskeletal elements during attack by fungal pathogens revealed that rapid morphological changes in the plant cytosol included reorganization of the cortical cytoskeletal elements. Plant cytoskeletal microtubules undergo extensive reorganization in response to symbiotic and pathogenic organisms. Intense GFP-tubulin fluorescence have been observed beneath the invading fungus in *Arabidopsis* inoculated with *Phytophthora sojae* (Gross *et al*., 1993). Localized microtubule depolymerisation at the contact site occurs in the interactions between *Phytophthora sojae* and *Glycine max* (Cahill *et al*., 2002).

Earliest response of plants to penetration by fungi or oomycetes is the rapid translocation of cytosol and subcellular components to the infection site (Takemoto and Hardham, 2003). This site-directed cytoplasmic streaming is achieved by reorganisation of cortical and transvacuolar cytoskeletal actin elements such that actin microfilaments and cables form a radial array focused on the infection site (Kobayashi and Hakuno, 2003; Takemoto *et al*., 2003). Microtubule cytoskeletal changes have been observed in response to attempts by a potential pathogen to invade the plant cell in interactions between *Hordeum vulgare* and *Blumeria graminis*. Radial arrays of microtubules have been observed beneath the fungal appressoria (Hoefle *et al*., 2011).

# MATERIALS AND METHODS

# 3. Materials and methods

The study on 'Inheritance of molecular markers linked to vascular streak dieback disease resistance in hybrid progenies of cocoa (*Theobroma cacao* L.)' was accomplished at Department of Plant Biotechnology, College of Horticulture, Kerala Agricultural University, during the period of 2016-2018. The materials used and methodologies adopted are discussed in this chapter.

## 3.1 Plant materials

Nineteen hybrid trees that have been developed by breeding efforts at Kerala Agricultural University and exhibited resistance to VSD in the field for over 13 years were used as parent materials. These VSD resistant hybrids were crossed between each other based on the availability of flowers and hybridization was not done in the tree with stand no. 17.2 due to the unavailability of flowers. Details of the parents in which hybridization was carried out is given in Table 2 (Plates 1a, 1b and 1c). Two hundred and sixty nine seedlings were obtained and they were raised in the nursery and served as the material for the study.

**Table 2. Details of the parents in which hybridization was carried out**

| Sl No. | Hybrids | Parentage |
|---|---|---|
| 1 | 2.3 | M 13.12 × GVI 55 |
| 2 | 4.1 | GII 19.5 × GVI 55 |
| 3 | 5.11 | GVI 4 × GVI 55 |
| 4 | 10.8 | GVI 126 × GIV 18.5 |
| 5 | 11.10 | |
| 6 | 11.23 | |
| 7 | 13.11 | GVI 126 × GVI 55 |
| 8 | 13.13 | |
| 9 | 14.6 | GVI 126 × GVI 55 |
| 10 | 14.15 | |

| | | |
|---|---|---|
| 11 | 15.7 | GVI 137 × GVI 55 |
| 12 | 16.10 | GVI 140 × GVI 55 |
| 13 | 16.11 | |
| 14 | 19.6 | GVI 143 × GVI 55 |
| 15 | 21.27 | GVI 148 × GVI 55 |
| 16 | 10.10 | GVI 126 × GIV 18.5 |
| 17 | 15.10 | GVI 137 × GVI 55 |
| 18 | 22.1 | GVI 167 × GIV 18.5 |

### 3.2 Hand pollination

The hybridization was done as per the procedure described by Mallika *et al*. (2002). The flower bud that will open on the following day was recognized by whitish colour and swollen appearance. The bud was covered with a hood of plastic tube of size 5cm × 1.5-2.0 cm, which was sealed to the bark by using glaze putty. This tube was covered with a muslin cloth at the top which was kept in place by a rubber band. This ensured circulation of air and exclusion of insects.

On the next day, opened flowers were collected from the desired male parent and its stamen was carefully taken out by pushing the corresponding petal. One entire anther with a part of the filament was deposited on the stigma of the flowers which were covered on the previous day (Plate 2). One or two staminodes of the flower to be pollinated was pinched off to give access to the stigma. Emasculation was not necessary for self incompatible hybrids. The pollinated flowers were labelled by using tin foil pieces and fixed by using ball pins. The hoods were removed 24 h after pollination and the fertilization was confirmed by the visual swelling of the ovaries in 3-5 days.

In order to prevent undue shedding and wilting of fruits from hand pollination, all the developed fruits from the tree produced by open pollination were removed. The developed pods were covered with a wire mesh after 6-8


GVI 126 × GIV 18.5 (10.10)


M 13.12 × GVI 55 (2.3)


GVI 126 × GIV 18.5 (10.8)


GII 19.5 × GVI 55 (4.1)


GVI 126 × GVI 55 (13.13)


GVI 126 × GVI 55 (13.11)

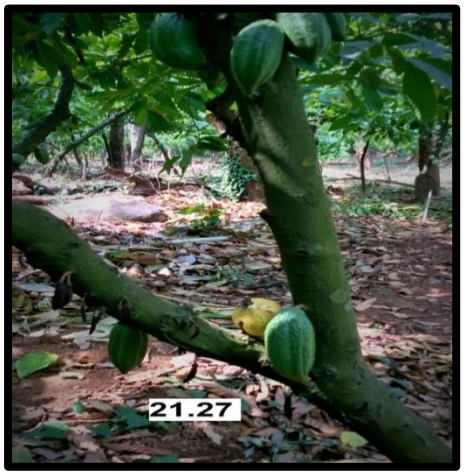
**Plate 1a. Parents used in hybridization programme**


GVI 126 × GIV 18.5 (11.10)


GVI 126 × GVI 55 (14.6)


GVI 4 × GVI 55 (5.11)


GVI 126 × GVI 55 (14.15)




GVI 148 × GVI 55 (21.27)

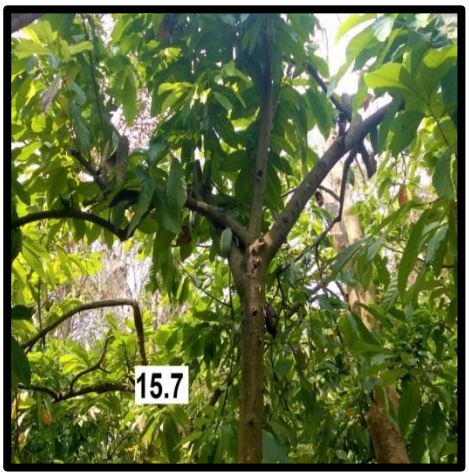

GVI 126 × GIV 18.5 (11.23)

**Plate 1b. Parents used in hybridization programme**


**GVI 140 × GVI 55 (16.11)**


**GVI 140 × GVI 55 (16.10)**


**GVI 137 × GVI 55 (15.10)**




**GVI 137 × GVI 55 (15.7)**


**GVI 143 × GVI 55 (19.6)**


**GVI 167 × GIV 18.5 (22.1)**

**Plate 1c. Parents used in hybridization programme**

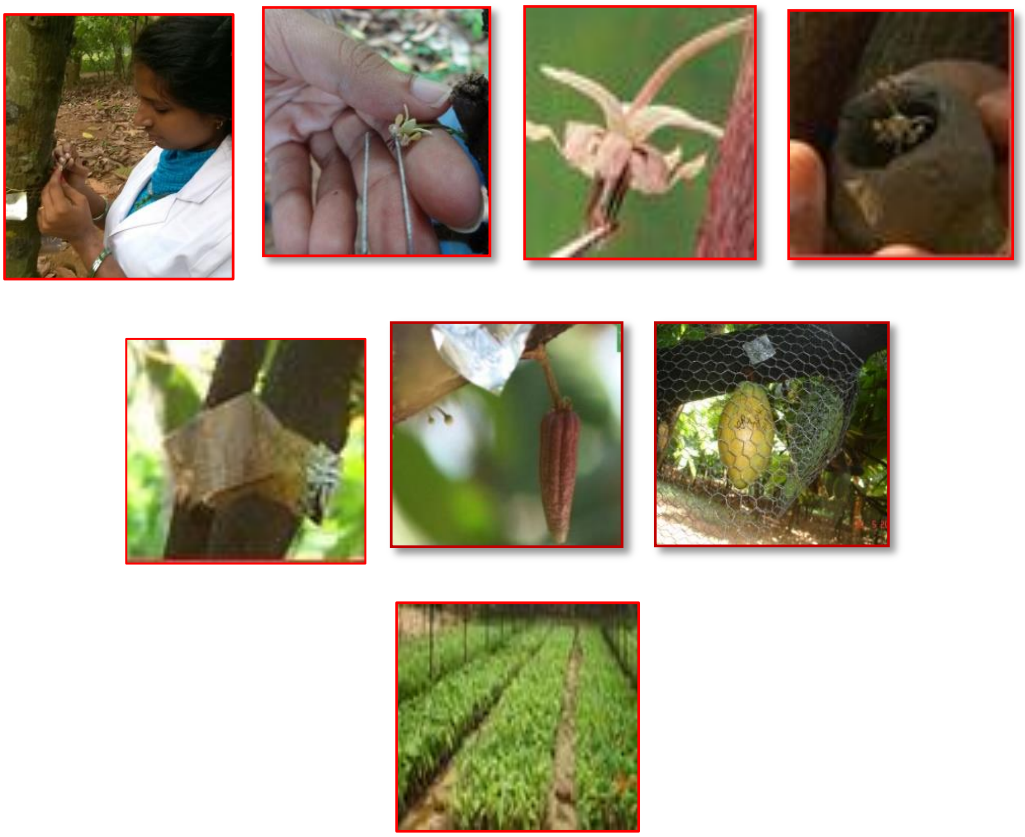


**Plate 2. Stages in hand pollination**

**Plate 3. Seedlings in the nursery**

weeks to protect them from mammalian pests. The pods were collected at maturity and beans were extracted and raised in the nursery (Plate 3).

### 3.3 Screening for disease resistance

Seedlings were exposed to natural inoculum as per the procedure described by Mallika *et al*. (2002) and classified as susceptible or resistant. The hybrid seedlings were arranged in five rows with five plants in each row. The VSD infected seedlings were used for providing natural inoculum by arranging them in two rows of five seedlings each around the hybrid seedlings (Plate 4). Overhead sprinkler system was provided throughout the period to ensure moisture and water splash that will promote the transfer of disease from infected ones to others.

### 3.4 DNA extraction

Tender leaves (light green in colour) from five months old seedlings (Plate 5) were selected as the ideal part of extraction of genomic DNA. Pale green tender leaves yielded sufficient quantity of good quality DNA. Tender leaves from the seedlings were collected on ice early in the morning. The leaves collected were quickly covered in aluminium foils and were transported to the laboratory in ice box. The leaf surface was cleaned by washing with sterile water and wiping with 70 per cent ethanol. Extraction method developed by Dellaporta *et al.* (1983) was used for genomic DNA isolation from leaves.

### 3.5 Equipment and machinery

This research work was carried out by using the facilities and equipment available at the Department of Plant Biotechnology, College of Horticulture. The centrifugation was done in high speed refrigerated floor model centrifuge (KUBOTA 6500, Japan). For the estimation of quantity and quality of DNA,

**Plate 4. Screening the hybrids for VSD resistance using infected seedlings**

**Plate 5. Five months old cocoa seedling used for DNA extraction**

Nanodrop ND-1000 spectrophotometer was used. PCR was done in Agilent Technologies (SureCycler 8800) and Life Technologies (Proflex). For agarose gel electrophoresis, horizontal gel electrophoresis system (Bio-Rad) was used and for documenting the gel, Gel Doc (Bio- Rad) was used. The gel picture was analysed by using Quantity one software (Bio- Rad). The list of laboratory equipments used in this study is provided in Appendix I.

### 3.5.1 Reagents

a. Extraction Buffer 1

- 0.5 M EDTA
- 1 M Tris
- 4 M NaCl

b. Extraction Buffer 2

- 0.5 M EDTA
- 1 M Tris

c. 20 per cent SDS

d. 5M Potassium acetate

e. 3M Sodium acetate

f. Chilled isopropanol

g. 80 per cent Ethanol

h. Liquid nitrogen

i. Sterile distilled water

Composition of reagents are provided in Appendix II

### 3.5.2 Protocol

- 0.08-0.1g of leaf tissue was ground in to fine powder using liquid nitrogen
- 1mL of extraction buffer 1 and 50 µL of β- Mercaptoethanol was added on to it and transferred to a 2mL eppendorf tube
- 100 µL of 20 per cent SDS was added to the tube and mixed thoroughly by vigorous shaking
- The tube was incubated at $65^0$C for 10 minutes in a waterbath
- 500 µL of 5M potassium acetate was added to it and mixed by vigorous shaking
- The tube was incubated at $0^0$C for 20 minutes
- The tube was spun at 13,000 rpm for 20 minutes
- The supernatant was poured on to a 2 mL tube containing 500 µL isopropanol and mixed by inverting
- The tube was incubated at $-20^0$C for 30 minutes
- The tube was centrifuged at 12,000 rpm for 15 minutes

- The supernatant was discarded and the DNA pellet was dried by inverting the tube on tissue paper for 10 minutes

- The pellet was redissolved in 200 µL extraction buffer 2

- The tube was centrifuged at 12,000 rpm for 10 minutes to remove the insoluble debris

- The supernatant was transferred to a 1.5 mL tube and 75 µL 3M sodium acetate and 500 µL isopropanol were added to it and mixed by inverting

- The tube was spun for 2 minutes at 10,000 rpm

- The pellet was washed with 500 µL of 80 per cent ethanol and dried thoroughly

- The pellet was redissolved in 50 µL sterile distilled water

### 3.5.3 Quality assessment of isolated DNA

The assessment of quality of isolated DNA was done by electrophoresis on 0.8 per cent agarose gel (Sambrook *et al.,* 1989).

#### 3.5.3.1 Equipment

a. Electrophoresis unit- Bio-Rad power pack, gel casting tray, comb

b. Gel documentation system- BioRad Gel DOC imaging system

### 3.5.3.2 Reagents

a. Agarose ( Promega) 0.8 per cent (w/v)

b. TAE buffer - 50X

- Tris base -242 g
- Glacial acetic acid -57.1 mL
- 0.5 mM EDTA -100 mL

c. Loading dye (Bangalore Genei)- 6X

d. Ethidium bromide (SRL)- stock concentration 10 mg/mL ; working concentration 0.5 μg/ mL

Composition of reagents is provided in Appendix III.

### 3.5.3.3 Procedure

- 0.8g agarose was dissolved in 100 mL 1X TAE buffer by heating it in oven for about 1 minute
- About 4 μL of ethidium bromide was added from the stock solution when the agarose solution attained about 42 to 45$^{o}$C and mixed well
- The gel casting tray was prepared by fixing it in gel casting apparatus

- The comb was placed in gel tray about 1 inch from one end of the tray and was positioned vertically such that the teeth are about 1 to 2 mm above the inner surface of the tray

- The warm gel solution was poured into the casting tray to a depth of 5 mm and was allowed to solidify for about 30-45 minutes at room temperature

- The comb was removed gently and the casting tray was placed in electrophoresis chamber

- The gel was covered with 1X TAE buffer till the wells were fully submerged

- About 1 µL of 6X gel loading dye was added for every 5µL of DNA sample and this DNA-dye mixture was loaded on to each well

- A suitable DNA ladder was also loaded in one well

- Electrophoresis was carried out at 70 V until dye has migrated to about two-third the length of the gel.

- The bands were visualized and documented under gel documentation system. The gel profile was examined for intactness of the DNA and presence of contamination with RNA and protein

- Intact DNA appeared as thick white band just below the well. The degraded ones appeared as a smear because of the presence of a large number of DNA fragments which differed only in few bases. The presence of protein was observed as a thick white patch which was restricted in the wells itself. The RNA contamination appeared as thick band with size less than 100 bp.

### 3.5.4 Quantity assessment of DNA using spectrophotometer

DNA purity was further confirmed and its quantity was assessed by using nano drop spectrophotometer (NanoDrop-1000). Nucleic acids and proteins show its peak absorbance at 260 nm and 280 nm respectively. Purity was indicated by the ratio OD260/OD280 by recording the absorbance at both the wavelengths. If the value was between 1.8 and 2.0, it indicated that the DNA was pure and free from contaminations. When the ratio was less than 1.8, it meant that the sample was protein contaminated and if it was greater than 2.0, it showed that the sample was RNA contaminated.

#### 3.5.4.1 Procedure

- The installed operating software, ND-1000 in the system was opened which is connected to the nano drop spectrophotometer

- The option nucleic acid was selected

- The sampling arm of the spectrophotometer was opened and 1μL sterile distilled water was added onto its lower measurement pedestal

- The sampling arm was closed and the spectral absorption measurement was initiated by using the operating software

- The absorption reading was set to zero with blank measurement

- 1μL of sample was added onto the lower measurement pedestal and the option ‘measure’ was selected

- After the completion of the measurement, the sampling arm was opened and wiped both the upper and lower measurement pedestals by using a laboratory tissue paper

### 3.6 Markers used for the inheritance study

Three ISSR and one SSR marker have been reported and validated for VSD resistance in cocoa (Chandrakant, 2014; Tulshiram, 2016). These markers namely, UBC 811, UBC 815, UBC 857 (ISSR) and mTcCIR42 (SSR) was used in this study to evaluate the inheritance of VSD resistance in cocoa. DNA from the progeny obtained from the successful crosses was amplified using PCR with the above primers.

#### 3.6.1 DNA amplification/ PCR conditions

An appropriate reaction mixture with accurate proportions of the reagents was essential for proper amplification of ISSR and SSR markers. The components of the reaction mixture included sterile distilled water, *Taq* buffer B, $MgCl_2$, dNTPs, primers, *Taq* DNA polymerase and template DNA. A mastermix of the reaction mixture was made with all these components excluding the template DNA. The aliquot of this mastermix was dispensed to 200 µL PCR tubes to which the template DNA was added.

The PCR was carried out in thermal cycler (Applied Biosystems, Agilent) which was previously programmed with appropriate temperatures for denaturation, annealing and extension steps with desired number of cycles for efficient amplification, as temperature profile in these steps depicts the pattern of amplification.

### 3.6.1.1 Inter Simple Sequence Repeats (ISSR) assay

Good quality genomic DNA isolated from the leaves of cocoa seedlings was diluted to 25 ng/µL before using it in the analysis. Previously reported ISSR markers linked with VSD resistance in cocoa were used for the ISSR assay. PCR amplification was performed in 20 µL reaction mixture and the components of the reaction mixture includes,

| | |
|---|---|
| a) Genomic DNA (25ng/µL) | - 2.0 µL |
| b) 10X *Taq* assay buffer B | - 2.0 µL |
| c) $MgCl_2$ | - 2.0 µL |
| d)dNTPs mix (10 mM each) | - 1.5 µL |
| e) *Taq* DNA polymerase (3U) | - 0.4 µL |
| f) Primer (10 pM) | - 1.5 µL |
| g) Autoclaved distilled water | - 10.6 µL |
| Total volume - | 20.0 µL |

The thermal profile used in the PCR amplification was,

Initial denaturation – $94^0$C for 4 minutes
Denaturation – $94^0$C for 45 seconds
Primer annealing – $43.3^0$C to $52.1^0$C for 1 minute } 35 cycles
Primer extension – $72^0$C for 2 minutes
Final extension – $72^0$C for 8 minutes
Incubation: -$4^0$C for infinity to hold the sample

Three reported ISSR primers screened for the analysis are listed in Table 3.

**Table 3. Details of ISSR primers used in the inheritance study**

| Sl. No. | Primer name | Nucleotide sequence | Annealing temperature ($^0$C) |
|---|---|---|---|
| 1 | UBC 811 | 5'- GAGAGAGAGAGAGAGAC- 3' | 43.3 |
| 2 | UBC 815 | 5'- CTCTCTCTCTCTCTCTG- 3' | 44.9 |
| 3 | UBC 857 | 5' -ACACACACACACACACYG- 3' | 52.1 |

The amplified products were electrophoresed on 1.8 per cent agarose gel stained with ethidium bromide, along with 3 kb DNA ladder (Sigma, USA). The gel profile obtained was documented with gel doc imaging system for further analysis. The documented ISSR profiles were carefully examined for amplicons.

### 3.6.1.2 Simple Sequence Repeats (SSR) assay

Good quality genomic DNA isolated from the leaves of cocoa seedlings was diluted to 25 ng/µL before using it in the analysis. Previously reported SSR marker linked with VSD resistance in cocoa was used for the assay. PCR amplification was performed in 20 µL reaction mixture and the components of the reaction mixture includes,

a) Genomic DNA (25 ng/µL) - 2.0µL
b) 10X Taq assay buffer B - 2.0µL
c) $MgCl_2$ - 2.0 µL
d) dNTPs mix (10mM each) - 1.5µL
e) Taq DNA Polymerase (3U) - 0.4µL
f) Forward Primer (10pM) - 0.75µL
g) Reverse Primer (10pM) - 0.75µL
h) Autoclaved Distilled Water - 10.6 µL

Total volume - 20.0µl

The thermal profile used in the PCR amplification was,

Initial denaturation – $94^0$C for 4 minutes
Denaturation – $94^0$C for 45 seconds
Primer annealing – $55^0$C for 1 minute
Primer extension – $72^0$C for 2 minutes
} 30 cycles
Final extension – $72^0$C for 8 minutes
Incubation: -$4^0$C for infinity to hold the sample

The reported SSR primer used for screening and analysis is listed in Table 4.

**Table 4. Details of SSR primer used in the inheritance study**

| Sl. No. | Primer name | Nucleotide sequence | Annealing temperature ($^0$C) |
|---|---|---|---|
| 1 | mTcCIR42 | **Forward primer-**<br>5'- TTGCTGAAGTATCTTTTGAC- 3'<br>**Reverse primer-**<br>5'-GCTCCACCCCTATTTG- 3' | 55 |

The amplified products were electrophoresed on 2.0 per cent agarose gel stained with ethidium bromide, along with 3 kb DNA ladder (Sigma, USA). The gel profile obtained was documented with gel doc imaging system for further analysis. The documented SSR profiles were carefully examined for amplicons.

### 3.7 Inheritance of molecular markers

The inheritance of molecular marker was calculated as the percentage of resistant seedlings over total resistant seedlings in which the marker is expressed.

Inheritance percentage of marker in each cross =

$$\frac{\text{No. of resistant seedlings with marker expressed} \times 100}{\text{Total No. of resistant seedlings}}$$

### 3.8 Flanking sequence analysis

Argout *et al*. (2011) had done the whole genome sequencing of cocoa by using the flow cytometry. The flanking sequences of the ISSR markers UBC 811 and UBC 857 and the SSR marker was extracted from the whole genome sequence database and were analysed with the bioinformatics tools (ORF finder and BLASTp).

#### 3.8.1 ORF finder

Open Reading Frames (ORFs) from the flanking sequences were obtained by using NCBI ORF finder.

#### 3.8.2 BLASTp

The ORF obtained was used as query to run BLASTp for similarity searches.

# RESULTS AND DISCUSSION

# 4. RESULTS AND DISCUSSION

The study entitled 'Inheritance of molecular markers linked to vascular streak dieback disease resistance in hybrid progenies of cocoa (*Theobroma cacao* L.)' was carried out in Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, and Cocoa Research Centre, Kerala Agricultural University during 2016-2018. The objective was to study the inheritance of identified ISSR and SSR markers linked to VSD resistance in hybrid progeny of cocoa (*Theobroma cacao* L.). Chandrakant (2014) identified one SSR marker and five ISSR markers which were said to be linked to the gene contributing to the resistance for VSD in cocoa. Tulshiram (2016) validated these markers and proved that one SSR marker (mTcCIR42) and three ISSR markers (UBC 811, UBC 815, UBC 857) were useful in identifying resistance to VSD.

Cocoa being a cross pollinated crop, the progeny in subsequent generation will show segregation. The present study is based on the inheritance of these markers in diverse population, to confirm their efficiency in identifying VSD resistant genotypes. This will enable the exploitation of these markers in Marker Assisted Selection (MAS).

One SSR marker, mTcCIR42 and three ISSR markers, UBC 811, UBC 815 and UBC 857 which were reported to be linked with VSD resistant gene in cocoa were used for the inheritance study. The results obtained during the study are presented and discussed in this chapter.

## 4.1 Hybridization programme

The hybrids furnished in the Table 2 were crossed between each other according to the availability of flowers. However, due to old trees, much flowers were not available. Hence, hybridization was carried out on hybrids in which sufficient flowers were present. Hybridization was not done on the hybrid 17.2 with the parentage GVI 140× GVI 55 due to the unavailability of flowers. Due to

cross compatibility problems, most of the flowers pollinated were lost. Hybridization was carried out by selecting eighteen hybrids as female parents (4.1, 2.3, 5.11, 10.10, 10.8, 11.10, 13.11, 13.13, 14.15, 11.23, 21.27, 15.10, 16.10, 16.11, 15.7, 14.6, 22.1 and 19.6). A total of one hundred and ninety three cross combinations were tried. Two thousand two hundred and thirty seven flowers were pollinated. Details are furnished in the Appendix IV.

Even though two thousand two hundred and thirty seven flowers were pollinated, only seven pods were obtained from seven crosses. This huge difference between number of flowers pollinated and pod set is due to compatibility issues. Like self incompatibility, cross incompatibility is also reported in cocoa. The reason for the same is due to the similar genetic constitution of the crossed genotypes (Mallika *et al*., 2002).

The seven crosses which yielded pods included 16.11× 4.1 [(GVI 140 × GVI 55)× (GII 19.5 × GVI 55)], 14.6× 4.1 [(GVI 126 × GVI 55)× (GII 19.5 × GVI 55 )], 14.6× 16.11 [(GVI 126 × GVI 55)× (GVI 140 × GVI 55)], 16.10× 2.3 [(GVI 140 × GVI 55)×(M 13.12 × GVI 55)], 4.1× 15.7 [(GII 19.5 × GVI 55)×(GVI 137 × GVI 55)], 11.10× 16.11 [(GVI 126 × GIV 18.5)×(GVI 140 × GVI 55)] and 16.11× 13.13 [(GVI 140 × GVI 55)×(GVI 126 × GVI 55)].

**Table 5. Germination percentage of different successful crosses**

| Sl No. | Cross | No. of pods | No. of seeds sown | No. of seeds germinated | Germination percentage (%) |
|---|---|---|---|---|---|
| 1 | 16.11× 4.1 | 1 | 61 | 56 | 91.8 |
| 2 | 14.6× 4.1 | 1 | 14 | 14 | 100 |
| 3 | 14.6× 16.11 | 1 | 58 | 48 | 82.8 |
| 4 | 16.10× 2.3 | 1 | 49 | 22 | 44.9 |
| 5 | 4.1× 15.7 | 1 | 57 | 50 | 87.7 |
| 6 | 11.10× 16.11 | 1 | 32 | 26 | 81.3 |
| 7 | 16.11× 13.13 | 1 | 54 | 53 | 98.1 |

The details of germination of seeds from successful cross combinations are given in the Table 5. Maximum number of beans was obtained from the pod of the cross 16.11× 4.1, and it was 61. Minimum number of beans was obtained from the pod of the cross 14.6× 4.1 and it was 14. Maximum germination percentage was found in the cross 14.6× 4.1 (cent per cent) and minimum was found to be in the cross between the hybrids 16.10 and 2.3 (44.9 per cent). A minimum of 7- 8 days and a maximum of 10 days were taken for the seeds to attain fifty per cent germination. More than 90 per cent germination had been achieved in cocoa under normal conditions (Amma *et al*., 2009). A total of one hundred and twenty seedlings were selected for molecular analysis which represents all the seven successful crosses.

**4.2 Classification based on VSD disease incidence**

A valid score chart for scoring disease incidence was available only for scoring disease on trees. Hence, seedlings which were lost due to heavy VSD incidence within five months age were classified as susceptible. Seedlings which showed symptoms like drying of new sprouts and mild roughening of the stem, increased number of yellow leaves and the appearance of interveinal chlorosis and terminal necrosis (Plate 6) were classified under partially resistant category. Whereas the seedlings with no symptoms of VSD were classified as resistant ones (Plate 7). The details of visual screening and classification of plants are furnished in the Table 6.

Maximum percentage of infected seedlings was seen in the cross 14.6× 16.11 and the infection percentage was 45.8 per cent. Lowest percentage of infected seedlings was seen in the cross 16.11× 13.13 and it was 15 per cent. Variation in percentage of infected seedlings was observed among the seven crosses. The reason was that the hybrid population itself is segregating due to the heterozygous nature of its parents (Minimol *et al*., 2016).

**Plate 6. Symptoms of VSD observed for partially resistant hybrids in the nursery**

**Plate 7. Resistant seedlings in the nursery**

| Sl. No. | Cross combinations | Total no. of seedlings | No. of resistant seedlings | No. of susceptible seedlings | No. of partially resistant seedlings | Percentage of infected seedlings (%) |
|---|---|---|---|---|---|---|
| 1 | 16.11× 4.1 | 56 | 34 | 20 | 2 | 39.2 |
| 2 | 14.6× 4.1 | 14 | 8 | 4 | 2 | 42.8 |
| 3 | 14.6× 16.11 | 48 | 26 | 18 | 4 | 45.8 |
| 4 | 16.10× 2.3 | 22 | 18 | 3 | 1 | 18.1 |
| 5 | 4.1× 15.7 | 50 | 28 | 20 | 2 | 44.0 |
| 6 | 11.10× 16.11 | 26 | 21 | 4 | 1 | 19.2 |
| 7 | 16.11× 13.13 | 53 | 45 | 6 | 2 | 15.0 |
| Total | | 269 | 180 | 75 | 14 | |

**Table 6. Classification of seedlings based on VSD disease incidence**

### 4.3 Screening for initial vigour

Seedlings were evaluated for their initial vigour at the third month stage based on $HD^2$ value obtained by measuring the height and diameter of the individual plants (Enriquez, 1981). $HD^2$ values of the selected seedlings for molecular analysis are given in the Appendix V.

### 4.4 Molecular analysis

#### 4.4.1 Isolation of DNA

Isolation of good quality DNA is essential for molecular analyses regardless of the marker or the plant samples. Vigorous plants were selected and DNA was isolated from them. For the extraction of good quality DNA from plant tissues, several protocols were available (Saghai-Maroof *et al*., 1984; Doyle and Doyle, 1987; Haymes, 1996; Sheperd *et al*., 2002; Mogg and Bond, 2003). For all the plant species, similar isolation protocols for DNA isolation cannot be reproduced (Porebski *et al*., 1997; Ribeiro and Lovato, 2007).

Modified Doyle and Doyle (1987) method suggested by Chandrakant (2014) was tried however found unsuitable due to the high level of mucilage content, as it interferes with the PCR techniques. Genomic DNA isolation method developed by Dellaporta *et al.* (1983) was used for DNA extraction from leaves.

**Table 7. Details of the seedlings selected for analysis with markers**

| Sl No. | Cross | Seedlings selected for molecular analysis | Resistant | Partially resistant |
|---|---|---|---|---|
| 1 | 16.11× 4.1 | 19 | 17 | 2 |
| 2 | 14.6× 4.1 | 7 | 5 | 2 |
| 3 | 14.6× 16.11 | 14 | 10 | 4 |
| 4 | 16.10× 2.3 | 13 | 12 | 1 |
| 5 | 4.1× 15.7 | 22 | 20 | 2 |

| | | | | |
|---|---|---|---|---|
| 6 | 11.10× 16.11 | 8 | 7 | 1 |
| 7 | 16.11× 13.13 | 37 | 35 | 2 |
| Total | | 120 | 106 | 14 |

A total of one hundred and twenty samples depending upon initial vigour and availability of new flush leaves were used for DNA isolation representing all the seven crosses. From the cross 16.11×4.1, the samples selected for molecular marker analysis were nineteen. In the cross between the hybrids 14.6 and 4.1, marker analysis was done in seven seedlings. In 14.6× 16.11, fourteen seedlings were selected for molecular marker analysis. Thirteen seedlings were selected from the cross 16.10× 2.3 for screening with primers. The seedlings selected from the crosses 4.1× 15.7, 11.10× 16.11, 16.11× 13.13 for analysis with the primers were twenty two, eight and thirty seven respectively. Seedlings were selected in such a manner that both resistant and partially resistant types were ensured from each cross. The details of the seedlings selected for screening with primers from all the seven successful crosses are furnished in the Table 7.

### 4.4.2 Quantification of DNA

Quantification of extracted DNA was done by spectrophotometric method using spectrophotometer. Spectrophotometric analysis gave the acceptable ratio of UV absorbance (A260/280) between 1.8 and 2.0 in most of the samples. RNase was not used because it was observed that, extraction of sufficient quantity of good quality DNA after RNase treatment was not possible.

**Table 8. Quality and quantity of genomic DNA isolated from seedlings**

| Cross | Seedlings | Quantity (ng/µL) | $A_{260/280}$ |
|---|---|---|---|
| 4.1× 15.7 | 1.1 | 67.1 | 1.94 |
| | 1.2 | 810.9 | 1.97 |
| | 1.3 | 102.2 | 1.98 |
| | 1.4 | 83.6 | 1.94 |

| | | | |
|---|---|---|---|
| 4.1× 15.7 | 1.5 | 460.3 | 1.95 |
| | 1.6 | 111.9 | 1.90 |
| | 1.7 | 102.7 | 1.92 |
| | 1.8 | 77.5 | 1.90 |
| | 1.9 | 357.8 | 2.01 |
| | 1.10 | 1006.6 | 2.02 |
| | 1.11 | 112.2 | 1.96 |
| | 1.12 | 86.3 | 1.91 |
| | 1.13 | 173.7 | 1.97 |
| | 1.14 | 132.9 | 1.97 |
| | 1.15 | 123.2 | 1.99 |
| | 1.16 | 182.8 | 1.98 |
| | 1.17 | 97.4 | 1.93 |
| | 1.18 | 299.9 | 2.03 |
| | 1.19 | 165.9 | 1.94 |
| | 1.20 | 148.2 | 2.02 |
| | 1.21 | 163.8 | 2.00 |
| | 1.22 | 110.4 | 1.90 |
| 11.10× 16.11 | 2.1 | 75.7 | 1.95 |
| | 2.2 | 491.3 | 2.03 |
| | 2.3 | 88.8 | 1.93 |
| | 2.4 | 330.7 | 2.01 |
| | 2.5 | 69.1 | 1.98 |
| | 2.6 | 112.0 | 1.89 |
| | 2.7 | 79.9 | 1.93 |
| | 2.8 | 205.5 | 1.96 |
| 14.6×4.1 | 3.1 | 305.8 | 1.89 |
| | 3.2 | 234.8 | 1.86 |
| | 3.3 | 464.5 | 1.91 |
| | 3.4 | 380.4 | 1.91 |

| | | | |
|---|---|---|---|
| 14.6×4.1 | 3.5 | 301.0 | 1.86 |
| | 3.6 | 361.5 | 1.80 |
| | 3.7 | 168.2 | 1.85 |
| 14.6×16.11 | 4.1 | 240.7 | 1.90 |
| | 4.2 | 1125.7 | 1.94 |
| | 4.3 | 265.7 | 1.97 |
| | 4.4 | 372.7 | 1.94 |
| | 4.5 | 74.7 | 1.95 |
| | 4.6 | 130.3 | 1.95 |
| | 4.7 | 291.7 | 2.01 |
| | 4.8 | 426.0 | 2.01 |
| | 4.9 | 116.2 | 1.86 |
| | 4.10 | 156.8 | 1.92 |
| | 4.11 | 227.8 | 2.03 |
| | 4.12 | 264.4 | 2.03 |
| | 4.13 | 343.8 | 1.90 |
| | 4.14 | 265.8 | 1.81 |
| 16.10× 2.3 | 5.1 | 629.5 | 1.94 |
| | 5.2 | 130.2 | 1.90 |
| | 5.3 | 30.9 | 1.94 |
| | 5.4 | 102.1 | 2.01 |
| | 5.5 | 53.7 | 1.90 |
| | 5.6 | 54.9 | 1.97 |
| | 5.7 | 80.3 | 1.99 |
| | 5.8 | 50.2 | 1.99 |
| | 5.9 | 700.7 | 1.97 |
| | 5.10 | 75.4 | 1.91 |
| | 5.11 | 41.2 | 1.95 |
| | 5.12 | 65.2 | 1.97 |
| | 5.13 | 63.0 | 1.99 |

| | | | |
|---|---|---|---|
| 16.11× 4.1 | 6.1 | 82.1 | 2.01 |
| | 6.2 | 112.2 | 1.94 |
| | 6.3 | 223.9 | 2.01 |
| | 6.4 | 131.2 | 2.00 |
| | 6.5 | 529.1 | 1.94 |
| | 6.6 | 126.9 | 1.97 |
| | 6.7 | 106.6 | 1.94 |
| | 6.8 | 203.7 | 1.97 |
| | 6.9 | 552.5 | 1.94 |
| | 6.10 | 134.2 | 1.95 |
| | 6.11 | 126.4 | 1.99 |
| | 6.12 | 253.4 | 1.96 |
| | 6.13 | 83.8 | 1.96 |
| | 6.14 | 163.3 | 1.99 |
| | 6.15 | 75.6 | 1.94 |
| | 6.16 | 102.6 | 1.97 |
| | 6.17 | 152.6 | 1.96 |
| | 6.18 | 113.9 | 1.98 |
| | 6.19 | 115.0 | 1.95 |
| 16.11× 13.13 | 7.1 | 179.3 | 1.93 |
| | 7.2 | 141.6 | 1.97 |
| | 7.3 | 193.5 | 1.95 |
| | 7.4 | 167.7 | 1.98 |
| | 7.5 | 177.3 | 1.97 |
| | 7.6 | 77.2 | 1.93 |
| | 7.7 | 92.8 | 1.92 |
| | 7.8 | 103.8 | 2.02 |
| | 7.9 | 400.2 | 1.92 |
| | 7.10 | 144.1 | 2.01 |
| | 7.11 | 171.6 | 2.01 |

| | | | |
|---|---|---|---|
| 16.11× 13.13 | 7.12 | 163.5 | 2.03 |
| | 7.13 | 512.6 | 2.02 |
| | 7.14 | 197.5 | 2.02 |
| | 7.15 | 170.3 | 2.03 |
| | 7.16 | 318.3 | 2.01 |
| | 7.17 | 488.6 | 2.02 |
| | 7.18 | 217.2 | 2.01 |
| | 7.19 | 160.7 | 1.97 |
| | 7.20 | 287.3 | 1.92 |
| | 7.21 | 343.5 | 1.94 |
| | 7.22 | 508.5 | 1.89 |
| | 7.23 | 318.9 | 1.84 |
| | 7.24 | 490.6 | 1.88 |
| | 7.25 | 68.4 | 1.97 |
| | 7.26 | 136.7 | 1.98 |
| | 7.27 | 191.5 | 1.96 |
| | 7.28 | 197.9 | 1.92 |
| | 7.29 | 154.0 | 1.95 |
| | 7.30 | 596.8 | 1.97 |
| | 7.31 | 150.3 | 1.93 |
| | 7.32 | 177.7 | 1.99 |
| | 7.33 | 173.1 | 1.99 |
| | 7.34 | 156.7 | 1.92 |
| | 7.35 | 152.6 | 1.92 |
| | 7.36 | 267.6 | 1.93 |
| | 7.37 | 220.0 | 1.96 |

DNA isolated from twenty two seedlings of the cross 4.1× 15.7 were quantified by using spectrophotometer and slight RNA contamination was observed in DNA from five seedlings. In the cross between 11.10 and 16.11,

DNA from eight seedlings were extracted and quantified. DNA samples from out of the eight seedlings, two were with slight RNA contamination. DNA was isolated from seven seedlings of the cross 14.6× 4.1, and was quantified. None of them had shown the presence of RNA contamination. In the cross 14.6× 16.11, DNA extraction and quantification was done from fourteen seedlings, out of which four were detected with slight RNA contamination.

In 16.10× 2.3, extraction and quantification of DNA was done from thirteen seedlings. In this cross, one sample showed slight contamination with RNA. In the cross between 16.11 and 4.1, DNA was extracted from nineteen seedlings out of which three were detected with slight RNA contamination. In 16.11× 13.13, DNA from thirty seven seedlings was quantified by using spectrophotometer and RNA contamination was detected in ten samples.

When the samples with and without RNA contamination were screened with the primers, it was observed that there was no difference in the banding pattern, indicating the slight RNA contamination had not interfered with the PCR amplification of the bands (Raval *et al*., 1998). Hence, these samples were used for screening with the primers for disease resistance. Details of the quality and quantity of DNA extracted are given in the Table 8.

**4.5 Molecular marker analysis**

Molecular markers have already turned out to be an efficient means in genetic analysis (Milach, 1998). PCR based molecular marker systems amplifies a specific part of genomic DNA based on the primer. In this study, for screening the seedlings with VSD resistance, identified ISSR and SSR marker systems from earlier studies were used. Genomic DNA of one hundred and six resistant and fourteen partially resistant seedlings from seven crosses were amplified with these primers by using the previously standardized protocols (Chandrakant, 2014) and were analysed.

### 4.5.1 Inter Simple Sequence Repeats (ISSR) analysis

ISSR markers are PCR based dominant, reproducible and highly sensitive marker system which does not require any prior information about the genomic DNA sequence which is to be amplified. These primers only need a small amount of target DNA for amplification. The ISSR primers target a subset of 'simple sequence repeats' (SSRs) and amplify the ISSR region between two closely spaced oppositely oriented SSRs (Zietkiewicz *et al*., 1994). Due to its high reproducibility, these markers offer the potential for direct usage in Marker Assisted Selection (MAS) (Bornet and Branchard, 2001; Reddy *et al*., 2002).

Three ISSR primers (UBC 811, UBC 815, UBC 857) which were identified to be linked with VSD resistance from the previous studies and are capable of producing polymorphic bands in segregating population were used for the amplification of extracted DNA from the seedlings. Amplified fragments were electrophoresed in 1.8 per cent agarose gel. The details of the ISSR primers analysed in this study are furnished in Table 3. The result of the analysis with these primers are detailed and discussed here under.

#### 4.5.1.1 Inheritance of ISSR marker UBC 811 in segregating progeny

Genomic DNA diluted to 25 ng/µL from all the seedlings were amplified with the primer UBC 811. In the earlier studies, screening with the primer UBC 811 had yielded distinct polymorphic band of 0.950 kb size in all the resistant hybrids analysed (Chandrakant, 2014; Tulshiram, 2016). An average of seven amplicons were obtained from each cross. The molecular weight of the bands varied from 0.3 to 1.5 kb. The details of screening with the primer UBC 811 for the presence of marker band on one hundred and twenty seedlings from seven crosses are listed in Table 9.

**Table 9. Inheritance of ISSR marker UBC 811 in segregating progeny**

| Sl No. | Cross | Seedlings | Reaction | UBC 811 (950 bp) |
|---|---|---|---|---|
| 1 | 4.1× 15.7 | 1.1 | R | + |

| | | | | |
|---|---|---|---|---|
| 2 | 4.1× 15.7 | 1.2 | R | + |
| 3 | | 1.3 | R | + |
| 4 | | 1.4 | R | + |
| 5 | | 1.5 | R | + |
| 6 | | 1.6 | R | - |
| 7 | | 1.7 | R | - |
| 8 | | 1.8 | R | + |
| 9 | | 1.9 | R | - |
| 10 | | 1.10 | R | - |
| 11 | | 1.11 | R | + |
| 12 | | 1.12 | R | + |
| 13 | | 1.13 | R | + |
| 14 | | 1.14 | R | + |
| 15 | | 1.15 | R | + |
| 16 | | 1.16 | R | + |
| 17 | | 1.17 | R | + |
| 18 | | 1.18 | R | + |
| 19 | | 1.19 | R | - |
| 20 | | 1.20 | R | - |
| 21 | | 1.21 | PR | + |
| 22 | | 1.22 | PR | - |
| 23 | 11.10× 16.11 | 2.1 | R | + |
| 24 | | 2.2 | R | + |
| 25 | | 2.3 | R | + |
| 26 | | 2.4 | R | + |
| 27 | | 2.5 | R | + |
| 28 | | 2.6 | R | + |
| 29 | | 2.7 | R | + |
| 30 | | 2.8 | PR | - |
| 31 | 14.6× 4.1 | 3.1 | R | + |

| | | | | |
|---|---|---|---|---|
| 32 | 14.6× 4.1 | 3.2 | R | + |
| 33 | | 3.3 | R | + |
| 34 | | 3.4 | R | - |
| 35 | | 3.5 | R | - |
| 36 | | 3.6 | PR | - |
| 37 | | 3.7 | PR | - |
| 38 | 14.6× 16.11 | 4.1 | R | + |
| 39 | | 4.2 | R | - |
| 40 | | 4.3 | R | + |
| 41 | | 4.4 | R | + |
| 42 | | 4.5 | R | - |
| 43 | | 4.6 | R | - |
| 44 | | 4.7 | R | - |
| 45 | | 4.8 | R | - |
| 46 | | 4.9 | R | - |
| 47 | | 4.10 | R | - |
| 48 | | 4.11 | PR | - |
| 49 | | 4.12 | PR | - |
| 50 | | 4.13 | PR | - |
| 51 | | 4.14 | PR | - |
| 52 | 16.10× 2.3 | 5.1 | R | + |
| 53 | | 5.2 | R | + |
| 54 | | 5.3 | R | + |
| 55 | | 5.4 | R | + |
| 56 | | 5.5 | R | + |
| 57 | | 5.6 | R | + |
| 58 | | 5.7 | R | + |
| 59 | | 5.8 | R | + |
| 60 | | 5.9 | R | + |
| 61 | | 5.10 | R | + |

| | | | | |
|---|---|---|---|---|
| 62 | 16.10× 2.3 | 5.11 | R | + |
| 63 | | 5.12 | R | + |
| 64 | | 5.13 | PR | + |
| 65 | 16.11× 4.1 | 6.1 | R | + |
| 66 | | 6.2 | R | + |
| 67 | | 6.3 | R | + |
| 68 | | 6.4 | R | + |
| 69 | | 6.5 | R | + |
| 70 | | 6.6 | R | - |
| 71 | | 6.7 | R | + |
| 72 | | 6.8 | R | + |
| 73 | | 6.9 | R | - |
| 74 | | 6.10 | R | + |
| 75 | | 6.11 | R | + |
| 76 | | 6.12 | R | + |
| 77 | | 6.13 | R | - |
| 78 | | 6.14 | R | + |
| 79 | | 6.15 | R | - |
| 80 | | 6.16 | R | - |
| 81 | | 6.17 | R | - |
| 82 | | 6.18 | PR | - |
| 83 | | 6.19 | PR | + |
| 84 | 16.11× 13.13 | 7.1 | R | + |
| 85 | | 7.2 | R | + |
| 86 | | 7.3 | R | + |
| 87 | | 7.4 | R | + |
| 88 | | 7.5 | R | + |
| 89 | | 7.6 | R | + |
| 90 | | 7.7 | R | + |
| 91 | | 7.8 | R | + |

| | | | | |
|---|---|---|---|---|
| 92 | 16.11× 13.13 | 7.9 | R | + |
| 93 | | 7.10 | R | + |
| 94 | | 7.11 | R | - |
| 95 | | 7.12 | R | + |
| 96 | | 7.13 | R | + |
| 97 | | 7.14 | R | - |
| 98 | | 7.15 | R | + |
| 99 | | 7.16 | R | + |
| 100 | | 7.17 | R | + |
| 101 | | 7.18 | R | + |
| 102 | | 7.19 | R | + |
| 103 | | 7.20 | R | + |
| 104 | | 7.21 | R | + |
| 105 | | 7.22 | R | + |
| 106 | | 7.23 | R | - |
| 107 | | 7.24 | R | + |
| 108 | | 7.25 | R | + |
| 109 | | 7.26 | R | - |
| 110 | | 7.27 | R | - |
| 111 | | 7.28 | R | + |
| 112 | | 7.29 | R | - |
| 113 | | 7.30 | R | + |
| 114 | | 7.31 | R | + |
| 115 | | 7.32 | R | - |
| 116 | | 7.33 | R | + |
| 117 | | 7.34 | R | - |
| 118 | | 7.35 | R | + |
| 119 | | 7.36 | PR | - |
| 120 | | 7.37 | PR | - |

In the cross between the parents 4.1 and 15.7, the polymorphic band of 950 bp was detected in the fourteen out of twenty resistant seedlings screened. It was present in one out of two partially resistant seedlings (Fig. 1a and 1b). The polymorphic band of 950 bp was present in both the parents (Tulshiram, 2016).

In the cross between the parents 11.10 and 16.11, the polymorphic band of 950 bp was present in all the seven resistant seedlings and was not detected in the partially resistant seedling screened (Fig. 2). The marker band of 950 bp was detected in both the parents in earlier study (Tulshiram, 2016). In the cross 14.6× 4.1, the marker band of 950 bp was found in three out of five resistant seedlings. Its presence was not detected in any of the two partially resistant seedlings screened (Fig. 3). The polymorphic band of 950 bp was present in both the parents (Tulshiram, 2016).

The number of seedlings screened with UBC 811 from the cross between the parents 14.6 and 16.11 were ten resistant and four partially resistant seedlings out of which the 0.950 kb marker band was found only in three resistant seedlings (Fig. 4). Tulshiram (2016) detected the presence of the polymorphic band of 950 bp in both the parents. In the cross between 16.10 and 2.3, the polymorphic band of 0.950 kb was found in all the twelve resistant and one partially resistant seedlings (Fig. 5). The polymorphic band of 950 bp was present in both the parents 16.10 and 2.3 (Tulshiram, 2016).

In the cross 16.11× 4.1, 0.950 kb marker band was detected in eleven out of seventeen resistant seedlings and was present in one partially resistant seedling screened (Fig. 6a and 6b). It was present in both the parents according to the study conducted by Tulshiram (2016). In the cross between the parents 16.11 and 13.13, out of the thirty five resistant seedlings, the 950 bp marker band was found in only twenty seven seedlings and was not detected in the two partially resistant seedlings screened (Fig. 7a, 7b and 7c). The polymorphic marker band of 950 bp was present in both the parents (Tulshiram, 2016).

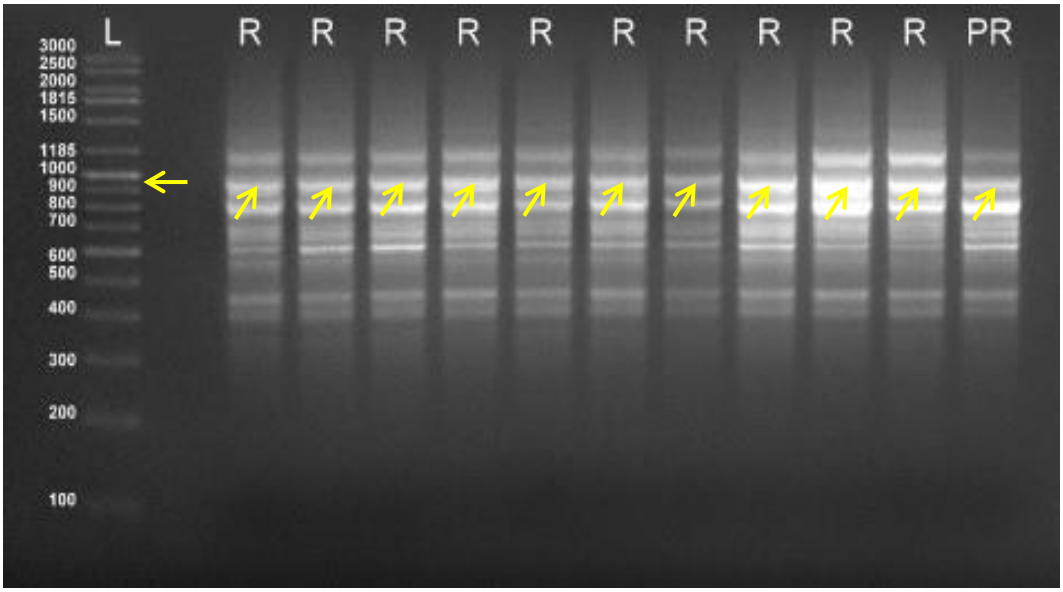



Fig 1a. Amplification pattern of the ISSR marker UBC 811 in segregating generation of the cross 4.1 X 15.7 showing resistant/ partially resistant reaction to VSD

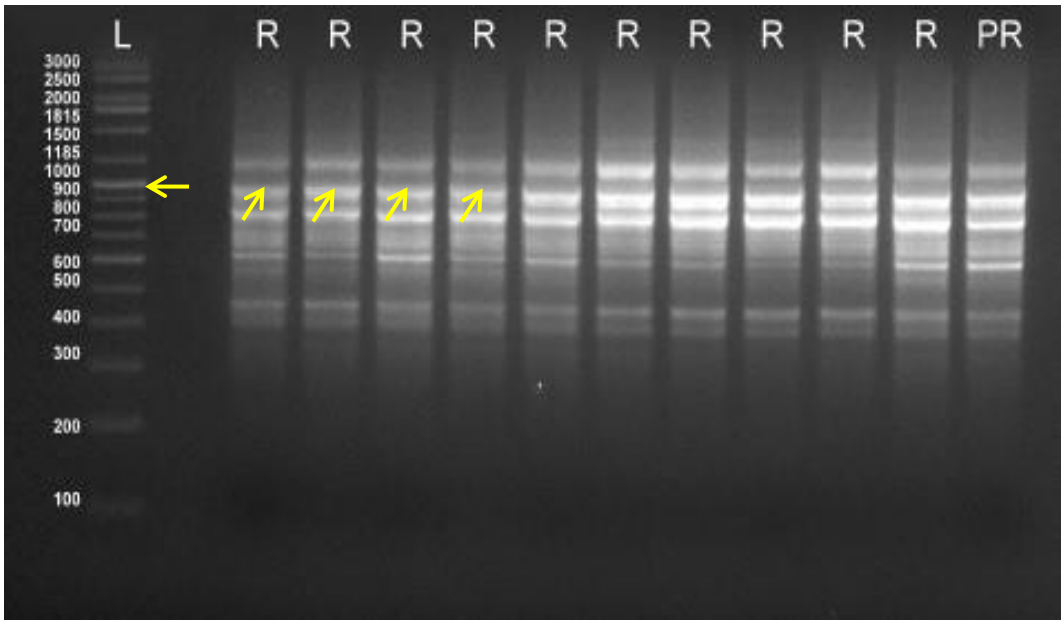



Fig 1b. Amplification pattern of the ISSR marker UBC 811 in segregating generation of the cross 4.1 X 15.7 showing resistant/ partially resistant reaction to VSD

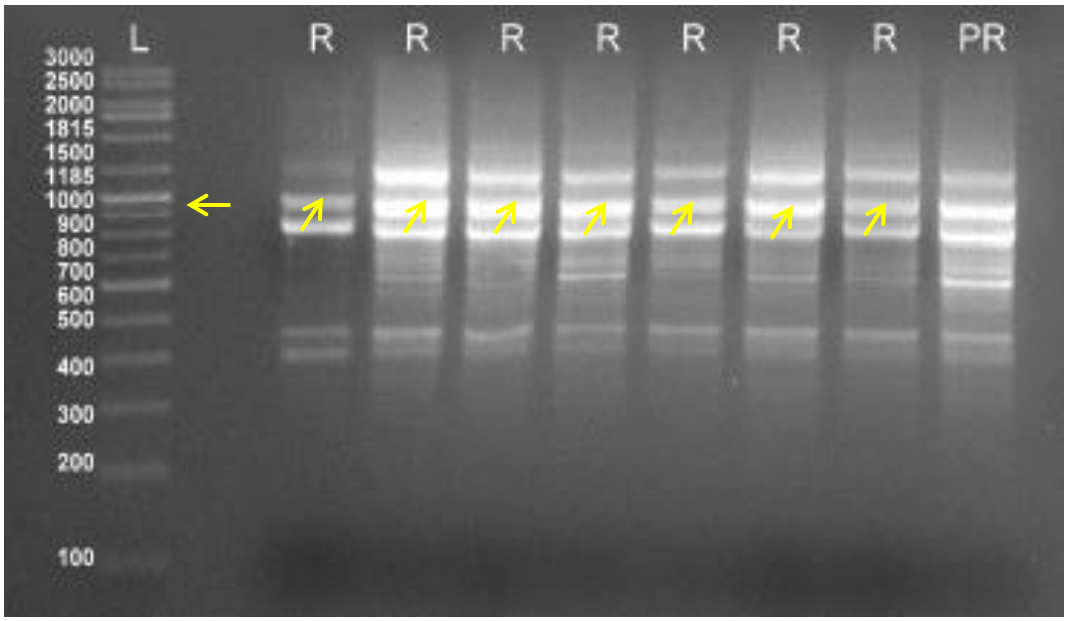



Fig 2. Amplification pattern of the ISSR marker UBC 811 in segregating generation of the cross 11.10 X 16.11 showing resistant/ partially resistant reaction to VSD

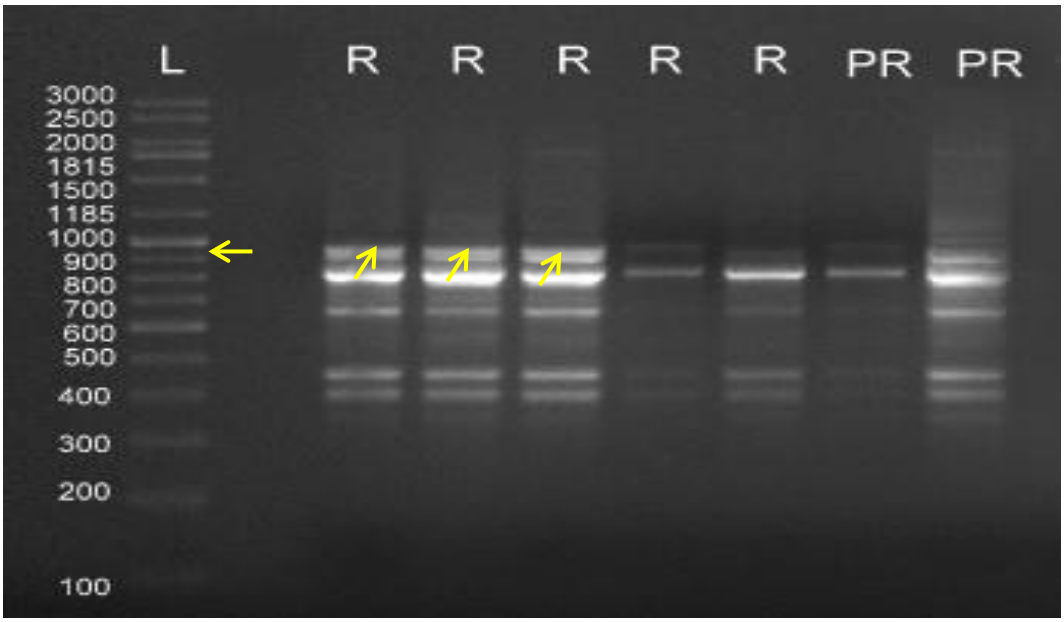



Fig 3. Amplification pattern of the ISSR marker UBC 811 in segregating generation of the cross 14.6 X 4.1 showing resistant/ partially resistant reaction to VSD

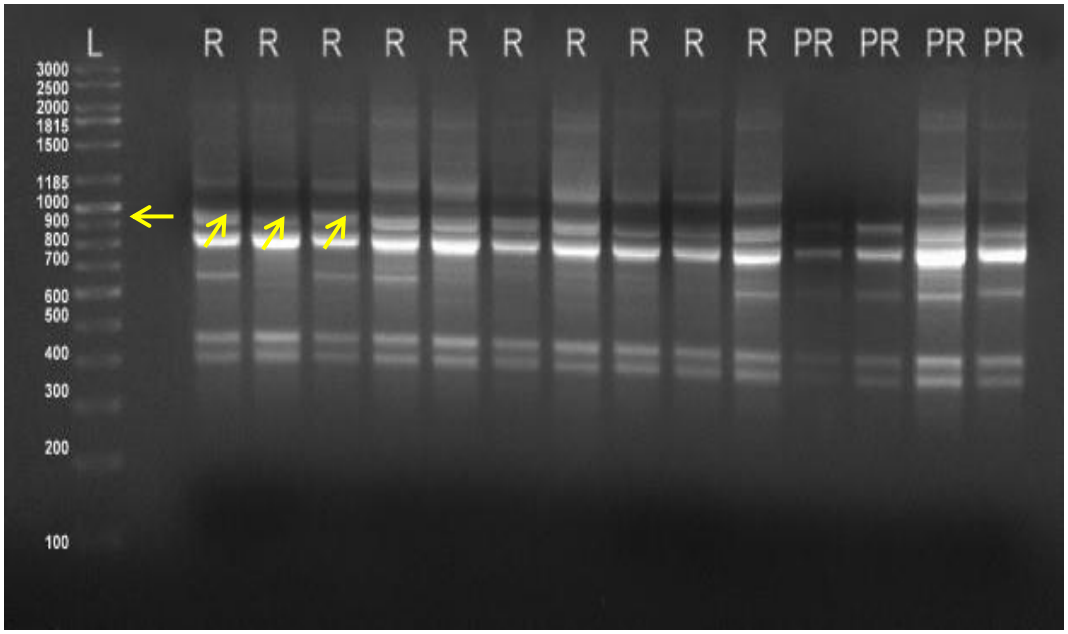



Fig 4. Amplification pattern of the ISSR marker UBC 811 in segregating generation of the cross 14.6 X 16.11 showing resistant/ partially resistant reaction to VSD

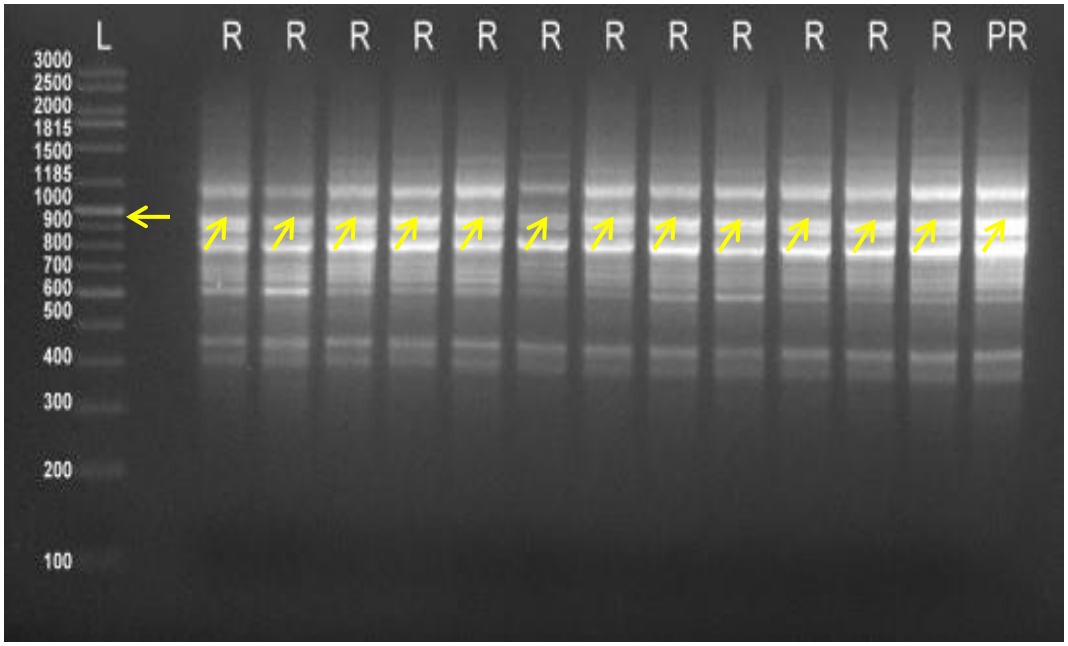



Fig 5. Amplification pattern of the ISSR marker UBC 811 in segregating generation of the cross 16.10 X 2.3 showing resistant/ partially resistant reaction to VSD

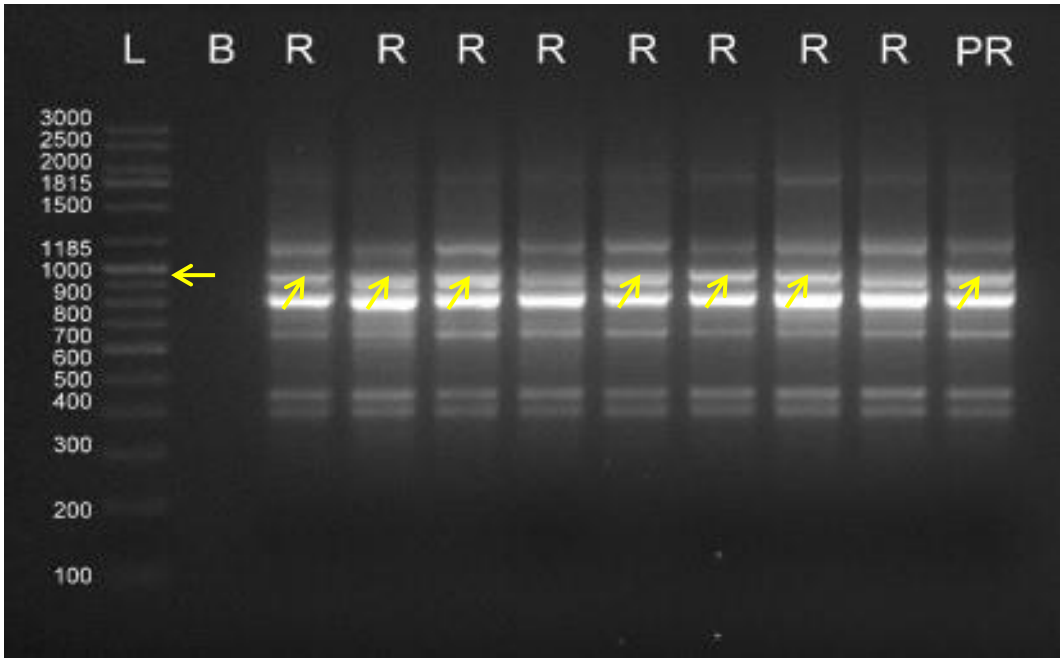



Fig 6a. Amplification pattern of the ISSR marker UBC 811 in segregating generation of the cross 16.11 X 4.1 showing resistant/ partially resistant reaction to VSD

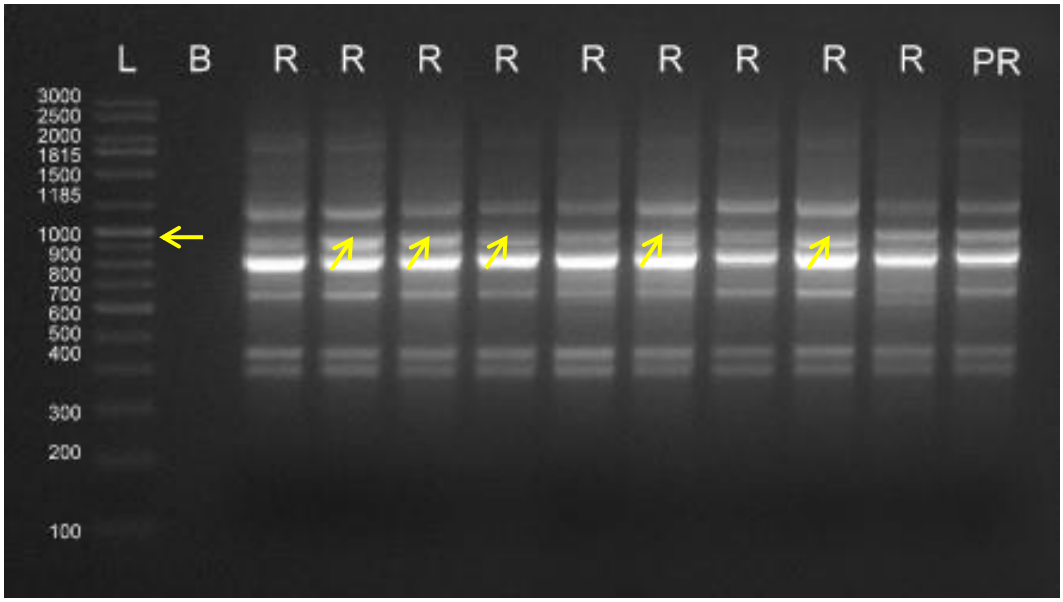



Fig 6b. Amplification pattern of the ISSR marker UBC 811 in segregating generation of the cross 16.11 X 4.1 1 showing resistant/ partially resistant reaction to VSD

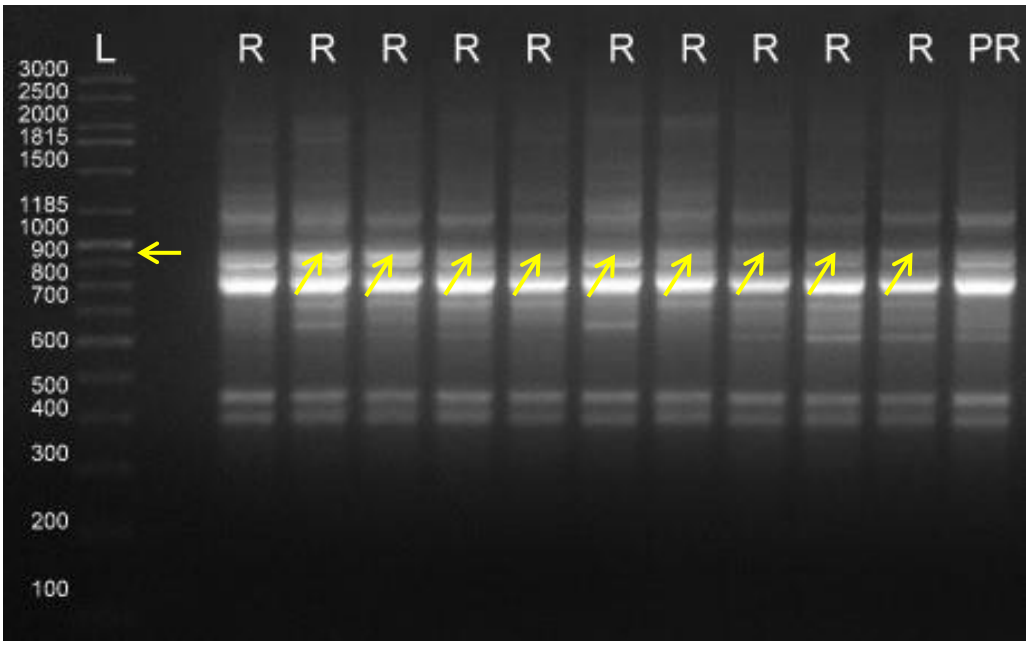



Fig 7a. Amplification pattern of the ISSR marker UBC 811 in segregating generation of the cross 16.11 X 13.13 showing resistant/ partially resistant reaction to VSD

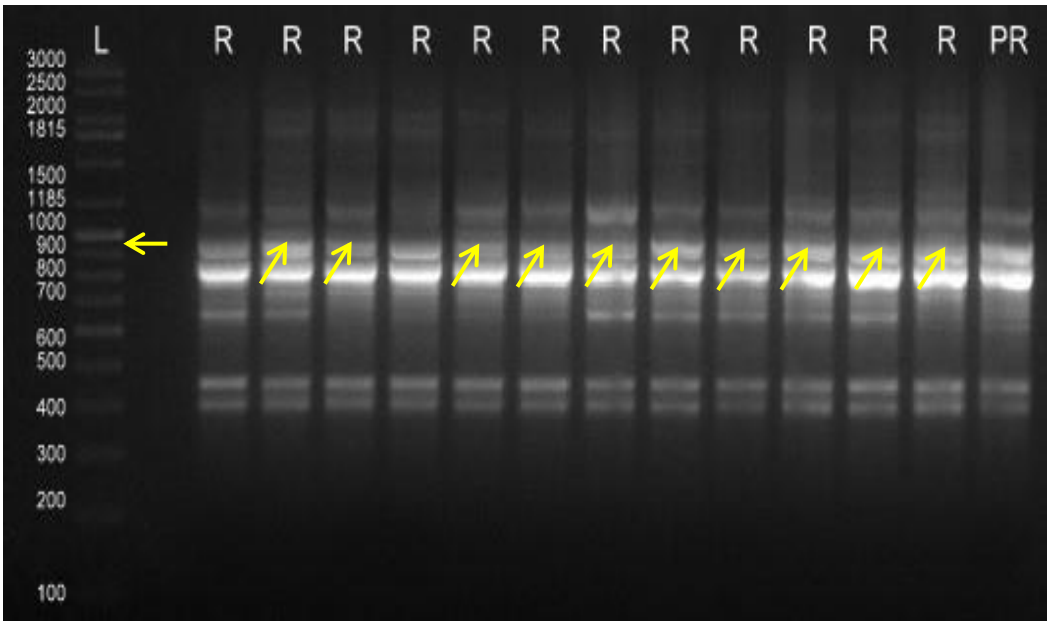



Fig 7b. Amplification pattern of the ISSR marker UBC 811 in segregating generation of the cross 16.11 X 13.13 showing resistant/ partially resistant reaction to VSD

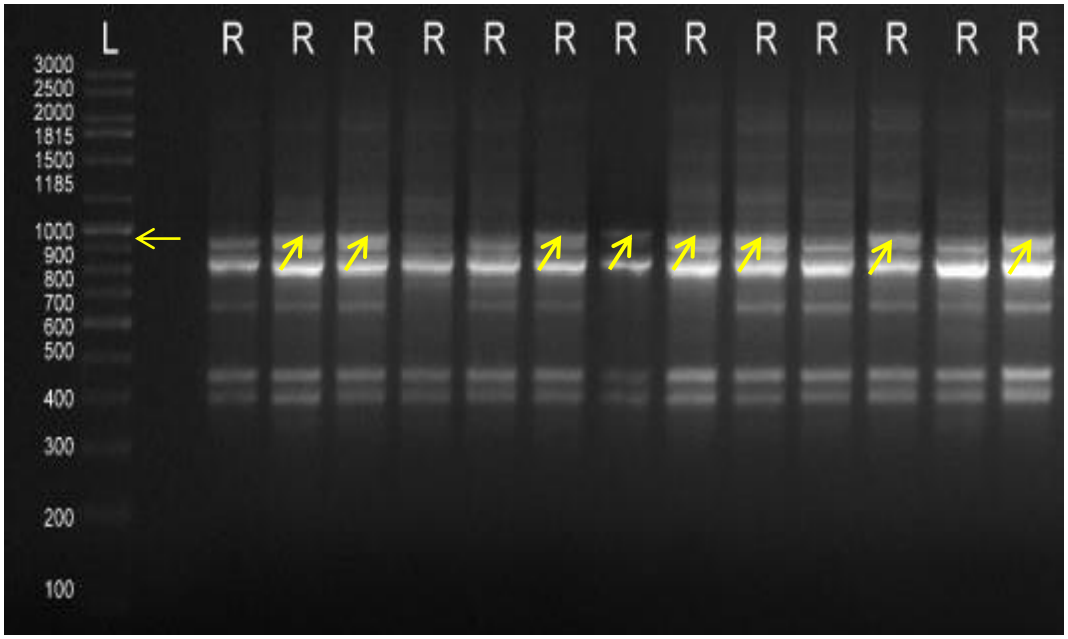



Fig 7c. Amplification pattern of the ISSR marker UBC 811 in segregating generation of the cross 16.11 X 13.13 showing resistant/ partially resistant reaction to VSD

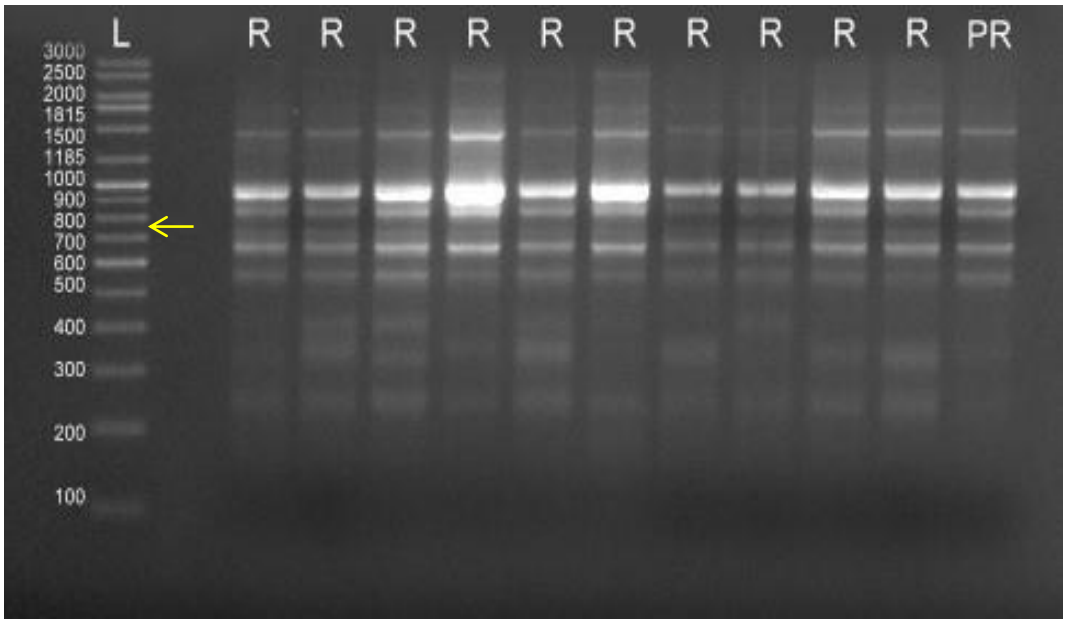



Fig 8a. Amplification pattern of the ISSR marker UBC 815 in segregating progeny of the cross 4.1 X 15.7 showing resistant/ partially resistant reaction to VSD

Among all the seven crosses, one hundred and six resistant seedlings and fourteen partially resistant seedlings were screened with the primer UBC 811, out of which the marker band was observed in seventy seven resistant and three partially resistant plants. The presence of marker band was not detected in all the resistant seedlings. The primer UBC 811 is linked to only a single gene and thus the 950 bp band represents the presence of only a single gene conferring resistance to VSD. The resistance to VSD is provided by multiple genes *ie*., VSD resistance is polygenic in nature (Tan and Tan, 1988; Van der Vossen, 1997). Also, Additive gene effect is observed for VSD resistance in cocoa ie., contribution to resistance is made by the combined effect of presence of each of the single gene conferring resistance to VSD.

Hence, in the twenty nine resistant seedlings in which the presence of marker band was not observed, the resistance to VSD might be provided by other genes which are not yet identified. Also, out of the fourteen partially resistant seedlings screened, the marker band was observed in three seedlings. These seedlings became partially resistant due to the absence of the major genes conferring resistance to VSD. Hence, the resistant gene linked to the marker UBC 811 might be present only in the three partially resistant seedlings.

#### 4.5.1.2 Inheritance of ISSR marker UBC 815 in segregating progeny

Genomic DNA of one hundred and twenty seedlings from seven crosses were screened with the primer UBC 815. A band of size 0.750 kb was found to be present in the resistant seedlings when amplified with the primer in the previous studies (Chandrakant, 2014; Tulshiram, 2016) and thus indicating that the marker is linked with the gene contributing to VSD resistance. An average of seven amplicons were obtained from the seedlings of each cross and their molecular weight varied from 0.2 to 2.5 kb. Details of the presence of the marker band by amplifying with the primer UBC 815 on one hundred and twenty seedlings are listed in Table 10.

**Table 10. Inheritance of ISSR marker UBC 815 in segregating progeny**

| Sl. No. | Cross | Seedlings | Reaction | UBC 815 (750 bp) |
|---|---|---|---|---|
| 1 | 4.1× 15.7 | 1.1 | R | - |
| 2 | | 1.2 | R | - |
| 3 | | 1.3 | R | - |
| 4 | | 1.4 | R | - |
| 5 | | 1.5 | R | - |
| 6 | | 1.6 | R | - |
| 7 | | 1.7 | R | - |
| 8 | | 1.8 | R | - |
| 9 | | 1.9 | R | - |
| 10 | | 1.10 | R | - |
| 11 | | 1.11 | R | - |
| 12 | | 1.12 | R | - |
| 13 | | 1.13 | R | - |
| 14 | | 1.14 | R | - |
| 15 | | 1.15 | R | - |
| 16 | | 1.16 | R | - |
| 17 | | 1.17 | R | - |
| 18 | | 1.18 | R | - |
| 19 | | 1.19 | R | - |
| 20 | | 1.20 | R | - |
| 21 | | 1.21 | PR | - |
| 22 | | 1.22 | PR | - |
| 23 | 11.10× 16.11 | 2.1 | R | - |
| 24 | | 2.2 | R | - |
| 25 | | 2.3 | R | - |
| 26 | | 2.4 | R | - |
| 27 | | 2.5 | R | - |

| | | | | |
|---|---|---|---|---|
| 28 | 11.10× 16.11 | 2.6 | R | - |
| 29 | | 2.7 | R | - |
| 30 | | 2.8 | PR | - |
| 31 | 14.6× 4.1 | 3.1 | R | - |
| 32 | | 3.2 | R | - |
| 33 | | 3.3 | R | - |
| 34 | | 3.4 | R | - |
| 35 | | 3.5 | R | - |
| 36 | | 3.6 | PR | - |
| 37 | | 3.7 | PR | - |
| 38 | 14.6× 16.11 | 4.1 | R | - |
| 39 | | 4.2 | R | - |
| 40 | | 4.3 | R | - |
| 41 | | 4.4 | R | - |
| 42 | | 4.5 | R | - |
| 43 | | 4.6 | R | - |
| 44 | | 4.7 | R | - |
| 45 | | 4.8 | R | - |
| 46 | | 4.9 | R | - |
| 47 | | 4.10 | R | - |
| 48 | | 4.11 | PR | - |
| 49 | | 4.12 | PR | - |
| 50 | | 4.13 | PR | - |
| 51 | | 4.14 | PR | - |
| 52 | 16.10× 2.3 | 5.1 | R | + |
| 53 | | 5.2 | R | + |
| 54 | | 5.3 | R | + |
| 55 | | 5.4 | R | + |
| 56 | | 5.5 | R | + |
| 57 | | 5.6 | R | + |

| | | | | |
|---|---|---|---|---|
| 58 | 16.10× 2.3 | 5.7 | R | + |
| 59 | | 5.8 | R | + |
| 60 | | 5.9 | R | + |
| 61 | | 5.10 | R | + |
| 62 | | 5.11 | R | + |
| 63 | | 5.12 | R | + |
| 64 | | 5.13 | PR | - |
| 65 | 16.11× 4.1 | 6.1 | R | + |
| 66 | | 6.2 | R | + |
| 67 | | 6.3 | R | + |
| 68 | | 6.4 | R | + |
| 69 | | 6.5 | R | + |
| 70 | | 6.6 | R | - |
| 71 | | 6.7 | R | + |
| 72 | | 6.8 | R | + |
| 73 | | 6.9 | R | - |
| 74 | | 6.10 | R | - |
| 75 | | 6.11 | R | + |
| 76 | | 6.12 | R | + |
| 77 | | 6.13 | R | - |
| 78 | | 6.14 | R | - |
| 79 | | 6.15 | R | - |
| 80 | | 6.16 | R | - |
| 81 | | 6.17 | R | - |
| 82 | | 6.18 | PR | - |
| 83 | | 6.19 | PR | - |
| 84 | 16.11× 13.13 | 7.1 | R | + |
| 85 | | 7.2 | R | + |
| 86 | | 7.3 | R | + |
| 87 | | 7.4 | R | + |

| | | | | |
|---|---|---|---|---|
| 88 | 16.11× 13.13 | 7.5 | R | - |
| 89 | | 7.6 | R | - |
| 90 | | 7.7 | R | - |
| 91 | | 7.8 | R | - |
| 92 | | 7.9 | R | - |
| 93 | | 7.10 | R | - |
| 94 | | 7.11 | R | - |
| 95 | | 7.12 | R | - |
| 96 | | 7.13 | R | - |
| 97 | | 7.14 | R | - |
| 98 | | 7.15 | R | - |
| 99 | | 7.16 | R | - |
| 100 | | 7.17 | R | - |
| 101 | | 7.18 | R | - |
| 102 | | 7.19 | R | - |
| 103 | | 7.20 | R | - |
| 104 | | 7.21 | R | - |
| 105 | | 7.22 | R | - |
| 106 | | 7.23 | R | - |
| 107 | | 7.24 | R | - |
| 108 | | 7.25 | R | - |
| 109 | | 7.26 | R | - |
| 110 | | 7.27 | R | - |
| 111 | | 7.28 | R | - |
| 112 | | 7.29 | R | - |
| 113 | | 7.30 | R | - |
| 114 | | 7.31 | R | - |
| 115 | | 7.32 | R | - |
| 116 | | 7.33 | R | - |
| 117 | | 7.34 | R | - |

| | | | | |
|---|---|---|---|---|
| 118 | 16.11× 13.13 | 7.35 | R | - |
| 119 | | 7.36 | PR | + |
| 120 | | 7.37 | PR | - |

The polymorphic band of 0.750 kb, which was identified to be linked with VSD resistance from the earlier studies was not at all detected in four crosses *viz*., 4.1× 15.7, 11.10× 16.11, 14.6× 4.1 and 14.6× 16.11 (Fig. 8a, 8b, 9, 10, 11). However, the polymorphic band of 0.750 kb was present in all the parents (Tulshiram, 2016).

Out of the twelve resistant and one partially resistant seedling screened in the cross between the parents 16.10 and 2.3, the marker band was detected in all the resistant seedlings (Fig. 12). When the parents were screened, the 750 bp band was present in both the trees (Tulshiram, 2016). In the cross 16.11× 4.1, only nine resistant seedlings were detected with the presence of marker band, out of the seventeen resistant and two partially resistant seedlings screened (Fig. 13a, 13b). Marker band was detected in both the parents (Tulshiram, 2016).

In the cross 16.11×13.13, DNA isolated from thirty five resistant and two partially resistant seedlings were amplified with the primer UBC 815. Presence of the 750 bp band was found only in four resistant seedlings and one partially resistant seedling (Fig. 14a, 14b, 14c). The polymorphic band of 750 bp was present in both the parents (Tulshiram, 2016).

Out of the one hundred and six resistant and fourteen partially resistant seedlings screened, the marker band of 750 bp was detected only in twenty five resistant and one partially resistant seedling. In a polygenic gene action, the level of contribution given by each gene differs a lot (Tan and Tan, 1988). Hence, the gene to which the marker UBC 815 is linked, may not be contributing much to VSD resistance and hence in majority of the plants, even though this gene was not present, they expressed complete resistance. However, they turned out to be resistant because of the presence of other genes conferring VSD resistance. Also,

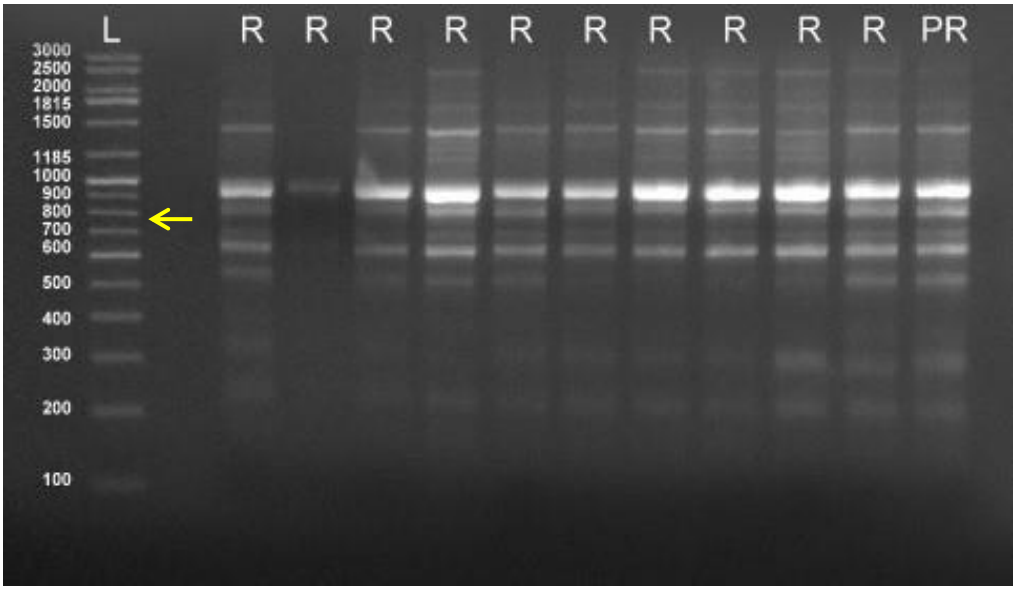



Fig 8b. Amplification pattern of the ISSR marker UBC 815 in segregating progeny of the cross 4.1 X 15.7 showing resistant/ partially resistant reaction to VSD

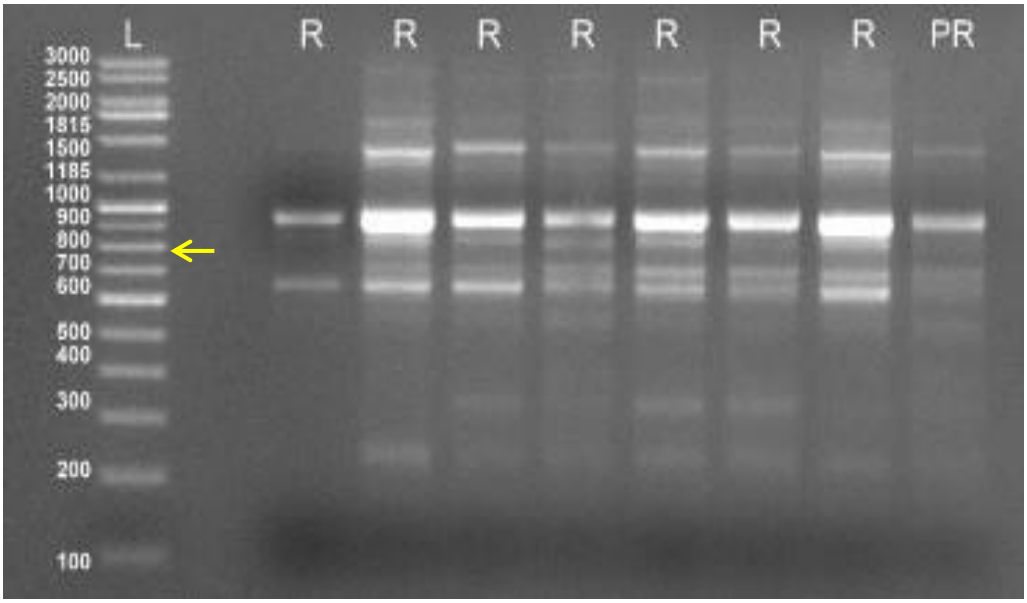



Fig 9. Amplification pattern of the ISSR marker UBC 815 in segregating generation of the cross 11.10 X 16.11 showing resistant/ partially resistant reaction to VSD

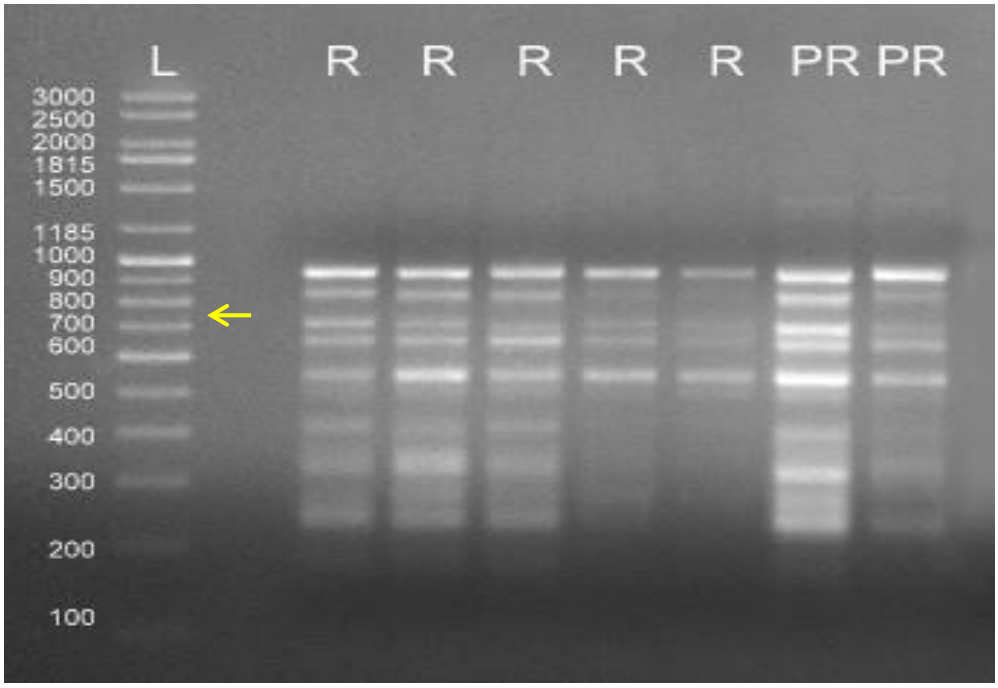



Fig 10. Amplification pattern of the ISSR marker UBC 815 in segregating generation of the cross 14.6 X 4.1 showing resistant/ partially resistant reaction to VSD

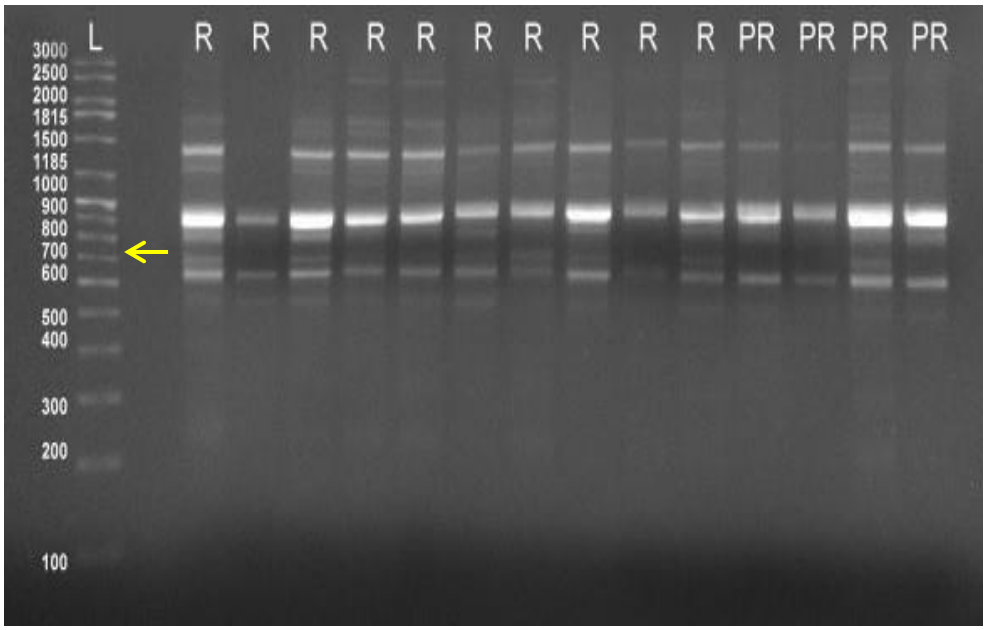



Fig 11. Amplification pattern of the ISSR marker UBC 815 in segregating generation of the cross 14.6 X 16.11 showing resistant/ partially resistant reaction to VSD

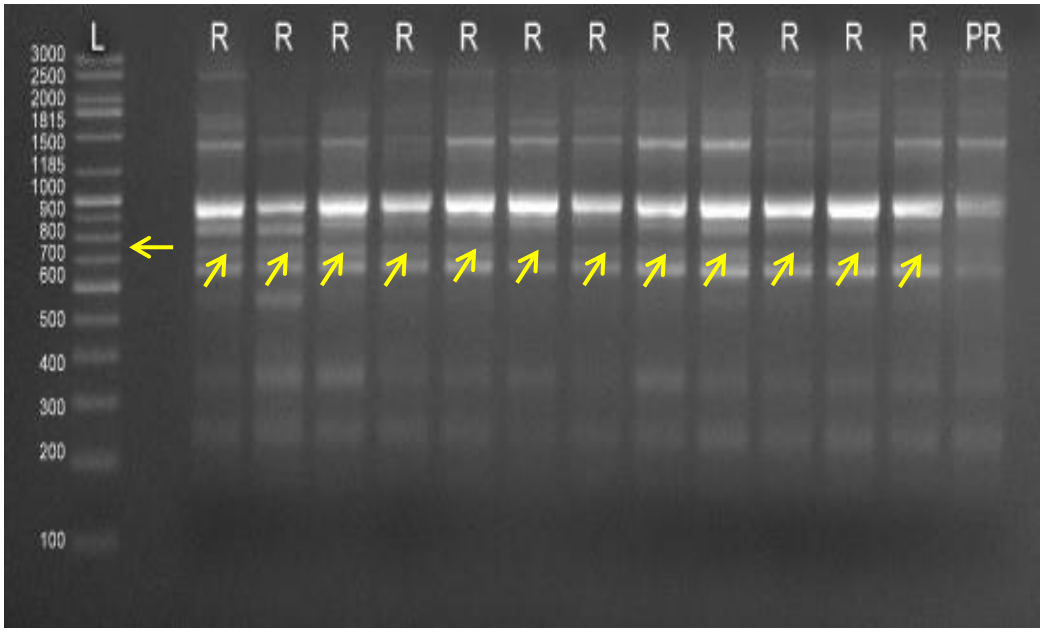



Fig 12. Amplification pattern of the ISSR marker UBC 815 in segregating generation of the cross 16.10 X 2.3 showing resistant/ partially resistant reaction to VSD

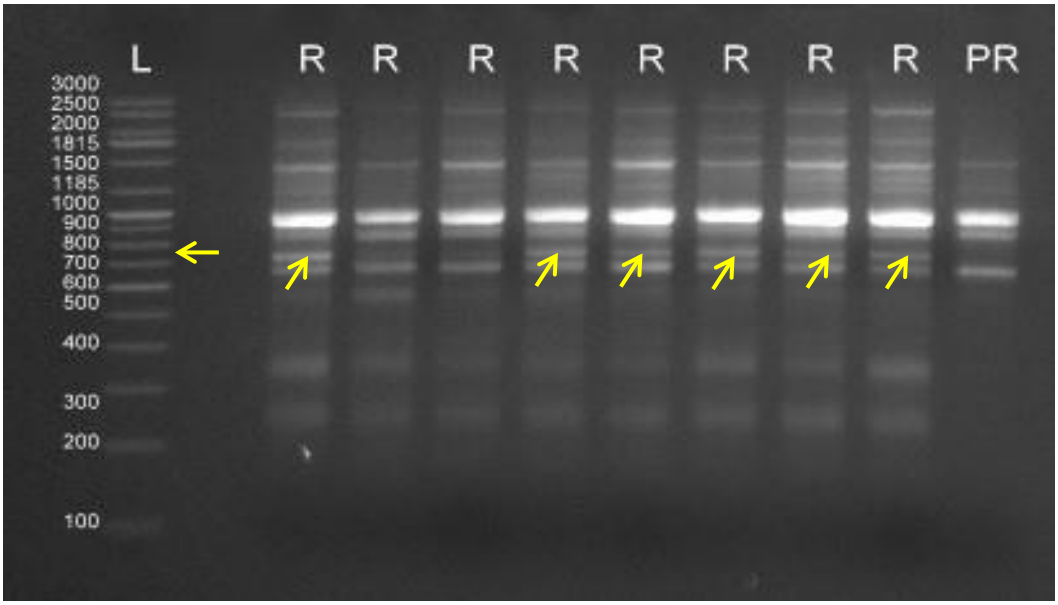



Fig 13a. Amplification pattern of the ISSR marker UBC 815 in segregating generation of the cross 16.11 X 4.1 showing resistant/ partially resistant reaction to VSD

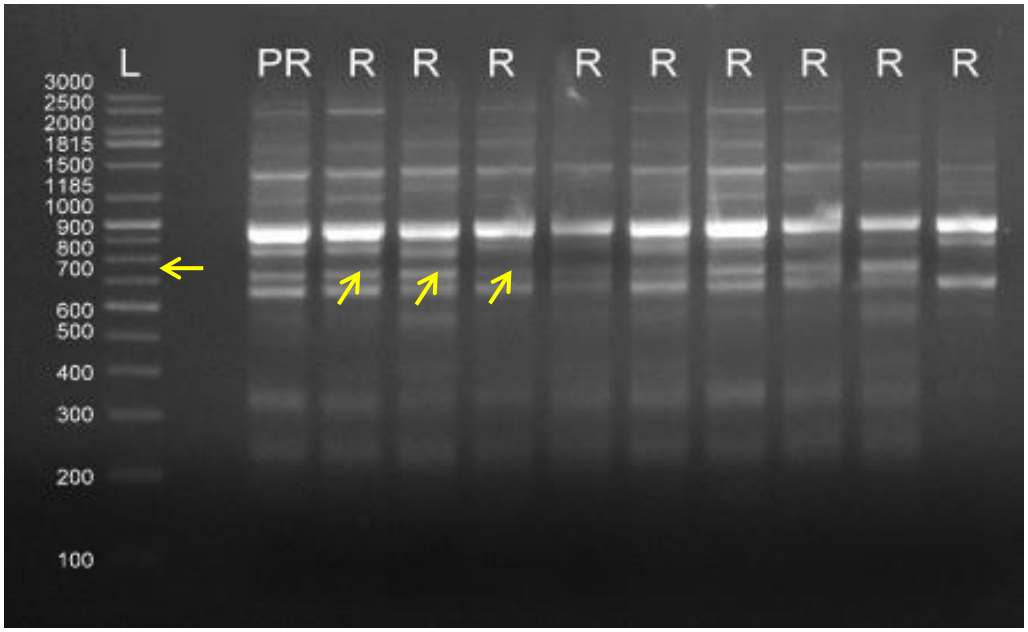



Fig 13b. Amplification pattern of the ISSR marker UBC 815 in segregating generation of the cross 16.11 X 4.1 showing resistant/ partially resistant reaction to VSD

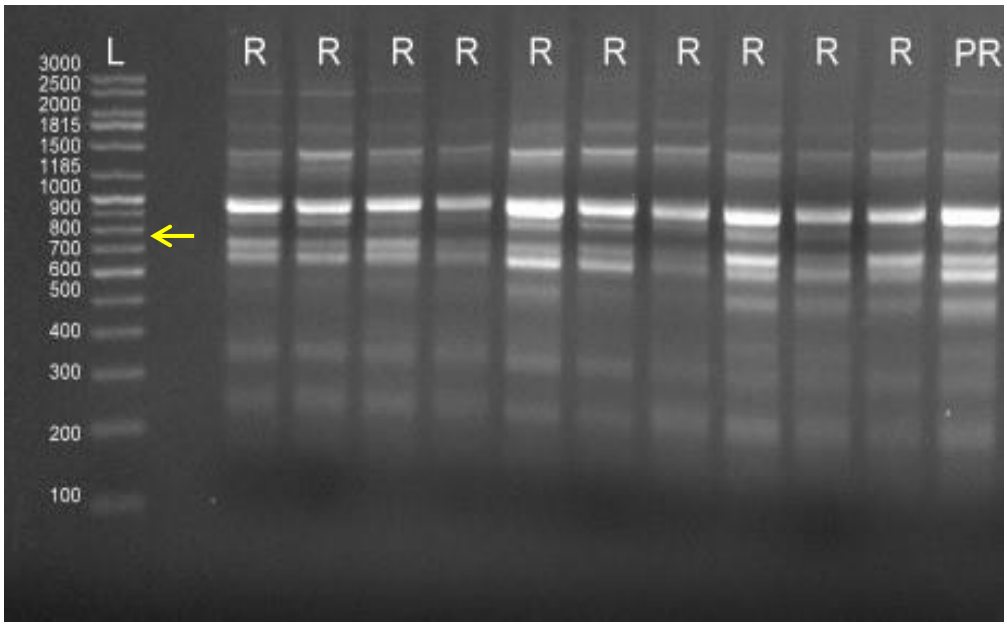



Fig 14a. Amplification pattern of the ISSR marker UBC 815 in segregating generation of the cross 16.11 X 13.13 showing resistant/ partially resistant reaction to VSD

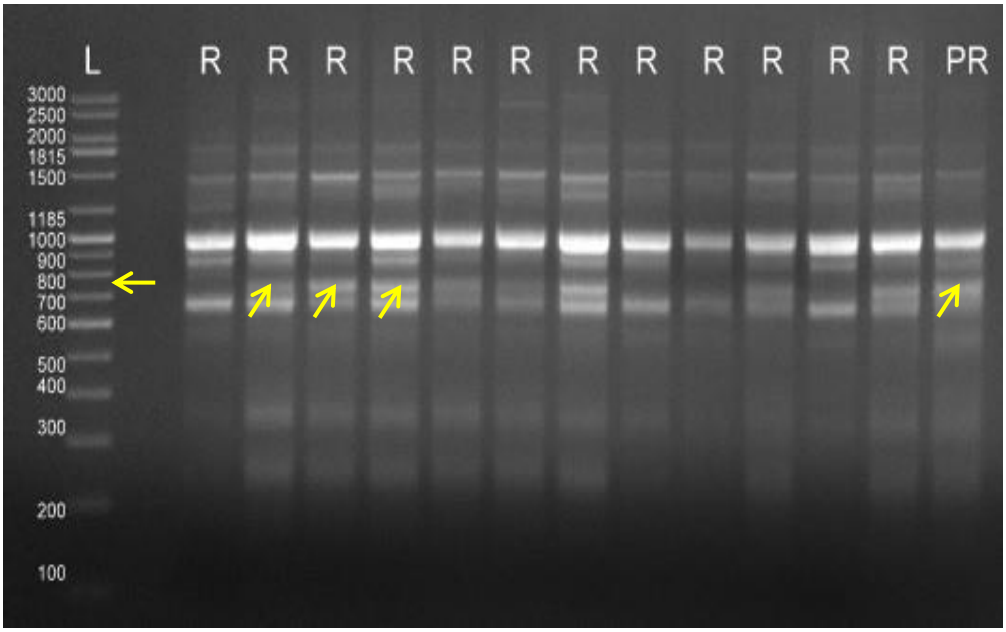



Fig 14b. Amplification pattern of the ISSR marker UBC 815 in segregating generation of the cross 16.11 X 13.13 showing resistant/ partially resistant reaction to VSD

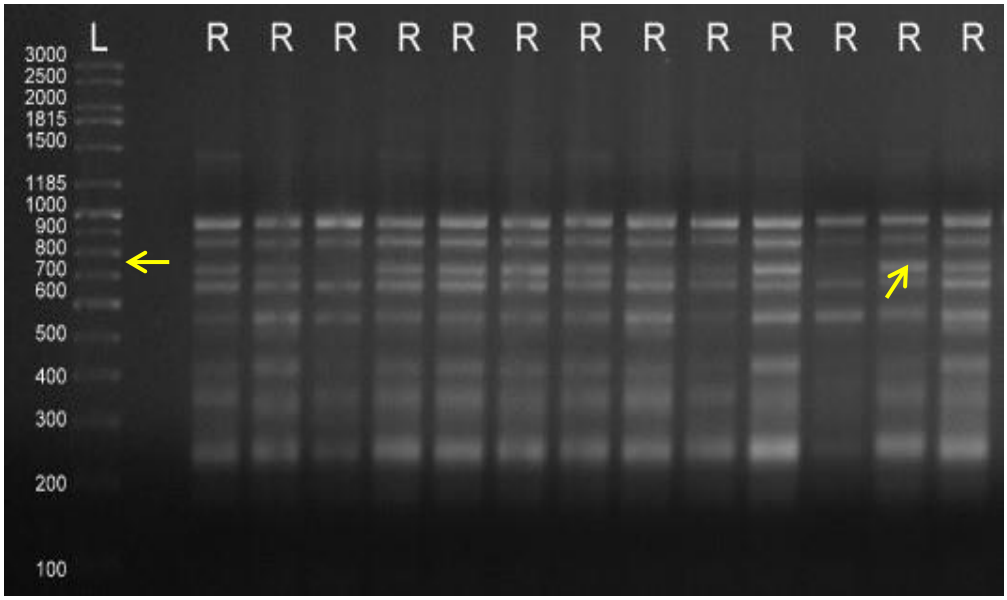



Fig 14c. Amplification pattern of the ISSR marker UBC 815 in segregating generation of the cross 16.11 X 13.13 showing resistant/ partially resistant reaction to VSD

in one of the partially resistant seedling, the presence of this gene was observed. Since its contribution to VSD resistance is very low, even though it is present, that seedling was not able to express complete resistance because of the absence of other major resistant genes.

#### 4.5.1.3 Inheritance of ISSR marker UBC 857 in segregating progeny

Diluted DNA from the seedlings of seven crosses were amplified with the primer UBC 857. Earlier studies using this primer had shown that the molecular band of size 0.450 kb was present in all the resistant seedlings screened (Chandrakant, 2014; Tulshiram, 2016), thus validated that the marker UBC 857 is linked with VSD resistance in cocoa. On an average, nine amplicons were obtained from each cross. Molecular weight of the amplicons varied from 0.3 to 2.5 kb. Details of the screening with the primer UBC 857 are furnished in the Table 11.

**Table 11. Inheritance of ISSR marker UBC 857 in segregating progeny**

| Sl. No. | Cross | Seedlings | Reaction | UBC 857 (450 bp) |
|---|---|---|---|---|
| 1 | 4.1× 15.7 | 1.1 | R | - |
| 2 | | 1.2 | R | - |
| 3 | | 1.3 | R | - |
| 4 | | 1.4 | R | - |
| 5 | | 1.5 | R | - |
| 6 | | 1.6 | R | - |
| 7 | | 1.7 | R | - |
| 8 | | 1.8 | R | - |
| 9 | | 1.9 | R | - |
| 10 | | 1.10 | R | - |
| 11 | | 1.11 | R | - |
| 12 | | 1.12 | R | - |
| 13 | | 1.13 | R | - |

| | | | | |
|---|---|---|---|---|
| 14 | 4.1× 15.7 | 1.14 | R | - |
| 15 | | 1.15 | R | - |
| 16 | | 1.16 | R | - |
| 17 | | 1.17 | R | - |
| 18 | | 1.18 | R | - |
| 19 | | 1.19 | R | - |
| 20 | | 1.20 | R | - |
| 21 | | 1.21 | PR | - |
| 22 | | 1.22 | PR | - |
| 23 | 11.10× 16.11 | 2.1 | R | + |
| 24 | | 2.2 | R | - |
| 25 | | 2.3 | R | - |
| 26 | | 2.4 | R | - |
| 27 | | 2.5 | R | + |
| 28 | | 2.6 | R | - |
| 29 | | 2.7 | R | - |
| 30 | | 2.8 | PR | - |
| 31 | 14.6× 4.1 | 3.1 | R | + |
| 32 | | 3.2 | R | + |
| 33 | | 3.3 | R | + |
| 34 | | 3.4 | R | + |
| 35 | | 3.5 | R | - |
| 36 | | 3.6 | PR | - |
| 37 | | 3.7 | PR | - |
| 38 | 14.6× 16.11 | 4.1 | R | - |
| 39 | | 4.2 | R | - |
| 40 | | 4.3 | R | - |
| 41 | | 4.4 | R | - |
| 42 | | 4.5 | R | - |
| 43 | | 4.6 | R | - |

| | | | | |
|---|---|---|---|---|
| 44 | 14.6× 16.11 | 4.7 | R | - |
| 45 | | 4.8 | R | - |
| 46 | | 4.9 | R | - |
| 47 | | 4.10 | R | - |
| 48 | | 4.11 | PR | - |
| 49 | | 4.12 | PR | - |
| 50 | | 4.13 | PR | - |
| 51 | | 4.14 | PR | - |
| 52 | 16.10× 2.3 | 5.1 | R | - |
| 53 | | 5.2 | R | - |
| 54 | | 5.3 | R | - |
| 55 | | 5.4 | R | - |
| 56 | | 5.5 | R | - |
| 57 | | 5.6 | R | - |
| 58 | | 5.7 | R | - |
| 59 | | 5.8 | R | - |
| 60 | | 5.9 | R | - |
| 61 | | 5.10 | R | - |
| 62 | | 5.11 | R | - |
| 63 | | 5.12 | R | - |
| 64 | | 5.13 | PR | - |
| 65 | 16.11× 4.1 | 6.1 | R | - |
| 66 | | 6.2 | R | - |
| 67 | | 6.3 | R | - |
| 68 | | 6.4 | R | - |
| 69 | | 6.5 | R | - |
| 70 | | 6.6 | R | - |
| 71 | | 6.7 | R | - |
| 72 | | 6.8 | R | - |
| 73 | | 6.9 | R | - |

| | | | | |
|---|---|---|---|---|
| 74 | 16.11× 4.1 | 6.10 | R | - |
| 75 | | 6.11 | R | - |
| 76 | | 6.12 | R | - |
| 77 | | 6.13 | R | - |
| 78 | | 6.14 | R | - |
| 79 | | 6.15 | R | - |
| 80 | | 6.16 | R | - |
| 81 | | 6.17 | R | - |
| 82 | | 6.18 | PR | - |
| 83 | | 6.19 | PR | - |
| 84 | 16.11× 13.13 | 7.1 | R | - |
| 85 | | 7.2 | R | + |
| 86 | | 7.3 | R | + |
| 87 | | 7.4 | R | + |
| 88 | | 7.5 | R | + |
| 89 | | 7.6 | R | + |
| 90 | | 7.7 | R | + |
| 91 | | 7.8 | R | + |
| 92 | | 7.9 | R | - |
| 93 | | 7.10 | R | + |
| 94 | | 7.11 | R | + |
| 95 | | 7.12 | R | + |
| 96 | | 7.13 | R | + |
| 97 | | 7.14 | R | - |
| 98 | | 7.15 | R | + |
| 99 | | 7.16 | R | + |
| 100 | | 7.17 | R | + |
| 101 | | 7.18 | R | + |
| 102 | | 7.19 | R | - |
| 103 | | 7.20 | R | - |

| | | | | |
|---|---|---|---|---|
| 104 | 16.11× 13.13 | 7.21 | R | - |
| 105 | | 7.22 | R | - |
| 106 | | 7.23 | R | - |
| 107 | | 7.24 | R | - |
| 108 | | 7.25 | R | - |
| 109 | | 7.26 | R | - |
| 110 | | 7.27 | R | - |
| 111 | | 7.28 | R | - |
| 112 | | 7.29 | R | - |
| 113 | | 7.30 | R | - |
| 114 | | 7.31 | R | - |
| 115 | | 7.32 | R | - |
| 116 | | 7.33 | R | - |
| 117 | | 7.34 | R | - |
| 118 | | 7.35 | R | - |
| 119 | | 7.36 | PR | - |
| 120 | | 7.37 | PR | + |

In the cross between 4.1 and 15.7, genomic DNA isolated from twenty resistant and two partially resistant seedlings were amplified using this primer out of which the previously identified polymorphic marker band of 0.450 kb was not detected in any of them (Fig. 15a and 15b). The marker band of 450 bp was present in both the parents (Tulshiram, 2016). In 11.10× 16.11, the presence of 450 bp band was found in two out of seven resistant seedlings screened. The marker band was not detected in any of the partially resistant plants screened (Fig. 16). In both the parents crossed, the marker band was present when screened with the primer UBC 857 (Tulshiram, 2016).

In the cross between 14.6 and 4.1, the marker band was found to be present in only four out of five resistant seedlings screened. It was absent in the partially resistant seedlings screened (Fig. 17). The polymorphic band of 450 bp was

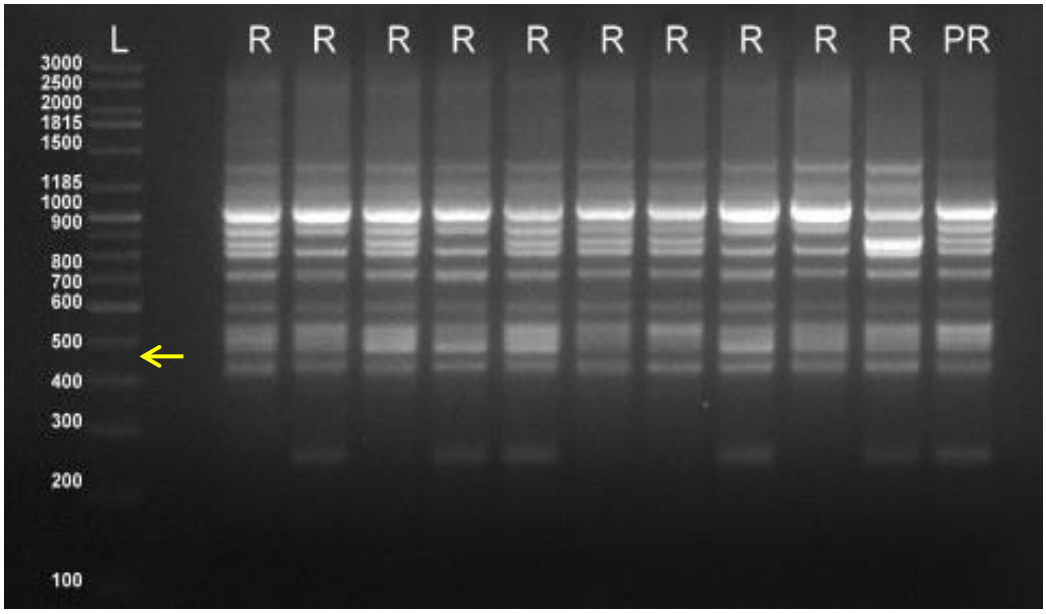



Fig 15a. Amplification pattern of the ISSR marker UBC 857 in segregating progeny of the cross 4.1X 15.7 showing resistant/ partially resistant reaction to VSD

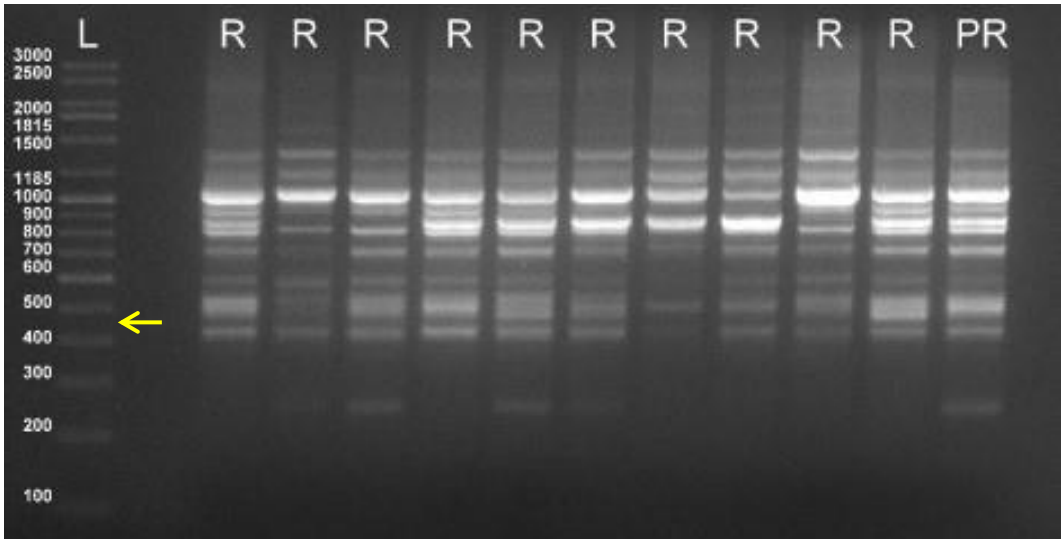



Fig 15b. Amplification pattern of the ISSR marker UBC 857 in segregating progeny of the cross 4.1X 15.7 showing resistant/ partially resistant reaction to VSD

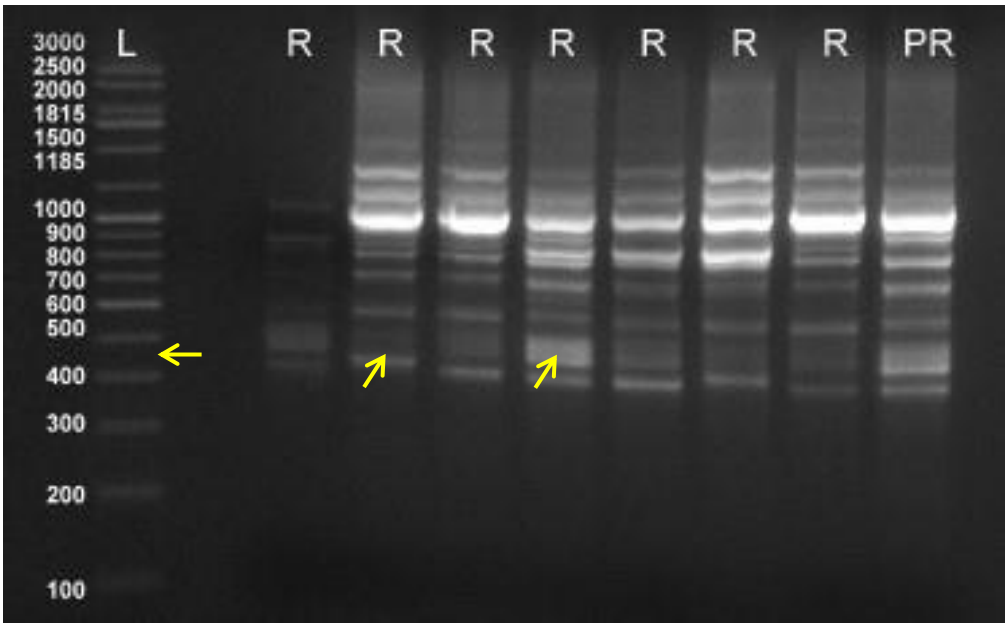



Fig 16. Amplification pattern of the ISSR marker UBC 857 in segregating progeny of the cross 11.10X 16.11 showing resistant/ partially resistant reaction to VSD

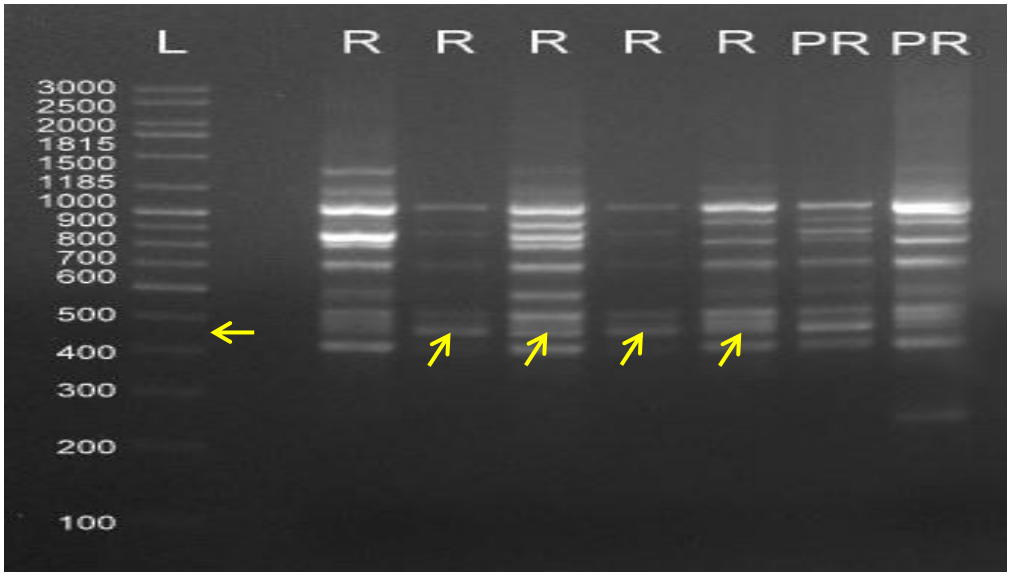



Fig 17. Amplification pattern of the ISSR marker UBC 857 in segregating progeny of the cross 14.6X 4.1 showing resistant/ partially resistant reaction to VSD

present in both the parents (Tulshiram, 2016). In the cross 14.6× 16.11, none of the ten resistant and four partially resistant seedlings screened were detected with the presence of marker band (Fig. 18). The polymorphic band of 450 bp was present in both the parents (Tushiram, 2016).

In the cross between the parents 16.10 and 2.3, twelve resistant and one partially resistant seedling were screened out of which none of them showed the presence of marker band (Fig. 19). The marker band was detected in both the parents (Tulshiram, 2016). In the cross between 16.11 and 4.1, none of the seventeen resistant and two partially resistant seedlings screened were detected with the presence of marker band (Fig. 20a and 20b). However the polymorphic band of 450 bp was present in both the parents (Tulshiram, 2016).

In 16.11× 13.13, genomic DNA from thirty five resistant and two partially resistant seedlings were amplified with the marker UBC 857. The presence of marker band was there in only fifteen resistant and one partially resistant seedlings (Fig. 21a, 21b, 21c). In the study conducted by Tulshiram (2016), the marker band was present in both the parents.

Twenty one resistant and one partially resistant seedlings were detected with the presence of marker band. Since resistance to VSD in cocoa is a contribution of many genes, presence of a single gene contributing to VSD will not make that plant completely resistant. The primer UBC 857 is linked to a single gene which confers VSD resistance. In eighty five resistant plants, the marker band was not observed. In those plants, majority of the other genes which confers VSD resistance might have been present. So those eighty five plants were resistant to VSD disease, even though the marker band was not present in them. Out of the fourteen partially resistant plants screened, one plant was showing the presence of marker band. The gene linked to the marker UBC 857 might be present only in that seedling. These plants behaved as partially resistant as they were not able to express complete resistance due to the absence of majority of genes conferring disease resistance.

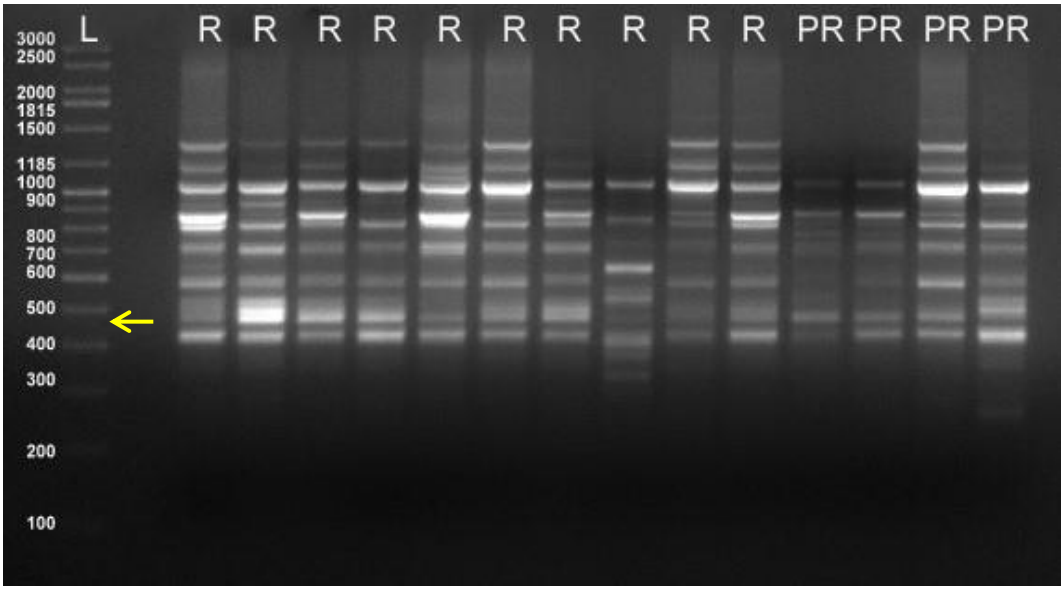



Fig 18. Amplification pattern of the ISSR marker UBC 857 in segregating progeny of the cross 14.6X 16.11 showing resistant/ partially resistant reaction to VSD

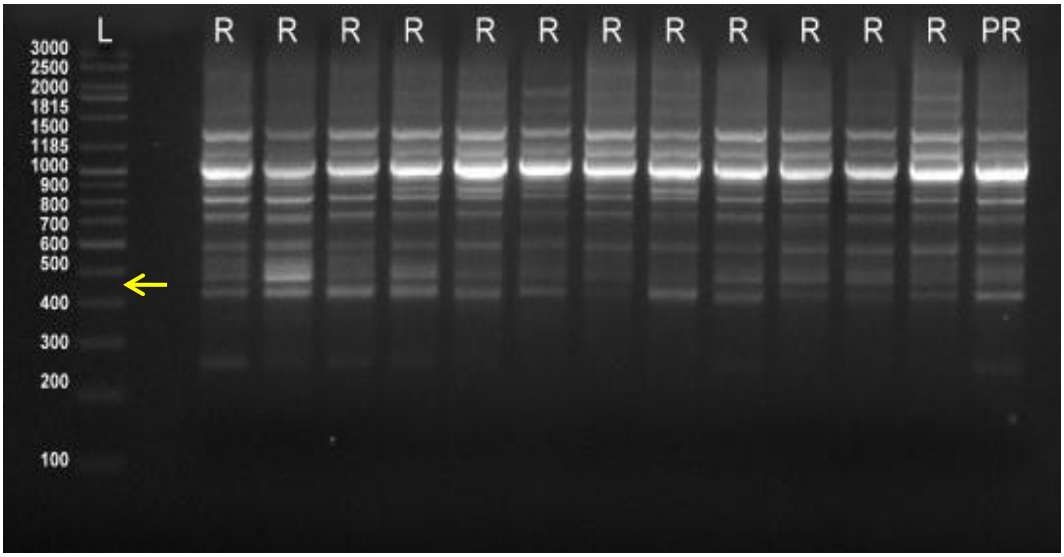



Fig 19. Amplification pattern of the ISSR marker UBC 857 in segregating progeny of the cross 16.10X 2.3 showing resistant/ partially resistant reaction to VSD

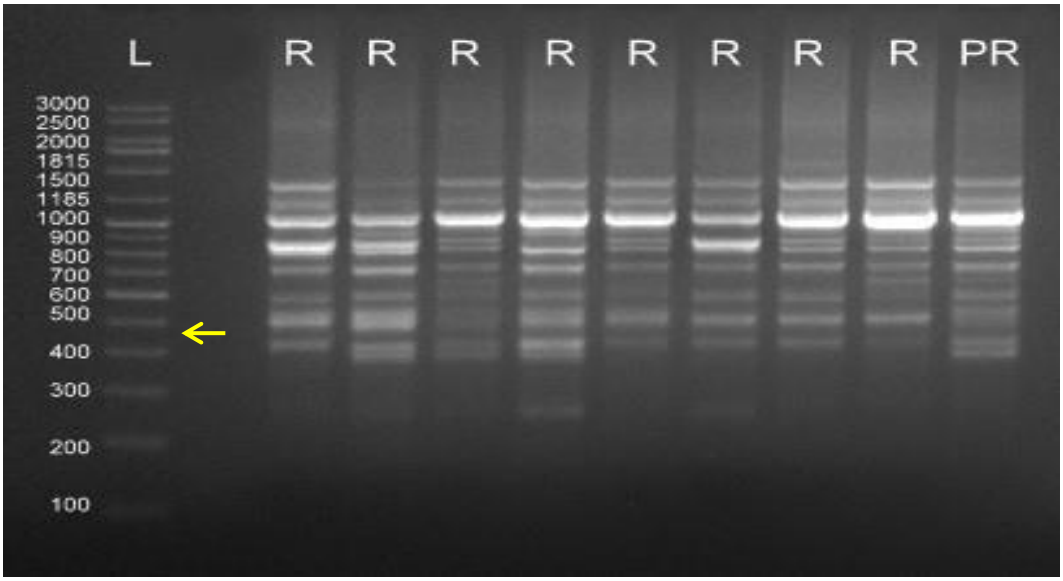



Fig 20a. Amplification pattern of the ISSR marker UBC 857 in segregating progeny of the cross 16.11 X 4.1 showing resistant/ partially resistant reaction to VSD

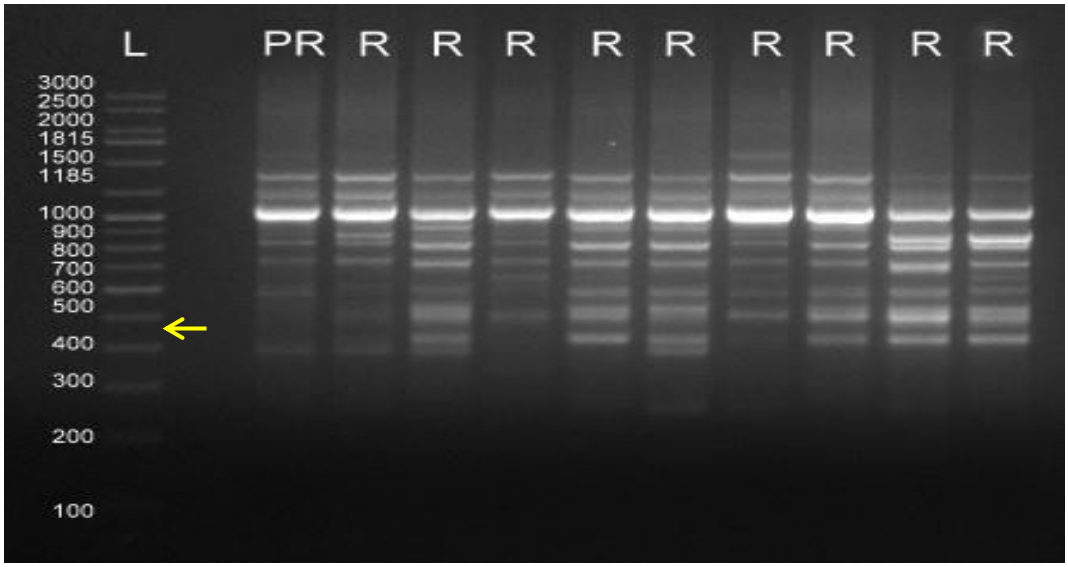



Fig 20b. Amplification pattern of the ISSR marker UBC 857 in segregating progeny of the cross 16.11 X 4.1 showing resistant/ partially resistant reaction to VSD

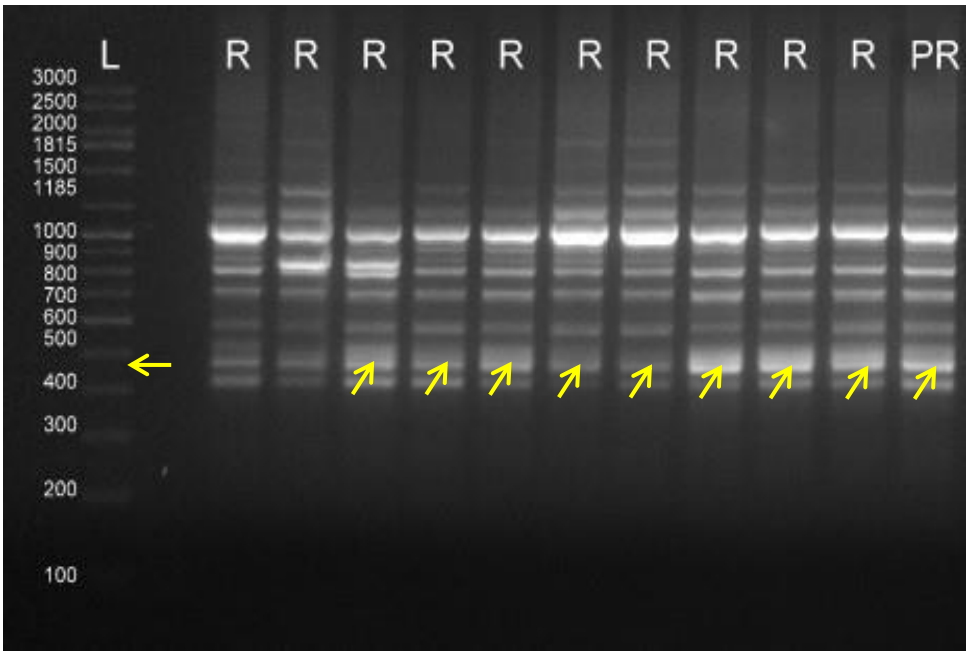



Fig 21a. Amplification pattern of the ISSR marker UBC 857 in segregating progeny of the cross 16.11 X 13.13 showing resistant/ partially resistant reaction to VSD

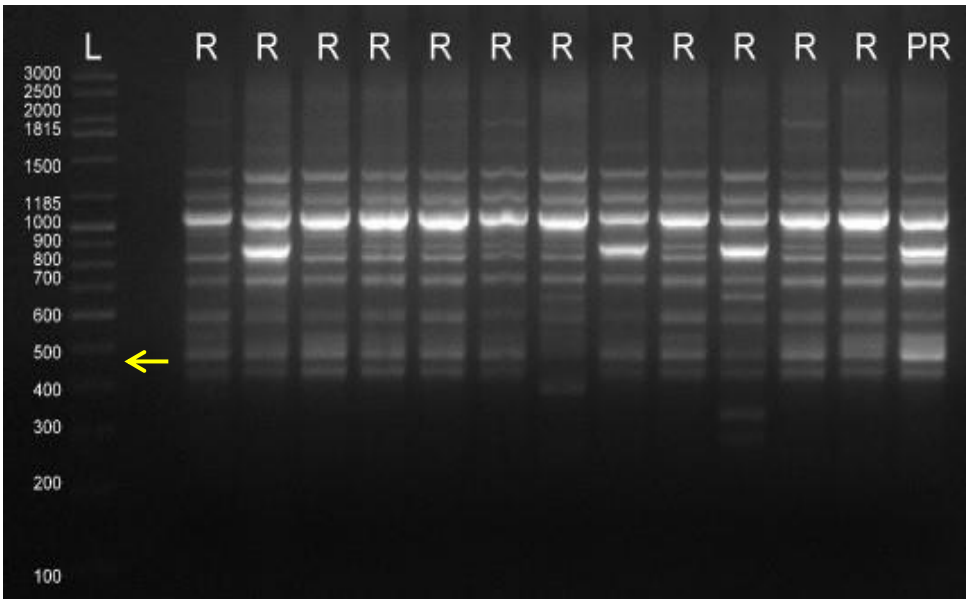



Fig 21b. Amplification pattern of the ISSR marker UBC 857 in segregating progeny of the cross 16.11 X 13.13 showing resistant/ partially resistant reaction to VSD

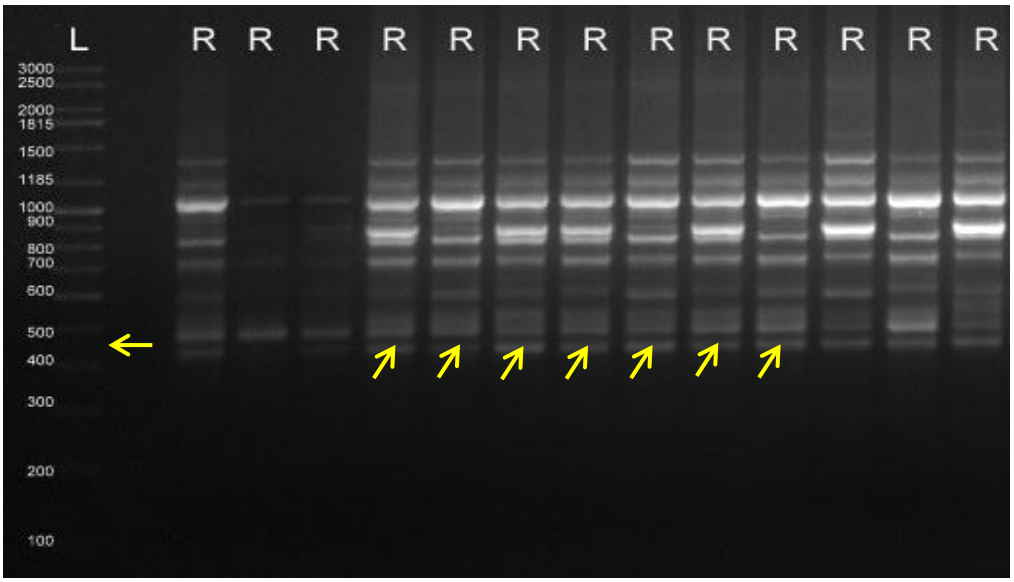



Fig 21c. Amplification pattern of the ISSR marker UBC 857 in segregating progeny of the cross 16.11 X 13.13 showing resistant/ partially resistant reaction to VSD

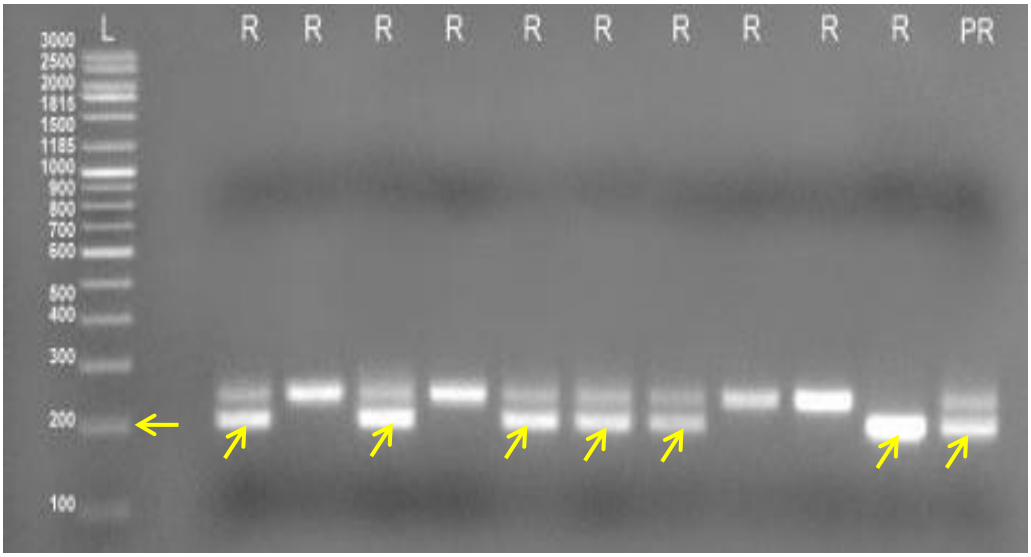



Fig 22a. Amplification pattern of the SSR marker mTcCIR42 in segregating progeny of the cross 4.1X 15.7 showing resistant/ partially resistant reaction to VSD

### 4.5.2 Simple Sequence Repeats (SSR) analysis

Simple Sequence Repeats are DNA sequences with repeat lengths of a few base pairs. Variation in the number of repeats can be detected with PCR by developing primers for the conserved DNA sequence flanking the SSR region. Microsatellites have become the molecular markers of choice for a wide range of applications in genetic mapping, genotype identification and variety protection, seed purity evaluation and germplasm conservation, diversity studies, paternity determination and pedigree analysis, gene and quantitative trait locus analysis, and marker-assisted breeding due to its abundance, level of polymorphism suitability on automated systems, locus specificity and high level of reproducibility. SSRs are present in a majority of the eukaryotic genomes. Molecular characterization of cocoa germplasm has increased significantly because of the development of SSR markers in cocoa.

Chandrakant (2014) eluted the polymorphic 450 bp amplicon from the ISSR primer UBC 857 and subjected it for reamplification. After confirming that it yielded only a single band, it was sequenced and it yielded 246 nucleotides, which on standard nucleotide BLAST had shown 94 per cent identity with the *Theobroma cacao* microsatellite DNA clone of mTcCIR42 SSR. Thus it was identified and validated to be linked with the gene contributing to VSD resistance and was found to clearly differentiate between the VSD resistant, susceptible and partially resistant cocoa clones. According to this study, screening with this primer has yielded a clear band of 650 bp in all the resistant lines screened. The partially resistant clones screened were characterized with the presence of a specific marker at 400 bp. In susceptible accessions, the marker was found to be slightly heavier at 450 bp.

Tulshiram (2016) has done SSR assay with the primer mTcCIR42 on resistant and susceptible lines and yielded a band of 200 bp in majority of the resistant lines indicating that it is associated with VSD resistance. The

amplification conditions for this primer used in the present study is mentioned in the materials and methods section. Amplified fragments were electrophoresed in 2.0 per cent agarose gel. The details of the SSR primer analysed in this study are furnished in Table 4. The result of the analysis with this primer in the present study is detailed and discussed here under.

**4.5.2.1 Inheritance of SSR marker mTcCIR42 in segregating progeny**

SSR amplification was done on the extracted DNA of seedlings of seven crosses and was electrophoresed on two per cent agarose gel. Two amplicons were observed in many crosses but in some seedlings three distinct amplicons were found. The molecular sizes of the bands detected were 0.2, 0.250 and 0.450 kb. According to previous studies, the polymorphic band of size 200 bp was found to be tightly linked to VSD resistance (Tulshiram, 2016). Details of the presence of marker band by screening with the SSR marker are furnished in Table 12.

**Table 12. Inheritance of SSR marker mTcCIR42 in segregating progeny**

| Sl No. | Cross | Seedlings | Reaction | SSR (200 bp) |
|---|---|---|---|---|
| 1 | 4.1× 15.7 | 1.1 | R | - |
| 2 | | 1.2 | R | + |
| 3 | | 1.3 | R | + |
| 4 | | 1.4 | R | + |
| 5 | | 1.5 | R | + |
| 6 | | 1.6 | R | + |
| 7 | | 1.7 | R | + |
| 8 | | 1.8 | R | + |
| 9 | | 1.9 | R | + |
| 10 | | 1.10 | R | + |
| 11 | | 1.11 | R | - |
| 12 | | 1.12 | R | + |
| 13 | | 1.13 | R | + |
| 14 | | 1.14 | R | + |

| | | | | |
|---|---|---|---|---|
| 15 | 4.1× 15.7 | 1.15 | R | + |
| 16 | | 1.16 | R | - |
| 17 | | 1.17 | R | - |
| 18 | | 1.18 | R | - |
| 19 | | 1.19 | R | - |
| 20 | | 1.20 | R | - |
| 21 | | 1.21 | PR | + |
| 22 | | 1.22 | PR | + |
| 23 | 11.10× 16.11 | 2.1 | R | + |
| 24 | | 2.2 | R | + |
| 25 | | 2.3 | R | + |
| 26 | | 2.4 | R | - |
| 27 | | 2.5 | R | + |
| 28 | | 2.6 | R | - |
| 29 | | 2.7 | R | - |
| 30 | | 2.8 | PR | - |
| 31 | 14.6× 4.1 | 3.1 | R | + |
| 32 | | 3.2 | R | + |
| 33 | | 3.3 | R | + |
| 34 | | 3.4 | R | + |
| 35 | | 3.5 | R | + |
| 36 | | 3.6 | PR | - |
| 37 | | 3.7 | PR | - |
| 38 | 14.6× 16.11 | 4.1 | R | + |
| 39 | | 4.2 | R | - |
| 40 | | 4.3 | R | - |
| 41 | | 4.4 | R | + |
| 42 | | 4.5 | R | + |
| 43 | | 4.6 | R | + |
| 44 | | 4.7 | R | + |

| | | | | |
|---|---|---|---|---|
| 45 | 14.6× 16.11 | 4.8 | R | - |
| 46 | | 4.9 | R | - |
| 47 | | 4.10 | R | - |
| 48 | | 4.11 | PR | - |
| 49 | | 4.12 | PR | + |
| 50 | | 4.13 | PR | + |
| 51 | | 4.14 | PR | + |
| 52 | 16.10× 2.3 | 5.1 | R | - |
| 53 | | 5.2 | R | - |
| 54 | | 5.3 | R | - |
| 55 | | 5.4 | R | - |
| 56 | | 5.5 | R | - |
| 57 | | 5.6 | R | - |
| 58 | | 5.7 | R | - |
| 59 | | 5.8 | R | - |
| 60 | | 5.9 | R | - |
| 61 | | 5.10 | R | - |
| 62 | | 5.11 | R | - |
| 63 | | 5.12 | R | - |
| 64 | | 5.13 | PR | - |
| 65 | 16.11× 4.1 | 6.1 | R | + |
| 66 | | 6.2 | R | + |
| 67 | | 6.3 | R | + |
| 68 | | 6.4 | R | + |
| 69 | | 6.5 | R | - |
| 70 | | 6.6 | R | - |
| 71 | | 6.7 | R | - |
| 72 | | 6.8 | R | + |
| 73 | | 6.9 | R | - |
| 74 | | 6.10 | R | - |

| | | | | |
|---|---|---|---|---|
| 75 | 16.11× 4.1 | 6.11 | R | - |
| 76 | | 6.12 | R | - |
| 77 | | 6.13 | R | - |
| 78 | | 6.14 | R | - |
| 79 | | 6.15 | R | - |
| 80 | | 6.16 | R | - |
| 81 | | 6.17 | R | - |
| 82 | | 6.18 | PR | - |
| 83 | | 6.19 | PR | - |
| 84 | 16.11× 13.13 | 7.1 | R | - |
| 85 | | 7.2 | R | + |
| 86 | | 7.3 | R | + |
| 87 | | 7.4 | R | + |
| 88 | | 7.5 | R | + |
| 89 | | 7.6 | R | + |
| 90 | | 7.7 | R | + |
| 91 | | 7.8 | R | + |
| 92 | | 7.9 | R | + |
| 93 | | 7.10 | R | - |
| 94 | | 7.11 | R | - |
| 95 | | 7.12 | R | + |
| 96 | | 7.13 | R | + |
| 97 | | 7.14 | R | - |
| 98 | | 7.15 | R | - |
| 99 | | 7.16 | R | - |
| 100 | | 7.17 | R | - |
| 101 | | 7.18 | R | - |
| 102 | | 7.19 | R | - |
| 103 | | 7.20 | R | + |
| 104 | | 7.21 | R | - |

| | | | | |
|---|---|---|---|---|
| 105 | 16.11× 13.13 | 7.22 | R | + |
| 106 | | 7.23 | R | - |
| 107 | | 7.24 | R | - |
| 108 | | 7.25 | R | - |
| 109 | | 7.26 | R | - |
| 110 | | 7.27 | R | - |
| 111 | | 7.28 | R | - |
| 112 | | 7.29 | R | - |
| 113 | | 7.30 | R | + |
| 114 | | 7.31 | R | + |
| 115 | | 7.32 | R | - |
| 116 | | 7.33 | R | - |
| 117 | | 7.34 | R | - |
| 118 | | 7.35 | R | - |
| 119 | | 7.36 | PR | + |
| 120 | | 7.37 | PR | - |

In the cross between 4.1 and 15.7, the marker band of 0.2 kb size was found to be present in thirteen out of twenty resistant seedlings and in the two partially resistant seedlings screened (Fig. 22a, 22b). Tulshiram (2016) screened the SSR primer mTcCIR42 in both the parents 4.1 and 15.7 and detected the presence of marker band in 4.1 only.

In the cross 11.10× 16.11, the presence of marker band of 0.2 kb was observed in four resistant seedlings, where the total number of resistant seedlings were seven. Only one partially resistant seedling was screened in this cross and the marker band was not detected in it (Fig. 23). When the SSR primer mTcCIR42 was screened in both the parents 11.10 and 16.11, the presence of marker band was detected in 16.11 only (Tulshiram, 2016). In the cross between the parents 14.6 and 4.1, genomic DNA of five resistant and two partially resistant seedlings

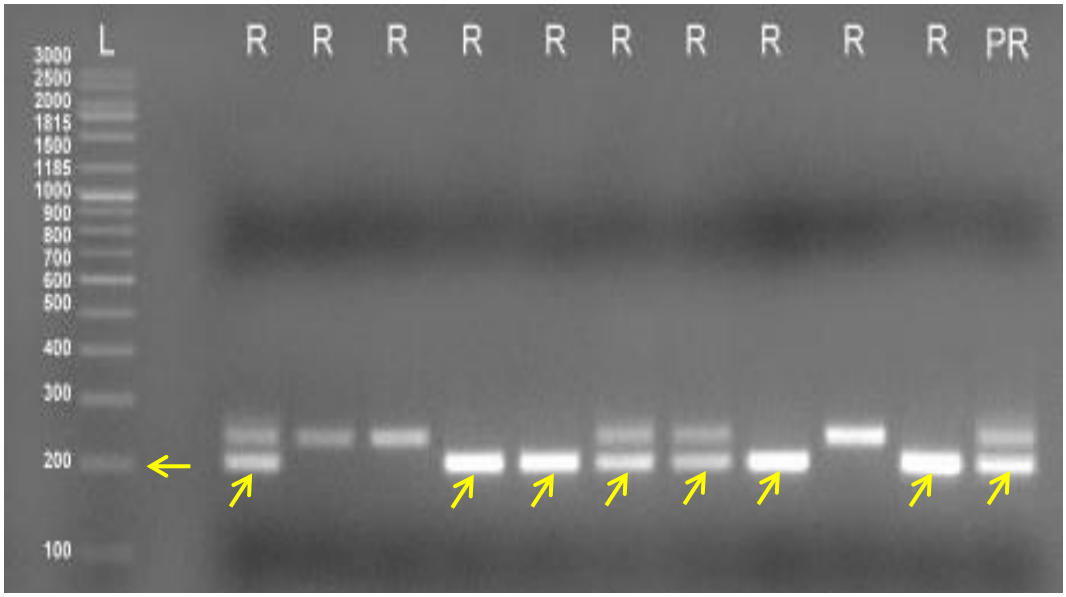



Fig 22b. Amplification pattern of the SSR marker mTcCIR42 in segregating progeny of the cross 4.1X 15.7 showing resistant/ partially resistant reaction to VSD

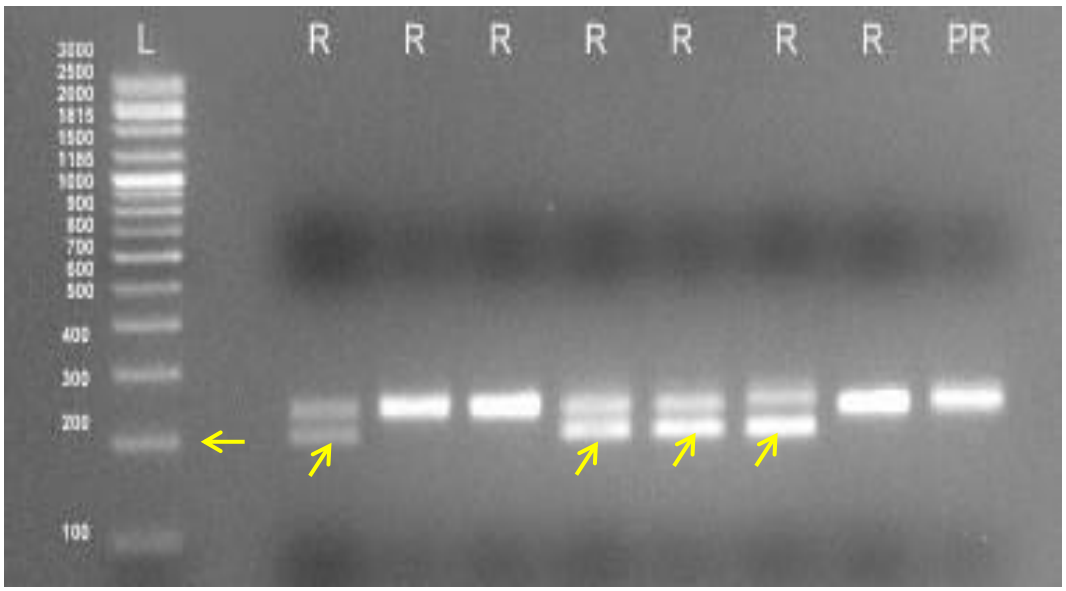



Fig 23. Amplification pattern of the SSR marker mTcCIR42 in segregating progeny of the cross 11.10X 16.11 showing resistant/ partially resistant reaction to VSD

were amplified with the concerned primer. 0.2 kb band was observed in all the five resistant seedlings. Its presence was not detected in the partially resistant seedlings screened (Fig. 24). Tulshiram (2016) screened the SSR primer mTcCIR42 in both the parents and detected the presence of marker band of 0.2 kb in both.

In the cross 14.6× 16.11, out of the ten resistant and four partially resistant seedlings screened, the 200 bp polymorphic band was found to be present in five resistant seedlings and three partially resistant seedlings (Fig. 25). The marker band of 200 bp was detected in both the parents (Tulshiram, 2016). In the cross between the parents 16.10 and 2.3, twelve resistant and one partially resistant seedling was screened with the primer. The marker band of 200 bp was not detected in any of the seedlings (Fig. 26). In the study conducted by Tulshiram (2016), the marker band was detected in 16.10 only.

In the cross 16.11× 4.1, 0.2 kb polymorphic band was observed in only five resistant seedlings, where as the total number of resistant plants screened were seventeen. Only two partially resistant seedlings were screened with the primer in this cross and the marker band was not detected in it (Fig. 27a, 27b). The marker band was present in both the parents (Tulshiram, 2016). In the cross between the parents 16.11 and 13.13, genomic DNA from thirty five resistant and two partially resistant seedlings were amplified with the SSR primer. The marker band was detected in fourteen resistant seedlings only and it was present in one partially resistant seedling screened (Fig. 28a, 28b, 28c). The marker band of 200 bp was present in both the parents (Tulshiram, 2016).

Out of the one hundred and six resistant plants screened, fourty six were detected with the presence of the SSR marker. The marker band was absent in sixty plants. Even though the gene linked with the SSR primer was not present in these sixty seedlings, they behaved as resistant. This may be due to the reason that major genes conferring resistance to VSD might be present in those plants and the resistant gene tagged by the SSR marker would have been the only gene that was

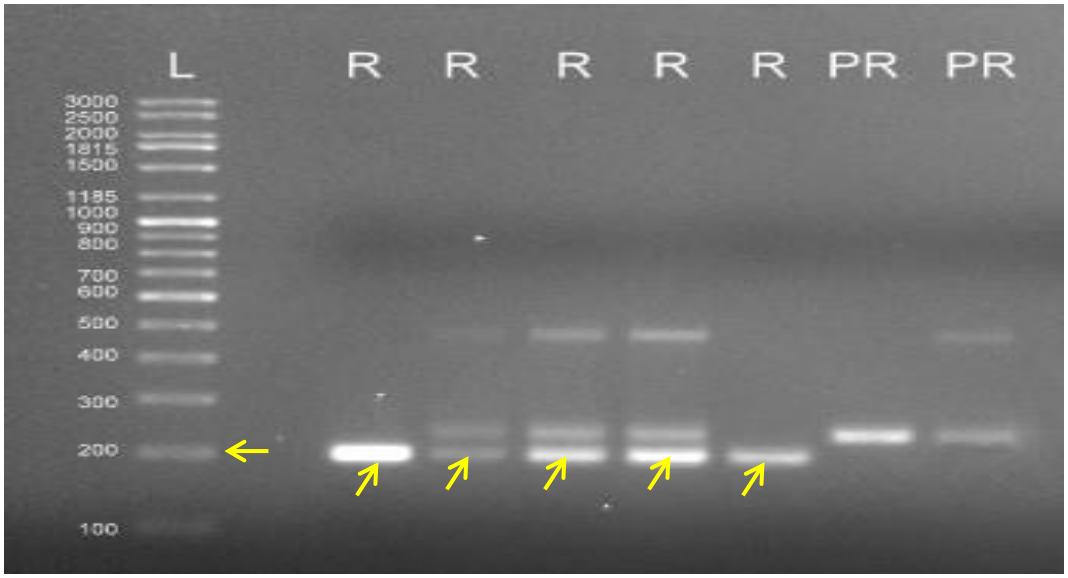



Fig 24. Amplification pattern of the SSR marker mTcCIR42 in segregating progeny of the cross 14.6X 4.1 showing resistant/ partially resistant reaction to VSD

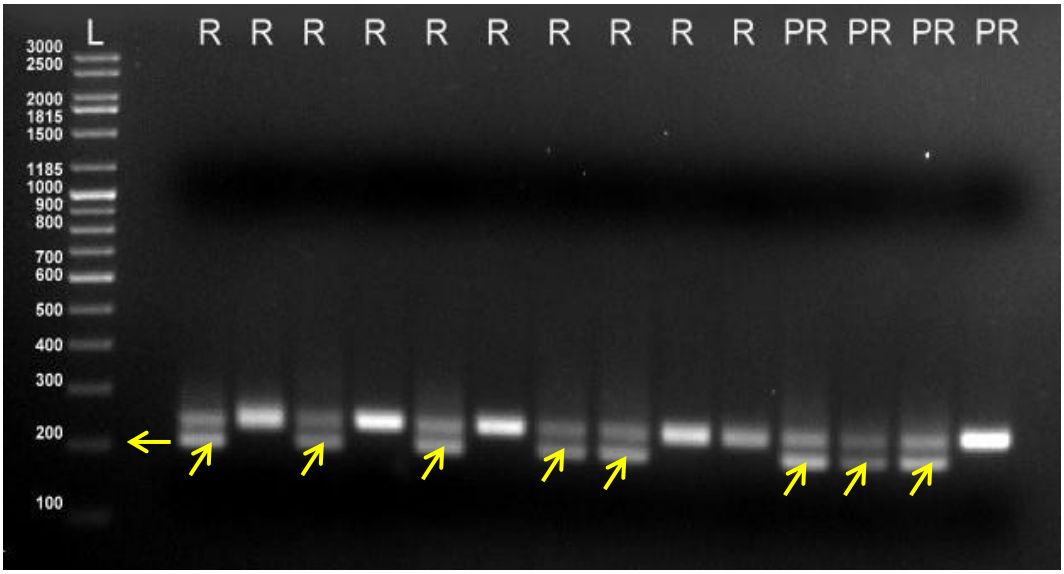



Fig 25. Amplification pattern of the SSR marker mTcCIR42 in segregating progeny of the cross 14.6X 16.11 showing resistant/ partially resistant reaction to VSD

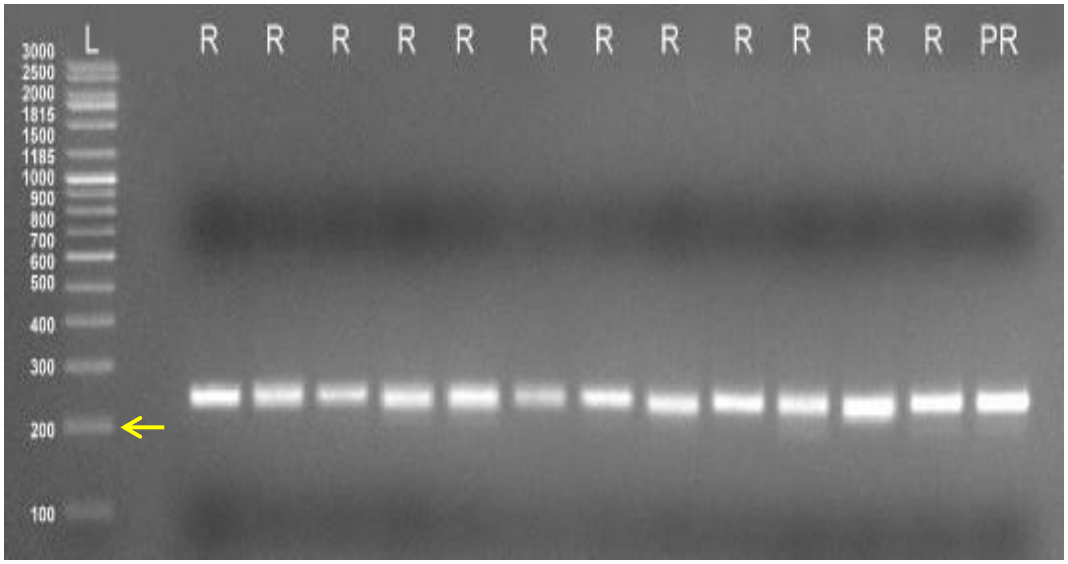



Fig 26. Amplification pattern of the SSR marker mTcCIR42 in segregating progeny of the cross 16.10X 2.3 showing resistant/ partially resistant reaction to VSD

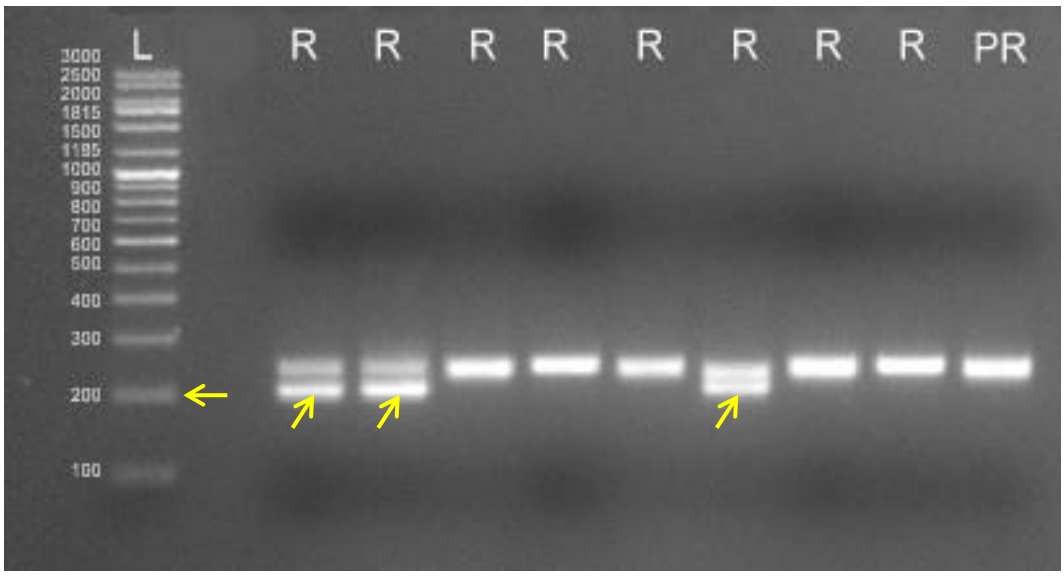



Fig 27a. Amplification pattern of the SSR marker mTcCIR42 in segregating progeny of the cross 16.11X 4.1 showing resistant/ partially resistant reaction to VSD

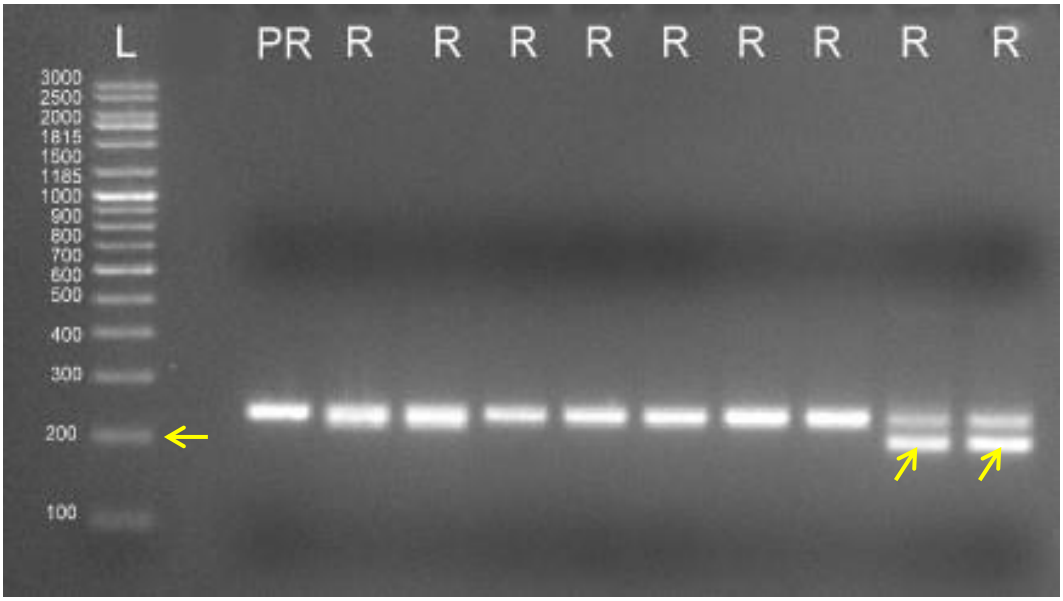



Fig 27b. Amplification pattern of the SSR marker mTcCIR42 in segregating progeny of cross 16.11X 4.1 showing resistant/ partially resistant reaction to VSD

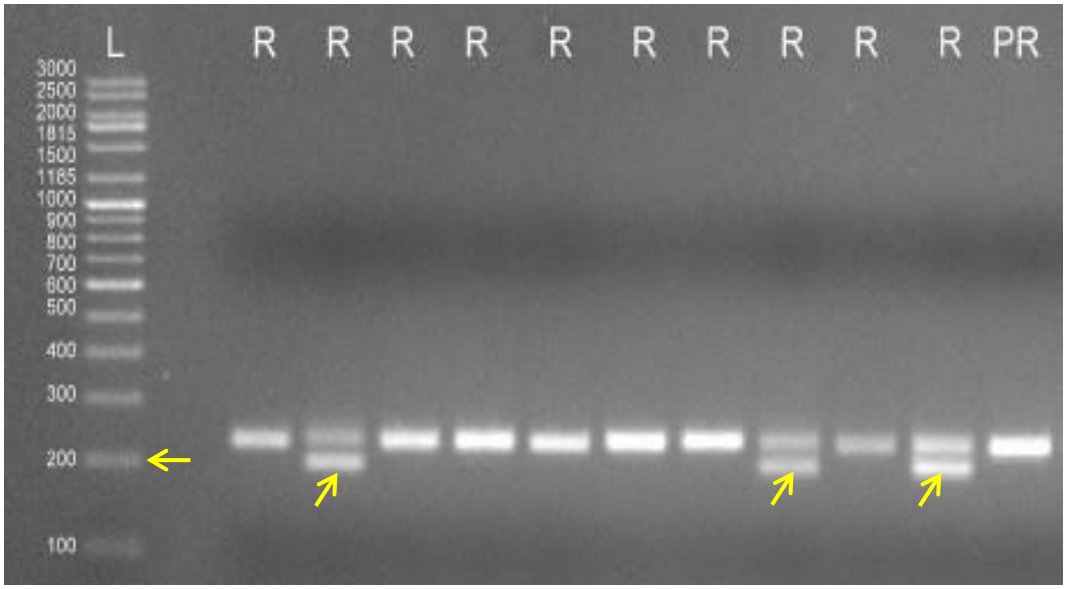



Fig 28a. Amplification pattern of the SSR marker mTcCIR42 in segregating progeny of the cross 16.11X 13.13 showing resistant/ partially resistant reaction to VSD

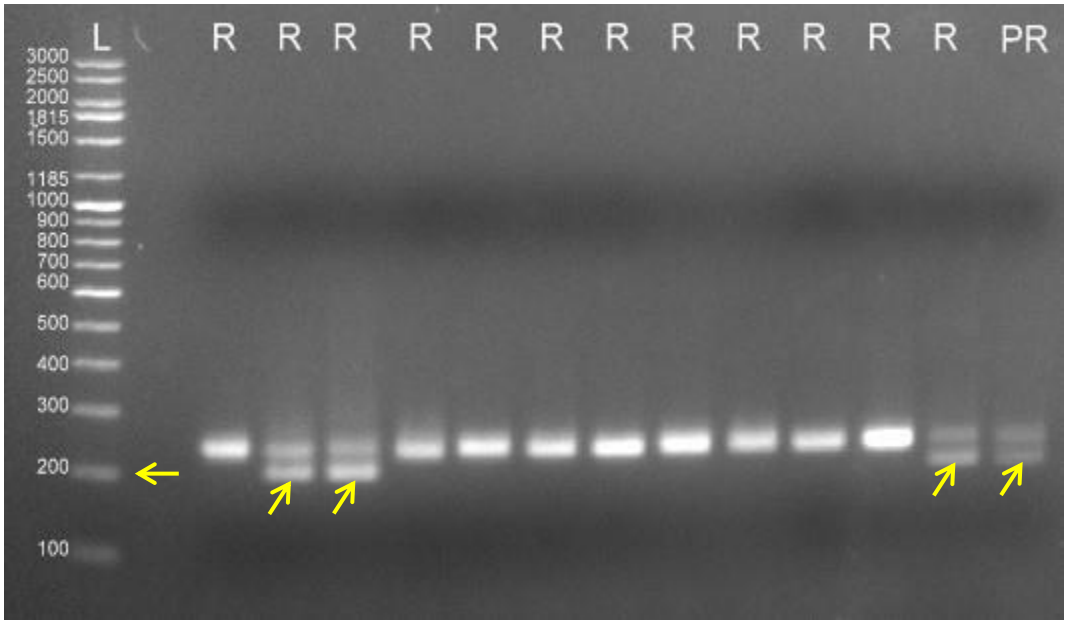



Fig 28b. Amplification pattern of the SSR marker mTcCIR42 in segregating progeny of the cross 16.11X 13.13 showing resistant/ partially resistant reaction to VSD

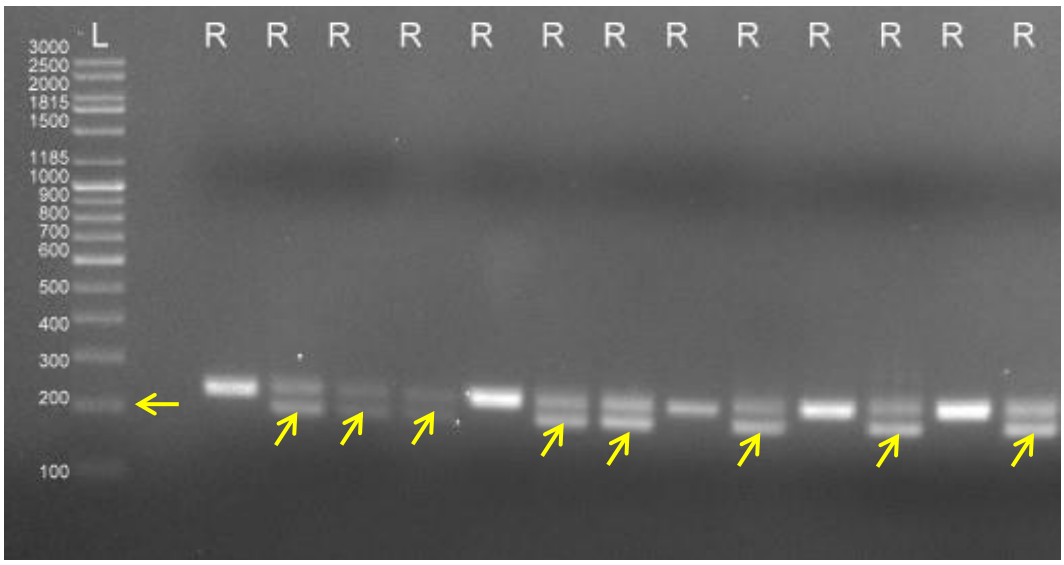



Fig 28c. Amplification pattern of the SSR marker mTcCIR42 in segregating progeny of the cross 16.11X 13.13 showing resistant/ partially resistant reaction to VSD

absent. Hence, even though the presence of the gene linked with the SSR marker was not present, those plants behaved as resistant. Same is the case with the partially resistant plants. Six out of fourteen partially resistant plants were detected with the presence of SSR marker. Since majority of the genes conferring resistance to VSD was not present in those plants and the resistant gene tagged by the SSR marker might have been the only gene present to contribute to resistance, they behaved as partially resistant and were not able to express complete resistance.

### 4.6 Percentage of inheritance of the markers

The susceptible seedlings were completely lost before five months of sowing due to heavy disease incidence. Hence, only partially resistant seedlings with some symptoms and resistant seedlings without any symptoms were screened to study the inheritance of marker from each cross. The details of the percentage of inheritance of the markers in the screened crosses has been given in the Table 13.

**Table 13. Inheritance percentage of the markers in resistant seedlings**

| Sl No. | Cross | Inheritance percentage (%) | | | |
|---|---|---|---|---|---|
| | | ISSR | | | SSR |
| | | UBC 811 | UBC 815 | UBC 857 | mTcCIR42 |
| 1 | 4.1× 15.7 | 70 | Nil | Nil | 65 |
| 2 | 11.10× 16.11 | 100 | Nil | 28.5 | 57.1 |
| 3 | 14.6× 4.1 | 60 | Nil | 80 | 100 |
| 4 | 14.6× 16.11 | 30 | Nil | Nil | 50 |
| 5 | 16.10× 2.3 | 100 | 100 | Nil | Nil |
| 6 | 16.11× 4.1 | 64.7 | 52.9 | Nil | 29.4 |
| 7 | 16.11× 13.13 | 77.1 | 11.4 | 42.9 | 40 |
| Mean (%) | | 71.70 | 23.47 | 21.62 | 48.78 |

Percentage of inheritance of the marker UBC 811 was cent per cent in two crosses *viz*., 11.10× 16.11 and 16.10× 2.3, 77.1 per cent in 16.11× 13.13, 70 per cent in 4.1× 15.7, 64.7 per cent in 16.11× 4.1, 60 per cent in 14.6× 4.1 and 30 per cent in 14.6× 16.11. UBC 811 was showing comparatively good mean percentage of inheritance of 71.70 per cent among the ISSR markers screened. The number of partially resistant seedlings that has shown the presence of marker band while screening was three out of fourteen in case of this marker.

The marker UBC 815 was showing poor mean percentage of inheritance of 23.47 per cent compared with the other markers. The percentage of inheritance of this marker was zero in four crosses *viz*., 4.1× 15.7, 14.6× 4.1, 11.10× 16.11 and 14.6× 16.11. Cent percentage inheritance was observed in the cross 16.10× 2.3. In the cross between the hybrids 16.11 and 4.1, the inheritance percentage was observed to be 52.9. The inheritance percentage was found to be 11.4 per cent in the cross 16.11× 13.13.

However in the earlier studies conducted, the marker UBC 815 has yielded a distinct polymorphic band of size 0.750 kb when screened on resistant hybrids. Inheritance of this gene to the progeny may be less and may be the reason not possible to tag this gene in segregating population.

The inheritance percentage of the marker UBC 857 was zero in four crosses *viz*., 4.1× 15.7, 14.6× 16.11, 16.10× 2.3 and 16.11× 4.1. In the cross 14.6× 4.1, the inheritance percentage was 80, whereas, it was 42.9 and 28.5 per cent in the crosses 16.11× 13.13 and 11.10× 16.11 respectively. The mean percentage of inheritance was 21.62 per cent and it was the lowest for the marker UBC 857 among the other markers. In the study conducted by Chandrakanth (2014) in VSD resistant hybrids, UBC 857 was showing a clear amplicon of 450 bp in all the resistant lines, and was absent in all the susceptible lines screened. Since it was not possible to tag this gene in segregating population, inheritance of the gene to which this marker is linked might be low in the progeny.

The inheritance of the SSR marker mTcCIR42 is 65 per cent in the cross 4.1× 15.7, 57.1 per cent in 11.10× 16.11, cent per cent in 14.6× 4.1, 50 per cent in 14.6× 16.11, 29.4 per cent in 16.11× 4.1 and 40 per cent in 16.11× 13.13. The inheritance percentage was zero in the cross 16.10× 2.3. The mean percentage of inheritance of the SSR marker was found to be 48.78 per cent.

Tulshiram (2016) conducted a study on validating mTcCIR42 marker and found out that the polymorphic band of size 200bp was linked with VSD resistance. In the present study, presence of 0.2 kb band was detected in majority of the resistant seedlings. Cent percentage of inheritance was also found in one of the crosses.

In the current study of 'Inheritance of molecular markers linked to vascular streak dieback disease resistance in hybrid progenies of cocoa (*Theobroma cacao* L.), percentage of inheritance of the identified three ISSR markers (UBC 811, UBC 815 and UBC 857) and one SSR marker (mTcCIR42) were studied by screening these markers with one hundred and twenty seedlings from seven crosses which are segregating in nature. The ISSR marker UBC 811 was showing the highest mean percentage of inheritance of 71.70 per cent. Least percentage of inheritance was shown by the ISSR marker UBC 857 and it was 21.62 per cent followed by the UBC 815, whose inheritance percentage among the resistant seedlings is 23.47 per cent .

Nineteen resistant seedlings were identified (Plate 8) from one hundred and twenty seedlings in which three or more markers were expressing. The details of the hybrid seedlings with three or more markers expressed are given in the Table 14.

**Plate 8. Resistant seedlings identified with three or more markers expressed**

**Table 14. List of hybrids with three or more markers expressed**

| Sl No. | Hybrid No. | Markers | | | |
|---|---|---|---|---|---|
| | | ISSR | | | SSR |
| | | UBC 811 | UBC 815 | UBC 857 | mTcCIR42 |
| 1 | 2.1 | + | - | + | + |
| 2 | 2.5 | + | - | + | + |
| 3 | 3.1 | + | - | + | + |
| 4 | 3.2 | + | - | + | + |
| 5 | 3.3 | + | - | + | + |
| 6 | 6.1 | + | + | - | + |
| 7 | 6.2 | + | + | - | + |
| 8 | 6.3 | + | + | - | + |
| 9 | 6.4 | + | + | - | + |
| 10 | 6.8 | + | + | - | + |
| 11 | 7.2 | + | + | + | + |
| 12 | 7.3 | + | + | + | + |
| 13 | 7.4 | + | + | + | + |
| 14 | 7.5 | + | - | + | + |
| 15 | 7.6 | + | - | + | + |
| 16 | 7.7 | + | - | + | + |
| 17 | 7.8 | + | - | + | + |
| 18 | 7.12 | + | - | + | + |
| 19 | 7.13 | + | - | + | + |

These nineteen seedlings with three or more markers expressed belong to four crosses *viz*.,11.10× 16.11, 14.6× 4.1, 16.11× 4.1 and 16.11× 13.13, with two, three, five and nine seedlings respectively. These seedlings will be planted in the field and will be further evaluated for yield and yield contributing characters since double cross hybrids were found to be superior to single cross and can overcome

the yield reduction phenomena expressed in resistance breeding (Sriani *et al*., 2003; Ghanwat *et al*., 2016).

### 4.7 Identification of genes in flanking sequences of markers

#### 4.7.1 Identification of genes in flanking sequences of ISSR markers

The polymorphic bands of 0.950 kb and 0.450 kb generated by the ISSR markers UBC 811 and UBC 857, when screened on VSD resistant genotypes of cocoa has been sequenced by Tulshiram (2016). The flanking sequences of these two ISSR markers were extracted from NCBI. Five thousand nucleotides each were extracted from upstream and downstream region of both the ISSR markers to check for any genes related to disease resistance in cocoa.

The sequence extracted from the upstream region of the ISSR marker UBC 811 is 5'-

AGCAAAAGTGAAAATCATAAAAATGTCATCACGTTTGAACTTGTTTTA
AAGTTTTTCTTCAAATGGTACCTGCATAAACTTTGGTGCGCTTCTGGGA
TGCATTTTAGATTGCTTTCTTAATGCCGAGATACTGATCAGAAATGAAC
ATAGTATTTTCAGGACAACCAGCTGCATCACTCAGCTTGCTAAGAAAC
ACGTCCATAAGTCTTCGTCCTCAACATGGTCGATGCCAAACGCAATTG
ATATACATAGTCGTTCGCATCTTTGCATACAGCGACAAACAAAACACC
CTTGAACCTGCCTTTCAGATGTGTCGCATCAATTACAACCGTAGGACA
CATTACATCCCTAAACCCCCGAATACAAGCCCCGTATGCTCAGAAACA
ATATTTGAATCTTTCTGCCTCATCAGTAGCCACTGCAATGACTGTGTCA
GGATTTTCTTGTTTCAACATGCAAAAATATGAGGGTAGTAGTTGAAAT
GACTCCTCCGGGGAATCGAAAACAAGCTTCTCTGCATACTCTTTCCCTT
GCCAGGCCTTGCCATATAGGCATTCCAATCCCCACTGAACCTTCATCTC
ACCAATTATGTCCTTAGGTCTCAATGCTACTCCATTAGCCTGAAGCTTG
TGTGACATAAGTTCACCAATTATCTTCGCACTGGCAGTTGCAAATCGC
CCTTGCAAACCATCGATAGTACATGTGTGTACTTTATGGAACTTTTGAA
CTTTCCAATATTCTCTTCCTTCAAGTAACTTCGTTGCACGCACATTGAA
CTTGCATGCCTTGTCCTTGCAACCAACCTCATAACGAGCTTTACATTAC

CTTTTTACTCTTATCCCAAATTACTCTTTTAGAGCCAACATGTTCAAAA
CTCGTTTCAACTCAACCTTCGAGGAAAATATTCTTCATTCGTATAAGCG
ATTATCGGCACATGTCGACTCCTCAGTTGTAATTGTTTGGAAGGAGAA
CCTTTCTGCACTTGGAATTACCCACGTACGAGATATCCCTGCATTTCCC
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TTCTCATCAACGATAAGGTTATTGTATACGGGGTCATCACACTGAACA
TCTCCTACATCAACACCTCCAATAGGTTCTGTTTCACCTTCAACATTAT
TGCAAATTAGAATGTCACTGTCATAAAGCCAGTCCTCGTCTGAACTCT
CATCATGCCATTCAACAGGCCCATCATCCGAATTGTCATTAAATTTATC
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TCCTCAAATGTCGCAGTGTCACCCTCCAACATCATAGTGTTGTCCTCAA
GTGTCGTATTCTCATTTGAAAATGCCATTACACCCTGTACAGTTTCACT
CGATCGTTACGTTTGTTGGACACAAGTAACTAATTGGTTCGATGCACA
TCCTGATAGGAATTGTCCAGCCAACTGTTTTGTGAATGTCATATGTCTA
CCAGGCTGCACAAACGCTTGTGCATACGTCAATTACCATTGTTGTGGG
TTACGGAACGAATGTTGTGGTATATGTGAAGCATTGTATATCTCATTTT
GATTGAGCTAATTACCATGTTGTTCAGCTTCTTCATGTGACATAACATT
TGTTTGGCATCCTTTAATGCTGACATACACAGCCGGAACATTCCTCTAT
TCTAGCAAAATTAATGCAACCTCCTCATTGTCCTTGATAATTGGCTGTG
ATAGCTCTCCGGGTGTACCGATTAATGCATGTAACTCAATCTCGTCAAT
TTCTGAGTTTACCCCAACAACATCTTCAACCAGCTTTATCAATCCCACA
AACGACAAATCACTCCTAACCCCCTACATTTGTGACTCACCACCCTTGT
AAATGCCATCCACCCACTGACCACCGTGTCTTATCACAAGTCACACAA
TTGGAACCCCACTGAAATTTTGAAAACAACAATATTTTGTCAATCATTT
AACATATTACATGACATGCTGTGTTCTAGAATTTGTCAGTCATGTTGTA
ACATGCCATGCATATAACGTAAAATGTTGTAACACGCCATGCATATAA
CGTAAAATGTTGTAACACGCCATGCAGATAATATAAAATGTTGTAATA
CGCCATGCATATAACGTAAAATGTTGTAACACGCCATGCATATAATGT
AAAATGTTGTAACACGCCAACGCTGACATGCCAATGACTATAATTACT
GTGTTTTTACATGAACTGGCCTAAAATACCCATGGCGTGTGACACGCC
AATTAGTGTAATTATTGACTCGTTACATGAACTGTCCTAAAATAACCA

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CGTGATATCATCTCTACTATAGGGTCCGTTAATCTACAGTAATATGGCC
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AGAGTTGACAGAGCTCATACAATTCAGAGAGCTGATGACAATGGTGCT
TAATAGAGAGTTGACAAAGTTGAGGCAGTTCAAAGAGTTGAGAGAGA
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TTCTAATTAAATTAGTAAATGACTTTTTATTCAATAATTTTTTTTTCTTT
TTATATGATTTCATTATTTTTGAAAATTTGTTTTATAATATCAAAAAAT
TAAAATTGGAGATATGCTACAAGTTGATGAAAATGTGACTTTGAATAA
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TGCTTCAAATCTAAATAACTTGAATGTCTACTCCGAATAACACAAACT
CGAGATAATCCAAACCCGACTCAACAAATTGTCACTTCTAAAAAGAGG
TAAGTGTCTCATTCTCAGAAATCCCTTTCCATTATTTCTCAAGATTTTA
ACTTAAAACACTTTTAAATTAGGTCTCAAATTGTAGATGCTATAATACC
TTTGGAAACAAAGCTTATTATTATTAGTTTTTGAAAATGAAATTTATTT
ATTTTTAAAGAAAGACTTTTAATAAGTACTATATGCTAATATAACATA

CTGCAATTGATTTGGAAAAAGGAAAGGTATCTTTTTTTAAAAAAAAAA
AATTATAGAAACCATTTCCAATTGCTTAGAGTTTTGTTTTCATTTGTTC
GTGGAAAATTGTTTCTAGAAATTGTTTTACAATAAGTGTAATACTATA
GTTTATATGACATATGTATATGAATTTAGACTAGTGTGACCATGGGTTA
AAGTCAGACTGGTGTGGGCTGAAATTGGATTTGGCATGGGTGGGATTT
GAATCCGCAGAAGAAGAGTTTGAACTGTCCATCTCGTTTGGGCCTTCT
GGACTAGGCTGGGTTATTCTAGTACCCCTCTTGGGTATTTGAGTTAGGT
TGGATTCTTCTGAATCCTTCTTGTAGGTTGGGTTCTTAAAATATCTTTTT
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GATTAAATAATATTTTATTATTAAAATTAACTAATTATTTCTAGTGATT
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TGTCTAGTGATATAAAATGATAATTATTGATTTGACCAAAGTAATTTAT
CAATAGTTAATTATAAAAAGTTATTTGATATAAAATGAAAATATAAAA
ATTTAATTGGATGTAATATAACAAATAAATATTTACTTAGAGTTTCAA
AAATAATTTAAACTTTTTTTAGATATGATGTATTTTTCTTTCAATATAA
GAAAGCTTAAAATATTTCACGTGGCAACTACCAATCAGGGGAAAAGTC
AATTTCTTAATCCTCAAGTGCTGCGCAACGGATTTATCCATTCGCAAAG
TTTCTTGGCTTGGCGTGGCTCTCTTAACAGGGAAAGGCCCCAAAATGT
GTAAGATAAAATCAGAATTTGGAAATTAAACAAAAGAGAGAGAGAGA
CAGAAAAAAAGGGGCATAAATATCAAGCTAAACCCCCGTCAAAATT-3’

The sequence extracted from the downstream region of the ISSR marker UBC 811 is 5’-

CCTCTTTGTAATTGTGTTGGAGTAATAGGGATTCAAAATTGTCAACATA
AATATTTTTCAAATATTACTCATGTTGATAATATTCTAATTTCATAGGT
GGTCTCTCTGTCTCCCCTCTTTCCTTACTTTTCTCCTTCTAATTGCTTTG
GCTAATTGTACATTTTTACCTGTTTCTAATAGTTTTCTAATATCGATGG

CTATGCTTTTGACTTGGTCCTTCTGTTTTTTCTAAGCCTGATTTTGGTTT
CTTTGTTACTTTTGTGTTCCTAATCATGAGTCATAGTCATTCTAATGGTC
TTCACAGCGCTTGTTTAGTATTTCCATCACTGCTGGTAATGCATCCGTC
TAATACCTTCTATGCAATGTATCTGGATACTAGAAGTGCCTGTTAGCCC
TTCCCTATGAGTAATTAAAGCATTAAACTTTTCAGTTAAAATACTAAA
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TGTTATGGTGGCTCAAACTCAACGATGGTTGTCTTTGCAGCATCATTGG
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TAGAGCAGTGTTTGGAGGTTTGTCTGTGTTTCCTTAACCCTTTTTCTATT
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TAGCTCTACCGCCTGGCATTTCTTTAGTGGGCTATAGGCTGTGTGTGTT
TAATTATTTGCATTTTCTCTGGTTTAGTGCTCTAAATTCTTCTGTGTCTA
CCTTTAAACTTTTTGATAGCACTATGTTTTAACTTGCACAACATGACAC
CGTAGGTCTGTGTCTTCCCTCCGTTAGAACTTTGGTATTTTGTCTTTATT

AGTCCAACACTGCCCAGTGTGGTGCATTTCTTACACATTTGCTTTATTG
AAACGTTTGTTTTTGGTGTTTTATTTTGGTTTCTTATCTTATGTCTTATG
TGACTACTGGTTTTGTAGATTGCACCAGATTGGAGGCAAGCTCTTGCC
CTGAAGAAGACAGTTGAGGATCGTATTGCTAAAGGTGATAACTATAAT
TTTCTTACAAATTTTAGAATTAATTATAGCATAGACTGAAAGAATGAT
GAATATATTTTGTGTTAATATAGATGGTGTTATTGGAATTGGCATCACT
GCTACTGCTGTGGGACTTATTGCGGGTGGAATTGCCGCAGCATTGTCT
CGCAAGAAATGATAAAACCAAAATTGTGCCCATGAGATTGCGCCCCC
CAAGTATCTTGCTGATTTTACTGGAAAAATAAGTGACAAAAGTAAAGT
TGTAGTATCTCTTGGTTTTAGAATGTATTGCTACAGGTGGTTTTGATCC
GGTTTGGCATGCCTAGCTGATAGAAATGAGAGTTTCGTTAATAGATAT
GACAAAGACACTATTCTCTGACCATATTGTAAATGCTTTTTTTTTGGGG
GGGCGTTTTGTGTGATCAACTTGAGTTACTGTTAGCATTTTGCAGTTTG
CTGTCATGGGGAAATTGCATGAATTTGCTACCTGTGAAAAGGTATATA
GATATGCATGGACAAGATTAAACTGTAATTATTCCATTTAGCATACGC
TGATAGTTCTTTGATGCGGTACGTCAGTAGTGCCTACAAAGAATCCAG
ATAGTAAAAAGAGTGATGGCACGCATGGCAGTAATGATGCCTCCAAC
GGCATGATAGGAAATCCTTCCTTTTCTGTTTATTTTTAAGGATGAAAGA
CTTGTTGCAGACGGTGCAGCGAAAACGAGATATTGAAGTTACAGCATG
TCAATTGCTGGATGCTTTCGCAATTGTACGAGTTTTAGCAACAAGGCC
AGCAAGAGAGCGGTTGGCACCCATTGTTGAATAGTAACTTTTTCGTTT
CTTTAGCCCCAGCATGCTTTTTATCAATGGTACCATATCTCCAATTTCG
AAACTATTTGCCAACCAACTAGACCCAAGTTGATCCAAAAAGTCAAAC
AAAATCCAAATCCAAAATGACCTGACACAAACTTAAAGAAACCCTCAT
CTGCAACAACATGAACCCAAGATTGATTCAATCTAAATCCATAATAAT
CCAAGCTTAATACTGAAAATTAAAACGATCCAATTTGTATAAAATGAT
ATTTTACAAATGCAAAATTTAATTACCACTTTTATTCTCAAGACAACTG
GACTTGAAAACGACCTGAATCCATCAAGATCCAAACTCGAGGTGATCC
GAATCCAAAATAACTGACCTGAACCCGCCGGTTTGCCACCTCTAATTT
TCCCTCATCTATCTCTGTGGATGGTGATGGAAGCCATTCCACCCACAAT
TCTTGTTTTACCTTGAGTAGGAGTCACAACCTATTTTCTAAAACTAAAA

CATTAGTTCTACATTTTGCACCGTCAGCTGCTTGATTAAAGATAGAAA
GACTTGGTAGACTCAACCAGTGTTCATAAAGAGTAAACAGATGTCACA
CGAAGCAACCACAAAAGAAGGATGGGCAAAAACTTAACAAGTACGCC
TGCAACTTCCCACAGGAAGATACAAAGGGAAACCGCATCACGGCATC
ACCAAACGCATCGGTAGGTGACATGTGACTGGGTGATCTCACCATCAT
ACATAACTTTGACCACCTGACCCCTTTCATAACCATAATATCTTGCAAT
GGCATCTTTCTTTAACAATCTAGGAAGCTGGAAATAATTATAAATTCA
AATCAAATGCCAAACAGTTAGTGACTGTAAGTCTGTAACCACTAAAGC
CTACCAAAAATAAACATAAAGATGCAATATTTTGTCATGCAGCTGCCC
TAAAATTTGGTATAGGTTTAAATCTTCACAACTAACATTGCATATATGA
TGTATGCACATCTTTTTAGTGCAACATATGTCTGAACGACAACAACAA
TATGAAAGCTAACGATGACATGTGTATGTAATATTTGACCAAAGCTCT
TAATGTTTAAAGACTAACCTGTTTCTCTTCTATACTGTACTTCTGCAAG
AGCTTTTGTTTCTCTTGTTCTGTCAATACCTGATGCTTGGGCTTTAACAC
ATGCTTCGTAATATTAACTAGTAAGTCAGTGATCTGAGAACCAAGAAA
CACAAAGTAAAGGAGATATTAGGGGTACAAGATGGTTTGAACAAGTT
GCATATTAGCTATGAGGAGACAAAAGGAGTTGGCATCCCACAACCAT
AATGGATGTAAAGAACTAATTTCTTGACTTAAATGTACAATGCCATGT
AAAGCCTGCAAAAGTTACAAATTTATCCTTGAGAGATTTGACAAGGAT
TACATATTATCATAAGATGGAAAGATTATGAGAATAATTAACCAATTT
CTAACAGTATGTGGTCTATAAACTAATAAAGACTAATACTGTAAATAG
AAGATTTCTTTCAAAAAAAAGAAAAAGTAAAAATTTTCATTTTTCAGG
GACAAAAAACCAATGATCAAGCCAATCGTCTATAGCTGATTGTCTATA
TCTATGTCCAGAGTTTACTTCACTCGGTGGACTAGCTGCTGGAAGGAA
AGAAACTTACAATCTTATAGGGACAGAATCAAGCTCGGCAAATTCTTT
TGAATTTATAGAATTGGCACAGGAAATATAAGGTCAGTTGAAGATTGC
AGACTGTAGTACAATTTTTAAGCTTTACATTGTTGATAACAATATAGA
GAACTTATAACCTATGACTAGAACTAGTAGTGCAAAATAACATCTCAA
TTTTGGTTAGTACAAACCACCTGAAGAAAAGACTGTCAAGATAAGCCG
CATGTTAGAAAAAAATGCAAGTTCCTTGCATGCCGATGGCTATATCTC
CTGAGGTAATCTAGAGACAAGGATGTGTAAGCAATTCCACAAGGATGT

GGCACCTTCTGAAACTATTAAAAAAATGTTTAAATTCAAAATTGAATC
ATTAAATGAGTATAAAAATAAAATGACGCACATAATCTTGCTTTTAGT
TCCCATAGAAACTTCAATAGTGAAATGCAATACACTCATTAGCAGTTT
TCATCCTTCATATACCAACCTTAAGCTCTAAGTTCAAAATTGCCACCAC
AAGTAGGCTAAAGAGATCAACTAATAAGCATGTTATCTCTGCCTTTAT
TATAATAAACTACTGAAAAATATCAAATAATATGAACCAAAGAGAGA
GAGAAGCATTGA-3’

By using NCBI ORF finder, one ORF was obtained from the upstream sequence (Fig. 29) and nine ORFs were obtained from the downstream region (Fig. 30) of the ISSR marker UBC 811. Details of the ORFs from both the upstream and downstream flanking sequences is given in the Table 15.

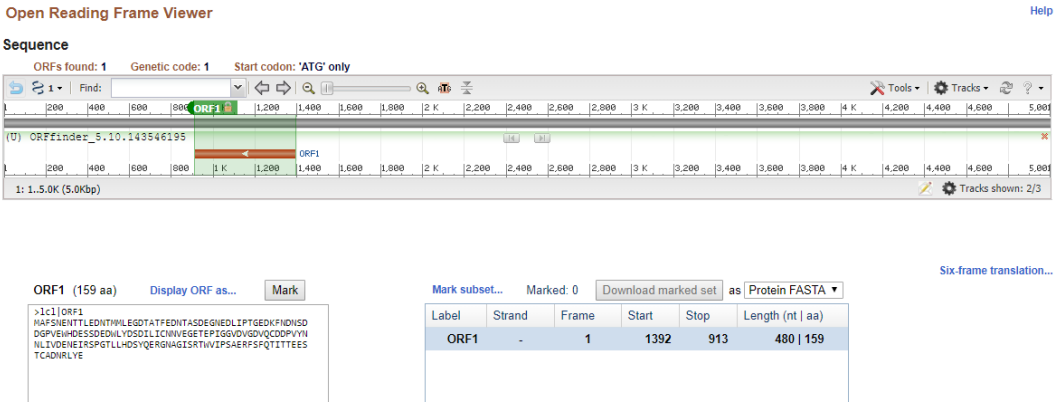


**Fig. 29 ORF from the upstream flanking sequence of UBC 811 marker**

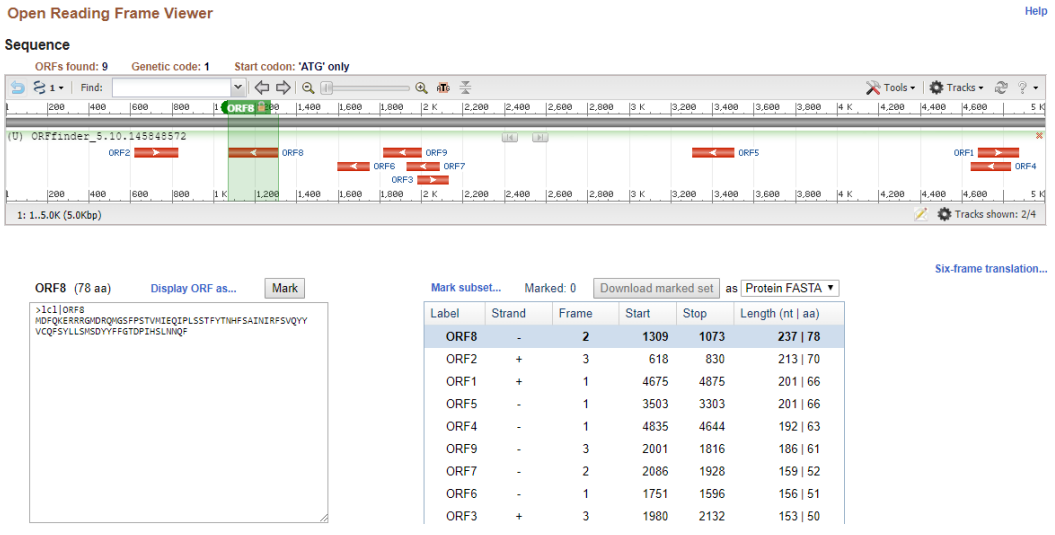


**Fig. 30 ORFs from the downstream flanking sequence of UBC 811 marker**

**Table 15. Details of ORFs from both the upstream and downstream flanking sequences of UBC 811 marker**

| Sl No. | Region | ORF | No. of amino acids | Sequence identity (%) | Protein |
|---|---|---|---|---|---|
| 1 | Upstream region | ORF 1 | 159 | 69.66 | Uncharacterized protein |
| 2 | Downstream region | ORF 1 | 66 | 29.63 | Hypothetical protein DU-187 |
| 3 | | ORF 2 | 70 | 68.85 | Mitochondrial fission protein A |
| 4 | | ORF 3 | 50 | 65.23 | Uncharacterized protein |
| 5 | | ORF 4 | 63 | 54.29 | Uncharacterized protein |
| 6 | | ORF 5 | 66 | 60 | Uncharacterized protein |
| 7 | | ORF 6 | 51 | 62 | Uncharacterized protein |
| 8 | | ORF 7 | 52 | 71.59 | Uncharacterized protein |
| 9 | | ORF 8 | 78 | 54.13 | Uncharacterized protein |
| 10 | | ORF 9 | 61 | 62.39 | Uncharacterized protein |

The single ORF from the upstream region and last seven ORFs from the downstream region of the ISSR marker UBC 811 had shown similarity with uncharacterized protein in *Theobroma cacao* with an identity of more than 54 per

cent when analysed with BLASTp tool. The first two ORFs identified from the downstream region had shown identity with hypothetical protein DU-187 and mitochondrial fission protein A with a similarity per cent of 29.63 and 68.85 per cent respectively.

The sequence extracted from the upstream region of the ISSR marker UBC 857 is 5'-

AATGCCATTCATTATGCACATTAAAACCTATTCTTGAGTAACCGTGTCA
CGACCCGGTACTCCCTTCGAGCCCATAACAACCGTCTCGACGTCCCAA
TAGACATTTATTGCTCGGAATGCCAACCAGAACCTCGGAAGGCTTTAA
TATCAGTTTTGGCATCTCCCCTATAAGCAACAATTGTTAAGTATCATGT
TTTGAGAGATTATAAACTATTTCCAAATCAAAACAAAAGAAAAATATT
TTTTATCTCATAGGGGCATTTTGATCATTTTTTCTCAAAAATCTCGAAA
CCAGCTAAAAATGTAGTATGCAAGTCAAAAACACATATTTCATAATCA
AAAATAAGTTTAGATGTCTAAAACATTCATTTAAAGTGAAACTATAAC
TATTTGAATATAATAAAATTATTCAATAAAATATTCTATTTTCTTATAA
AACAATATTTATAGAGTTACCGGTAAATAATTTTTGTAGCGAAAAAGC
TAAATAAATTCATACATACTGTAATACAGAGAACCAAAGTATAAAATT
TAATTTACAGTGGCACGTGGGCCCACGAACGACCAAAAGTATCAAAA
GCGATAAACCTATAGTTTTTGAGGATGCAAGTCCAACTGTAACCCTTA
ACAAAATGAGCTATATACCGACTATCCTAAGCCTAAAATGAGTGGAAA
GGAAAGTGATGAGATTATATAATCCTAGTGAGTAAACAAATACCATCT
AAAATATCTAAAGCTGAGTAAATAGAGACAAATAAAATAGATTAGCA
TAAAAATGATCCACAGCATTCCGAACTCATTTTAATAAGATAAAATAA
ATTCATTTCACCCAAATAAACAATGAGGCTTATGATTTTCTGAAAAGC
TTGACTCGTGCCAAAATCATTCTCATACTCAGTTATCAGGGACACCTTG
GCCTGGGGCTATCCCAACCGAGTCCACCAAGGCTATTAAAAAAGTCAT
GTACGCATAAATCATGTTTTTATTTGAAAACATAGTCATTCCTTGGTGT
TGGCTGAGTTCACCCTCACGTGGTGGTTTGGCGTGAAGTGAACTCTAA
AATTGTACCTCGAGAGTTACTCTATACACCTCTCCAATCGAGGTAAGA
ATATAACGAGATTCCATGCCCCTCAAAAAGGCTGGTCCACAGAACCAA

CCCACTGCAGCAAGCTAAACCTACCATACAATAAAATTTACAATAGCA
TGACATGGCAATTATTTTATTTTACAGCCTCTCAAGGCAAATCAACAGT
TCATACATTTTCAGCACATTTACCAATTCAACCATTCATGCCAATATTA
TCATAAGTCATTCAATTCAAACAATGATTTATAACACAGCAATTAGTC
TCCGATTACTCCATTTTCAAAACCATCTTTCAATTTCAAGATAATTTTA
TAAAATCATTTCATTTAATCACATTTTGCTTATAAAACATAAAGATTTA
CCATTTAAACTCATTTTTCTATCAAATCACAAAAACGAATAAAAACTA
CTTTCGATAATTAAGATGATAATAAGGTTACTCACTATTTCGGGAGTC
AAATTTCGAGTACTCCGATTCTTGATCCTCGGGTACTATCTCTTTACTT
TTGCTAGAAAGCTCACTACTTAGACATAATGCACAATAAGCCATTTAC
TACATTTGTGCTAAACCGTTGTAAAACCAATTCAGGCTTTATACTAATG
CATGAAATTGGATGTGAAATACTCGGGTAACAATCTAAATACGATGTT
CAATCTAAGTTCAATTGTGTACCTAAATAAAATGGTATTGGCCTAATTC
GATCCATCACCCAATTGATTGGCCAATATGTGCAATTTAACTCCAAAT
AATTCCATTTTTCTTCCAATTCTCCAAACCATCAAACACAAGTCAAATA
GCATAAAATTTAGCAAAATCATCTTCACATTTTCTTTTGGGAATTTCGG
CCATGGTGGGTGCATAAATACTATAATTTTTCCTACTTGATTTTCTCAT
TGTAATTCATCATTTCCTAACCAATTCACTTAAAATAAAATCAAATCC
AGCATCCACACTCTGCATGGAAGATTCGGCCAATGATGGGTGATGGGA
GGAAGTGAATTTTTCTTGTTGGCTTTTCATGCTACACATCACTAACTAA
CTAAAAACACTAAAATACATTGAAATCTAACCAAATCAAGCATCAAA
ACTTCCCTCTAAGTGTTCGGCCAGCAAGGGATTCATCATGATTTCATGT
GATTCAACCATGGAAAATAAGAAAAAATGCTTAAGAAAGAGAGATCT
CAAGTTTAATTGAAAAAATCTTACCTTTTTTTCACTTGTTTCCTTGAATT
TTCACACTTTTCCTTTGAATTTTTCTACCTAGGGTTTTTTTCTTCCTTTTT
TTTTGCTTCTCCTTGGTGAGACCGACCATAATGAAGAGAAATGGGCTA
GTTTTGTGCTAATTTATAAGGAAAATAATAAAGAAATAAAAGCTTGAC
ACTTGTCACCTTACCATTGGTCTATTTTTAAATTCTTAACTAGTAAGCT
TTCTTTCTCCACCACATAAAATCCTTCAATTATCCATGATAATAATGGA
TGAAATTCATGTGCTGGACAAGTGTTGGGTGGTGTAAAATTACAATTT
TACCCCTGGGGTGGCAAAATGATTATTTTGCCCTCTATGCTTGAAAATG

CCGGAATTAAAATTCTTCACTTCTCAAACTCAAATTATAATCCAAATG
ATCAATCTTAGTCAAAAAATTTCATCCAACACTCACATCTTAACCTCGG
GTAGCAAAATGACCATTTTGCCCCTAGGTCATGAAAATTTCAATTAGA
CTCCAAATCGGTTCTCGAACTCCGAATCACCATTTTAAGTCGTTTTGGG
GTTTTAAAATTCTCAATTTCATCTTAAAATCTCTATTTGGACTAGTTCG
AGGCTTAATTCAACTTAATTGTACTATTTGGTACAATACCATCATTAAC
GATTTTCAAGATACCCAAGTAAACAAATATTCATTCTTATGTAAAAAT
GGTATAATAAACATATTGTTGAACTAGGCTTGACAAACCGCCACTTCT
AACATTATATATAGAACTTTCACAATTCATTTAAGGGACTTTCACTTTT
CGAAACTACCTTAATCTCACTCAAAGCTTCTCTCTCTAGGCCTCTTTTT
ATTCTCTTCCACTTATTATGACACTCAGTACTTAAGAACTGTTTTGATC
TTCCCTTCTGTCACTTCCAGTACATCCCTTACTTATTTGTAACACTTTCT
CTTCTTATCACAAAAACCCCTTTACAGCCATGACACAAGGACTAGATT
AGACCTTAAATATAATATAAATACCAGCTTCGTCTAGAGCTGTCTAGC
CACAAACAAAATCGCAACCCCACCTACAAGAGATTAAAACCCAAAAC
AAGAATAGAAAAAAGATAAAAAAAAAATACAAAATATTTATATTTATT
ATTTACATTTAAGGATTTTTAAAACTTAAATACTATCTCTCATTTACCG
AGAATGTATAGAATCATTATACGACAATCCATCAAATATAAACTTGAA
AATTTTAAAATATATCATTGCATTATCCAATACCCCAATATAATTACCA
AGCATAGAAAAATCTATGGAAGAGTGTTCCCTGTCATTGCATAATATA
GTCCAAAACCCAATAGACCTCTGTATTGAAGAAGAAAAAAAGAGTAC
TCTGAATGGTTTTTGAAAGTGCAATACGCTTCGATTTATGCAAATTTTA
AGAAGTAAAATTTCTTATGTAGGGAGTTAGATTTGCTAATCCCAACGG
CAACAGTGCCCAAAAATGCTTCAAAAGCAAATGCTCTAGCATGAACTT
GGAAGACAATTCAAAAACTACACAAGACAACAGATTCATAAGGCTGT
ACAACCCATGCGATTTAATTATGTTGAAGCTTGAGCCCGATGTAATCA
GTTAAAAAGTCTAAGCCGCAGGGCCATCTCATGCATGGAGAACCCGAC
CTTACCTTCTGGGTAGAGCCACCACAAATTGAGAAATTTGATAGAATA
TTACATGACTAACAATCATACATAAAAACCAAAAAGGAAACATGTCC
GGAATACATAGAGGTACCACATCACTATTTCAAACATATAACCAACTT
CCCACTCATCCTATCCATCATACTGATTTTCAGGGAGAAACATTTTCTT

TTCACCCATAACAAACACAAACTCCGGAAAGGGAAAGACTCTTAATTA
CATACAAAGCAAACACAAGCTTGAATCTTATACCATAGTCGTACATTA
TTACCAAACGCTTTTTATAGATTTTGCCTCCTCACATGTGCACCTCTAC
TCCTCCTCCTCCTCCTCCTCCTCCTCCTCATACTCACCTTCCTCATCAGC
AGTGGCATCCTGGTATTGTTGATACTCGGACACAAGGTCATTCATGTT
GCTCTCCGCCTCTGTGAACTCCATCTCATCCATACCTTCACCAGTATAC
CAATGCAAGAAAGCCTTCCTCCTGAACATAGCAGTGAATTGTTCACTA
ACTCTCCTGAACATTTCCTGAATAGAGGTCGAGTTTCCAATAAAGGTT
GATGCCATGGAGAGCCCTCTAGGTGGAATGTCACACACACTGGATTTC
ACATTGTTTGGGATCCACTCCACAAAGTAGGAAGAATTCTTGTTTTGC
ACATTGATCATTTGCTCATCCACTTCCTTGGTGCTCATCTTGCCTCTGA
ACATGGCCGATGCAGTGAGGTAGCGGCCGTGCCTTGGGTCAGCAGCAC
ACATCATGTTCTTTGCATCCCACATTTGTTGGGTCAACTCAGGGACAGT
CAGAGCACGATACTGCTGGGATCCGCGGGAGGTCAGAGGAGCAAACC
CAACCATAAAAAAGTGAAGACGAGGGAAGGGAATGAGGTTCACTGCA
AGCTTTCGGAGGTCAGAGTTGAGTTGACCAGGGAACCTGAGACAGCA
AGTGACTCCACTCATGGTTGCCGAGATCAAGTGGTTCAAATCACCAAC
TGCAACATGTGTTAATACAAATAA-3’

The sequence extracted from the downstream region of the ISSR marker UBC 857 is 5’-

AGGATACTTGAATTTCATTAGGTCTTTAGCTACACAAAAAGGATAACT
AGCTCCACCCCTATTTGAACACACTGTATCTCAATTAAAATCTCCAAAT
AAATCATTGGATTGCGACTTGAAATTACATTTACAGCCCACACACTTTT
GGGGCCCCAAAATATTACTCAGATTTTATTTGAAATGCTAAGCCAACT
GTGTGTGTGTGTGTGCACGTAAAAAATAAAACCTAAAGTCAAAAGATA
CTTCAGCAACAAAGATATAAATAAGACCAACGGTCATTACGAACAGTT
AGTATAAAAAATTAAAAAAAAAAATTTTGCATGATAAAATTACTTTTC
CATTTTACAGCTACCCATCCATTATTCATTATAGCAGTCCTCATCTATA
ATAGTTGTTGGCCCAATTCAACCCCAAAATTTCAAGGCACCAAACTCT
TCAGCATGGGGGCAATGAACATTATGATCTAGCCACCACTAGTCAAGA

ATTGGCATGTAAAAAACATCACACCAAACAGCAACTGAAAACACAGA
AAACATATTTTTGTAGTCTCATATGTACTTACAGCTAGGAGTGGTCAAT
TTAAGAGTCCTGAAGCAGATATCGTACAAAGCCTCATTATCAAGAACC
ATGCACTCATCAGCATTCTCAACAAGCTGATGAACGGAAAGGGTAGCA
TTGTATGGCTCCACAACTGTATCTGAAACCTTTGGTGAGGGGAACACG
GAGAATGTGAGCATCATCCTGTCAGGGTACTCCTCTCTAATCTTTGAG
ATCAGTAAGGTTCCCATCCCAGACCCAGTTCCTCCACCCAATGAGTGG
CACACCTGGAAACCTGAAATGGCAGATTAGATAAGCTTCCACCAATAA
AGATAGCAACAAATAACTCCAAGAAACATGTTTGGCAACTACAATTAA
AAAGATATCAGCTTCTACCAGTCAGAACCTAAAAGAAACAACTTCCTT
ACCTTGCAGACAGTCACAGTTCTCAGCCTCCTTTCTCACAACATCAAG
AACTGCATCAATAAGCTCAGCTCCCTCAGTGTAATGGCCCTTAGCCCA
GTTGTTTCCAGCACCAGATTGTCCAAAAACAAAGTTATCCGGCCTGAA
AATCTGGCCATAAGGACCAGTTCGGACACTGTCCATGGTACCAGGCTC
CAAGTCCATGAGCACAGCACGCGGAACAAAGCGGCCACAGGAAGCCT
CATTATAGTATACATTGACACGCTCCAACTGCAGATCTGAGGAACCAG
TGTACCTTCCAGTTGGATCTATGCCGTGCTCATCGCAAACAACCTCCCA
AAACTTGGAACCAATCTGGTTCCCACATTGTCCACCTTGAACATGAAG
GATCTCTCTCATTTTTCCCCTAATTTACAACAATTGAGTTGAAATTAAA
TTCCAAATCATAAGACGAAATATTTTGATAAATCTATAACAATATATA
ACAGTATATATTTTGAAAAAAGTACCCGTCATGACAGCAATTGCATAT
TTAAAGTTTATTTTACAAATTCTGTTCATGTCATTTACCCTGTTTGCTGT
AACTGTGATTTATCATTGTTGTTACCTGATATTATATTGTTCTTTATGCA
GTTGATTACTTCACCCTTCTTAGCATGATGGATAAACTTTTATCAGACT
TTTTGTATTGATATGTGATTTTCAATAGTTCATTTCTCCTTATCCCAATT
TATTTTCATGCTATTATTCTTAGTAACAAATGACACTTCAATTATGACT
GTTAACGAATACAAAACTTAGTACATAATACACATGTTGCAACTTTAA
ATGATAATATCAAAAGATTCACTACATTTTTTCCCGAATGCAGTTTTAT
TTATGAACTTAACTTCCATTAACAATTACTTCAGGAGCCATTGGTTATC
AGCAGCCCATTAAAAATGCTACCATTACTTGATTACTAGATGCTGATC
TGTTGAAAATTCAAGCAAGTAGGGAGACAAAAAAATCCTTCTGTTTAC

AGTACATGATCATTGATGATTACGAATTATTCACAGCTTTTACTGTTTT
TGCTTACTTATTAATGCTGTTTTATAGTTTAAATAACATTTTTATCAATT
AATATCAACCACAATATCTTCAACTGTACCTTCTCTCTTAGTAGCTTAC
ATTTAAGCCATTATAGTTCTGTTTGCATAAAAAAAAAAAATTAGCTTTC
TCCTTTCCATTAACACTTCTTTTTAATATTTACAAAATGCTGAATATTC
ACTTCTGACTTTGCTCATTTATGCTAAGCTGTTCTTCGTGTCTTCCTTCA
TTGTTCATTTTGCTGTATTCTCCACTTCTATCAAAACTGTTCTCATTACC
CTTGCATGACAAAGTTAAGCTTTCTCTTCACTTTACATGATTCTTGAAT
CTTTATATTATATTAACCTATTCTATTTTGTTAACTTATCGTTAAAACTT
GCTTACTCATTCGGTCTGTCCATTCCTTTTCAATGCTGATTTCCATTATC
TGTTTGATCTTCTGAAAATACAGAATACTGTTCATTCATTACTTTCGTA
CACTAATTTATAAGCTTTTCCTGTTTTGATTCTGTTTCATGCGGGCAAA
ATCCTAGTTATGACAAAATGAATGCATATCTTAAGATGTAAAAACTTA
ACATATCAAAGAGATTCAAGATGTAATATCCAAGGCATCAAAATACAT
ACAGGCAATGCAGACTCGAACAAAAAAATTAGTTTTCGAATAGAAAA
CGATAATAATTTGAGCACAGTTTCAGTGAAAACGAGTAGAAAAGCTTG
CGCAAATCCAGATCTAAGCAAATGCCAGAACAAGTCTATCCACCACAA
ACGAAATGCAGAGACAAGAACGAGAAGCGAAAGCTTTAAACCGAAAA
TGCCATAAAAACTGTGAAGAAACTTCAGATCTACTTAATTGAATTCAA
CAAAAAGTAAGAGAACAAGCATTACCTGTGTTGGAATTACGGAATTTG
GGAGACTTCGCAGAAATTTTTGAGAGAATAATGAGGAGAGGAAAGGA
AAGGATGAGAGTGCTATATATAGGAAGTGAAGAGGGAGCAGTGAGAT
CGTAGGGTGAGCGGTTGTTCCCTCTTTTTTTAGTGATAGCCGTTGGATG
CCGTTCGATGGAGATAACGGACGGTCCAGATTGCAGAAGTTTGTTTGT
TTTAAAAATTTGTAAAATGTTTTGCGACAAATATACCCTTGGGGGAAG
TGACTGGATCGTCCTCTGAGTCCACCCCAGATTAAAATATCTGAGACC
TATTTGACTCTTGTACTATTTTACCAATTTCAAATCACCGCACATTCGC
CACGTGGCATTCAAACGTTCGGTTTAACTGAGTTGTCTTAAAATTAAC
ATCACGAGCCACTCATATTGAGTCAATGTTATAAGTAAGTTTTGAACC
GTGGATTAGCCCCGTGCCTTTTTATTTATTTATTTTTTATTGTATTATTA
TATTAGTTAGTTTGTTAATGAATAAATCATATATTGAAAATAAAAAGA

AATGAGAGACTTGTTGGTATATGCCTTTGAGCTAATTGAATTGATCAA
GAAATATATATTGTCATTTAATACATACTTAATTACATAATTATATGTG
ATAAAGGTTTTACTTCATGTTAGTACAATAATAACGTATTATTAAGTAC
TAATTGAATAAAGCCCATGAAACATAAAGGTCTTGTAAAAAAATTGTA
AAGTTGTTACAATTATGAGATCACTATGCATCAAAGCCATGTTTCTAA
AATTGTTCCTAGTCATTATATTATCAAGACAAGGTATTGACAATGCTCA
AAGACTAGTACATGTTAAGCCTCTTTATTTGAAAAGTAAACAATCAAT
CTCATAGATTGAAGTGTATGGGATATTTGAGGATAACATGTAGGTGCT
TGTTAAAGAACAAGTTCACTGAATATGACCCGCTATGAGAACTCCATT
TGGTATTTCACTTAAGTATCTATGAAAAATATTCTCATGTGATAGTCGT
GCAACTAGTCTTTGGACTTGAGGCACCAAGTCATCTTGTATGAGAAAT
GACATACTTTGATTTCACCCCTACGGGTCTGGAAGCGACCAGATGCTT
AAACAAGGTGTTTTTGAGTATGCCATAAAGCATGCAAAGACAAATGA
ATGGTCAAGAGAGGATTCATCACCCCAAGTGATATGAGGGGATGTATC
TCACTTGTTATCAATTTCTTGATTAATTTGAATAAAATCTTTGGCCAAA
GCATGATGGATTTGAGAAAAATTAGTTTCCTAAATTCATAAGGTTAGT
CATGAATTATGAGAACTAATATGGAATGTCATAAGTAGACACCGAGCC
ATAGTTAATGACATATTCGAGATATTTGATGAAAAGACCGTATTGCAC
TACGAGGCTTATCACTGAAGGGCTTTGTCAAATTTTTTAATTGACTCTC
TAACATTTGGGTAATCATGAGCATTGCTAGATGTCATTCATGATCTATA
AATATAAATTAATTATCCAATTGATATTTGACTATGATTTATATTTATT
GCCAACACGTTAAAAGCCTAATAGGTCACACACATTAAGTGACATGAT
TAGGATTAAATAAAGATAATTAATTAAATTAGACTTAATTAAAATAGA
CCAAAATAAAGTGAAAAATTAATTGAGTTCTTAAAGACTTAATTAAAT
TAAAATACAACTATTATATTTAGGGTTACAAATTAATTTGGCTATATAA
ATATGTTATGTTAGCAAACTAAAACTCTAAGCCTCAAACCACTTCTCA
GCCGTTTCATAAAATAAGAAAAGAAAAATAGTTTTCTTTGTCTGACAA
AATAAGATTTGGCATAAATTTTTGGTCATTTTGTTTTGAAAACAAAACT
TGCTAGCACAAGTAAAAAATCCTACCTTTGAGAAGGTCTTATTCAGTC
ACTGTGTGAATTACTGTTAAAGGCCAAACACTTGGGTGACTGAAGATT

TGACGAATCCTAAAAGTGAAAAAGCAAAAATTTGGATTCAATTGGCTT
TTAATTACAGTATCTAGA-3’

By using NCBI ORF finder, one ORF each was obtained from the upstream sequences (Fig. 31) and downstream sequences (Fig. 32) of the ISSR marker UBC 857. Details of the ORFs from the upstream and downstream flanking sequences is given in the Table 16.

**Table 16. Details of ORFs from the upstream and downstream flanking sequences of ISSR marker UBC 857**

| Sl. No. | Region | ORF | No. of amino acids | Sequence identity (%) | Protein |
|---|---|---|---|---|---|
| 1 | Upstream region | ORF 1 | 214 | 100 | Beta tubulin chain |
| 2 | Downstream region | ORF 1 | 140 | 84.30 | Hypothetical protein C4D60 |

The single ORF from the downstream region of the ISSR marker UBC 857 had shown 84.30 per cent similarity with hypothetical protein C4D60 when analysed with BLASTp tool. In the upstream region, the ORF identified had shown cent per cent identity with beta tubulin chain. It is spaced by a distance of fifty six nucleotides from the marker UBC 857. By deducing the spacing between the ORF and the marker it can be said that the marker UBC 857 is a part of beta tubulin gene. Thus UBC 857 is linked to beta tubulin gene which provides penetration resistance against the fungi causing VSD.

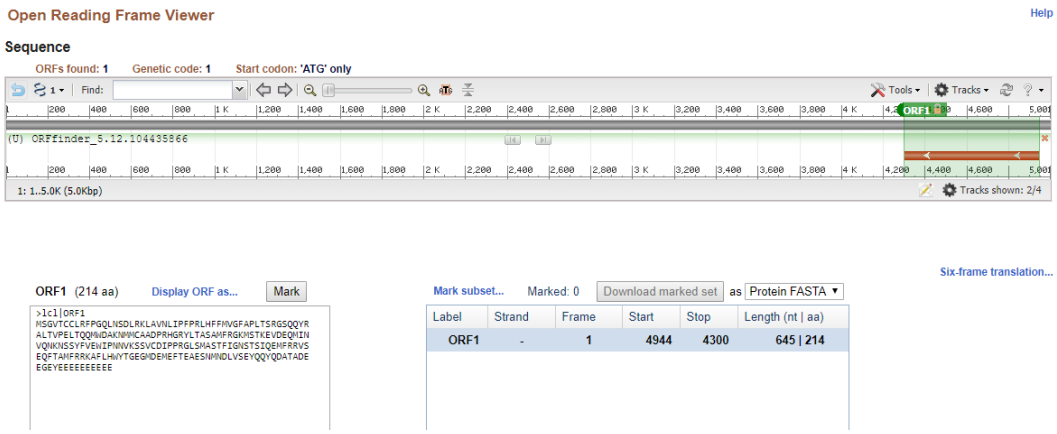


**Fig. 31 ORF from the upstream flanking sequences of the UBC 857 marker**

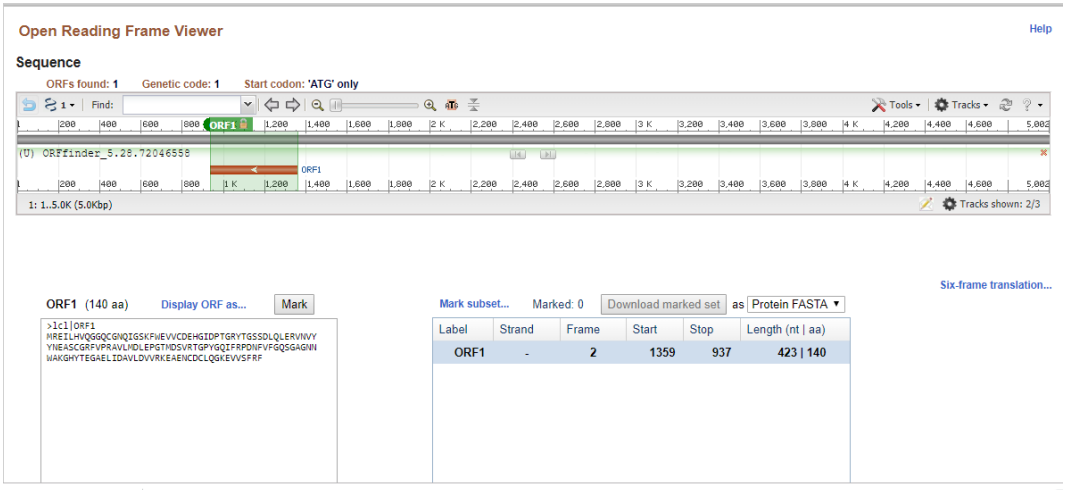


**Fig. 32 ORF from the downstream flanking sequences of the UBC 857 marker**

The ORF obtained from the upstream flanking region of the ISSR marker UBC 857 is

5’-

ATGAGTGGAGTCACTTGCTGTCTCAGGTTCCCTGGTCAACTCAACTCTG
ACCTCCGAAAGCTTGCAGTGAACCTCATTCCCTTCCCTCGTCTTCACTT
TTTTATGGTTGGGTTTGCTCCTCTGACCTCCCGCGGATCCCAGCAGTAT
CGTGCTCTGACTGTCCCTGAGTTGACCCAACAAATGTGGGATGCAAAG
AACATGATGTGTGCTGCTGACCCAAGGCACGGCCGCTACCTCACTGCA
TCGGCCATGTTCAGAGGCAAGATGAGCACCAAGGAAGTGGATGAGCA
AATGATCAATGTGCAAAACAAGAATTCTTCCTACTTTGTGGAGTGGAT
CCCAAACAATGTGAAATCCAGTGTGTGTGACATTCCACCTAGAGGGCT
CTCCATGGCATCAACCTTTATTGGAAACTCGACCTCTATTCAGGAAAT
GTTCAGGAGAGTTAGTGAACAATTCACTGCTATGTTCAGGAGGAAGGC
TTTCTTGCATTGGTATACTGGTGAAGGTATGGATGAGATGGAGTTCAC
AGAGGCGGAGAGCAACATGAATGACCTTGTGTCCGAGTATCAACAAT
ACCAGGATGCCACTGCTGATGAGGAAGGTGAGTATGAGGAGGAGGAG
GAGGAGGAGGAGGAGGAGTAG-3’

### 4.7.2 Identification of genes in flanking sequences of SSR marker

Chandrakant (2014) directly sequenced the amplicon of UBC 857, which was yielding good polymorphic band in resistant cocoa lines to VSD. The sequencing yielded 246 nucleotides, which on BLASTn had shown 94 per cent identity with the *Theobroma cacao* microsatellite DNA clone of mTcCIR42 SSR (NCBI number AJ271944). While screening, distinct polymorphic bands were obtained and this marker was able to differentiate between resistant and susceptible genotypes of cocoa.

The flanking sequences of this SSR marker which contains functional sequences were extracted from NCBI. Five thousand nucleotides each were

extracted from upstream and downstream of the SSR marker to check for any genes.

The sequence extracted from upstream region of the SSR marker-5’-
TGCCATTCATTATGCACATTAAAACCTATTCTTGAGTAACCGTGTCACG
ACCCGGTACTCCCTTCGAGCCCATAACAACCGTCTCGACGTCCCAATA
GACATTTATTGCTCGGAATGCCAACCAGAACCTCGGAAGGCTTTAATA
TCAGTTTTGGCATCTCCCCTATAAGCAACAATTGTTAAGTATCATGTTT
TGAGAGATTATAAACTATTTCCAAATCAAAACAAAAGAAAAATATTTT
TTATCTCATAGGGGCATTTTGATCATTTTTTCTCAAAAATCTCGAAACC
AGCTAAAAATGTAGTATGCAAGTCAAAAACACATATTTCATAATCAAA
AATAAGTTTAGATGTCTAAAACATTCATTTAAAGTGAAACTATAACTA
TTTGAATATAATAAAATTATTCAATAAAATATTCTATTTTCTTATAAAA
CAATATTTATAGAGTTACCGGTAAATAATTTTTGTAGCGAAAAAGCTA
AATAAATTCATACATACTGTAATACAGAGAACCAAAGTATAAAATTTA
ATTTACAGTGGCACGTGGGCCCACGAACGACCAAAAGTATCAAAAGC
GATAAACCTATAGTTTTTGAGGATGCAAGTCCAACTGTAACCCTTAAC
AAAATGAGCTATATACCGACTATCCTAAGCCTAAAATGAGTGGAAAG
GAAAGTGATGAGATTATATAATCCTAGTGAGTAAACAAATACCATCTA
AAATATCTAAAGCTGAGTAAATAGAGACAAATAAAATAGATTAGCAT
AAAAATGATCCACAGCATTCCGAACTCATTTTAATAAGATAAAATAAA
TTCATTTCACCCAAATAAACAATGAGGCTTATGATTTTCTGAAAAGCTT
GACTCGTGCCAAAATCATTCTCATACTCAGTTATCAGGGACACCTTGG
CCTGGGGCTATCCCAACCGAGTCCACCAAGGCTATTAAAAAAGTCATG
TACGCATAAATCATGTTTTTATTTGAAAACATAGTCATTCCTTGGTGTT
GGCTGAGTTCACCCTCACGTGGTGGTTTGGCGTGAAGTGAACTCTAAA
ATTGTACCTCGAGAGTTACTCTATACACCTCTCCAATCGAGGTAAGAA
TATAACGAGATTCCATGCCCCTCAAAAAGGCTGGTCCACAGAACCAAC
CCACTGCAGCAAGCTAAACCTACCATACAATAAAATTTACAATAGCAT
GACATGGCAATTATTTTATTTTACAGCCTCTCAAGGCAAATCAACAGTT
CATACATTTTCAGCACATTTACCAATTCAACCATTCATGCCAATATTAT

CATAAGTCATTCAATTCAAACAATGATTTATAACACAGCAATTAGTCT
CCGATTACTCCATTTTCAAAACCATCTTTCAATTTCAAGATAATTTTAT
AAAATCATTTCATTTAATCACATTTTGCTTATAAAACATAAAGATTTAC
CATTTAAACTCATTTTTCTATCAAATCACAAAAACGAATAAAAACTAC
TTTCGATAATTAAGATGATAATAAGGTTACTCACTATTTCGGGAGTCA
AATTTCGAGTACTCCGATTCTTGATCCTCGGGTACTATCTCTTTACTTTT
GCTAGAAAGCTCACTACTTAGACATAATGCACAATAAGCCATTTACTA
CATTTGTGCTAAACCGTTGTAAAACCAATTCAGGCTTTATACTAATGCA
TGAAATTGGATGTGAAATACTCGGGTAACAATCTAAATACGATGTTCA
ATCTAAGTTCAATTGTGTACCTAAATAAAATGGTATTGGCCTAATTCG
ATCCATCACCCAATTGATTGGCCAATATGTGCAATTTAACTCCAAATA
ATTCCATTTTTCTTCCAATTCTCCAAACCATCAAACACAAGTCAAATAG
CATAAAATTTAGCAAAATCATCTTCACATTTTCTTTTGGGAATTTCGGC
CATGGTGGGTGCATAAATACTATAATTTTTCCTACTTGATTTTCTCATT
GTAATTCATCATTTCCTAACCAATTCACTTAAAATAAAATCAAATCCA
GCATCCACACTCTGCATGGAAGATTCGGCCAATGATGGGTGATGGGAG
GAAGTGAATTTTTCTTGTTGGCTTTTCATGCTACACATCACTAACTAAC
TAAAAACACTAAAATACATTGAAATCTAACCAAATCAAGCATCAAAA
CTTCCCTCTAAGTGTTCGGCCAGCAAGGGATTCATCATGATTTCATGTG
ATTCAACCATGGAAAATAAGAAAAAATGCTTAAGAAAGAGAGATCTC
AAGTTTAATTGAAAAAATCTTACCTTTTTTTTCACTTGTTTCCTTGAATTT
TCACACTTTTCCTTTGAATTTTTCTACCTAGGGTTTTTTTCTTCCTTTTTT
TTTGCTTCTCCTTGGTGAGACCGACCATAATGAAGAGAAATGGGCTAG
TTTTGTGCTAATTTATAAGGAAAATAATAAAGAAATAAAAGCTTGACA
CTTGTCACCTTACCATTGGTCTATTTTTAAATTCTTAACTAGTAAGCTTT
CTTTCTCCACCACATAAAATCCTTCAATTATCCATGATAATAATGGATG
AAATTCATGTGCTGGACAAGTGTTGGGTGGTGTAAAATTACAATTTTA
CCCCTGGGGTGGCAAAATGATTATTTTGCCCTCTATGCTTGAAAATGCC
GGAATTAAAATTCTTCACTTCTCAAACTCAAATTATAATCCAAATGATC
AATCTTAGTCAAAAAATTTCATCCAACACTCACATCTTAACCTCGGGT
AGCAAAATGACCATTTTGCCCCTAGGTCATGAAAATTTCAATTAGACT

CCAAATCGGTTCTCGAACTCCGAATCACCATTTTAAGTCGTTTTGGGGT
TTTAAAATTCTCAATTTCATCTTAAAATCTCTATTTGGACTAGTTCGAG
GCTTAATTCAACTTAATTGTACTATTTGGTACAATACCATCATTAACGA
TTTTCAAGATACCCAAGTAAACAAATATTCATTCTTATGTAAAAATGG
TATAATAAACATATTGTTGAACTAGGCTTGACAAACCGCCACTTCTAA
CATTATATATAGAACTTTCACAATTCATTTAAGGGACTTTCACTTTTCG
AAACTACCTTAATCTCACTCAAAGCTTCTCTCTCTAGGCCTCTTTTTATT
CTCTTCCACTTATTATGACACTCAGTACTTAAGAACTGTTTTGATCTTC
CCTTCTGTCACTTCCAGTACATCCCTTACTTATTTGTAACACTTTCTCTT
CTTATCACAAAAACCCCTTTACAGCCATGACACAAGGACTAGATTAGA
CCTTAAATATAATATAAATACCAGCTTCGTCTAGAGCTGTCTAGCCAC
AAACAAAATCGCAACCCCACCTACAAGAGATTAAAACCCAAAACAAG
AATAGAAAAAAGATAAAAAAAAAATACAAAATATTTATATTTATTATTT
ACATTTAAGGATTTTTAAAACTTAAATACTATCTCTCATTTACCGAGAA
TGTATAGAATCATTATACGACAATCCATCAAATATAAACTTGAAAATT
TTAAAATATATCATTGCATTATCCAATACCCCAATATAATTACCAAGC
ATAGAAAAATCTATGGAAGAGTGTTCCCTGTCATTGCATAATATAGTC
CAAAACCCAATAGACCTCTGTATTGAAGAAGAAAAAAAGAGTACTCT
GAATGGTTTTTGAAAGTGCAATACGCTTCGATTTATGCAAATTTTAAG
AAGTAAAATTTCTTATGTAGGGAGTTAGATTTGCTAATCCCAACGGCA
ACAGTGCCCAAAAATGCTTCAAAAGCAAATGCTCTAGCATGAACTTGG
AAGACAATTCAAAAACTACACAAGACAACAGATTCATAAGGCTGTAC
AACCCATGCGATTTAATTATGTTGAAGCTTGAGCCCGATGTAATCAGT
TAAAAAGTCTAAGCCGCAGGGCCATCTCATGCATGGAGAACCCGACCT
TACCTTCTGGGTAGAGCCACCACAAATTGAGAAATTTGATAGAATATT
ACATGACTAACAATCATACATAAAAACCAAAAAGGAAACATGTCCGG
AATACATAGAGGTACCACATCACTATTTCAAACATATAACCAACTTCC
CACTCATCCTATCCATCATACTGATTTTCAGGGAGAAACATTTTCTTTT
CACCCATAACAAACACAAACTCCGGAAAGGGAAAGACTCTTAATTAC
ATACAAAGCAAACACAAGCTTGAATCTTATACCATAGTCGTACATTAT
TACCAAACGCTTTTTATAGATTTTGCCTCCTCACATGTGCACCTCTACT

CCTCCTCCTCCTCCTCCTCCTCCTCATACTCACCTTCCTCATCAGCA
GTGGCATCCTGGTATTGTTGATACTCGGACACAAGGTCATTCATGTTGC
TCTCCGCCTCTGTGAACTCCATCTCATCCATACCTTCACCAGTATACCA
ATGCAAGAAAGCCTTCCTCCTGAACATAGCAGTGAATTGTTCACTAAC
TCTCCTGAACATTTCCTGAATAGAGGTCGAGTTTCCAATAAAGGTTGA
TGCCATGGAGAGCCCTCTAGGTGGAATGTCACACACACTGGATTTCAC
ATTGTTTGGGATCCACTCCACAAAGTAGGAAGAATTCTTGTTTTGCAC
ATTGATCATTTGCTCATCCACTTCCTTGGTGCTCATCTTGCCTCTGAAC
ATGGCCGATGCAGTGAGGTAGCGGCCGTGCCTTGGGTCAGCAGCACAC
ATCATGTTCTTTGCATCCCACATTTGTTGGGTCAACTCAGGGACAGTCA
GAGCACGATACTGCTGGGATCCGCGGGAGGTCAGAGGAGCAAACCCA
ACCATAAAAAAGTGAAGACGAGGGAAGGGAATGAGGTTCACTGCAAG
CTTTCGGAGGTCAGAGTTGAGTTGACCAGGGAACCTGAGACAGCAAGT
GACTCCACTCATGGTTGCCGAGATCAAGTGGTTCAAATCACCAACTGC
AACATGTGTTAATACAAATAAGTAC-3’

By using NCBI ORF finder, an ORF (ORF 1) of 645 nucleotides (214 amino acids) was found from this upstream sequence at a distance of sixty one nucleotides from the SSR marker (Fig. 33). BLAST of this sequence had shown 97.20 per cent identity with Tubulin beta chain (Fig. 34). Details of the ORFs from the flanking sequences of the SSR marker is given in the Table 17.

**Table 17. Details of ORFs from the flanking sequences of SSR marker**

| Sl No. | ORF | No. of nucleotides | No. of amino acids | Start | Stop | Distance from SSR marker |
|---|---|---|---|---|---|---|
| 1 | ORF 1 | 645 | 214 | 4942 | 4298 | 61 |
| 2 | ORF 2 | 423 | 140 | 769 | 347 | 347 |

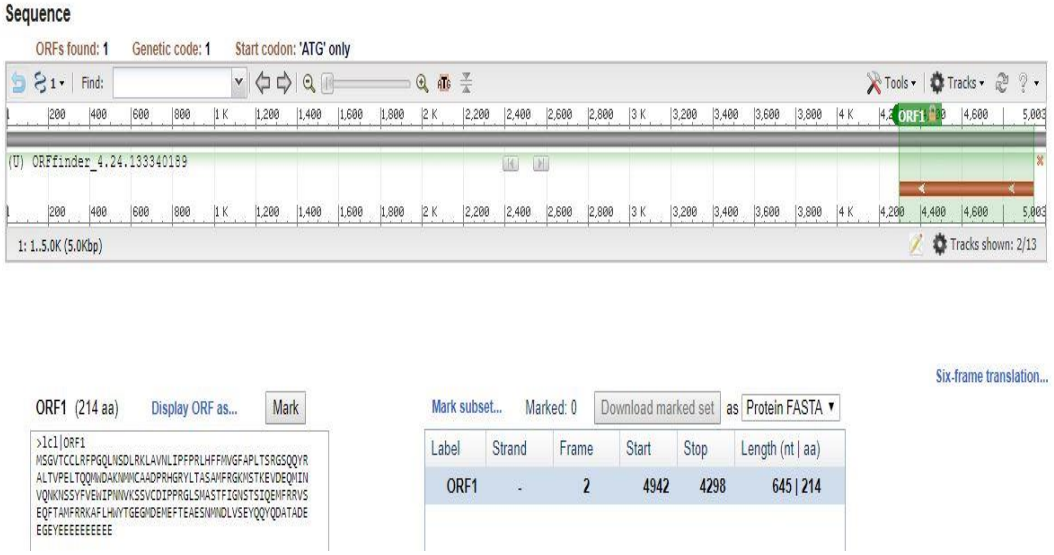


**Fig. 33 ORF from the upstream flanking sequences of the SSR marker**


**Fig. 34 Result of BLAST showing the sequence similarity of the ORF from upstream sequences of SSR marker**

ORF 1

5’-

ATGAGTGGAGTCACTTGCTGTCTCAGGTTCCCTGGTCAACTCAACTCTG
ACCTCCGAAAGCTTGCAGTGAACCTCATTCCCTTCCCTCGTCTTCACTT
TTTTATGGTTGGGTTTGCTCCTCTGACCTCCCGCGGATCCCAGCAGTAT
CGTGCTCTGACTGTCCCTGAGTTGACCCAACAAATGTGGGATGCAAAG
AACATGATGTGTGCTGCTGACCCAAGGCACGGCCGCTACCTCACTGCA
TCGGCCATGTTCAGAGGCAAGATGAGCACCAAGGAAGTGGATGAGCA
AATGATCAATGTGCAAAACAAGAATTCTTCCTACTTTGTGGAGTGGAT
CCCAAACAATGTGAAATCCAGTGTGTGTGACATTCCACCTAGAGGGCT
CTCCATGGCATCAACCTTTATTGGAAACTCGACCTCTATTCAGGAAAT
GTTCAGGAGAGTTAGTGAACAATTCACTGCTATGTTCAGGAGGAAGGC
TTTCTTGCATTGGTATACTGGTGAAGGTATGGATGAGATGGAGTTCAC
AGAGGCGGAGAGCAACATGAATGACCTTGTGTCCGAGTATCAACAAT
ACCAGGATGCCACTGCTGATGAGGAAGGTGAGTATGAGGAGGAGGAG
GAGGAGGAGGAGGAGGAGTAG-3’

The sequence extracted from the downstream region of SSR marker - 5’-

GAAGCAGATATCGTACAAAGCCTCATTATCAAGAACCATGCACTCATC
AGCATTCTCAACAAGCTGATGAACGGAAAGGGTAGCATTGTATGGCTC
CACAACTGTATCTGAAACCTTTGGTGAGGGGAACACGGAGAATGTGA
GCATCATCCTGTCAGGGTACTCCTCTCTAATCTTTGAGATCAGTAAGGT
TCCCATCCCAGACCCAGTTCCTCCACCCAATGAGTGGCACACCTGGAA
ACCTGAAATGGCAGATTAGATAAGCTTCCACCAATAAAGATAGCAAC
AAATAACTCCAAGAAACATGTTTGGCAACTACAATTAAAAAGATATCA
GCTTCTACCAGTCAGAACCTAAAAGAAACAACTTCCTTACCTTGCAGA
CAGTCACAGTTCTCAGCCTCCTTTCTCACAACATCAAGAACTGCATCA
ATAAGCTCAGCTCCCTCAGTGTAATGGCCCTTAGCCCAGTTGTTTCCAG
CACCAGATTGTCCAAAAACAAAGTTATCCGGCCTGAAAATCTGGCCAT
AAGGACCAGTTCGGACACTGTCCATGGTACCAGGCTCCAAGTCCATGA
GCACAGCACGCGGAACAAAGCGGCCACAGGAAGCCTCATTATAGTAT

ACATTGACACGCTCCAACTGCAGATCTGAGGAACCAGTGTACCTTCCA
GTTGGATCTATGCCGTGCTCATCGCAAACAACCTCCCAAAACTTGGAA
CCAATCTGGTTCCCACATTGTCCACCTTGAACATGAAGGATCTCTCTCA
TTTTTCCCCTAATTTACAACAATTGAGTTGAAATTAAATTCCAAATCAT
AAGACGAAATATTTTGATAAATCTATAACAATATATAACAGTATATAT
TTTGAAAAAAGTACCCGTCATGACAGCAATTGCATATTTAAAGTTTAT
TTTACAAATTCTGTTCATGTCATTTACCCTGTTTGCTGTAACTGTGATTT
ATCATTGTTGTTACCTGATATTATATTGTTCTTTATGCAGTTGATTACTT
CACCCTTCTTAGCATGATGGATAAACTTTTATCAGACTTTTTGTATTGA
TATGTGATTTTCAATAGTTCATTTCTCCTTATCCCAATTTATTTTCATGC
TATTATTCTTAGTAACAAATGACACTTCAATTATGACTGTTAACGAATA
CAAAACTTAGTACATAATACACATGTTGCAACTTTAAATGATAATATC
AAAAGATTCACTACATTTTTTCCCGAATGCAGTTTTATTTATGAACTTA
ACTTCCATTAACAATTACTTCAGGAGCCATTGGTTATCAGCAGCCCATT
AAAAATGCTACCATTACTTGATTACTAGATGCTGATCTGTTGAAAATTC
AAGCAAGTAGGGAGACAAAAAAATCCTTCTGTTTACAGTACATGATCA
TTGATGATTACGAATTATTCACAGCTTTTACTGTTTTTGCTTACTTATTA
ATGCTGTTTTATAGTTTAAATAACATTTTTATCAATTAATATCAACCAC
AATATCTTCAACTGTACCTTCTCTCTTAGTAGCTTACATTTAAGCCATT
ATAGTTCTGTTTGCATAAAAAAAAAAAATTAGCTTTCTCCTTTCCATTA
ACACTTCTTTTTAATATTTACAAAATGCTGAATATTCACTTCTGACTTT
GCTCATTTATGCTAAGCTGTTCTTCGTGTCTTCCTTCATTGTTCATTTTG
CTGTATTCTCCACTTCTATCAAAACTGTTCTCATTACCCTTGCATGACA
AAGTTAAGCTTTCTCTTCACTTTACATGATTCTTGAATCTTTATATTATA
TTAACCTATTCTATTTTGTTAACTTATCGTTAAAACTTGCTTACTCATTC
GGTCTGTCCATTCCTTTTCAATGCTGATTTCCATTATCTGTTTGATCTTC
TGAAAATACAGAATACTGTTCATTCATTACTTTCGTACACTAATTTATA
AGCTTTTCCTGTTTTGATTCTGTTTCATGCGGGCAAAATCCTAGTTATG
ACAAAATGAATGCATATCTTAAGATGTAAAAACTTAACATATCAAAGA
GATTCAAGATGTAATATCCAAGGCATCAAAATACATACAGGCAATGCA
GACTCGAACAAAAAAATTAGTTTTCGAATAGAAAACGATAATAATTTG

AGCACAGTTTCAGTGAAAACGAGTAGAAAAGCTTGCGCAAATCCAGA
TCTAAGCAAATGCCAGAACAAGTCTATCCACCACAAACGAAATGCAG
AGACAAGAACGAGAAGCGAAAGCTTTAAACCGAAAATGCCATAAAAA
CTGTGAAGAAACTTCAGATCTACTTAATTGAATTCAACAAAAAGTAAG
AGAACAAGCATTACCTGTGTTGGAATTACGGAATTTGGGAGACTTCGC
AGAAATTTTTGAGAGAATAATGAGGAGAGGAAAGGAAAGGATGAGAG
TGCTATATATAGGAAGTGAAGAGGGAGCAGTGAGATCGTAGGGTGAG
CGGTTGTTCCCTCTTTTTTTAGTGATAGCCGTTGGATGCCGTTCGATGG
AGATAACGGACGGTCCAGATTGCAGAAGTTTGTTTGTTTTAAAAATTT
GTAAAATGTTTTGCGACAAATATACCCTTGGGGGAAGTGACTGGATCG
TCCTCTGAGTCCACCCCAGATTAAAATATCTGAGACCTATTTGACTCTT
GTACTATTTTACCAATTTCAAATCACCGCACATTCGCCACGTGGCATTC
AAACGTTCGGTTTAACTGAGTTGTCTTAAAATTAACATCACGAGCCAC
TCATATTGAGTCAATGTTATAAGTAAGTTTTGAACCGTGGATTAGCCCC
GTGCCTTTTTATTTATTTATTTTTTATTGTATTATTATATTAGTTAGTTTG
TTAATGAATAAATCATATATTGAAAATAAAAAGAAATGAGAGACTTGT
TGGTATATGCCTTTGAGCTAATTGAATTGATCAAGAAATATATATTGTC
ATTTAATACATACTTAATTACATAATTATATGTGATAAAGGTTTTACTT
CATGTTAGTACAATAATAACGTATTATTAAGTACTAATTGAATAAAGC
CCATGAAACATAAAGGTCTTGTAAAAAAATTGTAAAGTTGTTACAATT
ATGAGATCACTATGCATCAAAGCCATGTTTCTAAAATTGTTCCTAGTCA
TTATATTATCAAGACAAGGTATTGACAATGCTCAAAGACTAGTACATG
TTAAGCCTCTTTATTTGAAAAGTAAACAATCAATCTCATAGATTGAAG
TGTATGGGATATTTGAGGATAACATGTAGGTGCTTGTTAAAGAACAAG
TTCACTGAATATGACCCGCTATGAGAACTCCATTTGGTATTTCACTTAA
GTATCTATGAAAAATATTCTCATGTGATAGTCGTGCAACTAGTCTTTGG
ACTTGAGGCACCAAGTCATCTTGTATGAGAAATGACATACTTTGATTT
CACCCCTACGGGTCTGGAAGCGACCAGATGCTTAAACAAGGTGTTTTT
GAGTATGCCATAAAGCATGCAAAGACAAATGAATGGTCAAGAGAGGA
TTCATCACCCCAAGTGATATGAGGGGATGTATCTCACTTGTTATCAATT
TCTTGATTAATTTGAATAAAATCTTTGGCCAAAGCATGATGGATTTGA

GAAAAATTAGTTTCCTAAATTCATAAGGTTAGTCATGAATTATGAGAA
CTAATATGGAATGTCATAAGTAGACACCGAGCCATAGTTAATGACATA
TTCGAGATATTTGATGAAAAGACCGTATTGCACTACGAGGCTTATCAC
TGAAGGGCTTTGTCAAATTTTTTAATTGACTCTCTAACATTTGGGTAAT
CATGAGCATTGCTAGATGTCATTCATGATCTATAAATATAAATTAATTA
TCCAATTGATATTTGACTATGATTTATATTTATTGCCAACACGTTAAAA
GCCTAATAGGTCACACACATTAAGTGACATGATTAGGATTAAATAAAG
ATAATTAATTAAATTAGACTTAATTAAAATAGACCAAAATAAAGTGAA
AAATTAATTGAGTTCTTAAAGACTTAATTAAATTAAAATACAACTATT
ATATTTAGGGTTACAAATTAATTTGGCTATATAAATATGTTATGTTAGC
AAACTAAAACTCTAAGCCTCAAACCACTTCTCAGCCGTTTCATAAAAT
AAGAAAAGAAAAATAGTTTTCTTTGTCTGACAAAATAAGATTTGGCAT
AAATTTTTGGTCATTTTGTTTTGAAAACAAAACTTGCTAGCACAAGTAA
AAAATCCTACCTTTGAGAAGGTCTTATTCAGTCACTGTGTGAATTACTG
TTAAAGGCCAAACACTTGGGTGACTGAAGATTTGACGAATCCTAAAAG
TGAAAAAGCAAAAATTTGGATTCAATTGGCTTTTAATTACAGTATCTA
GAGCAAGGTATGTAACTCTTAAACTTATTAGTGTTCATAATTAATTTAA
AAGTTACATAAAAAACACAAATATTGATTCTATAGGATTCTCTAATTT
AATACTAGTTTATTATTTCGCTGTGATATAATTTTAATTTATTGATTATT
TTCATGTTTGAACATGAGGTCGAATCCCAACAAGACTTCCATGAGTAA
AAATTTTATTTACTATTTTATATAAATTTTTTTCATTAATTGATCTTATT
TAACAATTATAAGTGACATAATCTATATGAAATAATTGAATAAAAATA
GTCAATGATATTAACCAGATATTTTTACATTCTCACTTTACCTTTTAAT
AATGCATAAAATTGTATTTGGTCCAAATTTTTCTATTGATTTTTTTTTAA
TTTTAGTTAACAAAAATATTTCTTTTTTATTTATTTGTACTTAAAAACTT
ATAATTTCGTATCTAAAGAAAGTTAAGTTTTTTATTTTTTATTCATTAA
AATTAACATAAATATATTAAATAGTTTTTCATATTATACAACACTTTTA
CATTAAATTTATCAAGAGCCATCTTAACTTCTCATTACCATTCTTTTTA
AATAATTCTATTGAATGATATTTCCTATAAAAGCTTCATCCAAATCAAA
CAAGCTATAAATTATAATATAAGATAATTCATTTAAATTATGTGGCAT
AA-3’

An ORF (ORF 2) of 423 nucleotides (140 amino acids) was identified from the downstream flanking sequence of the SSR marker at a distance of 347 nucleotides from the SSR marker (Fig. 35). BLAST of this sequence had shown 97.78 per cent with Tubulin beta chain (Fig. 36). Details of the ORF from the downstream flanking sequences has been given in the Table 17.

ORF- 2

5'-

ATGAGAGAGATCCTTCATGTTCAAGGTGGACAATGTGGGAACCAGATT
GGTTCCAAGTTTTGGGAGGTTGTTTGCGATGAGCACGGCATAGATCCA
ACTGGAAGGTACACTGGTTCCTCAGATCTGCAGTTGGAGCGTGTCAAT
GTATACTATAATGAGGCTTCCTGTGGCCGCTTTGTTCCGCGTGCTGTGC
TCATGGACTTGGAGCCTGGTACCATGGACAGTGTCCGAACTGGTCCTT
ATGGCCAGATTTTCAGGCCGGATAACTTTGTTTTTGGACAATCTGGTGC
TGGAAACAACTGGGCTAAGGGCCATTACACTGAGGGAGCTGAGCTTAT
TGATGCAGTTCTTGATGTTGTGAGAAAGGAGGCTGAGAACTGTGACTG
TCTGCAAGGTAAGGAAGTTGTTTCTTTTAGGTTCTGA-3'

Tubulin beta chain belongs to the microtubular component of cytoskeletal elements. It provides resistance by not allowing the fungi to penetrate the outer epidermal wall of the plants, hence protecting the plants from infection. This critically depends on fortification of the cell wall at the site of attempted penetration through the development of specialised cell wall appositions rich in antimicrobial compounds. Formation of cell wall appositions is achieved by rapid reorganisation of cytoskeletal elements (Kobayashi and Hakuno, 2003; Takemoto *et al*., 2003). Penetration resistance is a highly effective defence strategy rapidly mobilised by plants in response to attempted penetration by fungal and oomycete pathogens.

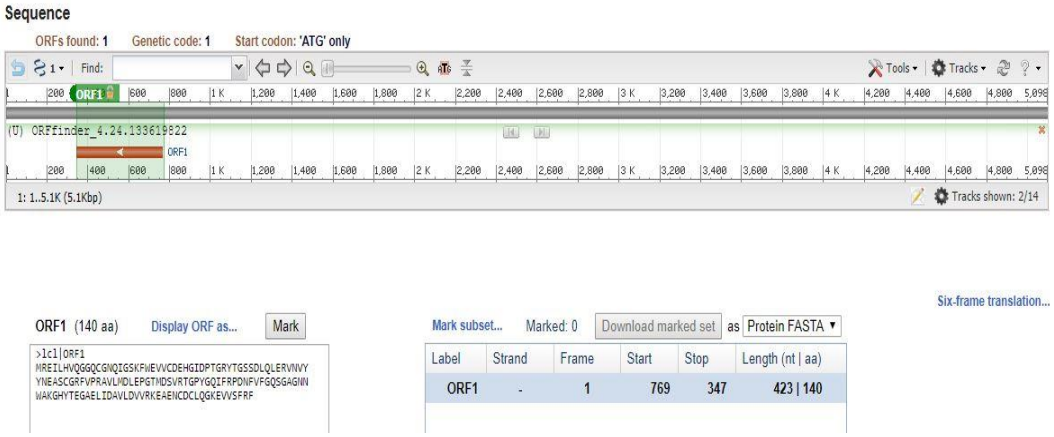


**Fig. 35 ORF from the downstream flanking sequences of the SSR marker**


**Fig. 36 Result of BLAST showing the sequence similarity of the ORF from downstream sequences of SSR marker**

The ORF 1 is on the left side of the SSR marker spaced by sixty one nucleotides. ORF 2 is on the right side of the SSR marker spaced by three hundred and forty seven nucleotides (Plate 9). Thus it is clear that the ORFs 1 and 2 are part of the same gene and the SSR marker mTcCIR42 lies within the beta tubulin gene, making it a functional marker. Thus it can be deduced that the SSR marker, which was proved to be linked with one of the VSD resistant genes from the earlier studies, is linked to the beta tubulin gene, which provides penetration resistance against the causal organism of VSD.

Further confirmation of the beta tubulin gene to which the SSR marker is linked can be done. Since the sequence of the gene is known, the primers of the resistant gene can be directly designed and it can be used for confirmation of resistant genotypes of cocoa.

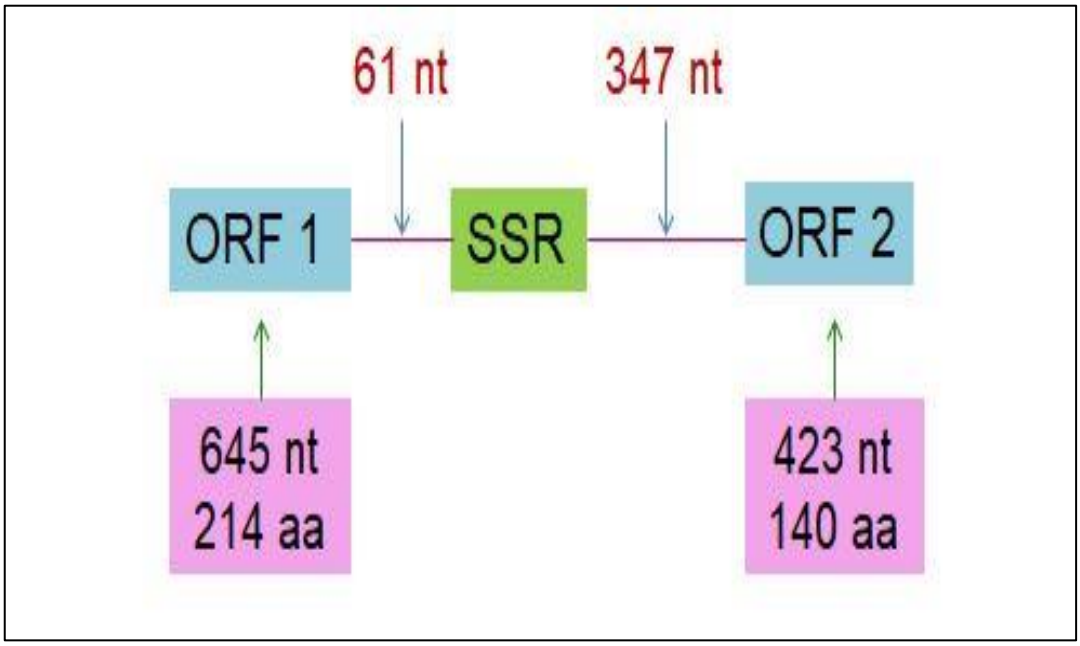


**Plate 9. Distance of ORFs from the SSR marker**

# SUMMARY

# 5. SUMMARY

The research work entitled 'Inheritance of molecular markers linked to vascular streak dieback disease resistance in hybrid progenies of cocoa (*Theobroma cacao* L.)' was carried out at Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, and Cocoa Research Centre, Kerala Agricultural University during 2016-2018. The objective of the research work was to study the inheritance of identified ISSR and SSR markers linked to VSD resistance in hybrid progeny of cocoa (*Theobroma cacao* L.).

The progeny from the hybridization between the VSD resistant hybrids *viz*., M 13.12 × GVI 55 (Stand no. 2.3), GII 19.5 × GVI 55 (Stand no. 4.1), GVI 4 × GVI 55 (Stand no. 5.11), GVI 126 × GIV 18.5 (Stand no. 10.8), GVI 126 × GIV 18.5 (Stand no. 11.10), GVI 126 × GIV 18.5 (Stand no. 11.23), GVI 126 × GVI 55 (Stand no. 13.11), GVI 126 × GVI 55 (Stand no. 13.13), GVI 126 × GVI 55 (Stand no. 14.6), GVI 126 × GVI 55 (Stand no. 14.15), GVI 137 × GVI 55 (Stand no. 15.7), GVI 140 × GVI 55 (Stand no. 16.10), GVI 140 × GVI 55 (Stand no. 16.11), GVI 143 × GVI 55 (Stand no. 19.6), GVI 148 × GVI 55 (Stand no. 21.27), GVI 126 × GIV 18.5 (Stand no. 10.10), GVI 137 × GVI 55 (Stand no. 15.10), GVI 167 × GIV 18.5 (Stand no. 22.1) maintained at Cocoa Research Centre, Kerala Agricultural University since 1998. Response to VSD disease and morphological characters of the hybrids were scored regularly at Cocoa Research Centre from 1999 onwards.

The salient outcome of the study is briefed as follows:

1. A total of two thousand two hundred and thirty seven flowers were pollinated during the period 2016- 2017. Seven pods obtained from the hybridization between the above said eighteen hybrids were sown in the nursery.

2. About two hundred and sixty nine seedlings were grown from the seven hybridized pods in which nursery screening for disease resistance was

done. Inoculum for disease incidence was provided naturally by placing disease infected seedlings all around the hybrid seedlings. High humidity is ensured by over head sprinklers.

3. Visual screening recorded one hundred and eighty seedlings as disease resistant, fourteen seedlings as partially resistant and seventy five seedlings as disease susceptible.

4. One hundred and twenty seedlings were selected for molecular analysis by using validated ISSR and SSR markers from the previous studies. Out of one hundred and twenty, one hundred and six were found to be resistant and fourteen were found to be partially resistant, when visually screened for disease symptoms.

5. The genomic DNA isolation protocol suggested by Dellaporta *et al*. (1983) was used for good quality genomic DNA extraction.

6. The quality and quantity of the isolated DNA was evaluated by using Nanodrop® spectrophotometer. The absorbance ratio ranged between 1.80-1.99 in majority of the samples. The quantity of DNA isolated ranged between 50.2 to 1125.7 ng/µL.

7. Three ISSR markers (UBC 811, UBC 815 and UBC 857) and one SSR marker (mTcCIR42) linked with VSD resistance gene, identified and validated from the previous studies (Chandrakant, 2014; Tulshiram, 2016) were used for screening the seedlings.

8. One hundred and twenty DNA samples were screened by using four primers to see the presence or absence of VSD resistant gene.

9. The polymorphic band of 950 bp, which was found to be linked with the gene conferring VSD resistance (Chandrakant, 2014; Tulshiram, 2016)

was found in seventy seven resistant seedlings and three partially resistant seedlings screened.

10. When screened with the primer UBC 815, the polymorphic band of 750 bp, which was found to be linked with VSD resistant gene from the previous studies, was present in only twenty five resistant and one partially resistant seedling.

11. Screening with the primer UBC 857, yielded the marker band of 450 bp which was validated to be tagged with the gene conferring VSD resistance, had shown the presence of marker band in twenty one resistant and one partially resistant seedling.

12. When the SSR marker was screened on one hundred and twenty seedlings, the 200 bp marker band, which was tagged with the VSD resistant gene was detected in fourty six resistant and six partially resistant seedlings.

13. The mean inheritance percentage of the markers UBC 811, UBC 815, UBC 857 and mTcCIR42 in all the one hundred and six resistant seedlings from seven crosses were found to be 71.70 per cent, 23.47 per cent, 21.62 per cent and 48.78 per cent respectively.

14. The ISSR marker UBC 811 and the SSR marker, mTcCIR42 were found to be having comparatively good percentage of inheritance among the segregating progeny screened.

15. Nineteen seedlings from four crosses were identified with three or more of the screened markers expressed.

16. Flanking sequences of the ISSR markers UBC 811, UBC 857 and the SSR marker mTcCIR42 were extracted from the whole genome database of cocoa which included five thousand nucleotides each from upstream and downstream region of each marker.

17. ORFs identified from both the upstream and downstream flanking sequences of the ISSR marker UBC 811 by using the ORF finder tool has turned out to be uncharacterized proteins

18. One ORF identified from the upstream region of the marker UBC 857 has shown identity with beta tubulin gene when analysed with BLASTp tool

19. By analysing the spacing between the ORF and the marker, it can be deduced that the marker UBC 857 is a part of beta tubulin gene

20. Two ORFs were identified from both the upstream and downstream flanking sequences by using the ORF finder tool.

21. With BLASTp tool, it was analyzed that both the ORFs showed more than 97 per cent identity to beta tubulin gene.

22. When the spacing between the marker and the flanking sequences were analysed, it was deduced that both the ORFs are part of the same gene and the SSR marker mTcCIR42 lies within the beta tubulin gene.

23. Tubulin beta chain belongs to the microtubular component of cytoskeletal elements which provides resistance by not allowing the fungi to penetrate the outer epidermal wall of the plants, hence protecting the plants from infection.

24. Penetration resistance is a highly effective defence strategy rapidly mobilised by plants in response to attempted penetration by fungal and oomycete pathogens.

25. The ISSR marker UBC 857 and the SSR marker mTcCIR42 is linked to the beta tubulin gene, which provides VSD resistance by giving resistance against penetration of the plant cell by the fungus.

The future line of the work includes field planting of the nineteen seedlings with three or more markers expressed. They can be further evaluated for yield and yield contributing characters since double cross hybrids were found to be superior to single cross and can overcome the yield reduction phenomena expressed in resistance breeding (Sriani *et al*., 2003; Ghanwat *et al*., 2016). Sequence of the beta tubulin gene can be used for primer designing, which can be used for confirmation by screening in resistant genotypes of cocoa.

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# APPENDIXES

## APPENDIX I

**List of laboratory equipments used for the study**

Refrigerated centrifuge : Kubota 6500, Japan

Horizontal electrophoresis system : BioRad, USA

Thermal cycler : Agilent Technologies (SureCycler 8800) and Life Technologies (Proflex)

Gel documentation system : BioRad, USA

Nanodrop® ND-1000 spectrophotometer : Nanodrop®Technologies lnc. USA

## APPENDIX II

### Composition of reagents required for DNA isolation

### 1. Extraction buffer 1

Tris : 1 M, pH 8.0
EDTA : 0.5 M, pH 8.0
NaCl : 4 M
Adjusted the pH to 8 and made up final volume up to 100 ml.

### 2. Extraction buffer 2

Tris : 1 M, pH 8.0
EDTA : 0.5 M, pH 8.0
Adjusted the pH to 8 and made up final volume up to 100 ml.

### 3. SDS (20 per cent)

SDS : 20 g
Distilled water: 80 mL

### 4. 5 M KOAC (Pottassium acetate)

KOAC : 49.1 g
Distilled water: Up to 100 mL

### 5. 3 M NaOAC (Sodium acetate)

NaOAC : 24.6 g
Distilled water: Up to 100 mL

### 6. Ethanol (80 per cent)

To the 80 parts of absolute ethanol (100 per cent), 20 parts of sterile distilled water was added to make 80 per cent ethanol.

## Appendix III

**Composition of reagents used for quality assessment of isolated DNA**

### 1. TAE Buffer 50X

Tris base : 242 g
Glacial acetic acid : 57.1 ml
0.5M 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.

## APPENDIX IV

### Details of hand pollination

| Plant No. | Cross | No. of flowers pollinated |
|---|---|---|
| 4.1 | 2.3 | 12 |
| | 11.10 | 18 |
| | 10.8 | 5 |
| | 14.6 | 4 |
| | 16.10 | 3 |
| | 15.7 | 29 |
| | 13.13 | 4 |
| | 21.27 | 2 |
| | 5.11 | 1 |
| | 13.11 | 3 |
| | 11.23 | 1 |
| | 14.15 | 1 |
| | 16.11 | 1 |
| | 22.1 | 1 |
| 2.3 | 11.10 | 47 |
| | 15.7 | 52 |
| | 10.8 | 10 |
| | 14.6 | 19 |
| | 4.1 | 67 |
| | 16.11 | 20 |
| | 21.27 | 8 |
| | 13.13 | 10 |
| | 13.11 | 17 |
| | 5.11 | 2 |
| | 15.10 | 2 |
| | 16.10 | 1 |

| | | |
|---|---|---|
| 2.3 | 10.10 | 3 |
| | 11.23 | 1 |
| | 19.6 | 2 |
| | 14.15 | 1 |
| 5.11 | 13.13 | 2 |
| | 2.3 | 2 |
| | 11.10 | 14 |
| | 15.7 | 2 |
| | 16.10 | 1 |
| | 16.11 | 1 |
| | 21.27 | 1 |
| | 11.23 | 3 |
| | 14.15 | 1 |
| | 14.6 | 5 |
| | 4.1 | 1 |
| | 10.8 | 3 |
| 10.10 | 4.1 | 7 |
| | 22.1 | 1 |
| | 16.10 | 5 |
| | 13.11 | 1 |
| | 2.3 | 2 |
| | 11.10 | 3 |
| | 10.8 | 2 |
| 10.8 | 11.10 | 19 |
| | 2.3 | 22 |
| | 5.11 | 5 |
| | 13.13 | 5 |
| | 14.6 | 5 |
| | 16.10 | 24 |
| | 13.11 | 9 |

| | | |
|---|---|---|
| 10.8 | 22.1 | 1 |
| | 16.11 | 4 |
| | 10.10 | 6 |
| | 21.27 | 1 |
| | 15.10 | 1 |
| | 4.1 | 18 |
| | 15.7 | 30 |
| 11.10 | 5.11 | 10 |
| | 4.1 | 99 |
| | 2.3 | 16 |
| | 10.8 | 24 |
| | 16.11 | 65 |
| | 16.10 | 14 |
| | 13.11 | 12 |
| | 21.27 | 2 |
| | 10.10 | 4 |
| | 14.6 | 7 |
| 13.11 | 2.3 | 16 |
| | 10.8 | 3 |
| | 14.6 | 13 |
| | 10.10 | 5 |
| | 11.10 | 6 |
| | 16.10 | 12 |
| | 4.1 | 3 |
| | 13.13 | 2 |
| | 16.11 | 8 |
| 13.13 | 2.3 | 39 |
| | 4.1 | 33 |
| | 10.8 | 12 |
| | 11.10 | 40 |

| | | |
|---|---|---|
| 13.13 | 5.11 | 6 |
| | 14.15 | 4 |
| | 16.10 | 11 |
| | 14.6 | 9 |
| | 16.11 | 9 |
| | 13.11 | 5 |
| | 21.27 | 3 |
| | 10.10 | 5 |
| | 15.7 | 7 |
| | 15.10 | 1 |
| | 22.1 | 3 |
| 14.15 | 11.10 | 3 |
| | 2.3 | 17 |
| | 13.13 | 4 |
| | 4.1 | 9 |
| | 10.8 | 6 |
| | 5.11 | 6 |
| | 15.7 | 1 |
| | 16.10 | 1 |
| | 21.27 | 1 |
| | 14.6 | 1 |
| | 16.11 | 1 |
| 11.23 | 5.11 | 4 |
| | 2.3 | 3 |
| | 10.8 | 1 |
| | 4.1 | 2 |
| | 16.11 | 1 |
| 21.27 | 11.23 | 1 |
| | 11.10 | 2 |
| | 10.8 | 1 |

| | | |
|---|---|---|
| 21.27 | 2.3 | 3 |
| | 16.11 | 1 |
| 15.10 | 10.10 | 9 |
| | 2.3 | 7 |
| | 13.11 | 2 |
| | 16.10 | 3 |
| | 4.1 | 2 |
| 16.10 | 13.13 | 47 |
| | 11.23 | 2 |
| | 21.27 | 4 |
| | 2.3 | 155 |
| | 11.10 | 29 |
| | 10.8 | 17 |
| | 5.11 | 9 |
| | 4.1 | 23 |
| | 15.7 | 2 |
| | 14.15 | 3 |
| | 14.6 | 1 |
| | 13.11 | 5 |
| | 16.11 | 6 |
| 16.11 | 11.23 | 4 |
| | 21.27 | 5 |
| | 2.3 | 48 |
| | 11.10 | 27 |
| | 10.8 | 18 |
| | 5.11 | 7 |
| | 13.13 | 55 |
| | 14.15 | 3 |
| | 4.1 | 46 |
| | 15.7 | 1 |

| | | |
|---|---|---|
| 16.11 | 16.10 | 9 |
| | 14.6 | 3 |
| | 13.11 | 3 |
| | 10.10 | 3 |
| | 15.10 | 2 |
| 15.7 | 21.27 | 2 |
| | 2.3 | 58 |
| | 10.8 | 8 |
| | 11.10 | 24 |
| | 14.15 | 3 |
| | 13.13 | 8 |
| | 4.1 | 53 |
| | 16.10 | 23 |
| | 16.11 | 18 |
| | 5.11 | 1 |
| | 11.23 | 2 |
| | 14.6 | 1 |
| | 13.11 | 5 |
| | 10.10 | 2 |
| 14.6 | 15.7 | 2 |
| | 11.23 | 5 |
| | 21.27 | 9 |
| | 2.3 | 38 |
| | 11.10 | 32 |
| | 10.8 | 13 |
| | 14.15 | 4 |
| | 4.1 | 47 |
| | 16.11 | 16 |
| | 16.10 | 22 |
| | 5.11 | 3 |

| | | |
|---|---|---|
| 14.6 | 13.11 | 12 |
| | 13.13 | 14 |
| | 19.6 | 4 |
| 22.1 | 15.10 | 41 |
| | 5.11 | 2 |
| | 4.1 | 2 |
| 19.6 | 11.10 | 19 |
| | 4.1 | 8 |
| | 13.11 | 3 |
| | 16.10 | 5 |
| | 14.6 | 8 |
| | 13.13 | 14 |
| | 2.3 | 4 |
| | 10.10 | 1 |
| | 10.8 | 3 |
| | 15.7 | 2 |
| | 16.11 | 4 |
| | Total | 2237 |

## APPENDIX V

## $HD^2$ value of selected hybrids

| Sl. No. | Cross | Plant No. | Height (H) | Diameter (D) | $H \times D^2$ |
|---|---|---|---|---|---|
| 1 | 16.11× 4.1 | 1.1 | 22 | 0.76 | 12.71 |
| 2 | | 1.2 | 20 | 0.71 | 10.10 |
| 3 | | 1.3 | 22 | 0.73 | 11.72 |
| 4 | | 1.4 | 23 | 0.76 | 13.28 |
| 5 | | 1.5 | 20 | 0.73 | 10.66 |
| 6 | | 1.6 | 19 | 0.75 | 10.69 |
| 7 | | 1.7 | 23 | 0.76 | 13.28 |
| 8 | | 1.8 | 23 | 0.73 | 12.26 |
| 9 | | 1.9 | 24 | 0.80 | 15.36 |
| 10 | | 1.10 | 22 | 0.76 | 12.71 |
| 11 | | 1.11 | 20 | 0.73 | 10.66 |
| 12 | | 1.12 | 23 | 0.75 | 12.94 |
| 13 | | 1.13 | 23 | 0.76 | 13.28 |
| 14 | | 1.14 | 22 | 0.76 | 12.71 |
| 15 | | 1.15 | 21 | 0.74 | 11.50 |
| 16 | | 1.16 | 24 | 0.80 | 15.36 |
| 17 | | 1.17 | 25 | 0.80 | 16.00 |
| 18 | | 1.18 | 21 | 0.73 | 11.19 |
| 19 | | 1.19 | 22 | 0.74 | 12.05 |
| 20 | 14.6× 4.1 | 2.1 | 23 | 0.80 | 14.72 |
| 21 | | 2.2 | 24 | 0.80 | 15.36 |
| 22 | | 2.3 | 23 | 0.76 | 13.28 |
| 23 | | 2.4 | 22 | 0.73 | 11.72 |
| 24 | | 2.5 | 23 | 0.74 | 12.60 |
| 25 | | 2.6 | 24 | 0.80 | 15.36 |
| 26 | | 2.7 | 24 | 0.76 | 13.86 |
| 27 | 14.6× 16.11 | 3.1 | 24 | 0.75 | 13.50 |
| 28 | | 3.2 | 27 | 0.78 | 16.43 |
| 29 | | 3.3 | 23 | 0.67 | 10.32 |
| 30 | | 3.4 | 22 | 0.76 | 12.71 |
| 31 | | 3.5 | 23 | 0.67 | 10.32 |
| 32 | | 3.6 | 22 | 0.74 | 12.05 |
| 33 | | 3.7 | 25 | 0.73 | 13.32 |
| 34 | | 3.8 | 25 | 0.74 | 13.69 |
| 35 | | 3.9 | 26 | 0.73 | 13.86 |
| 36 | | 3.10 | 26 | 0.76 | 15.02 |
| 37 | | 3.11 | 27 | 0.76 | 15.60 |
| 38 | | 3.12 | 25 | 0.73 | 13.32 |
| 39 | | 3.13 | 23 | 0.67 | 10.32 |

| | | | | | |
|---|---|---|---|---|---|
| 40 | 14.6× 16.11 | 3.14 | 25 | 0.73 | 13.32 |
| 41 | 16.10× 2.3 | 4.1 | 25 | 0.72 | 12.96 |
| 42 | | 4.2 | 24 | 0.73 | 12.79 |
| 43 | | 4.3 | 23 | 0.72 | 11.92 |
| 44 | | 4.4 | 25 | 0.75 | 14.06 |
| 45 | | 4.5 | 25 | 0.75 | 14.06 |
| 46 | | 4.6 | 24 | 0.72 | 12.44 |
| 47 | | 4.7 | 23 | 0.72 | 11.92 |
| 48 | | 4.8 | 26 | 0.82 | 17.48 |
| 49 | | 4.9 | 25 | 0.76 | 14.44 |
| 50 | | 4.10 | 28 | 0.78 | 17.04 |
| 51 | | 4.11 | 27 | 0.75 | 15.19 |
| 52 | | 4.12 | 26 | 0.79 | 16.23 |
| 53 | | 4.13 | 25 | 0.78 | 15.21 |
| 54 | 4.1× 15.7 | 5.1 | 23 | 0.72 | 11.92 |
| 55 | | 5.2 | 25 | 0.75 | 14.06 |
| 56 | | 5.3 | 22 | 0.75 | 12.38 |
| 57 | | 5.4 | 24 | 0.72 | 12.44 |
| 58 | | 5.5 | 26 | 0.75 | 14.63 |
| 59 | | 5.6 | 23 | 0.74 | 12.59 |
| 60 | | 5.7 | 22 | 0.74 | 12.05 |
| 61 | | 5.8 | 23 | 0.67 | 10.32 |
| 62 | | 5.9 | 22 | 0.70 | 10.78 |
| 63 | | 5.10 | 28 | 0.83 | 19.29 |
| 64 | | 5.11 | 26 | 0.80 | 16.64 |
| 65 | | 5.12 | 25 | 0.83 | 17.22 |
| 66 | | 5.13 | 24 | 0.75 | 13.50 |
| 67 | | 5.14 | 26 | 0.77 | 15.42 |
| 68 | | 5.15 | 24 | 0.73 | 12.79 |
| 69 | | 5.16 | 25 | 0.80 | 16.00 |
| 70 | | 5.17 | 22 | 0.76 | 12.71 |
| 71 | | 5.18 | 26 | 0.86 | 19.23 |
| 72 | | 5.19 | 24 | 0.76 | 13.86 |
| 73 | | 5.20 | 25 | 0.80 | 16.00 |
| 74 | | 5.21 | 29 | 0.92 | 24.55 |
| 75 | | 5.22 | 28 | 0.92 | 23.70 |
| 76 | 11.10× 16.11 | 6.1 | 23 | 0.71 | 11.59 |
| 77 | | 6.2 | 27 | 0.78 | 16.43 |
| 78 | | 6.3 | 23 | 0.71 | 11.59 |
| 79 | | 6.4 | 27 | 0.80 | 17.28 |
| 80 | | 6.5 | 29 | 0.60 | 10.44 |
| 81 | | 6.6 | 28 | 0.75 | 15.75 |
| 82 | | 6.7 | 21 | 0.54 | 6.12 |
| 83 | | 6.8 | 24 | 0.71 | 12.10 |

| | | | | | |
|---|---|---|---|---|---|
| 84 | 16.11× 13.13 | 7.1 | 25 | 0.73 | 13.32 |
| 85 | | 7.2 | 28 | 0.76 | 16.17 |
| 86 | | 7.3 | 24 | 0.73 | 12.79 |
| 87 | | 7.4 | 27 | 0.76 | 15.60 |
| 88 | | 7.5 | 28 | 0.60 | 10.08 |
| 89 | | 7.6 | 30 | 0.70 | 14.70 |
| 90 | | 7.7 | 25 | 0.60 | 9.00 |
| 91 | | 7.8 | 29 | 0.73 | 15.45 |
| 92 | | 7.9 | 22 | 0.70 | 10.78 |
| 93 | | 7.10 | 24 | 0.70 | 11.76 |
| 94 | | 7.11 | 25 | 0.73 | 13.32 |
| 95 | | 7.12 | 21 | 0.83 | 14.47 |
| 96 | | 7.13 | 22 | 0.83 | 15.16 |
| 97 | | 7.14 | 28 | 0.80 | 17.92 |
| 98 | | 7.15 | 23 | 0.76 | 13.28 |
| 99 | | 7.16 | 28 | 0.70 | 13.72 |
| 100 | | 7.17 | 26 | 0.67 | 11.67 |
| 101 | | 7.18 | 24 | 0.78 | 14.60 |
| 102 | | 7.19 | 30 | 0.80 | 19.20 |
| 103 | | 7.20 | 23 | 0.80 | 14.72 |
| 104 | | 7.21 | 26 | 0.76 | 15.02 |
| 105 | | 7.22 | 26 | 0.76 | 15.02 |
| 106 | | 7.23 | 19 | 0.68 | 8.79 |
| 107 | | 7.24 | 22 | 0.70 | 10.78 |
| 108 | | 7.25 | 24 | 0.67 | 10.77 |
| 109 | | 7.26 | 26 | 0.70 | 12.74 |
| 110 | | 7.27 | 30 | 0.73 | 15.99 |
| 111 | | 7.28 | 34 | 0.83 | 23.42 |
| 112 | | 7.29 | 25 | 0.80 | 16.00 |
| 113 | | 7.30 | 25 | 0.70 | 12.25 |
| 114 | | 7.31 | 24 | 0.83 | 16.53 |
| 115 | | 7.32 | 24 | 0.73 | 12.79 |
| 116 | | 7.33 | 21 | 0.80 | 13.44 |
| 117 | | 7.34 | 20 | 0.70 | 9.80 |
| 118 | | 7.35 | 24 | 0.76 | 13.86 |
| 119 | | 7.36 | 22 | 0.73 | 11.72 |
| 120 | | 7.37 | 24 | 0.67 | 10.77 |

# INHERITANCE OF MOLECULAR MARKERS LINKED TO VASCULAR STREAK DIEBACK DISEASE RESISTANCE IN HYBRID PROGENIES OF COCOA (*Theobroma cacao* L.)

**By**

**MIDHUNA M. R.**
**(2016-11-106)**

**ABSTRACT OF THESIS**

***Submitted in partial fulfillment of the***

***requirement for the degree of***

## MASTER OF SCIENCE IN AGRICULTURE

**(Agricultural Plant Biotechnology)**

**Faculty of Agriculture**

**Kerala Agricultural University, Thrissur**


**DEPARTMENT OF PLANT BIOTECHNOLOGY**
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**KERALA, INDIA**

**2019**

## Abstract

*Theobroma cacao* L. (also known as the chocolate tree) is a major cash crop and the costliest beverage crop. Andhra Pradesh is the leading cocoa producing state in India but Tamil Nadu ranks first with an area of 26,969 ha. Vascular Streak Dieback (VSD) caused by the fungus *Ceratobasidium theobromae* is a serious disease in cocoa. Since it is a vascular pathogen, chemicals have little effect on disease control. The most tenable and economic technique to tackle this disease is by evolving resistant materials. Kerala Agricultural University had initiated VSD resistant breeding since 1995. Seedlings from hybridization, exhibiting high levels of resistance were selected and field established. Nineteen hybrids, exhibiting resistance to VSD (after screening for a period of thirteen years), were selected for the present study.

The progeny obtained from these hybrids by crossing it among themselves were used as plant materials for the study. Two thousand two hundred and thirty seven flowers were pollinated and seven pods were obtained. About two hundred and sixty nine seedlings were grown from the seven hybridized pods in which nursery screening for disease resistance was done. Inoculum was dispensed by keeping already infected seedlings around the experimental materials. High humidity was ensured by providing over head sprinkler system. Visual screening recorded one hundred and eighty seedlings as disease resistant, fourteen seedlings as partially resistant and seventy five seedlings as disease susceptible.

Three ISSR markers (UBC 811, UBC 815 and UBC 857) and one SSR marker (mTcCIR42) linked with VSD resistance gene, identified and validated from the previous studies were used for screening of the one hundred and twenty seedlings out of which one hundred and six were resistant and fourteen were partially resistant.

The polymorphic band of 950 bp, which was found to be linked with the gene conferring VSD resistance was recorded in seventy seven resistant seedlings

and three partially resistant seedlings, when screened with the primer UBC 811. When screened with the primer UBC 815 (750 bp) and UBC 857 (450 bp), the polymorphic marker band which was found to be linked with VSD resistant gene from the previous studies, was present in only twenty five resistant and one partially resistant seedling and twenty one resistant and one partially resistant seedling respectively. When screened with SSR marker, the 200 bp marker band, which was tagged with the VSD resistant gene was detected in fourty six resistant and six partially resistant seedlings. The ISSR marker UBC 811 and SSR marker mTcCIR42 were found to be having comparatively good percentage of inheritance among the segregating progeny screened with a mean inheritance percentage of 71.70 per cent and 48.78 per cent respectively.

Flanking sequences of the ISSR markers UBC 811, UBC 857 and SSR marker mTcCIR42 were extracted from the whole genome database of cocoa. The ORFs from the flanking sequences of UBC 811 were identified to be uncharacterized proteins by using BLASTp tool. One ORF from the upstream sequence of the UBC 857 had shown identity with beta tubulin chain. Analyzing the distance between the marker and the flanking region, it was deduced that UBC 857 is a part of beta tubulin gene. Two ORFs were identified from both the upstream and downstream flanking sequences of the SSR marker. Using BLASTp tool, it was analyzed that both the ORFs showed more than 97 per cent identity to beta tubulin gene. Analysing the spacing between the marker and the flanking sequences, it was deduced that both the ORFs are part of the same gene and the SSR marker mTcCIR42 lies within the beta tubulin gene. Tubulin beta chain belongs to the microtubular component of cytoskeletal elements which provides resistance by not allowing the fungi to penetrate the outer epidermal wall of the plants, hence protecting the plants from infection. The ISSR marker UBC 857 and the SSR marker mTcCIR42 are linked to the beta tubulin gene, which provides VSD resistance by giving resistance against penetration of the plant cell by the fungus.

Nineteen seedlings were identified to be having three or more markers expressed. They can be planted in the field and can be further evaluated for yield and yield contributing characters. Sequence of the beta tubulin gene can be used for primer designing, which can be used for confirmation by screening in resistant genotypes of cocoa.