PRELIMINARY EVALUATION OF DOUBLE CROSS HYBRIDS FOR YIELD AND VASCULAR STREAK DIEBACK (VSD) DISEASE RESISTANCE IN COCOA (*Theobroma cacao* L.)

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KERALA, INDIA

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THESIS

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DEPARTMENT OF PLANT BREEDING AND GENETICS

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KERALA, INDIA

2020

DECLARATION

I, hereby declare that the thesis entitled **"Preliminary evaluation of double cross hybrids for yield and Vascular Streak Dieback (VSD) disease resistance in cocoa" (*Theobroma cacao* L.)**is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title of any other University or Society.

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Certified that the thesis entitled **"Preliminary evaluation of double cross hybrids for yield and Vascular Streak Dieback (VSD) disease resistance in cocoa" (*Theobroma cacao* L.)"** is a record of research work done independently by **Ms. Alfiya A. R.** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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ABBREVIATIONS

%	Percentage
=	Equal to
µg	Microgram
µL	Microlitre
AFLP	Amplified Fragment Length Polymorphism
PCR	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	Potassium 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
ºC	Degree Celsius
OD	Optical Density
pH	Hydrogen ion concentration
pM	Pico Mole
PVP	Poly vinyl pyrrolidone
QTL	Quantitative Trait Loci
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
SCAR	Sequence Characterized Amplified Region
SNP	Single Nucleotide Polymorphism
TAE	Tris Acetate EDTA
TE	Tris EDTA
U	Unit
UBC	University of British Columbia
V	Volts
β	Beta
VSD	Vascular Streak Dieback

Introduction

1. INTRODUCTION

Cocoa (*Theobroma cacao* L.) commonly known as 'Food of God' is one of the perennial trees of high economic value. Cocoa is a cross pollinated tree species belonging to family Malvaceae (Alverson *et al*., 1999). It is believed to be originated in the tropical rainforest of equatorial America specifically at the foot of the Andes near the Amazon basin (Mossu, 1992). The most important economically valuable part of cocoa is its beans which forms the principal raw material for confectioneries, beverages, chocolates and other edible products.

Breeding programmes generally focus on the development of high yielding varieties. However, the outbreak of novel pests and pathogens resulted in prioritizing more on development of pest and disease resistant varieties without compromising the yield potential.

Vascular Streak Dieback disease (VSD) caused by *Ceratobasidium theobromae* (Samuels *et al.*, 2012), pose a great threat to cocoa growers. The disease causes complete defoliation and eventually death of the tree (Abraham *et al.*, 2002). The disease was first reported in Papua New Guinea (Shaw, 1962; Bridgland *et al.*, 1967), with a characteristic brown streaking in the xylem of infected shoots (Keane *et al.*, 1972) and was later reported in many parts of Southeast Asian countries (Keane and Guest, 2007).

In India, the disease was initially observed in Kottayam district of Kerala (Abraham, 1981, Chandramohan and Kaveriappa, 1982) and was later reported in other districts (Abraham and Ravi, 1991).

Ceratobasidium theobromae is a highly specialized, near-obligate parasite of cocoa (Keane and Guest, 2007). The characteristic symptoms of VSD include a green-spotted chlorosis and falling of leaves beginning on the second or third flush behind the stem apex, raised lenticels and darkening at the leaf scars and infected xylem. Eventually, complete defoliation occurs and lead to the death of the tree (Abraham *et al.*, 2002).

Different management strategies including quarantine, cultural practices, biological, botanical and chemical control are employed for the control of the disease. However, even high volume spray of chemicals was found to be ineffective in complete control of the disease (Prior, 2007), and the only way to tackle the disease is to breed resistant varieties.

Development of double cross hybrids can evolve pest and disease resistant varieties without sacrificing yield and with maximum exploitation of heterosis (Gallais and Guy, 1971). The double cross hybrid produced by crossing between Trinitario and upper Amazonian type cocoa found to be much superior to the single cross hybrids (Efron *et al.,* 2003). Average yield superiority of the double cross hybrids over the F_1 hybrids have been proven by many scientists (Sriani *et al*., 2003; Ghanwat *et al.,* 2016).

Molecular markers are strong tool in identifying genotypes with disease resistance since they are least affected by the environment (Efron *et al.,* 2002). ISSR and SSR primers are proven to be efficient in tagging resistant genes (Pinheiro *et al.,* 2012). Chandrakant (2014) identified three ISSR markers UBC811, UBC857, UBC826 and one SSR marker mTcCIR42 to be linked with VSD resistance. Tulshiram (2016) further validated their efficiency in VSD resistant plants.

Cocoa Research Centre (CRC), Kerala Agricultural University (KAU), had undergone massive breeding programme in developing double cross hybrids for VSD resistance using parents that expressed field resistant for VSD for more than 35 years. Selection of superior genotypes at two to two and half years age in cocoa can negate the long wait of eight years from planting for selection (Francies, 1998). Motilal *et al*. (2017) also suggested that selection at an early stage employing marker-assisted selection programme is desirable in perennial crops like cocoa.

With this background, twenty double cross hybrids at early bearing stage selected from the double cross hybrids bred for vascular streak die back disease resistance and field planted during 2017 at Cocoa Research Centre (CRC) farm served as the material for the study entitled ‘Preliminary evaluation of double cross hybrids for yield and Vascular Streak Dieback (VSD) disease resistance in cocoa

(*Theobroma cacao* L.)'. The objectives of the study was to evaluate the double cross hybrids bred for vascular streak die back disease resistance in early yielding stage and to select the superior double cross hybrids for comparative yield trial.

Review of Literature

2. REVIEW OF LITERATURE

The botanical name of cocoa is *Theobroma cacao*, first defined by Carolus Linnaeus, the father of modern-day taxonomic plant classification, and was published in his classic book, *Systema Naturae* in the mid-1700s. *Theobroma* is a Greek word that can be translated as 'Food of Gods': 'theos' meaning god and 'broma' meaning food.

The primary types of cocoa grown have traditionally been referred to as Forastero, Trinitario, and Criollo. Most of the world's production is based on Forastero-derived types (Hunter, 1990). Earlier, cocoa was categorized in the Sterculiaceae family and at present grouped in the family Malvaceae (Bayer and Kubitzki, 2003). The Forastero is further divided into upper Amazon and lower Amazon types. The Trinitario types are hybrids between Forastero and Criollo types (Motamayor *et al.,* 2003). *Theobroma cacao* is an economically important agricultural commodity for millions of people across the world. It is cultivated by about 6 million farmers globally, and livelihood for more than 40 million people (World Cocoa Foundation, 2012; Beg *et al*., 2017).

2.1. COCOA CULTIVATION IN INDIA

British introduced cocoa in India from the islands of Amboina during the 20th century (Ratnam, 1961). Cocoa forms a major component of complex agrosystem and thus contribute economic and ecological benefits and are mostly cultivated by smallholder farmers (Wood and Lass, 1985; Alves, 1990). In India cocoa is grown as an intercrop over an area of 94,008 hectares with a total production of 23,981 metric tonnes in the southern states namely Kerala, Karnataka, Andhra Pradesh and Tamil Nadu. Andhra Pradesh ranks first in area, production and productivity with an area of 32,949 hectares having a production of 9615 metric tonnes and 950 kg/ ha productivity. The average Indian productivity is 669 kg/ ha (DCCD, 2020).

2.2. DIVERSITY IN COCOA POPULATIONS

The cocoa population was reported to exhibit a high degree of variation in their morphological characters as well as in genetic level due to their outbreeding nature (Cuatrecasas, 1964). Cocoa was classified into different groups such as varieties, cultivars, types or populations based on morphological characters of pods and beans (Dias, 2001).

Based on the appearance of the pod, Bekele and Butler (2000) classified cocoa into four groups as follows: 1) Angoleta (deeply ridged, warty, square at the stalk end); (2) Cundeamor (similar to Angoleta however characterized by a bottleneck); 3) Amelonado (smooth, shallow furrows, melon-shaped with a blunt end and slight bottleneck); and (4) Calabacillo (small and nearly spherical).

A set of 15 microsatellites (SSRs) that can detect a high degree of polymorphism have been identified and are proposed as international molecular standards for DNA fingerprinting of *T. cacao* (Saunders *et al*., 2001, 2004).

2.3. MORPHOLOGICAL EVALUATION OF THE COCOA

Germplasm collections and families of related accessions can be efficiently utilized for their economic as well as breeding gains through morphological evaluation (Iwaro, *et al.,* 2003; Bekele *et al*., 2006; Maharaj *et al*., 2011). Effective utilization of crop germplasm can be achieved through morphological characterization of plant materials with desired traits (Santos *et al.,* 2012). Both qualitative and quantitative characters are evaluated for morphological characterization. Wide variability in morphology is exhibited by various genotypes of cocoa.

Cocoa breeders use morphological descriptors to choose the most suitable accessions for breeding programmes (Engels *et al.*, 1980). Leaf, flower, fruit and seed descriptors are used for the phenotypic characterization of the species utilizing the gene bank resources (Engels *et al.,* 1980; Bekele and Bekele, 1996). The flush colour of leaves in cocoa ranges from pale green to shades of red (Wood and Lass, 1985; Kochhar, 1998).

Characterization of 155 clones of wild cocoa trees (*Theobroma cacao* L.) belonging to two different rivers basins was carried out in French Guiana based on the four floral descriptors *i.e.* petal ligule width, sepal width, gynoecium length and number of ovules per ovary. They identified significant variability among the materials for the floral characters studied (Lachenaud *et al.,* 1999).

Asna (2013) carried out morphological and biochemical evaluation of fifty cocoa accessions (including exotic and indigenous) and based on the evaluation accessions were clustered. Indigenous accessions were clustered into three clusters based on quantitative characters whereas exotic accessions were clustered into nine clusters.

Cocoa types and populations are distinguished mainly based on fruit phenotype (Efombagn *et al.,* 2009). Matured fruit is berry but commonly known as pod. The shape of fruit varies from spherical to slightly elongated. The colour ranges from green to reddish for immature pods, whereas it ranges from yellowish to pinkish colour in matured pods.

Morphological variability in cocoa populations can be estimated using various morphological traits of fruits like apex form, immature colour and basal constriction (Engels, 1983; Bekele *et al*., 1994; Lachenaud *et al.,* 1999). A study was carried out in 23 accessions of cocoa to determine the role of pod apex on pod shape and confirmed the influence of fruit apex in fruit shape (Minimol *et al.,* 2011).

Fallo and Cilas (1998) reported high heritability and additive variance for cocoa bean weight. Generally, the pods comprise of 20-40 beans and the colour of the beans may be white, pink, brown or purple (Bartley, 2005). Rubeena (2015) conducted a study on variation in morphological and biochemical characters in twenty five hybrid progenies bred for bold beans to analyse bean character and concluded that bean weight, bean width and bean thickness contribute to the boldness of beans. A descriptor that confirmed to international standards to assess the performance of cocoa types was developed based on this study. As per the descriptor, threshold bean size indicated a bean having a weight, breadth and thickness in the range from 1.16 to

1.31 g, 10.29 to 11.22 mm and 4.35 to 4.98 mm respectively.

Morphological characters like the number of normal beans per pod, pod girth, pod weight, pod length, wet beans weight per pod, dry bean weight and the number of flat beans per pod were recorded in 14 cocoa accessions by Sari and Susilo (2013). They concluded that pod weight influenced the dry weight of normal beans and wet bean weight per pod character can be substituted by dry weight per normal bean as selection criteria.

Adenuga *et al.* (2015) conducted a study on phenotypic variation in nine early bearing cocoa genotypes based on pod and bean characters and reported significant genotypic variation for fruit length, weight of beans per pod, fresh weight of single bean, pod value, dry bean length and pod index. Genetic distance among the germplasms and population structures can be determined using the morphological and agronomic characters of leaf, flower, pod and bean (Thi *et al*., 2016).

Sumitha *et al.* (2018) studied the performance of the seven improved varieties (CCRP 1, CCRP 2, CCRP 3, CCRP 4, CCRP 5, CCRP 6 and CCRP 7) under Tamil Nadu condition. Mean pod weight among the varieties was 440.06 g. Pod weight exhibited variation from 399.45g in CCRP 7 to 502.19 g in CCRP 5. The number of beans were highest in CCRP 5 (48.31) and lowest in CCRP 7 (34.18). CCRP 5 excelled other varieties with a single dry bean weight of 1.02 g.

Enriquez and Soria (1967) recorded that variation in the dry bean weight of single bean ranged from 0.5 to 2.5 g. Based on the dry weight, they classified beans as small (< 0.99 g), medium (1-1.5 g) and large (>1.6 g). According to Monteiro *et al.* (2009), cocoa genotypes with bean weight more than one gram is regarded as superior. Since beans are the economic part of cocoa, genotypes with higher bean weight are more desirable (Oyedokun *et al.*, 2011).

Widyasary and Susandarini (2020) evaluated the morphological characters of leaf, pod and bean in nineteen cocoa clones in four districts of Suleswani, Indonesia and the variability was used to derive the taxonomic affinity using cluster analysis. They observed higher variability in pod and bean characters than leaf characters and

cluster analysis grouped the clones into two clusters based on similarity in morphology. Morphological parameters were used to study diversity in the KAU released varieties in cocoa (Sujith *et al.*, 2017).

2.4. BIOCHEMICAL CHARACTERS OF COCOA

2.4.1. Fat Estimation in Cocoa Beans

Among the crop plants, cocoa ranks second in having the highest fat content next to coconut. Cocoa butter, due to its physical, chemical and unique organoleptic nature is of significant demand in the food industry (Lipp and Anklam, 1998). It forms a key ingredient in most of the confectioneries. Physical properties of cocoa butter are exploited in the pharmaceutical and cosmetic industries.

Cost of production of chocolate depends on the fat content of the cocoa beans since lower the fat content, higher the cost of grinding the beans. Thus, the economic value of beans depends on their fat content (Duncan and Veldsman, 1994; Wood and Lass, 1985).

A study conducted on fat estimation of 490 Brazilian accessions concluded the average fat content as 53.2 per cent showing a variation of 45.4 per cent in accession CC 57 to 60.3 per cent in accession NA 312. A significant negative correlation between dry bean yield per plant and fat content was also reported. Significance of pollen in determining the fat content of beans was identified by a full diallel crossing of three genotypes having high fat with three genotypes of low fat (Pires, *et al*., 1998).

Moreno *et al*. (2015) determined the fat content and nutritional composition of cocoa beans from the different geographical origin (Ghana and Ecuador). They identified fat as the major nutrient (>40 %) in the cocoa beans irrespective of their origin.

Rubeena (2015) estimated fat content in hybrids and parents bred for bold bean trait and recorded a variation from 33.3 to 58.5 per cent in hybrids and 38.51 to 52.7 per cent among the parents.

2.4.2. Polyphenol Estimation in Cocoa

Polyphenols have gained great significance recently due to its antioxidant and anti-inflammatory properties that have preventive and/or therapeutic effects for cardiovascular disease, neurodegenerative disorders, cancer and obesity (Perez, *et al.*, 2010; Singh *et al.*, 2011).

Cocoa liquor, beans and powder showed variation in total polyphenol and ranged from 45-52 mg/ g, 34-60 mg/ g and 20-62 mg/ g, respectively (Nazaruddin *et al.,* 2006). D'Souza *et al*. (2017) conducted a detailed analysis of the polyphenols present in both fermented and unfermented cocoa beans from different geographic origins to understand their systematic difference based on their origin as well as fermentation process. To identify and quantify the proanthocyanidins and their glycosides, they employed ultra-high performance liquid chromatography (UHPLC) along with ultra-high resolution time of flight mass spectroscopy. They determined the number of biomarkers that can distinguish the fermented and unfermented beans as well as beans of different origins. The polyphenol content of cocoa products is determined by its place of origin and method employed in processing (Jalil and Ismail, 2008).

Cocoa is rich in polyphenols like flavanol monomers (epicatechin and catechin) and their oligomers namely procyanidins, and anthocyanidins (Hammerstone *et al.,* 1999). Unfermented cocoa beans consist of monomers and tetradecamers (Kelm *et al.*, 2006).

Polyphenol constitutes 6-8 per cent of dry bean weight (Wollgast and Anklam, 2000). Cocoa accessions were characterized based on phenol content (Asna and Presannakumari, 2016). They observed variation of phenol content between 2.25 and 9.09 per cent in the accessions studied.

2.5. HETEROSIS BREEDING IN COCOA

Development of superior hybrids has made a significant contribution to cocoa productivity. Hybrid vigour is exploited by crossing distant genotypes for the

production of superior hybrids in cocoa (Apshara, 2019).

Cocoa (*Theobroma cacao* L.) exhibited high hybrid vigour for yield and yield contributing characters (Atanda and Toxopeus, 1971; Dias and Kageyama, 1997). Development of high and early yielding cocoa hybrids with disease resistance resulted in increased production in Brazil (Dias and Kageyama, 1995). A study conducted in Nigeria revealed that hybrids exhibited high heterosis and heritability for yield traits than their parents (Sobowale *et al*., 2016).

Genetic improvement of cocoa was attained by the development of superior hybrids in Kerala (Minimol *et al.*, 2015). Quality traits, yield, adaptability as well as resistance to pest and diseases have been achieved through hybrid breeding programmes (Adewale *et al*., 2014). Hybrid (CCRP 15) resistant to vascular streak dieback disease with considerable yield was developed and resistance was confirmed by budding and by using molecular markers (Minimol *et al.*, 2018).

2.6. DOUBLE CROSS HYBRIDS

Heterosis can be fully exploited through the development of double cross hybrids to evolve pest and disease resistant varieties with maximum yield potential (Gallais and Guy, 1971). Efron *et al.* (2003) conducted a study on crosses between Trinitario and upper Amazonian type cocoa and found that double cross hybrids are much superior to the single cross hybrids. Sriani *et al*. (2003) evaluated the economic performance of ten double cross hybrids of maize developed from eleven inbred lines and recorded average yield superiority of the double cross hybrids over check variety. Ghanwat *et al.* (2016) also evaluated single and double cross hybrids developed from six parental inbred lines in maize and found three double cross hybrids excelled among them with high per se performance along with significant heterosis.

2.7. DISEASES IN COCOA

Microbes like fungi, virus and nematodes cause several diseases in cocoa (Wood and Lass, 1985; Bowers, 2001), however most of them possess only regional or local significance.

The diseases of global importance include black pod, caused by *Phytophthora* spp. (*P. palmivora*, *P. capsici*, *P. citrophthora*, *P. heveae*, and *P. megakaria*); witches'-broom, caused by *Crinipellis perniciosa*; cocoa swollen shoot virus (CSSV) and monilia pod rot, caused by *Moniliophthora roreri* (Dias, 2001). These diseases may cause an annual yield reduction of nearly 40 per cent and may extend up to 90-100 per cent in some locations (Wood and Lass, 1985). In West African countries CSSV transmitted by mealy bugs was found to be devastating (Muller and Sackey, 2005).

Diseases like vascular streak dieback (VSD) caused by *Oncobasidium theobromae,* cacao wilt (*Ceratocystis* blight), caused by *Ceratocystis fimbriata*, *Verticillium* wilt, caused by *Verticillium dahliae*, and pink disease, caused by *Corticium salmonicolo* are confined to certain regions, however, are of high economic importance.

Most of these diseases are controlled by the combined practice of phytosanitary pruning with adequate fungicide application (Wood and Lass, 1985; Dias, 2001; Krauss and Soberanis, 2001). However, such practices are uneconomic and non-ecofriendly. Thus, breeding for the development of resistant varieties is of prime important.

The limited sources of genetic resistance in cocoa against the pests and pathogens pose a hindrance (Eskes and Lanaud, 1997). However, advances in cocoa genetics and molecular techniques are contributing to resistance breeding (Eskes and Lanaud, 1997; Bennett, 2003; Risterucci *et al*., 2003; Clement *et al*., 2004). Transgenic resistance can also be achieved through transformation and regeneration (Maximova *et al.,* 2005).

2.8. DIEBACK IN COCOA

Dieback is the progressive death of plants due to the environmental factors like water stress, physiological and nutritional disorders, pathogens, insect attacks and or some other interactions between these different factors. However, the symptoms of dieback as typical of fungal infection indicate that the pathogens as the prime cause

for the disease (Archer and Adu-Acheampong, 2011). Globally, several pathogens are found to be directly linked to cocoa dieback. In Cameroon, a fungus namely *Lasiodiplodia theobromae* has been isolated and identified as the causal agent for cocoa dieback (Mbenoun *et al.,* 2008). The same fungus was later found to be the causative agent for dieback in the cocoa plantation that occurred in 2000 in the Karnataka state in India (Kannan *et al.,* 2010). Insect vectors like *Sahbergella singularis* (brown cocoa mirid) in association with pathogenic fungi also resulted in cocoa dieback (Anikwe and Otuonye, 2015).

Oncobasidium theobromae and *Verticillium* sp. also caused dieback symptoms in cocoa in Southeast Asia and Uganda respectively (Lass, 1985).

2.9. VASCULAR STREAK DIEBACK

In the early 1960s, a devastating disease was first reported in Papua New Guinea (Shaw, 1962; Bridgland *et al.,* 1967). It was named as vascular-streak dieback (VSD) due to the characteristic brown streaking in the xylem of infected shoots. This is visible when the dry surface of the leaf scars formed by the abscission of infected leaves were scraped off (Keane *et al.,* 1972).

Vascular streak dieback disease has severely affected the cocoa plantations in Malaysia during the 1960s and 1970s (Turner and Shepherd, 1980). It was reported as a new encounter interaction between an indigenous fungus *Ceratobasidium (Oncobasidium) theobromae,* and cocoa introduced into Southeast Asia and parts of Melanesia (Keane and Guest, 2007). Samuels *et al.* (2012) reported another species of *Ceratobasidium, Ceratobasidium ramicola* as causal organism for VSD in cocoa in regions of Java, East Java, Kaliwining and Papua localities of Indonesia and Florida of USA.

In the initial stage of infection, white basiodicarps were seen on leaf scar and further the diseased leaves fell off during the wet weather. The fungus was identified belonging to a new genus and species of basidiomycete, *Oncobasidium theobromae* (Talbot and Keane, 1971), and was present only in the xylem vessels of infected parts. It is an obligate parasite and its isolation is difficult. They can be transferred to an

agar medium but cannot be sub-cultured. They do not produce spores in artificial medium (Keane *et al.,* 1972; Keane and Prior, 1991). Avocado (*Persea americana*: Lauraceae) is the only known alternative host of *C. theobromae* (Anderson, 1989). Infection is lethal when affected on seedlings and clonal plants. In matured plants, it is fatal in the susceptible genotypes.

VSD has resulted in heavy economic losses in most of cocoa growing areas in South and Southeast Asia and Melanesia (Ploetz, 2007). Even though VSD is a geographically restricted disease, it causes a yield loss of about 30,000 tonnes (Ploetz, 2007).

Malaysian cocoa industry was adversely affected by VSD, because the farmers substituted cocoa with alternatives like oil palm due to high cost of management of the disease. VSD expanded its spread in the cocoa plantations of East and West Java, Africa and Latin America.

The fungus was renamed as *Thanatephorus theobromae* by Roberts (1999). Based on the DNA sequence data, Samuels *et al.* (2012) placed the species within *Ceratobasidium*, the genus to which currently it belongs to. Oberwinkler *et al.* (2013) proposed that the fungus belongs to *Rhizoctonia* genus.

Abraham (1981) and Chandramohan and Kaveriappa (1982) reported this disease for the first time in India in the Kottayam district of Kerala. It further spread to other districts including Trivandrum, Pathanamthitta, Kozhikode, Idukki *etc.* (Abraham and Ravi, 1991).

2.9.1. Symptoms of VSD

The foremost visible typical symptom of VSD is the single leaf chlorosis generally the second or third flush behind the shoot apex, with scattered islets of green tissues, 2-5 mm in diameter (Keane *et al.,* 1972; Keane and Prior, 1991). As the infection spread, the infected leaves become fully chlorotic and fall. It takes several months from the initial infection to the visible expression of symptoms in the plants. Three discoloured vascular traces are visible at the point of attachment of leaves when they shed off. It gradually spread to adjacent leaves and the stem. Infected xylem is

visible as dark streaks inside the vascular tissues when the stems are cut longitudinally. 'Broomstick' symptoms are developed by the proliferation of lateral buds which later die due to the infection from the main stem. Lenticels expand thus makes the bark rough. In susceptible genotypes, the fungus spread internally and ultimately kills the tree.

The distinct symptoms expressed uniformly in different locations over several years. Many scientists reported the expression of symptoms of the disease from various countries including Papua New Guinea, India, Malaysia and Indonesia (Prior, 1980; Abraham, 1981, Ahmad, 1982 and Abraham and Ravi, 1991).

However, in 2004, a change in the expression of symptoms was observed around several distinct geographical locations like Malaysia (Peninsular Malaysia and Sabah) and Indonesia (East Java, Bali, Sulawesi, Papua and West Papua) (Keane and Guest, 2007; Purwantara *et al*., 2009). Instead of the formation of green islands on the yellowing leaves, necrotic patches were developed and the leaves tended to remain attached. Sometimes, both the traditional and novel symptoms expressed together.

2.9.2. Epidemiology of VSD

During wet weather conditions, when the affected leaf falls off, hyphae emerge out of the leaf scar and develop into basidiocarp. However, if leaf shed off in a dry condition the scar heals and hyphae fail to emerge. Thus, only during wet weather conditions basidiocarps are continuously formed. It forms a white, flat, velvety coating over the leaf scars and nearby bark. Having a short life span, basidiocarps release spores at night only. Basidiospores remain viable for only a few hours in the morning and need free water for germ tube growth (Prior, 1979). On attached branches, basidiocarp remains viable for nearly one week whereas only for one or two days on cut branches. Basidiocarp formation, basidiospore release, dissemination of spores and infection of leaves depend on extended periods of wetness, rather than intense rainfall. Thus, there is a critical relationship between wetness, as influenced by rainfall and humidity, and infection periods (Keane, 1981; Dennis and Keane, 1992).

Dispersion of basidiospores is mediated through the wind, however, effective dispersal occurs at high humidity and wet conditions (Keane, 1981). The infection found to be fatal to seedlings less than ten month old (Keane, 1981).

In West Malaysia and Papua New Guinea, the disease spread was more in the wetter region having an annual rainfall of more than 2500 mm (Dennis and Keane, 1992). Sporulation and infection were higher in the fields where there was high canopy humidity and leaf wetness due to heavy shade and unpruned cocoa plants (Dennis and Keane, 1992).

2.9.3. Management of VSD

Management of VSD is carried out through the following practices.

2.9.3.1. Quarantine

Quarantine measures have to be taken to restrict the transport of *C. theobromae* by humans since the natural spread of the fungus is very restricted. Movement of cocoa vegetative planting material from VSD infected areas to unaffected areas is prohibited in Malaysia (Byrne, 1976). However, under strict quarantine procedures cocoa planting materials can be exchanged. Superior clones from New Britain was transferred to the disease-free islands of the North Solomons and New Ireland under strict quarantine supervisions (Prior, 1985).

Studies on the seeds of pods from infected branches revealed that there was no transmission of disease through them (Keane and Prior, 1991). Thus, seeds do not pose a threat for importing countries since seeds in their fresh or dried form or fermented condition does not act as a carrier of the pathogen. Grafts are also safe for exchange since budwoods from infected branches do not produce basidiocarp. The infection and sporulation in the nursery seedlings can be avoided by protecting them with any structures to prevent the leaves from getting wet for more than one hour.

2.9.3.2. Chemical Control

In Malaysia and Papua New Guinea, several fungicides were applied as a soil drench and foliar application to control VSD caused by *Ceratobasidium theobromae*. However, most of them found to be ineffective in controlling the disease due to the inability of these compounds to accumulate in the shoot apex where the infection occurs (Ajayakumar, 1996; Prior, 2007).

Foliar application of bitertanol at 1500 ppm in two weeks interval was found to be effective in complete control of VSD disease in nursery seedlings (Ooi and Chew, 1985). Growing the plants in nurseries enclosed in plastic sheeting and hand watering in the morning so that the water dries out rapidly is one of the effective methods for controlling disease in seedlings (Keane and Guest, 2007).

Foliar spray of Bordeaux mixture and kitazin can control VSD in adult trees to some extent (Abraham and Ravi, 1991). Studies have been conducted in using systemic ergosterol biosynthesis-inhibiting fungicides, such as flutriafol, hexaconazole, propiconazole, tebuconazole and triadimenol. These fungicides were found to be successful in controlling VSD under nursery and experimental conditions. However, they were not commercially viable in established cacao plantations (Holderness, 1990).

A comparative study on the effect of foliar application of flutriafol at different concentrations (0.05, 0.1 and 0.15 %) and a combination fungicide (active compound combination of azoxystrobin and difenoconazole (0.1 %)) on seedlings, immature and mature cocoa was carried out (Nuraini, 2014). Both flutriafol at all the concentrations and the comparative fungicide found to be effective in controlling the disease at the seedling stage. On immature plants, flutriafol found to be ineffective whereas comparative fungicide was effective to some extent. On matured plants, both were ineffective.

2.9.3.3. Cultural Practices

Infected materials were cut down 30 cm below the discoloured xylem to prevent further spread of infection and thus reduced the inoculum level by removing the potential sites of sporulation. In Java, highly trained teams pruned out diseased branches of mature cocoa plants every two weeks for about two years and controlled the incidence of disease below one per cent. However, in a similar unpruned planting, disease incidence increased from about 30 to 90 per cent in ten months (Pawirosoemardjo and Purwantara, 1992).

Moist conditions are essential for sporulation and infection, thus shade and canopy management to increase aeration and sunlight on the foliage are of great significance to control pest and diseases (Dennis and Keane, 1992).

2.9.3.4. Biological Control

VSD is caused by *C. theobromae* which is a vascular pathogen that infects the young leaves. Epiphytic microbes can be used to reduce leaf infection. Vascular colonization can be avoided by using endophytic fungi and bacteria. (Guest and Keane, 2018).

Trichoderma species are endophytic fungi that can sustain living sapwood and leaves of cacao (*Theobroma cacao*). *Trichoderma asperellum* was introduced in two local clones of cocoa, MCC1 and M04 at the incision site in the bark for side grafting with the concentration of 4 g/ 10 ml, 4 g/ 100 ml, and 4 g/ 1000 ml (suspended in water). Observations after 21 weeks of grafting revealed that the occurrence of incidence of VSD was comparatively reduced in the local clones with that of untreated control (71.2 and 70.1 %). The concentration of 4 g/ 1000 ml was the most effective in controlling the disease, with the disease incidence of 18.2 and 15.6 per cent, respectively, on both the clones followed by 4g/ 10 ml with disease incidence of 20.6 and 21.7 per cent and 4 g/ 100 ml with disease incidence of 29.1 and 20.9 per cent respectively. Application of *T. asperellum* to cocoa scions while grafting not only reduces the VSD incidence but also enhances the growth of grafts (Rosmana *et al*., 2016).

2.9.3.5. Botanicals

Several doses of oil formula and nano emulsion of citronella was applied against vascular streak dieback (VSD) disease on cocoa plants in West Sumatra (in Padang Pariaman District and Limapuluh Kota District) resulted in reduction in VSD intensity. A dosage of 0.1 per cent of the nano emulsion of citronella formulation found effective to suppress the intensity of VSD disease on cocoa plants (≥ 30 % of effectiveness level) (Noveriza *et al.,* 2018).

2.9.3.6. Disease Resistance

Unavailability of regular supply of inoculum for bioassay since *in vitro* culture of the fungus is not possible act as a hindrance to study of the disease resistance in plants. However, Kerala Agricultural University has taken up a breeding programme to develop resistant hybrids against VSD (Mallika *et al.*, 2002; Minimol *et al.*, 2015).

2.9.3.6.a. Resistance Breeding for VSD in Cocoa

Xu *et al.* (2017) described immunity/ resistance as "a double-edged sword" because the defence protein produced against the pathogen can affect the plant itself thus result in yield reduction. Many scientists reported that disease resistance mechanism leads to yield reduction (Bazzaz, 1987: Marquis, 1991; Heil, 2000; Creissen *et al.,* 2016). Thus, resistance breeding should also focus on considerable yield improvement.

Cocoa Research Center, Kerala Agricultural University conducted an extensive breeding programme to develop disease resistant planting materials. Hybridization programme was initiated in 1995-96. Germplasm collections were field screened for VSD symptoms. Percentage of twigs infected in a tree was used as criteria to determine the VSD resistance as per the score chart given by Abraham *et al.* (2000) and Marfu *et al.* (2004). Thus, based on disease intensity, thirty one female and four male plants were selected as resistant parents.

Pollination hood was slightly modified (Amma *et al.*, 2009) and assisted hand pollination was carried as per the protocol suggested by Wood and Lass (1985). Based on the availability of flowers on the selected female parents on the trunks and thick fan branches at convenient height, cross pollination was tried during the dry period September 1995 to March 1996.

Among the 2949 flowers hybridized in different crosses, only 298 developed into matured fruits which were used to produce hybrid seedlings. From March 1996, the resultant hybrid pods were sown in the nursery under high humid conditions in the shade house. 5921 hybrid seedlings were raised from the 298 hybrid pods. Since the parents used were heterozygous, the hybrid populations were segregating in nature (Mallika *et al.*, 2002; Minimol *et al*., 2016).

Natural inoculum of the pathogen was supplied by placing VSD infected seedlings around the healthy seedlings with adequate supply of moisture for disease spread till May 1998. The susceptible seedlings dried off during the period of inoculation. Based on the continuous screening for two years, 566 resistant seedlings were selected and transplanted to the main field during June 1998.

The plants were well maintained in the field with good management practices for entire observation period (2004 to 2014). Disease scoring (Abraham *et al.*, 2000), as well as number of pods per tree per year, was recorded during the entire period.

Fifty resistant hybrids were selected by field screening in the peak periods of investigation for 15 years (Minimol *et al*., 2016). Among the 50, only 46 retained whereas 4 were lost due to natural calamities. Self-incompatibility of the selected hybrids was tested by selfing 100 flowers per tree as per the procedure suggested by Mallika *et al.* (2002) and observed their seed set. Out of the 46 hybrids, 37 hybrids found to be self-incompatible (Minimol *et al*., 2013). Evaluation of the yield contributing characters among the hybrids revealed VSD I 31.8 (CCRP 15) as superior. Thus, VSD I 31.8 is released as a novel variety (CCRP 15) with resistance to VSD coupled with a higher yield (Minimol *et al.,* 2018). Resistance in VSD I 31.8 was confirmed using the ISSR marker UBC857 and SSR marker mTcCIR42 that were

proved to be linked with VSD resistance by Chandrakant, (2014) and further validated by Tulshiram (2016).

2.9.3.7. Nature of Genes in VSD Resistance

Cocoa expresses horizontal resistance to VSD. VSD resistance is highly polygenic and largely inherited as additive genes (Tan and Tan, 1988; Keane, 1992; Samuels *et al*., 2012). Higher the number of additive genes, the higher the resistance in the genotype.

2.10. MOLECULAR MARKERS IN COCOA

Utilization of molecular markers made great significance in the study of cocoa genetics. The first group of molecular markers used in cocoa was isozymes. Genetic diversity and population structure studies were done using isozymes in cocoa (Lanaud, 1986). However, limitation in the number of polymorphic enzymatic systems restrict the broad use of isozymes in the studies of cocoa genetics even though they have a significant role in germplasm characterization, population studies, supported linkage mapping and cultivar identification (Figueria *et al.*, 2004).

Molecular markers commonly used in cocoa include restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphisms (AFLPs), microsatellites and single nucleotide polymorphisms (SNPs) (Laurent *et al.*, 1994; Lanaud *et al*., 1999; Motilal and Butler, 2003; Turnbull *et al*., 2004; Kuhn *et al*., 2012; Santos *et al*., 2012).

Development of molecular markers linked to major quantitative trait loci (QTL) has greatly accelerated breeding programmes in cocoa. Lanaud *et al.* (1995) constructed the first genomic map in cocoa using RFLP and RAPD markers. The genetic map thus developed consist of 193 loci covering 759 cM in 10 linkage groups.

Application of the highly polymorphic, PCR based codominant microsatellite markers enhanced the molecular studies in cocoa. They aid in marker assisted selection (Pugh *et al.,* 2004). Utilization of SSR markers has been done in genetic diversity studies of historical cocoa plantations in Brazil (Santos *et al.,* 2015) and

germplasm assessments in Indonesia (Dinarti *et al.*, 2015) and Cuba (Martınez *et al.*, 2017).

Genetic variability of cocoa grown in different ecosystems of Nigeria was evaluated using SSR markers (Aikpokpodion *et al*., 2006). The evolutionary relationship of species within the *Theobroma* genus was determined using a set of 20 EST-SSRs (Silva *et al.*, 2017) Allegre *et al.* (2012) developed the first SNP-based linkage map for cocoa. Alignment of RNA sequence (RNAseq) data of 16 cocoa cultivars to the assembled Matina 1–6 transcriptome resulted in the identification of a large number of SNPs (Livingstone *et al.*, 2015).SNP-based DNA fingerprinting utilized in the assessment of cocoa bean authentication (Fang *et al*., 2014), cocoa variety development (Padi *et al.*, 2015, 2017) and cacao genetic diversity (Cosme *et al.*, 2016). Efficient genotyping of cocoa trees at field conditions can be done using an optimized 50 -nuclease (TaqMan)-based SNP assay (Livingstone *et al.*, 2012)

Molecular markers have been identified to be linked with various agronomic (Clement *et al.*, 2003) and quality traits (Araujo *et al.*, 2009). Molecular markers have been identified to be linked with resistance to diseases such as resistance to *Ceratocystis* wilt (Santos *et al*., 2012) and resistance to *Phytophthora* species (Akaza *et al.*, 2016; Motilal *et al.*, 2016). Molecular markers were used to study the genetic basis of QTL for self- incompatibility (Silva *et al.*, 2016).

DNA barcoding can be done using cytoplasmic DNA also (Bieniek *et al.*, 2015). A study was conducted for the analysis of variation in the sequence of the trnH-psbA intergenic spacer in 28 cacao accession obtained from different farms in southern Mexico (Gutierrez-Lopez *et al.*, 2016). They identified indels in this region as potential markers for the development of a DNA barcoding system in cocoa.

2.11. MOLECULAR MARKERS LINKED TO VSD RESISTANCE IN COCOA

Microsatellite and inter-microsatellite markers were developed for tagging VSD resistant genes in cocoa (Chandrakant, 2014). The efficiency of identified ISSR and SSR primers was confirmed by tracking the VSD resistance by screening of four accessions each of highly resistant, highly susceptible and partially resistant cocoa clones. Among the 71 ISSR and 46 SSR primers used, only five ISSR primers UBC811, UBC815, UBC826, UBC857 and UBC866 gave reproducible results with polymorphic bands that distinguish the resistant and susceptible cocoa clones. However, none of the SSR primers gave reproducible result.

Thus, to develop an SSR primer, the distinct polymorphic band formed by UBC857 marker was eluted and carried out nested PCR. The product thus obtained was electrophoresed to confirm that it is yielding a single band (Chandrakant *et al.*, 2020).

Sequencing of the product from nested PCR was done and it generated 246 nucleotides. On BLASTn, these sequences expressed 94 per cent identity with the *Theobroma cacao* microsatellite DNA clone of mTcCIR42 SSR (GenBank AJ271944). SSR assay using the primer mTcCIR42 (Lanaud *et al.*, 1999, annealing 55°C) yielded distinct polymorphic band that distinguished the resistant clones from susceptible and partially resistant ones (Chandrakant *et al.*, 2020).

Tulshiram (2016) further validated these primers using twenty resistant and four susceptible genotypes on the reproducibility of the polymorphic band in the resistant plants. The study revealed that the ISSR markers UBC811, UBC815, UBC826 and UBC857 and SSR marker mTcCIR42 can successfully distinguish the resistant and susceptible plants. The ISSR markers UBC811, UBC815, UBC826 and UBC857 produced distinct polymorphic band at 950, 750, 650 and 450 bp, respectively in all the resistant genotypes whereas they were absent in susceptible ones.

SSR assay using mTcCIR42 produced a characteristic band of 200 bp in the resistant genotypes. However, susceptible plant does not exhibit this band. VSD I 31.8 (CCRP 15) is a novel hybrid released in the world with VSD resistance and considerable yield in which the resistance gene is tagged using ISSR marker UBC857 and SSR marker mTcCIR42 (Minimol *et al*., 2018). The markers expressed in the hybrid as well as in resistant genotypes however were absent in susceptible genotypes.

Midhuna (2019) studied the inheritance of VSD resistance using ISSR marker UBC811, UBC815 and UBC857 and SSR marker mTcCIR42 in hybrid progeny of cocoa and also identified that beta tubulin gene is linked to the ISSR marker UBC 857 and the SSR marker mTcCIR42, that contributes resistance to VSD by providing resistance against penetration of the fungus into the plant cell.

2.12. CLUSTER ANALYSIS

Cluster analysis is an important statistical tool to understand the relationships between closely related accessions. Based on the structure of a complex pairwise genetic proximity measure, hierarchical ordering of accessions is carried out by an agglomerative algorithm. Proximity measures and clustering algorithm applied determines the hierarchical arrangement (Kaufman and Roussew, 1990).

Cluster analysis and principal component analysis was carried out in 294 cocoa cultivars based on 39 characters and the output revealed similarity to the conventional classification of cocoa as Criollo, Forastero and their subgroups (Engels, 1986)

Sujith *et al.* (2017) classified ten varieties released from Kerala Agricultural university into five clusters based on qualitative characters and quantitative characters separately.

2.13. CORRELATION ANALYSIS

Aikpokpodion and Dongo (2010) worked out correlation between eight quantitative traits of cocoa accessions including fruit weight, fruit length, number of beans per pod, fresh weight, cotyledon length, cotyledon width and nib weight and

recorded positive significant correlation between these characters.

Thondaiman and Rajamani (2014) studied phenotypic correlation with twenty traits of 151 cocoa trees. Wet bean weight per pod, dry bean weight per pod, single dry bean weight, number of pods per tree showed a highly significant positive correlation with the dry bean yield per tree.

Vithya *et al*. (2018) worked out correlation studies on various yield contributing characters in ten elite cocoa genotypes (TNAUCC 1-10) in Tamil Nadu. The number of pods per tree (pod yield) showed a significant and positive correlation with single dry bean weight and estimated dry bean yield per tree. Single dry bean weight exhibited a positive correlation with dry bean weight per pod and estimated dry bean yield per tree. Dry bean weight per pod was significantly and positively correlated with estimated dry bean yield per tree. Amitha *et al*. (2018) did correlation analysis on 13 characters of twenty genotypes at the early yielding stage in Tamil Nadu.

2.14. PATH ANALYSIS

Path analysis is used to estimate the direct and indirect effects of independent variables on a dependent variable (Li, 1975; Cruz *et al.*, 2006).

Investigation on the direct and indirect effect of ten characters in twelve cocoa hybrids cultivated in the experimental station in Medicilandia, Brazil revealed a direct effect of the number of healthy pods per tree and dry bean weight per pod on dry bean weight per tree (Almeida *et al.*, 1994).

Vithya *et al.* (2018) studied the direct and indirect effects of twenty traits on dry bean weight per tree in cocoa and reported that twelve traits showed positive direct effects on cocoa dry bean yield per tree. Wet bean weight per pod after fermentation (0.2358), dry bean weight per pod (0.7252), number of pods per tree (0.7015) showed positive direct effects on cocoa dry bean yield per tree.

2.15. DEVELOPMENT OF SELECTION CRITERIA

Based on path coefficient analysis, selection criteria have been developed in many crops. Sari and Susilo (2013) used path analysis to determine the yield component that have direct effect on bean weight. Characters considered include pod girth, pod length, pod weight, total wet bean weight per pod, number of beans per pod, number of flat beans per pod, dry weight per normal bean and shell content. They concluded that pod weight and wet bean weight per pod influence the dry weight of normal bean thus can be used for indirect selection of superior genotypes.

Materials and Methods

3. MATERIALS AND METHODS

The present study entitled 'Preliminary evaluation of double cross hybrids for yield and Vascular Streak Dieback (VSD) disease resistance in cocoa (*Theobroma cacao* L.)' was carried out in the Department of Plant Breeding and Genetics, College of Horticulture and the Cocoa Research Centre (CRC), Vellanikkara, during the period 2018-2020.

Twenty cocoa double cross hybrids which are in the early bearing stage, selected from the double cross hybrids bred for vascular streak die back disease resistance and field planted during 2017 at CRC farm served as the material for the study. These twenty hybrids were evaluated for their general vigour and yield performance. Screening for disease resistance and morphological and biochemical evaluations were carried out. CCRP 15 planted on the same field during the same period was included as check variety. All the hybrids were screened for VSD resistance under field condition and also in the screening net house. List of selected hybrids and their parentage are detailed in Table 1.

Table 1. List of hybrids selected for the study and their parentage

Sl. No.	Double cross hybrid	Parentage
1.	DC VSD 2.1	VSD 14.15 X VSD 16.11
2.	DC VSD 2.4	VSD 2.3 X VSD 4.6
3.	DC VSD 3.3	VSD16.10 X VSD 14.15
4.	DC VSD 6.11	VSD19.6 X VSD 2.3
5.	DC VSD 6.12	VSD 14.15 X VSD 2.3
6.	DC VSD 7.2	VSD 14.15 X VSD 2.3
7.	DC VSD 8.11	VSD 2.3X VSD 4.6
8.	DC VSD 8.10	VSD 14.15 X VSD 2.3
9.	DC VSD 9.6	VSD 2.3 X VSD 4.6

10.	DC VSD 9.13	VSD 2.3 X VSD 14.15
11.	DC VSD 12.5	VSD 14.15 X VSD 2.3
12.	DC VSD 15.2	VSD 16.10 X VSD 2.3
13.	DC VSD 15.5	VSD 16.10 X VSD 2.3
14.	DC VSD 18.3	VSD 14.15 X VSD 2.3
15.	DC VSD 18.6	VSD 2.3X VSD 4.6
16.	DC VSD 18.9	VSD 17.2 X VSD 19.6
17.	DC VSD 19.11	VSD 14.15 X VSD 2.3
18.	DC VSD 20.2	VSD 2.3 X VSD 4.6
19.	DC VSD 20.10	VSD 14.15 X VSD 16.11
20.	DC VSD 21.9	VSD 2.3 X VSD 4.6

3.1. MORPHOLOGICAL EVALUATION OF THE HYBRIDS

Quantitative and qualitative characters of the hybrids were evaluated for morphological characterisation. Completely Randomized Design (CRD) was used to carry out statistical analysis. Morphological observations include thirteen pod characters, twelve flower characters, six bean characters and flush colour of leaves. Five pods from each hybrid were used for morphological characterisation of pod and bean, during the period from September to February (2019-2020). To evaluate peeled bean characters, twenty beans were selected randomly from each pod and outer mucilage covering of beans was removed using forceps. Morphological characterisation of flower was done using five flowers from each hybrid. Qualitative characters were recorded using the descriptor developed by Bekele and Butler (2000). The descriptor and descriptor states are detailed in Table 2.

Table 2. Descriptor and descriptor states for qualitative character

Sl. No.	Characters	Descriptor State	Description
1.	Flush colour	0	Absent (green)
		3	Slight
		5	Intermediate(reddish green)
		7	Intense (red)
2.	Colour of pedicel	1	Green
		2	Reddish
		3	Red
3.	Colour of sepal	1	Cream
		2	Greenish cream
		3	Reddish
		4	Red
4.	Colour of petal	1	Cream
		2	Greenish cream
		3	Reddish
		4	Red
5.	Pod shape	1	Cundeamor
		2	Angoleta
		3	Amelonado
		4	Calabacillo
		5	Criollo
6.	Form of pod apex	1	Attenuate
		2	Acute
		3	Obtuse
		4	Rounded
		5	Mammelate
		6	Indented

Table 2. continued.

7.	Pod basal constriction	0	Absent
		1	Slight
		2	Intermediate
		3	Strong
		4	Wide shoulder
8.	Pod rugosity	0	Absent
		3	Slight
		5	Intermediate
		7	Intense
9.	Unripe pod colour	0	Absent
		3	Slight
		5	Intermediate
		7	Purplish green
10.	Colour of ripe pod	0	Absent (green)
		3	Slight (greenish yellow)
		5	Intermediate (yellowish green)
		7	Intense (yellow)
11.	Bean colour	1	White
		2	Grey
		3	Light purple
		4	Medium purple
		5	Dark purple
		6	Mottled
		7	Mixed

3.1.1. Evaluation of Qualitative Characters

3.1.1.1. Flush Colour

Observation on flush colour was taken on all the twenty hybrids as per the descriptor provided in Table 2.

3.1.1.2. Flower Characters

Ten flowers from each hybrid were collected and observations on colour of pedicel, sepal and petal were recorded as per the descriptor given in Table 2.

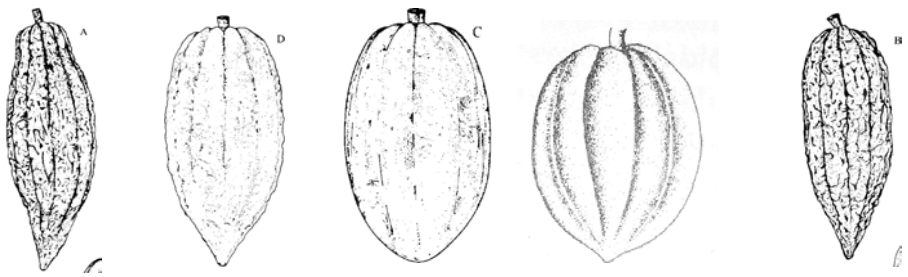
3.1.1.3. Pod and Bean Characters

Characters like colour of ripe and unripe pod, shape of the pod, pod rugosity form of pod apex, form of pod basal constriction, and cotyledon colour of peeled bean were observed. Five pods were collected from each hybrid and scoring of the characters were done based on the descriptor shown in Table 2.

3.1.1.3.a. Pod Shape

According to the descriptor (Figure 1.), pods were grouped as

1. Angoleta: deeply ridged and warty with square shape at stalk end
2. Amelonado: melon shaped fruits with slight bottle neck, having smooth and shallow furrows
3. Calabacillo: small spherical fruits
4. Cundeamor: intensively ridged, warty with characteristic bottleneck
5. Criollo: deeply ridged surface having acute apex

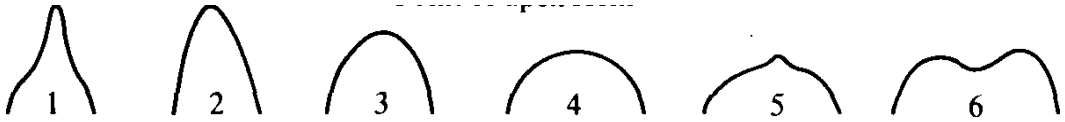


Cundeamor Angoleta Amelonado Calabacillo Criollo

Figure 1. Different shapes of cocoa pods

3.1.1.3.b. Pod Apex Form

Cocoa pods are observed with different forms of pod apex as represented in Figure 2. Observations for different pod apex were documented in all hybrids under the study.



Attenuate Acute Obtuse Rounded Mammelate Indented

Figure 2. Different pod apex forms in cocoa

3.1.1.3.c. Pod Basal Constriction

Shape of the basal constriction of pod was observed for each hybrid under study based on the descriptor provided in Table 2. Different forms of basal constriction generally observed are represented in Figure 3.

Absent Slight Intermediate Strong Wide shoulder

Figure 3. Different forms of pod base

3.1.1.3.d. Pod Rugosity

Unevenness or smoothness of the surface of pod is referred as pod rugosity. Based on the variation in smoothness of the surface, pods were grouped as shown in Table 2.

3.1.1.3.e. Unripe Pod Colour

Based on presence of anthocyanin pigmentation, colour of ridges and furrows of unripe pods were examined and categorized as the descriptor provided in Table 2.

3.1.1.3.f. Ripe Pod Colour

Based on the intensity of yellow and green colours on the pod ridges and furrows observations on the colour of ripe pods were recorded and classified as shown in Table 2.

3.1.1.3.g. Peeled Bean Colour

Colour of beans were observed after removing the outer mucilage and categorised based on the descriptor given in Table 2.

3.1.2. Quantitative Evaluation of the Characters

Quantitative characters of flower, pod and bean were evaluated in all the twenty hybrids under study. Ten flowers and five pods from each hybrid were used for recording observation.

3.1.2.a. Flower Characters

Collection of flowers (ten from each hybrid) was done in early morning. The following twelve characters were observed under laboratory microscope.

1. Length of pedicel (cm)
2. Diameter of flower (cm)
3. Length of sepal (cm)
4. Width of sepal (cm)
5. Length of petal(cm)
6. Width of petal (cm)
7. Number of stamens (cm)
8. Number of staminodes (cm)
9. Length of ovary (cm)
10. Width of ovary (cm)
11. Length of style (cm)

3.1.2.b. Pod Characters

The following eight pod characters were observed under laboratory conditions.

1. Pod weight (g)
2. Pod length (cm)
3. Pod width (cm)
4. Number of ridges per pod
5. Number of furrows per pod
6. Rind/ husk thickness (cm)
7. Number of beans per pod
8. Number of flat beans per pod

3.1.2.c. Bean Characters

Twenty beans were selected randomly from each pod and following measurements were recorded.

1. Wet weight of beans per pod (g)
2. Thickness of bean (cm)
3. Wet weight of single bean (g)
4. Dry weight of single bean (g)
5. Length of bean (cm)
6. Width of bean (cm)

3.2. EVALUATION OF BIOCHEMICAL CHARACTERS

Standard procedures were followed for the estimation of biochemical characters like fat content (%) and total polyphenol content (%).

Ripened pods were harvested from each hybrid and mucilaginous covering of each bean is removed by peeling and beans were taken out. Twenty beans were randomly selected from each pod. Bulking of the selected beans of each hybrid per replication was carried out and sun dried to reduce the moisture content below 8 per cent. In normal weather conditions, drying will be completed within a week.

Laboratory grinder was used to grind the dried beans and thus obtained fine powder was stored for further biochemical analysis.

3.2.1. Estimation of Fat Content

Fat content of cocoa beans were estimated using Soxhlet apparatus method explained by Sadasivam and Manickam (1996).Organic solvents like petroleum ether can dissolve fat present in cocoa beans and can be separated by evaporating the solvent. The procedure is detailed below:

Materials required for estimation:

- Cocoa beans powder – 10 g
- Petroleum ether (40-60 °C)
- Blotting paper

Procedure:

Cocoa bean powder was defatted using petroleum ether as solvent in Soxhlet apparatus.10 g of fine powdered cocoa beans were weighed and encased in blotting paper and tied using a twine. It was kept within the extraction tube of apparatus and adequate amount of solvent was poured. Fat accumulated at the bottom of round bottom flask by the siphoning of petroleum ether in Soxhlet apparatus. The extraction process can be completed in almost seven hours. When the extraction processes were over, the accumulated fat was transferred to a pre-weighed beaker. It was kept open to allow the evaporation of the remaining solvent. Then, weight of fat was measured and denoted as per cent.

$$\text{Fat (\%)} = \frac{\text{Weight of beaker with fat} - \text{Empty weight of beaker}}{\text{Weight of sample}} \times 100$$

3.2.2. Estimation of Total Phenol Content

Estimation of total phenol content was done using Folin – Ciocalteau (FC) reagent method explained by Malik and Singh (1980). The procedure followed is detailed below.

***Materials required for estimation*:**

- Cocoa beans powder (defatted) – 0.5 g
- Ethanol (80 %)
- Na_2CO_3 (20 %)
- FC reagent
- Catechol –100 mg

Procedure:

Defatted cocoa powder of 0.5 g was grinded with 10 ml of 80 per cent ethanol using a mortar and pestle. It was transferred to a centrifuge tube and centrifugation was done at 10,000 rpm for 20 minutes. Supernatant was collected in an evaporating dish. To collect the total phenol present in the sample, this procedure was repeated 2-3 times. Excess ethanol was removed by keeping the evaporating dishes in hot water bath for 60 minutes. Left-over residue was added with 40mL distilled water.0.2 mL aliquot was pipetted out from it and 13 mL distilled water was added to it. To this, 0.5 mL FC reagent was added. The test tubes were kept for 3 minutes incubation followed by addition of 2 mL of 20 per cent Na_2CO_3 solution. These test tubes were placed over boiling water bath for a minute and then incubated at room temperature for 60 minutes. Absorbance at 650 nm in the spectrophotometer was used against the reagent blank to quantify the total phenol content present in the sample.

Calibration of the detector was done using the following procedure. Catechin was used as the standard to estimate the total phenols in defatted cocoa powder.100 mg catechol was dissolved in 100 mL distilled water for the preparation of stock solution. One mL of aliquot was pipetted out from the stock solution and transferred into a ten mL standard flask and volume was made up. From this, 0.2 mL was pipetted out into a test tube and 13 mL of distilled water was added, followed by addition of 0.5 mL FC reagent and kept for 3 minutes incubation. After incubation, 2 mL of 20 percent Na_2CO_3 solution was added and mixed well. Absorbance of this solution was read at 650 nm.

The total phenol content present in the sample was determined in per cent by substituting the absorbance values in the given formula:

$$\text{Total phenol (\%)} = \frac{\text{OD of sample}}{\text{OD of standard}} \times \frac{\text{Concentration of standard}}{\text{Volume of sample}} \times 100$$

3.3. EVALUATION OF ECONOMIC CHARACTERS

To estimate the quantity of pod and bean yield obtained from each genotype, nine economic characters are considered. The economic characters determined include pod yield (number of pods harvested from a genotype in a year), pod value, pod index, efficiency index, conversion index, dry matter recovery, peeling ratio, total wet bean weight per tree and total dry bean weight per tree. The details of each economic characters is presented in Table 3.

3.4. GENETIC PARAMETERS

To understand the extent of variability present in the population, genetic parameters like Genotypic Coefficient of Variation (GCV), Phenotypic Coefficient of Variation (PCV) (Sivasubramanian and Madhavamenon, 1973), Heritability (H^2) and Genetic Advance (GA) (Johnson *et al*., 1955) were estimated.

3.4.1.Phenotypic Coefficient of Variation (PCV)

$$PCV = \frac{\sigma p}{\text{Grand mean}} \times 100$$

Where, σp = phenotypic standard deviation

3.4.2. Genotypic Coefficient of Variation (GCV)

$$GCV = \frac{\sigma g}{\text{Grand mean}} \times 100$$

Where, σg = genotypic standard deviation

According to Sivasubramaniam and Madhavamenon (1973) classification PCV and GCV values were ranked.

0– 10% – Low

10.1 – 20% – Moderate

>20% –High

3.4.3. Heritability (H^2)

$$\text{Heritability} = \frac{Vg}{Vp} \times 100$$

Where, Vg is the genotypic variance and Vp the phenotypic variance

Robinson *et al*., 1949 categorised range of heritability as follow:

0–30% –Low

31–60% –Moderate

>61% –High

3.4.4. Genetic Advance (GA)

$$GA = k\sigma p H^2$$

Where, k is a constant having value 2.06

3.4.5. Genetic Gain (GG)

$$GG = \frac{GA}{\text{Grand mean}} \times 100$$

Johnson *et al*. (1955) classified genetic gain as below:

0– 10% – Low

10.1 – 20% – Moderate

>20% –High

Table 3. Description of economic parameters of cocoa

Sl. No.	Economic characters	Description	Formula	Reference
1.	Pod Yield	No. of pods harvested from each hybrid in a year	-	-
2.	Pod Value (g)	Average dry weight of total beans	Average dry weight of bean x no. of beans per pod	Toxopeus and Jacob (1970)
3.	Pod Index	No. of pods required to get 1 kg dried beans	1000 g ÷ Pod value	Morera *et al*. (1991)
4.	Efficiency Index	Pod weight required to give 1 kg dry beans	Pod weight (g) ÷ Pod value	Jacob and Atanda (1971)
5.	Conversion Index	Amount of dry beans obtained from a given amount of wet beans	Pod value ÷ Wet bean weight per pod	Francies (1998)
6.	Dry Matter Recovery (%)	Ratio of dry bean weight to wet bean weight	$\frac{\text{Dry bean weight (g)}}{\text{Wet bean weight(g)}}$ X100	Francies (1998)

Table 3 continued

Sl. No.	Economic characters	Description	Formula	Reference
7.	Peeling Ratio (%)	Bean weight obtained after peeling	$\frac{\text{Peeled wet bean weight (g)}}{\text{Unpeeled wet bean weight (g)}} \times 100$	Ajmal (2016)
8.	Wet bean weight per tree	Total wet bean weight per tree obtained in a year	Average total wet bean weight per pod x pod yield	Minimol *et al.* (2014)
9.	Dry bean weight per tree	Total weight of dry beans obtained from a tree in a year	Pod value x pod yield	Minimol *et al.* (2014)

3.5. FIELD SCREENING FOR VSD RESISTANCE

Hybrids were screened visually at monthly interval in the field condition for disease symptoms. Number of twigs infected and total number of twigs were observed. Based on the intensity of infection the disease scoring was done as per the score chart given by Abraham *et al* (2000).The score chart is described in Table 3.4. Intensity of infection was calculated using the formula given below.

$$\text{Percentage of twigs affected (\%)} = \frac{\text{Number of twigs affected}}{\text{Total number of twigs in the plant}} \times 100$$

Table 4. Disease scoring for VSD symptoms

Disease scale	**Intensity of infection**
0	No infection
1	< 25 per cent of twig infected
3	25-50 percent infection
5	50-75 percent infection
7	>75 percent infection
9	Mortality of the plant

Net house screening for VSD was also carried out for a period of 6 months (September-February) by placing the budded plants of selected double cross hybrids in a 5x 5 arrangement and two rows of five VSD affected seedlings each in all the four sides as border rows. The VSD infected seedlings served as natural inoculum. Overhead sprinkler system was provided throughout the period to ensure moisture and water splash that favours the transfer of disease from the infected ones. Diagrammatic representation of the layout is given in Figure 4.

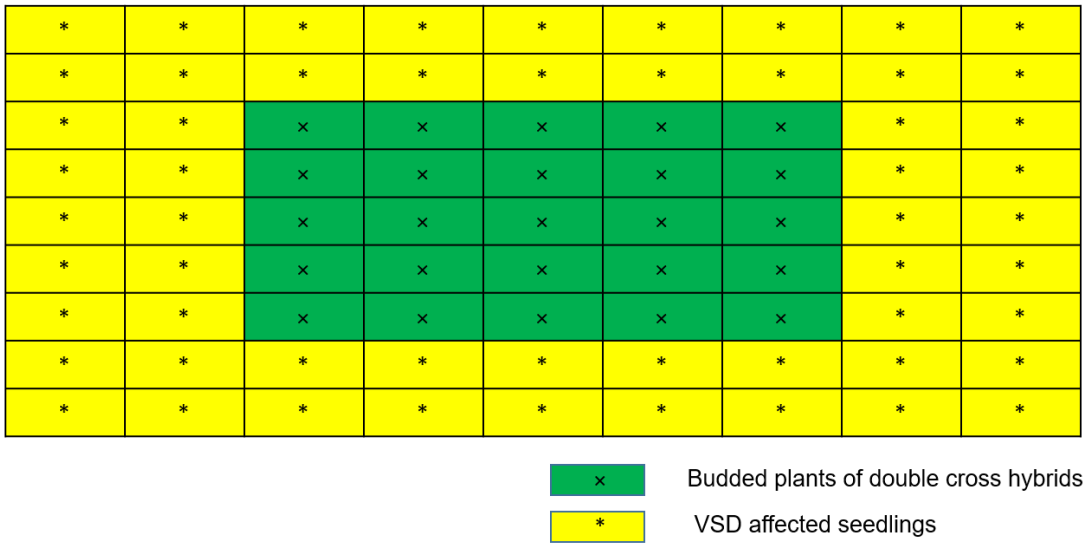


Figure 4. Layout of the 5x5 arrangement of the double cross hybrids with two rows of VSD affected seedlings as border rows

3.6. CONFIRMATION OF RESISTANCE WITH ISSR AND SSR MARKERS

Hybrids selected were subjected to screening at molecular level using markers. The ISSR markers UBC811, UBC826, UBC857 and SSR marker mTcCIR42 figured to be linked with VSD resistance by Chandrakanth (2014) and further validated by Tulshiram (2016) were used for the study.

3.6.1. DNA Extraction

3.6.1.1.Equipment and Machinery

This research work was carried out by using the facilities and equipments available at the Department of Plant Breeding and Genetics and Hi-Tech lab, Department of Olericulture, College of Horticulture. High speed refrigerated floor model centrifuge (KUBOTA 6500, Japan) was used for centrifugation. Quantity and quality of DNA was estimated using Nanodrop ND-1000 spectrophotometer. Agilent Technologies (SureCycler 8800) and Life Technologies (Proflex) were used to run PCR. Horizontal gel electrophoresis system (Bio-Rad) was used for the gel run and

documentation of the gel was done using Gel Doc (Bio- Rad).Analysis of the gel picture was done using Quantity one software (Bio- Rad).The list of laboratory equipments used in this study is provided in Appendix I.

3.6.1.2. Sample Collection

Tender leaves (reddish/reddish green/light green in colour) from the selected double cross hybrids bred for vascular streak die back disease resistance used in the study were used as ideal source for genomic DNA.

Scientists revealed young leaves (pale green) as a better source of good quality DNA in plants (Perry *et al*., 1998; Faleiro *et al.*, 2002; Sarma and Tanti, 2017). Chandrakanth (2014) and Sujith (2016) also revealed that good quality DNA can be obtained from the tender reddish coloured leaves in cocoa. Thus, tender reddish leaves were used as source for DNA isolation in the current study.

Sufficient quantity of good quality DNA was yielded from the tender leaves. Tender leaves from the hybrids were collected early in the morning. The collected leaves were wrapped in aluminium foil, labelled and placed in the ice and transported to the laboratory in ice box. The leaves were cleaned by washing with sterile distilled water and then surface sterilised with 70 per cent ethanol.

3.6.1.3. DNA Isolation

Good quality DNA is the primary requisite for molecular analysis irrespective of the plant samples and markers involved. Many protocols are established for the extraction of good quality DNA from plant tissues (Saghai-Maroof *et al*., 1984; Doyle and Doyle, 1987; Haymes, 1996; Scott and Playford, 1996; Sharma and Lavanya, 2000; Pirttila *et al.*, 2001; Shepherd *et al*., 2002; Mogg and Bond, 2003). Dellaporta method of DNA extraction developed by Dellaporta *et al.* (1983) suggested by Midhuna (2019) with slight modifications was used for isolation of DNA in the present study.

One gram of leaf sample was homogenized with liquid nitrogen. Modifications in the Dellaporta method involved substitution of extraction buffer 1 with 5 per cent CTAB buffer and addition of anti-oxidant β-mercaptoethanol and PVP

to repress the polyphenol present in the sample. Additional centrifugation with chloroform isoamyl alcohol added to the supernatant of the initial centrifugation was carried out.

3.6.1.3.a. Reagents

a. Liquid nitrogen
b. PVP
c. Beta-mercaptoethanol
d. CTAB buffer (5 X)
   - 20 mM EDTA (pH-8.0)
   - 100 mM Tris (pH-8.0)
   - 1.4 M NaCl
   - CTAB (5 %)
e. TE Buffer
   - 0.5 M EDTA
   - 1 M Tris
f. SDS (20 %)
g. Chloroform isoamyl alcohol (24:1)
h. Chilled isopropanol
i. 5M Potassium acetate
j. 3M Sodium acetate
k. Ethanol (80 %)
l. Sterile distilled water
m. Ethanol (70 %)

Composition of reagents are represented in Appendix II

3.6.1.3.b. Protocol

- 1 g of tender leaf sample was ground into fine powder using liquid nitrogen in a sterilized pre-chilled mortar and pestle

- 500µl of pre-heated (60°C) CTAB buffer, 50 µl β-mercaptoethanol and a pinch of PVP was added to it and ground well and transferred to a 2 ml centrifuge

tube

- 100 µl of SDS (20 %) was added to it and mixed well and kept in water bath for incubation at 65°C for 30 minutes. The contents were mixed by occasional gentle inversions
- 500 µl potassium acetate was added and vortexed and incubated at 0 °C for 20 minutes
- The tube was centrifuged at 13000 rpm for 15 minutes
- Transparent supernatant solution was transferred to a 1.5 ml centrifuge tube carefully without getting contaminated with the upper mucilaginous layer and equal volume of chloroform: isoamyl alcohol (24:1) was added to it
- The tube was centrifuged at 13000 rpm for 15 minutes
- Among the three layers formed, upper transparent solution was carefully transferred to a 1.5 ml centrifuge tube and 500 µl of isopropanol was added to it
- The tube was kept for incubation at - 20 °C for 30 minutes
- The tube was centrifuged at 13000 rpm for 15 minutes
- Supernatant was discarded and pellet was dried by placing the tubes in a tissue paper in an inverted position for 10 minutes
- The pellet was redissolved in 700 µl of TE buffer and kept for incubation at 4 °C for 30 minutes
- The tube was spun for 10 minutes at 12000 rpm
- The supernatant was transferred to a 1.5 ml centrifuge tube and 75 µl sodium acetate and 500 µl of isopropanol was added to it
- The centrifuge tube was inverted 20 times
- The centrifuge tube was spun at 10000 rpm for 2 minutes

- Supernatant was discarded and pellet was washed with 70 per cent ethanol and dried
- The pellet was redissolved in 100 µl of distilled water

3.6.1.4. Evaluation of Quality of Isolated DNA

Quality of isolated DNA was checked through electrophoresis on 0.8 per cent agarose gel (Sambrook *et al.*, 1989).

3.6.1.4.a. Equipment

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

b. Gel documentation system: BioRad Gel DOC imaging system

3.6.1.4.b. 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

3.6.1.4.c. Procedure

- 100 mL of TAE buffer was measured in a graduated cylinder and transferred to a flask. 0.8 g agarose was weighed and added to it and microwaved for 60 seconds to prepare 0.8 percent agarose solution
- The solution was left to cool to 42-45 ^{o}C and 2.5 µL of ethidium bromide from the stock solution was added to it and swirled well
- The gel casting apparatus was assembled by fixing the gel casting tray
- Placed the comb vertically at about 1 inch away from an end in such a way that the tooth of the comb are about 1-2 mm above the base of the tray
- Poured the warm gel solution into the tray to a depth of 5 mm and allowed to solidify for about 30-45 minutes at room temperature
- Removed the comb carefully and the casting tray was placed in the electrophoresis chamber
- 1X TAE buffer was poured into the chamber until the gel is fully submerged
- Samples were loaded into each well after mixing 1 µL of 6X gel loading dye for every 5µL of DNA sample
- DNA ladder was also loaded in a well
- The gel was runned at 70 V until the dye covered about two-third the length of the gel
- Gel documentation system was used for the visualisation and documentation of the gel image. Intactness of the DNA as well as presence of contamination with RNA and protein were assessed in the gel profile
- Intact DNA can be identified as they appear as thick white band just below the well. Due to the presence of many DNA fragments, the degraded DNAs if present appeared as smear. Protein contamination if present can be identified as

thick white patch present within the well. Thick band with size less than 100 bp indicated RNA contamination

3.6.2. Evaluation of the Quantity of DNA using Spectrophotometer

Confirmation of the purity of DNA as well as its quantity was done using nano drop spectrophotometer (NanoDrop-1000).Maximum absorbance is exhibited by nucleic acids and proteins at 260 nm and 280 nm respectively. Absorbance is recorded at both wavelength and ratio between the absorbance at 260 nm and 280 nm (A_{260}/A_{280}) provided an estimate of purity of nucleic acid. The ratio was found between 1.8 and 2.0 for pure DNA that is free from contaminations. If the ratio is less than 1.8, it indicated protein contamination. A value greater than 2.0 indicated RNA contamination.

3.6.2.1. Procedure

- The nano drop spectrophotometer was connected to the system and the pre-installed operating software, ND-1000 was opened
- The option nucleic acid was chosen
- The sampling arm of the spectrophotometer was opened and 1µL sterile distilled water was pipetted on to its lower measurement pedestal
- The sampling arm was closed and the operating software was used to take spectral absorption measurement
- Blank was used to set the absorption reading to zero
- Cleaned the lower measurement pedestal and 1µL of sample was pipetted onto it and the option ‘measure’ was chosen
- When the measurement was taken, the upper and lower measurement pedestals were wiped well using a laboratory tissue paper

3.6.3. Markers used for the Evaluation of VSD Resistance

The study utilized three ISSR and one SSR marker which were reported and validated for VSD resistance in cocoa by Chandrakant (2014) and Tulshiram (2016) for the molecular analysis. The markers included three ISSR, *i.e.,* UBC811, UBC826, UBC857 and one SSR marker mTcCIR42. Quality DNA from the selected double

cross hybrids were amplified using PCR with the above primers.

3.6.3.1. DNA Amplification

Proper amplification of ISSR and SSR markers require a suitable reaction mixture with precise amount of reagents. The composition of the reaction mixture encompassed sterile distilled water, *Taq* buffer A, $MgCl_2$, dNTPs, primers, *Taq* DNA polymerase and template DNA. All these components except template DNA was added in proportion to form the master mix. The master mix was equally distributed into 200 µL PCR tubes and template DNA was added to it.

Previously programmed thermal cycler (Applied Biosystems, Agilent) was used to undergo PCR. The pattern of amplification depends on the temperature profile in the steps during PCR thus accurate temperatures for denaturation, annealing and extension steps with desired number of cycles are set for efficient amplification.

3.6.3.1.a. Inter Simple Sequence Repeats (ISSR) Assay

Good quality genomic DNA obtained from the tender leaves of double cross hybrids was diluted to 30 ng/ µL for PCR. ISSR assay utilised ISSR markers that were earlier mentioned, which were linked with VSD resistance in cocoa. PCR amplification was carried out in 20 µL reaction mixture and the composition of the reaction mixture involves:

a) Genomic DNA (30 ng/ µL) - 2.0 µL
b) 10X *Taq* assay buffer A - 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 ºC for 4 minutes

Denaturation – 94 ºC for 45 seconds

Primer annealing – 43.3 ºC, 52.1 ºC and 53.3ºC for 1 minute } 35 cycles

Primer extension – 72 ºC for 2 minutes

Final extension – 72 ºC for 8 minutes

Incubation – 4 ºC for infinity to hold the sample

`The reported ISSR primers used for the molecular study is given in Table 5.

Table 5. Details of ISSR primers used in the study

Sl. No.	**Primer name**	**Nucleotide sequence**	**Annealing temperature (ºC)**
1	UBC811	5'- GAGAGAGAGAGAGAGAC- 3'	43.3
2	UBC826	5'- ACACACACACACACACC-3'	53.3
3	UBC857	5' -ACACACACACACACACYG- 3'	52.1

Electrophoresis of the amplified products were carried out on 1.5 per cent agarose gel stained with ethidium bromide along with a 1 kb DNA ladder (Sigma, USA) in a well. Gel doc imaging system was used for the documentation of the gel profile obtained. Careful examination of the documented ISSR profiles was done for further evaluation of the amplicon.

3.6.3.1.b. Simple Sequence Repeats (SSR) Assay

Good quality genomic DNA obtained from the tender leaves of double cross hybrids was diluted to 30 ng/ µL for PCR.SSR assay utilised SSR marker that was earlier mentioned to be linked with VSD resistance in cocoa. PCR amplification was carried out in 20 µL reaction mixture and the composition of the reaction mixture involves

:

a) Genomic DNA (30ng/µL) - 2.0 µL
b) 10X Taq assay buffer A - 2.0 µL
c) $MgCl_2$ - 2.0 µL
d) dNTPs mix (10 mM each) - 1.5 µL
e) Taq DNA Polymerase (3 U) - 0.4 µL
f) Forward Primer (10 pM) - 0.75 µL
g) Reverse Primer (10 pM) - 0.75 µL
h) Autoclaved Distilled Water - 10.6 µL

Total volume - 20.0 µl

The thermal profile used in the PCR amplification is given below,

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

The SSR primer used for the molecular analysis is given in Table 6.

Electrophoresis of the amplified products were carried out on 1.8 per cent agarose gel stained with ethidium bromide along with a 1 kb DNA ladder (Sigma, USA) in a well. Gel doc imaging system was used for the documentation of the gel profile obtained. Careful examination of the documented SSR profile was done for further evaluation of the amplicon.

Table 6. Details of SSR primer used in the study

Sl. No.	Primer name	Nucleotide sequence	Annealing temperature
1	mTcCIR42	**Forward primer-** 5'- TTGCTGAAGTATCTTTTGAC- 3' **Reverse primer-** 5'-GCTCCACCCCTATTTG- 3'	55

3.6.4. Percentage Expression of Molecular Markers

The percentage of resistant double cross hybrids in which the marker is expressed over total number of resistant double cross hybrids indicated percentage expression of a molecular marker.

Percentage expression of a marker (%) =

$$\frac{\text{No. of resistant double cross hybrids with marker expressed}}{\text{Total no. of resistant double cross hybrids}} \times 100$$

3.7. STATISTICAL ANALYSIS

3.7.1. Experimental Design and Analysis of Variance

The experiment was carried out in Completely Randomised Design with twenty treatments each with five replications. WASP 2.0 (Web Agri Stat Package) software was used to determine analysis of variance of the obtained data using Fischer's method. Interpretation of the data was done as per the method given by Panse and Sukhatme (1967). The level of significance at P = 0.05 was considered in the F test. For significant F values, critical difference and coefficient of variation values were recorded.

3.7.2. Cluster Analysis

SPSS 16 software was used for hierarchical clustering of the double cross hybrids. Agglomeration schedule and proximity matrix was used to analyse the data. Average linkage (between groups) method was used for clustering with squared Euclidean distance as measure of interval.

3.7.3. Correlation Analysis

The value of correlation coefficient between phenotypic characteristics indicates the degree of association between the traits. Correlation analysis was carried to determine Karl Pearson's Correlation Coefficient. The coefficient of correlation r (x,y) between two variables x and y, for the bivariate dataset (xi,yi) is as follow:

$$r(x,y)=cov(x,y)/\sigma x\sigma y$$

where,

cov(x,y): is the covariance between x and y

σx = standard deviation of variable x

σy = standard deviation of variable y

Correlation analysis was carried out on all the pod and bean characters. Pod characters studied includes pod weight (g), pod length (cm), pod breadth (cm), rind thickness (cm), total number of beans per pod, number of flat beans per pod. Bean characters includes total wet bean weight per pod, single wet bean weight, single peeled bean weight, single dry bean weight, dry bean length, dry bean breadth, dry bean thickness, total wet bean weight per tree per year and total dry bean weight per tree per year.

3.7.4. Path Coefficient Analysis

It measures the quantifications of the direct and indirect effects of the explanatory variables on one basic variable from path coefficients obtained by regression equations. Path coefficient analysis as suggested by Dewey and Lu (1959) was used to partition the phenotypic correlation into components of direct and indirect effects. Wet bean weight per tree was kept as dependant variable and the six positively correlated traits were used as independent variables and simultaneous

equations which express the basic relationship between path coefficients were derived to estimate the direct and indirect effects. Identification of important component traits through path analysis is useful in indirect selection for complex traits like yield and yield related traits.

The equation followed is given below

$$rij = Pij+\Sigma rikpkj$$

The residual effect was estimated by thequation: $(1-R^2)^{-1}$

Where, $R^2 = \Sigma p_{ij} \times r_{ij}$

Where:

- $p_{ij=}$Component of direct effects of the independent character (i) and dependent character (j) as measured by the path coefficient
- rij = Mutual association between the independent character (i) and dependent character (j) as measured by the correlation coefficient
- Σrikpkj = Summation of components of indirect effect of a given independent character (i) on the given dependent character (j) via all other independent character (k)

3.8. DEVELOPMENT OF SELECTION CRITERIA FOR YOUNG DOUBLE CROSS HYBRIDS

Characters having direct effect on total wet bean per tree per year were selected for development of selection criteria for double cross hybrids at early bearing stage along with total wet bean weight per tree per year. The double cross hybrids were scored based on their superiority for the significant characters and ranked. Based on the mean values of significant characters of the top ranked eight double cross hybrids, selection criteria was developed.

Results and Discussion

4. RESULTS AND DISCUSSION

Twenty cocoa double cross hybrids bred for VSD disease resistance in their early bearing stage served as the material for the present study entitled ‘‘Preliminary evaluation of double cross hybrids for yield and Vascular Streak Dieback (VSD) disease resistance in cocoa (*Theobroma cacao* L.)”. The hybrids were field planted during 2017 at CRC farm. Evaluation of the hybrids was carried out for their general vigour, yield, morphological and biochemical characters along with their resistant reaction against Vascular Streak Dieback (VSD) disease. The results of the present study are presented and discussed below.

4.1. EVALUATION OF MORPHOLOGICAL CHARACTERS

Economic and genetic gain can be achieved from chosen genotypes through the assessment of morphological characters (Iwaro *et al*., 2003; Bekele *et al*., 2006). Selection is based on the variability present in nature. Crop diversity can be estimated from different measures of variability and among them, morphological traits are used for cataloguing and characterisation (Malhotra and Aphsara, 2017). Morphological evaluation was carried out based on the qualitative and quantitative evaluation of floral, pod and bean characters. Completely Randomised Design (CRD) with five replications was employed for statistical analysis.

4.1.1. Evaluation of Qualitative Characters

The descriptor developed by Bekele and Butler (2000) was used for scoring of qualitative characters. The observations taken on qualitative characters are shown in Tables 7, 8 and also in Plates 1, 2, 3, 4, 5, 6, 7, 8 and 9.

Evaluation of qualitative characters included eleven characters namely., flush colour, colour of pedicel, colour of sepal, colour of petal, colour of unripe pod, colour of ripe pod, pod shape, pod apex form, pod basal constriction, pod rugosity and colour of the peeled bean. Double cross hybrids exhibited variation for almost all the characters except for the petal colour, all the hybrids had cream colour petals.

4.1.1.1. Evaluation of Cocoa Leaf Flush and Floral Characters

The observations recorded on the qualitative characters of leaf flush and flower are presented in Table 7 and Plate 1. Kochhar (1998) investigated on variation in flush colour in cocoa and documented the variations from light green to red. Less variability was exhibited by the hybrids for flush colour in the present study. Except for hybrid DC VSD 9.6, all the hybrids (95%) showed red flush colour. The hybrid DC VSD 9.6exhibited green flush colour . Among the various flush colours (green, greenish red, reddish green and red) explained in the descriptor of Bekele and Butler (2000), only green and red were observed in the present study. Flush colour forms a prominent trait in cocoa (Bartley, 2005), for morphological characterization. Asna (2013) observed all the four variations of flush colour in forty accessions of cocoa.

Variability in the cocoa cultivars can be identified through the evaluation of their floral characters (Enriquez and Soria, 1967; Lachenaud *et al*., 1999 and Efombagn *et al*., 2009). Colour of pedicel, sepal and petal were the three characters used for the qualitative study of the flower. Colours of pedicel and sepal have expressed variability in this investigation. However, no variability was seen in petal colour. All the hybrids showed cream petal colour. Shilpa (2019) also reported no variability in petal colour among the hybrids studied.

All the variation in the pedicel colour (green, red and reddish) given in the descriptor of Bekele and Butler (2000) were observed in the current study. Ten hybrids (50 %) exhibited green, seven (35 %) showed reddish and three (15 %) showed red pedicel colour. A study conducted by Veeresh (2017) on the pedicel colour of cocoa concluded green as the prominent pedicel colour in cocoa.

Red **Green**

Plate 1. Variability in flush colour

Green **Reddish** **Red**

Plate 2. Variability in pedicel colour

Angoletta **Amelando** **Calabacillo**

Plate 3. Variability in pod shape

Table 7. Qualitative characters of leaf and flower of young double cross cocoa hybrids

Sl. No.	Hybrid	Flush colour	Pedicel colour	Sepal colour	Petal colour
1	DC VSD 2.1	Red	Reddish	Cream	Cream
2	DC VSD 2.4	Red	Red	Cream	Cream
3	DC VSD 3.3	Red	Red	Cream	Cream
4	DC VSD 6.11	Red	Green	Cream	Cream
5	DC VSD 6.12	Red	Green	Greenish cream	Cream
6	DC VSD 7.2	Red	Green	Greenish cream	Cream
7	DC VSD 8.10	Red	Green	Cream	Cream
8	DC VSD 8.11	Red	Reddish	Greenish cream	Cream
9	DC VSD 9.6	Green	Reddish	Greenish cream	Cream
10	DC VSD 9.13	Red	Reddish	Greenish cream	Cream
11	DC VSD 12.5	Red	Green	Cream	Cream
12	DC VSD 15.2	Red	Green	Greenish cream	Cream
13	DC VSD 15.5	Red	Reddish	Cream	Cream
14	DC VSD 18.3	Red	Green	Greenish cream	Cream
15	DC VSD 18.6	Red	Reddish	Cream	Cream
16	DC VSD 18.9	Red	Green	Cream	Cream
17	DC VSD 19.11	Red	Reddish	Cream	Cream
18	DC VSD 20.2	Red	Green	Cream	Cream
19	DC VSD 20.10	Red	Red	Cream	Cream
20	DC VSD 21.9	Red	Green	Greenish Cream	Cream

Bekele and Butler (2000) classified sepal colour as cream, greenish cream, red and reddish in their descriptor. Only cream and greenish cream were shown by the hybrids in the present study. Twelve hybrids (60 %) exhibited cream sepal colour and rest of the eight hybrids (40 %) exhibited greenish cream colour. A study on forty cocoa accessions recorded greenish cream, cream and reddish sepal colour among them (Asna, 2013).

4.1.1.2. Evaluation of Cocoa Pod and Bean

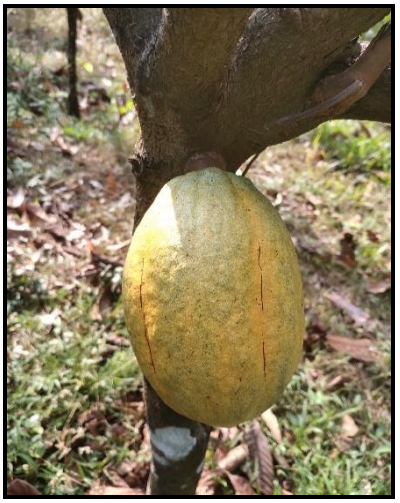
Cuatrecasas (1964) reported high variability in shape, colour and size of pods of *Theobroma* species, especially *Theobroma cacao*. Studies on Brazilian *Theobroma* species also revealed similar conclusion (Santos *et al*., 2012). In the present study, characters like pod shape, colour of unripe and ripe pod, pod apex form, pod basal constriction, pod rugosity and bean colour were recorded for the qualitative evaluation of pod. The observations recorded are shown in Table 8 and in Plates 3, 4, 5, 6, 7, 8 and 9. Bekele and Butler (2000) described five pod shapes (Cundeamour, Amelonado, Angoleta, Calabacillo and Criollo) in their descriptor.

The hybrids under this study exhibited only three types of pod shape. Twelve hybrids (60 %) exhibited Calabacillo, five hybrids (25 %) Angoleta and three hybrids (15 %) Amelonado pod shape. DC VSD 2.1, DC VSD 2.4, DC VSD 3.3, DC VSD 6.11, DC VSD 6.12, DC VSD 7.2, DC VSD 9.6, DC VSD 9.13, DC VSD 12.5, DC VSD 15.2 and DC VSD 15.5 are the twelve hybrids which have Calabacillo shaped fruits. Calabacillo types were smooth nearly spherical fruits. Hybrids having Angoleta shaped fruits were DC VSD 8.10, DC VSD 8.11, DC VSD 18.9, DC VSD 20.2 and DC VSD 18.3. Angoletta type fruits are defined by deeply ridged and warty surface with a square shape at the stalk end. Hybrids DC VSD 18.6, DC VSD 19.11, DC VSD 21.9 have Amelonado shaped fruits. Amelonado types are pods which are smooth melon shaped with shallow furrows having a blunt end and slight bottle neck. Asna (2013) studied pod shape in forty cocoa accessions and reported that majority (88 %) had cudeamour shape whereas amelonado and angoletta types were exhibited by three accessions each.

Light green

Intermediate green

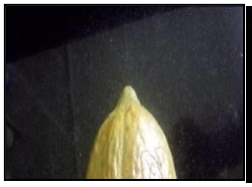
Plate 4.Variability in unripe pod colour



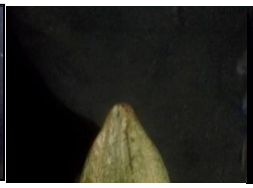
Yellowish green

Yellow

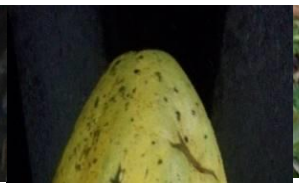
Plate 5. Variability in ripe pod colour



Attenuate



Acute



Obtuse

Rounded

Plate 6. Variability in pod apex


Absent **Slight**

Plate 7. Variability in pod basal constriction




Absent **Slight** **Intermediate** **Intense**

Plate 8.Variability in pod rugosity


Medium purple **Dark purple**

Plate 9.Variability in peeled bean colour

Pod colour and shape form prominent characters in identifying variability among genotypes (Thi *et al.,* 2016). Bekele and Butler (2000) denoted four types of variation in colour of unripe pods (dark green, intermediate green, light green and purplish green). However, only intermediate green (70 %) and light green (30 %) coloured unripe pods were observed under the present study. Hybrids DC VSD 6.11, DC VSD 6.12, DC VSD 9.13, DC VSD 15.2, DC VSD 15.5, DC VSD 18.6 expressed light green colour before ripening whereas the rest of the hybrids expressed intermediate green colour. Variability was expressed in ripe pod colour also. It was identified by observing the colour of ridges and furrows. Bekele and Butler (2000) described four types of variability in ripe pod colour (green, yellowish green, greenish yellow, yellow). However, only yellow (65 %) and yellowish green (35 %) was exhibited by the pods of the hybrids under study.

According to Bekele and Butler (2000), there are six types of pod apex including attenuate, acute, obtuse, rounded, mammelate and indented. However, only four among them were expressed in the present study. Majority (65 %) of the hybrids exhibited acute type of pod apex. Three (15 %) hybrids showed attenuate type of pod apex. Obtuse type of pod apex was observed in three hybrids (15 %) whereas round type of pod apex was expressed in a single (5 %) hybrid under study. Among the hybrids, DC VSD 3.3, DC VSD 9.13, DC VSD 15.5 had attenuate type, DC VSD 6.11, DC VSD 8.10 and DC VSD 21.9 had obtuse type of pod apex. DC VSD 18.6 expressed rounded pod apex and the rest of the hybrids exhibited acute pod apex. Shilpa (2019) observed that majority (45 %) of the hybrids had obtuse type of pod apex.

Observations were recorded for type of pod basal construction in all the hybrids under study. Among the five classes of pod basal constriction (absent, slight, intermediate, strong and wide shoulder) given by Bekele and Butler (2000) in their descriptor, only two classes were observed in the current study. Majority (65 %) of the hybrids expressed slight basal constriction in the pods whereas it was absent in the other hybrids. Hybrids DC VSD 2.1, DC VSD 3.3, DC VSD 6.11, DC VSD 7.2, DC VSD 18.3, DC VSD 18.6 and DC VSD 21.9 had no basal constriction and the remaining hybrids had slight basal constriction. Rubeena (2015) also observed similar

results in her study where majority (75 %) of the hybrids under study exhibited slight basal constriction.

Roughness or unevenness in the surface of pod is measured as pod rugosity. Bekele and Butler (2000) classified pod rugosity into four classes as absent, slight, intermediate and intense. All the four classes were observed in the pods of hybrids under study. Nine (45 %) hybrids had slight rugosity and five (25 %) hybrids exhibited intermediate rugosity. Only two hybrids (10 %) showed intense pod rugosity whereas the rest of the four (20 %) exhibited smooth surface. Hybrids DC VSD 2.4, DC VSD 6.12, DC VSD 7.2, DC VSD 9.6, DC VSD 15.2, DC VSD 15.5, DC VSD 18.9, DC VSD 19.11 and DC VSD 20.2 showed slight rugosity. DC VSD 3.3, DC VSD 8.10, DC VSD 12.5, DC VSD 20.10 and DC VSD 21.9 were the hybrids which exhibited intermediate rugosity.

Hybrids DC VSD 2.1, DC VSD 6.11, DC VSD 9.13 and DC VSD 18.6 showed absence of rugosity on the pod surface, whereas DC VSD 8.11 and DC VSD 18.3 exhibited intense pod rugosity. Rubeena (2015) observed slight rugosity in most of the hybrids developed for bold beans. Forastero genotypes are characterized by the smooth and slight rugous pod surface (Kochhar, 1998; Malhotra and Apshara, 2017).

Variability is expressed in the peeled bean colour in different genotypes of cocoa. Bekele and Butler (2000) grouped peeled bean colour into seven classes as white, grey, light purple, medium purple, dark purple, mottled and mixed. Twelve (60 %) hybrids exhibited dark purple colour and the rest (40 %) exhibited medium purple colour as the peeled bean colour. Veeresh (2017) observed variability in the bean colour and concluded that dark purple (40 %) and medium purple colour (40 %) are more prominent in bean colour among the hybrids studied.

Table 8. Qualitative pod and bean characters of cocoa early stage double cross hybrids

Sl. No.	Hybrids	Pod shape	Unripe pod colour	Ripe pod colour	Pod base	Pod apex	Pod rugosity	Bean colour
1	DC VSD 2.1	Calabacillo	Intermediate green	Yellow	Absent	Acute	Absent	Medium purple
2	DC VSD 2.4	Calabacillo	Intermediate green	Yellow	Slight	Acute	Slight	Dark purple
3	DC VSD 3.3	Calabacillo	Intermediate green	Yellow	Absent	Attenuate	Intermediate	Medium purple
4	DC VSD 6.11	Calabacillo	Light Green	Yellow	Absent	Obtuse	Absent	Dark purple
5	DC VSD 6.12	Calabacillo	Light green	Yellow	Slight	Acute	Slight	Dark purple
6	DC VSD 7.2	Calabacillo	Intermediate green	Yellowish green	Absent	Acute	Slight	Medium purple
7	DC VSD 8.10	Angoleta	Intermediate green	Yellow	Slight	Obtuse	Intermediate	Dark purple
8	DC VSD 8.11	Angoleta	Intermediate green	Yellowish green	Slight	Acute	Intense	Dark purple
9	DC VSD 9.6	Calabacillo	Intermediate green	Yellowish green	Slight	Acute	Slight	Medium purple
10	DC VSD 9.13	Calabacillo	Light green	Yellow	Slight	Attenuate	Absent	Dark purple
11	DC VSD 12.5	Calabacillo	Intermediate green	Yellow	Slight	Acute	Intermediate	Dark purple

Table 8. continued**.**

Sl. No.	Hybrids	Pod shape	Unripe pod colour	Ripe pod colour	Pod base	Pod apex	Pod rugosity	Bean colour
12	DC VSD 15.2	Calabacillo	Light green	Yellow	Slight	Acute	Slight	Dark purple
13	DC VSD 15.5	Calabacillo	Light green	Yellow	Slight	Attenuate	Slight	Dark purple
14	DC VSD 18.3	Angoletta	Intermediate green	Yellowish green	Absent	Acute	Intense	Dark purple
15	DC VSD 18.6	Amelonado	Light green	Yellow	Absent	Rounded	Absent	Dark purple
16	DC VSD 18.9	Angoletta	Intermediate green	Yellowish green	Slight	Acute	Slight	Medium purple
17	DC VSD 19.11	Amelonado	Intermediate green	Yellow	Slight	Acute	Slight	Medium purple
18	DC VSD 20.2	Angoletta	Intermediate green	Yellowish green	Slight	Acute	Slight	Medium purple
19	DC VSD 20.10	Calabacillo	Intermediate green	Yellowish green	Slight	Acute	Intermediate	Dark purple
20	DC VSD 21.9	Amelonado	Intermediate green	Yellow	Absent	Obtuse	Intermediate	Medium purple

4.1.1.2..a. Cluster Analysis of Double Cross Hybrids Based on the Qualitative Characters

Cluster analysis of the twenty double cross hybrids was carried out based on eleven qualitative characters and is represented in Table 9 and Figure 5. Less variability was exhibited by the double cross hybrids for the qualitative characters The twenty hybrids were broadly classified into four clusters based on proximity matrix value. Cluster I had the highest number of double cross hybrids (14) namely DC VSD 6.12, DC VSD 15.2, DC VSD 2.4, DC VSD 19.11, DC VSD 7.2, DC VSD 3.3, DC VSD 20.10, DC VSD 12.5, DC VSD 8.11, DC VSD 18.3, DC VSD 8.10, DC VSD 21.9, DC VSD 18.9 and DC VSD 20.2. Cluster II consisted of four double cross hybrids including DC VSD 6.11, DC VSD 18.6, DC VSD 2.1 and DC VSD 9.13. They showed yellow ripened pod colour. Hybrids DC VSD 15.5 and DC VSD 9.6 formed separate clusters, cluster III and cluster IV, respectively. DC VSD 15.5 remained distinct with calabacillo shape having attenuate pod apex, slight rough surface and dark purple bean colour. DC VSD 9.6 is distinct from other double cross hybrids with green flush colour whereas rest of them showed red flush colour.

Table 9. Hierarchical clustering of the twenty double cross hybrids based on qualitative characters

Cluster No.	**No. of double cross hybrids**	**Name of double cross hybrid**
I	14	DC VSD 6.12, DC VSD 15.2, DC VSD 2.4, DC VSD 19.11, DC VSD 7.2, DC VSD 3.3, DC VSD 20.10, DC VSD 12.5, DC VSD 8.11, DC VSD 18.3, DC VSD 8.10, DC VSD 21.9, DC VSD 18.9, DC VSD 20.2
II	4	DC VSD 6.11, DC VSD 18.6, DC VSD 2.1, DC VSD 9.13
III	1	DC VSD 15.5
IV	1	DC VSD 9.6

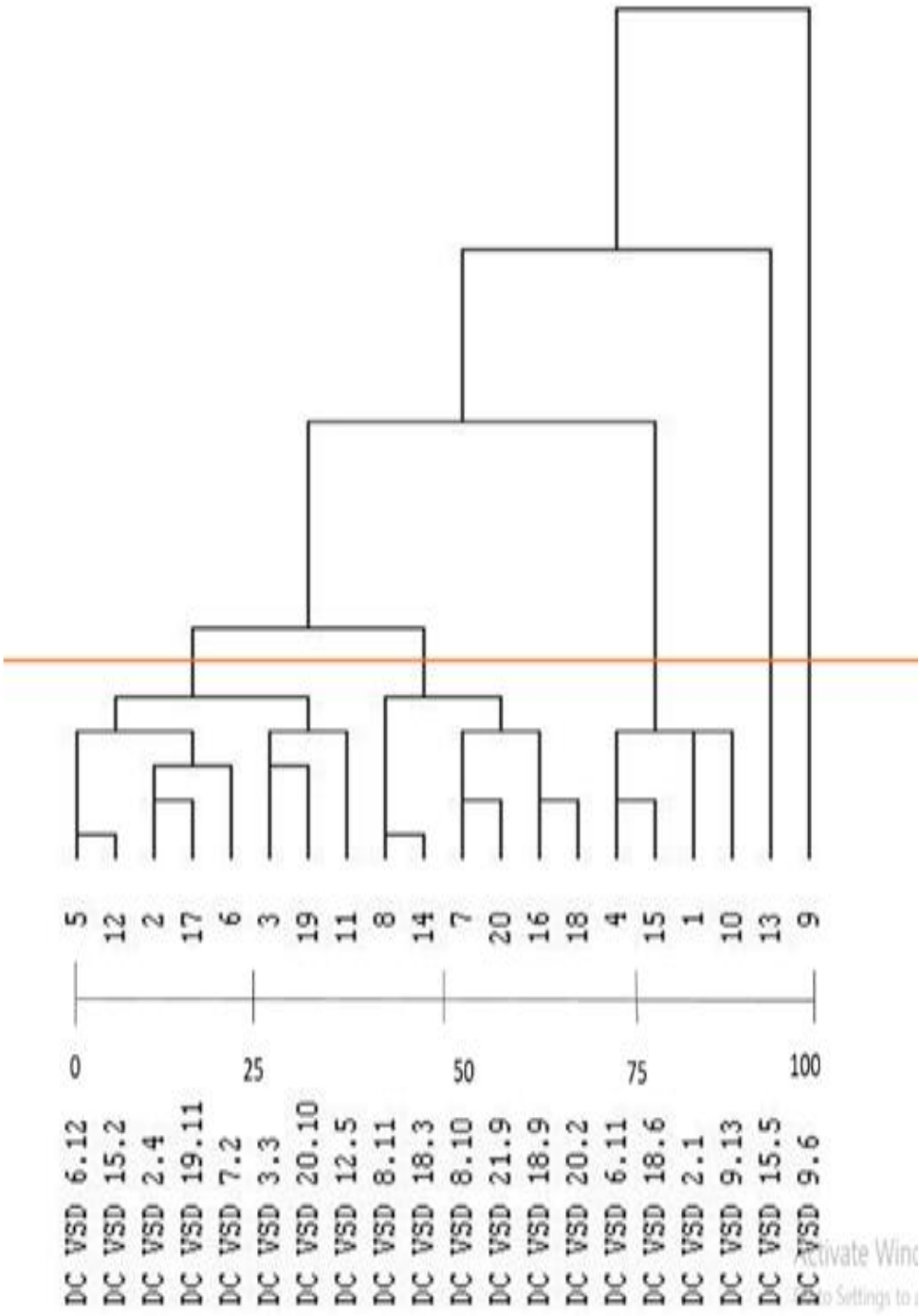


Figure 5. Dendrogram generated using the hierarchical clustering of average linkage (between groups) of double cross hybrids based on qualitative characters

4.1.2. Evaluation of Quantitative Characters

The twenty hybrids under study served as treatments and five pods from each hybrid as the replication of each treatment. For evaluation of floral characters, also considered ten flowers from each hybrid was considered as ten replication per treatment. The experiment was conducted in Completely Randomised Design (CRD).

4.1.2.1. Quantitative Evaluation of Floral Characters of Cocoa

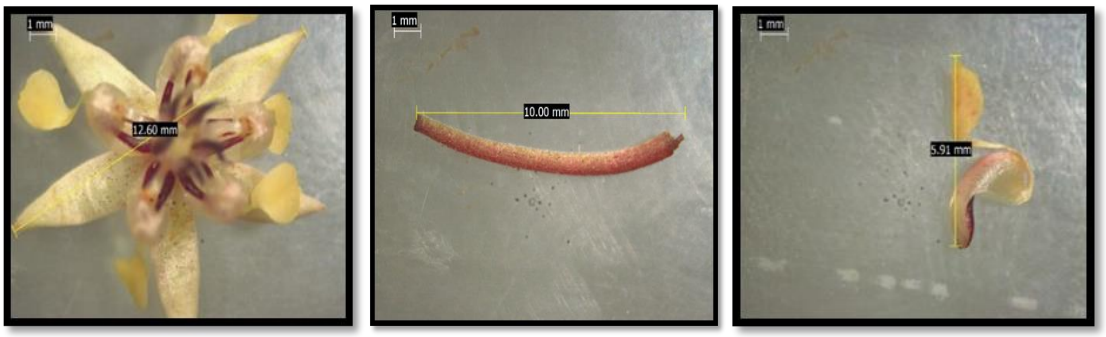
Significant variation was recorded among the hybrids for all the floral characters at five per cent level of significance. Mean values of all the quantitative characters of cocoa flower are enlisted in Tables 10.a, 10.b. and Plate 10.

Variation in diameter of the flower ranged from 11.84 mm to 15.5 mm among the twenty hybrids. Hybrid DC VSD 7.2 had the lowest value (11.84 mm) for flower diameter which was on par with hybrid DC VSD 21.9 (11.89 mm) whereas hybrid DC VSD 15.5 had the highest value (15.5 mm). Pedicel length showed variation from 6.58 mm to 15.10 mm and it was exhibited by DC VSD 9.6 and DC VSD 3.3 respectively. Sepal length varied from 5.33 mm in the hybrid DC VSD 2.1. to 7.84 mm in hybrid DC VSD 15.2. Sepal breadth ranged from 1.54 mm to 2.76 mm in DC VSD 18.3 and DC VSD 2.1 respectively.

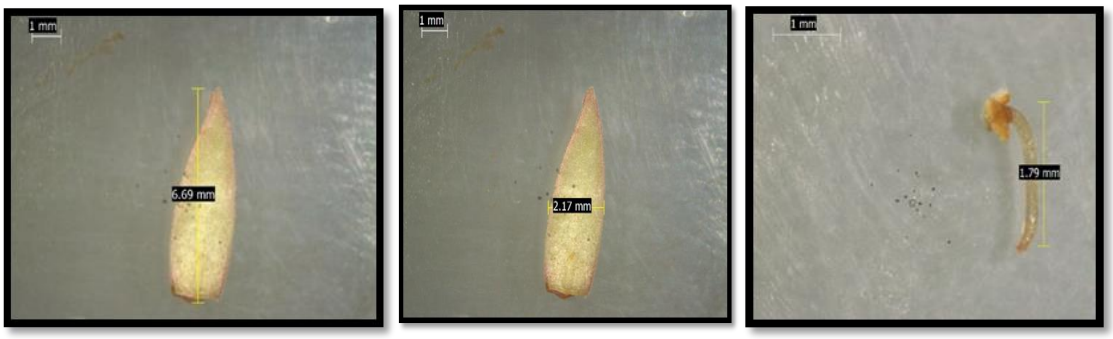
Petal length showed variation from 4.76 mm in DC VSD 6.11 to 6.56 mm in DC VSD 20.2. Petal breadth varied from 1.69 mm in DC VSD 19.11 to 2.54 mm in DC VSD 2.1. Stamen length varied from 1.64 mm in hybrid DC VSD 2.1 to 2.26 mm in hybrid DC VSD 19.11. Staminode length varied from 4.35 mm in hybrid DC VSD 20.2 to 6.63 mm in hybrid DC VSD 7.2. Characters like ovary length, ovary breadth as well as length of the style also exhibited significant variation among the hybrids. Ovary length varied from 0.99 to 1.75 mm in hybrid DC VSD 18.3 and hybrid DC VSD 2.1 respectively. Ovary breadth ranged from 0.96 to 1.29 mm in hybrid DC VSD 20.2 and hybrid DC VSD 2.1 respectively. Length of the style was highest in hybrid DC VSD 2.4 with a maximum value of 2.03 mm and lowest in DC VSD 18.3 with a value of 1.26 mm which is on par with DC VSD 15.5 (1.27 mm). The result obtained for all the eleven floral characters were found to be similar to the findings of earlier researchers.

Veeresh (2017) reported significant variation among thirty genotypes for all the floral characters. He recorded the range of variability for various characters including diameter of the flower, pedicel length, sepal length, sepal breadth, petal length, petal breath, length of staminode, length of stamen, ovary length, ovary breadth and length of style as ranged from 8.9 -14.7 mm, 8.2 -18.3 mm, 5.2 - 7.4 mm, 1.0 - 2.1 mm, 6.5 - 8.9 mm, 1.2 - 2.0 mm, 4.7 - 6.8 mm, 1.6 - 2.8 mm, 1.1 - 2.2 mm, 0.9 - 1.3 mm and 1.5 - 2.5 mm respectively. These results are on consonance with the present findings.

Similar study was carried by Shilpa (2019) on twenty hybrids of cocoa. Significant variation was exhibited by the hybrids for all the eleven floral characters under study. Based on her study, diameter of the flower, pedicel length, sepal length, sepal breadth, petal length, petal breadth, length of staminode, length of stamen, ovary length, ovary breadth and length of style ranged from 10.19 -15.14 mm, 8.32 - 19.80 mm, 5.34 - 7.52 mm, 1.38 - 2.78 mm, 2.35 - 5.76 mm, 2.64 - 1.29 mm,1.61 - 3.37 mm,4.16 -6.66 mm,1.36 - 2.54 mm,0.66 - 1.48 mm and 1.08 -2.67 mm respectively. These results are also on par with the present findings.



Diameter of flower (mm) **Length of pedicel (mm)** **Length of petal (mm)**



Length of sepal (mm) **Breadth of sepal (mm)** **Length of the stamen (mm)**

Length of style (mm) **Length of ovary (mm)** **Breadth of ovary (mm)**

Plate 10. Observations on the quantitative characters of cocoa flower of double cross hybrids

Table 10.a. Mean values of quantitative floral characters of early bearing cocoa double cross hybrids

Hybrids	Flower diameter (mm)	Pedicel length (mm)	Sepal length (mm)	Sepal breadth (mm)	Petal length (mm)
DC VSD 2.1	12.47	10.37	5.33	2.76	5.59
DC VSD 2.4	12.58	10.28	5.81	1.90	5.70
DC VSD 3.3	13.38	15.10	5.44	1.91	5.91
DC VSD 6.11	13.05	11.50	5.63	1.94	4.76
DC VSD 6.12	12.59	9.52	6.50	1.81	5.48
DC VSD 7.2	11.84	11.30	6.18	2.15	4.81
DC VSD 8.10	12.63	10.38	5.45	1.84	5.24
DC VSD 8.11	12.41	12.00	6.19	1.97	5.65
DC VSD 9.6	13.50	6.58	5.97	2.17	5.07
DC VSD 9.13	12.87	10.59	7.59	1.99	6.16
DC VSD 12.5	13.14	7.60	5.63	1.83	4.91
DC VSD 15.2	14.21	10.08	7.84	1.62	5.23
DC VSD 15.5	15.50	7.20	7.07	1.6	5.70

Table 10.a. continued**.**

Hybrids	Flower diameter (mm)	Pedicel length (mm)	Sepal length (mm)	Sepal width (mm)	Petal length (mm)
DC VSD 18.3	13.12	8.06	6.44	1.54	5.60
DC VSD 18.6	13.75	11.03	6.28	2.22	6.05
DC VSD 18.9	13.54	8.16	6.01	1.86	5.97
DC VSD 19.11	13.89	12.05	6.66	1.73	5.72
DC VSD 20.2	13.61	9.32	7.02	1.90	6.56
DC VSD 20.10	12.68	7.89	6.02	1.63	5.58
DC VSD 21.9	11.89	9.39	6.05	1.87	5.66
CV (%)	**1.56**	**1.66**	**1.48**	**2.41**	**2.14**
CD (5%)	**0.26**	**0.21**	**0.12**	**0.06**	**0.15**

Table 10.b. Mean values of quantitative floral characters of early bearing double cross cocoa hybrids

Hybrids	Petal breadth (mm)	Stamen length (mm)	Staminode length (mm)	Ovary length (mm)	Ovary breadth (mm)	Style length (mm)
DC VSD 2.1	2.54	1.64	5.77	1.75	1.29	1.92
DC VSD 2.4	2.28	1.75	4.87	1.44	1.15	2.03
DC VSD 3.3	2.34	1.83	6.24	1.26	1.09	1.75
DC VSD 6.11	1.83	1.67	4.84	1.08	1.20	1.84
DC VSD 6.12	2.44	1.79	5.05	1.36	1.00	1.44
DC VSD 7.2	2.09	1.85	6.63	1.13	1.07	1.52
DC VSD 8.10	2.21	1.74	5.05	1.21	1.05	1.44
DC VSD 8.11	1.84	2.07	5.12	1.35	1.16	1.35
DC VSD 9.6	1.93	1.96	4.96	1.30	1.13	1.36
DC VSD 9.13	1.91	1.76	5.56	1.35	1.23	1.77
DC VSD 12.5	1.88	1.93	4.94	1.12	1.13	1.50
DC VSD 15.2	1.71	1.84	4.86	1.33	1.05	1.82
DC VSD 15.5	1.81	1.84	5.05	1.00	1.09	1.27
DC VSD 18.3	2.03	1.84	4.58	0.99	0.97	1.26

Table 10.b. continued**.**

Hybrids	Petal breadth (mm)	Stamen length (mm)	Staminode length (mm)	Ovary length (mm)	Ovary breadth (mm)	Style length (mm)
DC VSD 18.6	1.82	1.94	5.45	1.22	1.16	1.60
DC VSD 18.9	1.76	1.84	5.08	1.22	0.99	1.53
DC VSD 19.11	1.69	2.26	5.07	1.20	1.13	1.83
DC VSD 20.2	1.84	2.03	4.35	1.16	0.96	1.64
DC VSD 20.10	1.78	1.94	4.81	1.21	1.05	1.57
DC VSD 21.9	1.83	1.85	4.62	1.50	1.05	1.74
CV (%)	**2.34**	**1.24**	**7.82**	**1.96**	**1.89**	**1.59**
CD (5%)	**0.06**	**0.03**	**0.51**	**0.03**	**0.03**	**0.03**

4.1.2.2. Evaluation of Quantitative Characters of Cocoa Pod and Bean of Double Cross Hybrids

Seven pod characters and six bean characters are included for studying quantitative characters of pod and bean. All the twenty hybrids along with CCRP 15 variety (check variety) planted in the same field was evaluated for the quantitative characters. Pod characters include pod weight (g), pod length (cm), pod breadth (cm), rind thickness (cm), number of beans per pod, number of flat beans per pod and total weight of wet beans per pod (g). Bean characters recorded include weight of single wet bean (g), weight of single peeled bean (g), weight of single dry bean (g), dry bean length (cm), dry bean width (cm) and dry bean thickness (cm). Average values for the quantitative characters of pod and beans are enlisted in Table 11.a. and 11.b. respectively.

Highest pod weight of 654.4 g was recorded by the hybrid DC VSD 18.6 followed by hybrid DC VSD 8.10 with a pod weight of 520.8 g. Hybrid DC VSD 9.13 had the lowest pod weight (195.34 g) among all the hybrids. However, all the hybrids had higher pod weight than that of the check variety (146.4 g). Adewale *et al*. (2013) revealed that higher pod weight can be considered for the selection of cocoa genotypes as like that of higher bean weight. Ajmal (2016) reported variability in pod weight among thirty cocoa hybrids with in a range between 249.64 g to 685 g. Kishor (2017) recorded a variation from 160 g to 724 g in twenty eight hybrids considered for his study.

Maximum pod length of 15.5 cm was observed in the hybrid DC VSD 8.10 which was on par with the hybrid DC VSD 6.12 (15.4 cm) whereas hybrid DC VSD 6.11 and hybrid DC VSD 9.13 had the shortest pod of 11.28 cm length. Highest mean pod breadth was exhibited by hybrid DC VSD 18.6 with a breadth of 9.4 cm and lowest by hybrid DC VSD 9.13 with a breadth of 5.5 cm which was on par with hybrid DC VSD 3.3 (5.56 cm). Almost all the hybrids found to be superior to the check variety having pod length 9.36 cm and pod breadth 6.4 cm.

Table 11.a. Mean values of quantitative pod and bean characters of double cross hybrids

Hybrids	Pod weight (g)	Pod length (cm)	Pod breadth (cm)	Rind/ husk thickness (cm)
DC VSD 2.1	388.84	13.86	7.94	0.78
DC VSD 2.4	292.20	11.80	7.68	0.93
DC VSD 3.3	264.40	12.34	5.56	0.79
DC VSD 6.11	329.48	11.28	6.62	0.91
DC VSD 6.12	358.12	15.40	7.56	1.04
DC VSD 7.2	321.42	12.30	6.66	0.99
DC VSD 8.10	520.80	15.50	7.70	1.27
DC VSD 8.11	382.00	13.50	6.40	0.94
DC VSD 9.6	330.94	12.72	7.52	1.11
DC VSD 9.13	195.34	11.28	5.50	0.51
DC VSD 12.5	351.62	13.46	6.64	0.96
DC VSD 15.2	337.64	12.72	7.64	1.12
DC VSD 15.5	324.06	12.48	6.92	0.99
DC VSD 18.3	334.52	13.18	6.62	1.17
DC VSD 18.6	654.40	14.30	9.40	1.84
DC VSD 18.9	438.40	14.10	6.78	1.26
DC VSD 19.11	262.84	12.42	6.40	0.77
DC VSD 20.2	364.00	13.78	6.82	0.80
DC VSD 20.10	283.08	12.86	6.94	0.75
DC VSD 21.9	439.22	13.32	8.46	1.19
CCRP 15 (Check variety)	146.40	9.36	6.40	0.75
CV (%)	**2.28**	**2.69**	**3.23**	**4.65**
CD (5%)	**9.98**	**0.44**	**0.29**	**0.06**

Table 11.b Mean values of quantitative pod and bean characters of double cross hybrids

Hybrids	No. of beans/pod	No. of flat beans/ pod	Total weight of wet beans/ pod (g)	Weight of single wet bean (g)	Single peeled wet bean weight (g)	Single dry bean weight (g)	Dry bean length (cm)	Dry bean width (cm)	Dry bean thickness (cm)
DC VSD 2.1	54.40	4.20	127.84	2.38	1.18	0.79	2.40	1.08	0.44
DC VSD 2.4	42.80	1.60	86.62	2.02	1.02	0.75	2.18	1.22	0.56
DC VSD 3.3	50.60	3.20	76.92	1.82	0.97	0.65	1.82	1.17	0.49
DC VSD 6.11	44.00	6.40	124.80	2.48	1.29	0.96	2.32	1.14	0.57
DC VSD 6.12	42.80	2.60	102.10	2.42	1.19	0.81	2.24	1.01	0.59
DC VSD 7.2	43.00	3.40	93.40	2.54	1.23	0.93	2.17	1.18	0.50
DC VSD 8.10	50.00	2.60	121.08	2.48	1.25	0.88	2.09	1.28	0.60
DC VSD 8.11	38.00	3.60	84.36	2.26	1.21	0.92	2.21	1.14	0.67
DC VSD 9.6	38.00	2.40	88.34	2.18	1.21	0.81	2.09	1.22	0.54
DC VSD 9.13	36.00	5.40	58.72	2.04	1.04	0.66	1.80	1.06	0.46
DC VSD 12.5	45.00	3.20	85.76	2.38	1.13	0.83	1.88	0.94	0.54
DC VSD 15.2	41.20	4.20	76.20	2.56	1.34	0.98	2.03	1.12	0.46
DC VSD 15.5	43.20	2.40	90.78	2.48	1.19	0.85	1.73	0.93	0.45

Table 11.b. continued.

Hybrids	No. of beans/pod	No. of flat beans/ pod	Total weight of wet beans/ pod (g)	Weight of single wet bean (g)	Single peeled wet bean weight (g)	Single dry bean weight (g)	Dry bean length (cm)	Dry bean width (cm)	Dry bean thickness (cm)
DC VSD 18.3	42.60	2.60	69.46	2.18	1.04	0.75	1.76	1.02	0.63
DC VSD 18.6	41.00	4.60	132.40	2.68	1.38	0.99	2.34	1.23	0.77
DC VSD 18.9	42.00	2.40	115.28	2.40	1.16	0.82	2.44	1.09	0.85
DC VSD 19.11	42.80	3.60	63.60	2.22	1.03	0.79	2.18	0.95	0.61
DC VSD 20.2	42.40	3.00	74.88	2.20	1.10	0.80	1.97	1.01	0.58
DC VSD 20.10	47.00	6.20	75.80	1.89	0.88	0.65	1.73	1.00	0.48
DC VSD 21.9	47.20	4.00	114.32	2.48	1.24	0.94	2.40	1.15	0.58
CCRP 15	41.20intro	2.40	55.43	1.56	0.64	0.45	1.45	0.64	0.45
CV (%)	**1.98**	**19.78**	**4.00**	**5.86**	**8.38**	**7.23**	**5.67**	**5.16**	**7.43**
CD (5%)	**3.38**	**0.88**	**4.60**	**0.17**	**0.12**	**0.07**	**0.15**	**0.07**	**0.05**

Highest value for rind/ husk thickness (1.84 cm) was observed in DC VSD 18.6 which had highest pod weight. Lowest husk thickness value of 0.51 cm was recorded in DC VSD 9.13 which had lowest pod weight. Hence, there is an influence of rind/ husk thickness in determining the pod weight. Rubeena (2015) and Shilpa (2019) also reported the effect of rind/ husk thickness in deciding pod weight. As per Enriquez and Soria (1966), rind thickness less than or equal to one centimetre is the most advantageous in cocoa. Only twelve hybrids among the twenty under study satisfied the desirable condition of rind thickness.

Various bean characters studied for the quantitative evaluation involves number of beans per pod, number of flat beans per pod, total wet weight of beans per pod (g), wet weight of single bean (g), peeled weight of single bean (g), dry weight of single bean (g), single dry bean length (cm), width (cm) and thickness (cm). Significant variation was expressed in all the characters among the twenty hybrids.

Thirty six beans per pod were the lowest number of beans per pod exhibited by hybrid DC VSD 9.13 which was on par with hybrids DC VSD 8.11 (38) and DC VSD 9.6 (38) whereas the hybrid DC VSD 2.1 exhibited the highest number of beans per pod having 54.40 beans per pod. Double cross hybrids showed significant variation for number of flat beans per pod. Among the hybrids number of flat beans per pod varied from 1.60 in hybrid DC VSD 2.4 to 6.40 in hybrid DC VSD 6.11. Unfertilized ovules give rise to flat beans in cocoa (Rubeena, 2015). Higher number of flat beans in the double cross hybrids may be because they have not attained the maturity for steady bearing.

Maximum wet bean weight of 132.40 g was observed in hybrid DC VSD 18.6 which was on par with hybrid DC VSD 2.1 (127.84 g) and the least was observed in hybrid DC VSD 9.13 with 58.72 g. Single wet bean weight was found to be highest in hybrid DC VSD 18.6 with a value of 2.68 g which was on par with hybrid DC VSD 7.2 (2.54 g) and DC VSD 15.2 (2.56 g) whereas it was lowest in hybrid DC VSD 3.3 (1.82 g) and was on par with DC VSD 20.10 (1.89 g). Variation in the single peeled wet bean weight ranged from 0.88 g in hybrid DC VSD 20.10 to 1.38 g in hybrid DC VSD 18.6. Nearly 50 per cent of the wet bean weight is due to pulp (white fleshy

substance) covering the beans. Beans with 30 per cent pulp are most desirable for fermentation.

Single dry bean weight was highest in hybrid DC VSD 18.6 with 0.99 g which was on par with hybrids DC VSD 15.2 (0.98 g), DC VSD 6.11 (0.96 g), DC VSD 21.9 (0.94 g), DC VSD 7.2 (0.93 g) and DC VSD 8.11(0.92 g). The least value for single dry bean weight was showed by the hybrids DC VSD 3.3 and DC VSD 20.10 having 0.65 g which was on par with DC VSD 9.13 (0.66 g). However, all the double cross hybrids were superior to check with 0.45 g single dry bean weight. Pound (1932) and Enriquez and Soria (1966), based on their study on cocoa genotypes reported variability in wet and dry weight of peeled bean in the range 0.5 to 2.5 g and 0.58 to 1.72, respectively and the current study is also on consonance with their results.

According to Monteiro *et al*. (2009) and Minimol *et al*. (2015) genotypes having single peeled dry bean weight more than 0.8 g is found to be superior. The same criteria (single dry bean weight more than 0.8 g) was utilized by Sumitha *et al*. (2018) and Deepa *et al*. (2019) for identifying superior genotypes.

Production of cocoa beans can be improved by selecting cocoa genotypes based on their high dry bean weight. Thirteen hybrids under study exhibited single dry bean weight more than 0.8 gram and hybrids DC VSD 18.6 (0.99 g), DC VSD 15.2 (0.98 g), DC VSD 6.11 (0.96 g) excelled among them having single dry bean weight near to one gram.

Dry bean length and breadth showed variation in a range from 1.73 cm in DC VSD 20.10 to 2.44 cm in DC VSD 18.9 and 0.93 cm DC VSD 15.5 to 1.28 cm in DC VSD 8.10 respectively. Dry bean thickness also showed variations which ranged from 0.44 cm in hybrid DC VSD 2.1 to 0.85 cm in DC VSD 18.9.

All the hybrids excelled the check variety in their early bearing stage in almost all the quantitative characters. Except for the number of ridges and furrows, all the characters exhibited significant variation among the hybrids. All the twenty hybrids under study exhibited ten ridges and furrows each. Shilpa (2019) also observed no variation in number of ridges and furrows in the hybrids.

4.1.2.2.a. Cluster Analysis of Double Cross Hybrids Based on the Quantitative Characters

Cluster analysis of the double cross hybrids was carried out on the basis of quantitative characters and is represented in Table 12 and Figure 6. There was no much variations in the quantitative characters considered. Maximum number of hybrids (9) belonged to cluster I including DC VSD 15.2, DC VSD 20.2, DC VSD 20.10, DC VSD 3.3, DC VSD 2.4, DC VSD 12.5, DC VSD 15.5, DC VSD 8.11 and DC VSD 9.6. The total wet bean weight per pod ranged between 70 to 90 g. Double cross hybrids DC VSD 6.12 and DC VSD 7.2 together formed a separate cluster. They had almost same number of beans per pod (42.80 in DC VSD 6.12 and 43 in DC VSD 7.2) and total wet bean weight per pod was 102.10 g and 93.4 g, respectively. Cluster III comprised of three double cross hybrids namely, DC VSD 18.3, DC VSD 19.11 and DC VSD 9.13. They had almost same single peeled bean weight (1.03 g in DC VSD 19.11 and 1.04 g in DC VSD 9.13 and DC VSD 18.3) and total wet bean weight per pod, was in the range of 55 g to 70 g (58.72 g in DC VSD 9.13, 63.60 g in DC VSD 19.11 and 69.46 g in DC VSD 18.3). DC VSD 8.10, DC VSD 18.9, DC VSD 21.9, DC VSD 6.11, DC VSD 18.6 and DC VSD 2.1 belonged to cluster IV. They had total wet bean weight per pod of more than 110 g and single wet bean weight of more than 2.4 g.

Sujith *et al.* (2017) classified ten varieties of cocoa (CCRP 1-10) based on quantitative characters into five clusters and Unweighted Pair-Group Method (UPGMA) was used to create dendrogram. More members belonged to cluster I including CCRP 1, CCRP 4, CCRP 7, CCRP 8 and CCRP 10. Varieties CCRP 2 and CCRP 3 constituted cluster II. CCRP 5, CCRP 6 and CCRP 9 formed specific clusters, cluster III, cluster IV and cluster V respectively.

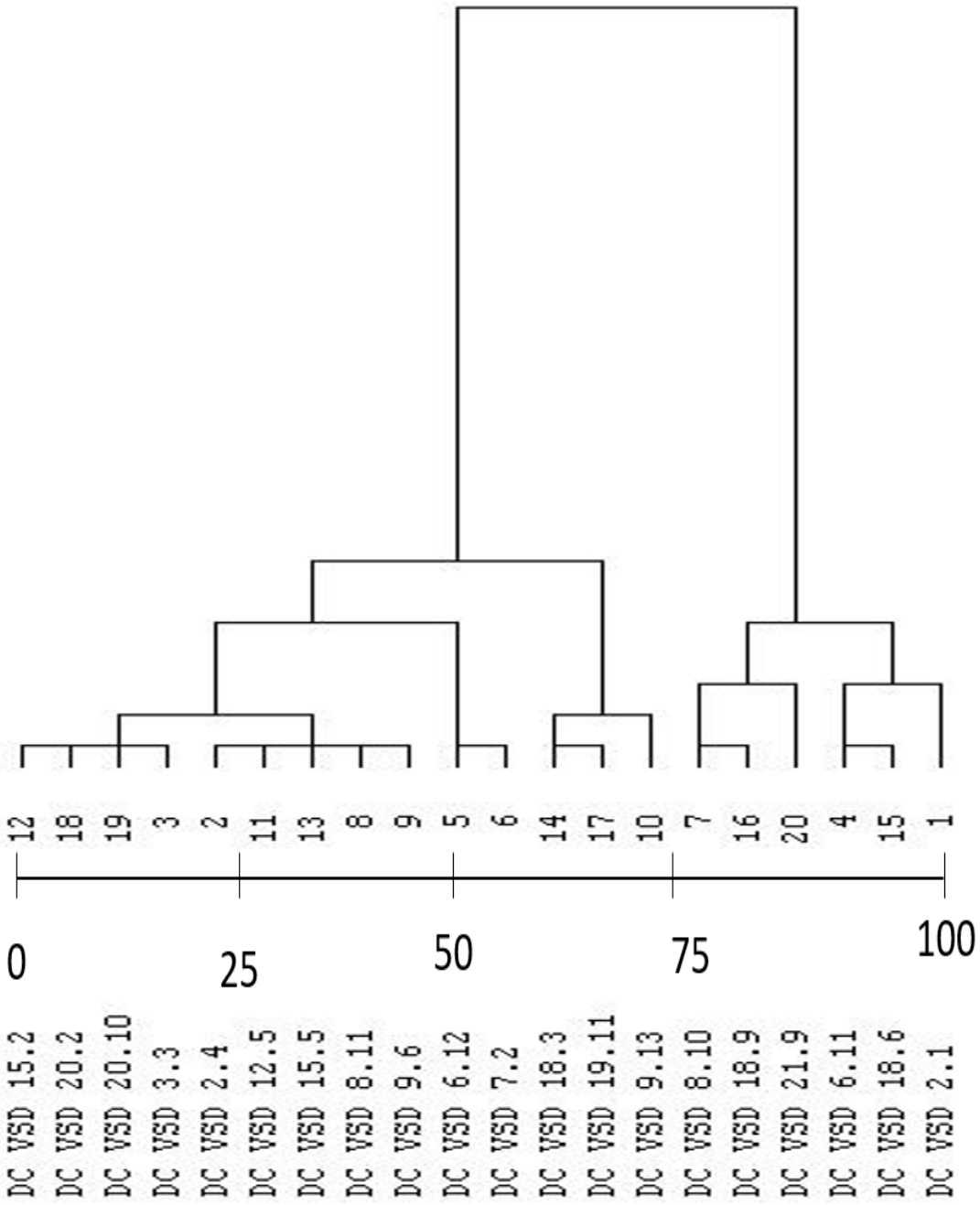


Figure 6. Dendrogram generated through the hierarchical clustering using average linkage (between groups) of double cross hybrids based on quantitative characters

Table 12. Hierarchical clustering of the twenty double cross hybrids based on quantitative characters

Cluster No.	No. of double cross hybrids	Name of double cross hybrid
I	9	DC VSD 15.2, DC VSD 20.2, DC VSD 20.10, DC VSD 3.3, DC VSD 2.4, DC VSD 12.5, DC VSD 15.5, DC VSD 8.11 and DC VSD 9.6
II	2	DC VSD 6.12 and DC VSD 7.2
III	3	DC VSD 18.3, DC VSD 19.11 and DC VSD 9.13
IV	6	DC VSD 8.10, DC VSD 18.9, DC VSD 21.9, DC VSD 6.11, DC VSD 18.6 and DC VSD 2.1

4.2. BIOCHEMICAL EVALUATION OF COCOA BEANS

Chemical composition of the cocoa beans determines the nutritional quality of the cocoa products (Adeyeye *et al*., 2010; Lettieri-Barbato *et al*, 2012). In the current study, fat and polyphenol content was estimated for the biochemical analysis. Completely Randomised Design with three replications was used to analyse the data derived through biochemical evaluation. Mean values of fat and polyphenol are given in Table 13 and represented in Figures 7 and 8.

According to Zak and Keeney (1976), fat comprises of 53 to 58 per cent of the cotyledon dry weight. In the present study, variation in fat content ranged from 42.76 per cent in hybrid DC VSD 20.2 to 61.57 per cent in hybrid DC VSD 18.3. Asna (2013) conducted a study to estimate variation in fat content in 50 cocoa accessions and recorded a variation between 40 to 65 per cent among the accessions which is

similar to the present findings.

Fat content of cocoa beans exhibited variation among genotypes (Rossini *et al*., 2011). Gu *et al*. (2013) carried out a comparative study on cocoa beans from China, Indonesia and Papua New Guinea and detected a variation in fat content which was ranged from 39.24 to 53.67 per cent for both fresh and fermented cocoa beans. A study on nutritional composition of cocoa beans from different regions of Cameroon observed a variation of fat content from 24.63 to 43.12 per cent (Caprioli *et al.,* 2016).

Fat content supplements flavour and aroma of the chocolates (Mossu, 1992). Quality improvement can be achieved through utilization of hybrids with high fat content (Monteiro *et al*., 2009). Afoakwa *et al.* (2013) developed a selection criteria based on fat content in cocoa beans *i.e.,* a genotype is regarded superior if it has a fat content of more than 45 per cent. Among the twenty hybrids studied, eighteen hybrids had more than 45 per cent fat content in cocoa beans.

Kim and Keeney (1984) reported that polyphenol contributes to the flavour and colour of chocolates. Polyphenols constitute an average of about 6-8 per cent of dry weight of cocoa beans (Grassi *et al.,* 2008). The hybrids studied exhibited a variation in polyphenol content which ranged from 4.09 per cent in hybrid DC VSD 3.3 to 9.54 per cent in hybrid DC VSD 21.9.

Many studies have been carried out in Kerala Agricultural University to identify the variation in total phenol content in the unfermented cocoa beans. Asna (2013) observed a variation from 2.25 to 9.09 per cent in fifty cocoa accessions whereas Rubeena (2015) observed a variation from 1.51 per cent to 5.64 per cent among forty hybrids in her study.

Veeresh (2017) also evaluated total polyphenol content in thirty cocoa accessions and reported a variation which ranged from 6.34 to 11.81 per cent. Shilpa (2019) also documented a variation in polyphenol content ranging from 3.71 to 9.36 per cent in hybrids under her study. Veeresh (2017) reported 4.5 per cent as the minimum level of polyphenol recommended in cocoa. All the double cross hybrids except DC VSD 3.3 exhibited polyphenol content of more than 4.5 per cent in the current study.

Table 13. Biochemical characters of cocoa beans of double cross hybrids

Hybrids	Fat (%)	Polyphenol* (%)
DC VSD 2.1	52.67	6.04 (14.22)
DC VSD 2.4	55.01	6.35 (14.59)
DC VSD 3.3	61.27	4.09 (11.66)
DC VSD 6.11	54.90	9.43 (17.89)
DC VSD 6.12	53.33	7.24 (15.61)
DC VSD 7.2	54.58	6.08 (14.28)
DC VSD 8.10	53.67	6.13 (14.33)
DC VSD 8.11	52.10	9.17 (17.62)
DC VSD 9.6	52.47	7.59 (15.99)
DC VSD 9.13	52.77	7.23 (15.59)
DC VSD 12.5	54.23	9.30 (17.75)
DC VSD 15.2	48.87	8.79 (17.23)
DC VSD 15.5	54.87	5.52 (13.58)
DC VSD 18.3	61.57	9.13 (17.59)
DC VSD 18.6	50.47	6.77 (15.08)
DC VSD 18.9	43.17	4.54 (12.30)
DC VSD 19.11	53.10	6.12 (14.32)
DC VSD 20.2	42.76	5.75 (13.87)
DC VSD 20.10	59.60	7.77 (16.18)
DC VSD 21.9	52.53	9.54 (17.99)
CCRP 15	43.03	6.08 (14.27)
CV (%)	**2.90**	**4.77**
CD (5%)	**3.37**	**0.74**

* Values in parenthesis are transformed values by using angular transformation

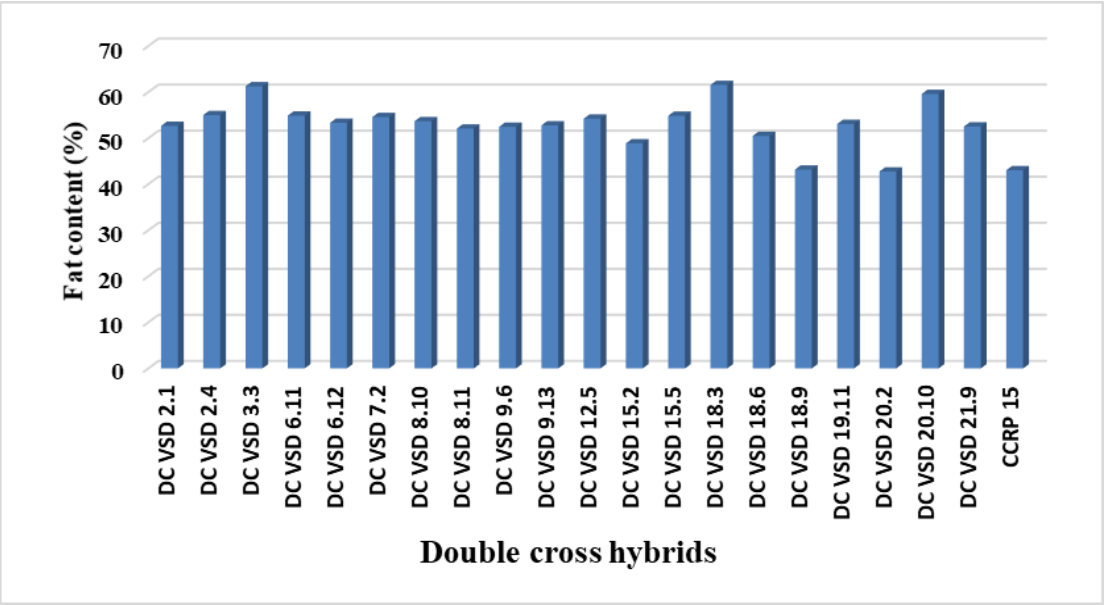


Figure 7. Variation in fat content among the double cross hybrids

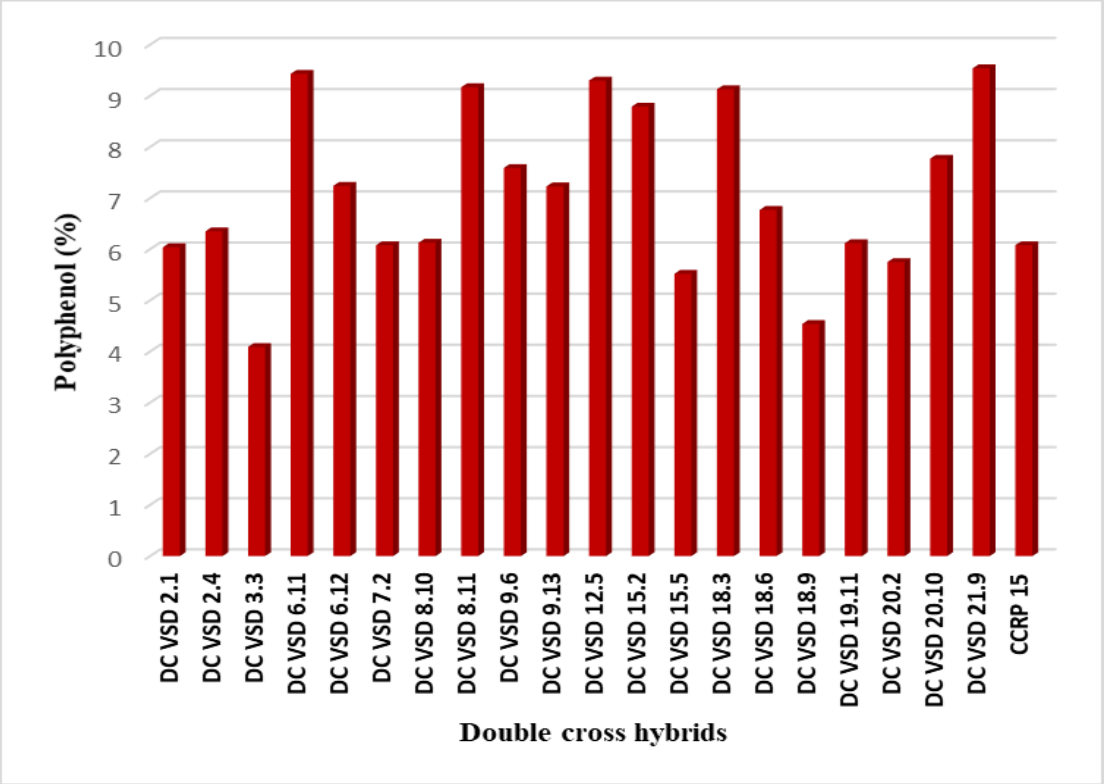


Figure 8. Variation in polyphenol content among the double cross hybrids

4.3. Evaluation of Economic Characters of Cocoa Double Cross Hybrids

Plant breeding aims at development of plants that are stable, high yielding and resistant to biotic and abiotic stresses (Djocgoue *et al*., 2006). Farmers prefer varieties based on their economic performance. Thus, plant breeder should develop varieties with high economic value coupled with added advantage of resistance to biotic and abiotic stresses as well as high quality. However, it's a strenuous job to the plant breeders.

The remunerative part in cocoa is its pod specifically its dried beans. Nine economic characters are generally used to understand the productivity of a genotype *i.e.* quantity of pod and bean yield. The economic characters comprise the number of pods harvested from a genotype in a year (pod yield), total wet bean weight obtained per plant, total dry bean weight obtained per plant and other six derived parameters like pod value (g), pod index, efficiency index, conversion index, dry matter recovery (%) and peeling ratio (%).

Mean value of economic characters calculated for each hybrid is given in Tables 14.a. and 14.b. Pod yield represents the number of pods obtained from 2019 to 2020. Pod yield ranged from 15 pods per tree per year in hybrids DC VSD 2.4, DC VSD 15.2, DC VSD 19.11, DC VSD 20.2, DC VSD 20.10 to 30 pods per tree per year in hybrid DC VSD 2.1. The pod yield obtained cannot be compared with that of the genotypes in the steady bearing stage since the double cross hybrids are in their early bearing stage (two and half years age). However, the variation ranged between 15 to 30 pods per year comparing to CCRP 15 having same age with 15 pods per year. CCRP 15 in their steady bearing stage produce about 80 pods per year thus the double cross hybrids showing higher pod yield in the early bearing stage are expected to be high pod producers when they reach their steady bearing stage. An average pod yield of about 20.4 pods per tree per year was observed in the hybrids under study. In Tamil Nadu, seven cocoa varieties were evaluated at the early stage (planted in 2010) for their yield and performance and reported an average pod yield of 25.36 pods/ tree/ year (Sumitha *et al.*, 2018).

The concept of pod value was developed by Toxopeus and Jacob (1970) as an economic parameter to indicate the average dry bean weight obtained from a pod. Average pod value of 35.27 g was exhibited by the twenty hybrids under study. Pod value ranged from 23.64 g in hybrid DC VSD 9.13 to 44.43g in hybrid DC VSD 8.10.

The results obtained were comparable to the findings of the previous researchers. In Tamil Nadu, a study was conducted to distinguish high yielding cocoa trees and reported variability in pod value ranging from 15.29 to 52.88 g (Velayutham *et al*., 2013).

The term pod index was initially used by Morera *et al*. (1991) to understand the number of pods needed to obtain one kilogram beans. Lower the pod index, higher the superiority of the genotype. Pod index varied from 22.58 (hybrid DC VSD 8.10) to 42.67 (hybrid DC VSD 9.13). Hybrids having a pod index equal to or less than 15 is suitable for breeding. However, none of the hybrids satisfied this criterion since the hybrids have not attained their steady bearing stage. Asna (2013) observed pod index ranging from 12 to 49 in exotic accessions of cocoa.

Jacob and Atanda (1971) put forward the concept of efficiency index in cocoa. It is the quantity of pod weight required to obtain one gram of dried beans. Lower the efficiency index, higher the superiority of the genotype. Efficiency index varied from 7.83 in hybrid DC VSD 6.11 to 16.15 in hybrid DC VSD 18.6. Shilpa (2019) documented efficiency index ranging from 8.29 to 16.18 in hybrids.

The quantity of dry beans derived from a specified quantity of wet bean is termed as conversion index (Francies, 1998). Higher the conversion index, superior the genotype. Conversion index ranged from 0.30 in DC VSD 18.9 to 0.47 in hybrid DC VSD 18.3.

The term dry matter recovery refers to the amount of dry bean obtained from a specific amount of wet bean weight when it is dried (Francies, 1998). Dry matter recovery is denoted as per cent. Dry matter recovery varied from 76.62 per cent in hybrid DC VSD 19.11 to 63.63 per cent in hybrid DC VSD 9.13. Higher the moisture content, lower the dry matter recovery (Rubeena, 2015).

Table 14.a. Mean values of economic characters of cocoa double cross hybrids

Hybrids	Pod yield*	Pod value (g)	Pod index	Efficiency index	Conversion Index	Peeling ratio (%)	Dry matter recovery (%)
DC VSD 2.1	30	43.08	23.28	9.05	0.34	49.64	67.29
DC VSD 2.4	15	31.96	31.74	9.27	0.37	50.53	73.15
DC VSD 3.3	20	32.78	30.56	8.08	0.43	53.76	67.09
DC VSD 6.11	27	42.14	23.75	7.83	0.34	52.19	74.06
DC VSD 6.12	23	34.52	29.08	10.41	0.34	49.19	68.09
DC VSD 7.2	25	40.16	24.96	8.03	0.43	48.56	76.53
DC VSD 8.10	16	44.43	22.58	11.76	0.37	50.45	71.08
DC VSD 8.11	23	35.12	28.51	10.89	0.42	53.66	76.33
DC VSD 9.6	18	30.63	32.79	10.84	0.35	55.54	66.88
DC VSD 9.13	25	23.64	42.67	8.33	0.40	51.21	63.63
DC VSD 12.5	17	37.16	26.95	9.47	0.43	47.53	73.21
DC VSD 15.2	15	40.56	24.90	8.40	0.45	52.34	73.31
DC VSD 15.5	18	36.67	27.88	9.02	0.43	47.77	71.40
DC VSD 18.3	24	31.93	31.33	10.48	0.47	47.76	72.18
DC VSD 18.6	23	40.66	24.67	16.15	0.31	51.40	72.17

Table 14.a. continued.

Hybrids	Pod yield*	Pod value (g)	Pod index	Efficiency index	Conversion Index	Peeling ratio (%)	Dry matter recovery (%)
DC VSD 18.9	17	34.50	29.02	12.73	0.30	48.27	71.08
DC VSD 19.11	15	33.65	29.78	7.84	0.44	46.24	76.62
DC VSD 20.2	15	33.99	29.47	10.73	0.45	50.18	72.75
DC VSD 20.10	15	30.38	33.27	9.39	0.40	46.66	73.52
DC VSD 21.9	27	44.36	22.86	10.02	0.39	50.08	75.44
CCRP 15	15	18.39	54.54	7.98	0.33	41.42	69.53
CV (%)	**-**	**8.31**	**8.4**	**8.06**	**8.67**	**7.37**	**5.83**
CD (5%)	**-**	**3.69**	**3.14**	**0.99**	**0.04**	**4.62**	**5.26**

*** Yield is expressed as no. of pods/tree/ year**

Table 14.b. Mean values of economic characters of cocoa double cross hybrids

Hybrids	Total wet bean weight per tree (kg)	Total dry bean weight per tree (kg)
DC VSD 2.1	3.83	1.29
DC VSD 2.4	1.30	0.48
DC VSD 3.3	1.54	0.66
DC VSD 6.11	3.37	1.14
DC VSD 6.12	2.35	0.79
DC VSD 7.2	2.33	1.00
DC VSD 8.10	1.94	0.71
DC VSD 8.11	1.94	0.81
DC VSD 9.6	1.59	0.55
DC VSD 9.13	1.47	0.59
DC VSD 12.5	1.46	0.63
DC VSD 15.2	1.36	0.61
DC VSD 15.5	1.53	0.66
DC VSD 18.3	1.67	0.76
DC VSD 18.6	3.04	0.93
DC VSD 18.9	1.96	0.59
DC VSD 19.11	1.14	0.50
DC VSD 20.2	1.12	0.51
DC VSD 20.10	1.14	0.46
DC VSD 21.9	3.09	1.20
CCRP 15	0.83	0.28
CV (%)	**4.25**	**8.37**
CD (5%)	**1.02**	**0.76**

Total wet bean weight per tree and total dry bean weight per tree were also estimated. Total wet bean weight per tree varied from 3.83 kg in hybrid DC VSD 2.1 to 1.12 in hybrid DC VSD 20.2. Total dry bean weight per tree varied from 1.29 kg in hybrid DC VSD 2.1 to 0.46 kg in hybrid DC VSD 20.10.

4.4. VARIABILITY ANALYSIS OF THE ECONOMIC CHARACTERS OF COCOA HYBRIDS

The success of any plant breeding programme in cocoa requires an understanding of genetic parameters (*i.e*, phenotypic coefficient of variation, genotypic coefficient of variation, heritability and genetic advance) present in the hybrids for the characters under improvement (Minimol *et al.*, 2014). Gain under selection can be predicted using heritability estimates along with genetic advance.

The proportion of variation in a phenotype that is caused by genetic variation is termed as heritability. Heritability estimate help to choose the selection method, create selection index and determine the minimum population required to carry out selection effectively and perceive response of various traits to selection (Theagricos, 2008).

Eight economic characters were analysed to determine the genetic parameters including phenotypic coefficient of variation, genotypic coefficient of variation, heritability, genetic advance and genetic gain. The derived results are depicted in Table 15.

According to Sivasubramanian and Madhavamenon (1973) phenotypic coefficient of variation and genotypic coefficient of variation is grouped into three classes; low (0-10 %), moderate (10.1-20 %) and high (>20 %). Three economic characters under study showed a high phenotypic coefficient of variation and genotypic coefficient of variation. They include wet bean weight per tree (43.14 % and 42.91 %), dry bean weight per tree (37.27 % and 36.22 %) and pod index (25.76 % and 24.35 %). High phenotypic coefficient of variation and moderate genotypic coefficient of variation was exhibited by pod value (20.03% and 18.22%) and efficiency index (21.53 % and 19.97 %).

Conversion index (14.95 and 12.56 %) expressed moderate phenotypic and genotypic coefficient of variation. Low phenotypic and genotypic coefficient of variation was exhibited by peeling ratio (9.07 and 5.28 %) and dry matter recovery (7.16 and 4.16 %). All the economic characters showed less difference between phenotypic coefficient of variation and genotypic coefficient of variation indicating that there is less influence of the environment (Johnson *et al*., 1955) and the variation exhibited is mostly contributed by the genotype of the double cross hybrids.

The ratio of genotypic variance to phenotypic variance expressed in percentage is termed as heritability. According to Robinson *et al*. (1949), heritability is classified into low (0-30 %), moderate (30.1-60 %) and high (> 60.1 %). Except for peeling ratio and dry matter recovery, the rest of the all economic characters under study exhibited high heritability (70.59 - 98.96 %). Wet bean weight per tree (98.96 %) and dry bean weight per tree (94.45 %) showed highest values of more than 90 percent. Peeling ratio (33.92%) and dry matter recovery (33.77 %) exhibited moderate heritability. High heritability revealed the least influence of environment on these traits and they are solely governed by genetic constitution of the hybrids (Soria, 1975).

Based on the scale suggested by Johnson *et al*. (1955), genetic gain was classified into low (0-10 %), moderate (10.1-20 %) and high (>20 %). The magnitude of progress to be made from selection in subsequent generation can be predicted based on the expected genetic gain estimated. Heritability and genetic gain estimated together considered are more effective in predicting the resultant effect of selection than heritability alone (Johnson *et al*., 1955). High genetic gain was expressed by wet bean weight per tree (87.94 %), dry bean weight per tree (72.51 %), pod index (47.42 %), efficiency index (38.14 %), pod value (34.16 %) and conversion index (21.74 %). Peeling ratio (6.34 %) and dry matter recovery (4.99 %) showed low genetic gain. High heritability along with high genetic advance revealed that heritability of these traits was due to additive gene action and selection of genotypes based on these characters can be effective (Johnson *et al*., 1955; Kashif *et al*., 2003).

Wet bean weight per tree, dry bean weight per tree, pod index, efficiency index, pod value and conversion index showed high heritability and high genetic gain indicating that they are controlled by additive genes and selection based on these characters can be effective.

Table 15. Variability analysis of economic characters of double cross cocoa hybrids

Character	**PCV (%)**	**GCV (%)**	**H^2 (%)**	**Genetic gain (%)**
Pod value (g)	20.03	18.22	82.79	34.16
Pod index	25.76	24.35	89.37	47.42
Efficiency index	21.53	19.97	85.99	38.14
Conversion index	14.95	12.56	70.59	21.74
Wet bean weight per tree (kg)	43.14	42.91	98.96	87.94
Dry bean weight per plant (kg)	37.27	36.22	94.45	72.51
Peeling ratio	9.07	5.28	33.92	6.34
Dry matter recovery (%)	7.16	4.16	33.77	4.99

4.5. SCREENING FOR VSD DISEASE RESISTANCE

All the twenty double cross hybrids under study were screened for VSD disease resistance in field as well as net house condition.

4.5.1. Field Screening for VSD Resistance in Cocoa

Screening of the double cross hybrids was carried out at monthly intervals. During the peak period of disease incidence (September to November) daily observations were recorded. Disease intensity was scored based on the score chart developed by Abraham *et al*. (2000). Observations and scores given to the hybrids are represented in Table 16. and Plates 11.a. and 11.b

Kamil *et al.* (2004) screened the clones and hybrids in Malayasia for VSD resistance. Scoring was done based on the disease severity of VSD in the field condition. Score chart comprised of 0-6 scale, the unaffected plants were given a score of 0 and the hybrid which show dieback or complete drying symptom was given a score of 6. Marfu *et al.* (2004) also screened the clones and hybrids in Papua New Guinea for VSD resistance. Individual plants were scored based on a 1-6 rating scale in which the VSD unaffected ones was given a score of 1 and completely lost plant due to VSD was given a score of 6.

Tulshiram (2016) followed the score chart given by Abraham *et al.* (2000) for field screening hybrids under natural conditions. A 0-9 scale was used based on the percentage of twigs infected in a tree. The present study also followed scoring based on the percentage of twigs infected in a tree on a 0-9 scale.

All the double cross hybrids exhibited considerable resistance to vascular streak dieback disease under field condition. The hybrids were exposed to various biotic and abiotic factors that favoured disease incidence in them. However, the plants were able to retain themselves even after infection without any management practices revealing the genetic resistance within in the hybrids.

Based on the field screening, the twenty double cross hybrids under study were classified as resistant hybrids (11) and partially resistant hybrids (9). The eleven double cross hybrids with no visual symptom of VSD infection includesDC VSD2.4, DC VSD6.11, DC VSD 7.2, DC VSD 8.10, DC VSD 8.11, DC VSD 12.5, DC VSD 18.3, DC VSD 19.11, DC VSD 20.2, DC VSD 20.10, DC VSD 21.9. Even though rest of the hybrids exhibited symptoms in some of the twigs, they reclaimed themselves indicating genetic resistance present within them and they were classified as partially resistant hybrids. Since VSD is controlled by polygenes (Tan and Tan, 1988; Keane, 1992; Samuels *et al*., 2012), lack of any one or few major genes will contribute to partial expression of disease.

Table 16. Scoring of double cross hybrids based on disease intensity

Hybrids	Total no. of twigs	No. of twigs affected	Percentage of twigs affected (%)	Scoring	Reaction to disease
DC VSD 2.1	5	2	40	3	Partial resistant
DC VSD 2.4	3	-	-	0	Resistant
DC VSD 3.3	4	1	25	1	Partial resistant
DC VSD 6.11	4	-	-	0	Resistant
DC VSD 6.12	4	1	25	1	Partial resistant
DC VSD 7.2	4	-	-	0	Resistant
DC VSD 8.10	5	-	-	0	Resistant
DC VSD 8.11	4	-	-	0	Resistant
DC VSD 9.6	5	3	60	5	Partial resistant
DC VSD 9.13	4	1	25	1	Partial resistant
DC VSD 12.5	3	-	-	0	Resistant
DC VSD 15.2	3	1	33.3	3	Partial resistant
DC VSD 15.5	4	1	25	1	Partial resistant
DC VSD 18.3	4	-	-	0	Resistant
DC VSD 18.6	5	2	40	3	Partial resistant
DC VSD 18.9	1	5	20	1	Partial resistant
DC VSD 19.11	4	-	-	0	Resistant
DC VSD 20.2	5	-	-	0	Resistant
DC VSD 20.10	5	-	-	0	Resistant
DC VSD 21.9	5	-	-	0	Resistant

.

DC VSD 15.2 **DC VSD 3.3**

DC VSD 6.12 **DC VSD 18.9**

Plate 11.a. Double cross hybrids expressed VSD symptoms in field condition

DC VSD 15.5

DC VSD 18.6

DC VSD 9.6

DC VSD 9.13

Plate 11.b. Double cross hybrids expressed VSD symptoms in field conditions

Partially resistant hybrids include hybrid DC VSD 2.1, DC VSD 3.3, DC VSD 6.12, DC VSD 9.13, DC VSD 9.6, DC VSD 15.2, DC VSD 15.5, DC VSD 18.6 and DC VSD 18.9.

4.5.2. Net House Screening for VSD Resistance

Budding was done for all the twenty double cross hybrids. They were kept for screening for VSD resistance along with the supply of natural inoculum through VSD infected seedlings for a period of six months (September – February). The budded plants were kept in a 5 x 5 arrangement with two rows of VSD affected seedling as border. Moisture and water splash were ensured through an overhead sprinkler system. However, none of the budded plants expressed any visual symptoms of VSD infection. This ensures that the genetic resistance present within the hybrids provided resistance to VSD infection. Absence of biotic and abiotic factors that favored the disease occurrence in the field condition may also have contributed to the absence of VSD infection under net house condition. The arrangement of budded plants (5x5) along with the VSD affected seedlings in the net house is shown in Plate 12.

4.6. MOLECULAR ANALYSIS

4.6.1. DNA Isolation

Modified Dellaporta method was used for isolation of good quality DNA. The procedure followed in the study is detailed in Materials and Methods section (3.6.1.3.b). Gel profile of good quality DNA thus obtained through modifications in the extraction method is presented in Plate 13.

Mucilage content in the Malvaceae leaf samples are very high (Bayer *et al*., 1999) and transfer of the middle aqueous layer without contamination of the mucilaginous supernatant after centrifugation posed a great hindrance. Intervention of the DNA extractions and PCR amplification due to the high amount of polysaccharides, polyphenols, tannins and other secondary metabolites in the leaf samples in cocoa have been reported by many scientists (Echevarría-Machado *et al*., 2005; Chandrakant, 2014). The viscous nature of polyphenols interferes in DNA isolation and also obstruct Taq polymerase activity during PCR amplification (Pinto

et al., 2007).

Conventional extraction procedures cannot purify DNA from contamination of these compounds. Scientists have reported many extraction methods for isolation of quality DNA from cocoa with high mucilage and polyphenol including modifications in CTAB-based protocols (Mace *et al.*, 2003; Chandrakant, 2014; Sujith, 2016; Tulshiram, 2016) as well as use of specific kits (Saunders *et al*., 2004).

A protocol was developed by Jose and Usha (2000) to extract quality DNA from okra (*Abelmoscus esculentus*), a crop that belongs to Malvaceae family. The protocol developed was an integration of Dellaporta *et al*. (1983), the CTAB method (Doyle and Doyle, 1990), and alkali lysis.

4.6.2. Quantification of DNA

Quantity and quality of the isolated DNA were estimated through spectrophotometer analysis using nanodrop ND-1000®. The ratio of optical density at 260 and 280nm indicated the quality of DNA. $A_{260/A280}$ value ranged from 1.84 to 2.0 in the samples under study. Absorbance ratio ($A_{260/A280}$) 1.8 to 2.0 is considered as good quality DNA (Thakur *et al*., 2014; Sujith, 2016). The quality and quantity of the isolated DNA from the twenty double cross hybrids are listed in Table 17.

The isolated DNA was diluted to 30 ng/µL to avoid the interference of polyphenols in the PCR reactions (Sujith, 2016). Both ISSR and SSR analysis was carried out using same concentration (30 ng/ µL).

4.7. MOLECULAR MARKER ANALYSIS

Amplification of primer specific genomic DNA takes place in PCR based molecular marker systems. Present study was based on three ISSR markers namely UBC811, UBC826, UBC857 and an SSR marker mTcCIR42 which were reported (Chandrakant, 2014) and validated (Tulshiram, 2016) to be linked with VSD resistance. Genomic DNA of all the twenty double cross hybrids were amplified with the previously standardized protocols (Chandrakant, 2014), which is detailed under.

Plate 12.a. Budded double cross hybrids in net house under screening

Plate 12.b. 5x5 arrangement of the budded plants along with two rows of VSD affected seedlings as border row supplying natural inoculum

Plate 12. Net house screening for VSD resistance

Materials and Methods (*3.6.3.1.a.* and *3.6.3.1.b.*), and analysed. Number and nature of amplicons is enlisted in Tables 18 and 19.

VSD resistance is a polygenic trait *ie*. multiple genes are offering resistance against VSD (Tan and Tan, 1988; Vossen, 1997). Each primer used in the study is linked to a specific gene that confers resistance to VSD. Higher the number of polymorphic band linked to the specific primers in a genotype, higher the resistance offered by it since VSD resistance exhibit additive gene effect.

4.7.1. Inter Simple Sequence Repeats (ISSR) Analysis

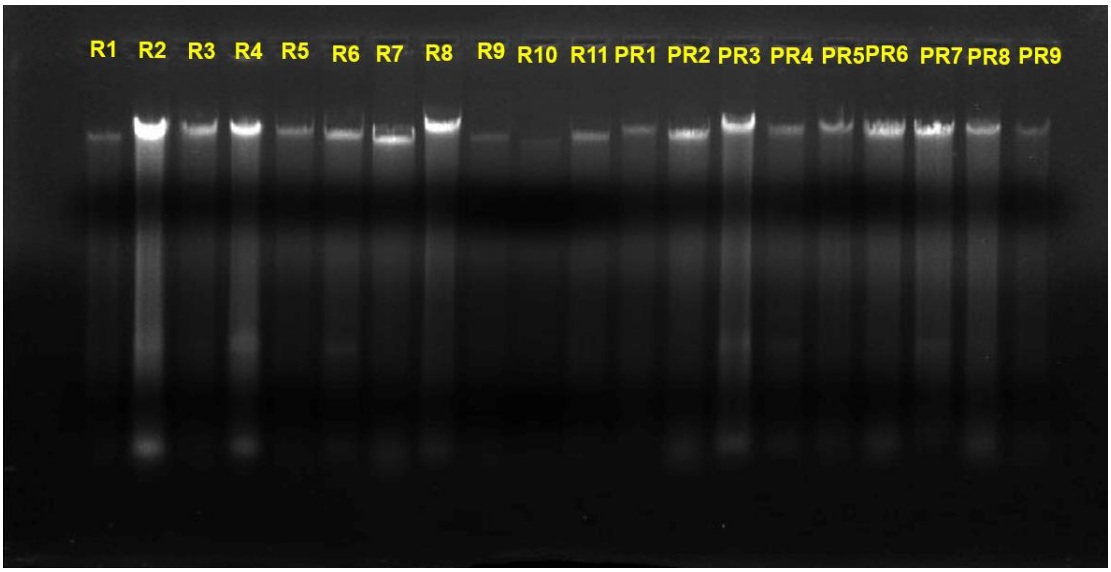
ISSR marker system involves single PCR primer to amplify DNA sequences demarcated by two inverted simple sequence repeats composed of the same units. To avoid unspecific hybridization to the microsatellite region and to reduce the slippage, the primer is constituted by few SSR units with an anchored end (Zietkiewicz *et al*., 1994; Blair *et al.,* 1999; Weising *et al.,* 2005). Reproducible, abundant and polymorphic multilocus patterns are generated by the PCR based ISSR markers in plant genomes (Bornet and Branchard, 2004). ISSR markers are greatly adapted in Marker Assisted Selection (MAS) due to its universality and ease of development (Agostini *et al*., 2008; Jabbarzadeh *et al*., 2010) along with high reproducibility and low cost of technique (Weising *et al.,* 2005; Li *et al.,* 2010).

Three ISSR primers (UBC811, UBC826, UBC857) which were recognised to be linked with VSD resistance from the earlier studies (Chandrakant *et al.*, 2020) was used for the molecular analysis of the genomic DNA isolated from the double cross hybrids. PCR conditions required for the primers are detailed under Materials and Methods (*3.6.3.1.a.*). Agarose gel (1.8 %) was used to run the PCR products. The details of the ISSR primers analysed in this study are furnished in Table 5

.

Table 17. Quantity and quality of DNA isolated from double cross hybrids

Double cross hybrids	**Concentration of DNA (ng/ µl)**	**$A_{260/280}$**	**Quality**
DC VSD 2.1	71.65	1.98	Good
DC VSD 2.4	321.4	2.00	Good
DC VSD 3.3	94.75	1.97	Good
DC VSD 6.11	204.32	1.99	Good
DC VSD 6.12	141.10	1.99	Good
DC VSD 7.2	65.1	1.97	Good
DC VSD 8.10	167.90	1.95	Good
DC VSD 8.11	187.9	1.93	Good
DC VSD 9.6	62	1.97	Good
DC VSD 9.13	31.85	1.84	Good
DC VSD 12.5	76.25	1.97	Good
DC VSD 15.2	115.3	1.98	Good
DC VSD 15.5	150.45	1.90	Good
DC VSD 18.3	87.5	1.99	Good
DC VSD 18.6	107.15	1.99	Good
DC VSD 18.9	97.5	1.88	Good
DC VSD 19.11	241.35	1.98	Good
DC VSD 20.2	123	1.99	Good
DC VSD 20.10	117.65	1.92	Good
DC VSD 21.9	66.4	1.95	Good



R1: DC VSD 2.4; R2: DC VSD 6.11; R3: DC VSD 7.2; R4: DC VSD 8.10; R5: DC VSD 8.11; R6: DC VSD 12.5; R7: DC VSD 18.3; R8: DC VSD 19.11; R9: DC VSD 20.2; R10: DC VSD 20.10; R11: DC VSD 21.9

PR1: DC VSD 2.1; PR2: DC VSD 3.3; PR3: DC VSD 6.12; PR4: DC VSD 9.6; PR5: DC VSD 9.13; PR6: DC VSD 15.2, PR7: DC VSD 15.5; PR8: DC VSD 18.6; PR9: DC VSD 18.9

Plate 13. Gel profile of the DNA isolated from the double cross hybrids

4.7.1.1. Expression of ISSR Marker UBC811 in the Double Cross Hybrids

Genomic DNA of both completely resistant hybrids, as well as partial resistant hybrids, were screened using UBC811. Earlier studies have reported a distinct polymorphic band of 950 bp in VSD resistant plants (Chandrakant, 2014; Tulshiram, 2016). All the double cross hybrids under study showed the characteristic polymorphic band. On an average, six amplicons were produced by UBC811 in the double cross hybrids. Gel profile obtained is represented in Plates 14 and 15.

Tulshiram (2016) validated the efficiency of UBC811 in four accessions each of highly resistant, highly susceptible and partially resistant categories. A distinct polymorphic band of 950 bp size was produced in all the resistant and three partially resistant clones however it was absent in susceptible ones thus confirmed that UBC811 is linked to the VSD resistant gene.

The presence of polymorphic band at 950 bp specific to VSD resistance in all the double cross hybrids (resistant and partially resistant) confirm the presence of a specific gene in all hybrids among the multiple genes that contribute to VSD resistance.

4.7.1.2. Expression of ISSR Marker UBC826 in the Double Cross Hybrids

Genomic DNA of all the twenty double cross hybrids was screened using UBC826. A distinct polymorphic band of 650 bp size was reported to be exhibited by UBC826 in resistant plants (Chandrakant, 2014; Tulshiram, 2016).

On an average, eight amplicons were produced by UBC826 in the double cross hybrids. Among the eleven double cross hybrids observed to be resistant under field condition, seven exhibited the characteristic polymorphic band. They include DC VSD 2.4, DC VSD 6.11, DC VSD 12.5, DC VSD 18.3, DC VSD 19.11, DC VSD 20.10 and DC VSD 21.9. Absence of the specific band indicated the absence of only the specific gene that is linked to UBC826. VSD resistance, being a polygenic character will be contributed by many other genes in these resistant hybrids that aid them resistance to VSD. Only hybrid DC VSD 15.2 and hybrid DC VSD 18.6 among

the nine partially resistant hybrids showed distinct polymorphic band of 650 bp size. The gene corresponding to UBC826 may have provided partial resistance in these hybrids. Gel profile obtained is represented in Plates 16 and 17.

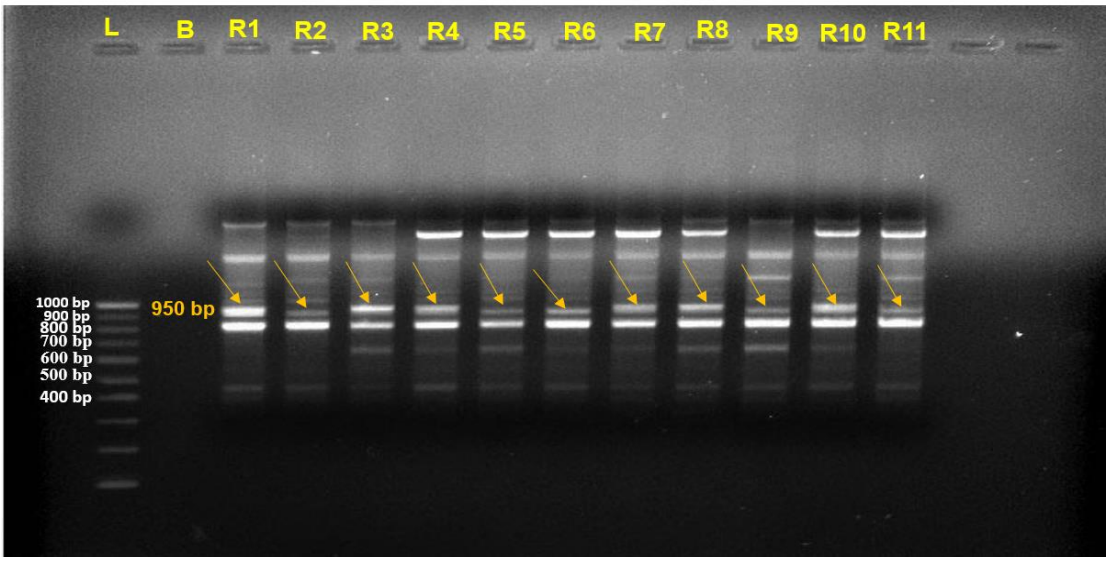
Tulshiram (2016) verified the efficiency of UBC826 and reported the presence of distinct polymorphic band of 650 bp size in resistant clone alone whereas it was absent in partially resistant and susceptible clones

4.7.1.3. Expression of ISSR Marker UBC857 in the Double Cross Hybrids

Molecular analysis was carried out using UBC857 in the genomic DNA of all the twenty double cross hybrids under study. On average, seven amplicons were produced by UBC857 in the double cross hybrids. Previous studies have reported a very distinctly polymorphic band at 450 bp produced by UBC857 that is linked to a specific gene conferring resistance to VSD (Chandrakant, 2014; Tulshiram, 2016).

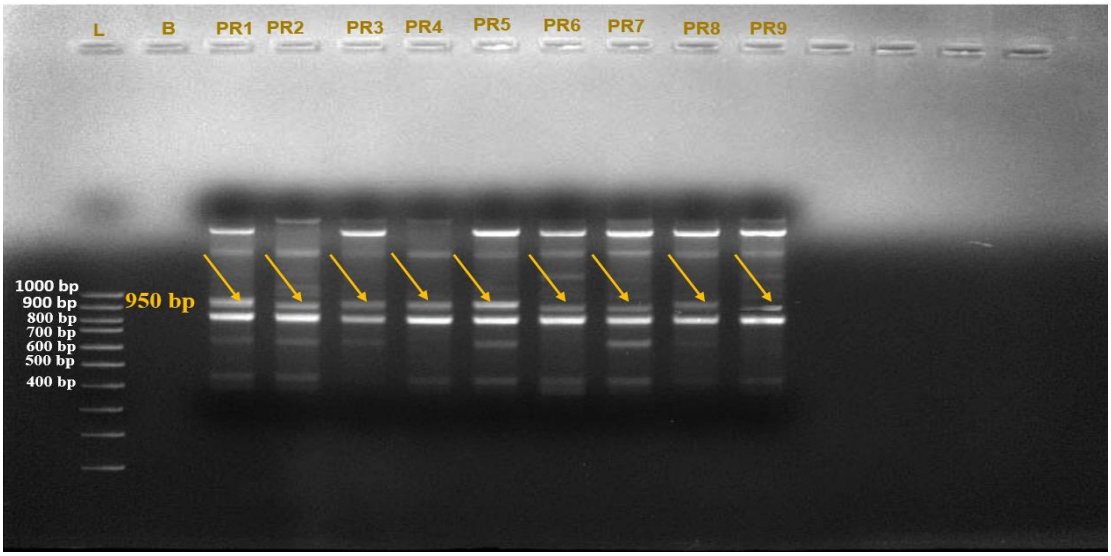
Among the eleven double cross hybrids that expressed complete resistance against VSD under field condition, seven showed the distinct polymorphic band at 450 bp when amplified with UBC857. Among the nine partially resistant double cross hybrids four exhibited the characteristic polymorphic band of 450 bp size. Gel profile obtained is given in Plates 18 and 19.

Tulshiram (2016) validated the efficiency of UBC857 and observed a very distinctly polymorphic band at 450 bp in all the resistant clones and three partially resistant clones whereas it was absent in all the susceptible clones. Thus, confirmed it to be linked with a gene that provides resistance to VSD.



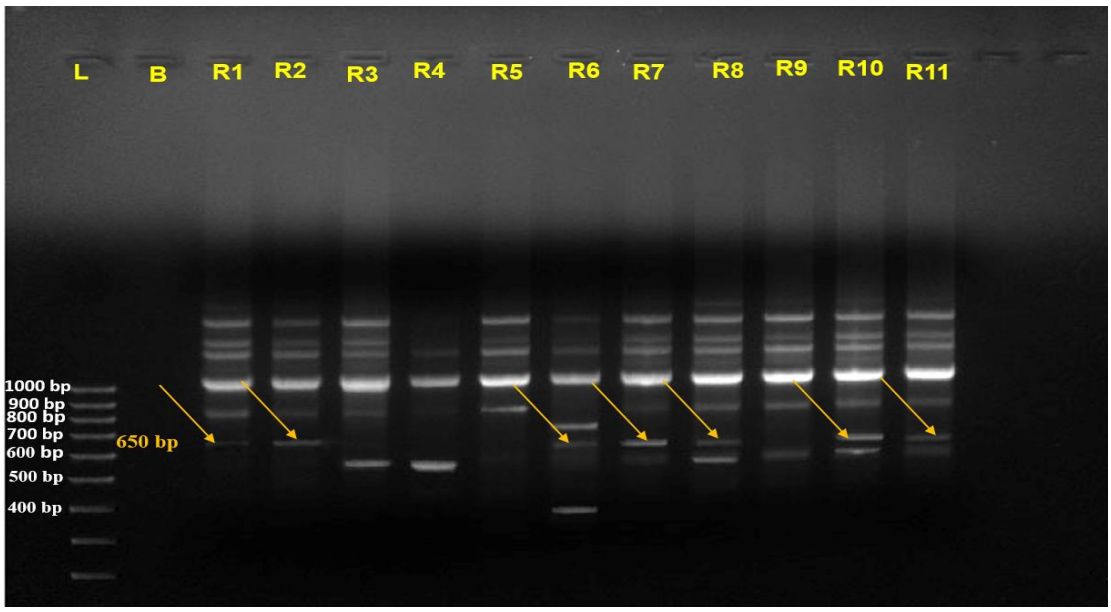
L: 1 kb ladder; B: Blank; R1: DC VSD 2.4; R2: DC VSD 6.11; R3: DC VSD 7.2; R4: DC VSD 8.10; R5: DC VSD 8.11; R6: DC VSD 12.5; R7: DC VSD 18.3; R8: DC VSD 19.11; R9: DC VSD 20.2; R10: DC VSD 20.10; R11: DC VSD 21.9

Plate 14. Amplification pattern generated with ISSR primer UBC811 in the double cross hybrids resistant under field screening for VSD



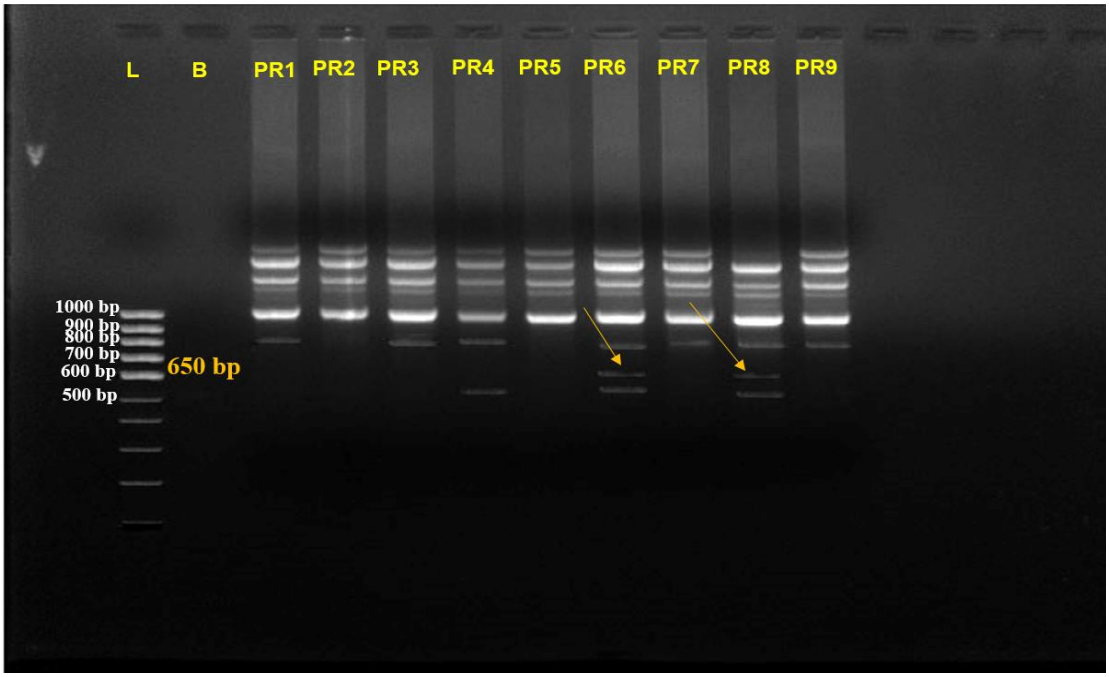
L: 1 kb ladder; B: Blank; PR1: DC VSD 2.1; PR2: DC VSD3.3; PR3: DC VSD 6.12; PR4: DC VSD 9.6; PR5: DC VSD 9.13; PR6: DC VSD 15.2, PR7: DC VSD 15.5; PR8: DC VSD 18.6; PR9: DC VSD 18.9.

Plate 15. Amplification pattern generated with ISSR primer UBC811 in the double hybrids partially resistant under field screening for VSD



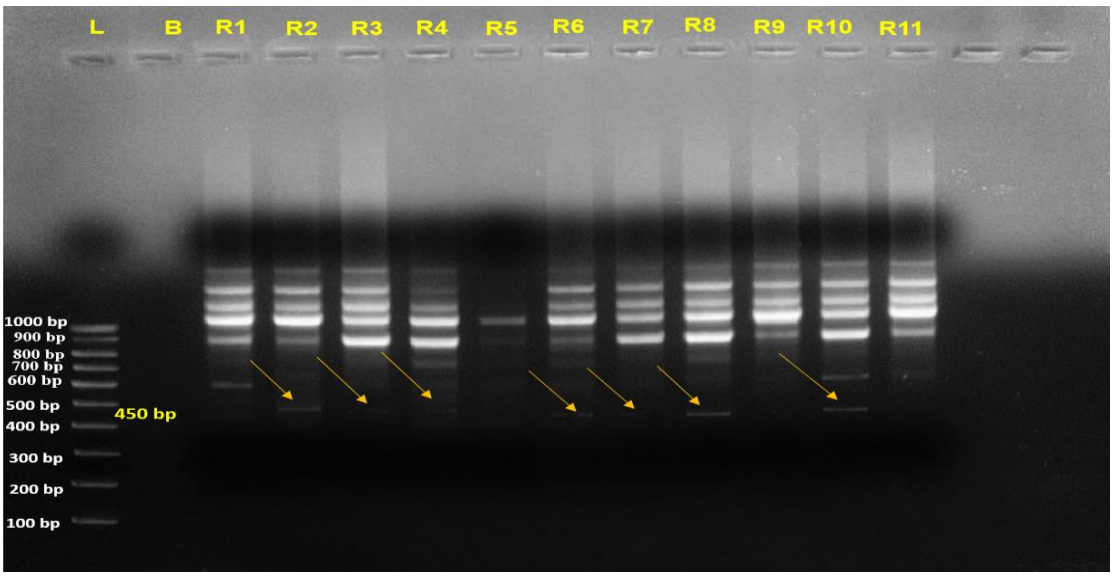
L: 1 kb ladder; B: Blank; R1: DC VSD 2.4; R2: DC VSD 6.11; R3: DC VSD 7.2; R4: DC VSD 8.10; R5: DC VSD 8.11; R6: DC VSD 12.5; R7: DC VSD 18.3; R8: DC VSD 19.11; R9: DC VSD 20.2; R10: DC VSD 20.10; R11: DC VSD 21.9

Plate 16. Amplification pattern generated with ISSR primer UBC826 in the double cross hybrids observed resistant under field screening for VSD



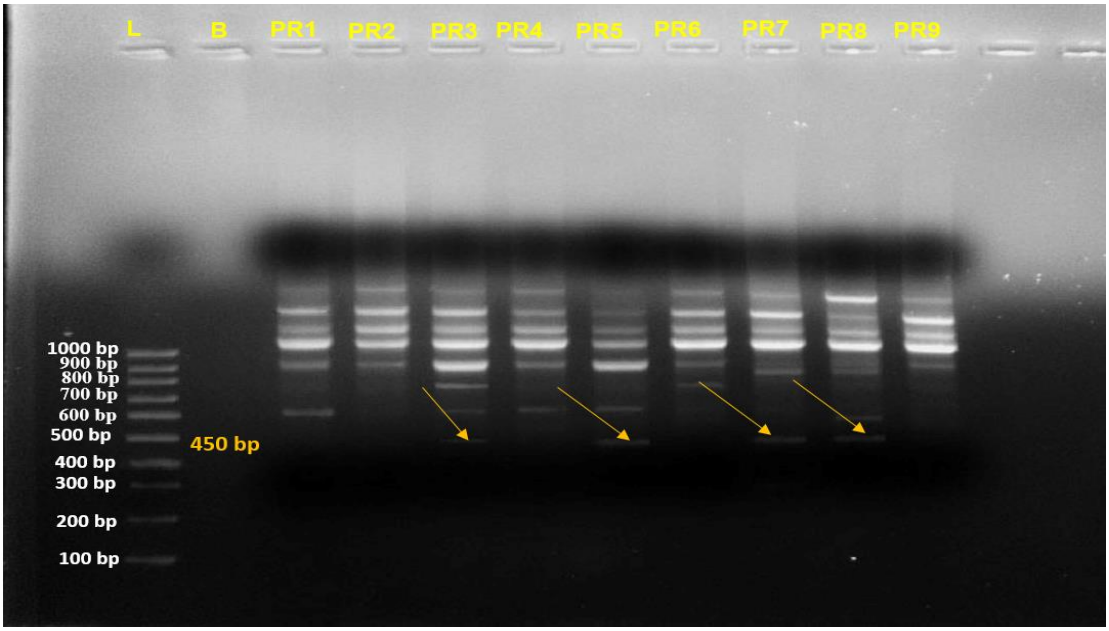
L: 1 kb ladder; B: Blank; PR1: DC VSD 2.1; PR2: DC VSD3.3; PR3: DC VSD 6.12; PR4: DC VSD 9.6; PR5: DC VSD 9.13; PR6: DC VSD 15.2, PR7: DC VSD 15.5; PR8: DC VSD 18.6; PR9: DC VSD 18.9.

Plate 17. Amplification pattern generated with ISSR primer UBC826 in the partially resistant double cross hybrids under field screening for VSD



L: 1 kb ladder; B: Blank; R1: DC VSD 2.4; R2: DC VSD 6.11; R3: DC VSD 7.2; R4: DC VSD 8.10; R5: DC VSD 8.11; R6: DC VSD 12.5; R7: DC VSD 18.3; R8: DC VSD 19.11; R9: DC VSD 20.2; R10: DC VSD 20.10; R11: DC VSD 21.9

Plate 18. Amplification pattern generated with ISSR primer UBC857 in the resistant double cross hybrids under field screening for VSD



L: 1 kb ladder; B: Blank; PR1: DC VSD 2.1; PR2: DC VSD3.3; PR3: DC VSD 6.12; PR4: DC VSD 9.6; PR5: DC VSD 9.13; PR6: DC VSD 15.2, PR7: DC VSD 15.5; PR8: DC VSD 18.6; PR9: DC VSD 18.9.

Plate 19. Amplification pattern generated with ISSR primer UBC857 in the partially resistant double cross hybrids under field screening for VSD

Table 18. Details of amplification pattern obtained with ISSR primers

Primers	Number of amplicons	Amplification pattern	
		Distinct	Faint
UBC811	6	4	2
UBC826	8	5	3
UBC857	7	5	2

4.7.2. Simple Sequence Repeat (SSR) Analysis

Simple sequence repeats (SSRs) are great source of genetic markers due to their abundance, high rate of polymorphism, ubiquitous distribution throughout the genome, codominant inheritance, high extent of allelic diversity and ease of assay by PCR (Gupta *et al.,* 1999; Kuleung *et al.,* 2004). Hence, SSR markers are widely utilized in germplasm characterization, genetic diversity and genetic mapping (Powell *et al.,* 1996; Varshney *et al.,* 2005). Scientists employed SSR markers for several genetic studies of cacao, including construction of various genetic and QTL maps (Brown *et al*., 2005; Faleiro *et al*., 2006; Chandrakant, 2014; Sujith, 2016).

Chandrakant (2014) eluted the polymorphic band at 450 bp and subjected it for nested PCR. The PCR product was run in agarose gel to confirm that it yielded only a single band. Sequencing of the nested PCR product was carried out which generated 246 nucleotides, and on BLASTn showed 94 per cent identity with *T. cacao* microsatellite DNA clone of mTcCIR42 SSR (GenBank AJ271944). Thus, it was confirmed to be linked with a gene conferring resistance to VSD. Resistant, partially resistant and susceptible clones were screened with this primer which clearly distinguished them with distinct polymorphic bands. All the resistant clones exhibited characteristic band at 650 bp whereas in partially resistant and susceptible clones the bands were formed at 400 bp and 450 bp respectively.

Tulshiram (2016) carried out SSR analysis using primer mTcCIR42 on resistant, partially resistant and susceptible clones and reported distinct bands at 200

bp in addition to the monomorphic bands at 230 bp in all the resistant and three partially resistant clones.

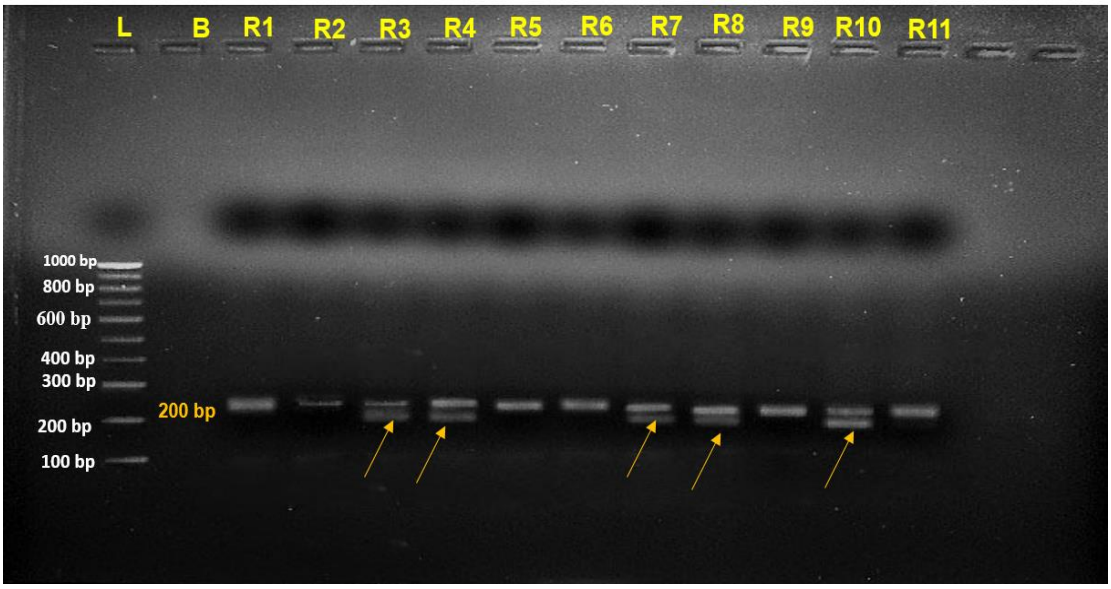
SSR locus mTcCIR42 have also been reported to be linked with resistance to lethal yellowing in cocoa (Rehem *et al.*, 2010). The polymorphic locus for lethal yellowing was reported at 230 bp whereas for VSD resistance it is at 200 bp. Tulshiram (2016) reported presence of monomorphic bands at 230 bp in all the VSD resistant hybrids indicated that different alleles of the linked gene are offering resistance for both diseases. PCR condition for the SSR marker mTcCIR42 is detailed in materials and methods (*3.6.3.1.b*). PCR products were electrophoresed in 1.8 per cent agarose gel. The details of the SSR primer analysed in this study are depicted in Table 6.

4.7.2.1. Expression of SSR Marker mTcCIR42 in the Double Cross Hybrids

Genomic DNA of all the twenty hybrids was analysed using the SSR marker mTcCIR42. Five hybrids among the eleven resistant double cross hybrids exhibited distinct polymorphic band at 200 bp. Two among the nine partially resistant hybrids also yielded distinct polymorphic band at 200 bp. Monomorphic band at 230 bp was present in all the twenty double cross hybrids. Gel profile obtained is given in Plates 20 and 21.

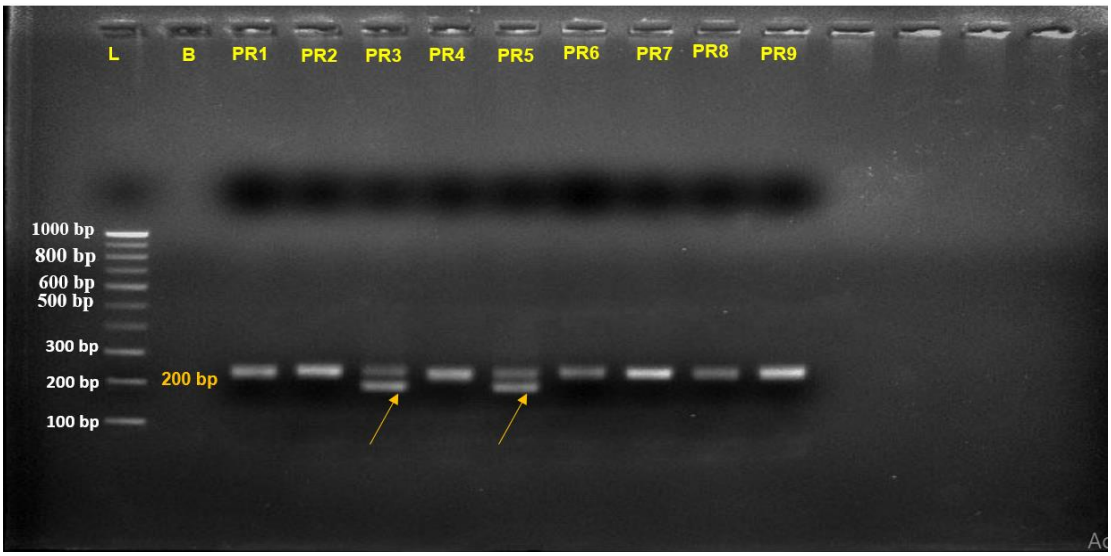
Table 19 Details of amplification pattern obtained with SSR primer

Primer	**Number of amplicons**	**Amplification pattern**	
		Distinct	**Faint**
mTcCIR42	2	2	-



L: 1 kb ladder; B: Blank; R1: DC VSD 2.4; R2: DC VSD 6.11; R3: DC VSD 7.2; R4: DC VSD 8.10; R5: DC VSD 8.11; R6: DC VSD 12.5; R7: DC VSD 18.3; R8: DC VSD 19.11; R9: DC VSD 20.2; R10: DC VSD 20.10; R11: DC VSD 21.9

Plate 20. Amplification pattern generated with SSR primer mTcCIR42 in the resistant double cross hybrids under field screening for VSD



L: 1 kb ladder; B: Blank; PR1: DC VSD 2.1; PR2: DC VSD3.3; PR3: DC VSD 6.12; PR4: DC VSD 9.6; PR5: DC VSD 9.13; PR6: DC VSD 15.2, PR7: DC VSD 15.5; PR8: DC VSD 18.6; PR9: DC VSD 18.9.

Plate 21. Amplification pattern generated with SSR primer mTcCIR42 in the double cross partially resistant hybrids under field screening for VSD

4.7.3. Scoring of Double Cross Hybrids Based on Molecular Analysis

VSD resistance shows polygenic inheritance, *i.e.* multiple genes are contributing towards resistance (Tan and Tan, 1988; Vossen, 1997). Contribution of each gene towards resistance differ very much (Tan and Tan, 1988). Due to the additive gene action of the VSD resistance, more the number of genes present in a genotype, higher the level of resistance offered by it. The present study considered four genes that are linked with UBC811, UBC826, UBC857 and mTcCIR42. Double cross hybrids DC VSD 18.3, DC VSD 19.11, DC VSD 20.10 expressed the presence of all the four genes linked to the markers. Scoring of the double cross hybrids based on molecular analysis is given in Table 20.

Hybrids DC VSD 6.11, DC VSD 6.12, DC VSD 7.2, DC VSD 8.10, DC VSD 9.13, DC VSD 12.5, DC VSD 18.6 yielded bands corresponding to three genes linked to the primers used. Only two genes linked with the primers were found in the hybrids DC VSD 2.4, DC VSD 15.2, DC VSD 15.5 and DC VSD 21.9. Hybrids DC VSD 2.1, DC VSD 3.3, DC VSD 8.11, DC VSD 9.6, DC VSD 18.9 and DC VSD 20.2 had one gene linked with the primers under study. Since VSD is a polygenic trait, absence of characteristic band in the resistant plants indicate the presence of some other genes that are also contributing to the resistance which are linked to some other molecular markers. Presence of characteristic bands in partially resistant hybrids indicated that the genes corresponding to the markers expressed were not strong enough to impart complete resistance against VSD disease.

Table 20. Scoring of double cross hybrids based on molecular analysis

Symbol	Double cross hybrids	ISSR primer			SSR primer
		UBC811	UBC857	UBC826	mTcCIR42
R1	DC VSD 2.4	+	-	+	-
R2	DC VSD 6.11	+	+	+	-
R3	DC VSD 7.2	+	+	-	+
R4	DC VSD 8.10	+	+	-	+
R5	DC VSD 8.11	+	-	-	-
R6	DC VSD 12.5	+	+	+	-
R7	DC VSD 18.3	+	+	+	+
R8	DC VSD 19.11	+	+	+	+
R9	DC VSD 20.2	+	-	-	-
R10	DC VSD 20.10	+	+	+	+
R11	DC VSD 21.9	+	-	+	-
PR1	DC VSD 2.1	+	-	-	-
PR2	DC VSD 3.3	+	-	-	-
PR3	DC VSD 6.12	+	+	-	+
PR4	DC VSD 9.6	+	-	-	-
PR5	DC VSD 9.13	+	+	-	+
PR6	DC VSD 15.2	+	-	+	-
PR7	DC VSD 15.5	+	+	-	-
PR8	DC VSD 18.6	+	+	+	-
PR9	DC VSD 18.9	+	-	-	-

4.7.4. Percentage of Expression of Molecular Marker among the Resistant Double Cross Hybrids

Three ISSR markers UBC811, UBC826, UBC857 and one SSR marker mTcCIR42 were employed for molecular analysis. UBC811 expressed at specific reported region in all the eleven double cross hybrids that expressed complete resistance on field screening. UBC826 and UBC857 expressed in seven double cross hybrids at specific reported regions among the eleven resistant hybrids. Expression of SSR marker mTcCIR42 at specific reported regions was observed in five double cross hybrids among the eleven resistant hybrids.

Table 21. Percentage of expression of molecular markers among the double cross hybrids

Sl No.	Primers	Number of resistant hybrids in which primer expressed at specific reported region	Total number of resistant hybrids	Percentage of expression at specific reported region (%)
1.	UBC811	11	11	100
2.	UBC826	7	11	63.63
3.	UBC857	7	11	63.63
4.	mTcCIR42	5	11	45.45

4.8. CORRELATION STUDIES ON POD AND BEAN CHARACTERS OF EARLY STAGE COCOA DOUBLE CROSS HYBRIDS

Correlation analysis was carried out for all the pod and bean characters. Data obtained is represented in Table 22. Significant positive correlation was exhibited by pod weight on ten quantitative characters of pod and bean including pod length (0.699), pod breadth (0.769), rind thickness (0.879), total wet bean weight (0.763), single wet bean weight (0.659), single peeled bean weight (0.658), single dry bean weight (0.619), dry bean length (0.527), dry bean thickness (0.618) and wet bean weight per tree (0.501).

Thondaiman and Rajamani (2014) documented positive significant correlation of pod weight with wet bean weight before fermentation (0.682), wet bean weight after fermentation (0.647), dry bean weight per pod (0.644), single wet bean weight (0.496), single dry bean weight (0.490) and dry bean yield per tree (0.398).

In the present study pod length expressed significant positive correlation with pod weight (0.699), pod breadth (0.492), rind thickness (0.521), total wet bean weight per pod (0.459) and dry bean thickness (0.445). Amitha *et al*. (2018) observed significant correlation of pod length with pod weight (0.761) and number of beans per pod (0.652). However, there was no correlation exhibited with the number of beans per pod in the current study.

Significant positive correlation was exhibited by pod breadth with nine characters including pod weight (0.769), pod length (0.492), rind thickness (0.737), total wet bean weight (0.665), single wet bean weight (0.582), single peeled bean weight (0.586), single dry bean weight (0.550), dry bean length (0.534), and wet bean weight per tree (0.491). Increase in pod breadth will result in increase in bean size and weight (Glendinning, 1963; Kumaran and Amma, 1982). The results obtained in the present study also confirmed the influence of pod breadth on bean size and weight.

Rind thickness showed positive significant correlation with pod weight (0.879), pod length (0.521), pod breadth (0.737), total wet bean weight (0.595), single wet bean weight (0.640), single peeled bean weight (0.651), single dry bean weight (0.627), dry bean width (0.445) and dry bean thickness (0.640).

Total number of beans per pod and number of flat beans per pod didn’t show any correlation with any other pod and bean characters. Earlier studies have reported positive correlation between number of beans per pod and total wet bean weight (Thondaiman and Rajamani, 2014; Asna, 2013 and Veeresh, 2017). However, such correlation was not observed in the current study.

Significant positive correlation was exhibited by total wet bean weight per pod with pod weight (0.763), pod length (0.459), pod breadth (0.665), rind thickness (0.595), single wet bean weight (0.644), single peeled bean weight (0.648), single dry bean weight (0.561), dry bean length (0.722), dry bean width (0.455), wet bean weight per tree (0.835) and dry bean weight per tree (0.687). Earlier studies also have reported positive correlation of total wet bean weight with single wet bean weight, bean length and bean width (Kumaran and Amma, 1982; Aikpokpodion, 2010).

Single wet bean weight exhibited positive significant correlation with pod weight (0.659), pod breadth (0.582), rind thickness (0.640), total wet bean weight per pod (0.644), single peeled bean weight (0.905), single dry bean weight (0.894), dry bean length (0.532), wet bean weight per tree (0.555) and dry bean weight per tree (0.539). Since peeling and drying of wet bean gives the peeled and dry bean weight, single wet bean weight is positively correlated to single peeled bean weight and single dry bean weight. Single peeled bean weight showed positive correlation with pod weight (0.658), pod breadth (0.586), rind thickness (0.651), total wet bean weight per pod (0.648), single bean weight (0.905), single dry bean weight (0.924), dry bean length (0.566), wet bean weight per tree (0.576) and dry bean weight per tree (0.537).

Single bean dry weight showed positive correlation with pod weight (0.619), pod breadth (0.550), rind thickness (0.627), total wet bean weight per pod (0.561), single bean weight (0.894), single peeled bean weight (0.924), dry bean length (0.568), wet bean weight per tree (0.491) and dry bean weight per tree (0.509).

Dry bean length expressed positive association with pod weight (0.527), pod breadth (0.534), total wet bean weight per pod (0.722), single bean weight (0.532), single peeled bean weight (0.566), single dry bean weight (0.568), dry bean thickness (0.511), wet bean weight per tree (0.697$^{)}$ and dry bean weight per tree (0.569). Veeresh (2017) documented positive correlation of bean length with bean weight.

Positive correlation was exhibited by dry bean width with rind thickness (0.445) and total wet bean weight per pod (0.455). Dry bean thickness showed positive significant correlation with pod weight (0.618), pod length (0.445), rind thickness (0.640) and dry bean length (0.511).

Wet bean weight per tree per year showed positive correlation with pod weight (0.501), pod breadth (0.491), total wet bean per pod (0.835), single wet bean weight (0.555), single peeled bean weight (0.576), single dry bean weight (0.491), dry bean length (0.697) and dry bean weight per tree (0.947).

Dry bean weight per tree showed positive correlation with total wet bean weight per pod (0.687), single wet bean weight (0.539), single peeled bean weight (0.537), single dry bean weight (0.509), dry bean length (0.569) and wet bean weight per tree (0.947).

Vithya *et al*. (2018) worked out correlation studies on various yield contributing characters in ten elite cocoa genotypes (TNAUCC 1-10) in Tamil Nadu. Single dry bean weight exhibited positive correlation with estimated dry bean yield per tree.

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Table 22. Correlation among pod and bean characters of double cross hybrids of cocoa during early bearing years

Traits	PW	PL	PB	RT	NBP	NFBP	TWWP	WWSB	SPBW	SDBW	DBL	DBW	DBT	WBWT	DBWT
PW	1	.													
PL	.699**	1													
PB	.769**	.492*	1												
RT	.879**	.521*	.737**	1											
NBP	.157	.281	.174	-.066	1										
NFBP	-.090	-.342	-.069	-.218	.035	1									
TWWP	.763**	.459*	.665**	.595**	.407	.077	1								
WWSB	.659**	.384	.582**	.640**	-.031	-.002	.644**	1							
SPBW	.658**	.301	.586**	.651**	-.140	.027	.648**	.905**	1						

Table 22. continued

Traits	PW	PL	PB	RT	NBP	NFBP	TWWP	WWSB	SPBW	SDBW	DBL	DBW	DBT	WBWT	DBWT
SDBW	.619**	.217	.550*	.627**	-.139	.059	.561*	.894**	.924**	1					
DBL	.527*	.299	.534*	.404	.096	-.005	.722**	.532*	.566**	.568**	1				
DBW	.434	.071	.389	.445*	.046	-.068	.455*	.140	.414	.334	.428	1			
DBT	.618**	.445*	.256	.640**	-.266	-.218	.363	.276	.248	.302	.511*	.203	1		
WBWT	.501*	.206	.491*	.332	.370	.317	.835**	.555*	.576**	.491*	.697**	.327	.135	1	
DBWT	.378	.114	.374	.225	.393	.309	.687**	.539*	.537*	.509*	.569**	.254	-.017	.947**	1

**. Correlation is significant at the 0.01 level.*. Correlation is significant at the 0.05 level.

PW	Pod weight (g)	**RT**	Rind thickness (cm)	**TWWP**	Total wet bean weight per pod (g)	**SDBW**	Single dry bean weight (g)	**DBT**	Dry bean thickness (cm)
PL	Pod length (cm)	**NBP**	Number of beans per pod	**WWSB**	Wet weight of single bean (g)	**DBL**	Dry bean length (cm)	**WBWT**	Wet bean weight per tree (g)
PB	Pod breadth (cm)	**NFBP**	Number of flat beans per pod	**SPBW**	Single peeled bean weight (g)	**DBW**	Dry bean width (cm)	**DBT**	Dry bean weight per tree (g)

4.9. PATH COEFFICIENT ANALYSIS

The concept of path analysis was proposed by Wright (1921). Dewey and Lu (1959) used it for the first time for plant selection. It is the standardized partial regression coefficient that involves division of the correlation coefficients into the measures of direct and indirect effects of set of independent variables on the dependent variable. It is also known as cause and effect relationship.

The direct and indirect effects of independent variable on dependent variable are categorized by Lenka and Misra (1973) as given below:

0.00 to 0.09	– Negligible
0.10 to 0.19	– Low
0.20 to 0.29	– Moderate
0.30 to 0.99	– High
More than 1.00	– Very high

4.9.1. Path Coefficient Analysis of Pod and Bean Characters on Total Wet Bean Weight Per Tree in Early Stage of Double Cross Hybrids

All the characters having positive significant correlation with total wet bean weight per tree per year were considered for path analysis to determine their direct and indirect effects on total wet bean weight per tree. Residual effects contribution of 0.0186 on total wet bean weight per tree indicated that 98.14 per cent of characters that contribute to the total wet bean weight per tree per year were considered in the present study. The results obtained are furnished in Table 23.

Vidhya *et al.* (2018) studied the direct and indirect effects of twenty traits on dry bean weight per tree in cocoa and reported that twelve traits showed positive direct effects on cocoa dry bean yield per tree. The parameters, wet bean per pod after fermentation (0.235), dry bean weight per pod (0.725) and number of pods per tree (0.701) showed positive direct effects on cocoa dry bean yield per tree.

Dry bean weight per tree exhibited high significant positive (0.717) direct effect on total wet bean weight per tree. Total wet bean per pod (0.280) and single peeled bean weight (0.241) expressed moderate significant positive direct effect on total wet bean weight per tree. Low significant positive direct effect on total wet bean weight per tree was showed by dry bean length (0.139). Pod breadth (0.031) showed negligible positive direct effect on total wet bean weight per tree. Single dry bean weight (-0.302) showed high negative direct effect on total wet bean weight per tree and single wet bean weight and pod weight showed negligible high negative direct effect on total wet bean weight per tree.

4.9.1.1. Indirect Effect on Total Wet Bean Weight Per Tree

4.9.1.1.a. Pod Weight

Pod weight showed moderate positive indirect effect on total wet bean weight per tree (0.271, 0.214) through high direct effect of dry bean weight per tree (0.717) and moderate direct effect of total wet bean per pod (0.280). Low positive indirect effect on total wet bean weight per tree was expressed by pod weight (0.159) through moderate direct effect of single peeled bean weight (0.241). Negligible positive indirect effect on total wet bean weight per tree (0.024, 0.073) was shown by pod weight through negligible direct effect of pod breadth (0.031) and low direct effect of dry bean length (0.139).

4.9.1.1.b. Pod Breadth

Pod breadth showed moderate positive indirect effect on total wet bean weight per tree (0.267) through high direct effect of dry bean weight per tree (0.717). Low indirect effect on total wet bean weight per tree was exhibited by pod breadth (0.186, 0.141) through moderate direct effect of total wet bean per pod (0.280) and single peeled bean weight (0.241). Negligible positive indirect effect on total wet bean weight per tree (0.074) was expressed through low direct effect of dry bean length (0.139). Low negative indirect effect on total wet bean weight per tree (-0.165) was exhibited by high negative direct effect of single dry bean weight (-0.302). Negligible negative indirect effect on total wet bean weight per tree (-0.025, -0.018) was shown by pod breadth due to negligible negative direct effect of pod weight (-0.025) and

negligible negative direct effect of single unpeeled bean weight (-0.031).

4.9.1.1.c. Total Wet Bean Per Pod

Total wet bean per pod exhibited high positive indirect effect on total wet bean weight per tree (0.492) through high direct effect of dry bean weight per tree (0.717). Low positive indirect effect on total wet bean weight per tree was exhibited by total wet bean per pod (0.156, 0.100) through moderate direct effect of single peeled bean weight (0.241) and low direct effect of dry bean length (0.139). Negligible indirect effect on total wet bean weight per tree was expressed by total wet bean per pod (0.021) through negligible direct effect of pod breadth (0.021).

4.9.1.1.d. Single Wet Bean Weight

Single wet bean weight showed high positive indirect effect on total wet bean weight per tree (0.387) through high direct effect of dry bean weight per tree (0.717). Moderate positive indirect effect on total wet bean weight per tree (0.218) was exhibited by single wet bean weight through moderate positive direct effect of single peeled bean weight (0.241). Low positive indirect effect on total wet bean weight per tree (0.180) was exhibited by single wet bean weight through moderate direct effect of total wet bean per pod (0.280). Moderate negative indirect effect on total wet bean weight per tree (-0.270) was expressed by single wet bean weight through high negative direct effect of single dry bean weight (-0.302).

4.9.1.1.e. Single Peeled Bean Weight

High positive indirect effect on total wet bean weight per tree was exhibited by single peeled bean weight (0.385) through high direct effect of dry bean weight per tree (0.717). Low positive indirect effect on total wet bean weight per tree was exhibited by single peeled bean weight (0.181) through moderate direct effect of total wet bean per pod (0.280). Moderate negative indirect effect on total wet bean weight per tree was exhibited by single peeled bean weight (-0.279) through high negative direct effect of single dry bean weight (-0.302).

4.9.1.1.f. Single Dry Bean Weight

Single dry bean weight exhibited high positive indirect effect on total wet bean weight per tree (0.365) through high direct effect of dry bean weight per tree (0.717). Moderate positive indirect effect on total wet bean weight per tree (0.223) was exhibited by single dry bean weight through moderate positive direct effect of single peeled bean weight (0.241).

4.9.1.1.g. Dry Bean Length

Dry bean length exhibited high positive indirect effect on total wet bean weight per tree (0.408) through high direct effect of dry bean weight per tree (0.717) and moderate indirect effect on total wet bean weight per tree (0.202)) through moderate direct effect of total wet bean per pod (0.280). Low positive indirect effect on total wet bean weight per tree was exhibited by dry bean length (0.136) through moderate direct effect of single peeled bean weight (0.241).

4.9.1.1.h. Dry Bean Weight Per Tree

Dry bean weight per tree exhibited low positive indirect effect on total wet bean weight per tree (0.192, 0.129) through moderate direct effect of total wet bean per pod (0.280) and through moderate direct effect of single peeled bean weight (0.241).

Table 23. Path analysis of total wet bean weight per tree

Character	C1	C2	C3	C4	C5	C6	C7	C8
C1	**-0.033**	0.024	0.214	-0.020	0.159	-0.186	0.073	0.271
C2	-0.025	**0.031**	0.186	-0.018	0.141	-0.165	0.074	0.267
C3	-0.025	0.021	**0.280**	-0.020	0.156	-0.169	0.100	0.492
C4	-0.022	0.018	0.180	**-0.031**	0.218	-0.270	0.074	0.387
C5	-0.0217	0.0181	0.181	-0.028	**0.241**	-0.279	0.079	0.385
C6	-0.020	0.017	0.157	-0.028	0.223	**-0.302**	0.079	0.365
C7	-0.017	0.016	0.202	-0.016	0.136	-0.171	**0.139**	0.408
C8	-0.0124	0.011	0.192	-0.016	0.129	-0.153	0.079	**0.717**

C1	Pod weight (g)	**C3**	Total wet bean per pod (g)	**C5**	Single peeled bean weight (g)	**C7**	Dry bean length (cm)
C2	Pod breadth (cm)	**C4**	Single unpeeled bean weight (g)	**C6**	Single dry bean weight (g)	**C8**	Total dry bean weight per tree (kg)

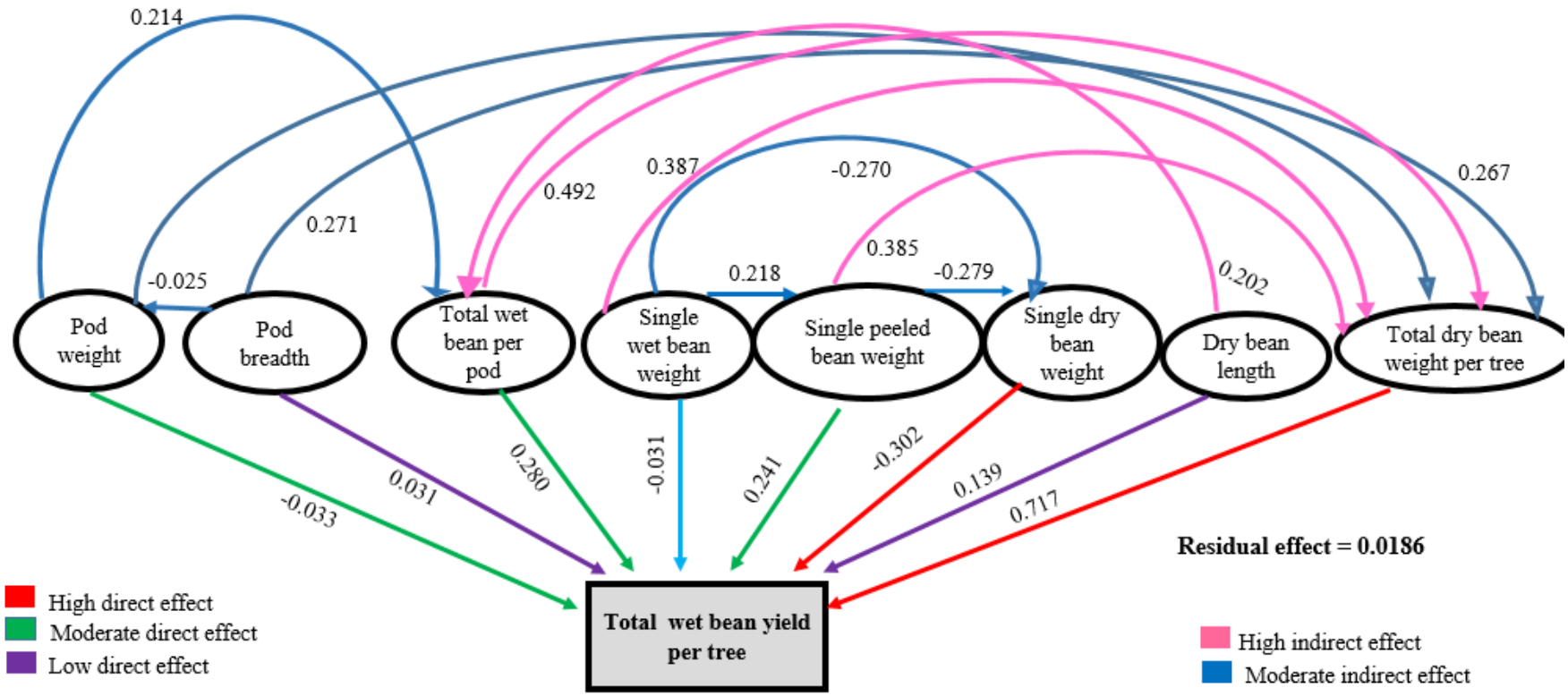

Figure 9. Path diagram on total wet bean yield per tree

4.10. SELECTION OF SUPERIOR DOUBLE CROSS HYBRIDS

The characters having high, moderate and low direct effect on total wet bean weight per tree were considered along with wet bean weight per tree for selection of superior double cross hybrids at early stage. Thus, wet bean weight per tree per year, dry bean weight per plant per year, wet bean weight per pod, single peeled wet bean weight and dry bean length were considered for selection. Single dry bean weight is also considered since it is considered as most important character for selection (Monteiro *et al.,* 2009). All the double cross hybrids were scored and ranked for the selected characters and are detailed in Tables 24 and 25.

Based on the ranking eight double cross hybrids were selected. Double cross hybrids, DC VSD 18.6 (Rank 1), DC VSD 6.11 (Rank 2), DC VSD 2.1(Rank 3), DC VSD 21.9 (Rank 4), DC VSD 8.10 (Rank 5), DC VSD 18.9 (Rank 5), DC VSD 6.12 (Rank 6) and DC VSD 7.2 (Rank 6) procured top ranks and were selected as superior genotype and are enlisted in Table 26 and Plates 22.a. and 22.b. Among the eight superior hybrids selected, four (DC VSD 6.11, DC VSD 21.9, DC VSD 8.10, DC VSD 7.2) recorded complete resistance and four (DC VSD 18.6, DC VSD 2.1, DC VSD 18.9, DC VSD 6.12) were with partial resistance. Hybrids DC VSD 18.6, DC VSD 6.11, DC VSD 8.10, DC VSD 7.2 and DC VSD 6.12 expressed three molecular markers whereas hybrid DC VSD 21.9 was with two molecular markers and hybrids DC VSD 2.1, DC VSD 18.9 had only one marker expressed. All these eight hybrids will be further evaluated in comparative yield trial (CYT).

Table 24. Scoring of early bearing double cross cocoa hybrids

Hybrid	Wet bean weight per plant (kg)	Score	Wet bean weight per pod (g)	Score	Dry bean weight per plant (kg)	Score	Single peeled wet bean weight (g)	Score	Dry bean length (cm)	Score	Single dry bean weight	Score
DC VSD 2.1	3.83	1	127.84	1	1.29	1	1.18	1	2.40	1	0.79	3
DC VSD 2.4	1.30	3	86.62	6	0.48	2	1.02	3	2.18	2	0.75	4
DC VSD 3.3	1.54	2	76.92	7	0.66	1	0.97	4	1.82	4	0.65	5
DC VSD 6.11	3.37	1	124.80	2	1.14	1	1.29	1	2.32	1	0.96	1
DC VSD 6.12	2.35	2	102.10	4	0.79	1	1.19	2	2.24	2	0.81	3
DC VSD 7.2	2.33	2	93.40	5	1.00	1	1.23	2	2.17	2	0.93	1
DC VSD 8.10	1.94	2	121.08	2	0.71	1	1.25	2	2.09	2	0.88	2
DC VSD 8.11	1.94	2	84.36	6	0.81	1	1.21	2	2.21	2	0.92	2
DC VSD 9.6	1.59	2	88.34	6	0.55	1	1.21	2	2.09	2	0.81	3
DC VSD 9.13	1.47	2	58.72	10	0.59	1	1.04	3	1.80	4	0.66	5
DC VSD 12.5	1.46	2	85.76	6	0.63	1	1.13	2	1.88	3	0.83	3

Table 24. continued.

Hybrid	Wet bean weight per plant (kg)	Score	Wet bean weight per pod (g)	Score	Dry bean weight per plant (kg)	Score	Single peeled wet bean weight (g)	Score	Dry bean length (cm)	Score	Single dry bean weight	Score
DC VSD 15.2	1.36	2	76.20	7	0.61	1	1.34	1	2.03	3	0.98	1
DC VSD 15.5	1.53	2	90.78	5	0.66	1	1.19	1	1.73	4	0.85	3
DC VSD 18.3	1.67	2	69.46	8	0.76	1	1.04	3	1.76	4	0.75	4
DC VSD 18.6	3.04	1	132.40	1	0.93	1	1.38	1	2.34	1	0.99	1
DC VSD 18.9	1.96	2	115.28	3	0.59	1	1.16	2	2.44	1	0.82	3
DC VSD 19.11	1.14	3	63.60	9	0.50	2	1.03	3	2.18	2	0.79	3
DC VSD 20.2	1.12	3	74.88	7	0.51	2	1.10	3	1.97	3	0.80	3
DC VSD 20.10	1.14	3	75.80	7	0.46	2	0.88	4	1.73	4	0.65	5
DC VSD 21.9	3.09	1	114.32	3	1.20	1	1.24	2	2.40	1	0.94	1

Table 25. Ranking of early bearing double cross cocoa hybrids

Hybrid	Wet bean weight per plant score	Wet bean weight per pod score	Dry bean weight per plant score	Single peeled wet bean weight score	Dry bean length score	Single dry bean weight score	Total score	Rank
DC VSD 2.1	1	1	1	1	1	3	8	3
DC VSD 2.4	3	6	1	3	2	4	19	10
DC VSD 3.3	2	7	1	4	4	5	23	13
DC VSD 6.11	1	2	1	1	1	1	7	2
DC VSD 6.12	2	4	1	2	2	3	14	6
DC VSD 7.2	2	5	2	2	2	1	14	6
DC VSD 8.10	2	2	2	2	2	2	12	5
DC VSD 8.11	2	6	2	2	2	2	16	8
DC VSD 9.6	2	6	1	2	2	3	16	8
DC VSD 9.13	2	10	1	3	4	5	25	14

Table 25. continued.

Hybrid	Wet bean weight per plant score	Wet bean weight per pod score	Dry bean weight per plant score	Single peeled wet bean weight score	Dry bean length score	Single dry bean weight score	Total score	Rank
DC VSD 12.5	2	6	1	2	3	3	17	9
DC VSD 15.2	2	7	1	1	3	1	15	7
DC VSD 15.5	2	5	1	1	4	3	16	8
DC VSD 18.3	2	8	1	3	4	4	22	12
DC VSD 18.6	1	1	1	1	1	1	6	1
DC VSD 18.9	2	3	1	2	1	3	12	5
DC VSD 19.11	3	9	2	3	2	3	22	12
DC VSD 20.2	3	7	2	3	3	3	21	11
DC VSD 20.10	3	7	2	4	4	5	25	14
DC VSD 21.9	1	3	1	2	1	1	9	4

Table 26. List of selected double cross hybrids

Selected double cross hybrids	**Rank secured**	**Reaction to disease**	**Number of molecular markers expressed**
DC VSD 18.6	1	Partial resistant	3
DC VSD 6.11	2	Resistant	3
DC VSD 2.1	3	Partial resistant	1
DC VSD 21.9	4	Resistant	2
DC VSD 8.10	5	Resistant	3
DC VSD 18.9	5	Partial resistant	1
DC VSD 6.12	6	Partial resistant	3
DC VSD 7.2	6	Resistant	3

DC VSD 18.6

DC VSD 6.11

DC VSD 2.1

DC VSD 21.9

Plate 22.a. Selected superior double cross hybrids

DC VSD 8.10

DC VSD 18.9

DC VSD 6.12

DC VSD 7.2

Plate 22.b. Selected superior double cross hybrids

4.11. DEVELOPMENT OF SELECTION CRITERIA FOR DOUBLE CROSS HYBRIDS AT EARLY BEARING STAGE

Based on the mean values for significant characters used for selection of superior genotypes, selection criteria have been developed for the double cross hybrids at early bearing stage. The mean values of the significant characters in the superior genotypes are given in Table 27.

Table 27. Mean values of significant characters in superior double cross hybrids

Sl. No.	**Rank**	**Hybrid**	**Wet bean weight per plant (kg)**	**Dry bean weight per plant (kg)**	**Wet bean weight per pod (g)**	**Single peeled wet bean weight (g)**	**Dry bean length (cm)**	**Single dry bean weight (g)**
1	1	DC VSD 18.6	3.04	0.93	132.40	1.38	2.34	0.99
2	2	DC VSD 6.11	3.37	1.14	124.80	1.28	2.32	0.96
3	3	DC VSD 2.1	3.83	1.29	127.84	1.18	2.40	0.98
4	4	DC VSD 21.9	3.09	1.20	114.32	1.24	2.40	0.94
5	5	DC VSD 8.10	1.94	0.71	121.08	1.25	2.09	0.88
6	5	DC VSD 18.9	1.96	0.59	115.28	1.16	2.44	0.82
7	6	DC VSD 6.12	2.35	0.79	102.10	1.19	2.24	0.81
8	6	DC VSD 7.2	2.33	1.00	93.40	1.23	2.17	0.93

4.11.1. Selection Criteria for Double Cross Hybrids at Early Stage

Table 28. Selection criteria for double cross hybrids at early stage

Sl. No.	Character	Range
1	Wet bean weight per plant per year (kg)	2 – 4
2	Dry bean weight per plant per year (kg)	0.60 – 1.30
3	Wet bean weight per pod (g)	95 -130
4	Single peeled wet bean weight (g)	1.16 -1.38
5	Dry bean length (cm)	2.09 -2.44
6	Single dry bean weight (g)	0.80-1.00

Early evaluation of the double cross hybrids can be done employing the selection criteria developed based on of yield contributing characters to save the years spend on preliminary evaluation and superior genotypes can be directly selected for comparatively yield trail. Thus, the time lag for release of a superior genotype as a variety in a perennial crop like cocoa can be reduced to some extent with the development of a selection of double cross hybrids at early bearing stage.

Future Line of Work

- The selected superior double cross hybrids can be further evaluated in comparative yield trial
- The selection criteria developed can be used for selection of superior double cross hybrids in their early bearing stage
- Molecular confirmation of the VSD resistance can be done using the ISSR primers UBC811, UBC826 and UBC857 and SSR marker mTcCIR42.

Summary

5. SUMMARY

The present study entitled ‘Preliminary evaluation of double cross hybrids for yield and Vascular Streak Dieback (VSD) disease resistance in cocoa (*Theobroma cacao* L.)’ was carried out in the Department of Plant Breeding and Genetics, College of Horticulture and Cocoa Research Centre (CRC), Vellanikkara, during the period 2018-2020. The objective was to evaluate the double cross hybrids bred for vascular streak dieback disease resistance in early yielding stage and to select the superior hybridsfor comparative yield trial. Twenty cocoa double cross hybrids which are bred for vascular streak dieback disease resistance field planted during 2017 at CRC farm served as the material for the study. These hybrids are in their early bearing stage.

The salient findings of the study are briefed below:

- Qualitative and quantitative evaluation of the morphological characters of flowers, pods and beans were carried out
- Double cross hybrids expressed significant variability for all the characters under study except petal colour and number of ridges and furrows
- Based on qualitative characters the double cross hybrids were classified into four clusters
- Based on quantitative characters the double cross hybrids were classified into four clusters
- Biochemical evaluation was carried out through fat and polyphenol estimation
- Fat content varied from 42.76 per cent in hybrid DC VSD 20.2 to 61.57 per cent in hybrid DC VSD 18.3.
- Variation in polyphenol content ranged from 4.09 per cent in hybrid DC VSD 3.3 to 9.54 per cent in hybrid DC VSD 21.9
- Economic parameters were estimated and all the hybrids were superior when compared with check (CCRP 15)
- All the twenty hybrids were screened for their resistance against vascular streak dieback (VSD) disease under field condition and were scored based on score chart given by Abraham *et al.* (2000)

- Eleven among twenty double cross hybrids expressed complete resistance whereas rest exhibited partial resistance against the disease
- Resistance conferred by the hybrids were confirmed using ISSR markers UBC811, UBC857, UBC826 and SSR marker mTcCIR42, figured to be linked with VSD resistance by Chandrakant (2014) and further validated by Tulshiram (2016)
- Dellaporta method (1983) of DNA extraction was used for isolation of good quality DNA from tender leaves of the double cross hybrids
- Quality and quantity of isolated DNA were estimated using Nanodrop® spectrophotometer. All the samples had good quality DNA with an absorbance value between 1.8 and 2.0 and quantity ranged from 62 to 321.4 ng/µl
- All the twenty hybrids were screened using the four primers to check existence of VSD resistant genes
- ISSR markers UBC811, UBC857, UBC826 and SSRmarker mTcCIR42 are reported to be linked with VSD resistance and produce distinct polymorphic band at 950 bp, 450 bp, 650 bp and 200 bp respectively (Chandrakant, 2014; Tulshiram, 2016)
- The characteristic polymorphic band at 950 bp was present in all the twenty double cross hybrids when screened with the SSR marker UBC811
- Screening with UBC857 yielded the marker band at 450bp in seven resistant and four partially resistant double cross hybrids
- The ISSR primer UBC826 produced distinct polymorphic band at 650 bp in seven resistant and two partially resistant double cross hybrids
- SSR marker mTcCIR42 developed characteristic band at 200 bp in five among eleven resistant and two among the nine partially resistant double cross hybrids
- Percentage of expression of UBC811, UBC857, UBC826 and mTcCIR42 among the eleven resistant double cross hybrids were 100, 63.63, 63.63 and 45.45 per cent, respectively

- Correlation and path analysis was carried out to identify the characters contributing to yield
- Based on the results of path analysis and considering the direct effects wet bean weight per tree, dry bean weight per tree, wet bean weight per pod, single peeled bean weight, dry bean length along with single dry bean weight were chosen as the six significant characters for development of selection criteria for double cross hybrids in the early bearing stage
- Selection criteria developed suggested for selection of superior genotypes with mean values in the following range for the significant characters
    - Wet bean weight per tree per year: 2 - 4 kg
    - Dry bean weight per tree per year: 0.6 -1.3 kg
    - Wet bean weight per pod :95 -130 g
    - Single peeled wet bean weight :1.16 -1.38 g
    - Dry bean length : 2.09 -2.44 cm
- All the twenty double cross hybrids were scored and ranked for the selected characters. Double cross hybrids DC VSD 18.6 (rank 1), DC VSD 6.11(rank 2) DC VSD 2.1(rank 3), hybrid DC VSD 21.9 (rank 4), hybrid DC VSD 8.10 (rank 5), DC VSD 18.9 (rank 5) DC VSD 6.12 (rank 6) and DC VSD 7.2 (rank 6) procured top ranks and were selected as superior double cross hybrids
- Among the eight superior genotypes, four double cross hybrids were showing complete resistance against vascular streak dieback disease *i.e.,* DC VSD 6.11, DC VSD 8.10, DC VSD 7.2 (with three markers expressed at specific reported regions) and DC VSD 21.9 (with two markers expressed at specific reported regions) and the four hybrids with partial resistance *i.e.,* DC VSD 18.6, DC VSD 6.12 (with three markers expressed at specific reported regions), DC VSD 2.1 and DC VSD 18.9 (with one marker expressed at specific reported regions) were chosen for comparative yield trial

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Appendix

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. CTAB buffer

CTAB : 5 per cent

Tris : 100 mM, pH 8.0

EDTA : 20 mM, pH 8.0

NaCl : 1.4 M

pH was adjusted 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

pH adjusted to 8 and made up final volume up to 100 ml.

3. SDS (20%)

SDS : 20 g

Distilled water : 80 mL

4. 5 M KOAC (Potassium 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 %)

To the 80 parts of absolute ethanol (100 %), 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% bromophenol blue
0.25% xylene cyanol
30% 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.

PRELIMINARY EVALUATION OF DOUBLE CROSS HYBRIDS FOR YIELD AND VASCULAR STREAK DIEBACK (VSD) DISEASE RESISTANCE IN COCOA (*Theobroma cacao* L.)

By

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ABSTRACT OF THE THESIS

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ABSTRACT

Cocoa is highly influenced by the climate change and growing environment, necessitating a long term and dynamic breeding programme. Even though the breeding programmes primarily focus on the development of high yielding varieties, outbreak of new pests and pathogens shift the priority to the development of resistant varieties. Vascular Streak Dieback disease (VSD) caused by *Ceratobasidium theobromae* (Samuels *et al.,* 2012), pose a great threat to cocoa crop, causing complete defoliation and eventual death (Abraham *et al.*, 2002). Even the high volume spray of chemicals was ineffective in disease control (Prior, 2007), and the only way to tackle it is to breed resistant varieties. Resistance breeding may result in yield reduction (Xu *et al*., 2017) however, breeding for double cross hybrids can overcome this situation (Gallais and Guy, 1971). Average yield superiority of the double cross hybrids over the F_1 hybrids has been shown by many scientists (Sriani *et al*., 2003; Ghanwat *et al.,* 2016).

Twenty double cross hybrids, bred for vascular streak dieback disease and planted during 2017, were used for the present study. Morphological characterization of the hybrids was carried out based on the quantitative and qualitative characters. Thirteen pod characters, twelve floral characters, six bean characters and flush colour of the leaves were studied. Except colour of the petal and number of the ridges and furrows, all other characters have expressed high variability among the double cross hybrids. The double cross hybrids have exhibited significant difference for fat and polyphenol content.

All the twenty double cross hybrids were screened and scored for the VSD resistance in the field condition, using the score chart (Abraham *et al.,* 2000). Based on the disease intensity, they were classified into eleven resistant and nine partially resistant.

Molecular marker analysis was carried out in all the twenty double cross hybrids to confirm the VSD resistance, using three ISSR markers UBC 811, UBC 857, UBC 826 and one SSR marker mTcCIR42, which were previously reported to be linked with VSD resistance (Chandrakant, 2014; Tulshiram, 2016). UBC 811 produced characteristic band at 950 bp in all the double cross hybrids. UBC 826 yielded distinct band at 650 bp in seven among the eleven resistant and two among the nine partially resistant hybrids. UBC 857 generated specific band at 450 bp in seven among the eleven resistant and four among the nine partially resistant hybrids. SSR marker mTcCIR42 developed characteristic band at 200 bp in five among the eleven resistant and two among the nine partially resistant hybrids.

Correlation analysis was carried out among all the pod and bean characters. Characters having significant correlation with total wet bean weight per tree per year were considered for path analysis. Dry bean weight per tree, single peeled bean weight, bean length along with total wet bean per tree and single dry bean weight were identified as significant yield contributing characters. Based on these characters along with single dry bean weight all the twenty double cross hybrids were scored and ranked. DC VSD 18.6 (Rank 1), DC VSD 6.11 (Rank 2), DC VSD 2.1(Rank 3), DC VSD 21.9 (Rank 4), DC VSD 8.10 (Rank 5), DC VSD 18.9 (Rank 5), DC VSD 6.12 (Rank 6) and DC VSD 7.2 (Rank 6) were the top ranked double cross hybrids. These hybrids will be further evaluated in CYT. Among the eight top ranked double cross hybrids, DC VSD 6.11, DC VSD 8.10, DC VSD 7.2 (three markers present at specific size) and DC VSD 21.9 (two markers expressed at specific size) have shown complete field resistance against VSD.

A selection criterion was developed based on the mean values of significant characters of eight top ranked double cross hybrids which will be used for selection of double cross hybrids at early bearing stage.