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IDENTIFICATION OF MARKERS LINKED TO POST-HARVEST PHYSIOLOGICAL DETERIORATION (PPD) RESISTANCE IN CASSAVA LINES

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THESIS

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KERALA, INDIA

2016

DECLARATION

I hereby declare that this thesis entitled "**Identification of markers linked to post-harvest physiological deterioration (PPD) resistance in cassava lines**" is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar title, of any other university or society.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF APPENDICES

ABBREVIATIONS

°C	-	Degree Celsius
%	-	Per cent
µl	-	Micro litre
ACCO	-	1-Amino Cyclo propane 1-Carboxylate Oxidase
AFLP	-	Amplified Fragment Length Polymorphism
AOX	-	Alternative Oxidase
APS	-	Ammonium Per Sulphate
APX	-	Ascorbate peroxidase
BAC	-	Bacterial Artificial chromosome
bp	-	Base pair
BSA	-	Bulk Segregant Analysis
CAPS	-	Cleavage Amplified Polymorphic Sequence
CAT	-	Catalase
CBSD	-	Cassava Brown Streak Disease
CIAT	-	International Centre for Tropical Agriculture
CMD	-	Cassava Mosaic Disease
CTCRI	-	Central Tuber Crops Research Institute
DNA	-	Deoxy-ribonucleic acid
DM	-	Dry Matter
EDTA	-	Ethylene Di-amine Tetra Acetate
EMBL	-	European Molecular Biology Laboratory
EST	-	Expressed Sequence Tag
et al.	-	And other co-workers
FAO	-	Food and Agricultural Organization
hr(s)	-	Hour(s)

ABBREVIATIONS

HRGP	-	Hydroxyproline-Rich GlycoProtein
IITA	-	International Institute of Tropical Agriculture
iTRAQ	-	isobaric Tagging for Relative and Absolute Quantification-based analysis
MAS	-	Marker Assisted Selection
m.a.s.l.	-	Metre above sea level
min	-	Minutes
mg	-	Milligram
Ml	-	Milli litre
NARO	-	National Agricultural Research Organization
NCBI	-	National Centre for Biotechnology Information
NTSYSpc	-	Numerical Taxonomy System for personal computer
Ng	-	Nanogram
OD	-	Optical Density
PAGE	-	Poly Acylamide Gel Electrophoresis
PCA	-	Principal Component Analysis
PCD	-	Programmed Cell Death
PCR	-	Polymerase Chain Reaction
PIC	-	Polymorphism Information Content
p-LIN	-	p-linamarase
POD	-	Peroxidase
PPD	-	Post-harvest Physiological Deterioration
PP pathway	-	Pentose Phosphate pathway
PPO	-	Poly Phenol Oxidase

ABBREVIATIONS

PRCRTC	-	Philippine Root Crop Research and Training Center
PSAT	-	Phospho Serine Amino Transferase
PVP	-	Poly Vinyl Pyrrolidone
QTL	-	Quantitative Trait Loci
RAPD	-	Random Amplified Polymorphic DNA
RFLP	-	Restriction Fragment Length Polymorphism
RH	-	Relative Humidity
ROS	-	Reactive Oxygen Species
SAS	-	System Analysis software
SDS	-	Sodium Dodecyl Sulphate
sec	-	Seconds
SOD	-	Super Oxide Dismutase
SMC	-	Simple Matching Coefficient
SNP	-	Single Nucleotide Polymorphism
SSR	-	Simple Sequence Repeat
TBE	-	Tris Borate EDTA
TDF	-	Transcript Derived Fragment
TEMED	-	Tetra Ethyl Methyl Ethylene Diamine
TRX	-	Thioredoxin peroxidase
UV	-	Ultra Violet
VS	-	Vascular Streaking

INTRODUCTION

Chapter 1

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is an important staple food which today ranks the fourth most energy-rich food crop due to high (>70%) carbohydrate content. It provides food for more than 800 million people worldwide (Falade and Akingbala, 2010) and forms an essential component of diet. Cassava is cultivated by farmers for its starchy storage roots and is mainly grown in marginally agricultural areas due to its greater adaptation to rain-fed conditions and low fertile soils. It also serves as a famine reserve crop because of its tolerance to abiotic stresses and adverse environmental conditions. However, the expansion of the crop is drastically reduced by the storage capacity of the roots, which is limited to only a short period (Wenham, 1995).

The short shelf-life of cassava tubers is directly linked to an endogenous physiological phenomenon known as post-harvest physiological deterioration (PPD). As soon as the tubers are uprooted from the ground, a blue or black discoloration in the xylem vessel develops and PPD sets within the next 24hrs. This rapid onset of decay shortens the shelf-life leading to poor yield and wastage of products and also hinders the commercial production of cassava by reducing the economic costs and market quality (Sanchez *et al.*, 2006). The extent of shelf life of cassava depends on the cultivar, harvest practices, handling and storage conditions. While storage conditions and management practices can go a long way in delaying PPD, the development of PPD resistant cassava cultivars remains the most challenging and desirable strategy.

Despite being a heterozygous cross-pollinating crop, it is more difficult to find a solution for PPD in cassava using traditional breeding methods. Development and deployment of the PPD resistant cultivars remains the most desired strategy with the involvement of molecular genomics and plant breeding approaches. Research organization such as, CIAT and IITA are focusing on improving the cassava resistance to PPD in hope of developing long-life cassava

roots and their recent research works are based on understanding the inherent biochemical and genetic factors regulating the PPD process.

To date, fewer attempts were made to control and delay PPD in cassava using molecular marker techniques. It is now known that DNA markers are widely used to identify and tag desired gene or genome regions in crop breeding program which will greatly assist in the preservation and exploitation of germplasm (Michelmore, 1995).

The simple sequence repeat (SSR) markers are one among the most reliable DNA markers used over the years for genetic diversity and cultivar analysis, taxonomic and phylogenetic studies, transgenics, gender identification, genome mapping and population genetics (Fregene *et al.*, 2003). Furthermore, allele specificity, high polymorphism rates, codominant and reproducible nature, high abundance and a broad distribution throughout the genome have made microsatellites (SSRs) one of the most popular genetic markers (Morgante *et al.*, 2002).

In the present study, an attempt was made to identify the putative marker linked to PPD resistance and study the variation among the PPD resistant and susceptible genotypes with the following main objectives:

1. Selection of long shelf-lived (PPD resistant) and PPD susceptible cassava lines for PPD study.
2. Assess the genetic variability among five PPD resistant and 23 susceptible lines using SSR markers.
3. Identification of markers associated with PPD resistance in selected cassava lines.

REVIEW OF LITERATURE

Chapter 2

REVIEW OF LITERATURE

2.1 Cassava

2.1 a) Background

Cassava (*Manihot esculenta* Crantz) is a perennial crop with starchy tuberous roots being the main harvested organ. It is commonly called as 'yuca' in Spanish, 'manioc' in French and 'mandioca' in Portuguese. Cassava is closely related to two sub-species *M. esculenta* ssp. *Peruviana* and *M. esculenta* ssp. *Flabellifolia* that are regarded as the wild cassava progenitors (Allem, 2002). One of the most valued features of this crop is its ability to produce acceptable root yields under marginal environmental conditions. Cassava is highly appreciated as a resilient crop that can grow under harsh conditions (annual rainfall of <500mm or >5000mm) and the roots can be restored for several months in ground without serious deterioration.

2.1 b) Origin and distribution

The cassava was first domesticated in Brazil and brought to West Africa by Portuguese in the sixteenth century, from where it expanded to sub-Saharan Africa (Hillocks, 2002; Nweke *et al.*, 2002). Today, cassava has become more important throughout the tropical and subtropical countries situated in the equatorial belt, between 30°N and 30°S. This crop requires an annual mean temperature of 20°C and above for optimum growth, although it is cultivated in diverse agro-ecological conditions (El-Sharkawy, 2007).

With West Africa, Brazil, Indonesia and Thailand being the major producers, cassava has achieved its worldwide importance in commercial production. Global production of cassava was increased to 291 million tonnes in 2014 of which over 167 million tonnes were from Africa (FAO, 2014).

2.1 c) Taxonomy and morphological characteristics

Cassava is a dicotyledonous root crop which is a member of Euphorbiaceae family. It belongs to the genus *Manihot*; sub-species *Manihot esculenta* Crantz and species (*ssp.*) *esculenta* (Allem *et al.*, 2001).

Cassava is a perennial and woody shrub having edible starchy tubers. The leaves are simple, with 3 and 9 leaf lobe arranged spirally around the stem and leaves in association with the inflorescence (Rogers, 1965). Cassava is monoecious with the development of male flowers near the tip and female flowers closer to the base of the inflorescence (Ekanayake *et al.*, 1997) and hence predominantly cross pollinated. Cassava contains cyanogenic glucosides which is toxic to humans and animals. Linamarin and lotaustralin are two cyanogenic glucosides that are identified in the leaves and roots of cassava plant (King and Bradbury, 1995).

A mature cassava plant grows from 1m to about 4m height with thick woody stems being topped by palmate, dark green or purplish leaves. The pigmentation of the stem varies from grey to yellow, orange, brown or purple providing one of the most stable characteristics for differentiation of cultivars.

Cassava is propagated vegetatively *via* mature stem. Seed propagation is commonly done under natural conditions and these seeds are used in various plant breeding programmes. In Africa, subsequent planting using spontaneous cassava seedlings are performed occasionally.

The cassava is a diploid species with a chromosome number of 2n=36 and have an estimated genomic DNA content of 1.67pg per cell nucleus (Awoleye *et al.*, 1994) and a high G+C content. This estimated value of DNA content corresponds to a haploid genome size of 772 megabase pairs (Bennet *et al.*, 1992).

2.2 Post-harvest Physiological Deterioration (PPD) in Cassava

2.2 a) Introduction

The biggest constraint to cassava production is the remarkable short shelf-life of cassava which arises due to rapid physiological deterioration that make

cassava tubers unacceptable for fresh human consumption and marketing (Reilly *et al.*, 2003).

The physiological deterioration during post-harvest period is associated with the development of dark bluish or brownish radial streaks near the xylem vessels. This unique root phenomenon occurring in the tubers of cassava plant is referred to as post-harvest physiological deterioration (PPD). The term vascular streaking or discoloration was used as alternatives for describing PPD which appears as blue-black fluorescence under UV light (Averre, 1967). PPD begins within 2-3 days (20-30°C and 65-80% RH) along with signs of peripheral streaking which later spread to the neighbouring parenchymatous tissues producing more brownish occlusions leading to a complete decay and rotting. The extent of deterioration depends on the handling and mechanical damage during harvest.

Vascular streaking is divided into two types which appear to occur independently. The first type, VS-I (vascular streaking-I or primary deterioration) is characterized by blue, black or brown streaks on cortical tissue which appears in a ring shape within 2-3 days and is free of microbial activity. The second type, VS-II (vascular streaking-II) also known as secondary deterioration is characterized by blue-black streaks along xylem vessel which occurs 5-7 days after harvest. The secondary deterioration is microbial and it commence when bacterial and fungal attack occurs and eventually lead to a general and complete root rotting (Plumbley and Rickard, 1991).

2.2 b) Economic impact of PPD

Cassava has reached more than 57% of its production potential (FAO, 2014) and is known to be a strategic food crop that can mitigate the rising demands for production of cassava for food, starch, animal feed and biofuel (Chauynarong *et al.*, 2009). Cassava comes in bulkier roots and this is an added advantage for commercial markets but the quantity of starch decreases soon after they have been harvested. This happens due to PPD which severely affect the starch producers and farmers.

Cassava production is prone to many biotic, abiotic and management constraints throughout the production cycle. The cassava tubers are highly susceptible to PPD causing a great reduction in the market quality of fresh cassava. Due to rise in population size and increased urbanization in developing countries, market places and processing centres are becoming more centralized adversely affecting the cassava value chains. A possible solution to this problem is the transportation of cassava roots from field to the market area, thereby increasing the time between producer and consumer.

Estimates on cassava PPD losses are rarely accurate and the reliable information is scarce to compute the actual worldwide losses due to PPD and poor post-harvest management. The farmers suffer from major economic losses like reduction in the quality of their produce and also the risk in timely supply of cassava as a raw material. The rapid root perishability has hampered cassava from becoming a commercial crop for human consumption and imposes a serious problem for large scale industrialization of cassava tubers for both animal feeding and other industrial uses.

In this respect, research on strategies applicable in retarding the onset of PPD must be employed and increase the storage life of cassava for its application in commercial and domestic purposes.

2.2 c) Biochemical mechanism and biosynthetic pathway involved in PPD

A biochemical level of understanding the deterioration process is necessary to identify the potential means to control and delay PPD. The mechanical damage caused during PPD process resemble certain wound signalling events in plants and triggers a series of biochemical pathways where reactive oxygen species (ROS) plays a pivotal role. Several studies suggest that oxygen mediate the initiation of PPD wound response and therefore it is an oxidative perhaps a peroxidase mediated process (Booth, 1977; Rickard, 1982).

PPD is associated with a cascade of biochemical changes such as increase in respiration, mobilization of starch into sugars, changes in lipid composition and increase in the rate of acid invertase. The production of phytohormone, ethylene

increases during PPD as ethylene play a key role in regulating wound and senescence response. The activity of various enzymes including catalases (CAT), peroxidases (POD), glucanases, chalcone synthase, dehydrogenases, phenylalanine ammonia lyase (PAL) and polyphenol oxidase (PPO) increase during PPD (Wenham, 1995; Tanaka *et al.*, 1983). The activity of PAL rapidly increases in cassava root as PPD increases. PAL is a key enzyme of the phenyl propanoid (PP) pathway (Petersen *et al.*, 1999) where PAL catalyzes the conversion of L-phenylalanine into hydroxycoumarins (a secondary metabolite) *via* E-cinnamic acid (Fig. 1) in the PP pathway.

Secondary metabolites like diterpenes, catechins, hydroxycoumarins (esculin, esculetin, scopolin and scopoletin) accumulate in cassava roots during PPD within 24-48hrs after harvest (Uritani, 1999). These secondary metabolites are derived from the PP pathway and are localized in the apoplast.

Wheatley and Schwabe (1985) identified a dark blue end product on a peroxidase mediated oxidation reaction with scopoletin and hydrogen peroxide (H_2O_2) which result in blue-streaking in cassava during PPD. A 150-200 fold increase in accumulation of scopoletin content was observed in highly susceptible roots of cassava (Buschmann *et al.*, 2000).

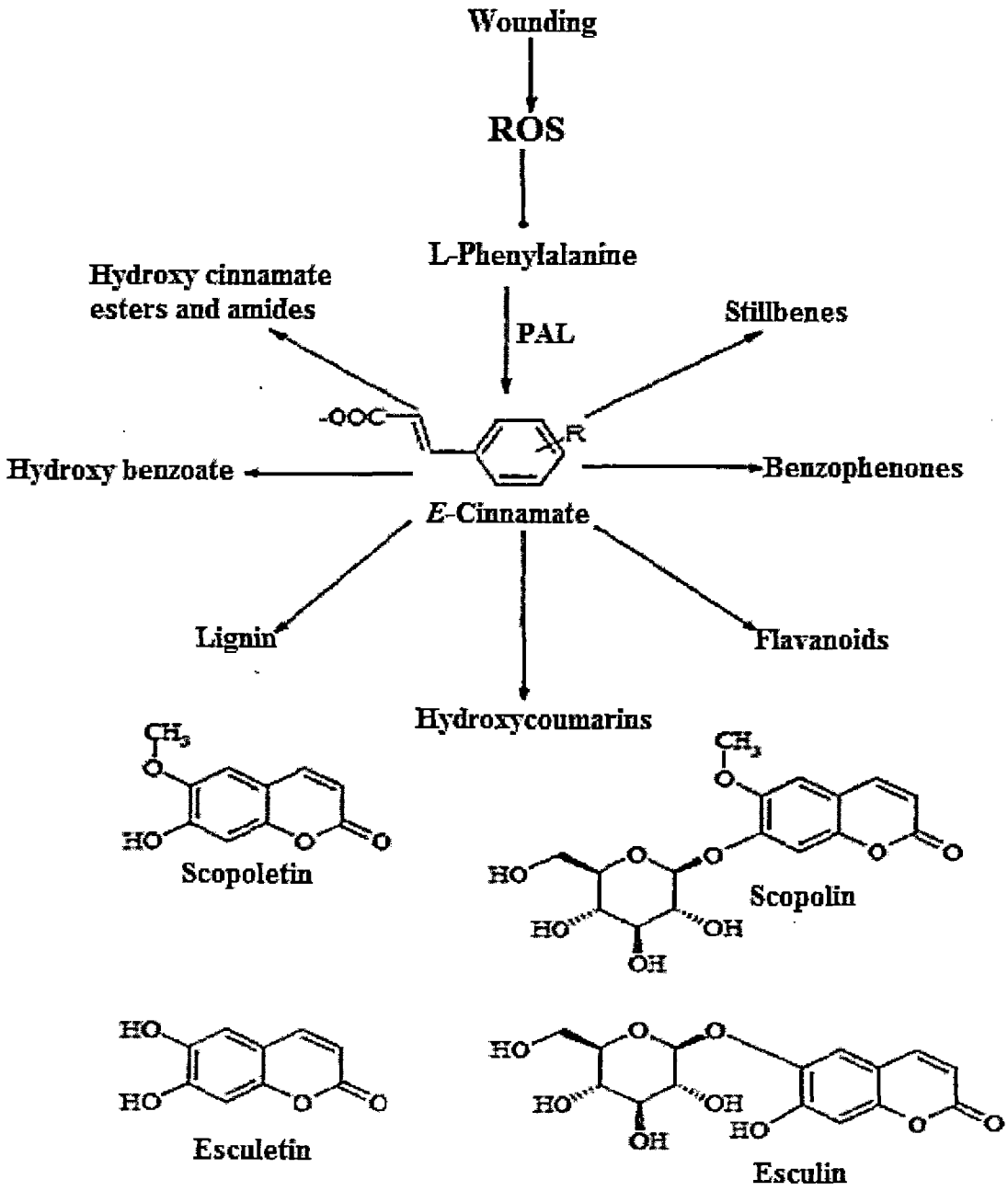


Fig 1. Metabolic pathway in the biosynthesis of phenyl propanoids in PPD

2.2 d) Molecular mechanism of PPD in cassava

A molecular insight on deterioration process is essential for choosing a suitable method for delaying and controlling PPD symptoms. Some research works based on genetic modification were attempted to isolate and characterize the genes that control or trigger PPD response (Reilly *et al.*, 2001; Li *et al.*, 2001). But prior knowledge on the molecular aspect of identifying PPD resistance is poorly understood. The development of conventional breeding methods could improve or modulate the PPD process and produce PPD resistant cultivars.

On a molecular basis, PPD is an oxidative process actively controlled by the expression of genes in the root which is triggered as the root detaches from the mother plant (Reilly *et al.*, 2003). The possible genes activated during early and late phase of PPD are associated with cellular processes such as modulation/scavenging of ROS, cell wall thickening and repair, PCD responses, transport of ion, water or metabolites, signal transduction, metabolism and biosynthesis, induction of defense-related genes and activation of protein synthesis and accumulation of secondary metabolites.

Beeching *et al.* (1997) studied on cyclohexamide inhibition, *in vivo* labeling of proteins and cDNA cloning in cassava and stated PPD as an active complex process involving up-regulation of genes and *de novo* synthesis of proteins. The metabolomic-chemometric approach used in the chemo-characterization study of cassava roots during PPD gave evidence for successful and easy screening of PPD susceptible genotypes (Uarrota *et al.*, 2014).

Huang *et al.* (2001) screened nearly 70 transcript derived factors (TDFs) using cDNA-AFLP, an RNA fingerprinting technique. The selected TDFs were involved in important biochemical and physiological processes of PPD such as oxygen stress, protein metabolism, carbohydrate metabolism and synthesis of secondary metabolites.

Specific genes involved in PPD have been identified, characterized and their expressions were evaluated based on a cDNA microarray analysis constructed from PPD susceptible roots. The study led to the identification of 72 non-redundant expressed sequence tags (ESTs) which showed differential regulation

during PPD. Of these, 63 genes were up-regulated (≥1.8 fold) which included CAT, ascorbate peroxidase (APX), secretory peroxidase, thioredoxin peroxidase (TRX) and glutathione S-tranferase and rest of the genes were down-regulated (Reilly *et al.*, 2007).

In a 2D gel-based proteomics study, several oxidative stress-related proteins like APX, TRX and small heat shock proteins were identified which exhibited differential expression during PPD. A proteome based technique known as isobaric Tagging for Relative and Absolute Quantification-based analysis iTRAQ, much better than the 2D gel approach, screened some novel proteins and detected an elevation in SOD activity during early phase (6-24 hrs) and an increase in CAT activity during the late phase (48-96 hrs) of PPD (Owiti *et al.*, 2011). Increase in the activity of two key enzymes, POD and CAT involved in H_2O_2 turnover was observed in the early stages of PPD (Beeching, 1998).

The expression of *cMeHRGP1* for hydroxyproline-rich glycoprotein (*HRGP*) produced during PPD process was isolated and characterized. This full length cDNA clone had unusually long 5' and 3' un-translated regions, proved to be an antisense pairing, coding for part of phosphoserine aminotransferase (*PSAT*) gene in its complementary strand (Han *et al.*, 2001). In detached cassava roots, the *cMeHRGP1* gene expression was lately induced when the root has reached an advanced level of deterioration. This adds to a fact that the PPD process is associated with inadequate wound responses for repair.

Recent strategies have focused on the reduction of ROS accumulation by over expressing cyanide insensitive mitochondrial alternative oxidase (AOX) in roots of transgenic cassava plants (Zidenga *et al.*, 2012). This strategy was able to delay PPD for approximately 3 weeks. The manipulation of ROS scavenging enzymes such as cytosolic SOD (*MeCu/ZnSOD*) and APX (*MeAPX2*) in transgenic cassava plants was conducted to study the tolerance against chilling and oxidative stresses. The transgenic cassava showed coupled expression of *MeCu/ZnSOD* and *MeAPX2* which resulted in improved tolerance to abiotic stresses (Xu *et al.*, 2014).

Comparative physiological and transcriptomic analyses revealed that exogenous application of melatonin could delay PPD by decreasing the H_2O_2 levels and increasing POD and CAT activities. These genes actively involved in the starch synthesis were repressed during PPD process indicating that melatonin is an important regulator of starch metabolism (Hu *et al.*, 2016).

2.2 e) Evaluation of PPD

The visible colouration of the root is an indication of PPD intensity. There are different subjective scoring methods for PPD evaluation; 1) subjective visual scaling of entire roots (Booth, 1976); (2) subjective analysis of biochemical and physiological changes in transverse sections of roots under ultraviolet (UV) light; (3) PRCRTC's method of numerical biochemical grading based on the severity of discoloration during PPD (Uritani *et al.*, 1983); and (4) visual inspection of peripheral PPD symptoms by scoring transverse sections of the root tissue blocks where the distal and proximal cut is given to increase deterioration (Wheatley, 1982). Buschmann *et al.* (2000) have suggested the measurement of UV fluorescent compound (hydroxycoumarins) as a biochemical marker to assess extent of PPD. Using HPLC, considerable differences of hydroxycoumarin content was observed among cassava varieties reporting that there was no significant relation between accumulation of hydroxycoumarins and the visual symptoms. This finding was supported by Salcedo *et al.* (2011) proving that hydroxycoumarins could not be used as a reliable biochemical marker for evaluation. Measurement of sugar/starch ratio is regarded as other alternatives for PPD evaluation (Oirschot *et al.*, 2000). However, it is not a common method since sugar/starch ratio in cassava is strongly depended on environmental and geographical conditions.

Booth's and Wheatley's methods are the two most common methods used for evaluating PPD. There is a need to identify and implement a quantitative and systematic phenotypic evaluation method for identifying cassava genotypes with delayed PPD (Han *et al.*, 2001). Identification of varietal differences in PPD susceptibility through evaluation of PPD symptoms can be a source of genetic

variation enabling crop improvement (Iglesias, 1996). Due to the difficulty in scoring PPD which is a destructive process, a root sampling device that can extract the core of root parenchyma has been developed for visually quantifying PPD and this method can be used effectively for biochemical and gene expression studies related to the control and delay of PPD (Garcia *et al.*, 2013).

2.3 Methods to delay and control PPD

Considering the large scale production of cassava, PPD becomes a serious issue since there is no technique available to store and preserve cassava roots commercially. The processing and market centres should be established very close to production area to ensure efficient and easy supply of raw material, processing of the raw material into storable forms and marketing of processed items. Currently, two main approaches have been chosen to overcome PPD i.e., utilization of improved storage techniques and exploitation of DNA markers through molecular breeding.

2.3 a) Utilization of improved storage techniques

The simplest method for delaying PPD commonly used on small farm lands is simply leaving the roots in the ground until needed (Scott *et al.*, 2000). The disadvantages of this method include increase in the risk of pathogen attack (Ingram and Humphries, 1972), unavailable land for other agricultural production, development of woody, fibrous roots with an impaired flavour (Westby, 2002) and increase in cooking time as well as reduction in starch quantity (Ravi *et al.*, 1996).

Pruning or cutting the leaves off from the cassava plant two to three weeks before harvesting reduce and delay PPD (Tanaka *et al.*, 1984). The effect of pruning was assessed in six cultivars having varying PPD susceptibility. In the MCOL 22 variety, roots after 20 days of storage that underwent pruning exhibited 4% deterioration while 96% deterioration was shown in unpruned plants (Rickard and Coursey, 1981). Studies at CIAT showed that PPD can be suppressed by pre-harvest pruning (Oirschot, 2000). The indirect involvement of ethylene in signal

transduction during PPD process was shown by Hirose (1984). It was reported that, after a wound response, pruning treatment had no significant relation with exogenous as well as endogenous application of ethylene.

Other traditional practices include storage of harvested fresh roots under field conditions such as in pits, boxes, clamp silos and trenches (Westby, 2002). Modern storage techniques include oxygen exclusion by storing in polyethylene bags, waxing and deep freezing (Lebot, 2009). Coating the roots in paraffin wax and wrapping in air-tight plastic bags to exclude oxygen can increase the shelf-life to four weeks but it is an expensive method (Wheatley and Schwabe, 1985). Alternatively, roots can be freezed (-20°C) in polyethylene bags for delaying PPD to about two weeks (Oirschot *et al.*, 2000).

2.3 b) Molecular breeding for PPD resistance

To date, fewer attempts were made to delay or reduce PPD in cassava using molecular marker based techniques. Use of molecular breeding methods for improving the resistance towards a deterioration response like PPD is a challenging task. But this can be achieved if there is more genetic variability present in cassava germplasm for PPD, which has yet to be proved. The high-throughput DNA marker genotyping are widely used for diversity analysis, genetic linkage mapping and marker-assisted selection.

The molecular marker technologies allow breeders to identify genetic loci controlling complex traits (e.g., drought resistance) without measuring phenotype (Babu *et al.*, 2003) and thus reduce the need for extensive field testing over space and time. Such genes controlling a trait of interest could be used in the selection and screening of genotypes in crop breeding programmes (Manickavelu *et al.*, 2006). The molecular markers are also used to dissect quantitative trait loci (QTLs) for physiological traits responsible for stress tolerance and some potential traits for drought tolerance.

Buschmann *et al.* (2000) observed high variability among roots of same cassava cultivars and pointed out significant relationship between PPD susceptibility and scopoletin accumulation. Morante *et al.* (2010) reported that

less work was attempted to improve PPD resistance through plant breeding methods. The authors have cited that lack of cassava germplasm with more genetic variation for PPD is important for implementing an effective breeding strategy for evaluating different sources of PPD resistance in cassava.

2.4 Reviews on improving PPD resistance in cassava

2.4 a) International review on PPD resistance

The strategy for improving the tolerance to PPD was recently considered as a significant bottleneck by many research organizations like CIAT (International Centre for Tropical Agriculture), IITA (International Institute of Tropical Agriculture), NARO (National Agricultural Research Organization), Uganda, and many project initiatives (Biocassava plus programme, Harvest plus initiative). In early 2009, CIAT scientists made a serendipitous discovery that a cassava variety conserved in the organization's gene bank had high levels of beta-carotene which showed PPD resistance for up to two months. CIAT has been screening cassava germplasm for identifying PPD resistance (Iglesias *et al.*, 1994). Some of the research work at CIAT related to cassava PPD and the strategies implemented for improving PPD resistance are briefly cited below.

A set of molecular markers significantly associated with the putative QTLs of PPD which will help in identifying the gene(s) that control and delay PPD were mapped (Cortes *et al.*, 2002). For this mapping study, 144 F_1 cassava plants were used to construct a mapping population. This population was obtained by crossing two elite cassava lines such as TMS 30572 and CM 2177-2 where TMS 30572 (or MNGA2) was the female parent raised at IITA, Nigeria, and CM 2177-2 was the male parent bred from CIAT.

Molecular analysis of TDFs which showed differential expression during PPD was studied in MCOL 22 (Columbian) and Faroka (Indonesian). The study gave the evidence for involvement of important physiological and biochemical process during PPD (Huang *et al.*, 2001).

Biosynthesis and metabolism studies on scopoletin showed that specific peroxidases can metabolize scopoletin into an insoluble coloured product

(Buschmann *et al.*, 2000). The study was analyzed in roots of seven different cassava cultivars such as CM 7033-3, MBRA 337, MCOL 22, MDOM 5, MNGA 1, MNGA 2 and SM 985-9.

PPD susceptible, CM 523-7 and MCOL 1505 and a tolerant MPER 183 cassava lines were used for evaluating the potential sources of PPD tolerance that CIAT has developed and quantified the level of tolerance at different intervals after harvesting (Morante *et al.*, 2010).

Sanchez *et al.* (2013) evaluated the fluctuations in weight, and also biochemical and functional properties of starch in storage roots of a PPD tolerant, AM 206-5 and a susceptible, HMC-1 that were stored for 14 days.

2.4 b) National review on PPD resistance

In ICAR-Central Tuber Crops Research Institute, 5 cassava lines (BR-2, BR-5, BR-6, BR-10 and BR-105) were identified as resistant to post-harvest physiological deterioration and they are at field testing. These lines exhibit characteristic leaf morphology with purple colored young leaves, medium branching and cylindrical tuber shape with purple rind (rich in anthocyanin) and produce white fleshed tuber. Among them, BR-105 recorded high dry matter (45-47%) content with more latex content in the rind portion even after 30 days. The number of tubers ranged from 4-6 per plant (3-5 kg/plant). The tubers were found hard and sticky after cooking. It is found that the tuber can be stored for more than a month without deterioration and discoloration under room temperature. The BR-105 genotype can therefore be used as a parental line for breeding varieties with longer shelf life. The molecular nature of PPD tolerance in this BR lines is under progress.

2.5 Use of Molecular markers in cassava

Molecular markers are stigmatized as constant landmarks in the genome. They are defined as identifiable DNA segments found at specific locations in the genome showing nucleotide sequence polymorphism in different individuals within and between species.

The different molecular marker systems include Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Single Nucleotide Polymorphism (SNP), and Simple Sequence Repeat (SSR).

Molecular markers are useful in construction of high-resolution genetic and linkage maps, identifying and tagging useful genes affecting traits of interest, QTL identification and gene pyramiding, genetic diversity analysis, marker assisted selection (MAS) and positional cloning of useful genes (Varshney *et al.*, 2005). Table 1 details the genetic diversity studies and molecular characterization in cassava using different molecular markers.

Fregene *et al.* (1997) constructed a molecular linkage map from the female parent of an intra-specific cross (TMS 30572 X CM 2177-2) using 132 RFLP, 30 RAPD, three SSR and three isozyme markers. The markers defined 20 linkage groups spanning 931.6 cM. Another genetic linkage map was constructed from the male parent using 107 RFLP, 50 RAPD, one SSR and one isozyme marker. Beeching *et al.* (1993) used RFLPs in assessing genetic diversity in a collection of African cassava cultivars and recommended the use of RFLPS in revealing genetic diversity within and between species.

The CMD resistance and susceptibility was assessed in about 20 cassava landraces and nine elite cassava lines from Africa and 11 cassava accessions from Latin America using AFLP markers (Fregene *et al.*, 2000) and found a genetic divergence among these cassava cultivars. de Oliveira *et al.* (2012) evaluated 358 cassava genotypes using 390 SNPs for different quantitative characters like weight of shoot, yield of fresh root, dry matter content, content of amylase in starch fraction and yield of starch.

Lopez *et al.* (2005) identified 136 SNPs from EST and 50 SNPs from BAC-end sequences of five cassava cultivars (each having 11 kb sequence length). The identified SNPs differentiated cultivars with a total frequency of 1 per 62 bp. Direct sequencing was performed to study the sequence based polymorphism in nine genes which regulate cyanogenesis, starch synthesis and

stress/defense related pathways (Kawuki *et al.*, 2009). 26 SNPs were identified and they were used for sequence characterization and diversity assessment.

2.6 Simple sequence repeat (SSR) Markers

Microsatellites or SSR markers are tandem repeats of short (1-6 bp) sequences such as $(GT)_n$ or $(CAC)_n$ (Gupta and Varshney, 2000). It represents a significant portion of higher eukaryotic genomes. The fragment polymorphism is due to genomic points of variation within a species and is based on the variable number of repeat units. SSR markers are multi-allelic which are dispersed throughout the genome, and are highly polymorphic and co-dominant. SSR analyses are low cost, highly reproducible, transferable between laboratories and amenable for high throughput genotyping (Hamwieh *et al.*, 2005). They are suitable option for assessing genetic diversity, mapping and molecular characterization of cassava (Tabbi *et al.*, 1997).

Several studies reported the use of SSR markers for genetic diversity assessment in cassava (Fregene *et al.*, 2003; Raji *et al.*, 2004; Lokko *et al.*, 2005). Genetic diversity was studied among 521 accessions from the cassava core collection at CIAT using SSR markers revealing the effectiveness of these markers in selection of unique genotypes for core collection (Chavarriaga-Aguirre *et al.*, 1999). Akano *et al.* (2001) used bulk segregant analysis (BSA) method for identifying dominant CMD resistant gene (*CMD2*) and the presence of an allele of SSRY 28 was identified in the resistant parent and bulk. Research work on genetic variability study using 36 SSR markers was carried out in 12 released cassava varieties and 24 central Kerala collections (Lekha and Pillai, 2011).

A full-sib population involving Namikonga (tolerant parent) and Albert (susceptible parent) were used for mapping SSR-defined QTLs putatively associated with resistance to cassava brown streak disease (CBSD) (Kulembeka, 2010). Pariyo *et al.* (2013) studied the SSR diversity of cassava in relation to CBSD resistance in South, East and Central Africa using 13 SSR markers. The genetic diversity and molecular characterization was studied among 162 cassava varieties from different locations across Puerto Rico using 33 SSR markers. This

study revealed the presence of high level of genetic diversity in the unknown samples. Moreover, the study assumes that traditional practices of intercropping and incorporation of volunteer seedlings have resulted in the contribution of recombinant genotypes to cultivated cassava collections (Montero *et al.*, 2011).

Table 1. Cassava genetic diversity studies using different molecular markers

Marker	Populations	References
RAPD	19 accessions	Marmey *et al.*, 1993
	31 accessions - Brazil	Colombo *et al.*, 1998
	126 accessions	Colombo *et al.*, 2000
	24 accessions - Brazil and CIAT collections	Carvalho *et al.*, 2001
	50 accessions - Ghana	Asante *et al.*, 2003
	24 landraces - Tanzania	Herzberg *et al.*, 2004
	30 accessions - Latin America	Ferreira *et al.*, 2008
RFLP	80 accessions (cassava and wild species)	Beeching *et al.*, 1993
	45 accessions and wild species	Fregene *et al.*, 1994
AFLP	Cassava wild species	Roa *et al.*, 1997
	521 accessions	Chavarriaga-Aguirre *et al.*,
	8 accessions	1999
	69 accessions - cassava wild species	Wong *et al.*, 1999
	40 accessions	Elias *et al.*, 2000
	48 *Manihot* species - 12 primer pairs	Fregene *et al.*,2000
		Whankaew *et al.*, 2012
SSR	Heterozygosity - 48 accessions	Chavarriaga-Aguirre *et al.*,
	24 accessions	1998
	220 accessions, 33 wild - 5 SSR	Carvalho *et al.*, 2001
	283 accessions - 67 SSR markers	Olsen and Schaal, 2001
	38 accessions -10 SSR markers	Fregene *et al.*, 2003
	270 accessions - Nigeria	Elias *et al.*, 2004
	245 accessions - 35 SSR markers - Uganda	Raji *et al.*, 2004

	138 CMD resistant accessions - Nigeria	Kizito *et al.*, 2005
	5 improved lines, 62 CMD resistant and 10 CMD	Lokko *et al.*, 2005
	susceptible landraces	Lokko *et al.*, 2006
	36 genotypes - 16 SSR markers	
	160 accessions	Moyib *et al.*, 2007
	18 cultivars - 3 SSR primers	Peroni *et al.*, 2007
	21 cultivars and 1 wild accession	Bi *et al.*, 2010
	96 accessions	de Bang *et al.*, 2011
	Cassava from 162 locations across Puerto Rico - 33	Ribeiro *et al.*, 2011
	SSR markers	Montero *et al.*, 2011
	43 Ghana in farmer preferred accessions - 20 SSR	
	primers	Asare *et al.*, 2011
	CTCRI varieties and Central Kerala varieties	
	93 accessions - 14 SSR primers	Lekha *et al.*, 2011
	51 farmer preferred cassava, 15 elite lines - 6 SSR	
	markers	Turyagyenda *et al.*, 2012
	21 Tanzanian farmer preferred landraces - 20 SSR	
	markers	Elibariki *et al.*, 2013
	69 accessions - Kenyan germplasm - 7 SSR	
	markers	Ndung'u *et al.*, 2014
ISSR	Cassava - four cultivar	Zayed *et al.*, 2013
SNP	Cassava - SNP	Lopez *et al.*, 2005
	74 cassava genotypes	Kawuki *et al.*, 2009
	53 accessions	Ferguson *et al.*, 2012

MATERIALS AND METHODS

Chapter 3
MATERIALS AND METHODS

The experiment on PPD was conducted at ICAR-Central Tuber Crops Research Institute (CTCRI), Sreekariyam, Trivandrum (annual rainfall of 1250 mm; altitude 50 m.a.s.l.; mean annual temperature of 22-31°C; coordinates: latitude of 8° 32'N and longitude of 76 °55'E).

3.1 Plant materials

Five PPD resistant and twenty-three PPD susceptible cassava lines (Plate 1 and 2, Table 2) were selected for phenotyping and genotyping analysis with the objective of identifying PPD resistance associated marker. The selected cassava genotypes were planted in the field premises of ICAR-CTCRI. The laboratory experiments were performed at the Division of Crop Improvement, ICAR-CTCRI.

Plate 1. Plot of PPD resistant BR-105 (left) and PPD susceptible H-1687 (right)

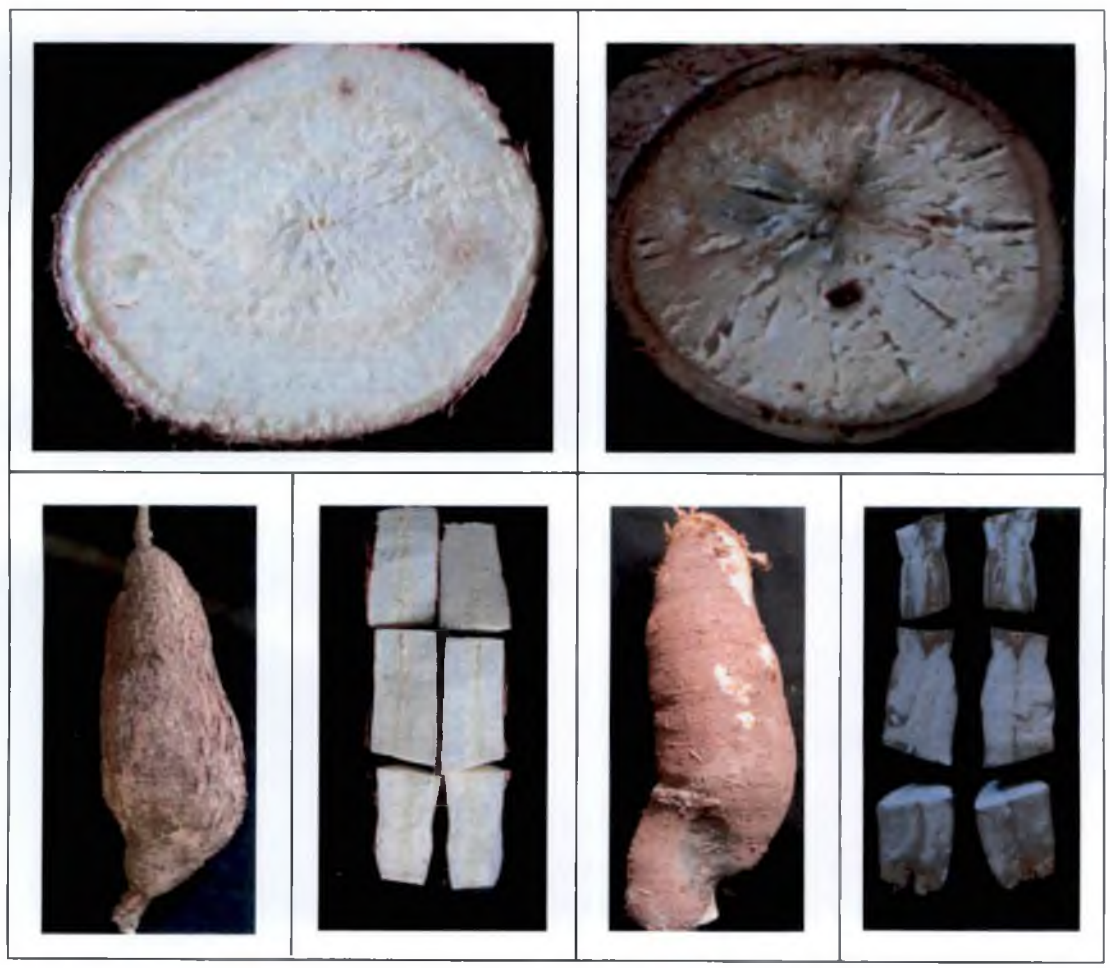


Plate 2. Cross-section and transverse section of the tuber, BR-105 (left) and H-1687(right) after 7 days of storage

Table 2. List of selected cassava accessions (PPD resistant and susceptible cultivars)

Sl. No.	Resistant Cultivar	Code
1.	BR 2	R1
2.	BR 5	R2
3.	BR 6	R3
4.	BR 10	R4
5.	BR 105	R5

Sl. No.	Susceptible Cultivar	Code	Sl. No.	Susceptible Cultivar	Code
6.	Albert	S1	18.	H-1687/Sree Visakh	S13
7.	Ambakadan	S2	19.	Harsha	S14
8.	CI-731/Sree Vijaya	S3	20.	MNGA	S15
9.	CI-732/Adukkumuttan	S4	21.	R-73	S14
10.	CI-848/Venjaramoodan	S5	22.	S-856/Sree Prakash	S15
11.	CI-849/Narayankappa	S6	23.	Sree Athulya	S16
12.	CO2	S7	24.	Sree Apoorva	S17
13.	CO4	S8	25.	TCH1/ Sree Rekha	S20
14.	CMR 100	S9	26.	TMS 30572	S21
15.	CR-43/11	S10	27.	Vellayani Hraswa	S22
16.	H-165	S11	28.	96/160	S23
17.	H-226	S12			

3.2 Phenotypic assessment of PPD

3.2.1. Evaluation of PPD intensity

Twenty five medium-sized tubers consisting of five PPD resistant and 20 susceptible lines were chosen and stored under ambient conditions in a room. For evaluating the scale of deterioration, the tubers were visually scored for PPD (Plate 3a and 3b) at different intervals of storage (3, 7 and 21 days). Scoring was performed with slight modifications from Wheatley's method (1985). A score ranging from 1 to 5 was assigned to each root slice, based on the intensity of PPD (1 = no damage, 2 = 25%, 3 = 26-50%, 4 = 51-75% and 5= 100% for fully damaged root slice).

The scoring of PPD was performed during first, third, seventh and twenty first day of storage. As the susceptible lines undergo complete deterioration after 3-5 days, PPD scores of these tubers were taken only until third day.

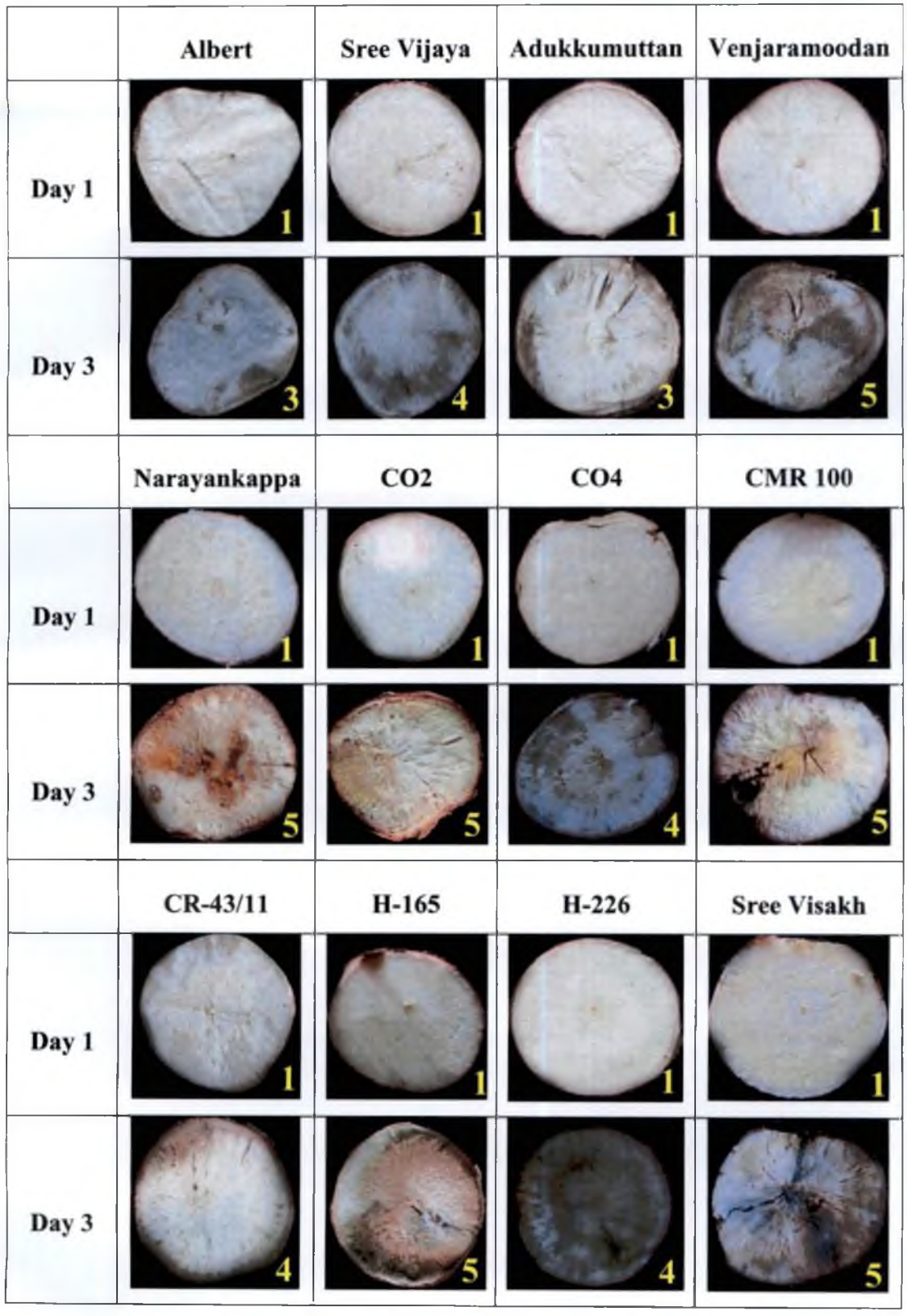


Plate 3a: Visual scoring in susceptible lines (1st and 3rd day)

(PPD score was shown in yellow in the right side corner of each section)

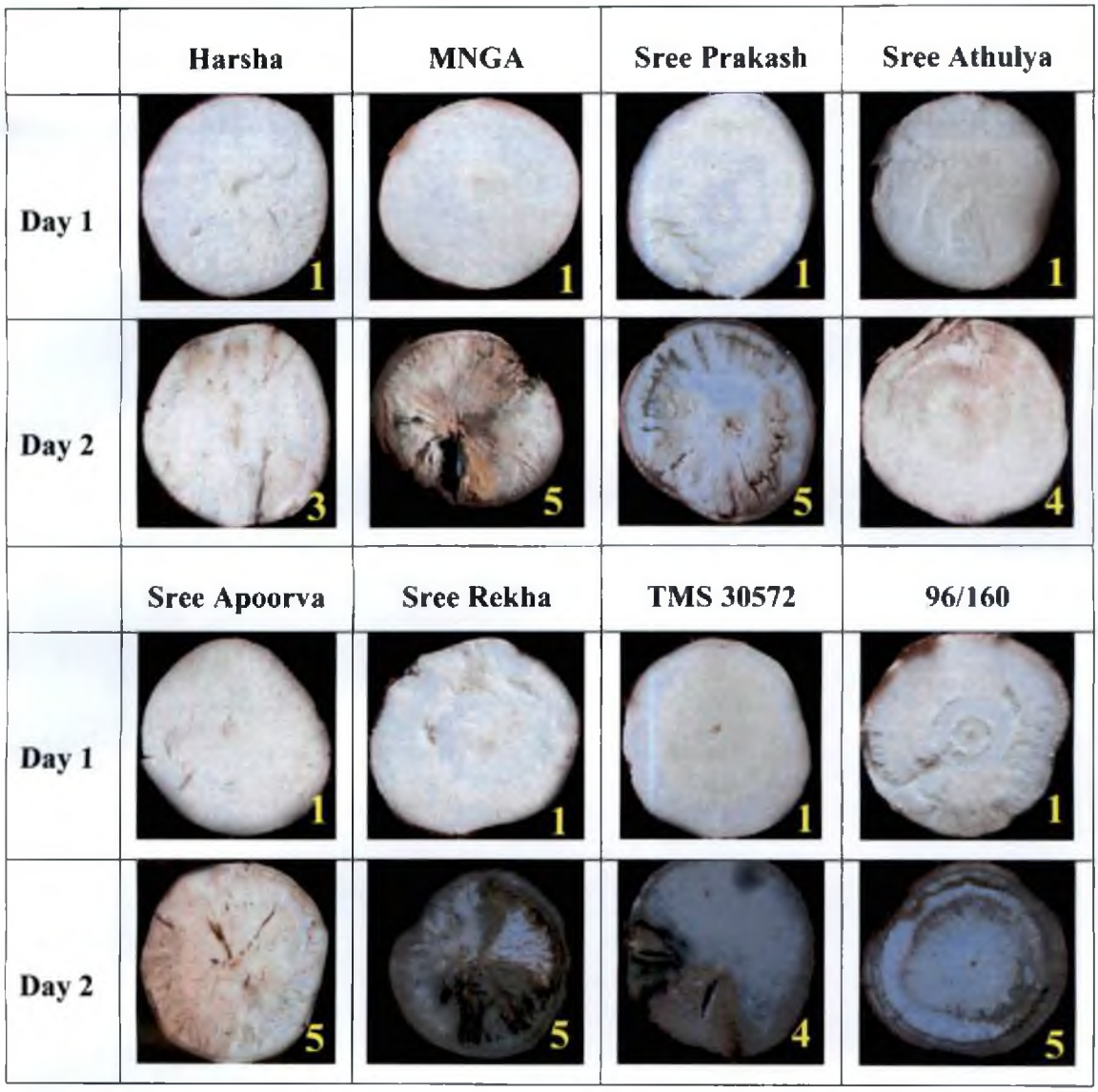


Plate 3a: Visual scoring in susceptible lines (1st and 3rd day)

(PPD score was shown in yellow in the right side corner of each section)

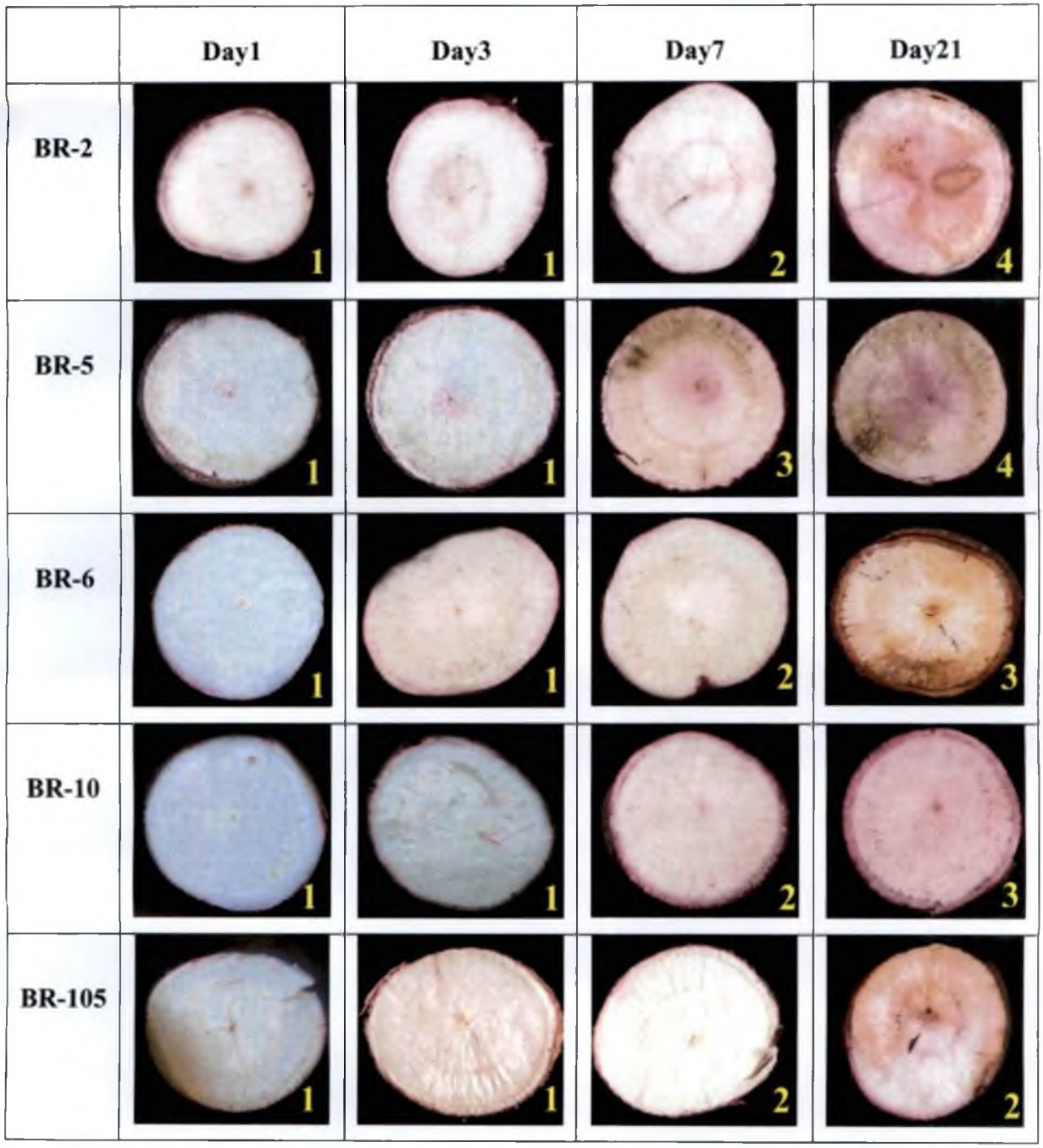


Plate 3b: Visual scoring in resistant lines (1st, 3rd, 7th and 21st day)

(PPD score was shown in yellow in the right side corner of each section)

3.2.2 Determination of dry matter and starch content

The dry matter and starch content of the tubers stored at different intervals (first, third, seventh and 21st day) were determined to analyze the variation in dry matter and starch content in PPD resistant/susceptible lines during storage.

To estimate the dry matter content, 50g of fresh cassava roots were chopped uniformly into small pieces and dried in an oven at 60°C for 72hrs with two replications. Dry matter was expressed as the percentage of dry weight relative to fresh weight.

Based on the dry matter data, the starch content of storage roots was calculated using a reported conversion formula: $y = 0.6945x - 6.34587$, where y is the starch content (%) and x is the dry matter content (Wang *et al.*, 1989).

3.3 Genomic DNA extraction

DNA was isolated from leaf samples by SDS method as described by Dellaporta *et al.*, (1983) with slight modifications. Fresh and young leaf samples were collected from the field. Approximately 1g of leaf tissue was frozen in liquid nitrogen, ground in a clean pestle and mortar along with 2% PVP. 20µl β-mercaptoethanol was added to 15ml extraction buffer (Appendix Ia) in a fresh 20ml centrifuge tube and mixed well. The ground tissue was added to the extraction buffer, mixed to form slurry. 1ml of 20% SDS was added and incubated in water bath (Memmert) maintained at 65°C for 1hr with gentle mixing. After incubation, 5ml of 5M potassium acetate was added, gently mixed and incubated on ice at 4°C for 20min. Samples were then centrifuged at 12000rpm (Sigma laborzentrifuge) for 20min at 4°C and supernatant was transferred to fresh 2ml tubes. DNA pellet was precipitated by adding 1ml chilled isopropanol (2/3 volume) and gently inverted 8-10 times. The samples were placed in a freezer at -20°C for 30min and centrifuged at 12000rpm for 15min at 4°C. The supernatant was decanted and the pellets were resuspended in 500µl TE buffer (Appendix Ii). A total of 5µl of RNase (10 mg/ml) was added and incubated in water bath at 37°C for 1hr. Equal volumes of chloroform and isoamyl alcohol (24:1) was added and mixed by inverting twice. Samples were centrifuged

at 12000rpm for 15min at 4°C and a fixed volume of upper aqueous phase was carefully transferred to fresh tubes.

To purify the DNA, 10µl of sodium acetate was added along with 500µl chilled ethanol, placed in -20°C for 2hrs and then centrifuged at 12000rpm for 10min at 4°C. Supernatant was discarded and the DNA pellet was re-precipitated in 500µl 70% ethanol, dried and washed again with 70% ethanol. The DNA pellet was air dried at room temperature for 1 hr and dissolved in 100µl sterile distilled water and stored in -20°C.

3.4 Agarose gel electrophoresis

Genomic DNA and PCR products were routinely separated using agarose gel electrophoresis. Agarose concentration varied from 0.8% to 2% (w/v) depending on the predicted size of DNA fragments. Agarose (Sigma) was suspended in Tris/Borate EDTA buffer (100ml 1X TBE; Appendix IIa) at the requisite concentration and boiled in microwave oven for 2min. The solution was allowed to cool and ethidium bromide was added (0.5µg/ml) and mixed. A sealed casting tray was already placed with a comb having the requisite number of wells. The gel was then poured in casting tray without any bubbles and allowed to set. When the gel gets solidified, comb was removed and transferred into gel tank which was filled with 1X TBE buffer. DNA samples were mixed with DNA loading buffer to a ratio of 1:2 and loaded into the wells of the solid agarose gel. The gels were run at 80 volts for 30min. The band was visualized on a UV transilluminator and the gel was imaged using a gel documentation system (Alpha Imager, USA).

3.5 DNA Quantification

DNA was quantified using Nanodrop (DeNovix-DS11+Spec) spectrophotometer. NanoDrop method determines absorbance at 260nm by taking reading at wavelength of 260nm. For double-stranded DNA,

OD at 260nm = 50ng/µl

The concentration and purity of DNA samples were determined by taking absorbance at 260/280nm. The tip of the nanodrop (where the sample should be placed) was cleaned using a drop of sterile distilled water and kimwipe. The spectrophotometer was calibrated with 1.5µl blank sample of sterile distilled water. After calibration, quantification was done by loading 1.5µl of each DNA sample onto the tip of the Nanodrop. The tip was cleaned after taking each reading. A260 and A260/A280 value for the successive readings was noted.

Based on the reading obtained from the Nanodrop, genomic DNA was diluted to a working concentration of 50ng/µl and stored at 4°C and the remaining stock DNA samples were stored in -20°C.

3.6 PCR Amplification using SSR and gene specific primers

3.6.1 Designing of gene specific primers

The gene specific primers were designed (Table 3) based on the genes that control PP (Phenyl Propanoid) pathway during PPD response. The PPD related genes selected for the study include different isoforms of ascorbate peroxidase (APX), catalase (CAT), phenylalanine ammonia lyase (PAL), p-linamarase (p-LIN), thioredoxin peroxidase (TRX), glutathione peroxidase (GPX), 1-amino cyclopropane 1-carboxylate oxidase (ACCO).

The nucleotide sequences of these PPD related genes were searched in the NCBI (National Centre for Biotechnology Information) database and 20 different gene sequences were retrieved. These sequences were used for designing PPD specific primers using Primer3plus software. The annealing temperature of the designed primers ranged from 54.4-59.4°C.

Table 3. Gene specific primers designed for the study

Sl. No.	Primer	Forward primer	Reverse primer	Tm (°C)	Size (bp)
1.	APX2-1	agcccatcaaggagcagttc	cctcagcaaatccgagctca	59.4	504
2.	APX2-2	ttgagcccatcaaggagcag	ctacagcttacgcctcagca	54.4	519
3	CAT2-1	agatgcttgcgttcgatcct	cagttactggtggtgccact	53.35	548
4.	CAT2-2	gctgctggcaactatcctga	atgcgagggtctgataaggc	54.4	599
5.	PAL1-1	aagtcgttagggcccaatgg	cccctggaaattgcctccat	54.4	540
6.	PAL1-2	ggcccaactccaagtcgtta	ttggggtcccctggaaattg	54.4	558
7.	PAL2-1	gaacaaggccttgcatggtg	tgagcaagtccttctcgcag	54.4	530
8.	PAL2-2	ggcttctttgagttgcagcc	tgatgcaatggccaaacgtg	53.35	519
9.	pLIN -1	agtttttgcccctggtcgat	gacgagtaagcctgtggacc	54.35	522
10.	pLIN-2	tcctcaagcactgcaagaca	ttggctgcttgcacatcaac	52.3	596
11.	TRX-1	cacgtcaaccactggtctca	tcccctcaaagcaattcccct	58.35	580
12.	TRX-2	gaagaaatggcttgctccgc	tctcctagaccgcctgactt	59.4	509
13.	GPX-1	tctgtgatgcctggtgaaga	ggaaggacgtttctctcagca	58.55	533
14.	GPX-2	cctgtatatctgtgatgcctggt	tgggaaggacgtttctctca	58.95	543
15.	ACCO1-1	tatggactgggagagcacct	cactgcatcacttccagggt	59.4	526
16.	ACCO1-2	cgccgtccaaactgagatca	ctggtaccgtctgtctgagc	60.4	507
17.	ACCO2-1	tgtgggaacaaaagtggcca	gagaaccctgtgaaggagca	58.35	501
18.	ACCO2-2	acttcatttggcaccgtcca	tcgcctgcaggattgtagaa	57.3	502
19.	ACCO3-1	aaagtctccagcacagacgg	gcctgtagggacttggcatt	59.4	548
20.	ACCO3-2	ggtccaaagtctccagcaca	tccacgttttgtcgcctgta	58.35	566

NB: NB: APX - Ascorbate peroxidase, CAT - Catalase, PAL - Phenylalanine ammonia lyase, p-LIN - p-Linamarase, TRX - Thioredoxin peroxidase, GPX - Glutathione peroxidase, ACCO - 1-Amino cyclopropane 1-carboxylate oxidase

3.6.2 SSR primer selection

17 different SSR primers (Table 4) were selected for identifying polymorphism among PPD resistant/susceptible cassava lines. The annealing temperature ranged from 55-59°C.

Table 4. SSR primers used for analysis of polymorphism

Sl. No.	Locus	Forward primer (5'-3')	Reverse primer (5'-3')	Product size (bp)
1.	SSRY6	tttgttgcgtttagaaaggtga	aacaaatcattacgatccatttga	298
2.	SSRY7	tgcctaaggaaaattcattcat	tgctaagctggtcatgcact	250
3.	SSR 8	agtggtttgagaagactggtga	tttccaaaatggaacttcaaa	288
4.	SSRY21	cctgccacaatattgaaatgg	caacaattggactaagcagca	192
5.	SSRY28	ttgacatgagtgatattttcttgag	gctgcgtgcaaaactaaaat	180
6.	SSRY32	caaatttgcaacaatagagaaca	tccacaaagtcgtccattaca	298
7.	SSRY34	ttccagacctgttccaccat	attgcagggattattgctcg	279
8.	SSRY35	gcagtaaaaccattcctccaa	ctgatcagcaggatgcatgt	282
9.	SSRY36	caactgtttcaaccaacagaca	attctcgtgaactgcttggc	134
10.	SSRY39	tcaatgcataggattttgaaagta	aatgaaatgtcagctcatgct	293
11.	SSRY40	tgcatcatggtccactcact	cattcttttcggcattccat	231
12.	SSRY44	ggttcaagcattcaccttgc	gactatttgtgatgaaggcttgc	194
13.	SSRY50	ccgcttaactccttgctgtc	caagtggatgagctacgcaa	271
14.	SSRY103	tgagaaggaaactgcttgcac	cagcaagaccatcaccagttt	272
15.	SSRY106	ggaaactgcttgcacaaaga	cagcaagaccatcaccagttt	270
16.	SSRY235	cagctttgccatccaatttt	cagcaaaatgacatgagtgtatctc	216
17.	SSRY324	cgcttacaacaccaccttca	gcttgatctcagccatgtca	206

PCR amplification using SSR and gene specific primers was performed initially in a small population of 10 cassava lines consisting of five PPD resistant (R1-R5) and five PPD susceptible (S16-S19, S21) lines (refer Table 2).

The PCR reaction was performed in an automatic Thermal Cycler (Bio-Rad: C1000^{TM}). The PCR reaction mixture cocktail is shown below:

Reagents	Concentration	Volume (µl)
Template DNA	50 ng/µl	2.0 µl
Primer	2 µM	2.0 µl
Taq buffer	10X	2.0 µl
dNTPs	0.2 mM	0.1 µl
Taq DNA polymerase	0.03 U	0.1 µl
Sterile water		13.8 µl
Total		20 µl

The following thermocyling PCR profile was set in PCR machine.

Initial denaturation	Denaturation	Annealing	Extension	Final extension	Hold
94°C	94°C	59°C	72°C	72°C	4°C
5min	1min	2min	2min	5min	∞
Run for 30 cycles					

The PCR products obtained were tested for amplification by 2% agarose gel electrophoresis.

3.7 Polyacrylamide Gel Electrophoresis (PAGE)

PCR amplified products were separated using DNA-PAGE (Sequi-Gen® GT, BIO-RAD sequencing gel, USA). The PCR products (3-4µl) were run in a 5-6% denaturing polyacrylamide gel at 100 watts for 1-2hrs and amplified fragments were visualized by silver staining (Panaud *et al.*, 1996).

i) Cleaning and Assembling the Glass Plates

- The small glass plates were soaked in 2% (w/v) sodium hydroxide overnight and the plates were cleaned in tap water using scrubber and rinsed in distilled water.
- Using absolute alcohol, the small and large plate/IPC was wiped neatly.
- 2ml of repellent (Laboline) was applied on large plate and spread uniformly with kimwipes. Similarly, 2ml of bind silane was applied on small plate and spread uniformly with kimwipe paper.
- Spacers were wiped with alcohol and each spacer was placed on either side edge of large plate and carefully shifted the small plate over the large plate/IPC (cleaned surface facing the larger plate) such that it is seated uniformly with the spacers.
- The plates together were gently lifted in vertical position and clamped on both sides using side lever clamp set and locked by moving the levers close to the IPC panel.
- The bottom of assembled unit was placed into the precision caster base with the bottom edge resting against the gray gasket inside. The cam pegs were pushed inside to connect the base to the clamps and locked the handles by turning in a perpendicular direction.
- The IPC assembly with the attached caster base was laid down flat on a bench and the comb (60 wells) was placed in a horizontal position between the plates.

ii) Gel matrix preparation and gel casting

- 100ml of 6% polyacrylamide denaturation solution was prepared in a conical flask and 600µl of 10% ammonium persulphate (APS) solution and 60µl of TEMED (Tetra Ethyl Methyl Ethylene Diamine) were added and mixed well by gentle swirling.

- The required gel volume was pulled into a 100ml syringe which was fitted to the injection port and slowly injected the gel solution until the gel matrix spreads uniformly throughout the plate without causing any bubble formation.
- The gel was allowed to polymerize for 30-60min.

iii) Sample loading and gel running (electrophoresis)

- After polymerization, the comb, syringe and precision caster base were detached and the gel plate unit was mounted into a universal base and a stabilizer bar was placed that allow the IPC assembly to stand vertical.
- The upper and lower buffer chambers were filled with 1X TBE running buffer. After flushing the wells, the top and bottom safety covers were fitted onto the appropriate electrode which was connected to a power pack. A temperature probe was also connected to monitor and control the gel temperature. The gel was kept for 15-30min pre-running.
- To 20µl PCR product, 8µl gel loading dye was added and mixed. Samples were denatured at 95°C for 5min and snap cooled on ice.
- After pre-run, the wells were flushed again; 3µl of each denatured PCR products were loaded onto the gel.
- The electrophoresis was resumed and allowed to proceed at 100 watts (constant) for 1-2hrs based on SSR product size (bp).
- Finally plates were dismantled from the electrophoresis apparatus for silver staining.

iv) Silver staining of PAGE gel

After careful removal of the small plate containing gel from the assembly, the following staining protocol was followed.

- **Fixing**

Gel was soaked in fixer (Appendix IIId) for 30min (until the dye disappears) with mild shaking in a shaker (RiVOTEK). Then the plates were washed twice in double distilled water for 15 minutes.

- **Staining**

Gel was soaked in staining solution (Appendix IIIe) for 30min with mild shaking followed by a single dip in double distilled water (10s).

- **Developing**

Gel was soaked in developer (Appendix IIIf) for 3-5min or till bands appeared. Again gel was soaked in fixer for 2-5min to stop the reaction followed by washing with double distilled water for 15-30min.

Gel documentation and scoring of bands

The gel was dried and scanned using a computer scanner. The clear bands showing polymorphism was identified. The polymorphic alleles obtained with each primer pair was scored for their presence (1) or absence (0).

3.8 Sequencing

a) Cycle Sequencing

Cycle sequencing is a modification of the traditional Sanger sequencing method. This method uses di-deoxynucleotides in a polymerization reaction to create a nested set of DNA fragments with di-deoxynucleotides at the 3' terminus of each fragment. It employs a thermostable DNA polymerase which can be heated to 95°C and retain the enzyme activity for repeated use.

The PCR reaction cocktail for cycle PCR is given below.

Reagents	Concentration	Volume (µl)
Ready reaction mix (BigDye Terminator)	2.5X	0.5µl
Dilution mix	5X	1µl
Template	100-200ng/µl	1µl
Primer (either forward/reverse)	2µM-10µM	2µl
MilliQ water	-	4µl
Total		10µl

The following thermocycling PCR profile was set for cycle sequencing.

Steps	Temperature	Duration
Initial denaturation	96°C	1min
Denaturation	96°C	10sec
Annealing	50°C	5sec
Extension	60°C	4min
Hold	4°C	∞
Run for 25 cycles		

b) Clean-up

The cycle sequencing products were subjected for cleanup prior to sequencing to remove unincorporated dye terminators and salts that may compete for capillary electrophoretic injection. The following protocol for clean-up (Appendix IV) was followed.

- A master-mix I (10µl sterile water and 2µl 125mM EDTA; per reaction) was prepared. 12µl of master-mix I was added to each PCR tubes containing 10µl product.
- A second master-mix II (2µl 3M sodium acetate; pH 4.6 and 50µl ethanol) per reaction was prepared. 52µl of master-mix II was added to each reaction.
- The contents were mixed well and incubated for 15min at room temperature.
- Then centrifuged at 12000g for 20min at room temperature.
- Supernatant was removed and 250µl 70% ethanol was added and centrifuged at 12000rpm for 10min at room temperature.
- Supernatant was removed and the pellet was dissolved in 10-12µl HiDi™ formamide and mixed by vortexing.
- 10µl of each sample was loaded onto respective wells. The 96 well-plate was sealed with septa strip and placed in a plate holder and the assembled plates were covered from above using a plate retainer. The assembled plate unit was then placed in auto-sampler of Genetic Analyzer (Applied Biosystems-3500).

- The sequencing analysis for the SSR primers were set in the machine and the samples were run. The data file ("filename.abi") was collected at the end of the program.

3.9 Data Analysis

3.9.1 Phenotypic analyses

a) PPD scoring

The mean PPD score for each root was calculated manually by averaging the scores of PPD resistant/susceptible lines for the different intervals of storage. The mean PPD score was compared among the resistant and susceptible lines and a bar diagram representing the PPD score assessment was plotted.

b) Dry matter and starch content analysis

The quantitative data of dry matter and starch content for PPD resistant and susceptible lines were analyzed separately using SAS system version 9 (SAS, 2010) software.

The first data included DM and starch content of resistant and susceptible lines until 3rd day of storage and the second data included resistant lines that remained as PPD resistant for 1, 3, 7 and 21 days. This classification of data was made to note that susceptible roots deteriorated after 3-5 days. Therefore, the data comparison of susceptible with the resistant was made for first and third day of storage. The significance of each treatment was tested at 5% probability level.

3.9.2 Molecular data analysis of SSR markers

a) Cluster analysis and PCA

The genotyping data were subsequently used to determine genetic relationships among the ten PPD resistant/susceptible lines which are screened using 17 SSR markers. The scoring data in the form of binary values was used for cluster analysis in NTSYSpc (Numerical Taxonomy System for personal computer). The PCA (2D plot-Principal Component Analysis) and the dendrogram clustering

were performed based on the UPGMA, unweighted pair-group method with arithmetic average using the NTSYS 2.21f software (Rohlf, 2000).

b) Simple Matching (SM)

The genetic associations between genotypes were calculated by Simple Matching Coefficient (SMC). For pair wise comparisons, SMC was calculated based on the similarity matrix generated using the SIMQUAL of NTSYSpc.

c) Percentage of polymorphism

The percentage of polymorphism was calculated based on the score data of 17 SSR primers. The total number of alleles and the number of polymorphic bands produced by each SSR primer was calculated.

d) Polymorphism Information Content (PIC)

The PIC values revealing the ability of each primer to distinguish the ten cassava lines were calculated for each of the 17 SSR primers.

Based on the scoring data, PIC value and the expected heterozygosity were calculated using a reported formula. The formula used for PIC calculation is:

$$PIC = 1 - \sum n(Pi)^2$$

where P is the proportion of number of alleles present in genotypes and n is the total number of alleles present in a primer. Primers that have PIC value ranging from 0.50-0.70 were selected.

3.10 Sequencing data analysis

The sequenced data obtained in the 'abi file format' was analyzed in DNASTAR version 9.0 software (LASERGENE) for sequence based polymorphism studies. Sequence assembly and contig search was done using CAP3 assembling software. The resulting conserved sequence was aligned and viewed in Clustal Omega software (EMBL) for identification of sequence based polymorphism.

3.11 Validation of SSR polymorphism

The primer which exhibited a polymorphic difference among the five resistant and five susceptible lines was validated among the total 28 cassava lines for further confirmation.

3.12 Attempts made for CAPS marker development

For the conversion of SSR markers into CAPS, the clustal alignment file ("filename.aln") showing the sequence polymorphism was used as an input file in CAPS designer tool of SGNCAPS software and searched for the corresponding restriction enzymes.

RESULTS

Chapter 4
RESULTS

The present study was carried out to identify the marker linked to post-harvest physiological deterioration (PPD) resistance in cassava lines. The results of the experiments conducted are described below.

4.1 Selection of plant material

Among the PPD resistant and susceptible cassava lines planted at ICAR-CTCRI, five PPD resistant and 23 PPD susceptible cassava lines were selected for phenotypic and genotypic assessment of PPD with the aim of identifying marker linked to PPD resistance in cassava.

4.2 Phenotypic analysis

4.2.1 PPD score analysis

The intensity of deterioration was examined in 25 cassava lines and the degree of PPD was scored visually (Wheatley *et al.*, 1985) by observing the level of deterioration at subsequent intervals of storage (1, 3, 7 and 21 days). The assessment of PPD was based on PPD score demonstrated in Plate 3a and 3b. The PPD evaluation results showed that BR lines are highly resistant to PPD for the first seven days and symptoms of discolouration was observable only after seventh day. The susceptible lines were PPD sensitive and showed symptoms of blue streaking from the second day, with the commencement of microbial rotting from third day after storage.

The mean PPD score after three days ranged from 1-3. The highest PPD score after three days (score-5) was observed for 55% of the selected lines which included susceptible lines (Venjaramoodan, Narayanakappa, CO2, CMR-100, Sree Visakh, MNGA, Sree Prakash, Sree Apoorva, Sree Rekha and 96/160) having a mean PPD score of 3. The lowest PPD score (score-1) after three days was observed for the resistant BR lines (BR-2, BR-5, BR-6, BR-10 and BR-105). The highest PPD score (score-3) for the BR lines after seven days accounted for

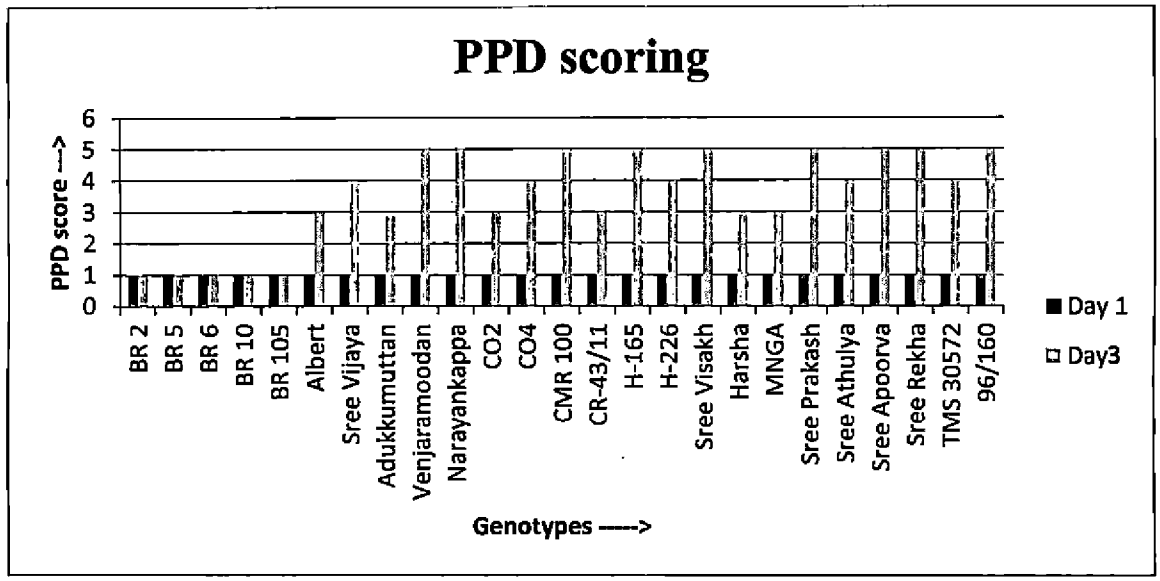


Fig 2. Distribution of PPD scores in resistant/susceptible lines during 1st and 3rd day

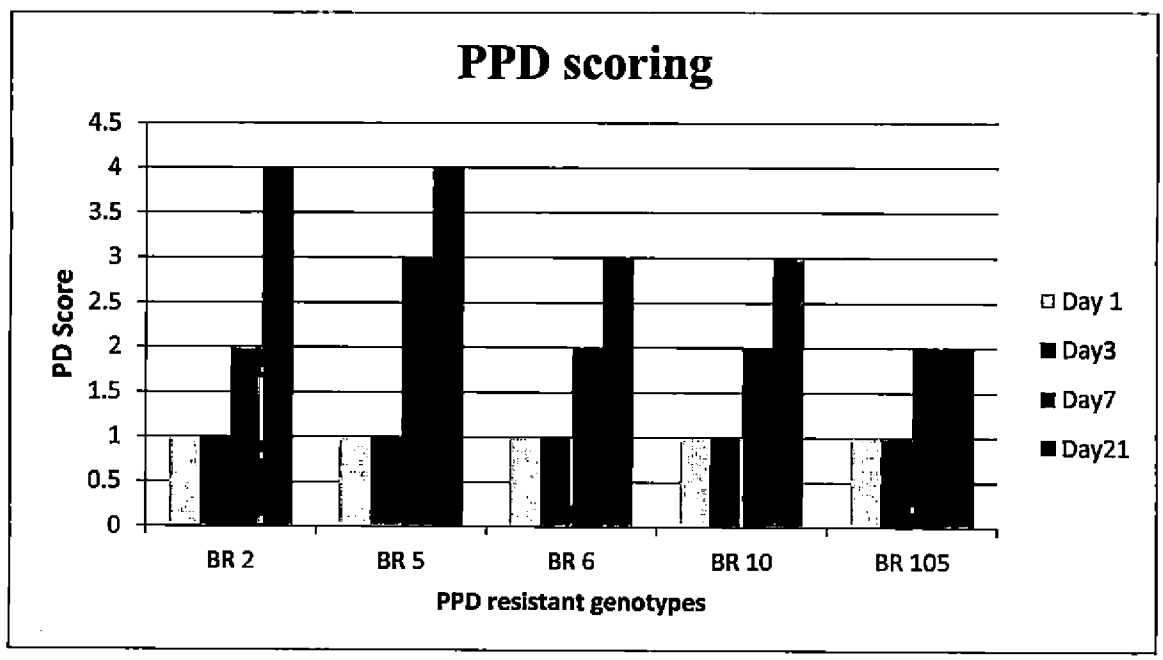


Fig 3. Distribution of PPD scores in resistant lines in 3, 7 and 21 days

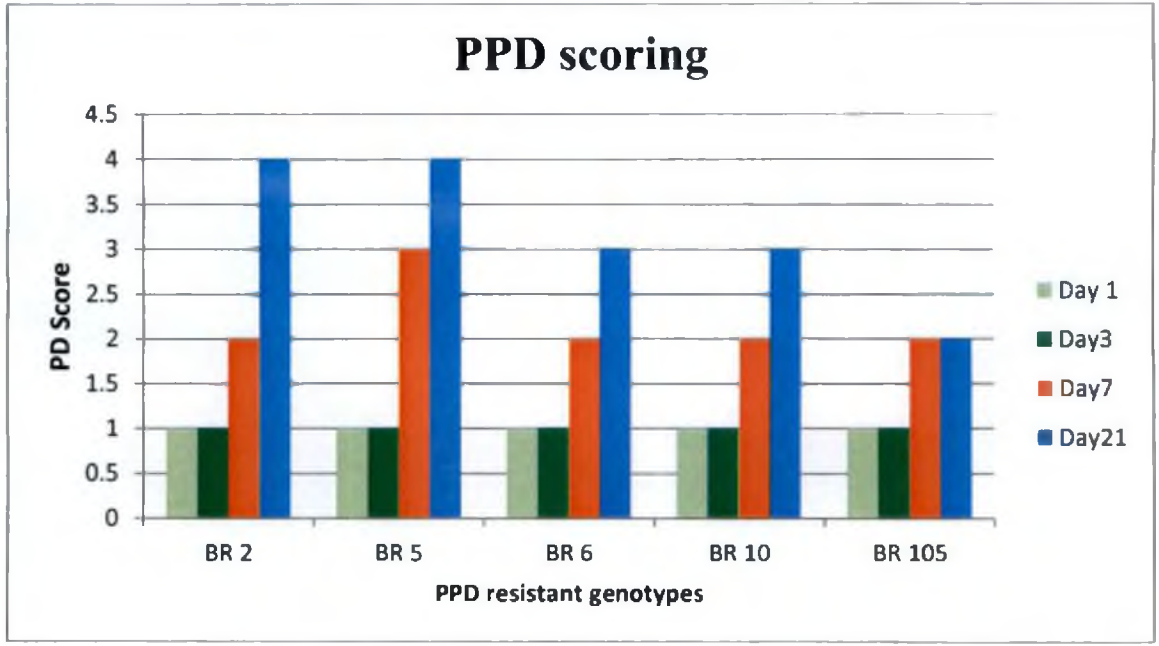


Fig 3. Distribution of PPD scores in resistant lines in 3, 7 and 21 days

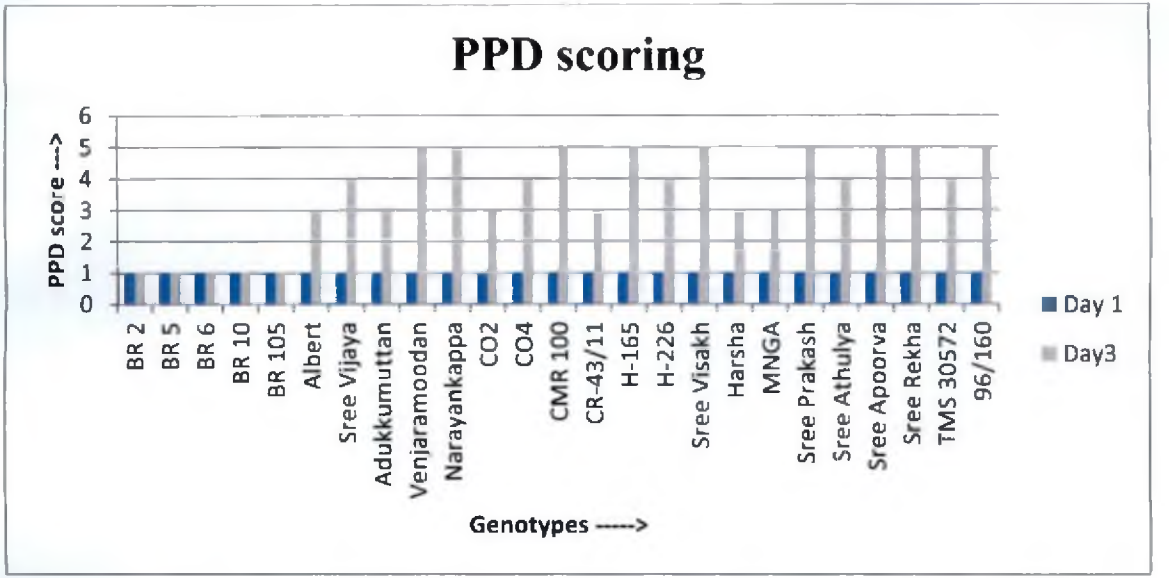


Fig 2. Distribution of PPD scores in resistant/susceptible lines during 1st and 3rd day

BR-5 line and after 21 days, the PPD score was maximum (score-4) for BR-2 and BR5 with a mean value of 2.25 and 2.0. The mean PPD after 21 days ranged from 1.5-2.25. BR-105 line was least susceptible with a mean value of 1.5 which survived from microbial attack even after 21 days and is visually identified to be the most resistant line. A bar diagram was also plotted based on the PPD scores in both resistant and susceptible lines (Fig. 2 and 3). The increase in PPD score observed in susceptible lines depends on the duration of storage.

4.2.2 Dry matter and starch content analysis

The dry matter content of five resistant and 25 susceptible cassava lines were determined by oven dry method. Based on the dry matter data, starch content was also calculated using a reported formula (Wang *et al.*, 1986). The starch and dry matter data was statistically analyzed using SAS system version 9.0 software to understand the variation in the quantitative data obtained in PPD resistant/susceptible lines during 1st, 3rd, 7th and 21st day after storage. The data comparing resistant and susceptible lines after three days of storage and the data of resistant tubers alone after 3, 7 and 21 days were analyzed separately because the data for susceptible lines were not determined due to its decay within three days. A significant difference between the resistant/susceptible genotypes was observed for both dry matter and starch content (Table 5). The dry matter and starch content showed significance in the data and the means were on par in the PPD resistant/susceptible lines at 5 % level of significance (P>0.05).

In the first data (3 days storage), BR-105 recorded high dry matter of 48.42% (starch-37.5%) whereas MNGA obtained the least value of 25.1% (starch-16%). In the second data (3, 7 and 21 days storage), a gradual decline in the dry matter and starch was observed for the five resistant lines at subsequent intervals. Among them, the highest value was occupied by BR-105 and the value showed a decrease from 48.6% (starch-35.91%) in the third day to a value of 46% (starch-33.69%) after 21 days.

Table 5: Data analysis of dry matter and starch content in different intervals

	1st day	3rd day		7th day		21st day	
Genotype	**FW**	**DM**	**Starch**	**DM**	**Starch**	**DM**	**Starch**
BR2	50	48.42^{a}	35.75^{a}	44.9^{d}	32.69^{d}	42.16^{e}	30.31^{e}
BR5	50	46.84^{bac}	34.38^{bac}	46.8^{abc}	34.34^{abc}	45.59^{bcd}	33.29^{bcd}
BR6	50	46.99^{ba}	34.51^{ba}	46.00^{bcd}	33.65^{bcd}	45.09^{cd}	32.85^{cd}
BR10	50	47.00^{ba}	34.51^{ba}	47.3^{ab}	34.78^{ab}	46.35^{bcd}	33.95^{bcd}
BR105	50	48.6^{a}	35.91^{a}	47.15^{ab}	34.65^{ab}	46.05^{bcd}	33.69^{bcd}
Albert	50	43.69^{fg}	31.64^{fg}				
Sree Vijaya	50	30.82^{n}	20.45^{n}				
Adukkumuttan	50	43.07^{fhg}	31.10^{fhg}				
Venjaramoodan	50	45.74^{bdec}	33.42^{bdec}				
Narayankappa	50	42.25^{ihg}	30.39^{ihg}				
CO2	50	29.05^{n}	18.91^{n}				
CO4	50	46.79^{bdac}	34.34^{bdac}				
CMR 100	50	30.81^{n}	20.44^{n}				
H-165	50	39.63^{lk}	28.11^{lk}				
H-226	50	39.87^{lkj}	28.31^{lkj}				
H-1687	50	34.32^{m}	23.49^{m}				
Harsha	50	44.84^{fdec}	32.64^{fdec}				
MNGA	50	25.81^{o}	16.09^{o}				
Sree Prakash	50	37.92^{l}	26.62^{l}				
Sree Athulya	50	44.09^{feg}	31.99^{feg}				
Sree Apoorva	50	42.50^{ihg}	30.61^{ihg}				
Sree Rekha	50	41.05^{ikj}	29.34^{ikj}				
TMS 30572	50	47.44^{ba}	34.9^{ba}				
96/160	50	44.93^{fdec}	32.71^{fdec}				

Means with same letters are not significantly different ($P<0.05$).

NB:

FW: Fresh Weight

DM: Dry Matter Content (%)

4.3 Isolation of Genomic DNA

Genomic DNA was isolated from fresh young leaves using Dellaporta method. The quality of the isolated DNA was checked in 0.8% agarose gel and the quantity was determined in Nano-spectrophotometer.

4.4 Quantification of DNA

The concentration of DNA present in each sample was determined by taking the absorbance reading at 260nm and 280nm in Nano-spectrophotometer. The quantity of DNA in different samples varied from 280-4500ng/µl. After quantification, all the samples were diluted to a uniform concentration of 50ng/µl and the uniformity was tested in agarose gel electrophoresis.

4.5 Checking the quality of DNA

The quality of each DNA sample was tested in 0.8% agarose gel with ethidium bromide dye. The gels were documented in a gel documentation system. The results showed intact genomic DNA without any sheared bands (Plate 4).

4.6 Designing of gene specific primers

In total, 20 primer sets were designed that corresponds to different PPD related genes (APX, CAT, PAL, TRX, GPX and ACCO) actively involved in PP pathway regulating PPD (refer Table 3). The nucleotide sequences of the respective primers were retrieved from NCBI database. The sequences were designed using Primer 3 Plus Software with GC content of 45-60%.

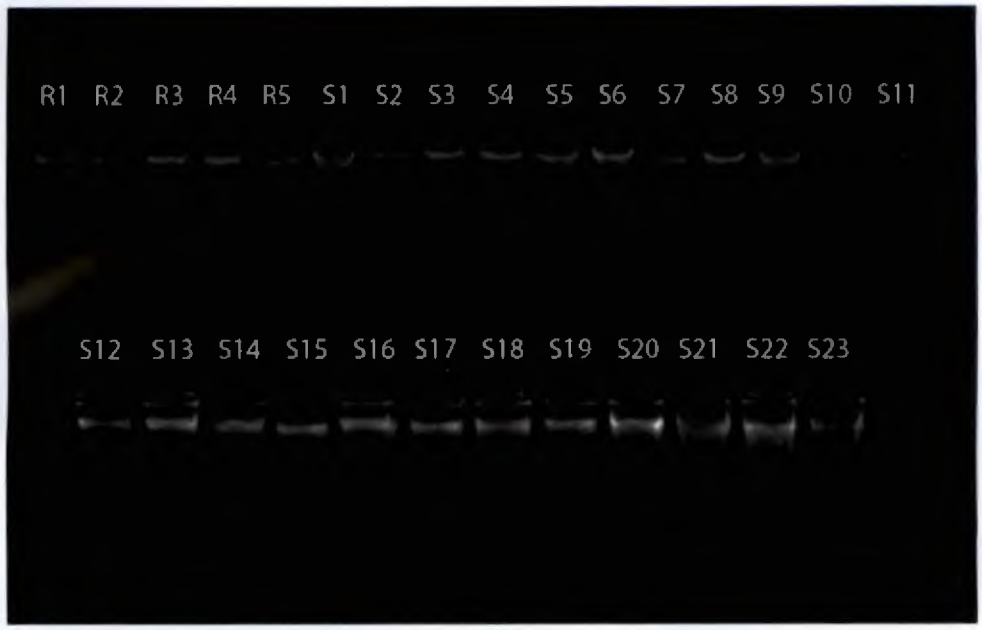
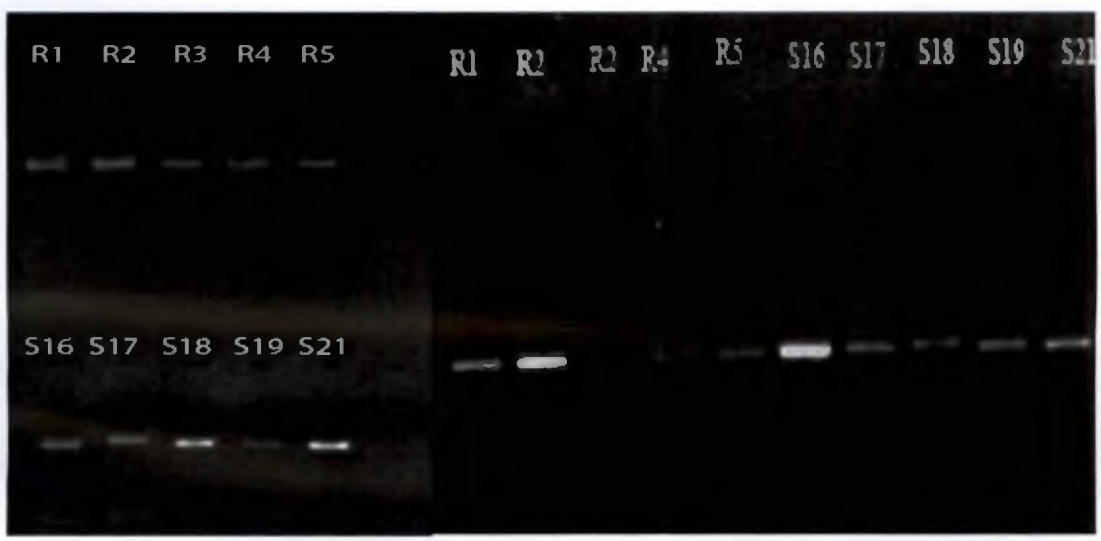


Plate 4: Checking the quality of isolated DNA by Agarose gel



a. b.

Plate 5: PCR amplification using a) gene specific primers and b) SSR primers

4.7 PCR amplification of gene specific and SSR primers

PCR amplification of respective primers was carried initially on a small genotype population comprising five PPD resistant (R1, R2, R3, R4, R5) and five susceptible lines (S16, S17, S18, S19, S21).

PCR amplification of gene specific primers (genes associated with PP pathway) were performed to optimize PCR conditions and the PCR products were run on 2% agarose gel to check for amplification. For 20 primers, the gel image showed the presence of good amplification in all the PCR products (Plate 5a) after optimization in annealing temperatures were performed, which varied between 54°C and 59°C.

Using seventeen randomly selected SSR primers, PCR amplification was done to optimize PCR conditions. The annealing temperature for SSR primers ranged from 55°C to 58°C. PCR products were run on 2% agarose gel to check for amplification. The gel image showed the presence of good amplification in all the samples (Plate 5b).

4.8 Analysis of polymorphism using PAGE gel

4.8.1 Gene specific primer analysis

The 20 gene specific primers showed presence of band in each samples. But, the expected polymorphism between selected lines was not observed in the PAGE gel image for the gene specific primers (Plate 6a).

4.8.2 SSR marker analysis

The selected 17 SSR markers used for marker analysis were polymorphic among the ten selected cassava lines and produced scorable, unambiguous results (Plate 6b). Polymorphic bands were used to assign loci for each primer and scored as present (1) or absent (0). The binary score data showed that the 17 primers produced a total of 34 alleles across the cassava lines selected for SSR analysis. But, the expected polymorphism distinguishing the five PPD resistant and five PPD susceptible cassava lines was shown by SSRY 8 primer (Plate 6b). SSRY 8 exhibited highly polymorphic SSR loci at 288 bp and distinguished PPD resistant and susceptible genotypes.

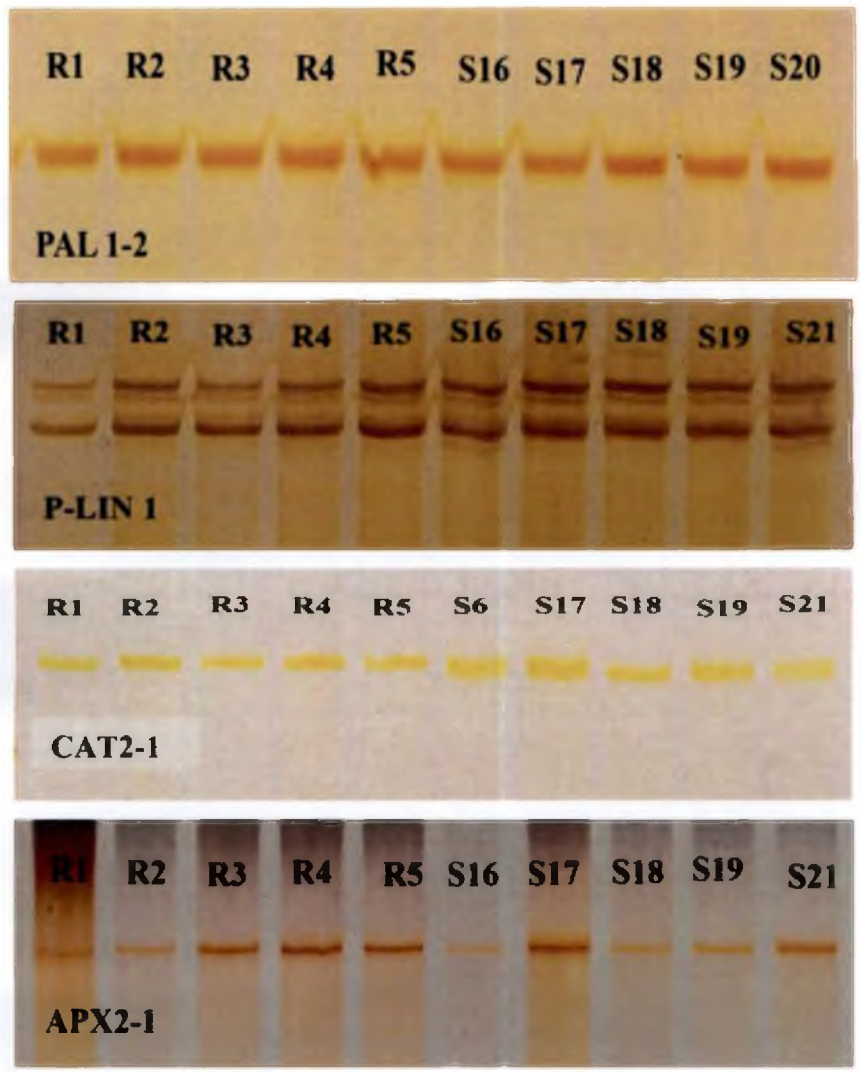


Plate 6a: PAGE gel banding pattern of gene specific primers

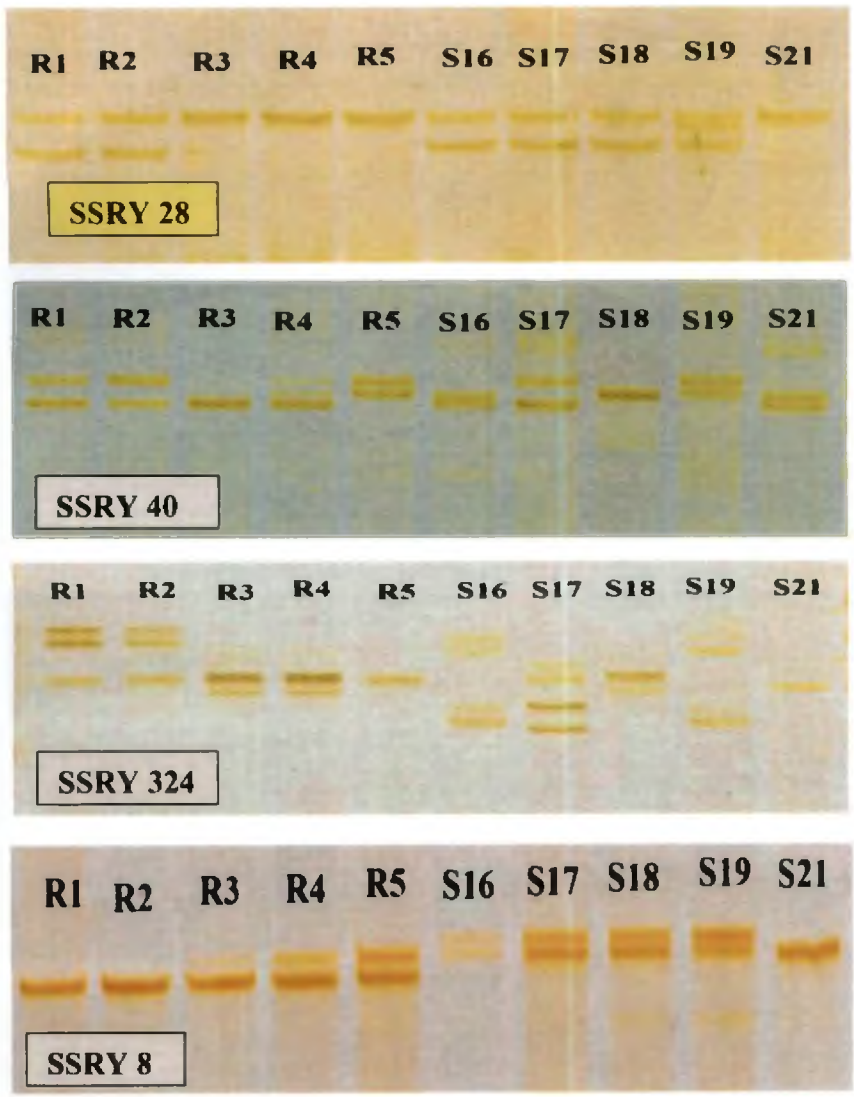


Plate 6b: PAGE gel banding pattern of SSR primers

4.9 Molecular data analysis

4.9.1 Cluster based dendrogram

The polymorphic bands produced were efficient in assessing genetic diversity among the cultivars. The number of scorable bands per primer ranged from 1 to 2. SSR primers used in DNA amplification resulted in scorable PCR bands or loci. The similarity matrix coefficient generated by the 17 SSR loci based on the cluster analysis ranged from 0.56 to 1.00 coefficients. The dendrogram obtained using UPGMA analysis in NTSYS software package revealed three major cluster groups (Cluster I, II and III) at 0.56 similarity coefficient (Fig. 4).

In the dendrogram shown (Fig. 4), cluster analysis of 17 SSR markers revealed the genetic similarities between ten cassava genotypes (five PPD resistant and five susceptible genotypes). The cluster I consisted of two sub-clusters (BR 2 and BR 5; BR-6 and BR-10) and a single BR-105 line. The cluster II is divided into H-165 and H-1687. Harsha and R-73 are grouped as sub-clusters of cluster III. H-226 existed as a single genotype in cluster III. BR 2 and BR 5 appeared to be closer to each other with 1.00 similarity coefficient. Cluster I is comprised of all the resistant genotypes (BR lines) which are distinct from other cluster groups. When the resistant and susceptible lines were compared separately, the similarity coefficient of resistant lines ranged from 0.56-1.00 whereas the susceptible lines exist in a range of 0.50-0.72.

4.9.2 Principal Component Analysis (PCA)

The binary data subjected to PCA investigated the structure of genetic diversity in the ten cassava lines. The 2D plot of PCA was obtained (Fig. 5) for the ten cassava genotypes using NTSYSpc which identified groups of lines which contributed to the variation among the genotypes. The result showed BR lines that were grouped together. BR-2 and BR-5 were having high similarity. Similar trend was shown by BR-10 and BR-6. The susceptible lines, H-1687 and H-165 were found close together. Also, H-226 and R-73 showed similar close association. Harsha remained as a single entity and was almost closer to other susceptible groups.

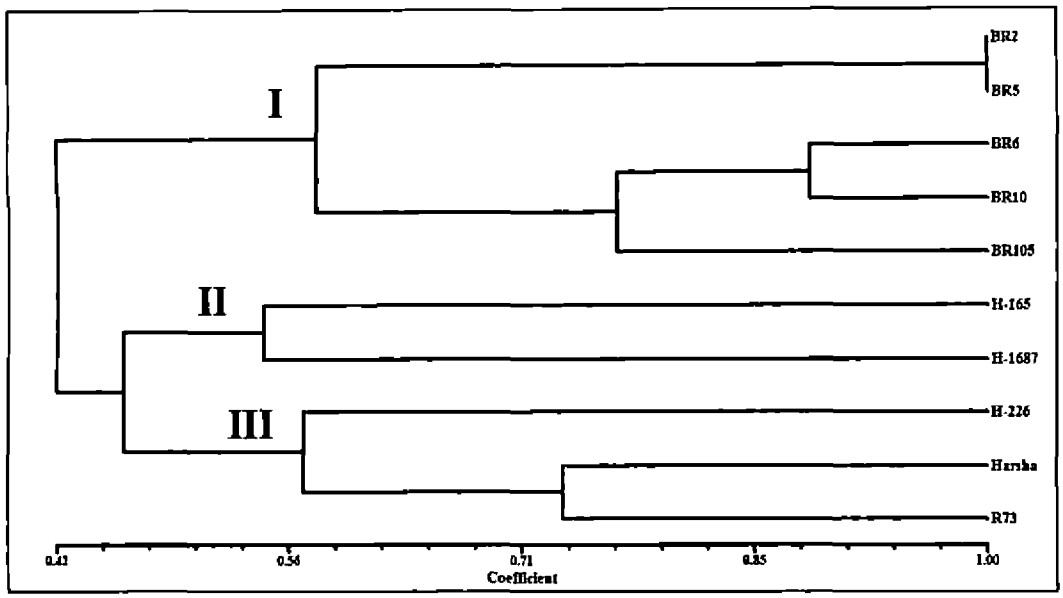


Fig 4. Dendrogram based cluster analysis

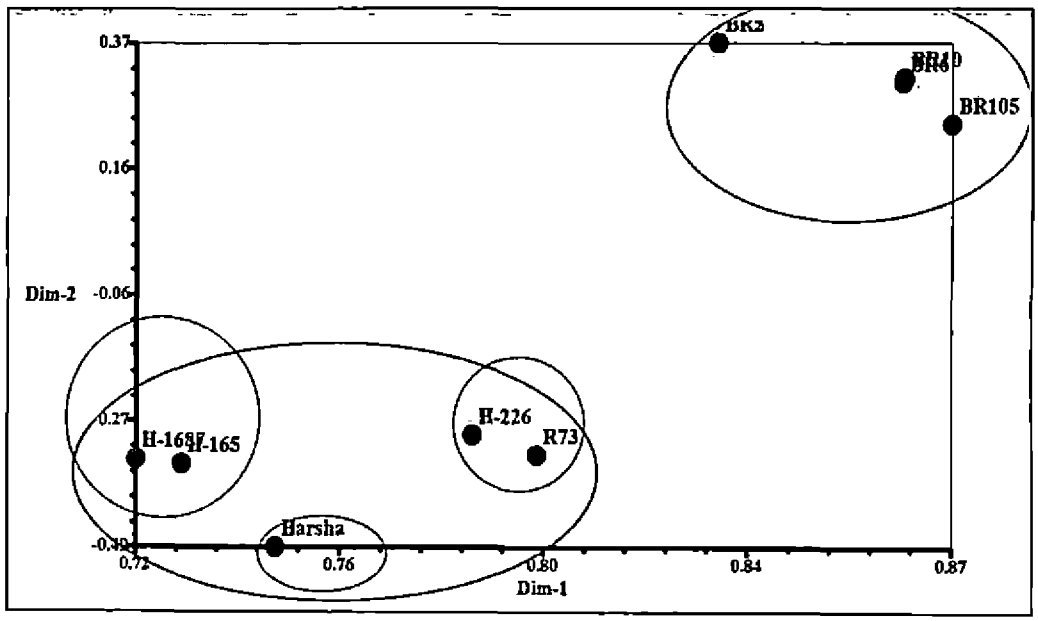


Fig 5. 2D plot of PCA

4.9.3 Similarity index

Using NTSYSpc software, the similarity values among the resistance/susceptible lines for 17 SSR primers obtained for each pair wise comparison is presented in Table 6. The similarity coefficient based on the markers obtained from simple matching ranged from 0.26-1.00. Among the selected genotypes of cassava, the lowest similarity index (0.26) was observed between PPD susceptible, H-1687 and resistant lines, BR-2 and BR-5. The highest similarity index value was between two resistant lines, BR-2 and BR-5.

Table 6. Similarity index values for each pair wise comparison among selected lines

	BR-2	BR-5	BR-6	BR-10	BR-105	H-165	H-226	H-1687	Harsha	R-73
BR2	1									
BR5	**1**	1								
BR6	0.58	0.58	1							
BR10	0.58	0.58	0.89	1						
BR-105	0.58	0.58	0.71	0.82	1					
H-165	0.43	0.43	0.37	0.41	0.4	1				
H-226	0.46	0.46	0.47	0.47	0.47	0.54	1			
H-1687	**0.26**	**0.26**	0.42	0.47	0.42	0.55	0.44	1		
Harsha	0.42	0.42	0.36	0.37	0.43	0.54	0.61	0.4	1	
R-73	0.37	0.37	0.47	0.47	0.47	0.38	0.53	0.44	0.73	1

4.9.4 Percentage of polymorphism

From the binary data obtained, the total number of alleles and percentage of polymorphism were calculated (Table 7). The number of alleles ranged from 2-3 (average 2.7 allele per marker). 100% polymorphism was exhibited by SSRY 6, SSRY 7, SSRY 8, SSRY 21, SSRY 28, SSRY 34, SSRY 36, SSRY 39, SSRY 40, SSRY 50, SSRY 235 and SSRY 324.

Table 7. Percentage of polymorphism generated by SSR primers

Sl. No.	Primer used	Number of alleles	Number of polymorphic bands	Pecentage of polymorphism (%)
1.	SSRY 6	2	2	100
2.	SSRY 7	3	3	100
3.	SSRY 8	3	3	100
4.	SSRY 21	3	2	66.7
5.	SSRY 28	3	3	100
6.	SSRY 32	3	2	66.7
7.	SSRY 34	3	3	100
8.	SSRY 35	2	1	50
9.	SSRY 36	2	2	100
10.	SSRY 39	2	2	100
11.	SSRY 40	3	3	100
12.	SSRY 44	2	1	50
13.	SSRY 50	3	3	100
14.	SSRY 103	3	2	66.7
15.	SSRY 106	3	2	66.7
16.	SSRY 235	2	2	100
17.	SSRY 324	3	3	100

4.9.5 Polymorphism Information Content (PIC)

The PIC value and the expected heterozygosity (He) are given in Table 8. The 17 polymorphic SSRs yielded 2-3 alleles and displayed PIC values between 0.28 and 0.66 with an average of 0.47. The lowest PIC value was showed by the primer SSRY 40 and highest PIC value by SSRY 44. Similarly, the expected heterozygosity ranged from 0.24 to 0.59. The average He value was 0.42.

Table 8. PIC value and heterozygosity of 17 SSR primers

Sl. No.	Primers	PIC	Expected Heterozygosity (He)
1.	SSRY 6	0.49	0.37
2.	SSRY 7	0.66	0.54
3.	SSRY 8	0.64	0.56
4.	SSRY 21	0.61	0.53
5.	SSRY 28	0.59	0.52
6.	SSRY 32	0.57	0.50
7.	SSRY 34	0.54	0.48
8.	SSRY 35	0.47	0.36
9.	SSRY 36	0.44	0.35
10.	SSRY 39	0.46	0.35
11.	SSRY 40	**0.66**	**0.59**
12.	SSRY 44	**0.28**	**0.24**
13.	SSRY 50	0.63	0.55
14.	SSRY 103	0.5	0.45
15.	SSRY 106	0.5	0.44
16.	SSRY 235	0.5	0.38
17.	SSRY 324	0.64	0.57

4.10 DNA sequencing of SSRY 8 marker

The sequencing of PCR products of SSR 8 primer was performed in ABI Genetic Analyzer. The sequenced data of the resistant and susceptible genotypes obtained in the 'abi file format' were used for identification of sequence based polymorphism through computational analysis.

4.10.1 Sequencing data analysis

The resulting sequence data of ten varieties were trimmed to remove contaminations and low complexity regions. Each sequence was assembled using their forward and reverse sequences to find the contig regions using CAP3 software. The conserved regions or contigs was used for finding sequence

polymorphisms. The contigs were aligned and viewed in Clustal Omega, an online sequence alignment software.

The Clustal Omega has aligned five contig sequences of PPD resistant line and three sequences of the susceptible line. The remaining two conserved sequences (susceptible line) were having short sequence length after contig assembly and alignment and therefore they did not meet the default requirements of minimum length of sequences. For further confirmation, sequenced data was assembled and aligned using SeqMan pro in DNASTAR version 9.0 software. The contigs obtained were aligned and viewed in SeqMan pro and polymorphisms were identified.

A dinucleotide CA repeat motif (CACACA) was identified (Fig. 6) in the three susceptible lines, which may be the reason for the difference in polymorphism shown by the SSRY 8 marker. This CA repeat was absent in the five resistant lines and corresponding to this repeat region, a deletion in sequence was identified which may be due to a change in the amino-acid sequences coding a particular PPD related protein.

Type of SSR	**Repeat**	**No. of SSR**	**Repeat region**
Di	(CA)6	1	197-200

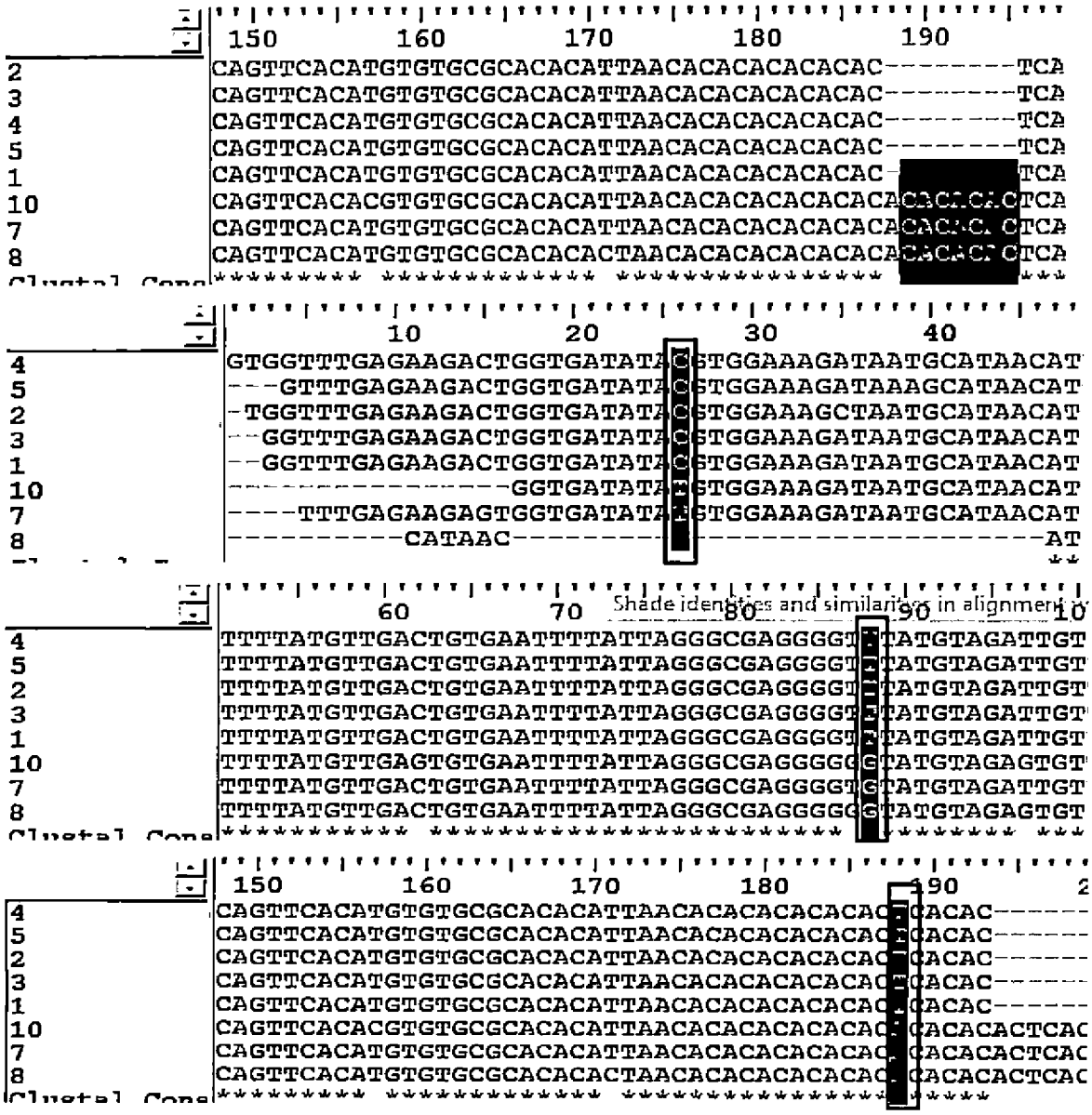


Fig 6. Clustal alignment of 8 conserved sequences showing polymorphism at four different regions (a 6 bp di-nucleotide (CA) repeat (top) present only in the susceptible lines and three SNP regions are highlighted)

A six base pair difference in the resistant/susceptible lines in the CA repeat of SSR region can be the reason for the differentiating polymorphism inspected. The above data clearly explains that **SSRY 8 marker** as a putative marker linked to PPD resistance.

From the aligned data, three potential SNP regions were identified (Fig. 6); two transition SNPs (G-T; C-T) and a transversion SNP (A-T) as shown below:

Characterization	**Type of SNP**	**Contig containing SNP**	**Conserved region**	**Total SNP**
Transition	T/G	TACATACACTTGATTATCAAG	77-97	2
	C/T	TTTATGTTGACTGTGAATTTT	13-38	
Transversion	T/A	TGCATGTTAATAGTGTAATAT	176-196	1

In future, the conserved sequence region containing identified SNPs can be annotated to find the gene corresponding to the PPD resistance.

4.11 Validation of SSR polymorphism

The sequence polymorphism shown by SSRY 8 marker in the five resistant and five susceptible lines were confirmed among the entire selected cassava lines using a 6% denaturing PAGE gel. The six di-nucleotide repeat identified explains the difference observed among the five resistant and 23 susceptible lines. The typical banding pattern shown by SSRY 8 marker was shown in plate 7.

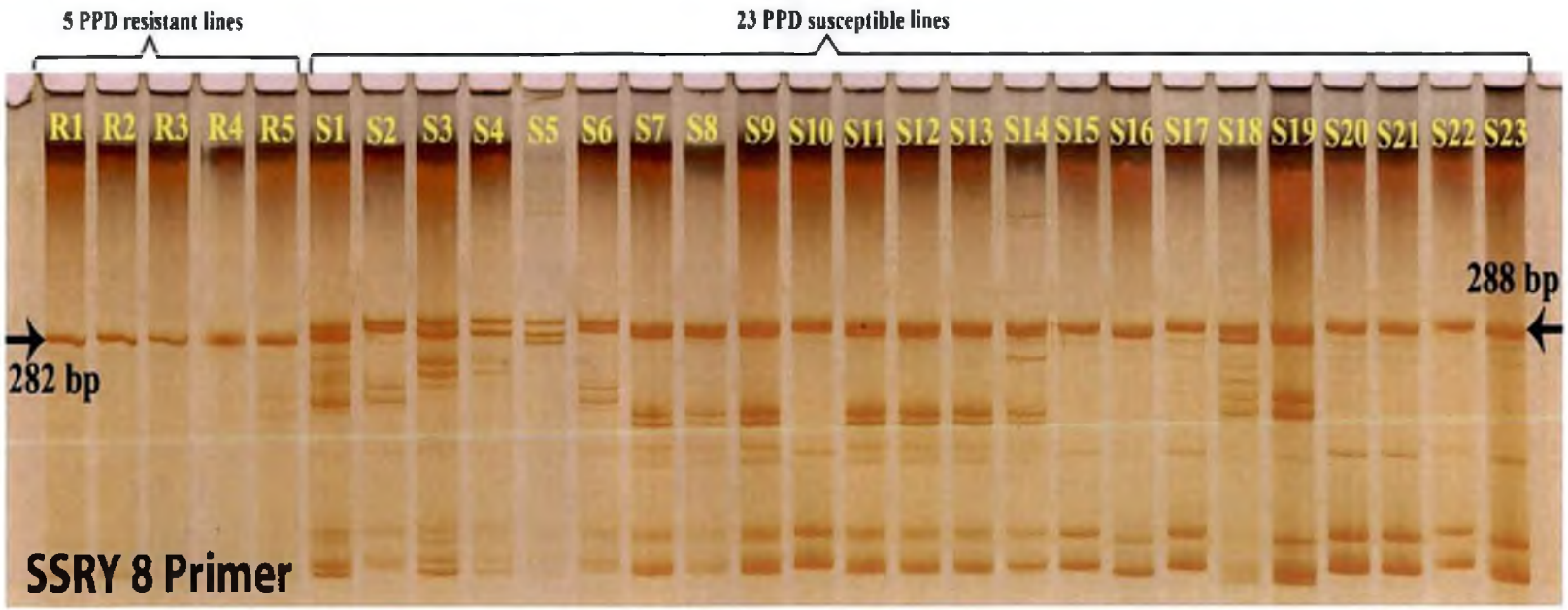


Plate 7. Validation of SSRY 8 in 28 genotypes

4.12 Attempt on CAPS marker construction

The attempt using CAPS designer tool of Sol Genome Network produced the following restriction sites for the CAPS marker development.

Sl. No.	Restriction Enzyme	Recognition Sequence
1.	AluI	AGCT
2.	CviJI	[A|G]GC[C|T]
3.	HincII	GT[C|T][A|G]AC
4.	PmlI	CACGTG
5.	Tsp509I	AATT

Due to the limited resources and availability of restriction enymes, further research work on development of CAPS marker was not performed.

DISCUSSION

Chapter 5

Discussion

PPD is a complex enzymatically mediated decay process which parallels the wound responses in plants. Many PPD related genes that include the up regulated and down regulated genes have been identified. Enhancing the storage potential of cassava would have substantial impact on resolving the deterioration related constraints associated with cassava marketing and utilization. It is expected that the molecular genetic approach combined with the selection procedures (molecular breeding) would lead to an increase in the shelf life of cassava roots to a minimum of 3 weeks without use of post-harvest treatments. This could be achieved by genetic manipulation to suppress the process involved in PPD and to enhance the wound healing response to prevent the onset of secondary microbial deterioration.

The molecular breeding for resistance is completely dependent on the genetics associated with the resistance, which may be difficult to understand and come up with conclusive results. The advent of molecular markers has opened avenues for plant breeding to exploit the marker and next generation sequencing technology that could predict a possible solution to the major constraint like PPD. Although substantial improvement have been made towards understanding the molecular and biochemical nature of PPD associated genes (Reilly *et al.*, 2003; Bayoumi *et al.*, 2010; Salcedo *et al.*, 2010), the entire story is still not clear due to the limited genetic variability. The objective of developing cassava varieties with longer shelf life will most likely be attained through genetic manipulation of regulatory mechanisms and signalling pathways that control the coordinate activation of multiple defense responses during PPD process.

The approach employed here has led to the identification of a marker linked to PPD resistance in cassava lines. In addition, the phenotypic and

genotypic assessment on variation between the resistant and susceptible lines clearly depicted the variability among PPD resistant and susceptible lines.

Based on the evaluation report on PPD scoring, the BR lines (BR-2, BR-5, BR-6, BR-10 and BR-105) exhibited high resistance after three and seven days of storage. Similar results were shown by MPER 13 and AM 206-5 which were tolerant to PPD (Garcia *et al.*, 2013). Also, a significant effect on the PPD-resistant/susceptible lines stored for different interval was observed. Among all, the resistant survived for 21 days without decay while the susceptible deteriorated after two days of storage. Salcedo *et al.* (2010) have highlighted the difficulties in assessing PPD in field-grown roots. They showed there was no correlation between the accumulation of hydroxycoumarins (fluorescent compounds) and visual assessment, suggesting the former is not a reliable technique and visual assessment or subjective visual scoring remains the preferred approach for PPD evaluation.

The percentage of starch content calculated is considerably higher in the roots of resistant lines than in the susceptible lines. The dry matter content was only about 30-35% (Sanchez *et al.*, 2013) after 4 days of storage. In a root sampling study, 44.2% dry matter after 14 days of storage was reported for a PPD tolerant cassava, AM 206-5 (Garcia *et al.*, 2013). It will be for the first time that a cassava tuber like BR lines recording high dry matter ranging 46-48.6%. However, the statistical data showed that the dry matter and starch levels were dependable and a gradual decline in both levels was seen on storage for 3 to 21 days. These results were consistent with the previous reports (Booth *et al.*, 1976; Osunsami *et al.*, 1989). The weight loss, from 42-46% observed in the resistant after 21 days explains the changes in dry matter and starch content in stored cassava roots that are well depended on storage conditions (Akingbala *et al.*, 2005). As reported in many studies (Rickard and Coursey, 1981; George and Browne, 1994), roots in this study lose weight gradually during the entire storage period.

However, this finding reinforces the long standing perception that mechanical damage or improper handling during harvest results in rapid decay of cassava roots. The study highlights the resistant line that gave better dry matter and starch data and showed least or no PPD susceptibility during PPD evaluation at particular intervals. This implies the need for development of cassava tubers with long shelf-life that can restore its freshness for atleast three weeks. Studying the improvement of these resistant lines is ultimate for further understanding the biochemical and molecular changes involved in conferring resistance to PPD.

Because of the less work attempted on PPD using molecular markers, PPD associated markers were not available and are not investigated. Hence, some primers were designed using Primer 3 plus software based on the PP pathway related genes controlling the PPD. The genes selected for primer designing included APX, CAT, PAL, TRX, GPX, ACC oxidase and pLIN (p-Linamarase). The results showed that the PCR products obtained were monomorphic for the designed gene specific primers. Similar monomorphic results were obtained for PCR primers used in mapping PPD related genes (Cortes *et al.*, 2002). Xu *et al.* (2014) have designed primers using Primer 3 plus software in real-time RT-PCR analyses of transgenic cassava plants.

In the future, gene specific primers used in this study can be sequenced by genotyping on a larger group of varieties which may show differences among the PPD resistant/susceptible cassava lines and may result in identification of potential sequence based polymorphism or insertion-deletions (in-dels). Such findings may assist in explaining the phenotypic difference among the cultivars. A cDNA clone (*cMeHRGP1*) for HRGP expression during PPD was isolated and characterized. *cMeHRGP1* showed antisense gene pairing coding for part of phosphoserine aminotransferase (*PSAT*). The deduced amino acid sequence of the coding region of phosphoserine aminotransferase (*PSAT*) contained 27 typical S(P)4 repeat motifs. Xu *et al.* (2014) confirmed that the transgenic approach is effective in improving the abiotic stress resistance in cassava *via* proper gene stacking of ROS scavenging enzymes.

Due to the monomorphic results shown by gene specific primers, 17 SSR primers were randomly selected and used for PPD molecular study. However, the 17 SSR primers were detected only in a small subset of genotypes (five PPD resistant and five susceptible). Considering the intricate methods in DNA sequencing of large population, the limited resources available and also the complex genetic background of cassava, only the small proportion of cassava genotypes were inspected in order to establish whether all of these primers could be used to identify PPD resistance trait in all kinds of cassava cultivars. In most cases, identifying molecular markers linked to traits such as resistance requires screening of relatively large populations because comparing bulk samples is easier than evaluating many individuals in different populations (Altinkut and Gozukirmizi, 2003; Barakat *et al.*, 2011). This approach showed similarity to the bulk sergeant analysis (BSA) where, a small bulk was thus selected for the PPD study prior to the validation of the identified polymorphism in all selected cassava genotypes.

The genetic variability study using 17 SSR primers among the selected resistant and susceptible lines has assessed significant results. The PCA and dendrogram clearly noted the difference among the selected lines where the resistant lines were clustered together. The similarity coefficient value of 0.56 indicated that a significant portion of the observed variation in PPD is attributable to inherent genetic factors contributing resistance. Cortes *et al.* (2002) have substantiated that the genotypic contribution for expression of final trait is highly significant even though PPD is a quantitative variable that is not significantly influenced by the environment. They have found significant differences between mapping populations from two different locations which explained the differential phenotypic expression. Verifying the ability of the SSR markers to distinguish between PPD resistant and susceptible cultivars confirmed that the markers had good discriminability and that combinations of these markers could be used in future to select plants with desirable resistant traits.

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Of the SSR primers surveyed, SSRY 8 marker showed unique polymorphic bands which distinguished the resistant and susceptible lines. Some of the bands differentially amplified in resistant and susceptible genotypes were isolated and sequenced. From the study, the differentiation in the length of the amplified bands resulted from units of SSR motifs (CA repeats) identified only in the susceptible genotypes.

The sequence based polymorphisms was identified after finding the conserved regions of the PPD resistant/susceptible sequences. Similar reports resulted in the identification of candidate genes, based on presence of conserved sequence region between species has been used for identification and mapping of genes of interest (Hu *et al.*, 2008; Muchero *et al.*, 2010). When the aligned contig sequences were screened for sequence polymorphisms; the DNA sequence alignments showed that some SSRs (CA repeats) in the susceptible line are polymorphic, and mainly involved di-nucleotide SSRs flanking region (refer Fig. 6). A six bp dinucleotide (CA) repeat was present only in the susceptible lines and absent in the five resistant lines. This deletion identified in the resistant lines may lead to change in the amino acid coding for a PPD resistance.

The BR lines exhibit a purple pigmentation in the inner cortex and towards the peripheral region which is more pronounced when stored for long duration. The anti-oxidant nature of anthocyanin, which is conferring this purple colouration, may be involved in delaying PPD. Moreover, high latex in the tuber rind was observed after 21 days of storage. The phenolic compound (latex) accumulation during these stages prevents the cassava tubers from microbial infection. These two peculiar features of the resistant lines may be contributing for the PPD resistance phenotypically. A molecular proteomic approach to study the genetics behind the presence of anthocyanin and high latex content in the resistant line is needed to identify the possible gene encoding the PPD resistance.

The potential role of SSR variation in the genome of PPD resistant and susceptible lines needs to be investigated. It can be hypothesized that these SSRs

may provide adaptive variation that is important to PPD resistance. The presence of polymorphic SSRs also reveals an important role for insertion-deletion mutations as a mechanism for producing genetic diversity in the cassava genome. Varshney *et al.* (2002) reported that tri-nucleotide repeats are the most common, followed by either di-nucleotide repeats or tetra-nucleotide repeats. Our result of identification of six di-nucleotide repeats $(CA)_6$ is in close agreement with previous studies reporting di-nucleotide repeats in jatropha (Wen *et al.*, 2010) and sweet potato (Zhang *et al.*, 2014). PPD resistance is not an exception, and although there are many cases in which this phenotype is controlled by numerous genes as reported by Reilly *et al.*, 2007, there are examples evidencing sequence changes of some genes that can explain differences between resistant and susceptible cultivars (Bryan *et al.*, 2000; Krattinger *et al.*, 2009).

The methodology used in this work is proposed as a strategy to identify genomic regions closely linked to PPD resistance genes. The difference in banding pattern of SSRY 8 marker observed in the five resistance/susceptible genotypes was validated in the 28 cassava lines. Results showed a unique polymorphism in five PPD resistant and 23 susceptible genotypes which was due to the presence of 6 bp di-nucleotide repeats. The attempt made to design a CAPS marker resulted only in identifying the four restriction enzymes from CAPS Designer of Sol Genomics Network. This work provides a way forward for developing a CAPS marker using these restriction enzymes. Due to lack of resources and insufficient restriction enzyme requirements, further work on CAPS marker development was not undertaken. The unique polymorphism obtained in this work are a valuable resource, since, CAPS markers can be designed from them to genotype the PPD resistant from susceptible lines, which can be used to identify these genetic loci in the cassava genetic map to establish associations with the regions involved in implying resistance. The use of restriction digestion for mapping the PPD related genes and identifying polymorphism with the development of CAPS markers for PPD related *ACCO* gene have been recently cited (Cortes *et al.*, 2002).

This study represents the first step toward the long-term goal of identifying marker linked to PPD resistance in cassava with a future perspective for developing cassava cultivars having longer shelf-life.

Furthermore, this research provides two significant results:

(1) SSRY 8 marker, confirmed to be associated with PPD resistance in cassava lines which can be used to track loci and genome regions or select specific phenotypes in crop breeding programs.

(2) A substantial set of potential single nucleotide polymorphisms (two transition SNPs and a transversion SNP) identified based on computational sequence analysis of SSRY 8 marker performed in a natural population comprised of ten cassava genotypes and is confirmed to have association with PPD resistance.

In addition, the SSR marker identified in this study can be subjected for validation in wet lab using a large cassava genotypes, annotate the gene associated for PPD resistance, identification of restriction sites for CAPS marker development, association mapping of PPD resistant genes and identification of candidate protein sequences involved in PPD resistance. The availability of complete sequenced genomes with the help of bioinformatics tools, allows the identification of resistant genes that code for proteins with conserved domains. However, functional genomic studies are essential to identify the specificity of the candidate protein sequences expressed during PPD resistance.

The identified SSR motif and the potential SNPs can be used in genetic analysis, germplasm characterization, linkage map construction and screening for QTLs controlling important traits in cassava. The detection of more molecular markers associated with resistance to PPD in cassava will enhance the development of cassava varieties with high and durable PPD resistance. Together, these findings enhance our understanding of the approaches for improving PPD resistance in cassava, and provide a valuable resource for discovering genes involved in PPD resistance in the storage roots of cassava.

SUMMARY

CHAPTER 6
SUMMARY

Cassava is an important staple food and a farmer preferred variety present in the genus *Manihot,* belonging to Euphorbiaceae family. Today, more than 800 million people feed on cassava as part of their dietary source of carbohydrates. Post-harvest physiological deterioration is an inherent root phenomenon resulting in root damage within 2-3 days upon harvest. Attempts to deduce the PPD resistance associated marker are quite known, though many genes involved in PPD and tolerance to the PPD trait have been studied. Identification of markers linked to the PPD resistance gene is the primary goal for prolonging the shelf life of cassava.

Present study entitled "Identification of markers linked to post-harvest physiological deterioration (PPD) resistance in cassava lines" was performed with the objective to understand the genetic variability among the PPD resistant and susceptible genotypes and identify the reliable marker linked to PPD resistance in this line.

The present study consisted of five PPD resistant and twenty-three PPD susceptible cassava lines. The study was conducted at ICAR-Central Tuber Research Institute (CTCRI), Trivandrum, Kerala. The findings obtained on this study were summarized as follows:

The phenotypic evaluations of PPD such as scoring of PPD, quantification of dry matter and starch content provided significant results showing that the resistant BR lines could extent the shelf life to almost three weeks. The scoring evaluated the PPD progression in resistant and susceptible lines. The dry matter and starch content undergoes gradual decline in subsequent intervals of storage.

The genomic DNA extracted from all the 28 leaf samples were of good quality with no RNA or protein contamination. Genetic polymorphism among five PPD resistant and five PPD susceptible lines were assessed using 20 designed gene specific primers and 17 SSR primers. All the SSR primers used for the study could reveal the polymorphism except the gene specific primers which produced

monomorphic bands. All the SSR primers showed 100% polymorphism except SSRY 21, SSRY 32, SSRY 103 and SSRY 106 which showed 67% polymorphism, while SSRY 35 and SSRY 44 exhibited only 50% polymorphism. A total number of 45 loci were amplified which exhibited 86.28% polymorphism. This was helpful for the genetic diversity analysis. The number of polymorphic bands ranged from 2 to 3 and the product size ranged from 134 to 298 bp.

The similarity coefficient values of ten genotypes ranged from 0.26 to 1.00. The lowest value of similarity index (0.37) was observed between H-1687 and resistant lines, BR-2 and BR-5 and the highest value was observed between two BR-2 and BR-5. The highest similarity was observed where the coefficient values ranged between 0.56 and 1.00.

Dendrogram based on similarity coefficient values obtained three major clusters consist of PPD resistant and susceptible lines at 56% similarity level. The cluster I contained 5 BR lines, cluster II included two susceptible lines, H-1687 and H-165 and the cluster III consisted of only three groups, a single H-226 line and sub-clusters of Harsha and R-73. In 2-D PCA scatter plot, the 5 resistant BR lines formed a separate cluster from the resistant species. The PIC values ranged from 0.28 to 0.66 (average 0.47). The lowest PIC value was showed by the primer SSRY 40 and highest value by SSRY 44. Similarly, the expected heterozygosity ranged from 0.24 to 0.59. The average He value was 0.42.

The molecular sequence data of ten varieties were assembled using CAP3 and aligned and viewed in Clustal Omega online sequence alignment software. The Clustal Omega has aligned five contig sequences of PPD resistant line and three contigs of the susceptible line. The sequence based polymorphism in the clustal-omega aligned data identified three SNPs and a di-nucleotide repeat (CA) motif. The data obtained was confirmed by assembling in SeqMan pro in DNASTAR. Using SeqMan pro, the assembled contigs were aligned and similar polymorphisms were identified.

A dinucleotide CA repeat motif (6bp) was identified in the susceptible lines and absent in the five resistant lines, which may be the reason for the difference in polymorphism shown by SSRY 8 marker. This deletion may be due

to the change in amino acid sequence conferring resistance to PPD. This unique polymorphism was validated among the five resistant and 23 susceptible lines using DNA-PAGE and the same polymorphism was observed in all genotypes. Thus, SSRY 8 marker is a reliable and efficient marker for identification of PPD resistance. Furthermore, sequence based polymorphism was detected producing three potential SNP regions such as two transition SNPs (G-T; C-T) and a transversion SNP (A-T). The restriction enzymes identified for CAPS can be used for CAPS marker design.

The identified SSR motif and the potential SNPs can be used in genetic analysis, germplasm characterization, CAPS marker development, mapping and screening for QTLs controlling important PPD traits in cassava. The SSRY-8 marker identified for resistance to PPD can be used in molecular breeding methods for selection and screening of PPD related genotypes and for improving the shelf life of cassava.

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APPENDICES

APPENDIX I

Chemical reagents used in genomic DNA extraction

a. **DNA extraction buffer (Dellaporta *et al.*, 1983)**

- Tris-HCl (pH 8.0) : 100mM
- EDTA : 5mM
- NaCl : 500mM
- β-Mercaptoethanol : 2% (v/v) - freshly added prior to DNA extraction
- PVP : 2% (w/v) - freshly added prior to DNA extraction

b. SDS - 20%

c. Potassium acetate - 500mM

d. Ice-cold ethanol

e. Sodium acetate (pH 5.2) - 300mM

f. RNase A - 10mg/ml

RNase A was dissolved in TE buffer and boiled for 15min at 100°C to destroy DNase and stored at -20 °C.

g. Chloroform:Isoamyl alcohol - 24:1

h. Ethanol - 70%

i. **Preparation of 10X TE buffer**

- Tris-HCl (pH 8.0) : 10mM
- EDTA : 1mM

Final volume was made upto 100ml with distilled water.

APPENDIX-II

Chemicals required in Agarose Gel Electrophoresis

a. Preparation of 10X TBE buffer

- Tris base : 107g
- Boric acid : 55g
- 0.5M EDTA (pH 8.0) : 40ml

Final volume was made upto 1000ml with distilled water and autoclave before use.

b. Ethidium bromide

Weigh 10mg of ethidium bromide powder (Sigma-Aldrich) and dissolve in 1ml of distilled water.

c. Preparation of gel loading dye

- Formamide : 50ml
- Xyline cyanol : 50mg
- Bromophenol blue : 50mg
- EDTA : 0.5M

d. Empty well dye

- Loading dye : 50µl
- Sterile water : 50µl

APPENDIX-III

Chemical reagents and solutions for DNA-PAGE

a. Bind saline

- Absolute ethanol : 99.5%
- Acetic acid : 0.5%
- Bind saline : 1µl

b. 40% acrylamide

- Acrylamide : 38g
- Bis-acrylamide : 2g

Final volume made upto 100ml using distilled water.

c. 6% Denaturing polyacrylamide gel containing 7M urea

42g urea was dissolved in a beaker containing 10ml TBE buffer (10X) and 15ml distilled water by heating in a microwave oven for 30-40s. 15ml acrylamide solution (19:1) was filtered and added to a measuring cylinder followed by the melted urea solution. The final volume was made up to 100ml using distilled water and stored in dark till use. 60µl TEMED (1:10) and 600µl APS (100mg/ml) was added and mixed just before casting the gel.

d. Fixer

- Acetic acid : 200ml
- Distilled water : 1800ml

Final volume made upto 2000ml

e. Silver stain

- Silver nitrate : 2g
- Formaldehyde : 3ml

Final volume made upto 2000ml

f. Developer

- Sodium carbonate : 60g

A final volume made upto 2000ml and stored in -20°C until use.

- Formaldehyde : 3ml
- Sodium thiosulphate (10mg/ml) : 400µl

The above reagents are freshly added and mixed thoroughly with the chilled sodium carbonate solution before use.

APPENDIX-IV

Reagents used in Clean-up for Sequencing

a.	EDTA	: 125mM
b.	Sodium acetate	: 3M (pH 4.6)
c.	Absolute ethanol	: 95%
d.	Ice cold ethanol	: 70%

IDENTIFICATION OF MARKERS LINKED TO POST-HARVEST PHYSIOLOGICAL DETERIORATION (PPD) RESISTANCE IN CASSAVA LINES

by

DEEPTHY CHANDRAN A.

(2011-09-110)

Abstract of the Thesis

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ABSTRACT

Cassava (*Manihot esculenta* Crantz) undergoes deterioration within a short period after harvest due to the occurrence of post-harvest physiological deterioration (PPD). To extend the shelf life, it is important to understand the molecular aspect of PPD associated resistance. In this study, five PPD resistant and 23 PPD susceptible lines were used for identification of a marker linked to PPD resistance. The phenotypic assessment on PPD by visual scoring showed the extent of deterioration in roots at different intervals. The dry matter and starch content was significant over 3, 7 and 21 days and produced a gradual decline in the content as PPD progressed over time. In the present study, 20 gene specific primers and 17 SSR primers were used, all the 20 gene specific primers produced monomorphic bands among resistant and susceptible group. The 17 SSR markers were used to survey polymorphism in a bulk of ten genotypes (5-resistant and 5-susceptible lines) and a unique polymorphism was identified in SSRY 8 marker that differentiated resistant and susceptible group. The genetic variability among the ten genotypes using 17 SSR markers was assessed by cluster analysis, PCA, simple matching and PIC value. Dendrogram constructed using 17 SSR primers produced three major clusters at 0.56 similarity coefficient, which clearly distinguished the PPD resistant from the susceptible lines. The PIC values ranged from 0.28 to 0.66 (average 0.47). The lowest PIC value was showed by the primer SSRY 40 and highest value by SSRY 44. The expected heterozygosity ranged from 0.24 to 0.59 (average-0.42). Computational sequence analysis of sequence data of SSRY 8 marker using DNASTAR software resulted in identification of (CA)6 repeat motif in the susceptible lines which explained the unique polymorphism in the resistant lines based on the deletion of (CA)6 repeats. In addition, 3 SNPs (two transition SNPs (G-T; C-T) and a transversion SNP (A-T) were also identified in the conserved regions of SSRY 8 sequence.