# BIOCHEMICAL AND MOLECULAR STUDIES ON POST-HARVEST PHYSIOLOGICAL DETERIORATION OF CASSAVA (*Manihot esculenta* Crantz)

by

SARAVANAN, R

(2012-21-118)

THESIS

Submitted in partial fulfillment of the requirements for the degree of

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DEPARTMENT OF PLANT PHYSIOLOGY

**COLLEGE OF AGRICULTURE**

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2015

# DECLARATION

I hereby declare that this thesis entitled **"BIOCHEMICAL AND MOLECULAR STUDIES ON POST-HARVEST PHYSIOLOGICAL DETERIORATION OF CASSAVA (*Manihot esculenta* Crantz)"** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title of any other University or Society.

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Date: 30th November, 2015

# CERTIFICATE

Certified that this thesis entitled "**BIOCHEMICAL AND MOLECULAR STUDIES ON POST-HARVEST PHYSIOLOGICAL DETERIORATION OF CASSAVA (*Manihot esculenta* Crantz)**" is a record of research work done independently by Sri. **Saravanan, R (2012-21-118)** under my guidance and supervision and it has not previously formed the basis for the award of any degree, fellowship or associateship to him.

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Date: 30th November, 2015

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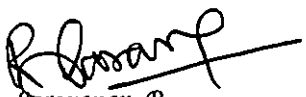

*Saravanan. R*

*30th November, 2015*

*Thiruvananthapuram,*

Dedicated to

My Beloved Parents

**Raju & Dhanalaxmi**

## List of abbreviations

| | |
|---|---|
| % | Percent |
| ABA | Abscisic acid |
| AOX | Alternate oxidase |
| APX | Ascorbate peroxidase |
| BHA | Butylated hydroxy anisole |
| BHT | Butylated hyroxy toluene |
| bp | base pair |
| CAT | Catalase |
| CKs | Cytokinins |
| cm | Centimeter |
| CV | Coefficient of variance |
| DAMPs | Damage associated molecular patterns |
| DAS | days after storage |
| DHAR | Dehydro-ascorbate |
| DNA | Deoxy-ribonucleic acid |
| et al. | and co-workers/ co- authors |
| EU | Enzyme units |
| FAO | Food and Agriculture Organization |
| FTIR | Fourier transform infrared analyzer |
| FT-NIR | Fourier Transform Near Infra-Red |
| GSH | Glutathione |
| $H_2O_2$ | Hydrogen peroxide |
| HPLC | High performance liquid chromatography |
| HPTLC | High performance thin layer chromatography |
| HRGPs | Hydroxy proline rich glycoproteins |
| i.e. | that is |
| JA | Jasmonic acid |
| kJ | Kilo joule |
| LOX | Lipoxygenase |
| MDARs | Monodehydro ascorbate reduced |
| MeJA | Methyl jasmonic acid |
| mg | Milligram |
| mL | Millilitre |
| MVA | Multi-variate analysis |
| NADPH | Nicotinamide adenine di-phosphate reduced |
| NBT | Nitro blue tetrazolium |

| | |
|---|---|
| NIRS | Near infrared reflectance spectroscopy |
| NO | Nitrous oxide |
| $^{o}$C | degree Celsius |
| OPDA | 12-oxo-phytodienoic acid |
| PAL | Phenylalanine ammonia lyase |
| PAMPs | Pathogen associated molecular patterns |
| PCA | Principal component analysis |
| PCD | Programmed cell death |
| PCs | Principal components |
| PLS | Partial least square |
| PMB | Potassium meta-bisulphate |
| POX | Peroxidase |
| PPD | Post harvest physiological deterioration |
| ppm | Parts per million |
| PR | Pathogenesis related |
| PS I | Photosystem I |
| PS II | Photosystem II |
| PTI | PAMPs triggered immunity |
| QTL | Quantitative trait loci |
| RNA | Ribonucleic acid |
| ROS | Reactive oxygen species |
| SA | Salicylic acid |
| SAM | S-adenosyl methionine |
| SNV | Standard normal variance |
| SNV | Standard normal variate |
| SOD | Superoxide dismutase |
| TBHQ | Tertiary butyl hydroquinone |
| UV | Ultraviolet |

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# Chapter 1

# Introduction

# 1 Introduction

Cassava (*Manihot esculenta* Crantz) is the fourth most important crop in developing countries. It is one of the most efficient producers of edible carbohydrates among the world's major food crops. The starchy roots are the staple food consumed by large populations in developing countries. Cassava is a subsistence crop in many parts of the world as well as an industrial crop for the production of starch and bio-fuel. The estimated increase in global harvested area of cassava was 44% between 1980 and 2011, from 13.6 million to 19.6 million hectares respectively, which was the biggest percentage increase among the world's five major food crops (Howeler *et al.*, 2013). Cassava is the cheapest source of calories for both human nutrition and animal feeding (Tonukari, 2004).

Cassava root is highly perishable and it deteriorates quickly (within 48 -72 hrs) after harvest and become unfit for both human and animal consumption and also for other industrial uses due to post harvest physiological deterioration (PPD)(Rickard, 1985). PPD of cassava is recognized as one of the major constraints for expansion of its cultivation in many parts of the world (Wenham, 1995). The traditional practice of partial harvesting of roots in subsistence farming communities can delay the complete use of the roots per cassava plant, but the practice remains unfavourable in commercial fields. Furthermore, the remaining roots after partial harvest are subjected to a loss of starch content, decline of palatability due to the increase of fibre content and an associated increase in cooking time (Rickard and Coursey, 1981). Increasing the shelf-life of cassava storage roots is desirable not only to solve problems of utilization and marketing but also to facilitate the conversion of cassava from a traditionally famine reserve crop and rural food staple to a cash crop. The PPD has become a major constraint (Janssen and Wheatley, 1985); Wenham, 1995) when the entry of rural farmers into the cash economy and processing on a larger or industrial scale which demands the roots to be transported long distances to reach the industry and consumers (Balagopalan, 2002; Westby, 2002). Therefore, research directed towards introducing resistance to PPD, or delaying the response, is considered a priority by international bodies such as the Food and Agriculture Organisation of

the United Nations (FAO) and the Cassava Biotechnology Network (Wenham, 1995).

Several lines of evidence suggest that the PPD response in cassava roots is an enzymatic, endogenous oxidative process. The initial symptoms of PPD generally starts with a bluish-black to black vascular discoloration (vascular streaking) which quickly spreads to the parenchyma tissue. The general discolouration is preceded by roots accumulating colourless deposits and intense fluorescence were observed to develop in the storage tissue after harvest. Immediately after harvest, increase in the activities of polyphenol oxidase and peroxidase and other oxidative enzymes were also reported (Rickard, 1981; 1985; Tanaka *et al.*, 1983).

PPD of cassava root is considered as a complex abiotic wounding stress response (Westby, 2002; Beeching *et al.*, 2002). Being a storage organ for photosynthates with no regenerative function as a propagule with reproductive function, cassava roots lack the biological need to repair wounds when detached from the plant (Reilly, *et al.*, 2004). The development of PPD throughout the storage tissues of cassava after harvesting suggests the transmission of intercellular signals from the sites of damage, and apart from the involvement of ethylene and hydrogen peroxide in this signalling network. It remains widely unknown which other signalling molecules are involved and how their interplay affect PPD in cassava. Research on the nature of mobile signals, their biochemical origin and the defence response genes they activate downstream are critical in developing a broader understanding of the PPD problem.

It was reported that inheritance of post-harvest root deterioration is at least partially controlled by additive and quantitative factors rather than qualitative ones (Kawano and Rojanaridpiched, 1983). Ten and three putative QTLs (quantitative trait loci), respectively, which explain between 5 and 13% of the phenotypic variance of PPD of cassava had already been identified. Hence variations among the genotypes for PPD can be exploited for breeding PPD tolerant varieties. Cassava breeding at CIAT (*Centro Internacional de Agricultura Tropical*) for PPD tolerance found genetic variability for this trait accounted for 52% of the total observed variability, indicating the possibility of progress in a

selection procedure. Tolerance to PPD in cassava may offer enormous benefit to both cultivators and processors and thus needs basic and applied research with a focus on long-term strategies such as improved varieties through conventional breeding and genetic transformation. A successful research programme demands the development of an appropriate and universal method to evaluate PPD in cassava that is both practical and quantitative to better link phenotypic tolerance to PPD. At present, PPD evaluation is subjective and mainly based on visual symptoms. A systematic work on PPD is the need of the hour to understand biochemical aspects of PPD and to exploit our genetic resources available at our country to identify potential genotypes for future breeding programmes.

The main aim of the study was to screen the cassava genotypes for post harvest physiological deterioration (PPD), to analyse the physiological biochemical and molecular mechanisms associated with PPD and to develop methods to delay the PPD in cassava.

# Chapter 2

# Review of Literature

# 2 Review of Literature

## 2.1 Cassava - A versatile crop of tropics

Cassava (*Manihot esculenta* Crantz) is ranked as the sixth most important food crop and consumed by 800 million people worldwide (Lebot, 2009). The storage roots are edible as they are the main carbohydrate storage organs in cassava and stores upto 85% starch on dry weight basis. It is an attractive cash crop for small-scale farmers with limited resources and it can grow well even in poor and marginal soil (Ceballos *et al.*, 2004; Wenham, 1995). Cassava crop is well adapted to traditional mixed cropping agricultural systems and subsistence farming system which helps the farmers to minimize the risk of total crop failure (Wenham, 1995). Moreover, cassava is being grown and processed for animal feed and material for various industrial applications (Beeching *et al.*, 2002). Cassava is also a climate resilient that responds to the priorities of developing countries with the changing trends in the world economy (Howeler *et al.*, 2013).

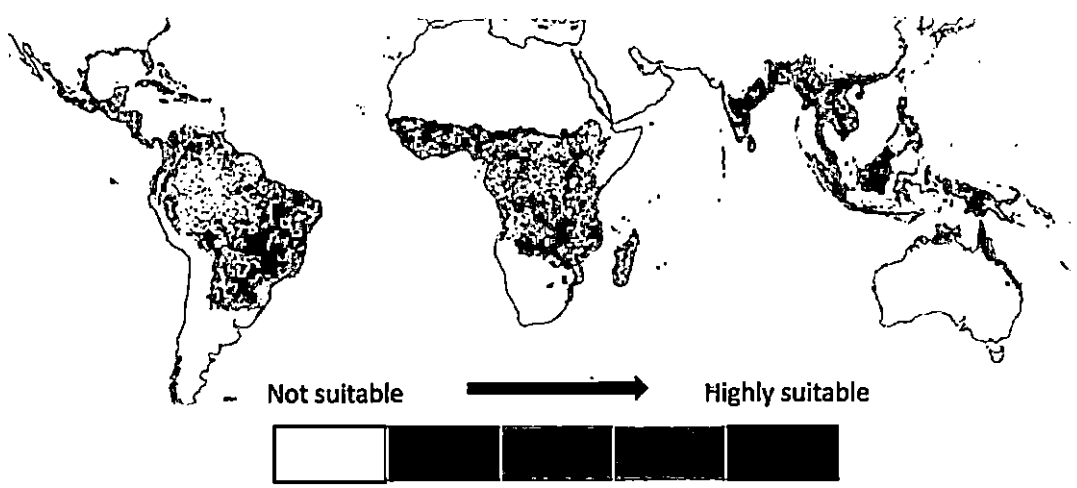



Figure 2.1. Land suitability for rain-fed cassava

(Source: FAO, Rome)

According to FAO estimates, the global harvest in 2013 was more than 278 million tonnes, representing a 60% increase since 2000 and an annual growth rate double that of the previous two decades. It is the cheapest source of calories for both human nutrition and animal feeding (Tonukari, 2004). Cassava production is expected to increase further primarily due to both higher demand as human food and its value as raw material for industrial proposes, particularly in Africa (Garcia and Dale, 1999; Tonukari, 2004). It is estimated that cassava yields more energy per hectare (1045 KJ hectare$^{-1}$) than other major crops, such as rice (652 KJ ha$^{-1}$ (Montagnac *et al.*, 2009). The starch rich storage roots are staple food several million people in developing world (Figure 2.2). Thus, in the developing world cassava is amongst the top four most important crops (with rice, sugarcane and maize). A variety of ways like boiling, mashing, frying and drying are widely used to produce granules, flour and chips that have a seemingly endless list of food and feed applications. In Asia and South East Asia the crop is grown mainly for animal feed and industrial purposes and for bio-fuel production. For example, cassava is used for production of sweeteners, acids, various alcohols, biodegradable plastics and there is also growing interest in using cassava as a source of bio-fuel mainly ethanol (Balat and Balat, 2009; Jansson *et al.*, 2009). Several advantages of cassava crop like high productivity even under minimal inputs in marginal soils, tolerance to abiotic and biotic stresses and also its flexible harvesting time make it an ideal crop for poor and marginal farmers. With increase in cassava production worldwide, there is likely to be more post harvest losses in term of quantum of roots harvested due to an inherent phenomenon called post harvest physiological deterioration (PPD) of cassava. The PPD of cassava is the principal cause of short shelf-life of harvested roots and has attracted considerable research interest for the last two decades.

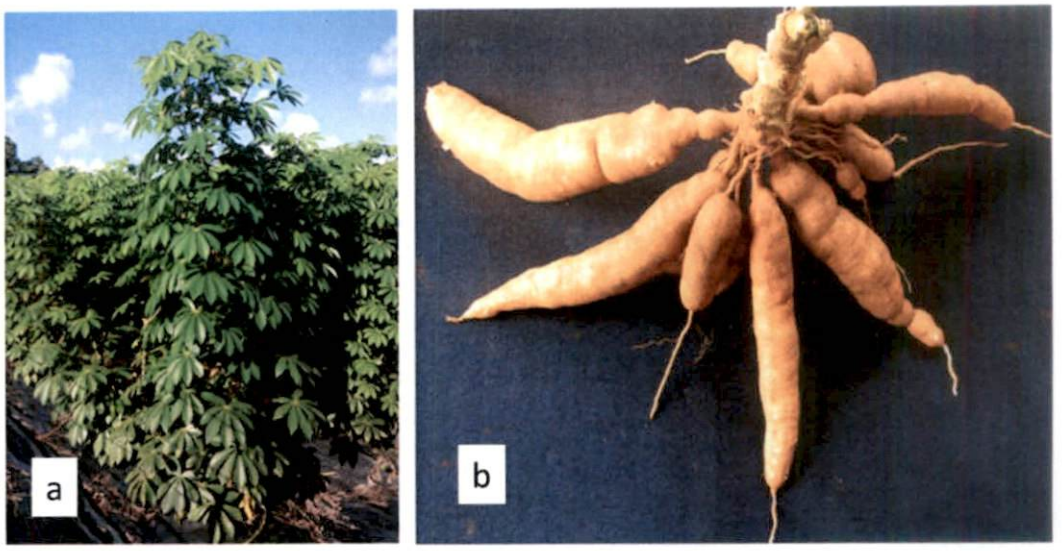
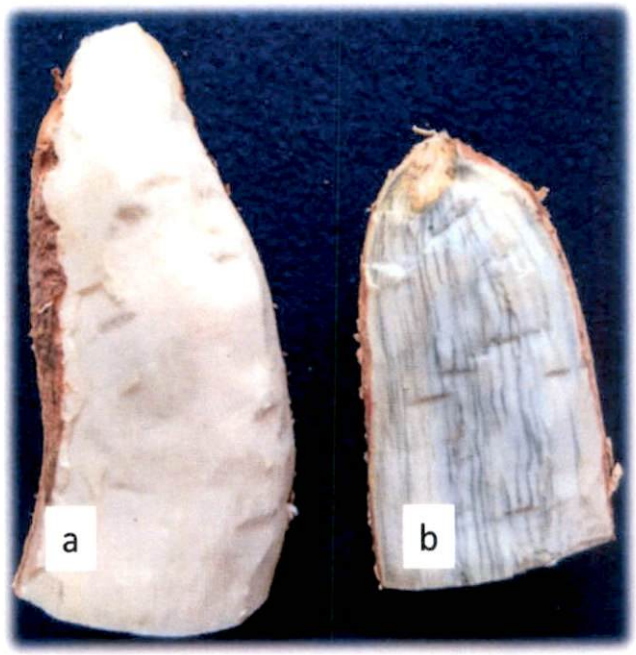



Figure 2.2. (a) Cassava field view and (b) fully matured storage roots.

## 2.2 Post harvest physiological deterioration of cassava roots and its implications

Harvested cassava roots undergo rapid deterioration and they can't be stored satisfactorily for more than two to three days under ambient conditions. This phenomenon, known as post-harvest physiological deterioration (PPD) depends on the genotypic as well as the environmental conditions and is followed by microbial infection (Noon and Booth, 1977; Ravi and Aked, 1996; Reilly *et al.*, 2004). The deterioration of cassava roots generally starts with a black-blue to black vascular discoloration (vascular streaking) which quickly spreads to the parenchyma tissue (Figure 2.3). Cassava PPD results in sizeable quantitative and marked qualitative post-harvest losses of the roots (Figure 2.4). Earlier studies of cassava post harvest deteriorations mainly concentrated on isolating microorganisms which were considered pathogenic to stored roots.




a) Healthy root b) PPD affected root

Figure 2.3. Initial symptom of PPD in cassava roots, visible as vascular streaking a) healthy fresh root and b) root showing the typical PPD symptoms

The entire root becomes unpalatable and unmarketable soon after the PPD symptoms develop.

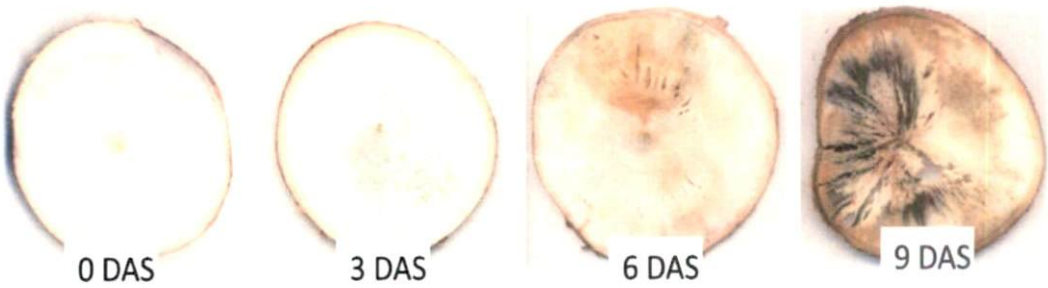



Figure 2.4. Progress of PPD symptoms in cassava variety Sree Padmanabha after 0, 3, 6 and 9 days after storage

PPD is considered one of the main post harvest constraints for farmers, traders and food and starch industries. The short shelf life of harvested roots makes the long distance transport of cassava for marketing or industrial purpose unviable either due to loss in transit or low deterioration in quality raw material for food

processing industry. Cassava root PPD limits the expansion of cassava production in developing countries, and has become a major constraint compared to other root crops, due to root discounting, waste and added costs (Reilly *et al.*, 2004; Wenham, 1995; Westby, 2002). PPD directly increases cost of production, processing and marketing and also burgeon the risks associated with cassava processing and marketing. Earlier estimates peg the losses due to PPD in cassava to be in the range of 5–25% of the total expected value of the crop (Wenham, 1995). Recently, an estimate by Rudi *et al.*, (2010) envisages the economic benefits of extending the shelf life of cassava to several weeks would reduce financial losses by $2.9 billion in Nigeria alone over a 20-year period.

## 2.3 Physiological, biochemical and molecular mechanism behind PPD

### 2.3.1 Wound induced signal transduction

Tissue damage in plants triggers a cascade of wound responses resulting in defence reactions of the wounded tissue and the subsequent sealing of exposed tissue by regeneration of a protective barrier (Bowles, 1990). Most common wound responses include increased activity of lytic enzymes (glucanase and chitinase), protease inhibitor proteins and production of hydroxyproline-rich glycoproteins. Enzymes associated with the phenylpropanoid pathway, such as phenylalanine ammonia-lyase and chalcone synthase, lead to the biosynthesis of phenolics which may act directly as defence compounds (quinones, phytoalexins) or can form polymers, such as lignin, that render cell walls more resistant to water loss and attack from microbial enzymes and changes to membrane lipids and the production of ethylene (Beeching *et al.*, 1998; Rickard, 1985). Specific genes involved in these mechanisms have been identified, characterized and their expression evaluated (Reilly *et al.*, 2001, 2007; Taki *et al.*, 2005). Currently, the evolutionary development of the plant immune system is represented as a zig-zag model (Jones and Dangl, 2006). Pathogen-associated molecular patterns (PAMPs) or microbe-associated molecular patterns (MAMPs), such as flagellin, chitin, glycoproteins, and lipopolysaccharides, or endogenous plant-derived signals that arise from damage caused by pathogen infection, called damage-associated molecular patterns (DAMPs), are recognized by pattern-recognition receptors. This results in the

activation of PAMP-triggered immunity (PTI). In response to tissue damage, endogenous plant signals (DAMPs) are produced that play an important role in the perception of herbivory.

Cassava, like all other plants rely on various defence mechanisms for protection against injury from insects, microorganisms or mechanical wounding. Cassava is inherently predisposed to wounding due to mechanical injury that usually accompanies harvesting and handling of the storage roots(Booth, 1976). Wounding presents a constant threat to plant survival because it not only physically destroys plant tissues, but also provides an entry point for pathogen invasion. To cope with wounding effectively, plants must prepare for pathogen attack while defending against insect predators. It is for this reason that it is hypothesized that plants may have evolved mechanisms that integrate the pathogen and wounding response. Mechanical wounding and pathogen infection in plants arising from localized tissue damage activates defence responses not only at the site of injury (local), but that are also transmitted to other parts of undamaged tissues (systemic). These immediate responses may involve the induction, translocation, perception and transmission of wound signals which are capable of inducing defence related gene expression. Wound activated responses are directed at preparing the plant for the potential extension of wounding, healing of damaged tissues and the activation of defence mechanisms that prevent further damage by inhibiting the defence signals (Bowles, 1998). The signalling pathways are mediated by many structurally different molecules including the oligopeptide systemin (Pearce *et al.*, 1991); molecules with hormonal activity such as jasmonic acid (JA), salicylic acid (SA), ethylene (Reymond and Farmer, 1998), and hydrogen peroxide ($H_2O_2$) (Orozco-Cardenas and Ryan, 1999) which further orchestrate the induction of defence responses.

Physiological deterioration in cassava parallels many features of plant wound responses since aspects of wound response are present in the harvested cassava root; however, the wound repair and subsequent down modulation of the signals necessary for restoration of integrity of damaged tissues are inadequate for wound healing. This deficiency leads to continuous cascades of wound responses that spread through the entire root. Interestingly, wounded cassava roots that

remain attached to the plant are capable of normal wound repair (Mwenje *et al.*, 1998) suggesting that efficient wound repair mechanism of the detached root was lost during evolution, since the root serves no biological function once detached from the plant. Unlike cassava roots, tubers of yam, sweet potato (modified roots) and potato (modified stem) can act as propagules, since they accumulate storage proteins which can be broken down to support sprouting. The storage proteins possess biological activities consistent with a role in protecting the tubers against pests, pathogens and abiotic stresses (Shewry, 2003). Cassava roots unfortunately lack true storage proteins which are necessary to mediate the stress responses associated with abiotic stresses, for example wounding (Shewry, 2003). The development of PPD throughout the storage tissues of cassava after harvesting suggests the transmission of intercellular signals from the sites of damage, and apart from the involvement of ethylene and hydrogen peroxide ($H_2O_2$) in this signalling network, it remains widely unknown which other signalling molecules are involved and how their interplay affect PPD in cassava. Research on the nature of mobile signals, their biochemical origin and the defence response genes they activate downstream are critical in developing a broader understanding of the PPD problem.

## 2.4 Role of Plant growth regulators in wound signal transduction and PPD expression

Diverse plant hormones act as central players in triggering the plant immune signalling network (Bari and Jones, 2009; Howe and Jander, 2008; Katagiri and Tsuda, 2010; Pieterse *et al.*, 2009, 2012). Analogous to animal hormones, plant hormones were originally recognized as regulators of growth and development (Santner and Estelle, 2009). Salicylic acid (SA) and jasmonic acid (JA) with its derivatives (collectively called jasmonates) are recognized as major defence hormones (Browse, 2009; Vlot *et al.*, 2009). However, the hormones ethylene (ET) (van Loon *et al.*, 2006), abscisic acid (ABA) (Ton *et al.*, 2009), gibberellins (GAs) (Navarro *et al.* 2008), auxins (Kazan and Manners, 2009), cytokinins (CKs) (Walters and McRoberts, 2006), brassinosteroids (Nakashita *et al.*, 2003), and nitric oxide (NO) (Moreau *et al.*, 2010) function as modulators of

the plant immune signalling network as well.

### 2.4.1 Role of ethylene in PPD of cassava

The phytohormone ethylene is a regulator of growth and development in plants and plays a key role in defence network (Ecker, 1995). Ethylene is synthesized from S-adenosyl methionine (SAM), which is a precursor substrate for many biochemical pathways, including polyamine biosynthesis (Ravanel *et al.*, 1998). Ethylene is involved in plant senescence, fruit ripening, abscission and the activation of senescence-related gene expression (Johnson and Ecker, 1998). It is induced upon a wide range of abiotic stresses such as wounding, flooding, chilling and desiccation or drought (O'donnell *et al.*, 1996; Wang *et al.*, 2002b). Ethylene also regulates plant responses to biotic stresses such as those induced by pathogens and enhances plant resistance against pathogen infection by the activation of the enzymatic activities of chitinase (Rakwal *et al.*, 2004), peroxidase, and phenylalanine ammonia lyase (Kato *et al.*, 2000). It is also thought to be responsible for inducing many biochemical processes that lead to programmed cell death (PCD) (Gunawardena *et al.*, 2001; Young *et al.*, 1997).

Wounding induces ethylene production in sweet potato roots within 24 hours (Sakai *et al.*, 1970) and was shown to precede wound lignifications and wound periderm formation by 24 and 48 hours respectively (St Amand and Randle, 1989). Similarly, ethylene levels in damaged cassava roots were reported to increase after an initial lag period of 6 hours and the levels remained high over a 22 hour period (Plumbley *et al.*, 1981). Hirose *et al.*, (1984) reported that ethylene levels in cassava peaked after 16 hour lag period with varietal differences influencing to the rate of ethylene production. Pre-harvest pruning, which involves the elimination of the aerial part of the cassava plant, has been shown to suppress PPD development (van Oirschot *et al.*, 2000). However, pre-harvest pruning has no significant influence on ethylene production following wounding and the exogenous application of ethylene on wounded roots does not affect the wound responses (Bleecker and Kende, 2000; Wang *et al.*, 2002a; Hirose *et al.*, 1984). All these observations have led to the speculation that ethylene may be involved

albeit indirectly in signal transduction during PPD. Accelerated ethylene biosynthesis in plants is frequently associated with the induction of ACS and ACO genes which are responsible at least in part for the increased levels of enzyme activity under some conditions (McKeon *et al.*, 1995; Bleecker and Kende, 2000; Wang *et al.*, 2002a). In a microarray study aimed at identifying genes expressed during cassava PPD, the ACC oxidase, was found to be up-regulated in cassava. Similarly in tomato, it has also been shown that ACC oxidase, is activated in response to wounding (Bowles, 1998). The individual members of the ACS and ACCO gene families are regulated differentially in response to specific stimuli, suggesting that they play different roles in enhanced ethylene production in response to different environmental or endogenous cues (Bleecker and Kende, 2000; Wang *et al.*, 2002a) . The involvement of ethylene in several agronomically and physiological important processes has contributed to its being targeted for manipulation by chemical and biotechnological methodologies (Abeles *et al.*, 1992; Mattoo and Suttle, 1991; Stearns and Glick, 2003). Detailed studies on the complex regulation of these pathways and of proteins involved still remains scarce and needs to be investigated in cassava.

### 2.4.2 Role of jasmonates and wound signalling and wound response

The plant hormone jasmonic acid (JA) is synthesized through the octadecanoid pathway from its α-linolenic acid precursor (Conconi *et al.*, 1996). Linoleic acid is a major fatty acid constituent of plant membranes and its application to tomato plants results in the expression of the same set of genes as the application of JA, suggesting that fatty acid release from complex membrane lipids is a critical checkpoint in JA biosynthesis (León *et al.*, 2001). The levels of endogenous jasmonates are elevated by stresses including mechanical wounding, herbivory damage or pathogen attack. These in turn signal the induction of expression of specific jasmonate responsive genes to counteract the stress. The wound induced synthesis of JA and its volatile derivative methyl jasmonate (MeJA) is one of the last steps in the signaling pathway which leads to activation of wound responsive genes including the induction of proteinase inhibitors (PIN) (Creelman and Mullet, 1997; Farmer and Ryan, 1992). Additionally, oligogalacturonides derived from plant cell walls upon injury have also been

shown to be elicitors of wound inducible PIN gene expression in solaneceous plants (Doares *et al.*, 1995).

In sweet potato the expression of the ipomoelin, a wound inducible protein, was shown to be enhanced by the application of methyl jasmonate (MeJA) and mechanical wounding (Imanishi *et al.*, 1997). The activation of the sporamin gene in sweet potato is similar to the wound activation of the proteinase inhibitor II gene (*pin* 2) in tomato, in that the *pin 2* gene was up-regulated by MeJA and absicic acid (ABA) and blocked by salicylic acid (SA) in the signal transduction pathway (Doares *et al.*, 1995). Currently there is no information regarding the role of MeJA as a possible signal leading to onset or progression of PPD in cassava roots. Research aimed at unravelling the major role of plant hormones such as jasmonic acid or its volatile derivative MeJA is necessary as this would shed more light into the contribution of these signalling molecules in initiating PPD. However, the rapid appearance of vascular streaking in harvested cassava roots is a result of membrane disorganization resulting in a loss of cellular compartmentalization (Tanaka *et al.*, 1983). One detailed study of lipid changes in harvested cassava roots has shown a progressive decline in phospholipid content indicating membrane degradation. It was speculated that these changes indicate structural alterations to membranes which might allow the interaction of substrates and enzymes producing dark streaking (Lalaguna and Agudo, 1989).

### 2.4.3 Role of salicylic acid

The signalling molecule (SA) is crucial for local hypersensitive responses and systemic acquired resistance against many plant pathogens (Maleck and Dietrich, 1999). SA interferes with wound-related gene expression by inhibiting the octadecanoid pathway (O'donnell *et al.*, 1996; Pena-Cortés *et al.*, 1993). It has been reported that the defence-signalling pathways salicylic acid (SA) and jasmonic acid (JA) can affect each other's responses (Niki *et al.*, 1998). SA mediates the oxidative burst that leads to cell death in the hypersensitive response, and activates the pathogenesis-related (PR) genes in the systemic leaves after infection with avirulent pathogens. SA is known to have an antagonistic effect on the JA signal (Doares *et al.*, 1995). Despite the many studies being done to determine the role of SA in signalling pathways, none has so far shown a

significant accumulation or reduction of SA during these responses indicating that SA is not the direct signal that induces these responses and further suggests that the pre-existing endogenous levels of SA may affect heat (Dat *et al.*, 1998), chilling (Janda *et al.*, 1999) and salt tolerance (Borsani *et al.*, 2001). It has also been shown that infection of tomato by *Pseudomonas syringae pv tomato* induces the accumulation of endogenous SA and enhances the wound-induced expression of phenylalanine ammonia lyase. This revelation shows that SA enhances the response of some wound-responsive genes after wounding. Although the relationships between SA and various responses have been described, the direct relationship between SA and wounding still remains obscure (Vlot *et al.*, 2009). Information on the involvement of SA and JA in wound response in cassava as well as in other root crops is still non-existent, necessitating the need to investigate the role of these signalling molecules in stress resistance in cassava as well as in different root crops.

## 2.5 Enzymatic activity during PPD

Several key enzymes of the phenylpropanoid pathway including phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase were induced in sweet potato and cassava, but the activity in cassava peaked much later (Tanaka *et al.*, 1983). Levels of peroxidase, which is involved in biosynthesis of lignin as well as detoxification of hydrogen peroxide, were also found to be higher in sweet potato than cassava. The high amount of poly-phenols and lignin produced in sweet potato tissue and taro on wounding as opposed to cassava tissue possibly explains why wound healing in cassava does not occur. A clear understanding of the molecular mechanisms involved in cassava in response to PPD is of fundamental importance for the development of rational breeding programs (molecular markers) and transgenic strategies to improve or modulate the process. To this end, several approaches have been developed to characterize PPD development based on analysis of dramatic changes in gene expression patterns and biochemical studies on the changes of secondary metabolite accumulation during PPD. Enzymes involved in the phenylpropanoid pathway, including PAL and 4-coumarate: CoA ligase, have been shown to be differentially regulated upon

wounding. The expression of PAL activity closely follows the progress of cassava root PPD, and peaks after approximately 2 days of wounding. The Peroxidase enzymes at the cassava root wound surface act upon the phenolic compounds generated by PAL. Apparently, accumulation of the enzyme occurs by de novo synthesis and is induced after a 24 h lag period.

Using sequentially sectioned cassava roots, it was found that superoxide dismutase (SOD), catalase, and peroxidase were predominantly expressed in regions closer to the wound site. Aldehydes have been detected from harvested root (Iyer *et al.*, 2010). These emissions are known to be derived from fatty acid oxidation, and were previously detected in cassava leaves. The commonly known pathway in fruits and vegetables leading to $C_6$ aldehyde and alcohol formation is derived from fatty acids via lipoxygenase (LOX) and alcohol dehydrogenase (ADH). The increase in aldehydes is associated with senescing plant tissues. Given the detection of aldehydes and only trace quantities of alcohols at 3 h after root detachment, the ADH pathway is seemingly operating during cassava root PPD.

## 2.6 Gene expression and molecular response to PPD

Using RNA fingerprinting analyses, Huang *et al.*, (2001) showed that transcripts involved in important biochemical and physiological processes, notably oxygen stress and carbohydrate and protein metabolism, are involved in PPD. In an attempt to identify the entire subset of genes that are differentially regulated in cassava during PPD, Reilly *et al.*, (2007) carried out a large-scale cDNA microarray analysis of the cassava root transcriptome. They found 72 differentially regulated ESTs, of which 63 were up-regulated and 9 were down-regulated. Many of the up-regulated PPD-specific ESTs were predicted to play roles in cell wall repair, reactive oxygen species (ROS) generation and turnover, programmed cell death, ion/water/metabolite transport, signal transduction, stress response and metabolism, and protein synthesis. ROS have been shown to increase very early during PPD and evidence for the involvement of ROS and associated turnover enzymes during PPD is accumulating. Several lines of evidence suggest that there is a controlled production of ROS in plant defence, especially in response to wounding and pathogen attack. (Reilly *et al.*, 2004) reported a rapid oxidative burst

within 15 min of harvest, signalling the start of PPD, predominantly due to a rapid production of superoxide and hydrogen peroxide. Several roles have been attributed to the accumulated ROS species, among which cell wall repair and remodelling, induction of defence-related genes, signal transduction, and triggering host cell death are significant.

## 2.7 Role of low molecular weight metabolites during PPD

The blue/black product synthesized during PPD is due to the oxidation of hydroxycoumarins by peroxidases and hydrogen peroxide ($H_2O_2$). Hydroxycoumarins are secondary metabolites that are involved in plant defense and include esculetin and scopoletin. Application of phenolic compounds to freshly harvested root sections revealed that only scopoletin caused a rapid and intense discolouration indicative of PPD (Wheatley and Schwabe, 1985). The synthesis of scopoletin *via* the phenylpropanoid pathway in cultivar MCOL22 increases during PPD, peaking 24 hours after harvest at 100 nmol $g^{-1}$ FW as measured using High Performance Liquid Chromatography (HPLC), before gradually returning to basal levels (approximately 20 nmol $g^{-1}$ FW) in subsequent days (Buschmann *et al.*, 2000a). Accumulation can also be visualised since hydroxycoumarins fluoresce under ultraviolet (UV) light. Interestingly, there was no correlation between quantification of fluorescence and subjective scoring of symptoms in 25 cultivars of cassava roots after five days storage. This discrepancy was attributed to stabilisation and gradual degradation of hydroxycoumarin content prior to symptom development (Salcedo *et al.*, 2010). The involvement of scopoletin in defence and PPD was implicated further since phenylalanine ammonia lyase (PAL), a key enzyme in its production, was up-regulated following treatment of cassava cell suspension cultures with pathogens including *Fusarium oxysporum* (Gómez-Vásquez *et al.*, 2004) and increased levels of the protein have also been detected within 24 hours post harvest (Owiti *et al.*, 2011). Similarly, a 17% increase in PAL activity has been reported in sweet potato following wounding and storage for two days at 15°C (Bayoumi *et al.*, 2008a, 2008b, 2010) (Reyes *et al.*, 2007). The biosynthetic pathway for scopoletin in harvested cassava roots is being elucidated using HPLC and mass spectrophotometer techniques (Bayoumi *et al.*, 2008a, 2008b, 2010) and which may offer insights into the factors affecting its production and regulation.

## 2.8 Role of reactive oxygen species (ROS) in PPD

Several studies indicated the role of ROS in cassava PPD. ROS are molecules that are derived from non-toxic molecular oxygen ($O_2$) and include singlet oxygen ($^1O_2$), superoxide anion radical ($O_2^{\bullet-}$), $H_2O_2$ and hydroxyl radical ($HO^{\bullet}$). In plants, ROS are produced during the normal metabolism of photosynthesis and respiration, involving photosystems I and II (PSI and PSII), mitochondrial electron transport chain, membranes and peroxisomes. Single oxygen oxidises amino acids and causes membrane damage and is unique amongst ROS since $O_2^{\bullet-}$, $H_2O_2$ and $HO^{\bullet}$ are generated *via* a series of reduction reactions. Although, $H_2O_2$ is less reactive than $O_2^{\bullet-}$, it readily permeates membranes and therefore it is capable of disrupting enzymes *via* oxidation of their thiol groups. $H_2O_2$ is removed by catalases (located in glyoxysomes and peroxisomes) and peroxidases (POX) located in different cellular compartments, but particularly chloroplasts. The final reductive stage (Fenton reaction) gives rise to $HO^{\bullet}$ that has extremely high oxidising potential compared to the other ROS and cause significant cellular damage (Garg and Manchanda, 2009). Importantly, although ROS are toxic by-products of aerobic reactions, $O_2^{\bullet-}$ and $H_2O_2$ serve crucial roles in signalling and defence gene activation (Galvez-Valdivieso and Mullineaux, 2010; Møller and Sweetlove, 2010). Adverse environmental conditions which can be biotic (imposed by other organisms) or abiotic (arising from an excess or deficit in the physical or chemical environment) disrupt the metabolic balance of cells resulting in enhanced accumulation of ROS (Mittler *et al.*, 2004). On wounding, plants transiently produce reactive oxygen species (ROS) including the superoxide anion in the damaged tissue, and hydrogen peroxide both locally and systemically (Orozco-Cardenas and Ryan, 1999). Wounding has been shown to stimulate the production of $H_2O_2$ in cassava (*Manihot esculenta* Crantz.), (Repka, 1999) perhaps

due to loss of compartmentalization within plant cells. A rapid oxidative burst which generates the initial signals composing particularly of high levels of damaging ROS has been shown to occur in cassava storage root upon wounding (Reilly *et al.*, 2004). Wheatley and Schwabe, (1985) suggested a possible correlation of PPD with an increase in phenolic compounds (e.g. scopoletin) and $H_2O_2$. In addition, it has been shown that a rapid increase of $H_2O_2$ occurs in cassava root over the first 24h of storage (Buschmann *et al.*, 2000a). The generation of $H_2O_2$ after wounding seems to be widespread in the plant kingdom and may act as an internal chemical mediator involved in the onset of signal transduction pathways (Doke 1991). Plants have evolved highly elaborate and an efficient ROS-scavenging mechanisms comprising of enzymatic and non enzymatic antioxidant molecules. The major ROS-scavenging enzymes in plants include superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), glutathione peroxidase (GPX) and peroxiredoxin. In combination with the antioxidants ascorbic acid and glutathione (Noctor and Foyer, 1998) these enzymes provide cells with efficient machinery to detoxify $O^{2-}$ and $H_2O_2$. SODs act as the first line of defense against ROS by catalyzing the dismutation of superoxide to hydrogen peroxide and oxygen. It acts in concert with CAT to detoxify potentially dangerous $H_2O_2$ transforming it to water. Similarly APX utilizes ascorbic acid as an electron donor to reduce $H_2O_2$ with the concomitant generation of monodehydroascorbate, which is spontaneously disproportionated back to ascorbic acid and dehydroascorbate via the NADPH dependent monodehydroascorbate reductase (MDAR). Dehydroascorbate reductase (DHAR) utilizes glutathione (GSH) to reduce dehydroascorbate thereby regenerating ascorbic acid. GPX is the principle cellular enzyme capable of membrane lipid peroxidation repair and is considered as the first line of defence against oxidative membrane damage (Kuhn and Borchert 2002). Unlike other organisms, plants have multiple genes encoding SOD and APX isoforms that are specifically targeted to chloroplast, mitochondria, peroxisomes, as well as to the cytosol (Asada, 1987).

Increases in enzyme activity of both peroxidase and catalase, the two key enzymes involved in turnover of $H_2O_2$, have been shown to occur during PPD (Beeching *et al.*, 1998). In a separate study to identify genes expressed during

PPD, the up regulation of genes involved in ROS turnover such as ascorbate peroxidase, catalase, secretory peroxidase, thioredoxin peroxidase, glutathione S-tranferase was demonstrated (Reilly *et al.*, 2007). The changes in expression patterns of the ROS regulating enzymes during PPD suggest the involvement of ROS in PPD development although no direct proof has been established. Although a complex interaction mechanism exists between the various ROS enzymes and general oxidative stresses in plants, interaction between these pathways provides key regulatory potential for activating multiple resistance mechanisms. The over-expression of individual ROS regulating genes may typically increase oxidative stress resistance in plants and could enhance the ROS scavenging capacity in cassava storage roots thereby delaying PPD. Generally the role various oxidants in changes in ROS levels vis-à-vis PPD are not clear and many of such actions are inferred and not proven. Role of antioxidants in modulation of PPD is yet to be ascertained accurately. It is highly probable that they may sometimes be casual rather than correlative event.

Cassava, like all other plants rely on various defence mechanisms for protection against injury from insects, microorganisms or mechanical wounding. Cassava is inherently predisposed to wounding due to mechanical injury that usually accompanies harvesting and handling of the storage roots (Booth, 1976). Wounding presents a constant threat to plant survival because it not only physically destroys plant tissues, but also provides an entry pathway for pathogen invasion. To cope with wounding effectively, plants must prepare for pathogen attack while defending against insect predators. It is for this reason that it is hypothesized that plants may have evolved mechanisms that integrate the pathogen and wounding response.

## 2.9 Modern tools for estimation of PPD

### 2.9.1 Application Metabolomics and Chemometrics in PPD research

PPD is a complex phenomenon which involves a plethora of pathways and an elaborate network of cellular functions occurring simultaneously in the harvested roots. Low molecular weight phytochemicals synthesized during PPD have significant role and hence monitoring them is gaining importance to

understand the mechanism behind PPD. Metabolomic approaches enable the parallel assessment of the levels of a broad range of metabolites and have been documented to have great value in both phenotyping and diagnostic analyses in plants (Osborne, 2006; Fernie and Schauer, 2008). A combination of metabolomic techniques with chemometric tools provides a fast, easy and reliable method for chemotaxonomy characterisation (Schulz and Baranska, 2007). Chemometrics is the use of mathematical and statistical methods to improve the understanding of chemical information and to correlate quality parameters or physical properties to analytical instrument data. Patterns in the data are modeled; these models can then be routinely applied to future data in order to predict the same quality parameters. Uarrota *et al.*, (2014) employed Mid-infrared vibrational Fourier transform spectroscopy (ATR-FTIR) to identify changes in the chemical and enzymatic composition of four cassava genotype samples during postharvest deterioration and to seek related biochemical markers for PPD. They found a clear trend with fresh samples and those with 3, 5, 8 and 11 days of PPD in terms of biochemical changes and the technique enabled discrimination by chemometric tools. There is a need for variable-reduction methods because of the vast amount of spectral information provided by NIR spectrophotometers, the substantial number of samples required to construct classification and calibration models, and the high number of correlated variables in the spectral data. The best known and most widely used variable-reduction method is the principal component analysis (PCA). PCA is a mathematical procedure which decomposes the data matrix with $n$ rows (samples) and $p$ columns (variables, e.g. wavelengths) into the product of a scores matrix, with $n$ rows (samples) and $d < p$ columns (principal components, PCs), and a loadings matrix, with $d < p$ rows (principal components, PCs) and $p$ columns (variables). Usually only a limited number $d < p$ of PCs are retained as the variability in the others is due to noise. This reduces the dimensionality of the data considerably, enabling effective visualisation, classification, and regression of multivariate data (Geladi, 2003).

### 2.9.2 Application of Near-infrared spectroscopy (NIRS)

The extent of PPD in roots is generally carried out using a qualitative measurement based on the visible changes observed in the parenchyma (Wheatley

*et al.*, 1982). These methods are largely subjective in nature and can result in biased estimate. Efforts to identify markers for PPD is met with little success as they had poor association with the PPD. Tools to objectively quantify the PPD with minimal effort and automation is need of the hour to identify PPD response among the cassava germplasm resources and to screen breeding population.

Application of Near-infrared spectroscopy (NIRS) together with chemometrics is routinely followed in several fields including agriculture. It is a well established technique for determining the components of many agricultural products (Belton *et al* 1994). It is a rapid, cost-effective, nondestructive method, allowing for simultaneous determination of principal constituents in a sample using multivariate data analysis (Osborne. 2006). In early studies, NIRS has been applied to determine the nutritional and functional components, such as protein, amino acids, crude fat, total starch, isoflavone, lutein, and carotene, in many crops including rice, soybean, maize, buckwheat, oats, wheat, common bean, and kale (Hacisalihoglu *et al.*, 2009; Bellon *et al.*, 1994; Chen *et al.*, 2009). The scope of NIR in analyzing the PPD of cassava is emerging as a tool due to its high precision, fast, accurate, easy and non-destructive technique that can be used as a replacement of time-consuming methods. NIR radiation covers the range of the electromagnetic spectrum between 800 and 2,500 nm (4,000-12,000$cm^{-1}$). NIR region contains information concerning the relative proportions of bands, which varies according to the structural components of the organic molecules. The change depends on the chemical composition of the product, as well as on its light scattering properties, which are related to the microstructure. In FT-NIR technique among different multivariate calibration tools partial least-squares (PLS) method can be used in food industry by extracting features from spectra to relate the changes in spectral data with the changes in the sample. The information extracted can be condensed in the latent variables or factors, which are used in the calibration and prediction steps. PLS makes use of the information of the NIR spectrum and the established analyte values associated with the spectrum. The developed equation applied to unknown samples and the analyte is determined.

## 2.10 Breeding and improvement of cassava for delayed PPD

Breeding for improved storability and delaying PPD has been an important theme for last few decades, however, the success for breeding cassava with longer shelf-life met with little success due to various reasons. The lack of reliable marker for identification of genotypes, lack of an objective scoring methodology, greater influence of environmental factors and quantitative nature of PPD trait, low heritability, lack of sufficiently accurate linkage map for PPD for locating PPD related markers etc., are some of the reasons for slow progress in breeding PPD tolerant varieties through either by conventional or marker aided selections in cassava.

### 2.10.1 Conventional breeding and selection for low PPD cassava

Breeding for longer shelf life has been hampered by a number of factors, such as the influence of environmental growth conditions and associated pre-harvest stress, limited genetic variability and a persistent but moderate negative correlation between low deterioration rate and high dry matter content (Wenham, 1995; Sanchez *et al.*, 2006). In addition, cassava roots are not organs of propagation, thus selection for postharvest conservation may not provide a selective advantage (Wenham, 1995). However, cassavas highly differentiated gene pools and the large percentage of dominant/recessive gene action loci make it highly heterotic (Fregene and Puonti-Kaerlas, 2002). The use of molecular markers for germplasm assessment is being employed to take advantage of this. Using 101 clones, Sanchez *et al.* (2006) showed a negative correlation between PPD and carotenoid content, suggesting that carotenoids may help delay PPD. (Morante *et al.*, 2010)) also identified genotypes with delayed PPD. The availability of these new sources of tolerance should make possible the identification of molecular markers linked to PPD.

Considerable effort has been devoted in improving cassava yield traits, nutritional qualities and resistance against diseases through conventional breeding. Since tolerance to PPD is a complex trait controlled by many genes and poor understanding of the QTL responsible for the trait, high heterozygosity, poor flowering and the cross-pollinating nature of cassava, limits the application of

breeding, especially for a multigenic trait such as PPD (Jennings and Iglesias, 2002). The negative correlation reported to exist between PPD and high dry matter (a desirable trait) could be difficult to separate via conventional breeding (Ceballos *et al.*, 2004) thereby suggesting the limitation of conventional breeding for delayed PPD. In addition, traditional breeding is usually difficult, time consuming and quite laborious. In light of these challenges to breeding for resistance to PPD, transgenic approaches look promising.

### 2.10.2 Molecular breeding

Biotechnological tools can be used to accelerate breeding programs through marker assisted selection (MAS) although the mapping of quantitative trait loci (QTLs) linked to PPD in cassava crosses has not yet identified strong markers. The evaluation of all biochemical components involved in PPD and further comparison of biochemically distinct cassava cultivars may reveal basic insights into the PPD problem and thereby lead to identification of candidates which can be tested by breeding and molecular approaches. Delayed PPD has been identified in *Manihot walkerae*, a wild relative of cassava, and the development of inter-specific hybrids between cassava and *Manihot walkerae*, offers possibilities for mapping PPD related genes. Cortes *et al.*, (2002) identified the major genome regions of cassava related to physiological post-harvest deterioration, thereby providing tools for the identification of gene(s) controlling this trait. Using molecular linkage map previously constructed based on the segregation of 240 RFLP, 100 RAPD, 85 microsatellite and five isoenzyme markers on 144 F1 individuals, they found a set of 10 molecular markers with a significant association with putative QTLs for PPD. Based on single-marker regression, eight putative QTLs were found to explain between 5–12% of the phenotypic variance of the PPD. In the male-derived framework map, two putative QTLs on linkage groups C and L explained 13% and 11% of this variance, respectively.

### 2.10.3 Transgenic approaches and genetic engineering to delay PPD

Analyses of genes expressed during cassava PPD reveals an altered regulation of genes and enzymes involved in signal transduction, reactive oxygen species (ROS) regulation, phytohormone synthesis, senescence, programmed cell

death (PCD) responses, and the formation of compounds involved in the synthesis of cell wall components. While these studies have contributed towards identifying some of the key genes whose regulation indicate a critical role in cassava wound response, they were restricted to the elements of the process that are regulated at the transcript level leaving out equally important information on regulation of their gene products.

(Siritunga and Sayre, 2004, 2003) developed transgenic acyanogenic plants, in which the expression of the *CYP79D1* and *CYP79D2* genes were selectively inhibited in the leaves, in a bid to eliminate cyanogens from cassava. However, the transgenic plants were unable to grow in the absence of reduced nitrogen ($NH_3$) suggesting that cyanide derived from linamarin is a major source of reduced nitrogen for cassava root protein synthesis (Siritunga and Sayre, 2004). Overexpression of the cyanide-insensitive alternative oxidase (AOX) in transgenic tobacco had been shown to decrease ROS accumulation generated by the respiratory chain electron transport. Over-expression of *Arabidopsis* AOX in transgenic cassava roots resulted in substantially reduced ROS accumulation and delayed the onset of PPD by as much as three weeks, enough time for the shipping or processing operations necessary after harvesting the crop. However, the variations in the field grown transgenic cassava plants were proved to be an impediment in this approach.

The second strategy to reduce PPD was to quench ROS production by over-expression of ROS-metabolizing enzymes (e.g., catalase, SOD, ascorbate peroxidase) or by the over accumulation of anti-oxidants, such as β-carotene. It was also previously observed that cassava varieties with elevated β-carotene content had extended shelf life. Indeed, the shelf life of transgenic plants with elevated β-carotene (40 ppm) content was extended to four weeks. Overall, these results suggest that ROS production from cyanide-poisoned mitochondria initiate PPD and that reduction in ROS accumulation will extend the shelf life of harvested cassava roots. PPD is a complex biochemical event; it will be interesting to observe how manipulation of a single pathway or a subset of genes will alter its expression in cassava roots. The challenges are many for modifying the PPD in a predictable and desired way through transgenic approach in years to come.

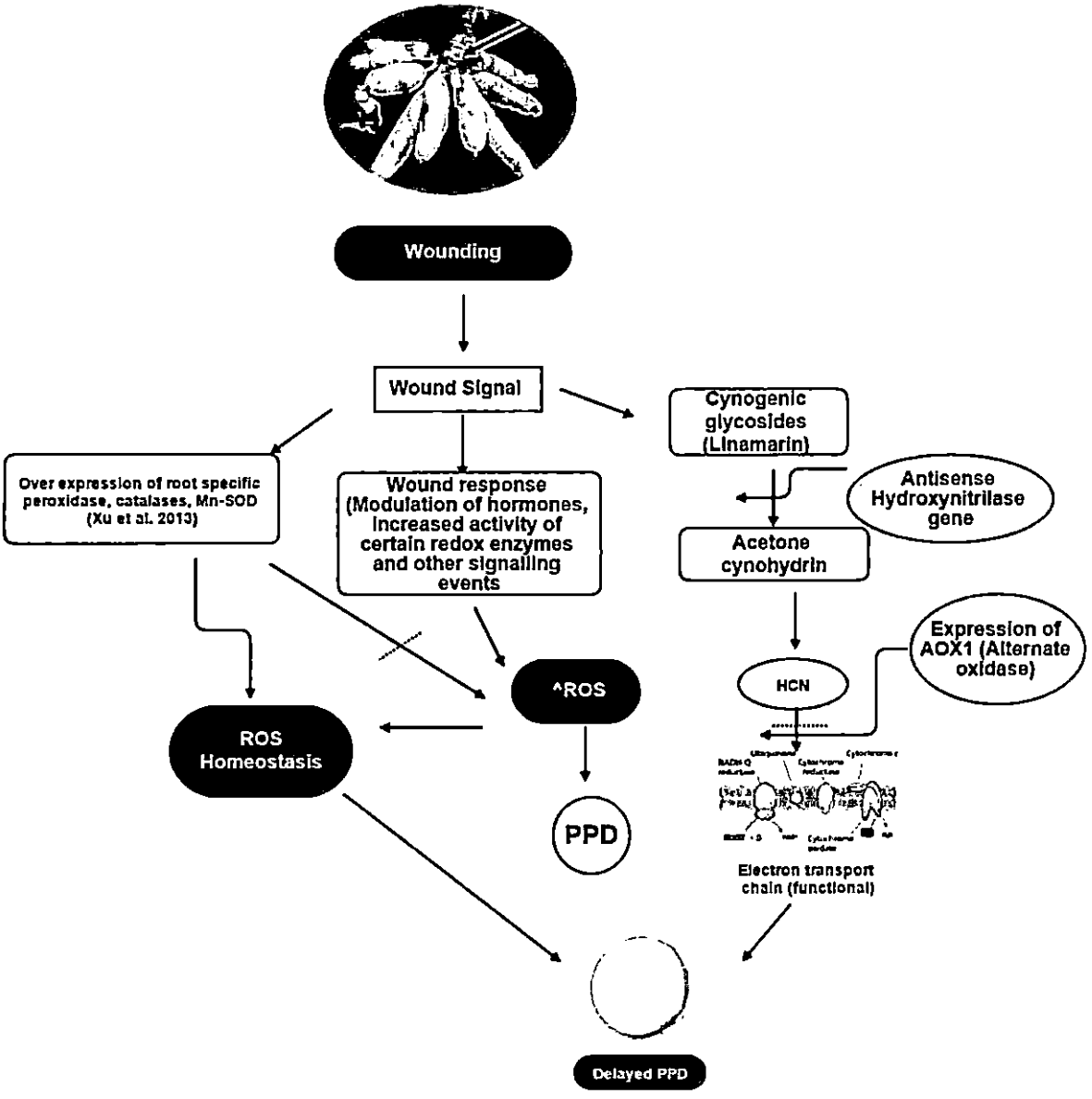



Figure 2.5 Schematic diagram of genetic manipulation of PPD by transgenic approaches

## 2.11 Management of PPD

### 2.11.1 Coating roots with paraffin wax and air tight packaging techniques

The most effective method for controlling cassava PPD is oxygen exclusion (Reilly *et al.*, 2004). Consequently, cassava for export markets such as Europe and the USA is treated with wax. Roots dipped in ordinary paraffin wax for 45 seconds at 90-95°C can be stored for a month (Lebot, 2009).

### 2.11.2 Delaying PPD through storage techniques

Studies on the deterioration of fresh cassava roots were conducted by the International Centre for Tropical Agriculture (CIAT) and the Natural Resources Institute (NRI) during the late 1970s. Methods such as a simple storage system based on root curing in polyethelene bags and treatment with a thiabendazole-based chemical to prevent the onset of deterioration (Wheatley, 1989), in-ground storage of roots until use are recommended for increasing the shelf-life. Thiabendazole prevents the secondary deterioration occurs when pathogens penetrate through wounds and bruises inflicted during harvesting and handling. Storage at high humidity encourages fungal rotting but is also necessary for effective wound healing. Processing of cassava into wide variety of products with a long shelf-life is traditionally followed (Lancaster *et al.*, 1982). The roots can be left in the ground for several months. The simplest method that many farmers use is in-ground storageor staggered harvesting i.e. harvesting the crop when it is needed. This is made possible by the fact that cassava does not have a distinct period of physiological maturity (Wenham, 1995). The harvesting window is thus flexible between 6-24 months. This strategy has several disadvantages. First, prolonged in-ground storage may increase chances of pathogen infection (Westby, 2002). At the same time the roots become woody (fibrous) due to lignifications or spongy and there can be loss of quality (Lancaster and Coursey, 1984). Lastly, in-ground storage locks up land that can otherwise be productively used. Storage in clamp silos, where roots are piled up on a layer of straw in conical heaps weighing between 300 and 500 kg and covered with straw and soil with openings left for

ventilation, has been found to be effective for four weeks (Rickard and Coursey, 1981; Westby, 2002). Box storage, with sawdust or coconut husk is also effective for four weeks (Westby, 2002). Moisture content of the sawdust requires careful control and lining the crates with plastic foil prevents drying out of the sawdust resulting in a storage period of 4–8 weeks (Rickard and Coursey, 1981). Low temperature storage is another strategy used to delay deterioration in cassava, although it is seldom practical for smallholder farmers. The most favorable temperature for storing fresh cassava is 3°C. At this temperature, the total weight loss after 14 days was 14% and was 23% after 4 weeks (Rickard and Coursey, 1981). Alternatively, roots, or pieces of root, can be stored frozen. Freezing changes the texture making it somewhat spongier, but the flavor is preserved (Rickard and Coursey, 1981). Roots stored at low temperature deteriorate faster when taken back to room temperature. Storage methods that cut off oxygen, such as storing in a water bath, are also practiced (Plumbley and Richard, 1991).

### 2.11.3 Post harvest processing

The processing of cassava helps to overcome problems with postharvest physiological deterioration, while also reducing the amount of cyanogens in the roots. Common processing techniques include peeling, grating, drying and fermentation (Westby, 2002). Fermentation of cassava roots under water is conducted in many cassava producing countries in Africa. A variety of products are produced. Roots are soaked in water for 3–5 days during which time they soften. This causes lactic acid fermentation and reduces pH to 4 (Lebot, 2009; (Oyewole and Odunfa, 1990). Pre-mold fermented roots are inserted during grating to act as starter colonies. Heap fermentation, achieved by heaping peeled roots and leaving them to ferment naturally, is practiced in Tanzania Uganda and Mozambique (Essers, 1995; Ndunguru *et al.*, 1999).

# Chapter 3

# Materials and Methods

# 3 Materials and Methods

The study on "Biochemical and molecular studies on post harvest physiological deterioration of cassava (*Manihot esculenta* Crantz)" was conducted at ICAR-CTCRI, Thiruvananthapuram and at the College of Agriculture, Vellayani. The study materials, procedures and storage conditions are presented in this section.

## 3.1 Plant material and cultural conditions

Cassava accessions comprising of released varieties, improved cassava clones and exotic accessions were collected from ICAR-CTCRI, Thiruvananthapuram farm (8° 29' N ; 76 ° 59' E). Cassava plants were grown one metre apart following standard cultural procedures. Harvest was done at 10-11 month after planting manually uprooting the plants and roots were collected with special care, not to cause any injury or damage. Commercial-size roots were selected and placed on shelves in a well-ventilated room. Roots were harvested separately for each experiment following similar procedure mentioned above and PPD studies were carried out in the harvested roots.

## 3.2 Root treatments and storage conditions

### 3.2.1 Screening of selected cassava genotypes for PPD performance

The carefully harvested roots were washed thoroughly in tap water to remove the soil and other dirt adhering to them. The roots were kept for 15-20 minutes for the excess water to evaporate and the roots were taken for experimental purpose. The roots were stored in ventilated rooms for the PPD study unless otherwise the treatments were high or low temperature or soil burial treatments. For the screening experiment, the roots were kept without any additional cut or wound other than severing them from plant as normally followed by the farmers. There were 61 cassava genotypes (Figure 3.2 & 3.3) and 10-12 roots of each genotype were kept for PPD experiment. Three roots were scored for PPD at 5, 10 and 15 days after storage (Chávez *et al.*, 2005).

### 3.2.2 Effect of different temperatures on PPD of cassava

To study the effect of different storage temperatures on PPD, roots of selected varieties such as Sree Athulya, Sree Jaya, Vellyani Hraswa, Kalpaka and Sree Padmanabha were kept at i) room temperature in steel rack, ii) 40 °C for high temperature treatment and iii) in refrigerated condition at 8 °C. The roots were kept for one week and PPD development was studied. Sampling was done with three roots for each treatment at 1, 3 and 6 days after harvest.

### 3.2.3 Effect of wax coating, poly-bag storage and burying the roots in soil on PPD development

For the experiment on the effect different root treatments to exclude the oxygen supply to roots i.e. wax coating, de-aerated poly-bag storage and burying the roots under the soil for two weeks, the treatments were imposed immediately after harvesting and cleaning roots without any delay. Paraffin clear wax pellets were taken in a steel vessel and heated on top of a hot plate to melt the wax. Cassava roots were immersed in the molten wax and quickly retrieved making a thin coating of wax over the entire root surface (Subramanyam and Mathur,1956). To store the cassava roots inside the de-areated poly-bags, the cleaned roots were taken in a polythene bag of suitable thickness. The bags containing cassava roots were vacuum sealed using a TALSA® vacuum packing machine. The wax coated roots and poly-bag sealed roots were kept in storage room for three weeks. Samples were taken at weekly intervals for three weeks. The sampling was done on three roots for each treatment.

### 3.2.4 Application of Phyto-hormone and its influence on PPD development in cassava

For the experiment on the effect of phyto-hormones on PPD development, salicylic acid and jasmonic acid at three different concentration (100, 200 and 300 ppm) were applied to the harvested roots by immersing the roots in specified concentrations for 10 minutes. The cut end of the roots were covered with polythene sheet and secured with rubber band. The roots were subsequently stored at room temperature in a ventilated room for 6 days. Three roots were sampled per treatment at 1, 3 and 6 days after treatment.

### 3.2.5 Application of preservative chemicals on PPD of cassava

For the experiment on the effect of preservative chemicals such as i) ascorbic acid, ii) potassium metabisulphite (PMB), iii) Butylated hydroxy toluene (BHT), iv) butylated hydroxyl anisole (BHA), v) tocopherol acetate and vi) tertiary butyl hydroquinone (TBHQ) were applied at 0.5 and 1% concentrations. The chemical solution was applied over the entire roots using a sprayer. The cut end of the roots were covered with polythene sheet and secured with rubber band. The roots were subsequently stored at room temperature in a ventilated room for 6 days. Three roots were sampled per treatment at 1, 3 and 6 days after treatment.

Figure 3.1 Cassava roots of different accessions kept in storage for PPD study

### 3.2.6 PPD evaluation

PPD evaluation was done at specified intervals by taking transverse sections at 25, 50 and 75% of the total length of the roots, starting from the proximal end. A slice (0.3 cm average thickness) was cut from the distal end of each transverse section. Digital pictures of each slice were obtained using scanner (HP Scanjet G2410). Three independent evaluations of PPD were carried out under laboratory conditions. For scoring of PPD expression, the roots of each genotype cut into three equal parts transversely from proximal end to distal end and visually scored in a scale of 1 to 5 following Salcedo *et al.*, (2010) with modifications. The PPD scores were given as no damage (score 1), upto 25% damage (score 2), 26-50% damage (score 3), 51-75% (score 4) and fully damaged root slice (score 5). The mean PPD score for each root was calculated by averaging the scores for the 3 transverse sections.

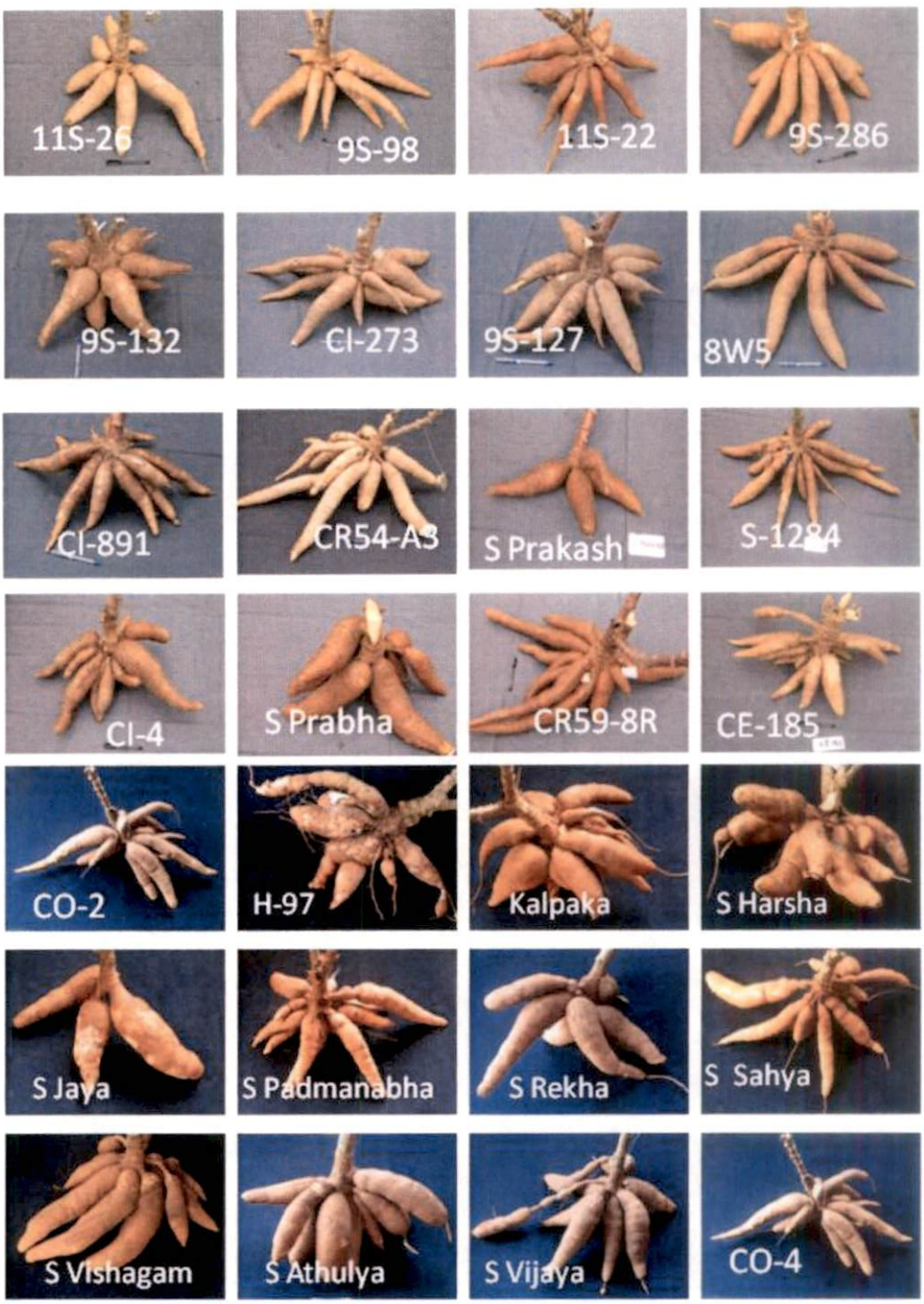



Figure 3.2. Photographs of selected cassava genotypes screened for biochemical aspects and PPD performance


Figure 3.3. Photographs of selected cassava genotypes screened for biochemical aspects and PPD performance

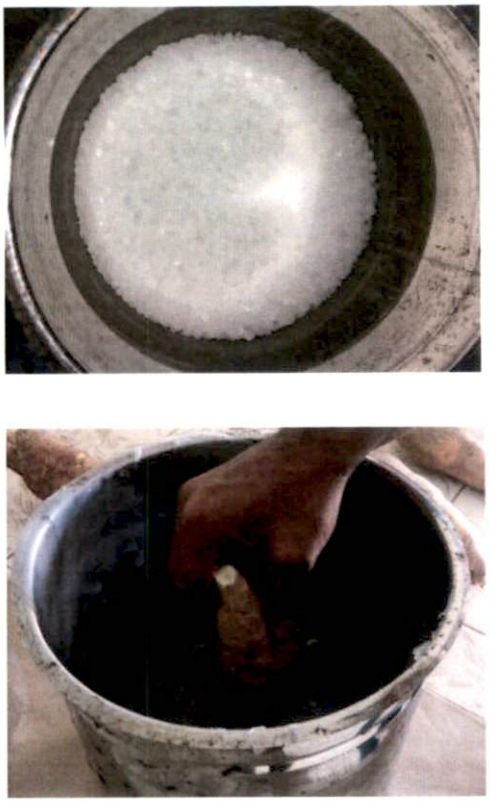


Figure 3.4. Paraffin wax pellets and molten wax used for coating of cassava roots

Figure 3.5. High temperature (40 °C) storage of cassava roots to delay PPD

## 3.3 Biochemical analysis

### 3.3.1 Determination of Starch and Total sugar

Dried cassava root tissue (2 g) was sliced and cut into small cubes of approximately 0.5 $cm^3$ and taken in 100 ml flask. The root samples were extracted with 20ml 80 % ethyl alcohol for overnight. The extract was filtered (Whatman No. 1) and collected separately for sugar estimation. To the residue 20 ml 2N HCl (Hydrochloric acid) was added and taken in a flask and kept on hot plate at 100 °C for 30 min. The content was cooled and volume was made up to 100 ml with double distilled water. The starch was completely hydrolyzed by treatment with 2N HCl while the non-reducing sugars were converted into reducing ones. Analysis of both the components is done in a similar manner, based on the number of reducing groups. The supernatant is directly used for starch estimation following Morthy and Padmaja, 2002.

**Reagents:**

A. Potassium Ferricyanide reagent (1%): Dissolve 1g of pot ferrricyanide in 100 ml dist water and store in brown bottle (can be stored for a month).
B. NaOH (2.5N): dissolve 10 g NaOH in 100 ml water.
C. HCl: Dilute concentrated HCl six times by adding 250 ml water to 50 ml HCl.
D. Methylene blue staining solution: Use diluted Methyl blue
E. Glucose standard: Dissolve 100 mg D-glucose in 100 ml water
F. 80% ethanol

**Titrimetric assay:**

To a 100 ml flask, 10 ml of Potassium ferricyanide was pipette out followed by 5 ml of NaOH and the contents were mixed thoroughly. The flask was kept over the flame for boiling. When the reagents began boiling, the flame was lowered and 3 drops of dilute methylene blue was added. The solution turned to blue-green. The starch hydrolysate was taken in a 2 ml blow pipette during starch estimation and added drop by drop to the boiling reagent, while for sugar estimation, the sugar extract was taken in a 10ml blow pipette (since the titre value will be 5-8 ml). The nearing of end point was

indicated by change of colour from blue-green to violet. A few more drops were added carefully, to reach the end point, which was indicated by the rapid disappearance of the violet colour. At this stage, the titre reading was noted, Titrations were repeated for each of the aliquots and starch content and total sugars were estimated based on method given by Moorthy and padmaja (2002) and the calculations are given below,

**Total Starch**

$$\frac{\textit{Volume of Potassium ferricyanide X make up volume X } 0.9 \textit{ X } 100}{\text{Titre volume x weight of sample x } 1000}$$

**Total Sugar**

$$\frac{\textit{Volume of Potassium ferricyanide X make up volume X } 100}{\text{Titre volume x weight of sample x } 1000}$$

### 3.3.2 Determination of carotene content

Carotene content was determined by taking 5 g from selected root tissue of cassava genotypes and extraction with petroleum ether, as described by Iglesias *et al.*, (1996). The modified protocol included several extractions with petroleum ether (35-65 °C). Approximately 5 g of tissue was obtained from representative and randomly selected roots from plants of each clone. The extracts were combined and evaporated using a rotary evaporator and the final residue was made upto 25 ml with Hexane. The quantification was done by measuring the absorbance at 455 nm using a Perkin-Elmer Lamda 25 UV-VIS spectrophotometer.

### 3.3.3 Determination of total phenol content

Root tissue was ground with pestle and mortar with 95% ethanol (2 ml·g-l ethanol and then once with 5% ethanol in the same procedure. Total soluble phenols in the combined supernatants were determined with Folin–Ciocalteu reagent by the method of Slinkard and Singleton (1977). Results were expressed as milligrams gallic acid equivalent (GAE) per gram fresh weight. The supernatant (0.2 ml) was taken in a test tube and 1 ml of Folin-Ciocalteu's reagent was added. This was allowed to stand for 8 min at room temperature. Next, sodium carbonate (7.5 %, w/v, 2 mL) was added, mixed and allowed to stand for 30 min. Absorption was measured at 765 nm (Perkin Elmer Spectrophotometer). The mean total

phenolic contents (n = 3) were expressed as milligram gallic acid equivalents per 100 g (mg GAE/100g dry mass or wet mass ± standard derivatives).

### 3.3.4 Determination of protein

The commercial protein assay reagent (5X - Bradford reagent, M/S Qualigens, India) was diluted to a working solution (1:5) with distilled water. Standards of known concentrations in the range of 0-50 µg protein (bovine serum albumin BSA) were prepared and 5 µl of the protein standards and of the samples were added to 1 ml of the diluted assay reagent. The samples were mixed by vortexing briefly and allowed to incubate for 15 minutes. Absorbance of the samples was then measured at 595 nm against a blank containing no proteins, and the readings noted. A standard curve of absorbance versus micrograms of protein was prepared and the concentration of protein in the samples determined from the curve.

### 3.3.5 Measurement of antioxidant enzyme activities

**Sample preparation for enzyme assays:** Cassava root sample 5 g was homogenized in 20 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM EDTA (ethylenediamine-tetraacetic acid), and 1% insoluble polyvinylpyrrolidone. The extract was centrifuged at 8000 rpm using Sorvell table top refrigerated centrifuge at 4° C for 10 minutes and the supernatant was stored in minus 20° C until for enzyme analysis.

3.3.5.1 Assay of Catalase:

Catalase (EC1.11.1.6) activity was measured following Cakmak *et al.* (1993). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 10 mM $H_2O_2$ and enzyme extract. Catalase was assayed at 25°C following the decomposition of $H_2O_2$ by the decline in the absorbance at 240 nm ($E=26.6\ mM^{-1}cm^{-1}$) using Lamda 50 (M/S Perkin Elmer, USA) UV-Vis spectrophotometer. Two technical replicates were carried out for each standard and experimental sample. Samples were standardised using the total protein content to account for differences in protein extraction efficiency between samples.

3.3.5.2 Assay of guaiacol peroxidase:

Peroxidase activity (EC1.11.1.7) was measured according to the method described by Cakmak *et al.* (1993). The reaction mixture contained 25 mM potassium phosphate buffer (pH7.0), 10 mM $H_2O_2$, 0.05% guaiacol, and the enzyme extract. Peroxidase activity was measured as the rate of the oxidation of guaiacol at 470 nm. The molar extinction coefficient of tetraguaiacol (26.6 $mM^{-1}cm^{-1}$) was used in calculating the enzyme concentration.

3.3.5.3 SOD activity

SOD activity was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT). The reaction mixture was 3 mL, which contained 50 mmol $L^{-1}$ phosphate buffer (pH 7.8), 13 mmol $L^{-1}$ methionine, 75 µmol $L^{-1}$ NBT, 2 µmol $L^{-1}$ riboflavin, 0.1 mmol $L^{-1}$ EDTA, and 0.1 mL of enzyme extract. Reaction was started by adding 2 µmol $L^{-1}$ riboflavin and placing the reaction tubes under 15 W fluorescent lamps for 15 min.

3.3.5.4 Determination of Phenylalanine ammonia lyase

PAL activity in the plant extract was determined by the production of cinnamate during 1 h at 30° C, as measured by the absorbance change at 290 nm. The assay mixture contained 15 mol L-phenylalanine, 30 mM sodium borate buffer (pH 8.8), and 0.2 to 0.5 ml of extract, depending on the PAL activity level, in a total volume of 3.0 ml. The substrate was added after 10 min of pre-incubation and the reactions stopped with 0.1 ml 6 N HCl. Assays were performed in triplicate. The molar extinction coefficient of cinnamic acid in assay buffer was 17,400. A unit of enzyme activity (kat) was defined as the amount of enzyme required for the formation of 1 mol of product in 1 sec under the assay conditions.

### 3.3.6 High Performance Thin Layer Chromatography

Roots of cassava accession at 10 days after storage (20 g each) were peeled then cut into approximately 0.5 $cm^3$ cubes and dried in oven at 55°C for 48 hours and crushed to fine powder. The root powder (5 g) was extracted with 10 ml hot methanol (60°C) thrice and the extracts were pooled. The extracts were filtered and evaporated under reduced pressure at concentrated to 2 ml and subjected to

TLC. A Camag HPTLC system comprising of Linomate V automatic sample applicator, Hamilton Syringe, Camag TLC Scanner-3, Camag Win CAT software, Camag Twin trough chamber and stationary phase precoated silica gel 60F 254 were used. The mobile phase comprised of Toulene: Ethylacetate: Formicacid: Methanol: Water (6: 5: 0.5: 3: 0.5). The solvent was run for 80 mm, band length 6 mm, slit dimension 6.00 x 0.30 mm. The Chromatogram was visualized using UV light 366 nm and scanned at 340 nm and 290 nm using TLC Scanner III CAMAG. The plate was subsequently derivatized using 1% vaniline/$H_2SO_4$ for visualization (Saravanan *et al.* 2015).

### 3.3.7 Evaluation of PPD through FT-NIR reflectance spectroscopy and chemometrics tools

Fourier Transform Near infra-red spectroscopic (NIRS) techniques are routinely employed for quick and easy tools to evaluate the various internal quality attributes of fruits and vegetable products. We employed NIR reflectance spectroscopy in the region of 10,000 - 4000 $cm^{-1}$ to measure the cassava root tissue to evaluate them for PPD expression. FT-IR spectrometer (M/S Perkin Elmer, USA) was used for measuring the diffused reflectance of cassava root samples. Cassava root samples were cut into 1cm thick slices using a sharp knife and IR data were collected and samples were dried and powdered for other chemical analysis. The PPD was assessed by visual scoring of roots under storage. The scoring was as mentioned above (3.2.6) and the samples were grouped as fresh, mild, high and severe based on the scale of 1 to 5. The spectra were subjected to standard normal variant (SNV) correction before calculating the principal component (PCA) models. SNV-corrected data were subjected to Savitzky-Golay smoothing and a mean normalization procedure before developing partial least-squares calibration (PLS) models. Other data transformations such as the Kubelka-Munk function [(1 – Reflectance) 2/(2 × Reflectance)], scatter and smoothing corrections, or derivative pretreatments, were also tested, but the results were not significantly better. Spectral data were compressed by using principal components analysis (PCA) and the scores of the first 15 principal components (PCs) were analysed. Scatter-plots of such components were used to visualise the structure of the multivariate data and to check for possible outliers.

## 3.4 Morphological and Physiological studies

Morphological and physiological parameters were studied for the roots used in the present investigations.

### 3.4.1 Root Characters and morphological parameters

Root fresh weight (g), root length (cm), root girth (cm) were taken after washing the roots with tap water. The weight of the roots are taken with a precision electronic balance and morphological measurements were taken with measuring tape.

### 3.4.2 Root dry matter content

. The root sample of cassava (50g) was made into small cubes (approximately 0.5 cm) with a sharp knife and dried in a hot air oven at 110° c for 48 hours and the dry weight was determined. The dry matter content was calculated as percentage.

### 3.4.3 Root respiration measurement

Root respiratory rate was recorded for intact roots using LI-7000 soil respiration system (Li-Cor Inc. Lincoln, USA) by covering the chamber bottom air tight.

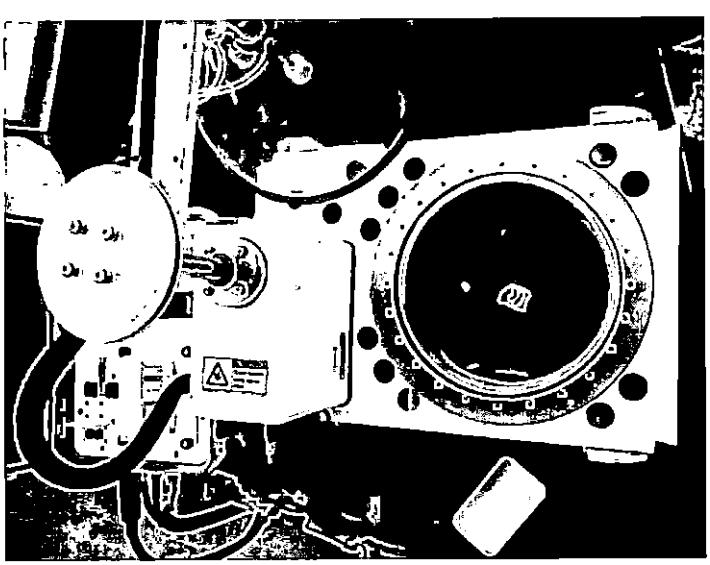


Figure 3.6. Root respiratory flux measurement using LI-COR LI-7000 soil respiratory system.

Intact roots of cassava were weighed and kept in the measuring chamber and the

respiratory $CO_2$ flux (µmol $CO_2$ per g) was measured for three minutes at 15 s interval. Three replicate measurements were made for each variety.

### 3.4.4 Tissue staining of cassava root sections

Tissue staining of cassava root tissue was carried out using stains such as erythrocin red, saffranin O, aniline blue, fast green and phlorglucinol for visualizing the PPD symptoms at early stages of storage. The stains were prepared in ethlyl alcohol at (1% w/v) concentrations. The transverse sections of cassava root roots (approximately 2-3 mm thick) were kept in the staining solutions ranging from 10 minutes to 30 minutes and washed with distilled water before being visualized and photographed.

### 3.4.5 Tissue printing for localization of peroxidase enzyme

Tissue printing of cassava root tissue for peroxidase enzyme was performed using guiacol as a substrate with added hydrogen peroxidase in the medium (Schopfer, 1994). Cassava root tissue from fresh as well as PPD affected roots were cut 3-4 mm thick sections transversely using a sharp knife. The tissue was quickly washed with distilled water and gently dried using a tissue paper to remove the excess water. The tissue imprints were taken in nitrocellulose paper (M/S Merck, India) on a paper towel. The imprinted paper is dried on a lab table and enzyme assay was performed in a petri-dish containing assay reagents. The reaction mixture contained 25 mM potassium phosphate buffer (pH7.0), 10 mM $H_2O_2$, 0.05% guaiacol. The reaction was performed for 2 min and assay was stopped by drying nitrocellulose paper on a paper towel. The images of tissue prints were captured using a flat bed scanner (HP, G2410).

### 3.4.6 Molecular studies

#### 3.4.6.1 Primers for PPD related genes

Gene specific primers were designed using the Primer 3 software. The various parameters like GC content, annealing temperature, product length (100-150bp) etc., were considered for selecting the appropriate primer combinations to amplify gene of interest. A set of two primers were synthesized per gene target gene (Table 3-1). A preliminary assessment was made using BLAST against the

NCBI GenBANK database and further by the Primer BLAST program to ascertain their suitability.

3.4.6.2 Extraction of total RNA from root tissues

Root tissue material was ground with liquid nitrogen in a sterile pestle and mortar treated with RNAase inhibitor. Total RNA was isolated from cassava root material using the Sigma RNA Isolation kit (Sigma-Aldrich). RNA was eluted from columns with 100 µl NFW. The concentration and quality of the RNA was determined by spectrophotometry readings at 230 nm, 260 nm and 280 nm. The concentration of RNA was calculated using the following equation:

RNA (ng/µl) = A260 x dilution factor x 40

The A260:A280 ratio assesses protein contamination of RNA samples and should ideally be greater than 1.8. The A260:A230 ratio assesses sample contamination by chemicals used in the RNA isolation procedure such as guanidine and phenol and should be greater than 2.0. After RNA quantification, 500 ng of each RNA sample w as electrophoresed to confirm its integrity.

3.4.6.3 cDNA synthesis

The RevertAid™ First Strand cDNA Synthesis kit (Fermentas) was used to create cDNA from RNA template. 1 µg total RNA for each sample was combined with 1 µl random hexamer primer, 1 µl oligo(dT)18 primer and DEPC-treated water to 12 µl in a 0.2 ml sterile thin-walled PCR tube. Samples were incubated at 70 °C for 5 mins and then chilled on ice. 4 µl 5x reaction buffer, 1 µl RiboLock™ RNase inhibitor (20 U/µl) and 2 µl 10 mM dNTPs were added in the order given. Samples were incubated at 25 °C for 5 mins. 1 µl RevertAid™ M-MuLV reverse transcriptase ( 200 U/µl) was added and the samples incubated at 25 °C for 10 mins, 42 °C for 60 m and 70 °C for 10 m. cDNA was stored at –20 °C.

Table 3-1. Complete set of primers related to wound response in plants designed for PPD studies

| Gene Name | Primer Name | Sequence |
|---|---|---|
| Phenylalanine ammonia lyase | PAL-F | CGCCATGCTTGTACGAATCA |
| | PAL-R | GAGGAACGAGATCTCCGGAG |
| | PAL F | TCAAGGGTGCTGAGATTGCC |
| | PAL R | GCACTGTGAACATGGTTTGTGA |
| | PALF | ATGGGTTGCCTTCAAATCTCAC |
| | PALR | CATCTTGGTTGTGCTGCTCAGC |
| Secretary Peroxidase | POXSF | GATGTGATGCTTCGTTGTTGCT |
| | POXSR | CACATGAAACTTTGTTCCTGCA |
| | POXSF | CTTCGCCGAGTAACAATGCG |
| | POXSR | AACACTATCAACGGCAGCCT |
| Respiratory Burst Oxidase Homologue | RBO F | GGCGAGATGACTCTACTTGGT |
| | RBO R | GTCAAAGCGACGACCAGGA |
| | RBO F | TGTTGGTAGTAATGATGCTTCGACT |
| | RBO R | CGACTTCATTCATGACCCCT |
| Wound responsive Transcriptional Factor Y | WRKY-F | GGCGTAAATATGGGCAGAAGCAG |
| | WRKY-R | CGCTCAACCTTTTTTTTGACAGG |
| | WYRK -F | TCAATCAACTGAGGCCACCA |
| | WRKY -R | GACAACCTACTGCCATGGTG |
| Senescence associated gene | SAG20-F | TCTCGCGACGTCAAATCTGT |
| | SAG20-R | ACGACGGAGAGAGGTACGAA |
| Extracellular xylulose transferase | XET-F | CTCTGCAGGGACTGTCACAA |
| | XET-R | CGTTGGATCAAACCAGAGGT |

3.4.6.4 Semi quantitative PCR assay

The specificity and sensitivity of the designed primer pairs were evaluated with a conventional PCR. Amplifications were performed in an Agilent Sure Cycler (Agilent Technologies, USA). The PCR reactions were conducted in a volume of 25 µl which included 100 ng of template DNA, 100 µM each deoxynucleotide triphosphate, 10 ng of each primer, 1.5 mM $MgCl_2$, 1 x Taq

buffer (10 mM Tris-HCl pH 9.0. 50 mM KCl, 0.01% gelatin), 1 U of Taq DNA polymerase (Merck GeNei, India). The following optimized PCR regime was used: denaturation at 95°C for 2 min, followed by 35 cycles of 95°C for 30 s, 55° C for 45 s, 72 °C for 1 min and final extension at 72 °C for 8 min. The amplified products were visualized in a 1.5% agarose gel and photographs were scanned through Gel Doc System (Alpha Imager, Alpha Innotech, USA). The agarose gel photograph was then processed through ImageJ software and area of the bands were calculated. The relative expression level of gene of interest (PAL and POX) is calculated with reference to α-tubulin transcript expression.

## 3.5 Statistical analysis, correlation and clustering procedure

All statistical analyses were carried out using RStudio statistical package (RStudio, 2014). Analysis of variance was used to evaluate the differences among cassava genotypes. PCA analysis and other statistical tests were conducted using RStudio Package.

### 3.5.1 Correlation studies and Cluster analysis

Correlation studies were conducted for the various data on root characters, biochemical parameters including enzyme activities with PPD scores. Chromatogram of each genotype was scored by the presence (1) or absence (0) of each band noted. Presence and absence of bands were entered in a binary data matrix. Based on chromatographic band spectra, cluster analysis was performed using *RStudio* statistical package. Dendrogram was constructed using Ward's method using squared Euclidean distance.

# Chapter 4

# Results

# 4 Results

The main objective of the study was to screen cassava genotypes for PPD performance, to explore the available techniques to identify a suitable method for objective quantification of PPD symptoms and to develop methods to prolong the storability of fresh cassava roots. The main findings of the experiments and results are presented here.

## 4.1 Screening of cassava genotypes for PPD tolerance and changes in biochemical constituents

### 4.1.1 Root characteristics of cassava genotypes

Cassava roots were stored for 20 days in a well ventilated room to study the development of PPD. The average temperature during the storage period was 28.0°C, with a range from 26 to 31°C. Average relative humidity at 7:00 h was 73%. The mean, range and distribution of the root characters like length, fresh weight, starch content and carotene content for the cassava genotypes studied are presented in Fig.4.1 and Fig 4.2 respectively. Mean root length of cassava genotypes was 34.9±7 cm. The accession 11S-4 had the longest root (51 cm), whereas, Sree Padmanabha had the shortest roots (20.7 cm). Mean root fresh weight was 752±280 g. The highest root fresh weight was observed for CR59-8 (2078.3 g), whereas CR35-8 had the lowest (288.3 g).

Root transverse sections of cassava accessions taken one day after harvest are presented in Figure 4.3 and Figure 4.4. The majority of the accessions studied did not show visible symptoms during this period and the vascular streaking was limited to only few genotypes. Parenchyma cells (with starch storing amyloplasts) were densely packed within the pericycle and the vascular bundles were distributed over the entire tissue. The peel (the cortex) thickness varied among the genotypes. There were small variations in the pattern of paranchyma arrangement and density of packing among the genotypes. The pith was arranged nearly at the centre in most cases. Harvested cassava roots when cut transversely, were either white or in shades of pale yellow in fresh conditions. The vascular

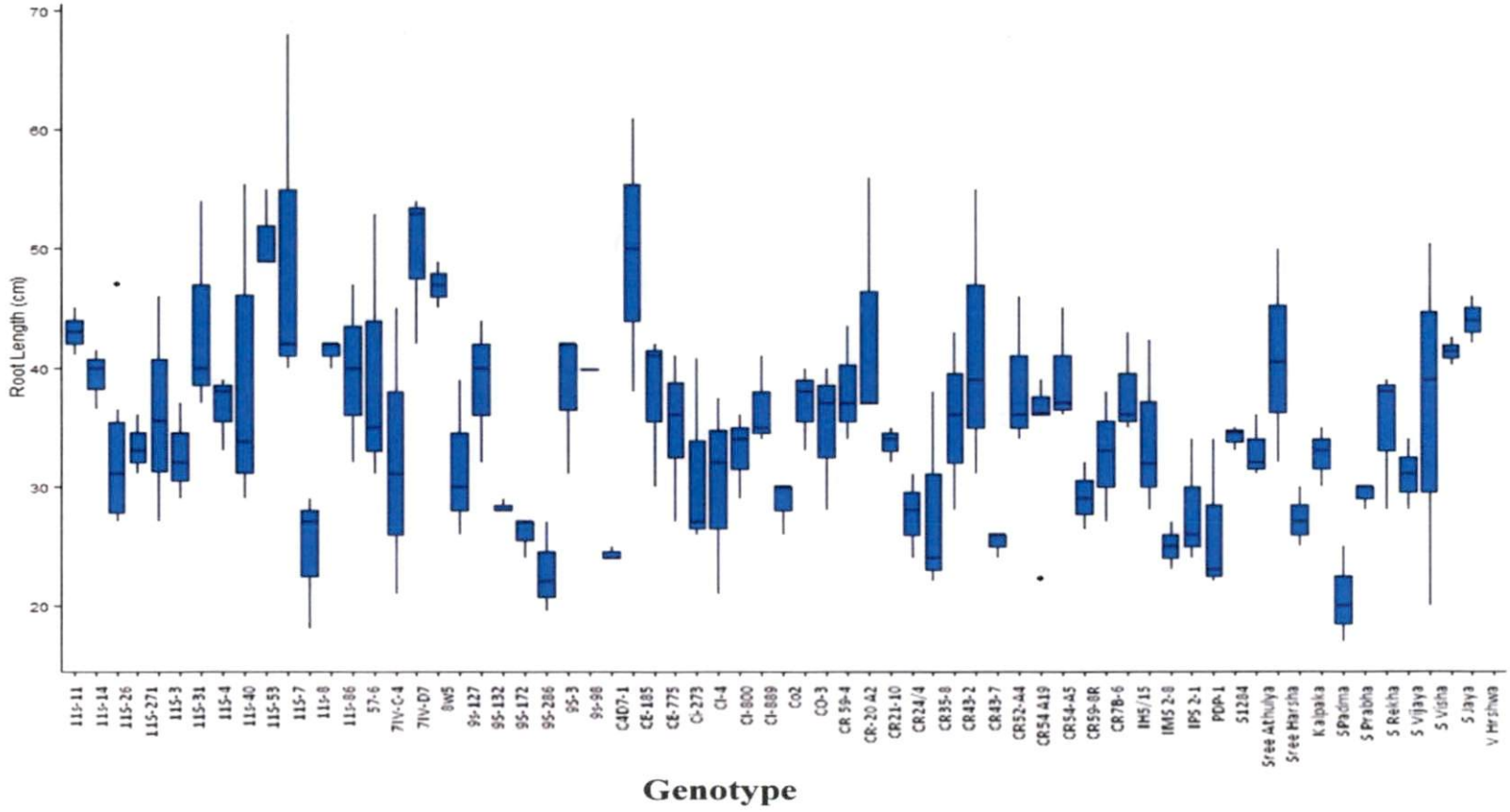



Figure 4.1 Box plot showing the mean, inter quartile range and distribution of root length of cassava genotypes studied for PPD expression. (The mean value is represented as dark line in the middle of the box, 75$^{th}$ percentile upper edge, 25$^{th}$ percentile lower edge and the range is represented by error bars).

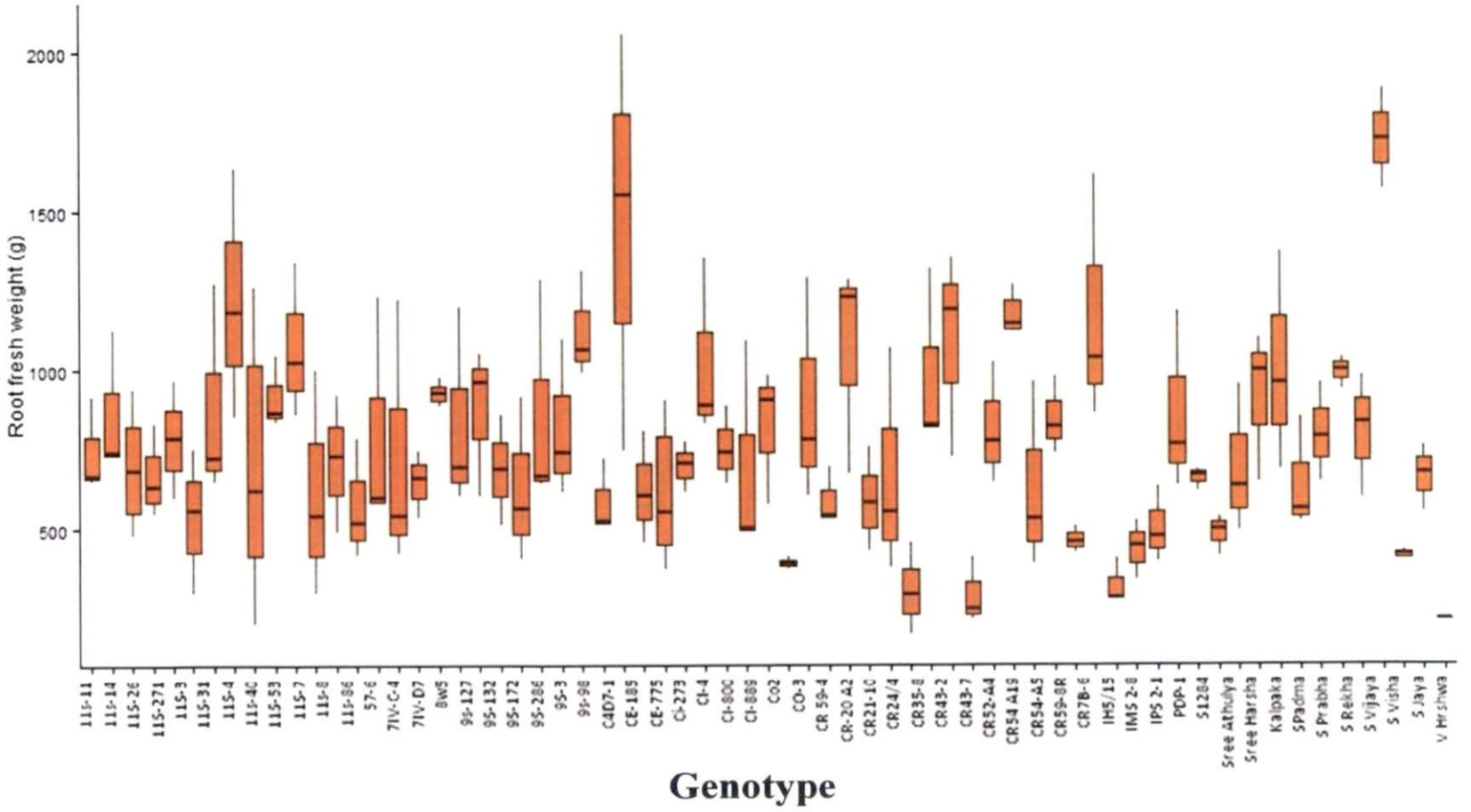



Figure 4.2. Box plot showing the mean, inter quartile range and distribution of fresh weight of root of cassava genotypes studied for PPD expression. (The mean value is represented as dark line in the middle of the box, 75$^{th}$ percentile upper edge, 25$^{th}$ percentile lower edge and the range is represented by error bars).

streaking started appearing after one to two days (in some cases much afterwards) of storage. Cassava accessions such as IMS2-8, 9S-172, 11S-53, IRS 2-10 and 9S-286 were started showing the visible discolouration in the parenchyma tissue one day after harvest. The initial PPD symptoms are characteristic of blue black discolouration of parenchyma tissue in the middle. The symptom appearance starts especially in the proximal cut end of the roots since this portion is invariably sustain the wounds. Accessions having neck portion at the proximal end are less prone to large wounds during harvest. The neck region or peduncle of the roots if present, prevents the exposure of the tissue to atmosphere and reduce the extent of wounding when the roots are severed from the plant. Those roots which have a neck were the ones which show lower streaking symptoms during the initial period and showed delayed onset of PPD. The PPD symptoms were normally started to appear in most accessions only after 3-5 days after harvest. However the speed of PPD symptom development hastened afterwards. The rapid phase of PPD development was marked with browning of the tissue with occasional watery lesions accompanied by foul smell. The root sections showing widely varying PPD symptoms in the selected accessions of cassava are presented in Figure 4.5 and 4.6. There was wide variation in the extent of discolouration, root tissue selectivity to PPD symptom development. However, the pith area of the root was generally had lower damage unless the secondary deterioration had started in the root tissues. The primary deterioration in the outer tissue favoured the development of secondary symptoms. In certain accessions like Ci273, 11S 271 and 11S-4 the secondary deterioration was severe and black discolouration due to rotting of the tissue was visible. It was interesting that some of the accessions such as 11S-86, CR 53-8 and CR 59-4 had healthy root tissue with low tissue damage though surrounding tissues showed deterioration. The genotypes categorized based on PPD score are presented in Table 4-2.


Figure 4.3 Root transverse sections of selected accessions showing (no visible symptoms or mild) symptoms of PPD at one day after harvest.


Figure 4.4 Root transverse sections of selected accessions showing (no visible symptoms or mild) symptoms of PPD at one day after harvest.


Figure 4.5 Root transverse sections of selected accessions 9 days after harvest showing varying intensity of visible PPD symptoms.

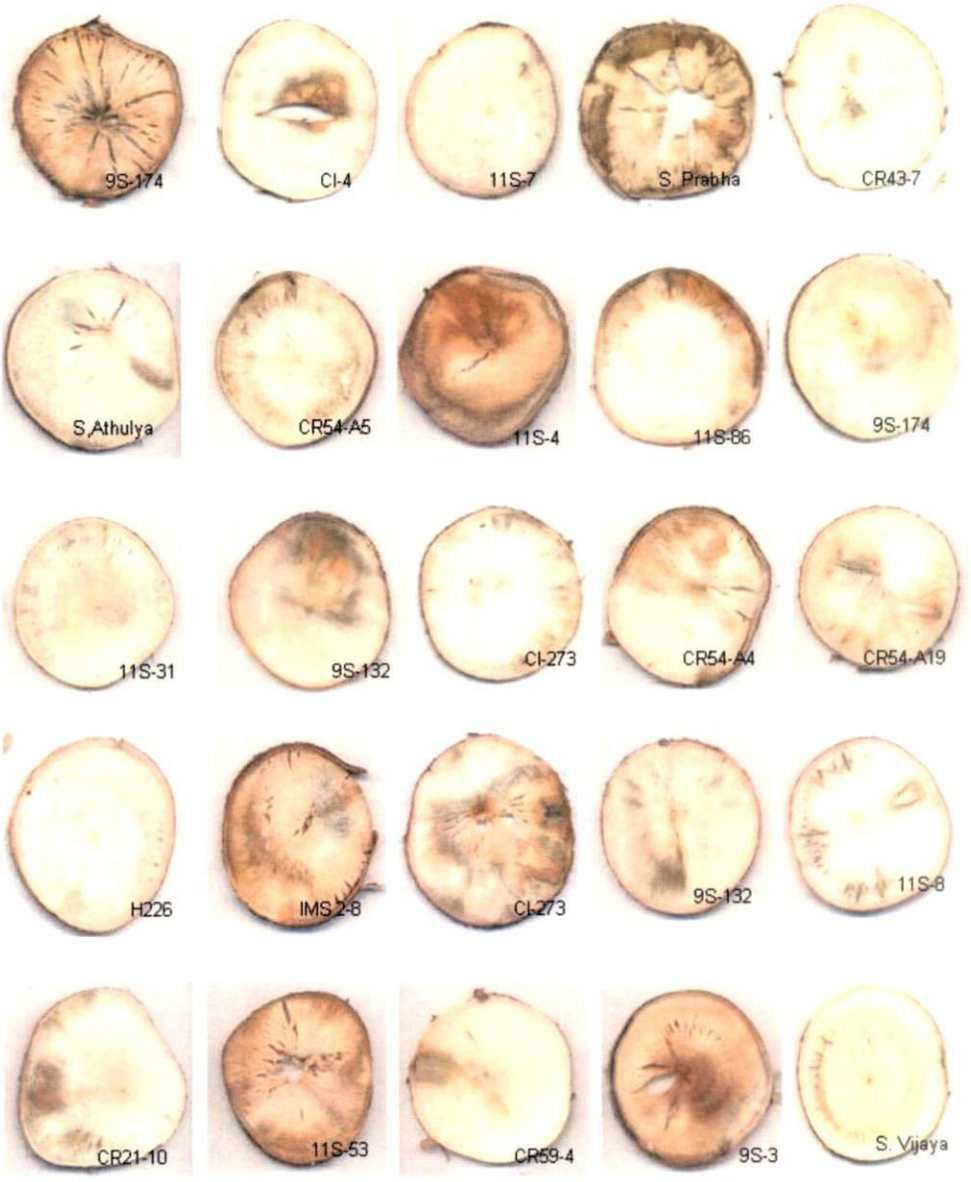



Figure 4.6 Root transverse sections of selected accessions 9 days after harvest showing varying intensity of visible PPD symptoms.

### 4.1.2 Relationship of root biochemical constituents with PPD

Average carotene and starch content were 0.34±0.1 µg/g and 82.1±4% respectively. Highest carotene content was recorded in CI-896 (0.78 µg/g) which had pale yellow root tissue. Genotypes such as CR 59-4, CR 42-3, IRS 2-1 had higher carotene content to the tune of 0.76, 0.72 and 0.68 ug g-1 respectively (Figure 4.7). Lowest carotene content were observed in 9S-174, 8W5 and 11S-271 accessions with a trace amount which was well below 0.15 µg and the root tissue was white devoid of pigmentation. In general, in the present study, the genotypes of cassava did not have appreciable amount of carotene content in root tissues as compared to high carotene levels observed in deep yellow fleshed varieties (Sánchez *et al.*, 2006). There was no correlation between the root morphological traits and PPD severity. Carotene and starch content of root did not influence the PPD in the genotypes studied. Starch content of cassava genotypes in root tissues showed significant variations. Highest starch content in roots was recorded for Sree Harsha and SB-56 and were close to 90% on dry weight basis (Figure 4.8). Nearly half of the total genotypes studied were having starch content of 80% and above. It is probably one of the reasons for genotypes showing higher PPD symptoms early in storage. Lowest starch content was recorded for genotypes such as 11S-42, 11S-4, 11S-86 and CR54-A5 with nearly 74% starch content on dry weight basis.

Earlier studies indicated that visible symptoms of PPD in intact cassava roots appeared after 5 days in most cases (Hirose *et al.*, 1984 and Salcedo *et al.*, 2009) and hence our initial PPD assessment on cassava genotypes was carried out on fifth day of storage by visual scoring. Since the roots were kept without additional cut other than detaching them from main stem, the PPD was noticed in most of the genotypes studied at the first sampling which was done on $5^{th}$ day of storage. The PPD progressed in most cases from the proximal portion of the root to distal end unless the tips are damaged during harvesting or handling. The tissue scans showed localized accumulation of carotene containing parenchyma cells near the pith region with slightly dark yellow colour visible as concentric ring around

the central pith. However, such higher accumulation of carotene in such cells does not delay the PPD expression in that region of root tissue. The scores of PPD in genotypes on 10$^{th}$ day of storage revealed that the PPD progressed quickly between 5 and 10 days of storage (Figure 4.9). Roots of several genotypes deteriorated rapidly and he PPD score reached to 3-4 during this period. Albeit there were differences among genotypes, severity of PPD scoring was not statistically significant.

### 4.1.3 Tissue imprinting peroxidase in cassava accessions

To visualize the changes in biochemical changes in terms of oxidative enzyme in root tissues of cassava after harvest, the tissue imprinting for peroxidase enzymes activity in healthy and roots with PPD were analyzed for selected genotypes and presented in Figure 4.10 to 4.13. There were wide variations in the activity and tissue pattern for peroxidase in the root tissue among the genotypes studied in fresh as well as roots affected by PPD. The imprints showed distinct pattern for peroxidase activity in the roots. Both intensity and extent of peroxidase activity varied in tissue samples. In general, higher peroxidase activity in the vascular regions within the xylem elements is observed. Though there was no clear pattern for peroxidase activity and PPD traits in cassava, the tissue imprinting showed genotype specific pattern for the activity. There was a remarkable increase in peroxidase activity in the root tissues with increasing PPD symptoms as seen by the strong staining of the tissue prints. There were intense colour developments in the affected tissues. The increased activity was accompanied by extensive spread to whole tissue makes peroxidase association to PPD either as a cause for ROS or as correlative phenomenon. Tissue printings after proper washing of the root tissue ensured that the cell wall bound apoplastic peroxidase are the major contributors for the colour development in our results. The cell wall bound peroxidases were believed to participate only in the lignin biosynthesis and the formation of cross-links in the cell wall and hence they might play an important role in wound healing.

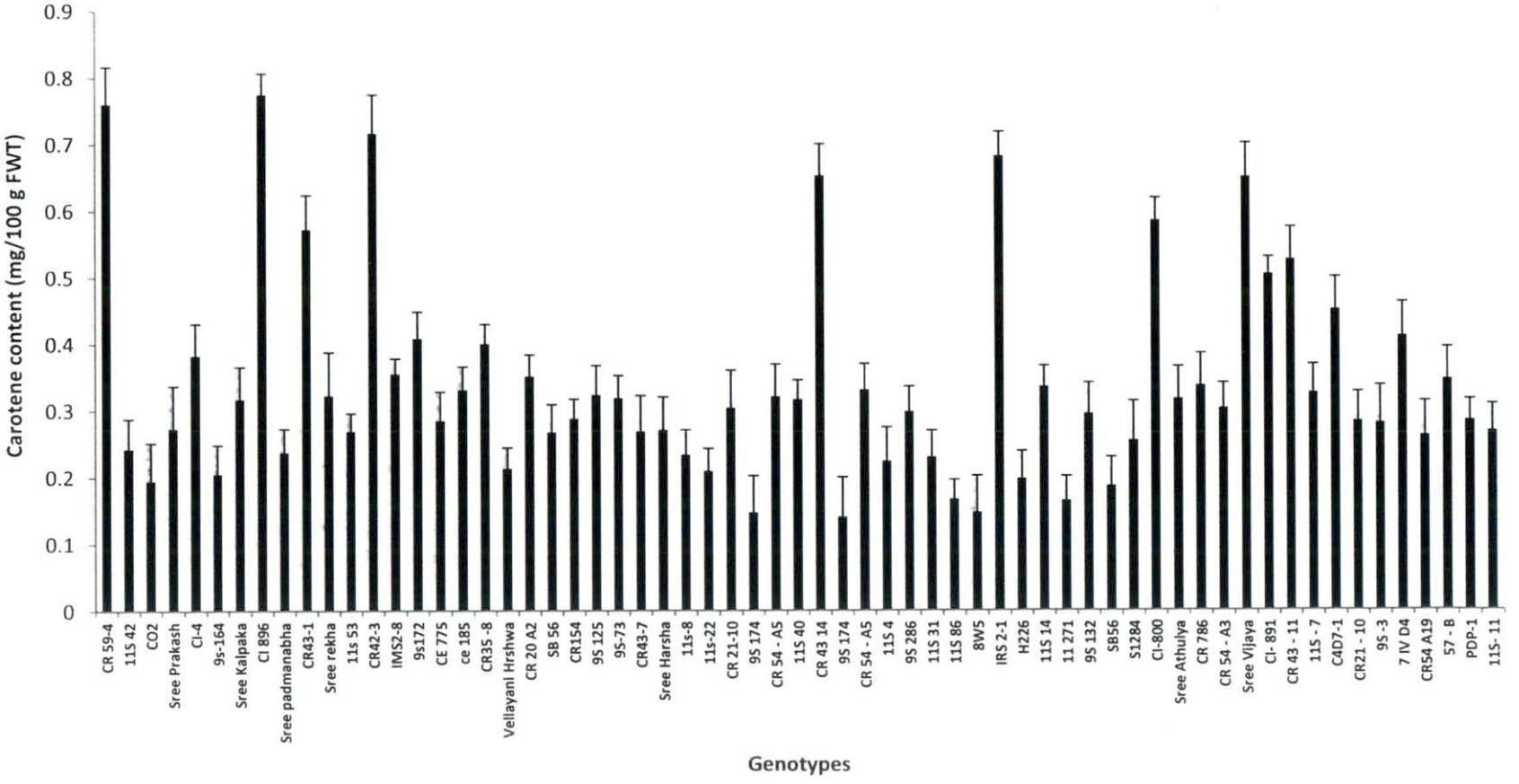



Figure 4.7. Total carotenoids content of cassava genotypes.

(Error bars indicate Std. Dev)

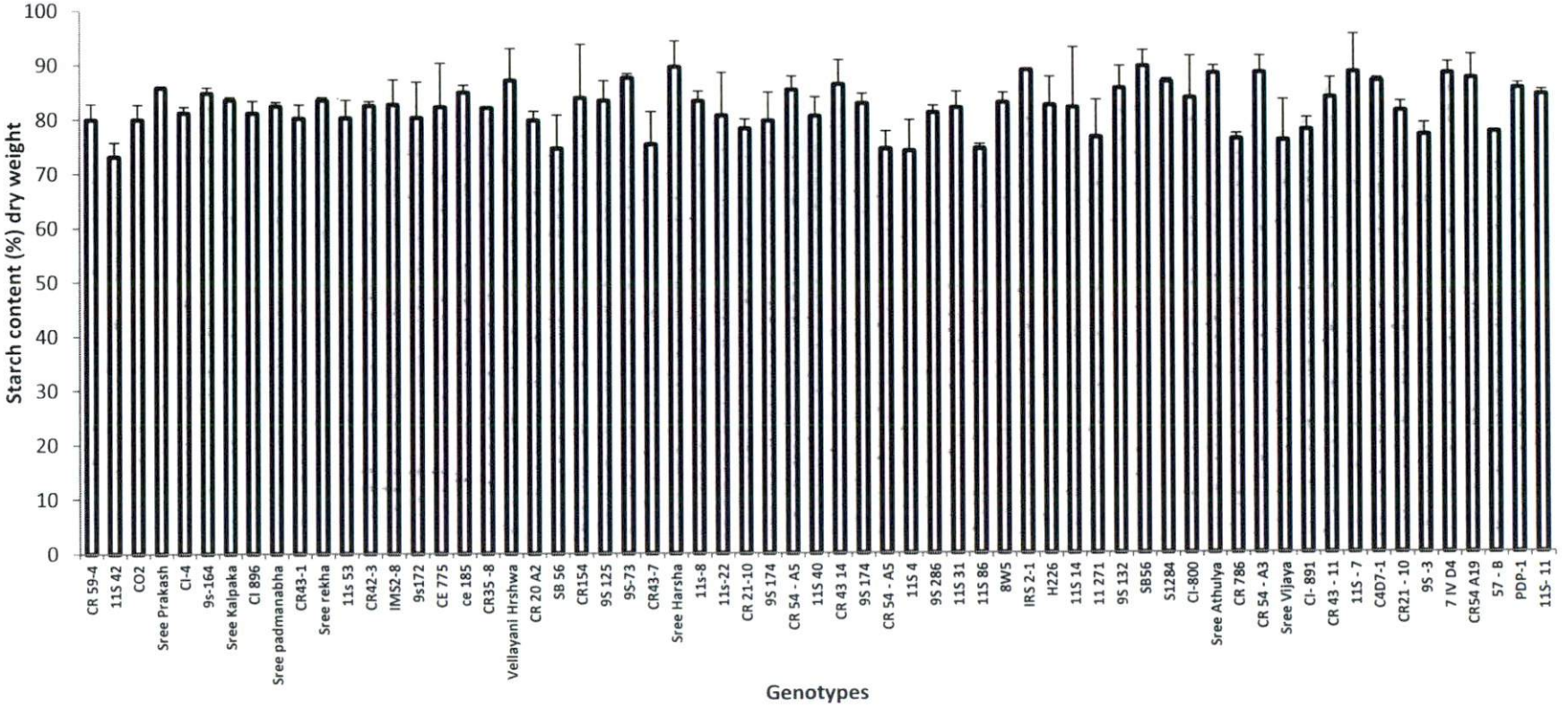



Figure 4.8. Starch content of cassava genotypes.

(Error bars indicate Std Dev.)

Table 4-1. Classification of cassava genotypes based on visual scoring of PPD symptoms

| **PDD category** | **Genotypes** |
|---|---|
| **Low** | 9S-7, 9S-98, 11S-31, 11S-86, 11S-14, CE63-3, CI43-2, CR43-2, CR54-A5, Sree Athulya and Kalpaka |
| **Moderate** | 11S-4, 11S-8, 7 IV D-7, CI-896, , 9S–127, CO-2, CR 43-7, CE-775, CR24-4, IMS 2-8, S-1284, H226, 57-6, Sree Vijaya and CR35-8 |
| **High** | 9S-3, 9S-56, 9S-174, 9S–172, 9S–286, 9S–132, 11S-3, 11S-7, 11S-11, 11S–22, 11S–26, 11S–53, 11S-40, 11S-271, 8W5, C4 D7-1, CE-7B6, CE-185, CI-4, CI-273, CI-800, CI-891, CI-889, CO-3, CR20 A2, CR21-10, CR52 A4, CR54 A3, CR54- A19, CR59-8R, CR-786, SB 56, PDP-1, Sree Prabha, Vellayani Hraswa and Sree Rekha |
| **Extreme** | CR43-11, CR59-4, CR-775, IH 5/15, IRS 2-1, Sree Harsha, Sree Padmanabha, 7 IV C4 and CR35-8 |

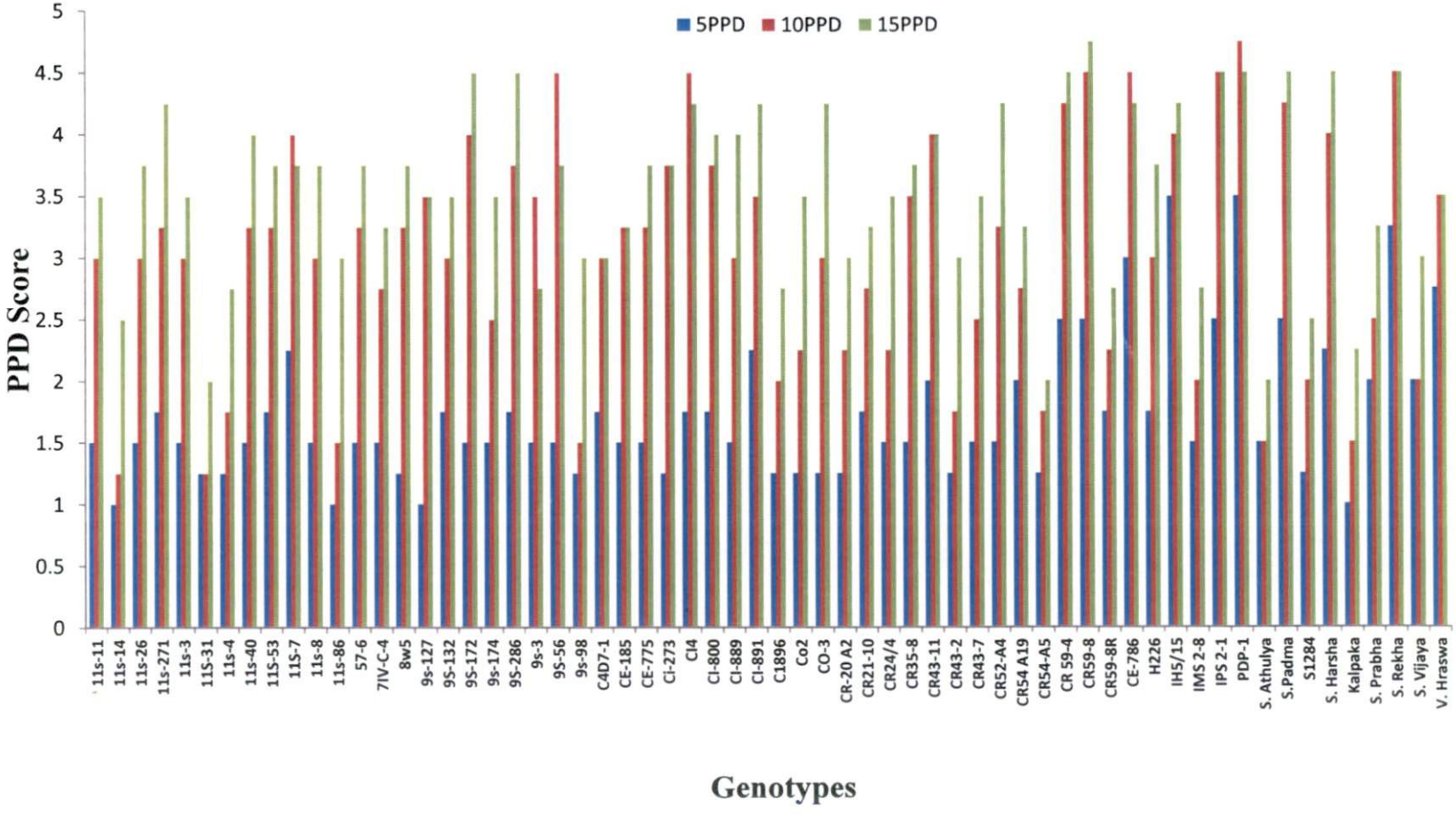



Figure 4.9. PPD score of 61 cassava accessions at 5, 10, 15 days of storage at room conditions

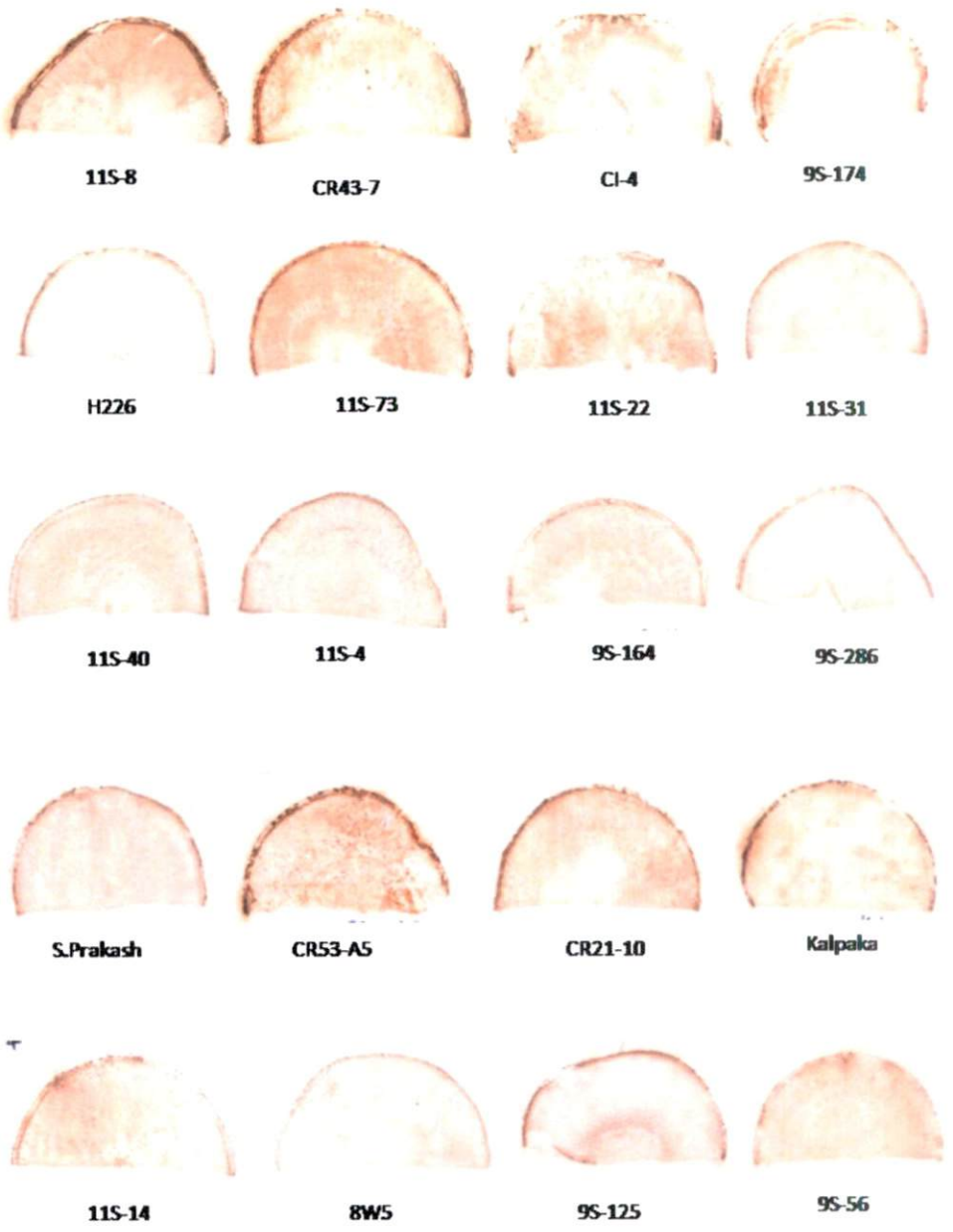



Figure 4.10. Tissue imprinting of peroxidase activity in the transverse sections of healthy cassava roots of selected accessions after harvest


Figure 4.11. Tissue imprinting of peroxidase activity in the transverse sections of healthy cassava roots of selected accessions after harvest

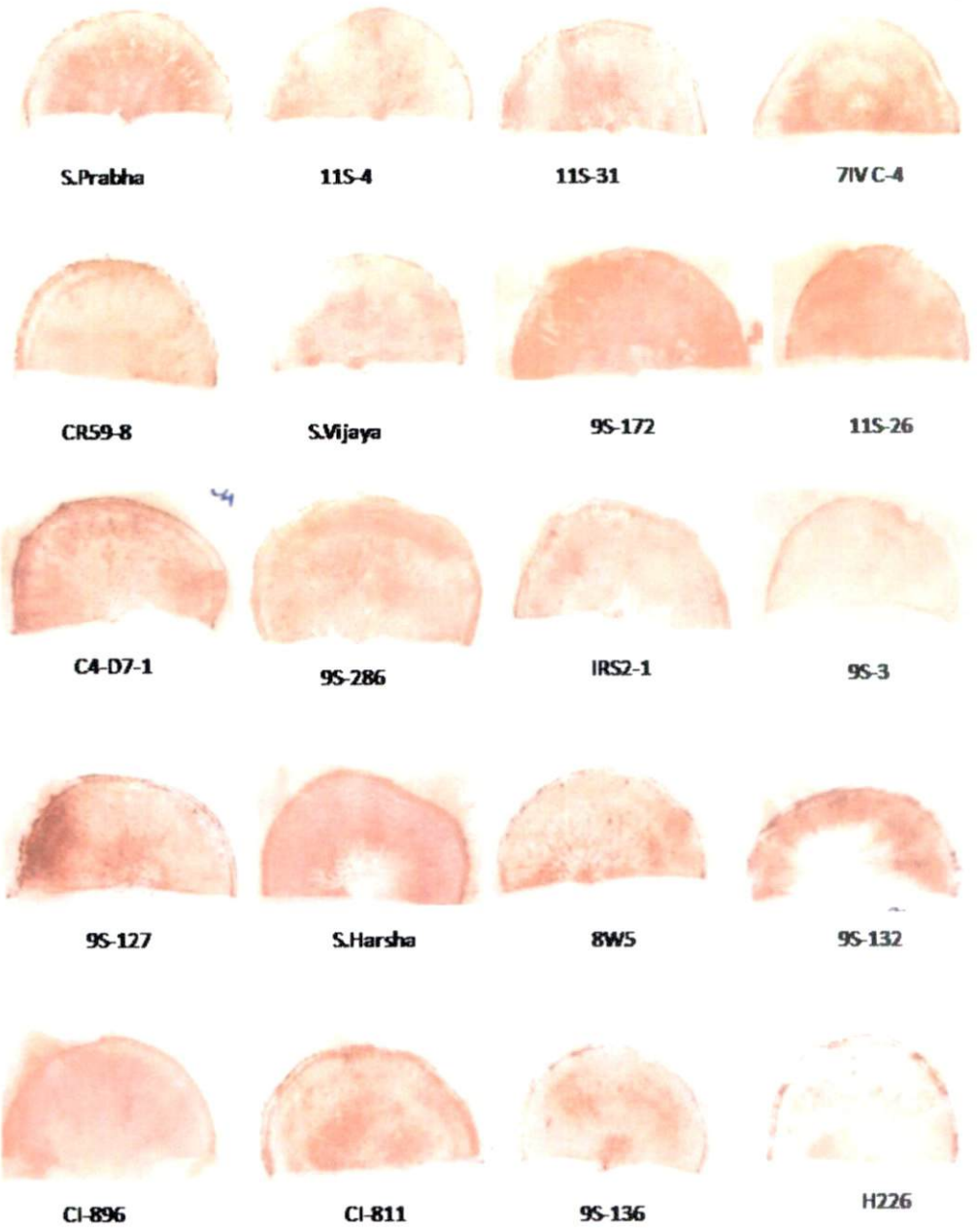



Figure 4.12. Tissue imprinting of peroxidase activity in the transverse sections of PPD affected cassava roots of selected accessions after harvest

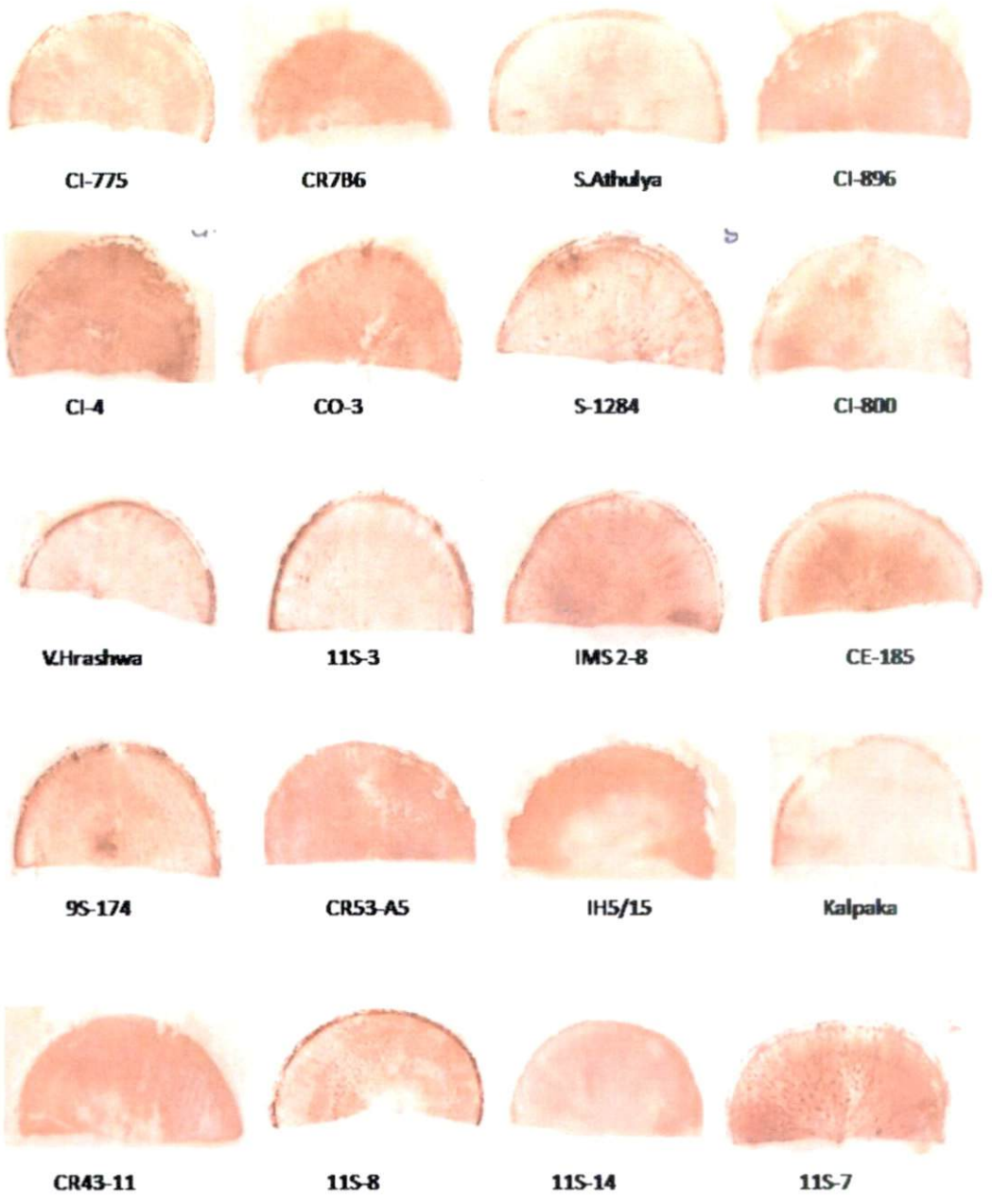



Figure 4.13. Tissue imprinting of peroxidase activity in the transverse sections of PPD affected cassava roots of selected accessions after harvest

Based on the visual scoring of roots, the cassava genotypes were grouped and segregated into different PPD category and presented in Table 4-1. Most of the genotypes studied were susceptible to early PPD and were either high or extreme category symptom groups. The moderate group also showed strong PPD symptoms with vascular streaking after a week and hence these genotypes will also show PPD symptoms at an early stage when the wounds are deep and storage conditions are favourable for deterioration.

Genotypes such as 9S-7, 9S-98, 11S-31, 11S-86, 11S-14, CE63-3, CI43-2, CR43-2, CR54-A5, CR59-8R, Sree Athulya and Kalpaka showed low PPD scores with lower discoloration and streaking compared to the rest of the genotypes. Roots of these genotypes were intact even at tenth day of storage and free from any foul smell which normally develops in cassava when PPD intensity is high. The progress of PPD in genotypes such as 11S-4, 7 IV D-7, CI-896, CO-2, CR 43-7, CR54- A19, CR24-4, IMS 2-8, S-1284 and Sree Vijaya was moderate. These genotypes were lesser susceptible to microbial deterioration compared to genotypes with high and extreme PPD. Genotypes such as CR43-11, CR59-4, CR-775, IH 5/15, IRS 2-1, Sree Harsha and Sree Padmanabha had high scores of 3 and 4 even at fifth day of storage and rapidly deteriorated with increased susceptibility to microbial attack.

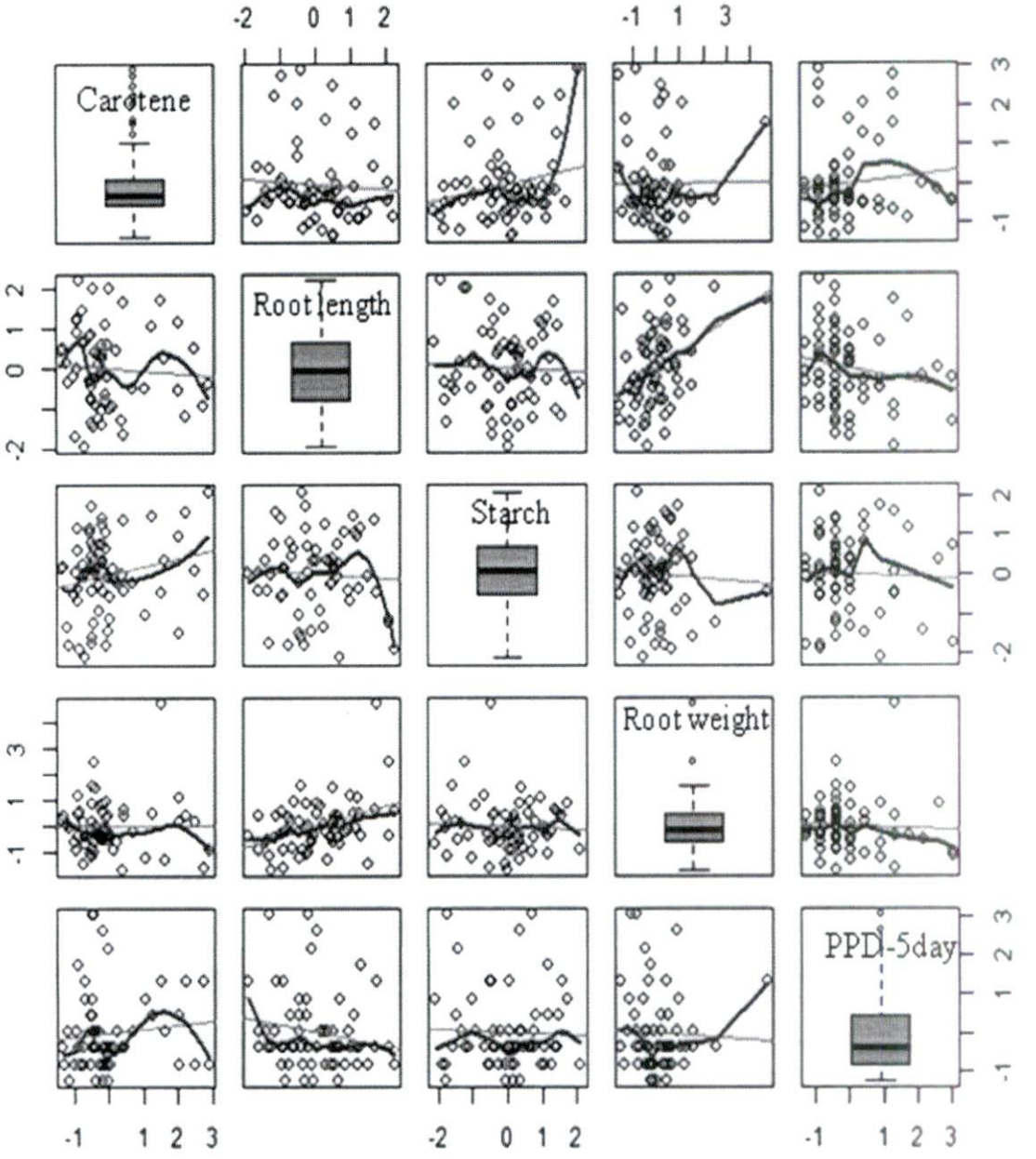



Figure 4.14. Correlation matrix of root characters, root carotene and starch content with PPD score of 61 cassava genotypes at 5 DAS. The box plot shows the mean and range value of specific parameter.

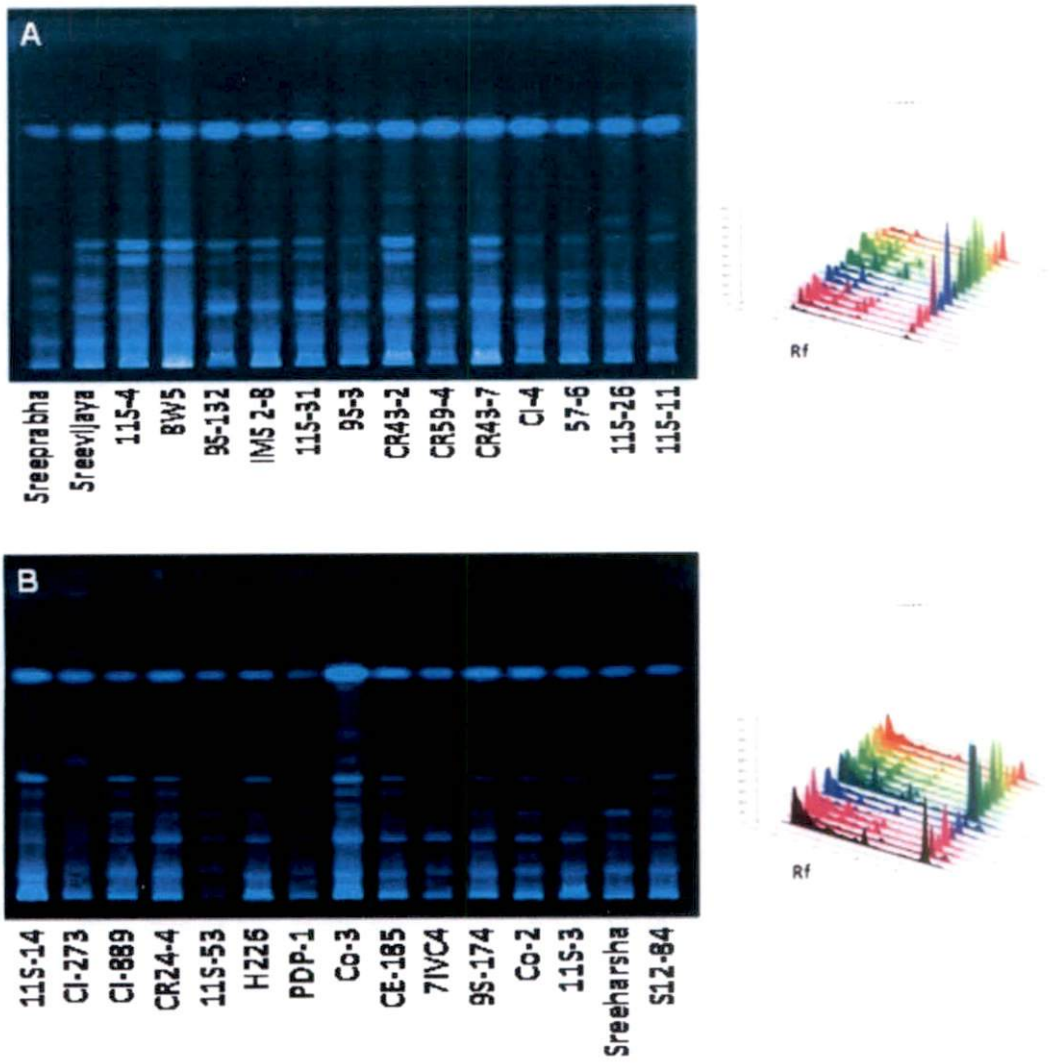



Figure 4.15. Thin layer chromatogram of phyto-chemical profile of methanolic extract of 61 cassava genotypes (A-B) at 10 days of storage. The image was taken using UV light of 350nm.

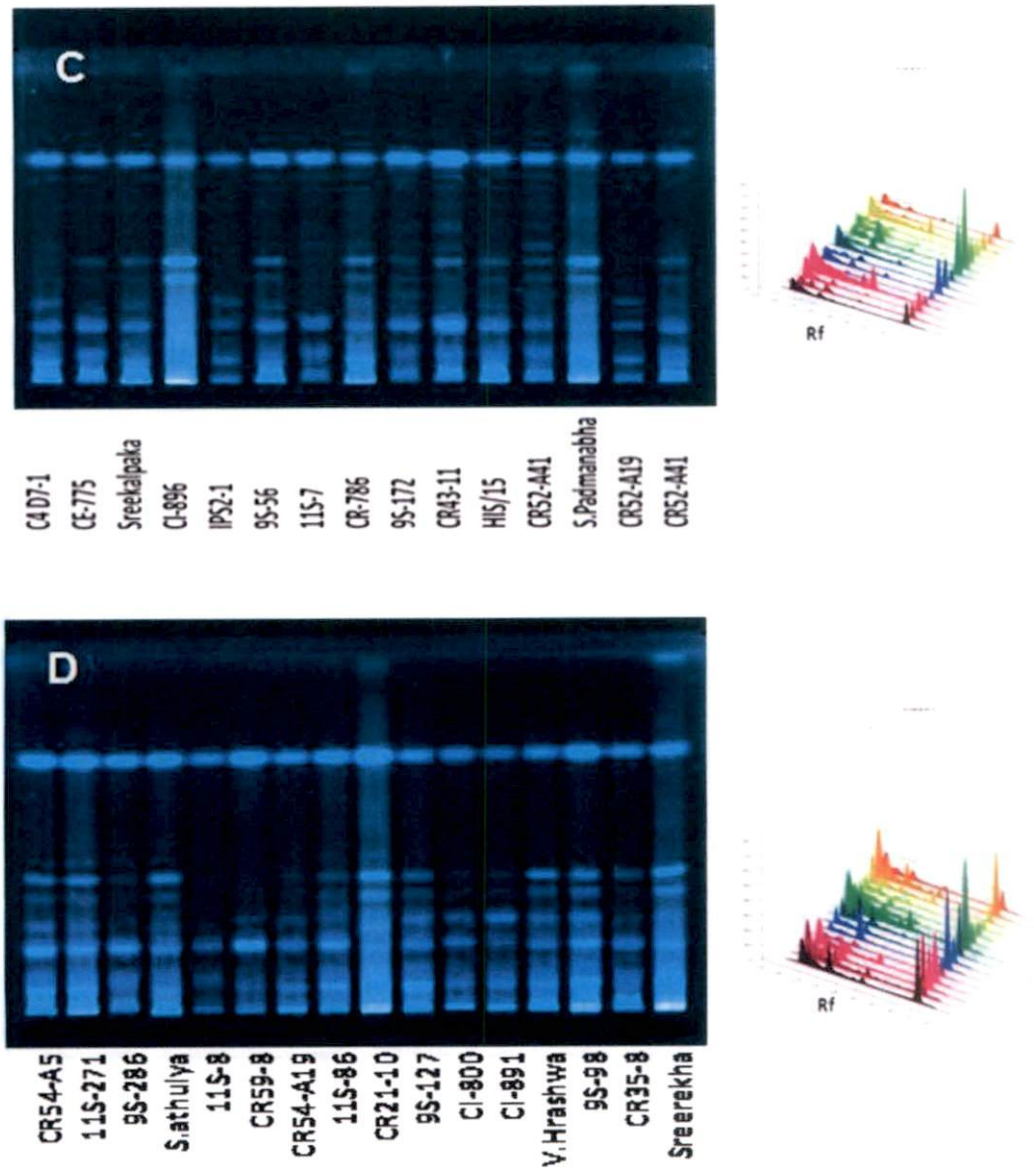



Figure 4.16. Thin layer chromatogram of phyto-chemical profile of methanolic extract of 61 cassava genotypes (A-B) at 10 days of storage. The image was taken using UV light of 350nm.

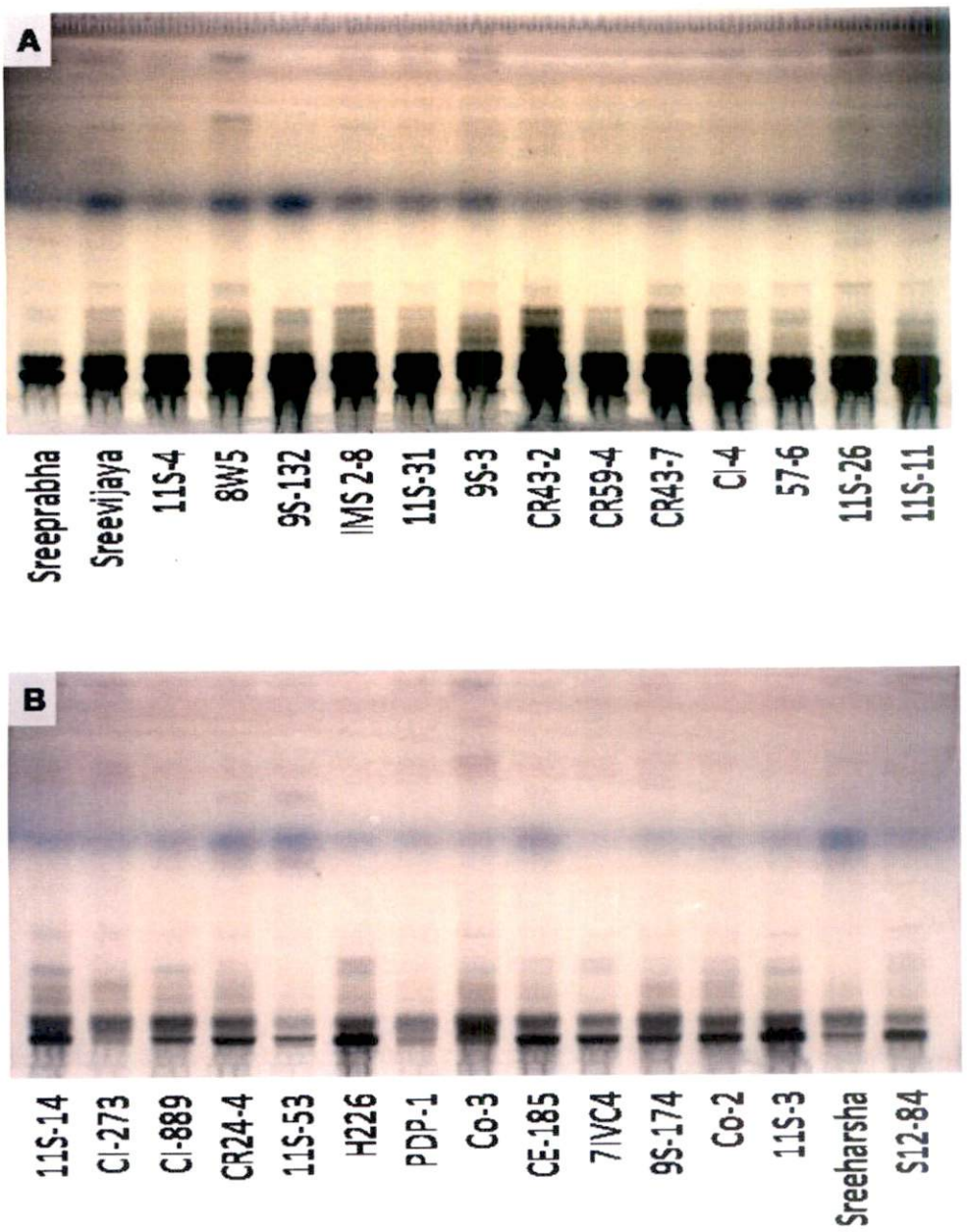



Figure 4.17. Thin layer chromatogram of phyto-chemical profile of methanolic extract of 61 cassava genotypes (A-B) at 10 days of storage. The image was taken using white light of after derivatization of plate using anisaldehyde.

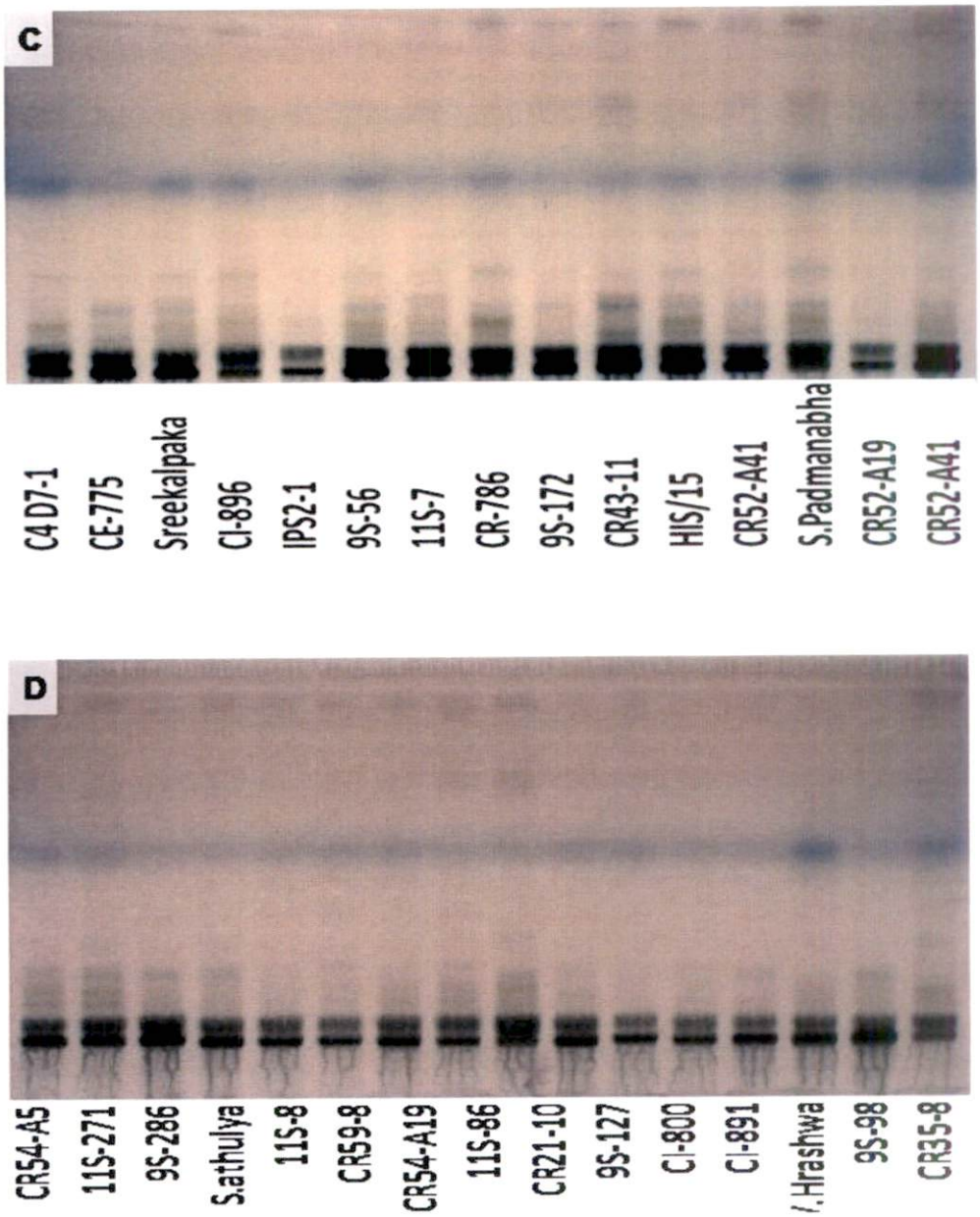



Figure 4.18. Thin layer chromatogram of phyto-chemical profile of methanolic extract of 61 cassava genotypes (C-D) at 10 days of storage. The image was taken using white light of after derivatization of plate using anisaldehyde

There were nine polymorphic bands and five monomorphic bands when both TLC plates were visualized with UV and visible light combined. The TLC plates were initially visualized with UV 366 nm and scanned at 290 nm and treated with 1% vaniline/$H_2SO_4$ for derivatization of the plates and then photographed for analysis (Figure 4.15 to 4.18). The polymorphic bands were scored and used for classifying the genotypes. Additional bands were visible after the derivatization of the plates. A dendrogram was constructed using Ward's method using squared Euclidean distance as it was intuitive and straight forward way of defining the similarity of objects. There were some degree of overlapping in I and II main groups, however there was a clear separation of genotypes having high and severe PPD in group III (Figure 4.19). The visual symptoms of deterioration during the advanced stages of PPD especially in the high and severe categories overlapped and assigning the scores to individual roots of genotypes were increasingly difficult. Furthermore, the genotypes in the high and severe PPD category showed similar symptoms of root deterioration and nearly identical chemical profile. The classification of genotypes based on chemical profile matched with their visual scoring to a great extent. In the present study, we found similarity in chemical profile and PPD intensity when the symptoms are at advanced stage. Genotypes like 11S-8, HI5/15, 92-286, 11S-7, 11S-40, IRS2-1, 9S-172, C4D7-1, CR54-A4, PDP-1, CI-273, 11S-53, Sree Harsha and Sree Prabha were grouped together (Cluster III) with similar chemical profile and PPD symptoms. The differentiation of cassava roots at the metabolites level corresponding to visual symptoms and chemotypic profile of PPD offer a rapid screen tool and can be incorporated into cassava breeding programmes on PPD tolerance.

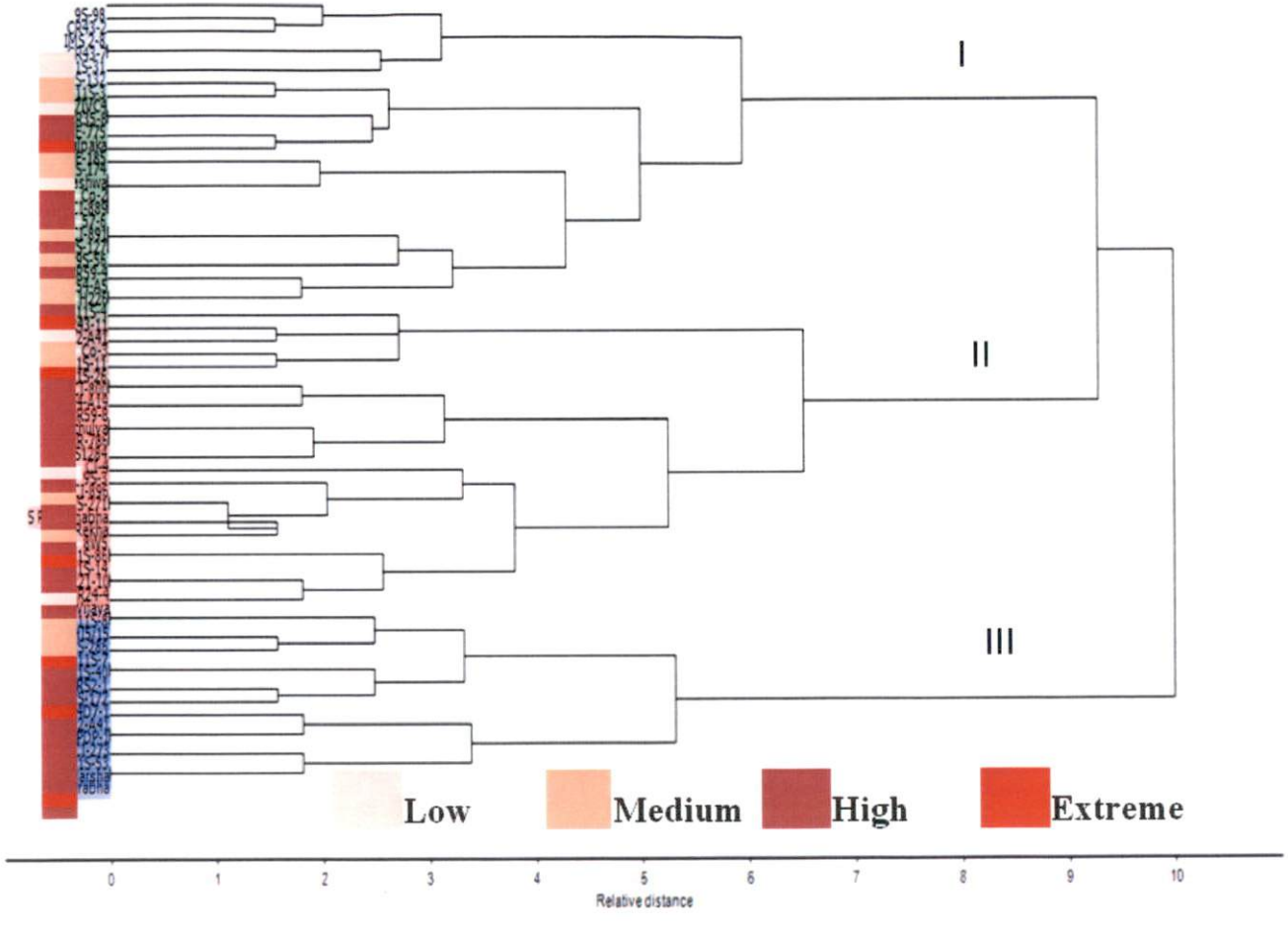



Figure 4.19. Dendrogram of cassava genotypes based on the chemotypic profile of roots under PPD by ward's method using squared Euclidean distance. Colored boxes indicate the cassava genotype with specific PPD characteristics.

## 4.2 Analysis of biochemical aspects of cassava roots with Near Infra-red (NIR) Spectroscopy

Near infrared spectroscopic analysis is routinely employed to determine functional composition of food products, biological matrices as it is rapid, on-line, minimalistic technique. NIR techniques are suitable to determine simultaneously several components in a food sample within a short time. NIR region of IR spectrum of samples are due to overtones and combinations of the fundamental vibrations observed in the Mid-IR region. Overtones has frequencies corresponding approximately to twice, three times etc. that of the fundamental, while combination bands arise by interaction of two or more vibrations taking place simultaneously and the frequency of a combination band is the sum of multiples of the relevant fundamental frequencies. Vibrations involving C–H, O–H, N–H and possibly S–H and C=O bonds are responsible for the majority of the observed absorption bands in the NIR region.

The samples were scanned in the NIR region of 10,000 $cm^{-1}$ to 4000 $cm^{-1}$ corresponding to 1100nm to 2400nm of NIR. However, the data were truncated to the range 6000 to 4000 nm, since no prominent spectral features that are to be observed in the residual part of the spectrum. The NIR spectroscopic analysis was done for cassava roots of field grown twenty genotypes. One set of plants were pruned one week before harvest and the other set was control. Both pruned and control plant roots were scored for PPD and respective category of roots were used for NIR analysis. The NIR absorbance spectra for cassava root slices for 6,000 - 4000 $cm^{-1}$ is presented in Figure (4.20- A & 4.23-A) for control and pruned plants, respectively. The NIR spectra showed a number of features which corresponds to various biochemical constituents like starch, moisture and protein and other minor constituents present in the root tissue. There was an increase in absorbance which peaked at 6100 cm-1 initially and a sharp decline afterwards. The steep decline bottomed out at 4500 cm-1. There was a large peak at 4000 cm-1. Cassava root slices showed strong absorbance at 5,180 cm-1 and a shoulder around 4716 cm-1 which can be attributed to the C–H and C=O stretching vibrations of -HC=CH-.

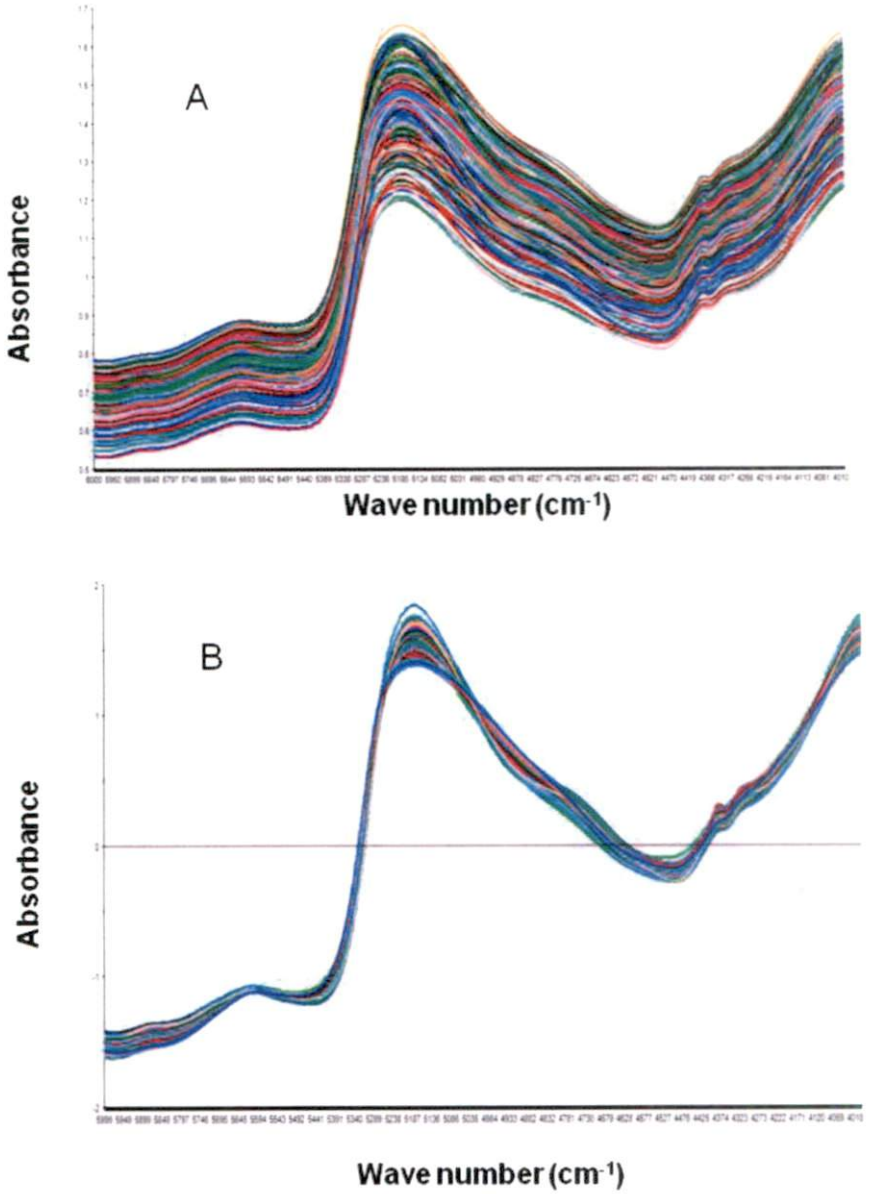



Figure 4.20. NIR spectral data of control cassava plants. A) Raw NIR spectra of control plants, B) SNV corrected spectral data

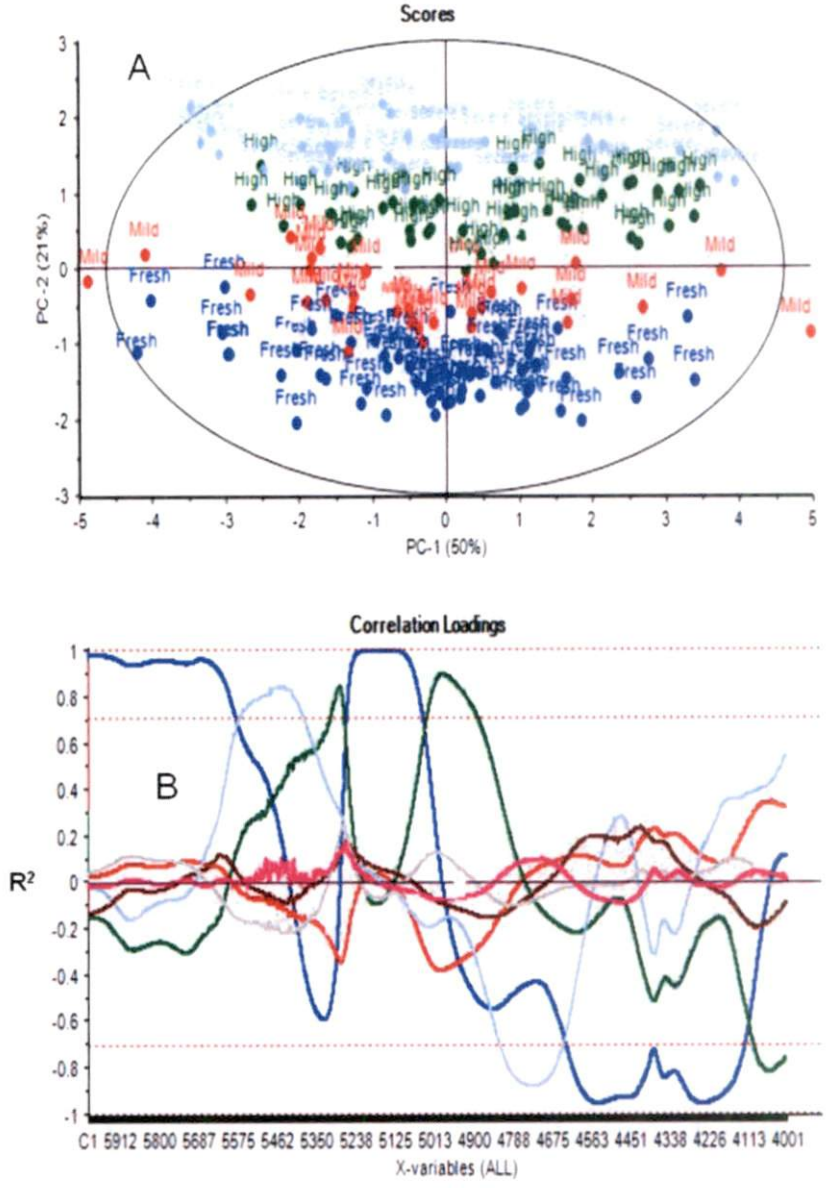



Figure 4.21. Principal component analysis based on spectral data of control plants. A) scores plot, B) loadings plot of PCs

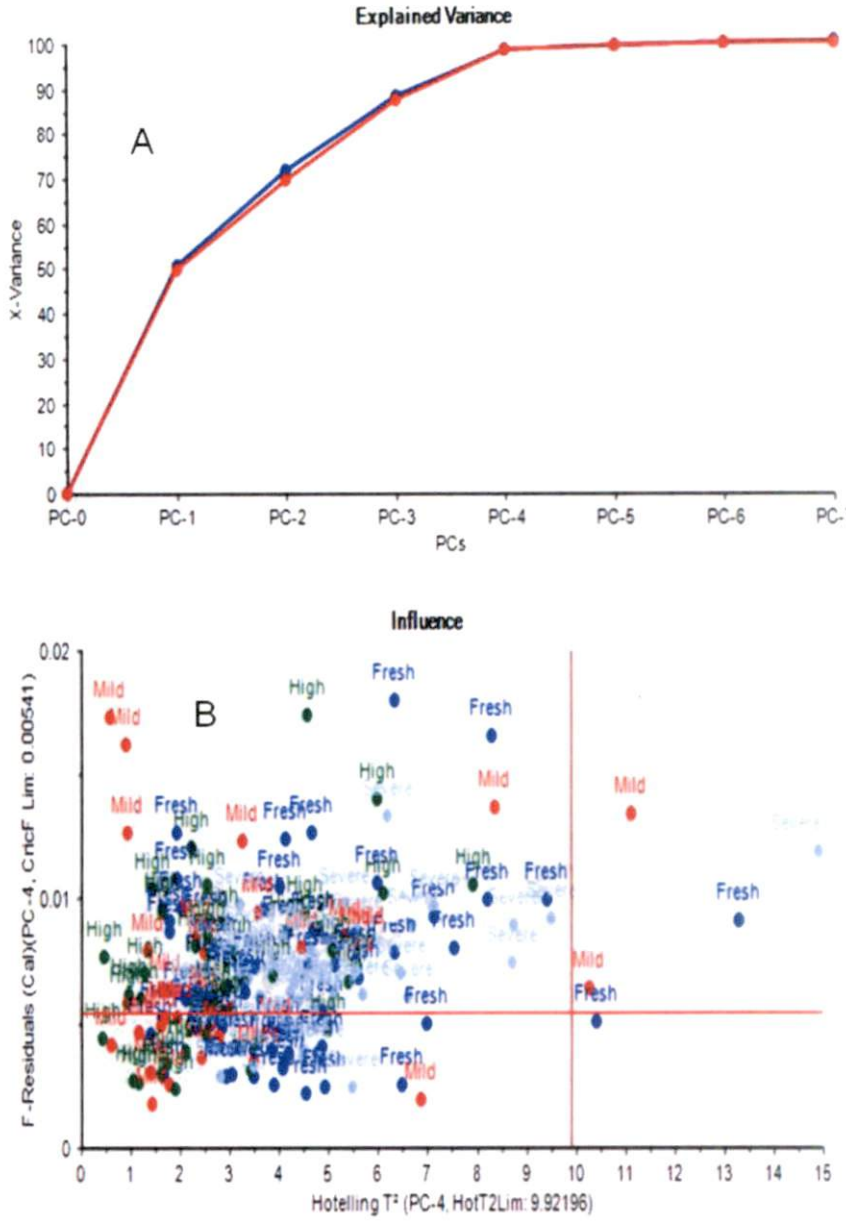



Figure 4.22 Principal component analysis of NIR spectra of control cassava plants A) plot of explained variance and B) residual or influence plot.

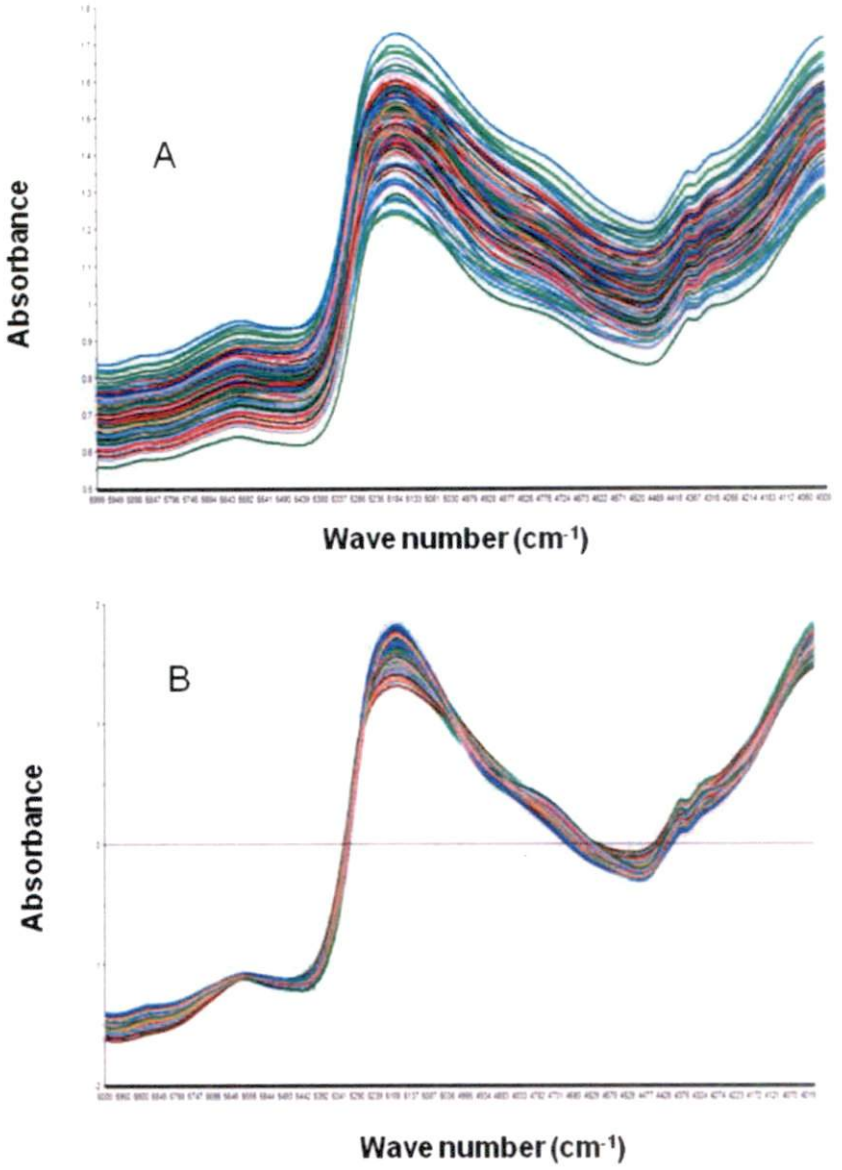



Figure 4.23. NIR spectral data of pruned cassava plants. A) Raw NIR spectra of pruned plants, B) SNV corrected spectral data

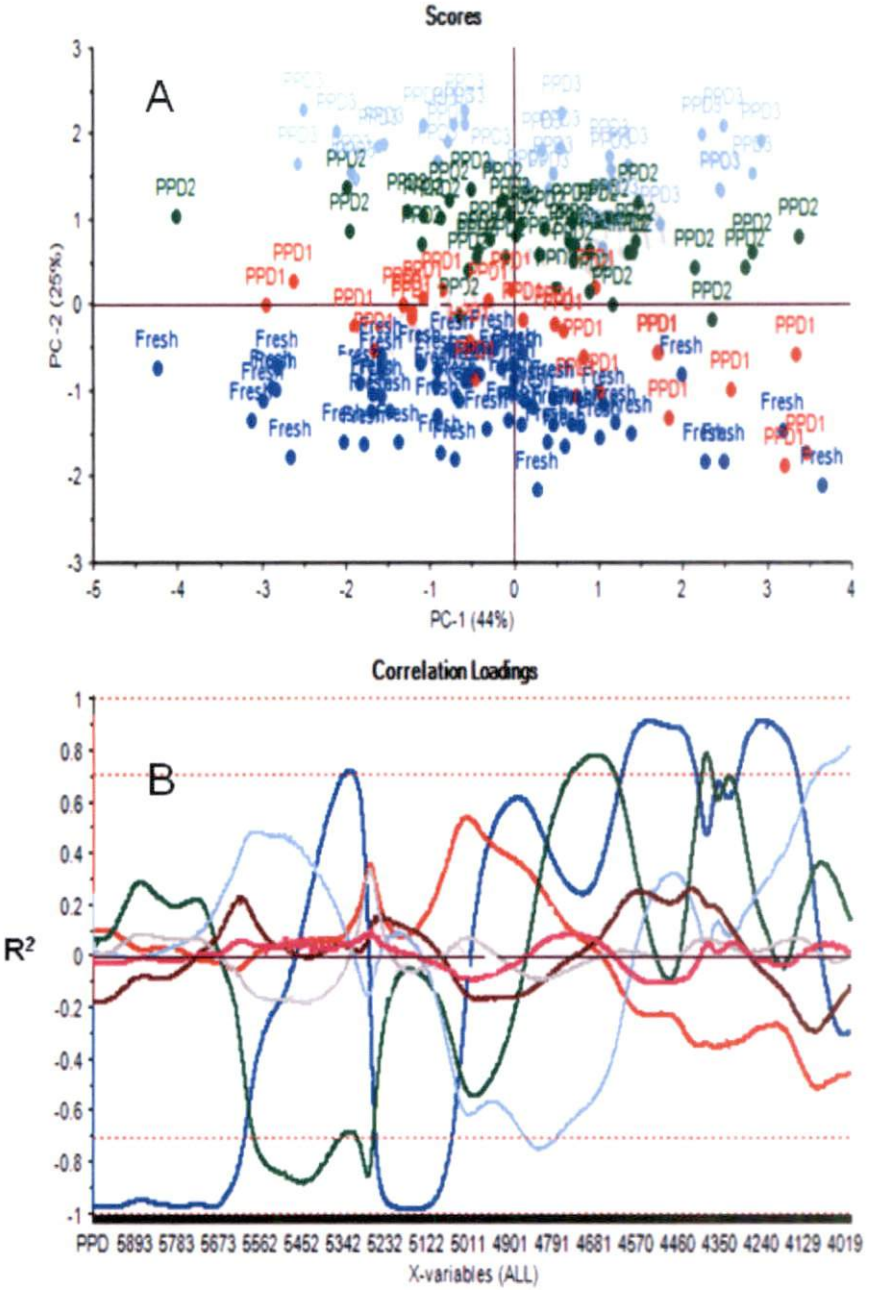



Figure 4.24. Principal component analysis based on spectral data of pruned plants. A) scores plot, D) loadings plot of PCs

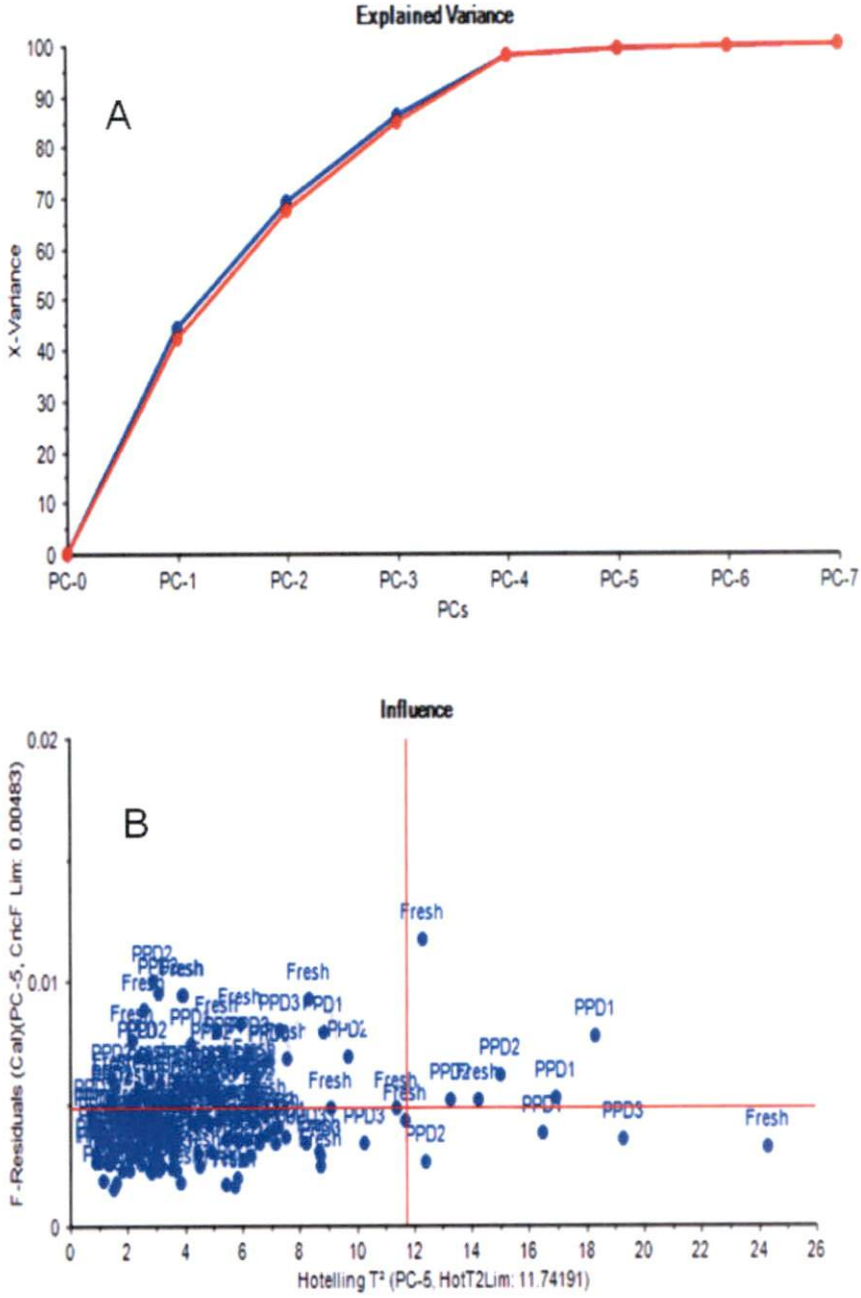



Figure 4.25. Principal component analysis of NIR spectra of pruned cassava plants A) plot of explained variance and B) residual or influence plot

### 4.2.1 PCA analysis

Biological matrices like root tissues are complex in nature and many chemical constituents are present and hence it is challenge to elucidate the data directly. Such data needs the multivariate data analysis (MVA) tools to fully describe them. MVA is a widely used tool for spectroscopic data (Xu *et al.*, 2013; Lupoi *et al.*, 2014). In MVA, the most commonly used tools are (a) Principal component analysis (PCA), and (b) Partial least square (PLS) analysis. Principal component analysis is mainly used for identifying outliers, sample comparison, and screening. The aim of principal component analysis (PCA) was to examine hidden structure of data, to visualize relationship (similarity and difference) between root samples with and without PPD and spectral wavelength (variables). PCA was used mainly to describe sample effect and as a descriptive tool. The PCA analyssis was conducted for the spectra obtained from various samples of fresh cassava roots and roots with typical PPD symptoms. The results of PCA are presented in Figures 4.21, 4.22 for control plants and 4.24, 4.25 for pruned plants. The scores plots depicted in Figure 4.21 A and Figure 4.24 A shows the distribution of various samples in principal components (PCs) 1 and 2. The first four principal components (PC) explained over 99% of the variance in the NIR spectrum. Only few outliers were detected among the samples. Scores plots revealing the overall structure of the control plants data are shown in Figure 4.21(A) and the loadings are presented in the Figure 4.21(B). The scores plot of pruned plants is presented in Figure 4.24 (A) and the corresponding loadings plot is presented in Figure 4.24 (B). Under normal conditions, 5% of the samples may lie outside the ellipse (CAMO, 2012) and samples far from center of the plot will have high leverage (potentially influential) (Naes *et al.*, 2002). Both control and pruned plants had less than 5% outliers and had sufficient model quality. Both the samples had the predictive ability as reflected in values of correlation (r), coefficient of determination ($R^2$) and residual prediction deviation (RPD) in both PLS and PCR models. Samples, which appear as potential outliners, were not removed in this study because they contain real root quality information. Comparison of variances showed the closeness of calibrated and validated curves, which reflected that models were true representative and there is absence of threat

from outliers. Together with the scores plot, PCA loadings plot allows for the determination of important chemical features responsible for the sample grouping. In the loadings plot, variables with large values are highly correlated with sample grouping (Sykes *et al.*, 2009).

Partial Least Square can be used to build prediction correlation model between spectral data and the property of sample matrices. The loadings plot showed clearly the influencing wave numbers for the differentiating the samples in various principal components such as 1$^{st}$ component wave numbers (1/cm) 5300, 5200-5100, 4600-4400,4240-4150 which are important for the variations in the first component. The second component had 5450, 5250, 4700 and 4400 $cm^{-1}$. The third component belonged to 4800 $cm^{-1}$.

## 4.3 Transcriptional analysis of cassava roots during PPD

Among the various wound response related genes like PAL, POX, RBOH, WRKY-TF, SAG 20 and XET tested with specific primers, two genes i.e. successful amplification was detected for PAL and POX and results are presented for these two genes (Fig 4.26). Comparative semi-quantitative PCR to measure PPD related gene expression requires reference genes whose expression is consistent throughout the differing experimental treatments, tissue and conditions. Transcriptional analysis works in cassava utilized tubulin as reference genes and hence it was taken as reference gene for our studies. Amplification primers for the proposed reference genes were used in PCR with standard *Taq* DNA Polymerase to check specificity. Template cDNA was derived from 1 µg total RNA from fresh cassava root tissue and the primers generated the predicted sized amplicons (~ 150 bp) observed following agarose gel electrophoresis. There was no non-specific priming was observed for primer pair, indicating good specificity to the target sequence. The expression of PAL and POX genes were differentially regulated during PPD in the cassava varieties. PAL gene product phenylalanine ammonia lyase is a key enzyme in the phenyl propanoid pathway and catalyses the biosynthesis of p-cinnamic acid. From p-cinnamic acid, various phenolic intermediaries and coumarins are synthasized. Expression of PAL is increased during storage during 1 and 3 days after harvest in Kalpaka whereas it remained at

same level at 1 day and decreased slightly at 3 days (~10%) in IH5/15. The increase in PAL was nearly 1.6 fold at 1 and 3 day compared to 0 day in Kalpaka (Fig 4. 27). The expression of POX modulated differently in genoytypes studied. POX expression reduced by 25% after one day of storage in Kalpaka and subsequently increased by 14% over 0 day level. In the case of IH5/15, POX expression increased marginally (up by 13%) in the initial stage of PPD and decreased and reached to 70% of 0 day expression.

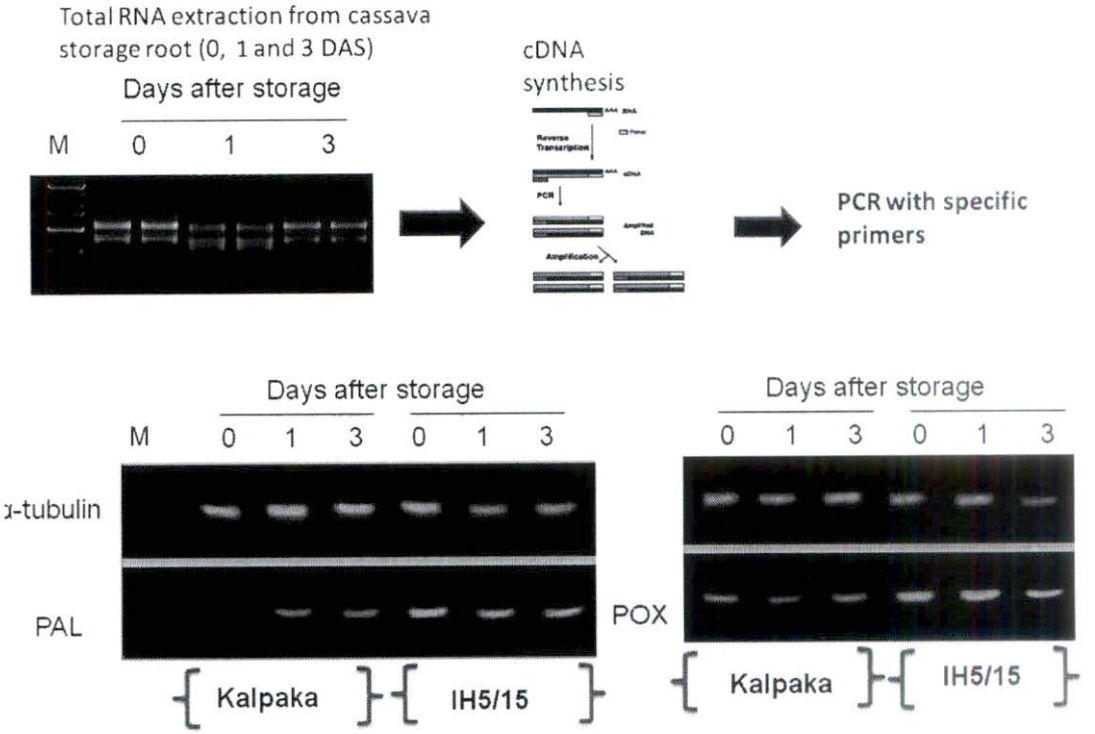



Figure 4.26. Semi-quantitative PCR analysis of PAL and POX gene expression in cassava root tissue at 0, 1 and 3 days after storage

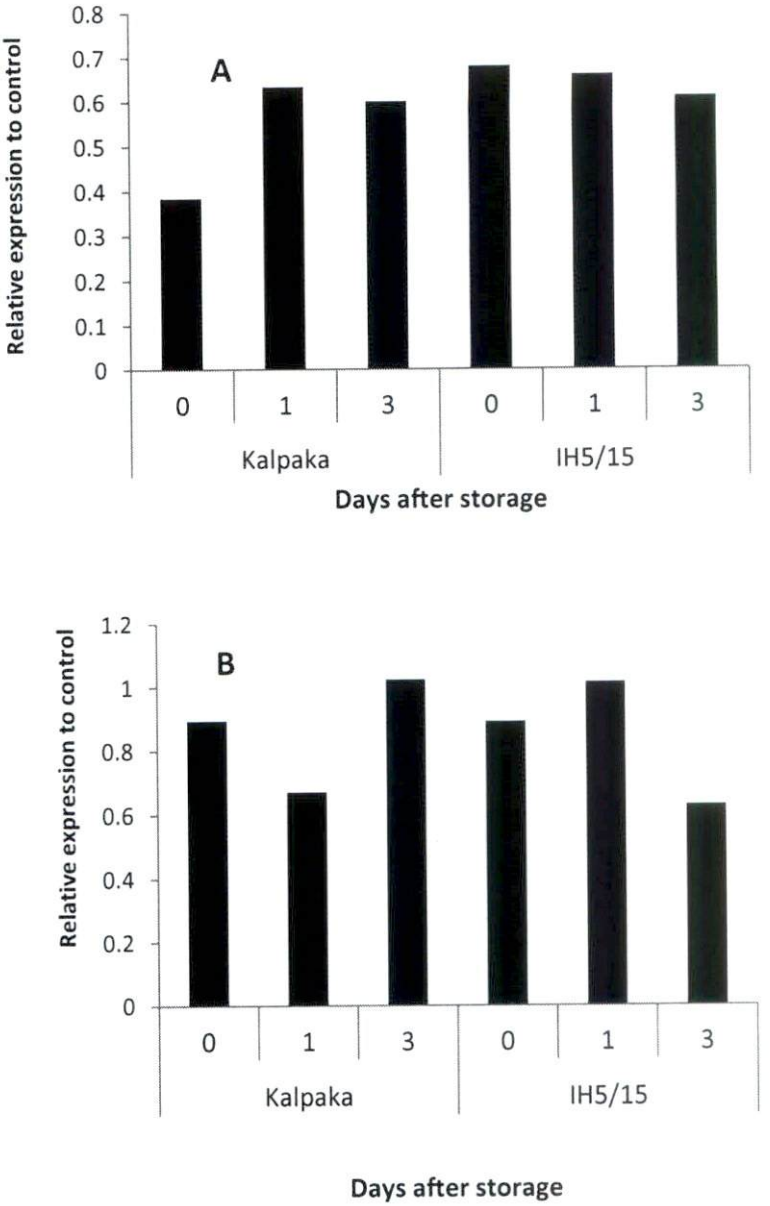



Figure 4.27. Relative expression level of PAL (A) and POX (B) gene at 0, 1 and 3 days after storage in Kalpaka and IH5/15 genotypes of cassava

## 4.4 Tissue staining of cassava roots for visualizing PPD symptoms

Free hand sections of root tissue of cassava were tested with different stains to characterize PPD symptoms and to explore the possibility of detecting the tissue anatomical changes before the visual symptoms appear in the tissue. Various stains like erythrocin red, saffranin O, aniline blue, fast green and phlorglucinol for visualizing the PPD symptoms at early stages of storage. The stains were prepared in ethyl alcohol at (1% w/v) concentrations. The root transverse sections were stained properly and stains distinguished the various tissue types like vascular tissue, parenchyma etc. The stains like saffranin O and erythrocin red differentiated the xylem vessels (Figure 4.28 B & A). However, the tissue damage due to PPD was not particularly highlighted by the staining.

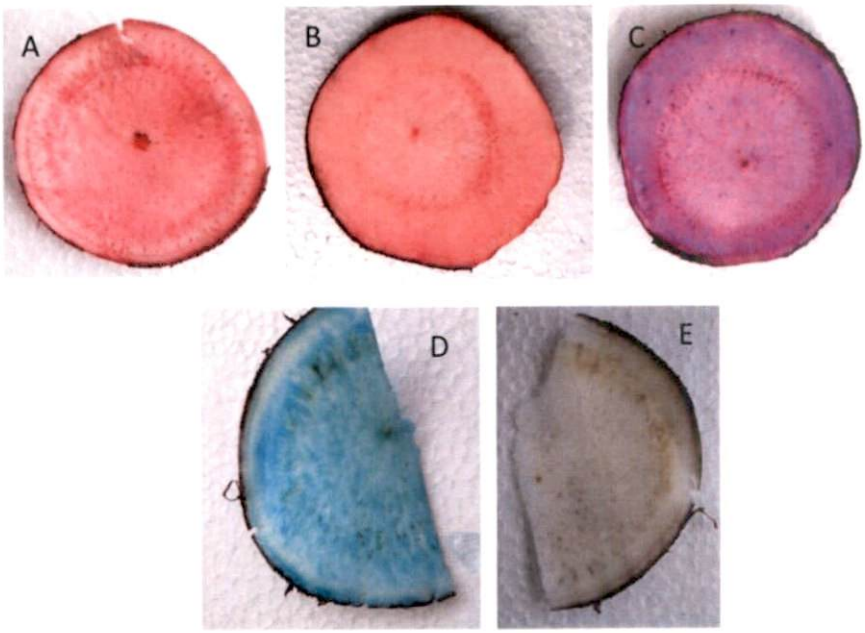



Figure 4.28. Staining of cassava root tissue with erythrocin red (A), saffranin O (B), aniline blue (C), fast green (D) and phlorglucinol (E) for visualizing the PPD symptoms at early stages of storage.

## 4.5 Delaying PPD through lowering oxygen concentration during storage

Cassava roots after harvest are susceptible to PPD, since the root tissue is alive and undergo various biochemical changes which ultimately results in loss of root quality. The main aim of this experiment was to minimize the exposure of roots to atmospheric oxygen and prevent the oxidative enzymes to come in contact with oxygen. There are several techniques available for reducing the oxygen concentration under laboratory conditions and cassava being a bulky agricultural commodity which is easily perishable; we employed economically viable techniques such as storage in de-aerated poly-bags, wax coating and burying harvested roots under the soil to delay the progress of PPD.

Morphological data and dry matter content of cassava roots of selected varieties of cassava used for studying the storage performance under oxygen exclusion are presented in Table 4.2. The root fresh weight of the varieties Roots of different cassava varieties used in this experiment differed significantly for the root morphological, fresh weight, dry matter content, root total sugar content and starch to sugar ration. Root fresh weight significantly differed. Among the varieties, Sree Athulya had the highest root fresh weight (1377.5g), whereas Sree Jaya had the lowest (736.7 g). Roots of S Jaya were smallest among them with a maximum root girth of 15.8 cm. Root sizes of Vellayani Hraswa, Kalpaka and Sree Padmanabha were at par each other. Root dry matter content highest in Sree Athulya (40.7%) and lowest root dry matter content was recorded for S. Jaya (34%). The dry matter content of Vellayani Hraswa and Kalpaka and Sree Padmanabha were at par with each other. Interestingly, starch content in the roots of different varieties was not significantly different. But the total sugar content varied significantly.

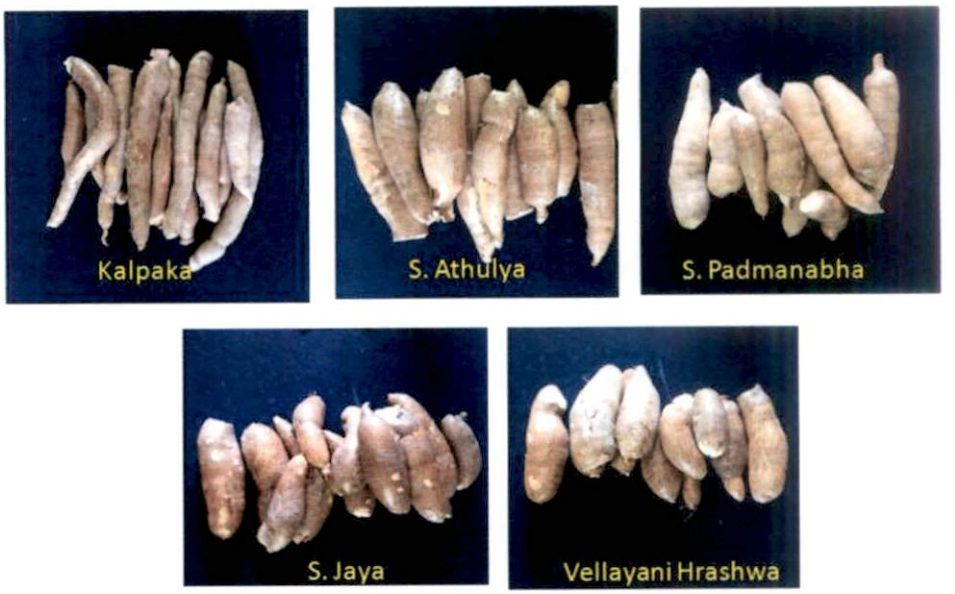



Figure 4.29. Wax coated roots of selected cassava varieties for prolonging the shelf-life and to delay the PPD


Figure 4.30. Storing of selected cassava varieties in deaerated polythene bags to increase the shelf-life and to delay the PPD

Figure 4.31. Storing of cassava roots under the soil to delay the onset of PPD

Table 4-2. Morphological and dry matter content of cassava varieties used for studying different methods of storage

| Variety | Root fresh weight (g) | Root length (cm) | Root Max. girth (cm) | Dry matter content (%) |
|---|---|---|---|---|
| V.Hrashwa | $1010.5^{ab}$ | $39.4^{b}$ | $22.8^{bc}$ | $34.6^{ab}$ |
| Sree Athulya | $1377.5^{c}$ | $44.3^{b}$ | $18.2^{ac}$ | $40.7^{b}$ |
| Kalpaka | $1113.9^{bc}$ | $37.2^{ab}$ | $23.0^{c}$ | $36.6^{ab}$ |
| S. Jaya | $736.7^{a}$ | $38.9^{b}$ | $15.8^{a}$ | $34.0^{a}$ |
| S Padmanabha | $864.6^{ab}$ | $27.1^{a}$ | $17.4^{ab}$ | $38.8^{ab}$ |

(* The superscript letter indicates the significant differences between the mean values at p=0.05)

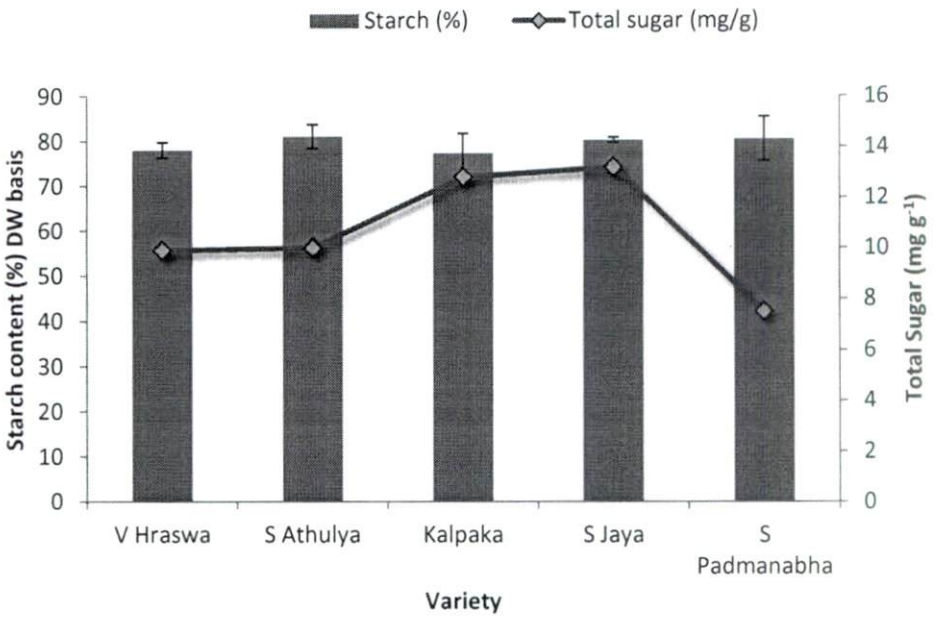



Figure 4.32. Content of starch and total sugar in selected varieties of cassava roots used for storage study on oxygen exclusion during storage

(Error bars indicate Std error)

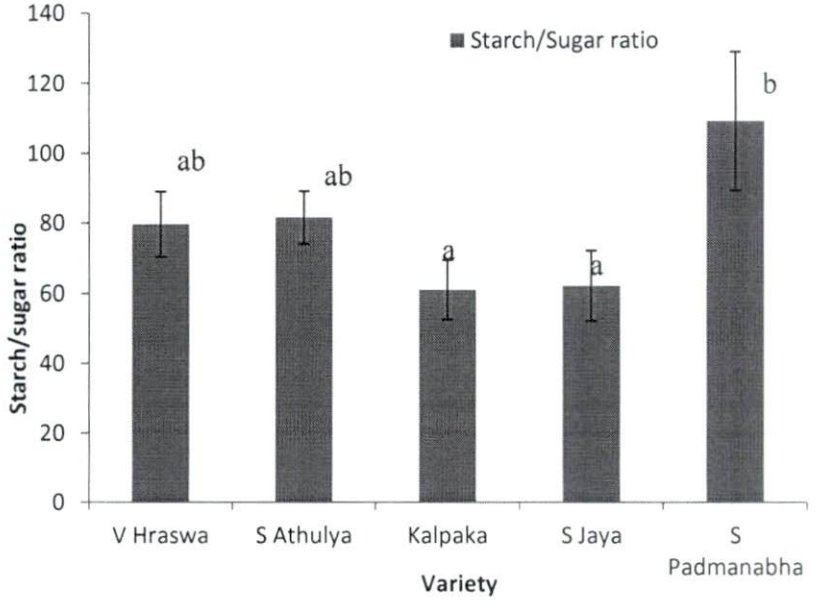



Figure 4.33. Starch to sugar ratio in selected varieties of cassava roots used for storage study on oxygen exclusion during storage

(Error bars indicate Std error)

Table 4-3. Percentage of roots damaged during storage by various storage methods in selected varieties of cassava

| Variety | Percentage of roots damaged | | | | | |
|---|---|---|---|---|---|---|
| | Wax Coated | | Polybag stored | | Stored under soil | |
| | 1st week | 2nd weeks | 1st week | 2nd weeks | 1st weeks | 2nd weeks |
| V Hraswa | 13.3$^{c}$ | 20.0$^{b}$ | 16.7$^{ns}$ | 33.3$^{b}$ | 23.3$^{ns}$ | 40.0$^{b}$ |
| S Athulya | 6.7$^{a}$ | 16.6$^{a}$ | 16.7$^{ns}$ | 23.3$^{a}$ | 26.7$^{ns}$ | 33.3$^{a}$ |
| Kalpaka | 10.0$^{b}$ | 23.3$^{b}$ | 20.0$^{ns}$ | 30.0$^{b}$ | 23.3$^{ns}$ | 36.7$^{ab}$ |
| S Jaya | 13.3$^{c}$ | 16.7$^{a}$ | 16.7$^{ns}$ | 26.7$^{ab}$ | 23.3$^{ns}$ | 36.7$^{ab}$ |
| S Padma | 13.3$^{c}$ | 23.3$^{b}$ | 16.7$^{ns}$ | 26.7$^{ab}$ | 26.7$^{ns}$ | 40.0$^{b}$ |

(* The superscript letter indicates the significant differences between the mean values at p=0.05, ns-non significant)

Total sugar content in Kalpaka (12.8 mg $g^{-1}$) and Sree Jaya (13.2 mg $g^{-1}$) were higher compared to other varieties tested (Figure 4.32). Lowest sugar content was noticed in roots of Sree Padmanabha (7.5 mg $g^{-1}$ tissue). Sree Athulya and V Hraswa had the intermittent sugar content of 10 mg $g^{-1}$. In line with the sugar content, the starch – sugar ratio also differed among the cassava roots in the varieties studied. Highest sugar to starch ratio was recorded for S Padmanabha with a ratio of 109.1 whereas, lower ratio was recorded for Kalpaka (61.0) and S Jaya (62.1) (Figure 4.33). Roots kept under storage following different methods like wax coating, poly-bag storage and buried under soil were showed varied response in terms of damage and deterioration during storage. Roots tend to lose shelf-life more easily when buried under soil. The microbial attack started where the root tissue was damaged due to harvesting and handling. More number of roots started showing damage symptoms due to microbial action compared to other method of storage. Nearly fifth of the roots buried in soil got damaged irrespective of the variety used. It tended to increase up to 35-40% after 2 weeks with more or less same effect in all the varieties (Table 4-3). The poly-bag stored roots tended to get spoiled after a week primarily due to water vapour accumulation inside the bag as a result of transpiration by roots. The condensation of water inside the bags created a favourable environment for the microbial growth. The damage was more

pronounced after 2 week of storage in poly-bag with increasing damage nearly upto 30% of stored roots. The roots tended to soften at cut ends with prolonged storage after few days and produced foul smell after taken out from the bags. Least percentage of roots damaged with wax coating and the roots damaged where comparatively lower during even after two weeks of storage.

## 4.6 Effect of different storage temperatures on PPD

Morphological parameters like root fresh weight, root length, root girth of the cassava varieties investigated were presented in Table 4-4. The root characters significantly differed for the varieties. However, the root dry matter (DM) content of the varieties did not vary significantly even though the starch content differed among them. Root fresh weight was highest for Kalpaka (1235 g) with maximum root girth (24.7 cm) and Sree Athulya had the longest roots. The dry matter content was ranging from 35%-40% among the varieties studied, however individual tubers differed greatly for DM. Starch content of Sree Padmanabha and Sree Athulya were higher compared to other varieties.

Table 4-4. Root characters, dry matter and starch content of selected cassava varieties used for storage studies

| Variety | Root fresh weight (g) | Root length (cm) | Root girth (cm) | Dry matter content (%) | Starch content (%) |
|---|---|---|---|---|---|
| **Vellayani Hraswa** | $768^{b}$ | $34.3^{bc}$ | $20.7^{b}$ | $35.4^{ns}$ | $80.2^{ab}$ |
| **Sree Athulya** | $866^{b}$ | $37.8^{c}$ | $15.8^{a}$ | $40.3^{ns}$ | $85.4^{c}$ |
| **Kalpaka** | $1235^{c}$ | $32.6^{b}$ | $24.7^{c}$ | $37.8^{ns}$ | $79.8^{a}$ |
| **Sree Jaya** | $412^{a}$ | $31.2^{b}$ | $14.2^{a}$ | $35.6^{ns}$ | $84.5^{bc}$ |
| **Sree Padmanabha** | $523^{a}$ | $26.4^{a}$ | $15.0^{a}$ | $35.5^{ns}$ | $86.0^{c}$ |

(* The superscript letter indicates the significant differences between the mean values at p=0.05, ns-non significant)

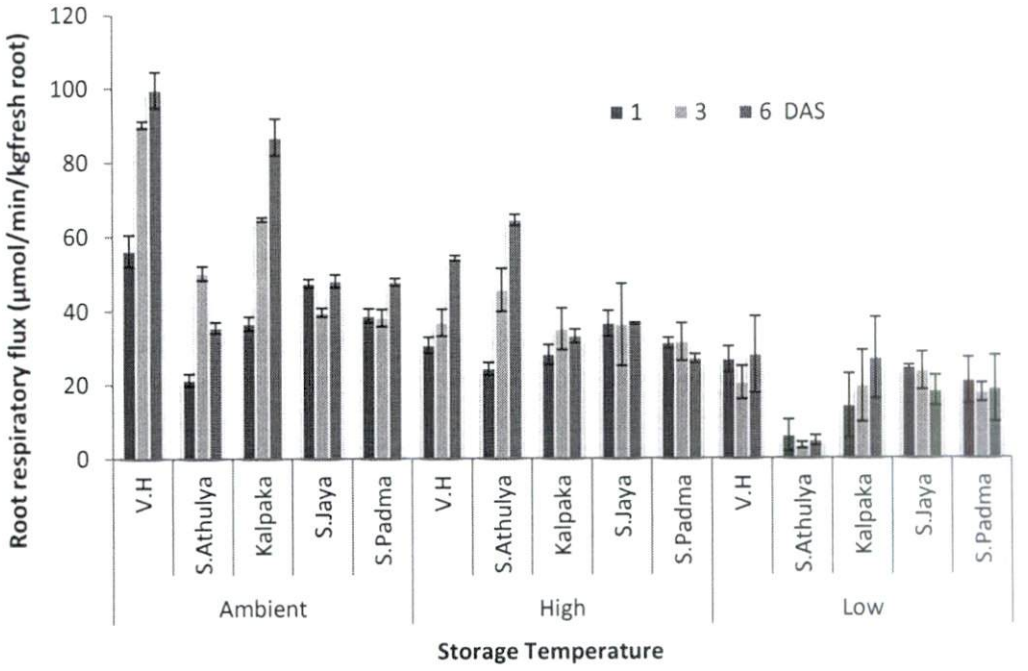



Figure 4.34. Respiratory $CO_2$ flux of storage roots of cassava varieties as affected by temperature. Results are average of three samples.

(Error bars indicate Std error)

The respiratory flux was measured for intact cassava roots after 1, 3 and 6 DAS and presented in Fig 4.34. Root respiratory flux was higher in roots stored at ambient temperature compared to high or low temperature. Among the varieties studied, Vellayani Hraswa and Sree Jaya showed higher respiratory activity of 99.6 and 90.1 µmol/min/kg fresh root respectively, under ambient/room temperature storage at 6 DAS. Sree Athulya was consistently having low respiratory $CO_2$ flux under all three temperature regimes. Respiratory rate during storage increased under both ambient and high temperature from 1 to 6 days in most of the varieties studied except Sree Padmanabha which showed nearly 30% in decrease in $CO_2$ flux at 6 DAS under high temperature.

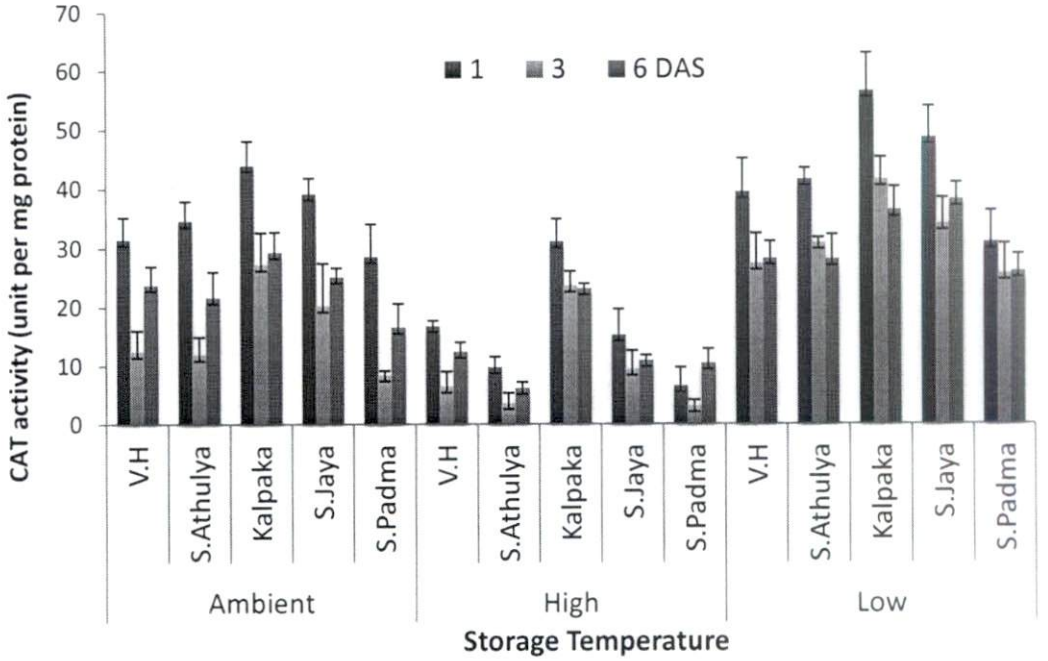



Figure 4.35. CAT activity measured in the roots of cassava after 1, 3 and 6 days after storage as affected by temperature regimes..

(Error bars indicate Std error)

Catalase activity measured in the roots after 1, 3 and 6 days after storage were presented in Fig 4.35. Cassava varieties showed variations in catalyse activity over the storage period as well as due to storage temperature. Temperature had a marked influence on catalase activity. Maximum catalase activity recorded at 1 DAS in all the varieties studied irrespective of temperature regime. Highest CAT activity of 56.32 units $mg^{-1}$protein was detected in Kalpaka under low temperature storage and the least (3.06 units mg-1 protein) was in S Padmanabha roots stored under high temperature on 3 DAS. CAT activity decreased drastically during storage under all temperature regimes. Under room temperature storage, Sree Padmanabha recorded highest reduction (~70%) of CAT activity at 3 DAS compared to 1 DAS. Catalase activity was highest in roots stored at low temperature followed by ambient/room condition. Root showed lowest CAT activity when stored at high temperature.

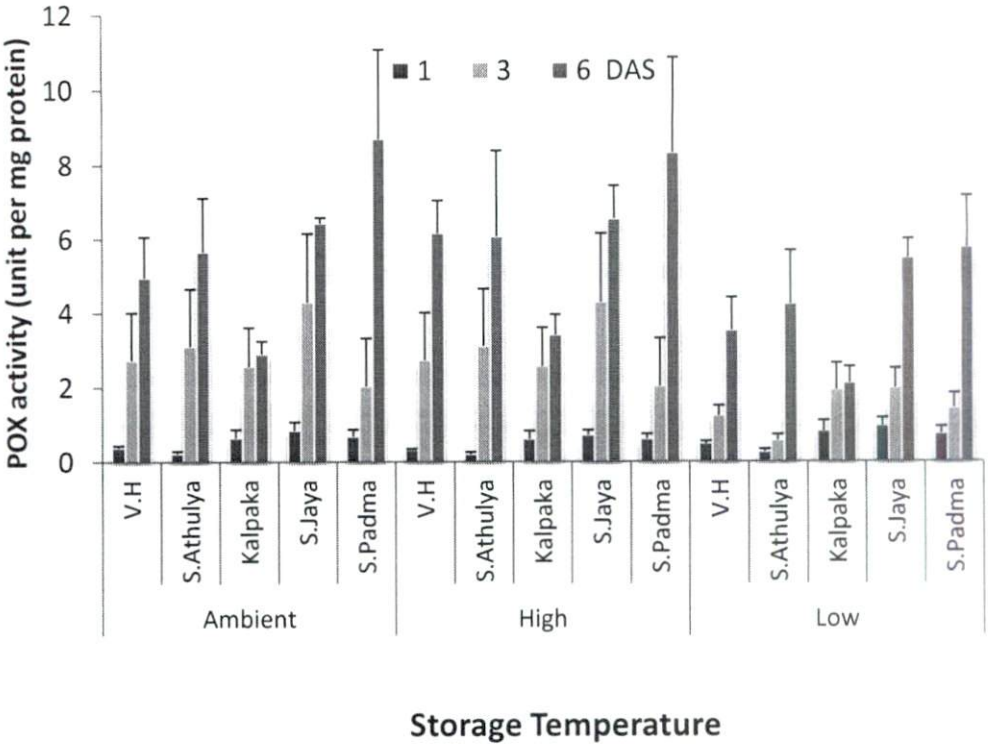



Figure 4.36. POX activity measured in the roots of cassava after 1, 3 and 6 days after storage in different temperature regimes.

(Error bars indicate Std error)

Peroxidase activity in root tissue of cassava showed a clear increasing trend from initial storage to later stages (Figure 4.36). The POX activity in cassava varieties significantly increased from 1 to 6 days after storage. Highest peroxidise activity was recorded in variety S Padmanabha at 6 days after storage with tenfold increase (8.8±1 unit per mg protein) at 6 DAS compared to POX activity at 1 DAS. The increase in POX activity was less prominent in Kalpaka compared to other varieties studied. Low temperature storage markedly affected the POX activity with lower increase compared to other storage temperature treatments.

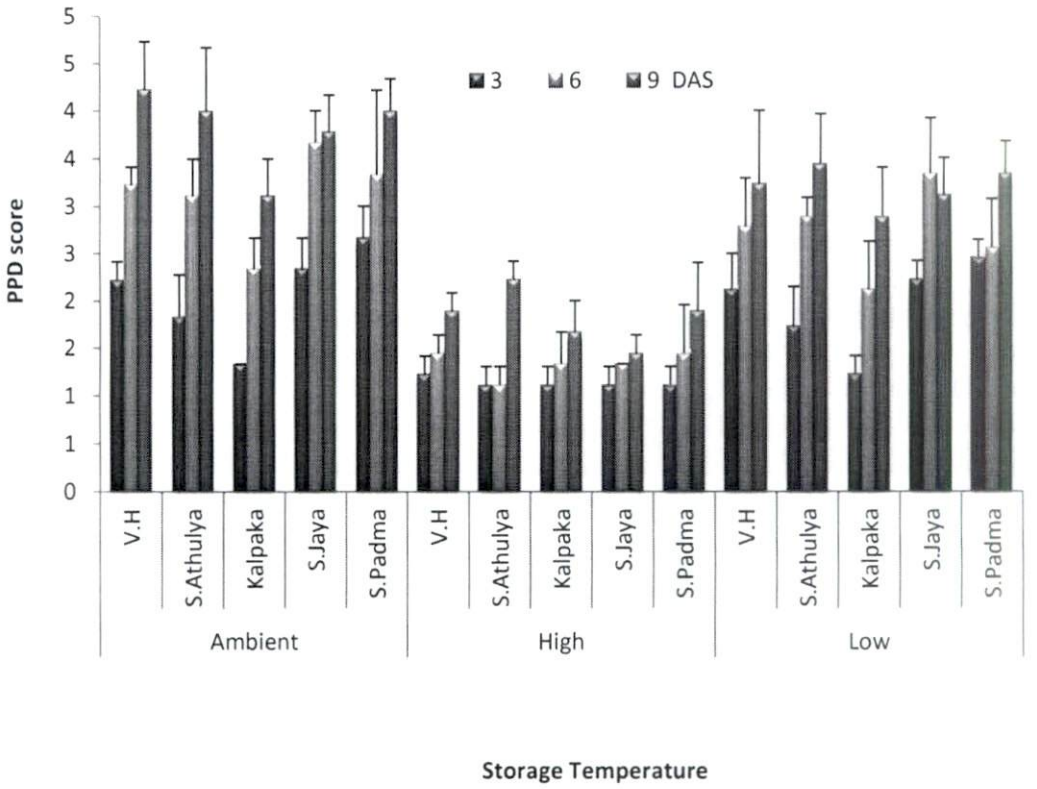



Figure 4.37. PPD scores of cassava roots after 3, 6 and 9 days after storage in different temperature regimes.

(Error bars indicate Std error)

PPD scores of roots under different storage temperatures significantly varied. Low PPD metablolic rate in low and high temperature storage resulted in lower PPD symptom development during storage (Figure 4.37). Significantly higher scores for all the varieties was under ambient storage, whereas, high temperature resulted in low streaking and root damage. Correlation between cassava root respiratory rate, enzyme activities like CAT and POX and PPD intensity were calculated for cassava roots stored at different length of time. The correlation values are presented in Table 4-5. There were significant positive between root respiratory flux at 3 and 9 days of storage to the CAT and POX activities studied in different cassava varieties. However, root respiration does not show correlation with the PPD symptom development during storage. There were significant positive correlation for activities of both CAT and POX enzymes and PPD at 6 and 9 DAS of storage.

Table 4-5. Pearson correlation co-efficient for cassava root respiratory flux, activity of oxidative enzymes and PPD intensity during storage

| Para-meter | CAT1 | CAT3 | CAT6 | POX1 | POX3 | POX6 | PPD-D3 | PPD-D6 | PPD-D9 | Res-D1 | Res-D3 | Res-D6 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| CAT1 | 1.00 | | | | | | | | | | | |
| CAT3 | 0.87* | 1.00 | | | | | | | | | | |
| CAT6 | 0.91* | 0.89* | 1.00 | | | | | | | | | |
| POX1 | 0.29* | 0.39* | 0.35* | 1.00 | | | | | | | | |
| POX3 | -0.24 | -0.25 | -0.33* | 0.23 | 1.00 | | | | | | | |
| POX6 | -0.55* | -0.59* | -0.59* | 0.10 | 0.32 | 1.00 | | | | | | |
| PPD-D3 | 0.34* | 0.17 | 0.39* | 0.20 | -0.15 | 0.14 | 1.00 | | | | | |
| PPD-D6 | 0.54* | 0.30* | 0.49* | 0.26* | -0.04 | 0.11 | 0.74* | 1.00 | | | | |
| PPD-D9 | 0.46* | 0.19 | 0.41* | 0.03 | -0.04 | -0.03 | 0.67* | 0.79* | 1.00 | | | |
| Res-D1 | -0.21 | -0.40* | -0.22 | 0.13 | 0.42* | 0.29 | 0.24 | 0.20 | 0.14 | 1.00 | | |
| Res-D3 | -0.16 | -0.41* | -0.22 | -0.16 | 0.37* | 0.07 | -0.00 | 0.08 | 0.21 | 0.73* | 1.00 | |
| Res-D6 | -0.16 | -0.36* | -0.23 | -0.16 | 0.33* | -0.01 | -0.03 | -0.01 | 0.16 | 0.71* | 0.91* | 1.00 |

## 4.7 Effect of different food preservatives on PPD of cassava roots

Root morphological parameters, root fresh weight, starch content at the initial as well as at the end of sampling, total phenol and PPD scores of the stored cassava are presented in Table 4-6. The roots were selected for uniformity in size and morphological characters to test the effect of food preservatives on their keeping quality and PPD incidence. Hence root morphological characters were not significantly different among them. Root fresh weight ranged from 927 g to 1213 g among the treatments. The mean fresh weight was 1098 g for the treatments used. Mean root length was 36.78 cm among the roots studied with a range starting from 27 cm to a maximum of 45 cm. Maximum root girth ranged from 13.6 to 21.2 cm among the treatment.

The starch content ranged from 76.4% to 84.1% in the roots used for the storage studies with an average starch content of 80.1% on dry weight basis at the beginning of the experiment. The starch content was not significantly different among the roots used for this study (Table 4-6). There was a notable decrease in storage at the end the sampling period with mean starch content decreased by 4.8%. Starch content decreased to 75.3% among the treatments. The starch content reduced invariably in all the treatments with no significant response to chemical treatments.

Total phenol content was measured in the roots after 6 days of storage. The phenol content of roots differed significantly among the treatments after the treatment with food preservatives. Lower phenol content was recorded for 1% PMB (9.9 mg g-1) which was at par with other treatments such as ascorbic acid (0.5 & 1%), Tocopherol (0.5 & 1%) and Whey protein (0.5 & 1%). Highest total phenol content was present in BHA (0.5%) and BHT (1%). Total phenol had a negative effect on PPD. PPD score recorded were lower for the treatments which increased the phenol content of roots under storage (Figure 4.38).

Table 4-6. Morphological and biochemical parameters and PPD scores during storage of cassava roots treated with food preservatives.

| Treatment | Root FW (g) | Root length (cm) | Root girth (cm) | Starch (%) Initial | Starch (%) Final | Total phenol ($mgg^{-1}DW$) | PPD score 3 DAS | PPD score 6DAS |
|---|---|---|---|---|---|---|---|---|
| **Ascorbic acid(0.5%)** | 976 | 34.8 | 21.8 | 80.5 | 76.67 | $12.8^{a}$ | 1.7 | $2.7^{b}$ |
| **Ascorbic acid (1%)** | 1026 | 34.5 | 22.9 | 79.0 | 72.49 | $11.2^{a}$ | 1.7 | $2.7^{b}$ |
| **Pot.MBS (0.5%)** | 1213 | 40.6 | 22.6 | 81.0 | 75.83 | $13.7^{a}$ | 1.7 | $3.3^{a}$ |
| **Pot.MBS (1%)** | 927 | 33.1 | 19.6 | 78.5 | 74.65 | $9.9^{a}$ | 1.7 | $2.7^{b}$ |
| **BHT (0.5%)** | 1138 | 40.5 | 22.1 | 79.1 | 74.37 | $29.0^{b}$ | 1.3 | $1.7^{c}$ |
| **BHT (1%)** | 992 | 35.0 | 23.0 | 81.1 | 76.80 | $36.5^{c}$ | 1.3 | $2.0^{c}$ |
| **BHA (0.5%)** | 1075 | 37.1 | 23.1 | 80.2 | 75.81 | $39.6^{c}$ | 1.0 | $2.0^{c}$ |
| **BHA (1%)** | 1168 | 39.5 | 23.8 | 81.4 | 77.61 | $36.0^{bc}$ | 1.3 | $2.0^{c}$ |
| **Tocopherol (0.5%)** | 1054 | 38.0 | 22.1 | 77.8 | 73.72 | $14.0^{a}$ | 1.7 | $3.3^{a}$ |
| **Tocopherol (1%)** | 1143 | 37.1 | 23.5 | 80.1 | 77.07 | $15.6^{a}$ | 1.3 | $3.3^{a}$ |
| **TBHQ (0.5%)** | 1226 | 41.0 | 24.0 | 81.6 | 76.67 | $14.0^{a}$ | 1.3 | $2.7^{b}$ |
| **TBHQ (1%)** | 1169 | 39.5 | 22.5 | 80.2 | 75.43 | $15.5^{a}$ | 1.7 | $3.3^{a}$ |
| **Whey Protein (0.5%)** | 1202 | 35.2 | 20.8 | 81.2 | 75.33 | $12.2^{a}$ | 1.3 | $2.7^{b}$ |
| **Whey Protein (1%)** | 1178 | 34.8 | 20.5 | 79.9 | 73.83 | $11.9^{a}$ | 1.7 | $2.7^{b}$ |
| **Control** | 978 | 30.5 | 18.4 | 79.7 | 73.4 | 11.3 | 1.7 | $3.3^{a}$ |

(* Supercript letters indicate significant differences between mean values at p=0.05)

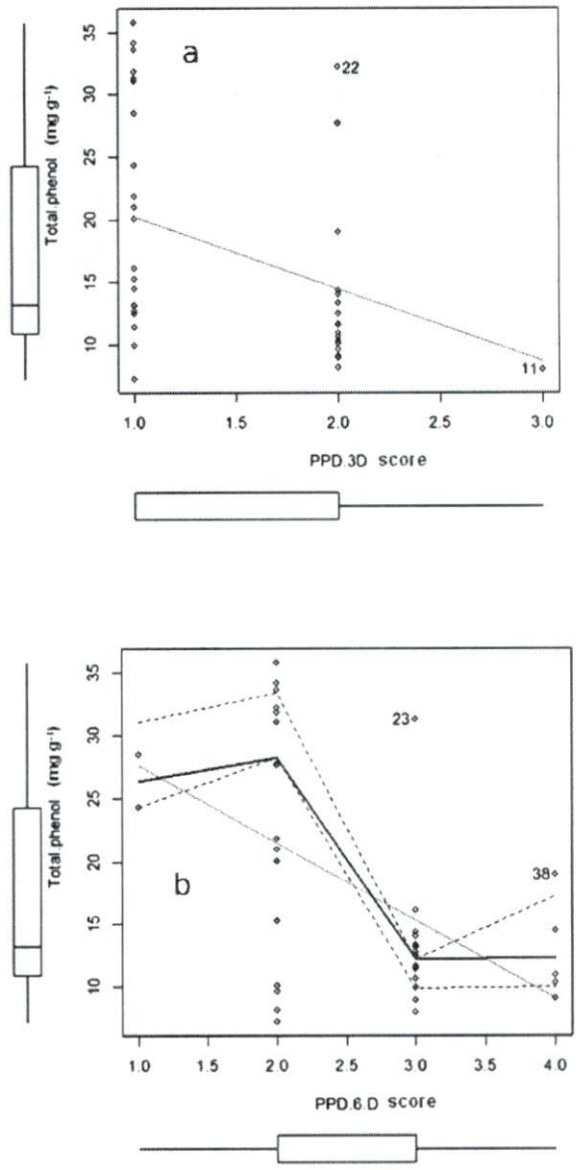



Figure 4.38. Relationship of total phenol content and PPD of cassava roots at 3 (a) and 6 days (b) after storage

Food preservative had a marginal influence on PPD incidence in the cassava roots. There was a marginal reduction in PPD symptoms in the tested preservatives such as BHT and BHA. The PPD score was lower (1.7) for BHT (0.5%) whereas, BHT at 1% and BHA at both 0.5 and 1% level produced similar PPD score of 2.0. Other treatments such as ascorbic acid, whey protein, TBHQ at

0.5% and PMBS at 1% produced similar visual symptoms and PPD score.

## 4.8 Effect of hormone application on cassava root PPD

Influence of wound related hormones such as jasmonic acid and salicylic acid on cassava PPD was studied with the application of 100, 200 and 300 ppm concentrations on wounded cassava roots. Cassava roots treated with different hormonal concentrations are presented in Figure 4-39. The roots were sampled at 0, 3 and 6 DAS to study the biochemical changes including the oxidative enzymes like CAT, POX and PAL activity.

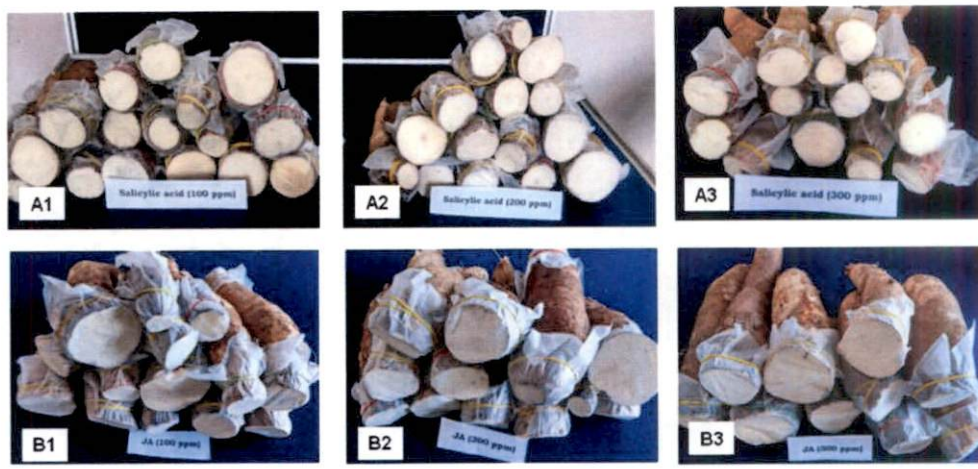



Figure 4.39. Cassava root treated with phytohormones salicylic acid (A1 to A3) and jasmonic acid (B1 to B3) at three different concentrations, 100, 200 and 300 ppm respectively.

Root characters of cassava used for studying the effect of wound hormones Jasmonic acid (JA) and Salicylic acid (SA) are presented in Figure 4-40. Length of cassava roots used for investigating ranged from 25.06 to 39.47 cm with a mean value of 32.56 cm. The average root length did not significantly differ among the different treatments. Root fresh weight ranged from 948 to 1794 g among the treatments and did not vary significantly. The average root fresh weight was 1357 g. Maximum girth of the roots ranged from 17.83 cm to 25.55 cm and average of maximum girth was 21.67 cm among the root used.

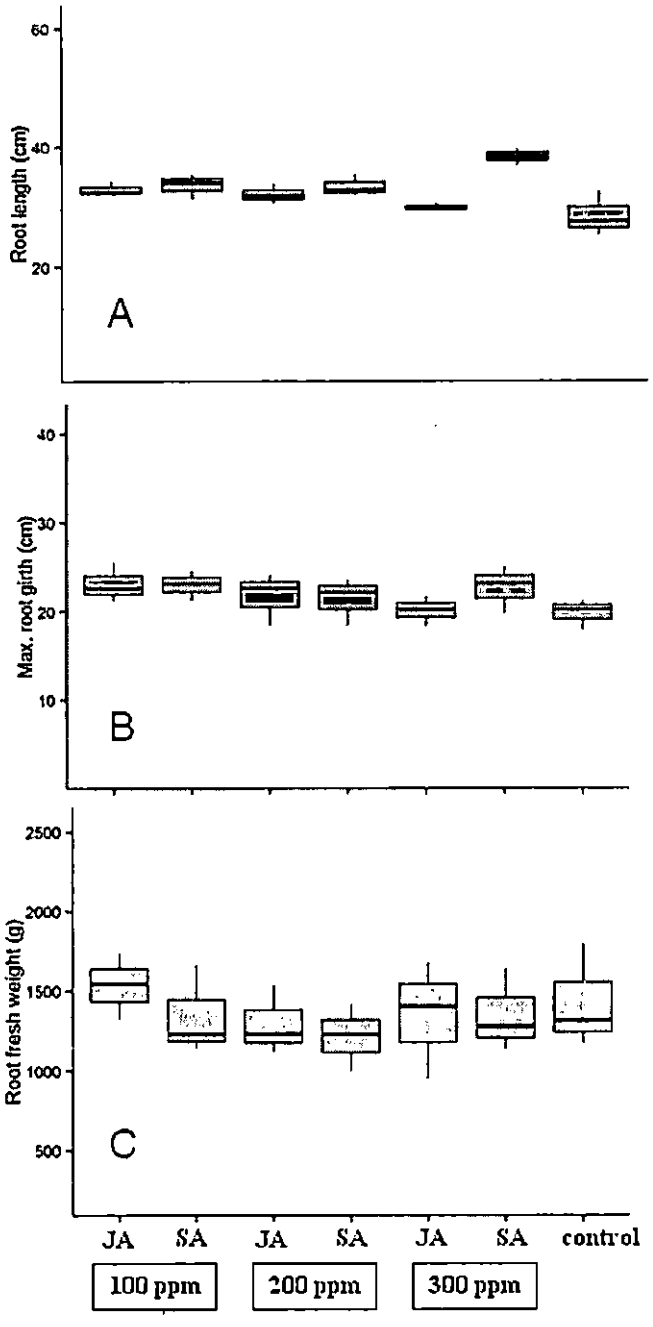



Figure 4.40. Root characters of cassava studied for the effect of different doses (100, 200 and 300 ppm) of jasmonic acid and salicylic acid on PPD. A) root length, B) max root girth and C) root fresh weight.

(Error bars indicate std err.)

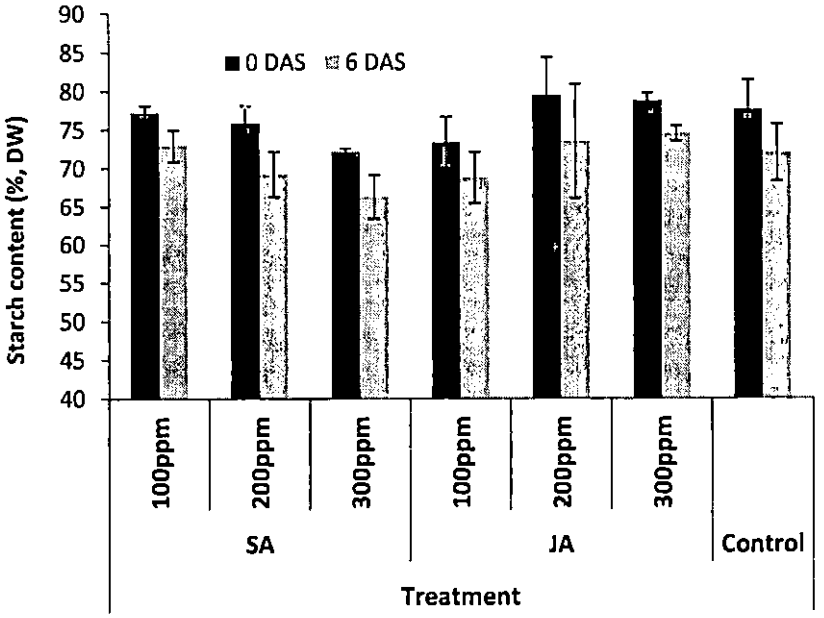



Figure 4.41. Starch content of cassava roots applied with 100, 200 and 300 ppm) of jasmonic acid and salicylic acid at 0 and 6 DAS.

(Error bars indicate std err.)

The PPD scoring was done at 3 DAS and 6 DAS in the hormone treated roots and the control. There was no significant difference observed for the PPD scores even and hence the data on PPD was not presented. However, there was biochemical changes in terms of oxidative enzymatic activity (CAT, POX and PAL) were observed in the roots with the application of hormones.

Starch content of the roots at the start of the experiment ranged from 70.21 to 83.33% among the roots samples and mean starch content was 76.35% (Figure 4.40). Tukey's test did not find significant differences in initial starch content. Starch content of roots decreased in all the treatments and application of JA and SA did not affect the starch loss in roots at all the tested concentrations of 100, 200 and 300 ppm at 6 DAS (Figure 4.40). The mean starch content at 6 DAS was 71.04 % with a reduction of 5.31% during the 6 day period.

Among the oxidative enzymes studied, CAT activity of roots decreased significantly from 0 to 6 DAS in control and in hormonal treatments (Figure 4.41). Highest CAT activity was found in SA-300 ppm treated roots 0, 3 and 6 DAS. Mean CAT activity was 53.27 units mg-1 protein in SA-300 ppm at 0 DAS. There

was a minor decrease CAT activity during storage in SA-300 ppm from initial storage. The rate was 51.96 and 44.15 units mg-1 protein in SA-300 ppm at 3 and 6 DAS respectively. Lowest enzyme activity was found among the treatment such as SA-100, SA-200 ppm, 300 ppm JA along with control at 3 DAS. In general, lowest CAT activity was recorded at 6 DAS. Minimum activity was recorded in control (30.86 units mg-1 protein) which was on par with 100 & 200 ppm SA and 300 ppm JA treatment.

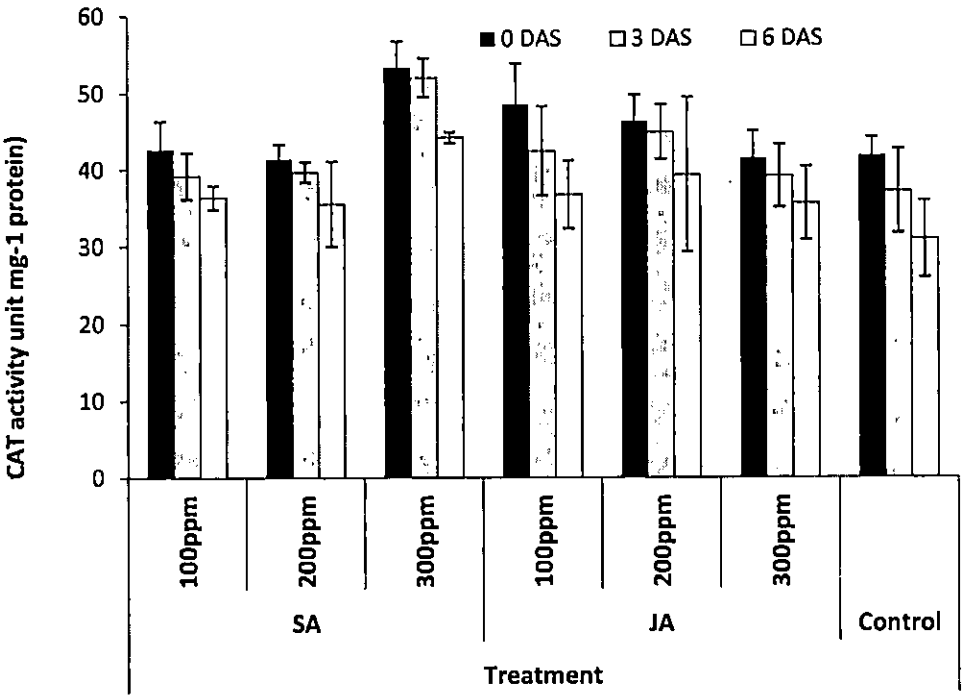



Figure 4.42. Effect of wound hormone SA and JA at 100, 200 and 300 ppm concentrations on CAT activity in cassava roots at 0, 3 and 6 days of storage.

(Error bars indicate std err.)

POX activity contrasted with the CAT activity during storage with minimum at initial days of storage to maximum at 6 DAS (Figure 4.42). POX activity was not significantly different in control as well as with hormonal treatments at 0 DAS. It increased steadily in all the treatments subsequently reaching maximum activity at 6 DAS. Hormonal treatments resulted in changes in

POX activity at 3 DAS itself with 200 & 300 ppm SA and 300 ppm JA recorded lower POX activity compared to control. Control roots POX activity (2.31 units mg-1 protein) was at par with 100 ppm SA and 100 ppm JA. POX activity increased drastically in control and reached highest activity of 10.22 at 6 DAS whereas, SA-200, 300ppm and JA-300ppm had the least POX during this period.

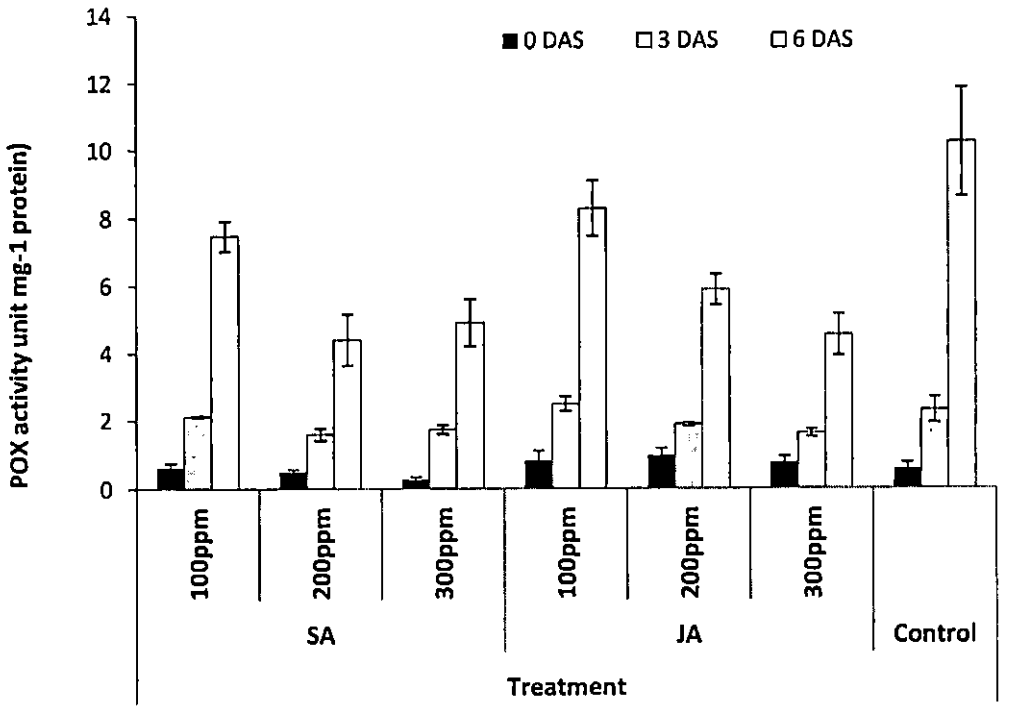



Figure 4.43. POX activity changes in cassava roots at 0, 3 and 6 days of storage with of wound hormone treatment SA and JA at 100, 200 and 300 ppm concentrations.

(Error bars indicate std err.)

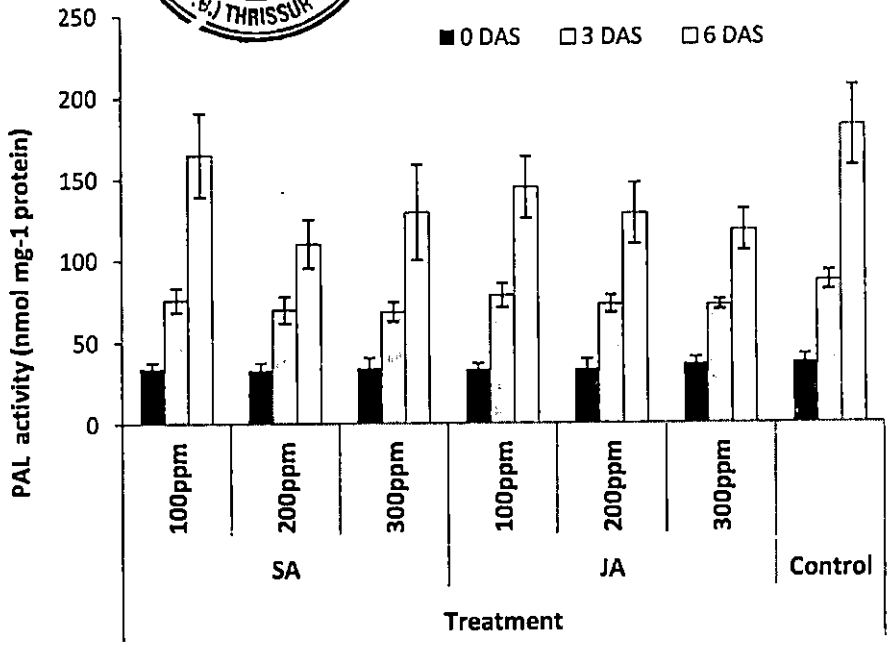



Figure 4.44. PAL activity changes in cassava roots at 0, 3 and 6 days of storage with of wound hormone treatment SA and JA at 100, 200 and 300 ppm concentrations

(Error bars indicate std err.)

The initial PAL activity was lowest in all the roots treated with hormones and in control among the sampling interval. From the initial low PAL activity, roots showed increased activity over the storage period in all the treatments as well in control (Figure 4.43). There was no significant difference in PAL among the treatments in O DAS, However, PAL activity was altered in the roots with hormone application at 3 and 6 DAS. At 3 DAS, PAL activity in control roots was at 87.19 n mol $mg^{-1}$ protein $min^{-1}$ and it was significantly higher compared to other treatments. Nearly five folds increase in PAL activity was noticed in roots at 6 DAS compared to 0 DAS in control as well as in 100 ppm SA treated roots. Lowest PAL activity was recorded in SA-200 ppm (110.0 ng mol $mg^{-1}$ protein) at 6 DAS followed by SA-200 ppm (118.28 n mol $mg^{-1}$ protein $min^{-1}$).

# Chapter 5

# Discussion

# 5 Discussion

The research project on biochemical and molecular studies on PPD of cassava comprised of experiments such as physiological screening of cassava genotypes for PPD performance, employing screening tools such as NIR spectroscopy and various storage treatments to increase the shelf life of fresh cassava roots. The discussions on the experimental results are presented in this section.

## 5.1 Genotypic variability for PPD response in cassava

Cassava roots undergo rapid deterioration, generally known as PPD after detached from the parent plant as a result of physiological and biochemical changes which lead to tissue damage. Once the PPD develops in the harvested roots, it quickly loses its integrity and rendered unpalatable. Additionally the roots develop foul smell due to subsequent microbial decay. The short shelf life of cassava roots is considered as one of the major limitations for its expansion in cultivation and marketing as well as processing (Wenham, 1995). Wounding caused during the harvest was reported to be the main reason for the PPD. The endogenous nature of cassava root primary deterioration was confirmed observations of Booth (1976). The initial deterioration later develops into secondary (microbial) deterioration by invasion of microbes and extensive tissue softening and discolouration (Wenham, 1995). There is a strong correlation between on-set of PPD and wounding caused during cutting roots from the plants during harvesting. Additional damages caused during handling of harvested roots, bruises created in the root surface and breaking of root tips are also increases the speed of PPD development. Booth (1976) found that primary deterioration was essentially a wound response being initiated near the region of mechanical damage; unlike in other storage organs (e.g. sweet potato), the response is not localised at the surface, but spreads down the root. Wounds and bruises also constitute points of entry for micro-organisms leading to the second stage of cassava root spoilage, known as "secondary deterioration". In general, the wounded portion of the roots are the first to show the typical PPD symptoms and the extent of damage caused to the roots vary due to endogenous and by external factors such as storage

temperature and humidity etc,. Root characters like root shape, root length, presence of root peducles, peel (periderm) characters, soil compaction and harvesting methods tend to influence the speed and intensity of PPD occurrence (Diamante, 1986; Wheatley, 1982). Genetic as well as environmental causes for variation in PPD response in cassava have already been reported (Buschmann *et al.*, 2000b; Iglesias *et al.*, 1996). When PPD severity was studied at three different locations in Colombia (Kawano and Rojanaridpiched, 1983) found significant interaction between location and season on PPD severity.

In the present study, it was found that cassava root morphological traits such as root length, root fresh weight and root girth did not correlate to the degree of PPD under storage. Correlation for root dry matter content and high starch content and the degree of PPD had been reported (Plumbley and Richard, 1991; Sánchez *et al.*, 2006). Wheatley and Gomez, (1985) who found no correlation between PPD and starch content of the roots, however, Van Oirschot *et al.*, (2000) found a negative correlation between PPD and sugar/starch ratio. Carotene content in the roots was reported to have negatively correlated with the level of PPD due to the antioxidant properties of the carotenoids which quench reactive oxygen species (ROS). Yellow fleshed roots of cassava having high carotene tended to have a delayed onset of PPD by 1 or 2 d (Sánchez *et al.*, 2006). High dry matter content enhances the PPD in the roots. Whereas pruning the plants a few days before harvest also delays PPD, but at the expense of a reduction in dry matter content of the root (Van Oirschot *et al.*, 2000).

β-carotene is a quencher of ROS (reactive oxygen species) produced during PPD and β-carotene has been reported to extend the storage life of cassava roots by up to 4 weeks (Sayre *et al.*, 2011). Gloria and Uritani (1984) reported that a low in β-carotene in the root tissues of cassava hastened the PPD. Chavez *et al.*, (2000) reported that carotenoid content in the root above 5 mg per kg fresh weight delayed the PPD symptom development. In the present study, carotene contents in roots of genotypes were below the threshold level and hence did not exert any positive effect on modulation of ROS and PPD. We found no correlation for root carotene content with PPD. However, carotenoids are implicated to delay or reduce postharvest deterioration in cassava roots (Morante *et al.*, 2010; Sánchez *et al.*,

2006). However, there was a weak negative correlation for root length and starch content with PPD in the genotypes studied. Van Oirschot *et al.*, (2000) reported negative correlation between PPD and sugar/starch ratio, in contrast with Wheatley and Gomez (1985), who found no correlation between PPD and starch content.

The cassava root is a storage organ for photosynthates and since it is not a propagule with reproductive function there is no biological need to repair wounds. Peaks of ROS as well as increased activity of antioxidative enzymes that modulate ROS have been detected during deterioration (Reilly *et al.*, 2001). Signal molecules synthesized or released during wound response trigger protective or defensive responses locally as well as systemically. These signals induce the production of defensive enzymes and secondary metabolites such as glucanases and chitinases, phytolaexins and antioxidants and help wound repair and sealing molecules (such as callose, lignin and suberin) (Buschmann *et al.*, 2000a).

Secondary metabolites (carotenoids, phenolics, flavonoids, and anthocyanins) are found in many species of the plant kingdom and are well recognized as potential antioxidants. Physiological deterioration of cassava roots resulted in altered enzyme activities, which generate phenols and leucoanthocyanins (Rickard, 1981). Accumulation of secondary metabolites like hydroxycoumarins e.g. scopoletin and its glucoside scopolin may help in quenching reactive oxygen species. Oxidation and polymerization of these coumarins may give rise to the blue/black discolouration that is typical of PPD. Under *in vitro* conditiions, scopoletin and hydrogen peroxide give a dark bluish colour in a peroxidase-mediated reaction (Edwards *et al.*, 1997).

Studies on inheritance of PPD with moderately and highly susceptible genotypes showed that PPD is under the control of quantitative and additive gene action with estimated heritability of 0.64 (Kawano and Rojanaridpiched, 1983). Further extending the genetic studies, Cortés *et al.* (2002) and Estevão (2007) identified three QTL regions in cassava genome which contributed for 5 and 13% respectively of the phenotypic variance. Among the genotypic difference for PPD, carotene content of roots clearly accounted for the tolerance for PPD and extends the shelf life for few days in yellow fleshed roots. Since PPD is largely accepted to be due to imbalance ROS production and quenching (Reilly *et al.*, 2003), carotene

may play an important role in quenching the reactive oxygen and help delay the PPD expression. It was proposed that a thresh hold level of 10.2 μg g−1 of carotene would be required for delaying the PPD symptoms in cassava roots.

## 5.2 Changes in biochemical constituents during PPD

There is a rapid accumulation of hydroxycoumarins like scopolin(6-methoxy-7-hydroxy-coumaroyl-7β-D-glucoside), scopoletin (6-methoxy-7-hydroxy-coumarin) and esculin (6, 7-dihydroxycoumaroyl-6-β-D-glucoside) in roots during the initial stages of PPD and phenolic compounds such as catechin and (+)-gallocatechin (Buschmann *et al.* 2000a). These metabolites were found to increase during the first 24 to 48 hours after wounding (Wheatley and Schwabe, 1985: Buschmann *et al.*,2000a) and present in low concentrations in intact roots or roots collected from pruned plants suggests that they have a role in PPD symptom development. It was also found that the concentration of phenolics modulated during the first few days. The first increase of scopoletin was followed by a decline and a second, comparatively smaller increase at 4 to 6 days after harvest. Pruning of plants few days prior to harvesting of roots was found to increase the shelf life of cassava and it was found that the roots deteriorated quickly with the exogenous application of scopoletin (Wheatley and Schwabe, 1985). It was proposed that the peroxidase-mediated oxidation of scopoletin resulted in the blue/black vascular streaking observed as PPD. Supporting the hypothesis, presence of all required components of the reaction, namely scopoletin, $H_2O_2$, and peroxidase enzyme activity were reported near the root xylem parenchyma vessels where vascular streaking symptoms occur (Reilly *et al.*, 2004). Recent experimental data appeared during last decade demonstrated that the cell-wall peroxidases could accomplish many other functions related to their superoxide-producing activity. The localization of cell wall peroxidases mostly in the out parenchyma of cassava roots and increased activity during storage indicated their role in PPD symptom development. Varietal differences could be more clearly understood by tissue printing and also for screening the varieties for important physiological studies involving oxidative enzymes and also for plant defense reactions. Increased $H_2O_2$ production in apoplatic region which is connected to vascular region may provide a

conduit for their action elsewhere in the root and hence spread of reactive oxygen for PPD in distal tissues. Genotypic difference for $H_2O_2$ production indicated that POX activity can contribute for ROS production during PPD and also as a quenching mechanism for reactive oxygen as there are several types of cell wall peroxidases with different specificity and mode of action. It will be interesting to understand whether the observed POX activity in tissue is due to de novo synthesis of POX enzymes or enhanced activity due to modulation of enzyme activity in cassava roots.

## 5.3 Wound response of cassava roots and PPD development

PPD is triggered in cassava roots after wounding caused by detaching the roots from the plant. The wound signal pathway and defensive responses takes place locally as well as systematically through the entire root. Multiple signaling pathways regulate the wound response in plants (Onkokesung *et al*, 2010). The wound signal transduction is studied in detail in other plant species and is still not completely understood in cassava. Signal transduction involve wound induced cell wall degradation products (oligosaccharides), lipid constituents from cell membrane (especially phosphatidic acid, PA), reactive oxygen species (ROS), electrical signals, hydraulic signals, and various plant growth regulators such as jasmonic acid, salicylic acid, ethylene, systemin etc., *(Heil and Ton, 2008; León et al.*, 2001; Zimmermann *et al.*, 2009). Various reactive oxygen scavenging systems operate in plants. Enzymes like peroxidase, catalase, superoxide dismutase, glutathione reductase etc., are differentially activated during wound response. Primary defense through enhanced SOD activity during wounding was reported to protect membrane lipids from degradation and reduction in CAT activity was also noted. Increased $H_2O_2$ production in later stages of PPD may directly act on membrane lipids and increase the peroxidation leading to membrane leakage.

## 5.4 Screening tools and PPD quantification

A systematic and objective method of evaluation of PPD and its quantification are important to screen the germplasm and breeding lines. Most of the available techniques are subjective in nature such as subjective visual scales

using entire roots. Methods such as scoring visual symptoms or fluorescent patches under UV light and with appropriate scores for the extent of discoloration during PPD (Uritani *et al.*, 1983) are recommended. Scoring transverse sections of the root by visual inspection of the ring pattern of zone B formed in the root pith during PPD was employed by Wheatley, 1982 and commonly followed for PPD studies. An objective quantitative and systematic evaluation which is quick and easy is the need of the hour (Han *et al.*, 2001; Estevao, 2007). Measuring of UV fluorescent compounds known as hydroxycoumarins as biochemical marker to assess the PPD susceptibility had been proposed by Buschmann *et al.*, (2000a) and Van Oirschot *et al,*. (2000) even though studies indicated that there were no clear correlation with PPD susceptibility. Salcedo *et al.*. (2010) found similar results using field grown roots of 26 different varieties, where the accumulation of hydroxycoumarin measured as fluorescence emitted in transversal root sections did not correlate with basic visual symptoms of PPD in cassava roots. Alternatively measurement of the sugar/starch ratio as described by Van Oirschot *et al.*, (2000) who found a strong correlation between them can be useful to PPD scoring.

NIR data showed that there was an increase in absorbance which peaked at 6100 $cm^{-1}$ initially and a sharp decline afterwards. The steep decline bottomed out at 4500 $cm^{-1}$. There was a large peak at 4000 $cm^{-1}$. Cassava root slices showed strong absorbance at 5,180 $cm^{-1}$ and a shoulder around 4716 $cm^{-1}$ which can be attributed to the C–H and C=O stretching vibrations of -HC=CH-. The varying spectral regions indicated that these regions may be associated with important biochemical constituents which are suitable for cassava screening by PCA. Metabolomic approaches are advantages and help parallel assessment of the levels of a broad range of metabolites and offer great value in both phenol-typing and diagnostic analyses in plants (Fernie and Shauer, 2008). A combination of metabolomic techniques with chemometric tools provides a quick and reliable method for chemotaxonomy characterization (Schultz and Baranska, 2007). Uarrota *et al.* (2014) applied metabolomic-chemometrics approach for screening cassava for PPD and distinguished the fresh samples and those with 3, 5, 8 and 11 days of PPD through principal component analysis (PCA), partial least square discriminant analysis and other modeling techniques. They found a positive

correlation for flavonoids and anthocyanins with PPD (y = 0.003x + 1.09, r2 = 0.38 for flavonoids and y = 0.1x + 3.4, r2 = 0.27 for anthocyanins), while the total phenolic and carotenoid amounts negatively correlated to PPD (y = _0.01x + 8.01, r2 = 0.53 for total phenolics and y = _0.28x + 6.96, r2 = 0.12 for carotenoids. It was possible to rapidly screen cassava during PPD and successfully achieved the required classification by combining the visual inspection of cassava roots to PPD, their biochemical profiles and ATR-FTIR spectral data set via chemometric tools.

## 5.5 Gene expression analysis during PPD and signaling during PPD

A large number of genes controlling various biochemical pathways are involved in the PPD. Genes related to various components of signal transduction network, defense molecules, enzymes, phytohormones, ROS modulators, components involved in production and alteration of cell wall, programmed cell death etc,. were found to be modulated during PPD. Using microarrays and a cDNA library of cassava varieties with early and late PPD, Reilly *et al.* (2007) detected 72 genes with significantly altered expression during PPD. Among them, a subset of 21 storage-root-wounding-specific transcripts, which potentially are PPD specific, was identified by a comparison between the expression in harvested storage roots and unwounded and wounded leaves. Earlier, Huang *et al.*, (2001) separated 70 transcript derived fragments (TDFs) related with the regulation of gene expression during PPD. They were catalogued as metabolism (24%), Wounding and stress (22%), signal transduction (12%), development (8%) and programmed cell death (6%). Wound related genes which are expressed during PPD have been studied for their expression characteristics and reported earlier. Transcription of ACC oxidase (involved in ethylene biosynthesis), phenylalanine ammonia-lyase (PAL- key enzyme of the phenyl propanoid metabolism pathway), catalase (Reilly *et al.*, 2002) and SOD (Lee *et al.*, 2007) were studied in cassava and in other plants.

In our study, it was found that expression of PAL and POX genes were differentially regulated during PPD in the cassava varieties studied. The expression of PAL is increased during storage during 1 and 3 days after harvest in Kalpaka whereas it did not vary in IH5/15 which is susceptible to PPD. The POX

expression was also modulated differentially with initial reduction in Kalpaka and subsequently increased by 14% over 0 day level. It increased marginally (up by 13%) in the initial stage of PPD and decreased and reached to 70% of 0 day expression in susceptible IH5/15. Development of PPD is evidently a complex phenomenon involving multiple cellular components. Wheatley and Schwabe (1985) proposed that vascular streaking observed as PPD may result from peroxidase-mediated oxidation of scopoletin. It a convincing hypothesis as the required components of the reaction, namely high level of scopoletin, $H_2O_2$, and peroxidase activities are initially present at the vicinity of the xylem vessels where vascular streaking symptoms occur. Also peroxidase isoforms with activity towards scopoletin are reported in the cassava storage root. Scopoletin is produced via the phenylpropanoid pathway and PAL represents the key entry point into phenylpropanoid metabolism. Wound-induced oxidative burst in injured cassava storage roots, the accumulation of secondary metabolites with antioxidant properties, and the altered regulation of genes related to the modulation of reactive oxygen stress. The up-regulation of genes related to defence and wound healing such as PAL, $\beta$-glucanase and hydroxyproline-rich glycoproteins (HRGPs), and of proteases, protease inhibitors and other genes that have been implicated in senescence or programmed cell death responses in other plant systems, have been reported (Han *et al.*, 2001; Huang *et al.*, 2001; Reilly *et al.*, 2003). Increased activities of catalase and peroxidase isoforms, including the root peroxidase transcript MecPX1, were up-regulated during the post-harvest storage period. Differences in catalase levels at both the enzyme and the transcriptional level have been reported (Reilly *et al.*, 2001), with levels of MecCAT1 transcript and overall catalase activity being more pronounced in less susceptible cultivars. Enzyme assay and tissue printing studies have indicated that overall peroxidase activity is lower in roots of less susceptible cassava cultivars (Campos and Decarvalho, 1990; Reilly, 2001). In non-pruned plants or more susceptible cultivars, where catalase levels are relatively lower and peroxidase levels relatively higher, a significant proportion of $H_2O_2$ scavenging could occur via peroxidase mediated reactions requiring the participation of cellular components, including scopoletin, as an electron. Thus, relatively higher levels of peroxidase would lead to increased

oxidation of scopoletin, observed as increased vascular streaking. Of the defense related genes, PAL shows upregulation within 24 h after injury in cassava storage roots.

Rapid generation of reactive oxygen species (ROS) is considered to be an important component of the resistance response of plants to pathogen challenge and even in wounding caused by herbivory. ROS can serve as toxic candidate for pathogen and also for the cross linking of cell wall to protect the host cell (Baker and Orlandi, 1995).The wounding response is thought to progress through the release of systemin (an 18–amino acid wound signal) in the wounded leaf, subsequent activation of early-response signal relay genes, such as polygalacturonase, allene oxide synthase, and lipoxygenase, and synthesis of the long-distance signal jasmonic acid (JA). The wound-induced increase in $H_2O_2$ levels is JA dependent and diphenylene iodonium sensitive, suggesting that a NADPH-like oxidase activity is required for the activation of wound/systemin responsive genes (Orozco-Cardenas and Ryan, 1999; Orozco-Cardenas *et al.*, 2001). Potential sources of ROS include NADPH oxidase, cell wall peroxidase, other flavin-containing oxidases, and oxalate oxidase (Neill *et al.*, 2002). Thus, the exact source of wound-induced ROS remains unknown.

Sagi *et al.*, (2004) showed a requirement of RBOHs (respiratory burst oxidase homologs) for expression of certain wound response genes in tomato, whereas other wound-responsive genes were regulated in a RBOH-independent manner. In plants, prominent transcripts induced within the wounded tissue were pathogenesis-related (PR) proteins, EREBP and WRKY transcription factors, enzymes for phenylpropanoid metabolism, and components of the JA and ethylene pathways. As the key entry point into phenylpropanoid metabolism, altered PAL expression could affect diverse pathways including the synthesis of wound healing components such as lignin and suberin, the synthesis of wound- or stress-related secondary metabolites including scopoletin, and signalling compounds such as salicylic acid.

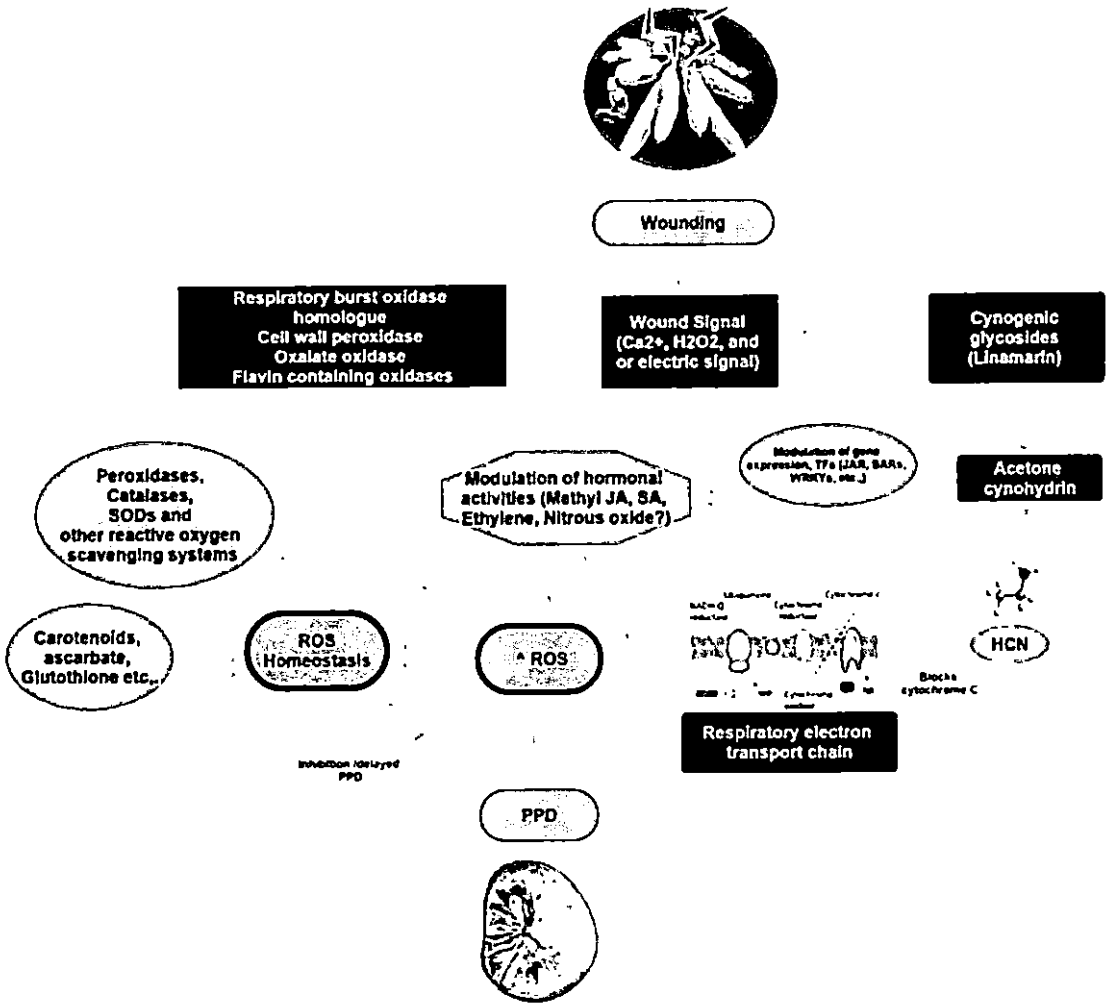



Figure 5.1. Relationship between reactive oxygen species (ROS) production and PPD in cassava roots.

Wound healing in cassava roots after harvest is slower compared to other tubers like potato or sweetpotato. Under normal storage conditions wound healing is not adequate to repair the harvested root and thus PPD sets in. The HRGPs are important class of proteins involved in wound healing and are expressed during PPD but do not show up-regulation until three days after harvest by which time PPD is well set in (Han *et al.*, 2001). Cassava roots with low HRGPs might be a factor which drives the wound response away from sealing of wounds towards continued defense responses causing the PPD symptoms. Gene expression studies during PPD had indicated that around 6% of genes expressed during PPD can be

classified as PCD (programmed cell death)-associated genes (Beeching, 2001; Huang *et al.*, 2001). In plants, PCD and Hypersensitive response towards pathogen infection show overlapping processes. However, no studies have been carried out to determine if such cell death reflects necrotic cell death or a form of PCD during PPD. HR and PCD have many common features. Both processes are active ones and can be inhibited by cycloheximide, increases in ethylene production have been noted before the onset of HR cell death, as have the accumulation of fluorescent coumarin components including scopoletin, up-regulation of PAL and β-glucanases, carotenoid degradation, membrane breakdown and increases in peroxidase and catalase activity (Pontier *et al.*, 1999). With respect to signals leading to the onset of PPD to date only two potential signal components that could lead to the modulation of PPD responses have been identified. Firstly, the plant hormone ethylene was detected within 6 h of injury of cassava storage roots, and lower ethylene levels were detected in less susceptible roots, although pre-harvest pruning did not prevent ethylene synthesis (Hirose, 1986), leading to the speculation that ethylene may play a role in signal transduction during PPD. Secondly, wound-induced oxidative burst measured as both superoxide anion and $H_2O_2$ accumulation. The $H_2O_2$ produced via the oxidative burst could potentially act to modulate PPD-related gene expression in the cassava storage root, as well as participating in a range of oxidative processes ranging from peroxidase-mediated oxidation of cellular components to cell wall cross-linking of HRGPs as part of the healing or curing response. Treatments known to inhibit PPD could act to impede the PPD progression pathway in various ways. One formed the superoxide anion ($O^{-2}$ ) could lead directly to cell membrane damage via the iron catalysed Haber-Weiss reaction. Spontaneous or SOD-mediated dismutation of $O^{-2}$ to $H_2O_2$ could lead to induction of PPD-related gene expression and to oxidation of cellular components including peroxidase-mediated oxidation of scopoletin. Exclusion of molecular oxygen (for example modified atmospheres, wax dipping) would act to prevent entry of molecular oxygen, thereby preventing its conversion into $O^{-2}$. Under such conditions of high humidity one would expect a higher proportion of intracellular spaces to be filled with water, thus effectively excluding oxygen. High-temperature treatments (such as dipping roots in hot water at 60 °C for 45 min) and

low temperature storage (Averre, 1967; Booth, 1976) also inhibit PPD and would be expected to impair enzyme activity perhaps preventing vascular streaking via inhibition of peroxidase activity and/or by inhibiting enzymes responsible for the conversion of molecular oxygen to $O^{-2}$. Several classes of plant peroxidases have been reported to be present in cytoplasm, cell organelle and in cell wall etc., The physiological role of plant POX has not been fully elucidated, but the enzyme is likely to function in the metabolism of phyto-growth hormones, lignification, crosslinking of cell wall polymers, and resistance to infection or wounding. The tissue printing and POX during PPD in our study clearly showed that POX is an important enzyme during the PPD of cassava roots. The positive correlation of POX and PPD score of root tissue is an important proof of their relationship. The exact nature of signal triggering the PPD is yet to be elucidated. Similarly, cycloheximide treatment would act to prevent synthesis of enzymes required for the development of PPD. The nature of the 'protectant' signal resulting from pre-harvest pruning is as yet unknown. Increased lignification, changes in starch and sugar composition, and reduced activities of PAL, acid invertase, and peroxidases, have previously been reported in roots from pruned plants (Data *et al.*, 1982; Kato *et al.*, 2000). Similarly, studies in cassava showed that pre-harvest pruning could result in changes in gene expression that may serve to protect the storage roots from subsequent injury. It is clear that PPD is a complex phenomenon involving both genetic and environmental factors. With respect to the considerable environmental induced variation in PPD it is tempting to speculate that such variation may reflect something as simple as the micronutrient status of the plant. For example, levels of available iron could affect progression of the Haber-Weiss reaction; similarly both the secretory peroxidases are haem-containing enzymes whose activity may be affected by relative levels of iron available within the plant.

## 5.6 Role of phytohormones in wound response and PPD

In plants, salicylic acid, jasmonates and ethylene were reported to play major role in wound response. SA works mainly antagonistically to jasmonates, ethylene can have either synergistic or antagonistic effects on certain subsets of genes. Jasmonic acid (JA) and its cyclic precursors and derivatives, collectively

referred to as jasmonates (JAs), constitute a family of bioactive oxylipins that regulate plant responses to environmental and developmental cues (Browse, 2009; Zhang and Turner, 2008; Wasternack, 2007). These signaling molecules affect a variety of plant processes including response to wounding (Zhang and Turner, 2008) and abiotic stresses, and defense against insects (Howe and Jander, 2008) There is evidence that the jasmonates 12-oxo-phytodienoic acid (OPDA), JA, and methyl-jasmonic acid (MeJA) act as active signaling molecules (Wasternack, 2007). SA plays a major role plant defense signaling especially in disease resistance (Vlot *et al.* 2009). SA is a phenolic compound that can be synthesized from the primary metabolite chorismate via two distinct enzymatic pathways, one involving phenyl alanine ammonia lyase (PAL) and the other isochorismate synthase (ICS) (Garcion and Metraux, 2006).

## 5.7 Storage conditions and physiological changes during PPD

The physiological and biochemical changes in detached cassava root lead to PPD. It is evident from the fact that cassava root indeed heal after wounding if the roots remain attached to the plants (Plumbley and Richard, 1991; Reilly *et al.*, 2004). However, the roots lose their down regulation of wound induced changes and subsequent repairing mechanism once they are detached from the plant. Unlike other tuber crops, cassava storage root lacks the active apical or intercalary meristem and hence wound healing and repairing of wounded tissue does not take place. The physiological processes during PPD are controlled by various enzymes and inhibition of those enzymes can alter the PPD progress.

In different method of storage employed, dry matter content of VH, Kalpaka and Sree Padmanabha were at par with each other. Interestingly, starch content in the roots of different varieties was not significantly different. But the total sugar content varied significantly. Highest sugar to starch ratio was recorded for S Padmanabha with a ratio of 109.1 whereas, lower ratio was recorded for Kalpaka (61.0) and S Jaya (62.1) indicating the varietal differences for starch breakdown during storage. The free sugar content might to some extent provide some protective role against PPD. Root respiratory flux was higher in roots stored at ambient conditions compared to high or low temperature storage. Among the

varieties studied, VH and Sree Jaya showed higher respiratory activity of 24.88 and 21.68 μmol $kg^{-1}$ per 15s respectively, under ambient/room temperature storage at 6 DAS. Sree Athulya was consistently having low respiratory flux under all three temperature storage. The increase in root respiratory rate during storage indicated the active cellular processes during storage. The flux of carbon skeletons required for various metabolic processes inside the cell are met from starch hydrolysis and changes in the enzymatic activities like POX and PAL corroborate the findings. Altered membrane peroxidation might be causing the increased permeability and leakage of lytic enzymes from cellular compartments. Thus increases the cellular damage during storage.

Modifying the storage conditions may help to alter the biochemical changes that happen during storage. Method such as clamp storage of cassava was reported by Richard and Coursey (1981) alter the storage physiology of cassava roots. In this system, a conical pile of 300-500 kg of fresh cassava roots is seated on a circular bed of straw and covered with more straw. However, clamp storage performs less well during the hot season. The temperature inside the clamp easily reaches 40°C, and heavy losses result even after 1 month of storage (Booth and Coursey, 1974). Cassava roots were packed in boxes containing adsorbent material such as sawdust (Rickard and Coursey, 1981) and resulted low PPD. The relative humidity inside the box was found to be critical for a successful storage. When the humidity was too high, deterioration due to bacteria and fungi rapidly caused damage to roots. If the humidity was too low, vascular deterioration was not prevented. Packing cassava roots in polyethylene bags was successful in preserving the roots for about 2 months (Ravi *et al.*, 1996). However, complete loss of the stored roots occurred as a result of microbial deterioration. Treating the roots with fungicides retarded the onset of spoilage (Rickard and Coursey, 1981).

Freshly harvested roots are treated with fungicides before the bags are closed to avoid the formation of mould and rot. When the roots which are packed airtight, breathe the oxygen content in the bags is reduced creating a preserving effect (Rickard and Coursey, 1981). High temperatures (above 40°C) as well as low temperatures (below 10°C) both have a positive effect on the duration of storage. Cold storage of cassava roots can also prevent and delay PPD. When kept

below 4°C, cassava roots do not show internal discoloration (Rickard and Coursey, 1981). Reduced temperatures extend the storage ability of cassava roots by delaying the rot processes which occur rapidly at normal storage temperatures. Experiments have shown that the most favourable temperature for the storage of fresh cassava roots is 3°C. Stored at this temperature, the total loss after 14 days amounted to 14% and after 4 weeks, 23% (Rickard and Coursey, 1981). But microbial damages can happen during storage if appropriate temperature regime is not maintained during storage. A bluish mould occurs on the surface of the roots at higher storage temperatures and the flesh of the roots turns brownish. Cassava roots could be kept satisfactorily under deep-freeze conditions but that changes in texture occurred in stored samples. Deep freezing of cassava has received little acceptance primarily due to its high-cost for a low-cost commodity such as cassava. In Columbia fresh cassava roots were simply dipped in paraffin at a temperature of 90° - 95°C. Without any fungicide being used, the storage duration could be extended to 1 - 2 months. Storage duration could be extended to about 10 days combining wax coating with suitable fungicide treatment (Rickard and Coursey, 1981). In our experiment, wax coating was preserving the roots for longer periods with lesser damage compared to poly-bag and storage under the soil. Prevention of oxygen entry and low moisture loss might have resulted in low metabolic rate and consequently low PPD development. Prevention of oxygen without microbial infection is the key to maintain the freshness of the root tissue. If initial microbial growth is not inhibited, the decay due to fermentation is more likely result in damage to roots.

Alteration of physiological and biochemical processes involving wound healing and periderm formation are implicated for the shelf-life extension in storage. Varying level of respiratory rate of detached roots of cassava under storage was reported. Previous reports by Hirose (1986) indicated that roots injured by removal of cortex or periderm have higher rates of respiration rates than intact roots after harvest. Injured roots showed highest rates on the first day of storage while respiratory rates of the intact roots and roots with minimal periderm removed reached their highest rates on the second day. In injured roots the respiratory rate decreased gradually until the fourth day when the rates began to increase again,

reaching their maximum approximately on the fifth day and rapid development of PPD on day 5 -6 coincide with the occurrence of the second respiration rate peak. Eight days after harvest the respiratory rate decreased to levels similar to time of harvest. Pruning of the cassava plant a few weeks prior to harvest is one of the most effective and low-cost measures to reduce PPD although the cellular mechanism involved is still unclear (Hirose *et al.*,1984b). Hirose *et al.* (1984a) found that, one day after harvest, intact roots from pruned plants showed a lower respiration rate compared with unpruned plants.

In conclusion, the study found significant genetic variations for PPD response among the 61 cassava genotypes studied. The classification of genotypes based on PPD carried out by physiological and biochemical analysis of root tissue resulted in identification of promising genotypes for low damage during the storage. Bio-chemical, physiological and molecular studies were carried out understand the PPD process in harvested roots. Peroxidase, catalase and phenyl alanine ammonia lyase are differentially regulated in the roots. Among the screening tools tested, FT-NIR yielded promising results and can be further tested for its suitability and ease and robustness as screening tool for PPD studies. The storage studies of selected varieties of cassava for improving the shelf-life and reduce the PPD incidence during storage were done with different storage temperature, oxygen exclusion during storage, hormonal application and preservative chemicals. The results were encouraging in terms of their suitability to prolong the storage of cassava with minimal PPD incidence for a period of two to three weeks. High temperature storage at 40° C for a week helped to reduce the onset of PPD, whereas coating of cassava PPD symptoms for few weeks. Wax coating of cassava is suitable for small scale marketing and is promising method for post harvest management of cassava roots. Future research may provide new insights into the causes of PPD in harvested cassava roots, biochemical and molecular mechanisms which control the PPD processes, new germplasm material for improving cassava for longer post harvest shelf-life.

# Chapter 6

# Summary

# 6 Summary

The research work on "Biochemical and molecular studies on post-harvest physiological deterioration of cassava (*Manihot esculenta* Crantz)" was aimed to understand physiological biochemical and molecular mechanisms associated with post harvest physiological deterioration (PPD) and to develop methods to prolong the storability of cassava storage roots. Cassava roots are an important source of daily diet to millions of population living in developing countries in African and Latin America and also an important industrial crop in the Asia Pacific region. PPD is a major post harvest problem and cause 15-25% loss to fresh cassava roots.

The study comprised of three parts i.e., i) screening of cassava genotypes ii) Study the feasibility of molecular, biochemical and visual markers for PPD response of storage roots and iii) Management of PPD through manipulation of storage conditions and application of hormones/chemicals.

The major findings of the study are given below,

- Significant differences were observed for PPD development and shelf-life of harvested roots. Cassava genotypes such as IMS2-8, 9S-172, 11S-53, IRS 2-10 and 9S-286 started showing the visible discolouration in the parenchyma tissue earlier (less than 3 days of storage). Genotypes such as 9S-7, 9S-98, 11S-31, 11S-86, 11S-14, CE63-3, CI43-2, CR43-2, CR54-A5, CR59-8R, Sree Athulya and Kalpaka showed low PPD scores and high storability.
- Tissue imprinting for peroxidase enzyme showed that there was a remarkable increase in peroxidase activity in the root tissues with increasing PPD symptoms.
- There was no correlation between the root morphological traits and PPD severity. Carotene and starch content of root did not influence the PPD in the genotypes studied. Chemotypic profile of roots with PPD symptoms was used to classify the genotypes based on PPD.

Near Infrared Spectroscopy (NIRS) was applied to characterize the cassava roots with varying PPD symptom levels. The NIR spectral features were

analysed using PCA and chemometric tools to differentiate PPD category in root tissue. The results showed that spectral features of fresh and deteriorated root tissue differed in the NIR regions of wave numbers from 5300, 5200-5100, 4600-4400, 4240-4150 $cm^{-1}$ in the first principal component, wave such as 5450, 5250, 4700 and 4400 $cm^{-1}$ for the second principal component. The third component belonged to 4800 $cm^{-1}$. Further studies in these regions will help identify the biochemical basis of PPD and also to quantify the PPD symptoms.

- Various stains such as saffranin, aniline blue, erythrocin, fast green and phloroglucinol stained the tissue specifically at vascular tissues and other cell components and were not suitable for detecting PPD.
- Storage techniques such as storing the harvested roots in de-aerated bags, wax coating and burying the roots under the soil were employed with selected cassava varieties like Sree Athulya, Sree Jaya, Vellayani Hraswa, Kalpaka and Sree Padmanabha to delay PPD.
- Wax coating was suitable to reduce PPD and increase the shelf life of roots for two weeks.
- Effect of different storage temperature on PPD was studied for five cassava varieties. Root respiratory flux was higher in roots stored at ambient conditions compared to high (40° C) or low temperature (8° C) storage.
- There were significant positive correlation between root respiratory flux at 3 and 9 days of storage to the CAT and POX activities studied in different cassava varieties.
- Roots were treated with various food preservatives at two different concentrations (0.5 and 1%). There was a weak, but significant reduction in symptom development in Butylated hydroxy touline – (BHT, at 0.5 and 1% level) treated roots compared to other treatments.
- Nearly three folds increase in total phenol content was noticed in BHT and Butylated hydroxyl aniline (BHA) treated roots.
- Salicylic acid and jasmonic acid did not show marked influence on PPD symptom development in cassava roots at 100, 200 and 300 ppm concentrations.
- The low PPD type genotypes such as 9S-7, 9S-98, 11S-31, 11S-86, 11S-14, CE63-3, CI43-2, CR43-2, CR54-A5, Sree Athulya and Kalpaka can be utilized

for breeding programmes.

- High temperature storage of cassava at 40 °C resulted in reduced respiratory rate and decreased the CAT activity. Whereas, POX increased in certain varieties thus helping the antioxidant system balance.
- Differentiation of cassava roots at the metabolites level corresponding to visual symptoms and chemotypic profile of PPD and NIR spectroscopy offer a rapid screening tool.
- Food preservatives like BHT and BHA have a significant, albeit marginal influence on PPD symptom development in cassava among the different storage treatments, wax coating with antiseptic pre-treatment is most suitable and economical for increasing shelf-life of roots.

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# Appendices

# Appendices

## Appendix-1. List of cassava genotypes screened for PPD

| | |
|---|---|
| 11s-11 | CR21-10 |
| 11s-14 | CR24/4 |
| 11s-26 | CR35-8 |
| 11s-271 | CR43-11 |
| 11s-3 | CR43-2 |
| 11S-31 | CR43-7 |
| 11s-4 | CR52-A4 |
| 11s-40 | CR54 A19 |
| 11S-53 | CR54-A5 |
| 11S-7 | CR 59-4 |
| 11s-8 | CR59-8 |
| 11s-86 | CR59-8R |
| 57-6 | CE-786 |
| 7IV-C-4 | H226 |
| 8w5 | IH5/15 |
| 9s-127 | IMS 2-8 |
| 9S-132 | IPS 2-1 |
| 9S-172 | PDP-1 |
| 9s-174 | Sree Athulya |
| 9S-286 | Sree Padmanabha |
| 9s-3 | S1284 |
| 9S-56 | Sree Harsha |
| 9s-98 | Sree Kalpaka |
| C4D7-1 | Sree Prabha |
| CE-185 | Sree Rekha |
| CE-775 | Sree Vijaya |
| Ci-273 | Vellyani Hraswa |
| CI4 | |
| CI-800 | |
| CI-889 | |
| CI-891 | |
| C1896 | |
| Co2 | |
| Co-3 | |
| CR-20 A2 | |

## Appendix-2. Reagents for total protein extraction and quantification

### a. Bradford reagent

Dissolve 100 mg of Coomassie Brilliant Blue G-250 in 50 ml 95% ethanol

100 ml 85% (W/V) phosphoric acid

Filter using Whatman filter paper

### b. Phosphate buffer

pH 7.0

Volume of 1M K2HPO4 – 61.5 ml

Volume of 1M KH2PO4 – 38.5 ml

Make up to 100 ml

# Abstract

# BIOCHEMICAL AND MOLECULAR STUDIES ON POST-HARVEST PHYSIOLOGICAL DETERIORATION OF CASSAVA

(*Manihot esculenta* Crantz)

by

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## ABSTRACT

The project entitled "Biochemical and molecular studies on post-harvest physiological deterioration of cassava *(Manihot esculenta* Crantz*)*" was conducted at the Dept of Plant Physiology, College of Agriculture, Vellayani and at ICAR-CTCRI, Thiruvananthapuram during 2013 to 2015. The main objective of the work was to analyse the physiological biochemical and molecular mechanisms associated with post harvest physiological deterioration (PPD) and develop methods to delay the PPD in cassava.

The initial screening of the cassava genotypes for their performance of PPD was done with 61 different genotypes including some released varieties. Significant differences were observed for PPD development and shelf-life of harvested roots. Cassava genotypes such as IMS2-8, 9S-172, 11S-53, IRS 2-10 and 9S-286 started showing the visible discolouration in the parenchyma tissue earlier (less than 3 days of storage). Genotypes such as 9S-7, 9S-98, 11S-31, 11S-86, 11S-14, CE63-3, CI43-2, CR43-2, CR54-A5, CR59-8R, Sree Athulya and Kalpaka showed low PPD scores and better shelf-life. Tissue imprinting for peroxidase enzyme showed that there was a remarkable increase in peroxidase activity in the root tissues with increasing PPD symptoms. There was no correlation between the root morphological traits and PPD severity. Carotene and starch content of root did not influence the PPD in the genotypes studied. Chemotypic profile of roots with PPD symptoms was used to classify the genotypes based on PPD.

To develop an objective screening tool, Near Infrared Spectroscopy (NIRS) was utilized to analyse root samples for PPD. Principal component analysis (PCA) and chemometric tools clearly grouped the different PPD category in root tissues. Various stains such as saffranin, aniline blue, erythrocin, fast green and phloroglucinol stained the tissue specifically at vascular tissues and other cell components and were not suitable for detecting PPD.

Storage techniques such as storing the harvested roots in de-aerated bags, wax coating and burying the roots under the soil were employed with selected cassava varieties like Sree Athulya, Sree Jaya, Vellayani Hrashwa, Kalpaka and Sree Padmanabha to delay PPD. Wax coating was suitable to reduce

PPD for few weeks. Effect of different storage temperature on PPD was studied for five cassava varieties. Root respiratory flux was higher in roots stored at ambient conditions compared to high (40° C) or low temperature (4° C) storage. There were significant positive correlation between root respiratory flux at 3 and 9 days of storage to the CAT and POX activities studied in different cassava varieties. Roots were treated with various food preservatives at two different concentrations (0.5 and 1%). There was a weak, but significant reduction in symptom development in butylated hydroxy touline – (BHT, at 0.5 and 1% level) treated roots compared to other treatments. Nearly three folds increase in total phenol content was noticed in BHT and butylated hydroxy anisole (BHA) treated roots. The plant hormones related to wound response such as Salicylic acid and jasmonic acid were used to study the PPD response in roots under storage. The roots did not show marked influence to hormone application.

Significant genetic variation was observed for PPD. The low PPD type genotypes such as 9S-7, 9S-98, 11S-31, 11S-86, 11S-14, CE63-3, CI43-2, CR43-2, CR54-A5, Sree Athulya and Kalpaka can be utilized for breeding programmes. High temperature storage of cassava at 40 °C resulted in reduced respiratory rate and increased antioxidant scavenging enzyme activity and also reduced the PPD. Differentiation of cassava roots at the metabolites level corresponding to visual symptoms and chemotypic profile of PPD and NIR spectroscopy offer a rapid screening tools. Among the different storage treatments, wax coating with antiseptic pre-treatment is most suitable and economical for increasing shelf-life of roots. Food preservatives like BHT and BHA have a significant, albeit marginal influence on PPD symptom development in cassava.