TIME COURSE GENE EXPRESSION PROFILING OF CASSAVA ROOTS (*Manihot esculenta*) UNDER PHYSIOLOGICAL POSTHARVEST DETERIORATION

by

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ABSTRACT

The economic expansion of tropical root crop cassava is threatened by the rapid physiological postharvest deterioration (PPD) developed within the first 48 hours after harvest. This phenomenon contributes substantially to economic losses for cassava farmers, and limits the production of cassava for industrial purposes. Genetic variation of cassava with potential to solve the problem has been scarce, as well as information about genes involved in PPD. In an effort to increase our understanding about PPD, Puerto Rican cassava germplasm collection was evaluated for its potential for tolerance to PPD testing both visual and digital image analysis methodologies. PPD evaluation identified two cassava accessions (MCol 2215, high PPD, and SM 494-1, low PPD) with contrasting levels of PPD. These accessions were used to study the changes in gene expression of a selection of 21 genes. Selected genes are associated with several biological processes include biosynthesis, plant defense, response to wounding, oxidative stress, program cell death, cyanide detoxification, cell wall, water transport and sulfur metabolism.

Expression patterns were evaluated in cortex and pith tissue from roots of contrasting accessions subject to varying times after harvest (0, 1, 24, 72, and 120 hours). Changes in gene expression were evaluated using real time PCR. Prior to the assessment of expression patterns of the target genes, the best reference genes for normalizing were obtained among five candidates. Multivariate analysis tools were used for the analysis of all data obtained, allowing the identification of coordinated gene expression patterns which were tested through a confirmatory statistical analysis. Results obtained suggest a model that explains the tolerance observed based in spatiotemporal changes of gene expression in cortex and pith tissue, these changes would be affecting Reactive Oxigen Species (ROS) activity, wound repair and hormone synthesis.

RESUMEN

La expansión económica de la yuca está amenazada por el rápido desarrollo del deterioro fisiológico postcosecha (DFP) en las primeras 48 horas después de la cosecha. Este fenómeno contribuye con pérdidas económicas para los agricultores, limitando la produción de yuca para la industria. La variación genética de la yuca con potencial para resolver este problema, asi como información disponible de los genes involucrados en DFP ha sido escasa. En un esfuerzo para incrementar nuestra comprensión acerca del DFP, el banco de germoplasma puertorriqueño de yuca fue evaluado en su potencial para la tolerancia al deterioro fisiológico postcosecha probando metodologías de análisis visual y análisis de imágenes digitales. La evaluación de PPD permitió la identificación de dos accesiones de yuca (MCol 2215, para alto DFP y SM 494-1, para bajo DFP) con niveles contrastantes de DFP. Estas accesiones fueron utilizadas para estudiar los cambios en la expresión génica de 21 genes. Los genes seleccionados están asociados con varios procesos biológicos, incluidos biosíntesis, defensa, respuesta a daño mecánico, estrés oxidativo, muerte celular programada, detoxificación de cianuro, pared celular, transporte de agua y metabolismo de azufre.

Los patrones de expresión fueron evaluados en el tejido cortical y medular en raíces de las accesiones contrastantes al deterioro fisiológico postcosecha, a diferentes tiempos después de la cosecha (0, 1, 24 72 y 120 horas). Los cambios en la expresión génica fueron evaluados usando PCR en tiempo real. Antes de la evaluación de los patrones de expresión de los genes blanco, los mejores genes de referencia fueron obtenidos entre cinco candidatos. Herramientas de análisis multivariado fueron usadas para el análisis de los datos obtenidos permitiendo la identificación de patrones de expresión génica coordinados, los cuales fueron evaluados a través de un análisis estadístico confirmatorio. Los resultados obtenidos sugieren un modelo que explica la tolerancia observada basada en cambios espacio-temporales de la expresión génica en el tejido cortical y medular. Estos cambios podrían estar afectando, las especies reactivas de oxígeno, reparación de daño mecánico y síntesis de hormonas.

I dedicate this thesis to my parents and brother

Yolanda Manuel and John

For existing, for educated me,
For encouraging, for love me
For supporting me.

There's *real* poetry in the *real* world. Science is the poetry of reality

Richard Dawkins

Biology is the science. Evolution is the concept that makes biology unique.

Jared Diamond

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Abbreviations

Abreviations	Term
AFLP	Amplified Fragment Length Polymorphism
bp	Base pair
cDNA	Complementary DNA
Ct	Cycle threshold
DNA	Deoxyribonucleic acid
E.C.	Enzyme commission number
Ha	Hectare
HCN	Hydrogen cyanide
HPLC	High-performance liquid chromatography
LOD	Limit of detention
mRNA	Messenger RNA
PCD	Program cell dead
PCR	Polymerase chain reaction
PPD	Physiological postharvest deterioration
QTL	Quantitaive trait loci
r	Pearson correlation coefficient
RH	Relative humidity
RNA	Ribonucleic acid
ROS	Reactive oxigen species
SE	Standar error
UV	Ultraviolet light

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1 INTRODUCTION

Cassava is the most important source of dietary carbohydrates for 750 millions of people around the world (Allen, 2002; Gleadow *et al*., 2009), with the main harvestd organ, being its starchy root. Due to its favorable agronomic traits, tolerance to abiotic stresses and adverse environments, cassava is produced mainly by small farmers in marginal agricultural areas. World cassava production has increased in the last years (García and Dale, 1999), and is expected to increase further with both the demand for human consumption and industrial proposes, particularly in Africa (Nyerhovmo, 2004). However, the expansion of the crop is restricted by the storage potential of the roots, which is limited to only a few days (Wenham, 1995).

The short storage life of cassava roots is directly linked to an endogenous physiological process called physiological postharvest deterioration (PPD). This process is characterized by the initial presence of blue-black streaks in the vascular tissue of roots, followed by a general brown discoloration which makes cassava roots unpalatable and unmarketable (Beeching *et al*., 2002). Cassava PPD has shown variation in speed of development and severity among different cassava cultivars or accessions, providing the opportunity for a better understanding of the phenomenon, and the use of natural genetic variability to improve this trait (Buschmann *et al*., 2000a)

Several strategies have been proposed to overcome the problem of PPD including the use of improved storage techniques and plant breeding. However, in the practice they have had a limited success. Hence, genetic modification is considered the most appropriate approach to solve PPD. However, studies in biochemistry and molecular biology focused on the genes

involved in cassava PPD need performed in order to identify potential strategies for the control of PPD in cassava (Westby, 2000).

PPD is a complex process which is not fully understood and is considered a deficient enzymatic stress response to wounding (Beeching *et al*., 2002). Several analyses of genes expressed during PPD (Huang *et al*., 2001; Really *et al*., 2001, 2004, 2007) show altered regulation in the expression of genes involved in the modulation reactive oxygen species, phytohormone synthesis, programmed cell death, synthesis of cells wall components and synthesis of anti-oxidant and other defensive compounds leading to the identification of potentially PPD specific genes.

The present study attempts to validate and evaluate the transcriptional activity of a set of genes described previously as potentially PPD specific and genes involved in metabolic pathways related with PPD. We take advantage of genetic variability of Puerto Rican cassava germplasm collection to identify contrasting PPD cassava accessions. Changes in gene expression of target genes were tested in root cortex and pith of accessions with contrasting PPD levels using real time PCR technology at different time points after harvest. The results of this research will help to validate the gene expression profile genes potentially involved in PPD and reach a clearer picture of the metabolic changes that lead to PPD in cassava and a possible tolerance mechanism which contributes towards strategies for reduction of PPD in cassava.

2 THEORETICAL BACKGROUND

2.1 Cassava Biology

2.1.1 Taxonomy

Cassava (*Manihot esculenta* Cranzt), also known as yuca (most Spanish-speaking countries) or tapioca (Asia), belongs to the Euphorbiaceae family. Members of this family are characterized by the presence of lacticiferous vessels, from which latex is synthesized and secreted. The Euphorbiaceae family includes crops with different plant architecture and economic importance such as oil producers (*Ricinus communis*, castor bean), edible roots (*Manihot* spp.), weeds (*Euphorbia spp*.), ornamentals and medicinals plants (Rodríguez, 2001; Ceballos and de la Cruz, 2002).

Within the *Manihot* genus, 98 species of herbs, shrubs and trees have been described, and those species are distributed across the neotropics (Olsen and Schaal, 1999).Only *Manihot esculenta* is commercially cultivated (Ceballos and de la Cruz, 2002). *M. esculenta* is placed in the Fruticosae section of the genus *Manihot*. The Fruticosae section contains low growing shrubs adapted to grassland, savannah or desert and is considered less primitive than the Arboreae section, which contains only tree species (Ojulong, 2006). The taxonomical classification of cassava is presented in table 2.1.

Class	Dicotyledoneae
Subclass	Archichlamydae
Order	Euphorbiales
Family	Euphorbiaceae
Subfamily	Crotonoideae
Tribe	Manihoteae
Genus	Manihot
Section	Fructicosae
Specie	Manihot esculenta

Table 2.1 Taxonomic classification of cassava

The scientific name of cassava was originally from Cranzt in1766, but later cassava was classified as two different species by Pohl in 1827 and Pax in 1910 depending on its bitter (*M. utilissima*) or sweet (*M. aipi*) flavor. However Ciferri in 1938 prioritized the current scientific name for cassava *M. esculenta* (Ceballos and de la Cruz, 2002). Allen (2002) recognized three related subspecies which form the primary gene pool (Table 2.2).

Basionym	Novel status	Category
M. esculenta Crantz	*M. esculenta Crantz* ssp. *esculenta*	Cultivated stock
M. flabellifolia Pohl	M. esculenta Crantz ssp. *Flabellifolia* (Pohl) Cifferi	Wild strain
M. peruviana Muller	*M. esculenta* Crantz ssp. *Peruviana* (Muller) Allem	Wild strain

Table 2.2 The subspecies of cassava according to Allen (2002)

Cassava cultivars have been classified according to morphology and presence of cyanogenic glycosides in their roots. Cyanogenic glycoside has been used to classify cassava cultivars into two major groups: the bitter cultivars, in which the cyanogenic glycosides are distributed in roots at high level (more than 100 mg/kg) and the sweet varieties, in which the cyanogenic glycoside are limited mainly to the peel at lower level. In general, sweet cassava tends to have a short growing season with mature roots in 6-9 months, and deteriorates fast if not is harvested after maturity. The bitter cassava cultivars in contrast, require 12-18 months to mature, and will not deteriorate greatly if left unharvested for several months after maturity (Nassar, 2004).

2.1.2 Origin and diversification

Until 1980's it was considered that cassava did not grow in the wild. However, a wild population, very similar from domesticated cassava, was found in the central Brazilian state of Goiás. This showed a relationship between wild populations and the crop origin (Allen, 2002).

The origin of cassava is still controversial, some authors consider that cassava was domesticated after the hybridization of closely related wild species (Rodriguez, 2001), while others like Allen (1994, cited by Halsey *et al*., 2008) suggest that modern cultivated cassava (*M. esculenta* ssp *esculenta*) and landraces arose from the domestication of the morphologically similar wild subspecie *M. escuelenta* subsp *flabellifolia*.

The hypothesis of the domestication from wild relatives has been supported by molecular studies using AFLPs (Roa *et al*.,1997) analysis of glyceraldehydes 3-phosphate dehydrogenase gene (Olsen and Schaal, 1999) and more recently by use of single nucleotide polymorphism (SNPs) and simple sequence repeats (SSR) molecular markers (Olsen, 2004).

Nassar (1978, cited by Nassar, 2002) identified four centers of diversity for species of *Manihot* genus in Mexico, Northeastern, Central and Southwestern of Brazil. Microcenters of diversity of these species with high concentration of species in small areas exist within central Brazil. In contrast, Halsey *et al*. (2008), reduces to two centers of diversity: northern South America (~80 species) and as secondary center of diversity Mexico and Central America (~ 17 species).

The archaeological evidence found showed that cassava was cultivated in those areas 3000 to 7000 years ago (Reichel-Dolmatoff, 1957, cited by Rodríguez 2001), and in the Peruvian coast earlier than 4000 B.C. (Nassar *et al*., 2008). It is believed that domestication of sweet cultivars (low-cyanogen cultivars) occurred as early as 6000 B.C. in the Amazonian rainforest Gibbons (1990, cited by El-Sharkawy 2004), contrasting with the opinion of Renvoize (1972, cited by El-Sharkawy, 2004), who suggested that sweet cultivars may have been domesticated in Mesoamerica.

Assuming that cassava was domesticated initially in one location, it was carried by Amerindian migrations in the 11th century to Central America, crossing the Caribbean Sea, where hybridization between the cultivated species and local wild species might have taken place, creating a new center of diversity and leading to the rise of numerous new species. This is the most probable explanation for the formation of a center of diversity in Mexico (Nassar, 2002). The other centers of diversity can also be attributed to migrations of the Amerindian Aruak group to the South Central and Northeastern Brazil (Nassar, 2002).

The Portuguese introduced cassava to Africa as processed forms, in the 17th century. Cassava then became a significant part of African subsistence farming after several simultaneous introductions (Benesi, 2005). Cassava spread first along the West African coast, and then in 18th century it moved to East Africa. European introduced cassava to Asia in the late 18th and 19th centuries, where it was cultivated mainly in marginal lands as an emergency crop. But by the 19th century cassava became an important food crop in India, Indonesia, Philippines, Malaysia, Thailand and China (Kizito, 2006).

2.1.3 Genetics, cytogentics and reproduction

The size of the nuclear cassava genome in cassava is small compared with other angiosperms, corresponding to 772 Mbp per haploid genome (Awoleye *et al*., 1994).The chromosome number in all species of genus *Manihot* investigated is 2n = 2x = 36 and *Manihot* species behave meiotically as diploids. It is believed that cassava is a product of an allopolyploidizing event; suggested by the possession of two sets of dissimilar nucleolar organizing regions, on the repetition of chromosome types, and on the basic chromosome numbers (Rodríguez, 2001).

Cassava is a monoecious species producing both male (pistillate) and female (estaminate) flowers in the same plant. Inflorescences are generally found in the reproductive branching, but sometimes can be found in the leaf axis on the upper plant. The number of female flowers is smaller than the number of male flowers, and is located on the lower part of the inflorescence (Alves, 2002). Wild *Manihot* species are typically allogamous and highly heterozygous, normally cross-pollinated by insects. However, in cultivated cassava a shift towards autogamy has occurred, explained by the cassava clonal system of cultivation and domestication history (Nassar, 2001).

Although cassava vegetative propagation is carried out by means of stem cuttings, known as stakes, seeds still are use by farmers to produce planting stocks, and breeding programs. One disadvantage of using seeds for propagation is that plants take longer to become established, grow smaller and less vigorous than plants from stakes (Alves, 2002; Nassar *et al*., 2008).

2.1.4 Cassava root system.

Roots are the main storage organ in cassava. Unlike the domesticated crop, the roots of wild species are fibrous and slender sometimes exhibiting a limited number of roots (Nassar, 2000). In plants propagated from seeds a typical primary tap root system is developed, similar to dicot species. The radicle of the germinating seed grows vertically and develops into a taproot, from which adventitious roots originate. Later, the taproot and some adventitious roots become storage roots. These roots develop radially around the base of the plant (Alves, 2002; Adetan *et al*., 2003).

Cassava plants grown from stakes develop adventitious roots from the basal cut surface of the stake and rarely from the buds under the soil. These roots develop to make a fibrous root system but only a reduced number of fibrous roots (between three and ten) start to accumulate starch to become storage roots. The other fibrous roots remain thin and help to water and nutrient absorption (Alves, 2002) (Figure 2.1A).

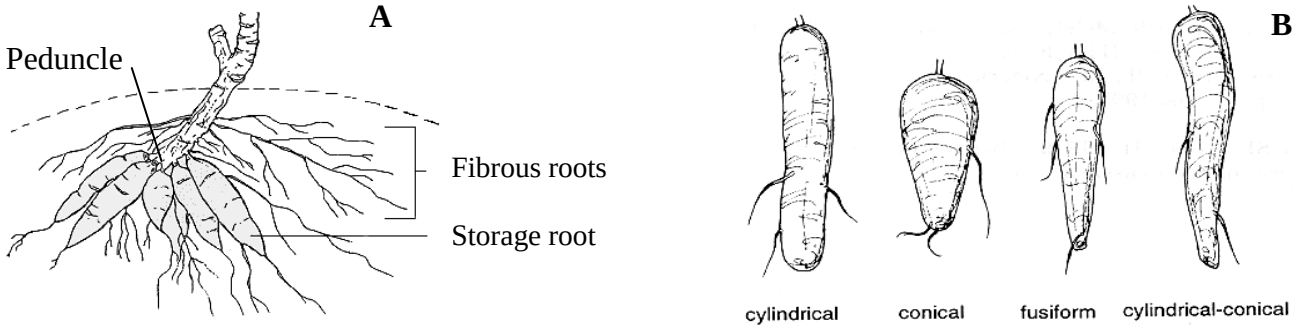


Figure 2.1 Cassava roots **A**. Root system **B**. Cassava storage root typical morphology. Source: Ekanayake *et al*., 1997.

The roots appear within ten days after the stakes are established. Storage roots develop by enlargement of the cambium and the formation of secondary xylem and outer periderm. About 26 days after planting starch starts to deposit in the secondary xylem parenchyma, and bulking process starts from the outer layers to the center of the storage parenchyma (Wheatley, 1982).Once a fibrous root becomes a storage root, its ability to absorb water and nutrients decreases significantly. The cassava radical system has low root density but a deep penetration, which confer the capacity for drought resistance (CIAT, 1981).

Anatomically, the cassava storage root is not a tuberous root and cannot be used for clonal propagation. The typical shape of cassava storage root is usually elongated, with depressions along its length. Usually, the middle part has a constant diameter. The root head and tail end are generally referred to as the proximal and distal ends, respectively. At its proximal end, the root is joined to the rest of plant by a short woody "neck" called peduncle with a variable size (<1cm to > 8cm long) (Onwueme, 1978, cited by Adetan *et al*., 2003).Variability in root size and form within a cultivar is greater than other crops and it is influenced by environmental conditions (CIAT, 1981; Alves, 2002) (Figure 2.1B).

The edible fleshy portion of the storage root is composed of 60 - 65% water, 30 - 35% carbohydrates, 1 -2% protein, up to 0.2% fat, 1- 2% fiber, and 1-1.5% mineral matter. The protein present in cassava tuber is rich in arginine but low in methionine and lysine. Protein content estimated from nitrogen analysis tends to be higher than estimation from amino acids;

some of this non protein nitrogen is originated from glycosides (Nassar, 2004). A list of morphological and agronomical characteristics of the cassava roots are showed in Table 2.3.

The mature cassava root has three different tissues. The outer *periderm* of dead cork cells (the skin or bark) with a few thick cells, which functions to seal the surface (Figure 2.2). It may be sloughed off and may represent 3% of the total weight. The thin *cortex* is composed of sclerenchyma, cortical parenchyma (with small starch grains), and phloem, constituting 11-20 % of the root weight. The detachable rind (peel) is the periderm and cortex. The *pith* composed of parenchyma cells (flesh), and is the edible portion of the flesh root. It contains starch as a matrix and radially distributed vascular strands of xylem. The flesh is approximately 85% of the total root weight (Alves, 2002).

Root characteristic	Variability
Morphological	
External colour	White or cream; yellow; light brown; dark brown
Cortex colour	White or cream; yellow; pink; purple
Pulp(parenchyma) colour	White; cream; yellow; pink
Peduncle	Sessile; pedunculate; both
Constriction	None or little; medium many
Shape	Conical; conical-cylindrical; cylindrical; fusiform
Agronomic	
No storage roots /plant	3-14 roots
Weight of one storage root	0.17-2.35 kg
Length of storage root	15-100 cm
Diameter of storage root	3-15 cm
DM in whole fresh roots	23-43%

Table 2.3 Some morphological and agronomic characteristics of cassava roots according to Alves (2002). **DM** Dry matter.

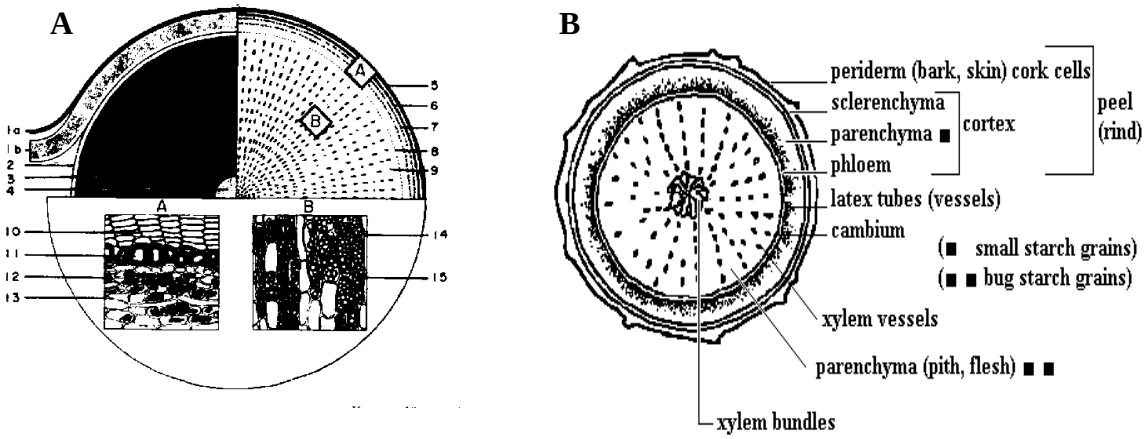


Figure 2.2 Cassava storage root cross sections. Source **A** Grace, (1977) **B** www. uq.edu.au/_School Science. *Left upper quadrant, after staining with iodine*: 1.Peel (periderm), 1a.Outer cork layer, 1b.Inner layer, 2.Cambium, 3.Centre, 4.Pith and primary xylem *Right upper quadrant, showing structural elements of the roots:* 5.Cork, 6.Sclerenchymatous fibres, 7.Latex vessels, 8.Cambium, 9.Xylem vessels *Inset A-Enlarged cross section of peel:* 10. Cork tissue, 11.Sclerenchymatous fibres 12.Starch 13.Parenchyma cell *Inset B-Enlarged cross section of centre:*14.Cell wall 15.Starch.

2.1.5 Agroecology and physiology

Currently cassava is cultivated in the regions between latitudes 30ºN and 30ºS and from sea level up to 1800m above sea level (Ceballos *et al*., 2004). Cassava adapts an extensive range of ecological environments, and is often found mixed with a variety of other cash crops (Leihner, 2002).

Cassava yield potential, in the absence of production constrains, is higher than other major staple food crops in the tropics, and the greatest potential for energy production after sugarcane (El-Sharkawy, 2004). Cassava is one of the most efficient starch producers (80 tons/ha per year), similar to rice and maize under optimal conditions, but under suboptimal conditions cassava exceeds the production of all others crops (Mejia de Tafur, 2002).

In the humid lowland tropics the roots can be harvested after 6-7 months. In regions with prolonged periods of drought or cold the farmers usually harvest after 18-24 months (Cock, 1984, cited by Alvez, 2002). Moreover, the roots can be left in the ground without harvesting for a long period of time, making it a useful security crop against famine (Cardozo and Souza, 1999; cited by Alvez, 2002).

Storage root yield in cassava is correlated with the rate of photosynthesis on the basis of total leaf nitrogen content (leaf nitrogen use efficiency). This indicates that the association between yield and leaf photosynthesis is mainly due to biochemical factors controlling carbon assimilation. In addition, another physiologically important trait that may contribute to yield is long leaf life and better leaf retention in cassava, particularly under stressful environments (El-Sharkawy, 2004).

Based on leaf anatomy, high photosynthetic rates, low photorespiration, and elevated PEP carboxylase activities in leaves of some wild *Manihot* species, El-Sharkawy (2004), suggested that cassava and wild *Manihot* species represent an intermediate photosynthesis between the typical C3 and C4 species. However such hypothesis has been contradicted by other authors (Edwards *et al*., 1990; Angelov *et al*., 1993; Calatayud *et al*. 2002, cited by El-Sharkawy, 2004) and more recently by Gleadow *et al.*, (2009).

2.2 Importance of cassava

Cassava, along with maize, and rice, constitute the most important sources of energy in the diet of approximately 750 million people in Africa, South America, Asia and the Pacific Islands

(Allen, 2002; Gleadow *et al.* 2009). This crop is ranked 6th in production among the staple foods (Figure 2.3A). In 2008 the main cassava producers were Nigeria, Thailand, Brazil, and Indonesia, with almost 51% of worldwide production (http://faostat.fao.org consulted in May 20, 2010) (Figure 2.3B). Cassava is produced for industrial purposes in Brazil and as an export crop in Thailand, but in Africa it is grown primarily for human consumption either fresh, boiled, baked, fried or in its processed forms. It is also used for animal feed, and as a raw material for producing starch, and starch derivate products (Fauquet and Fargette, 1990, Nassar, 2004).

Cassava leaves are sometimes consumed, but the main harvested organ is the starchy storage root. Although the actual yield is often low, sometimes less than 12.5 tons/acre, world production has increased by more than 35% in the last 20 years (García and Dale, 1999). Cassava is produced mainly by resource-limited small farmers in the tropics, contrasting with the capital intensive cereals such as wheat, rice and maize (El-Sharkawy, 2004).

Because most of the cassava grown is produced by small farmers in marginal agricultural areas, it is difficult to obtain exact data on production (Cock, 1985 cited by Rodriguez, 2001). According to FAO, cassava word production was estimated around 214 million tons in 2007, and the cultivated area was 18.5 million hectares. (http://faostat.fao.org consulted in May 20, 2010). More than two thirds of the total production of cassava is used as food for humans, and lesser amounts are used for animal feeding and industrial purposes (Nyerhovmo, 2004).

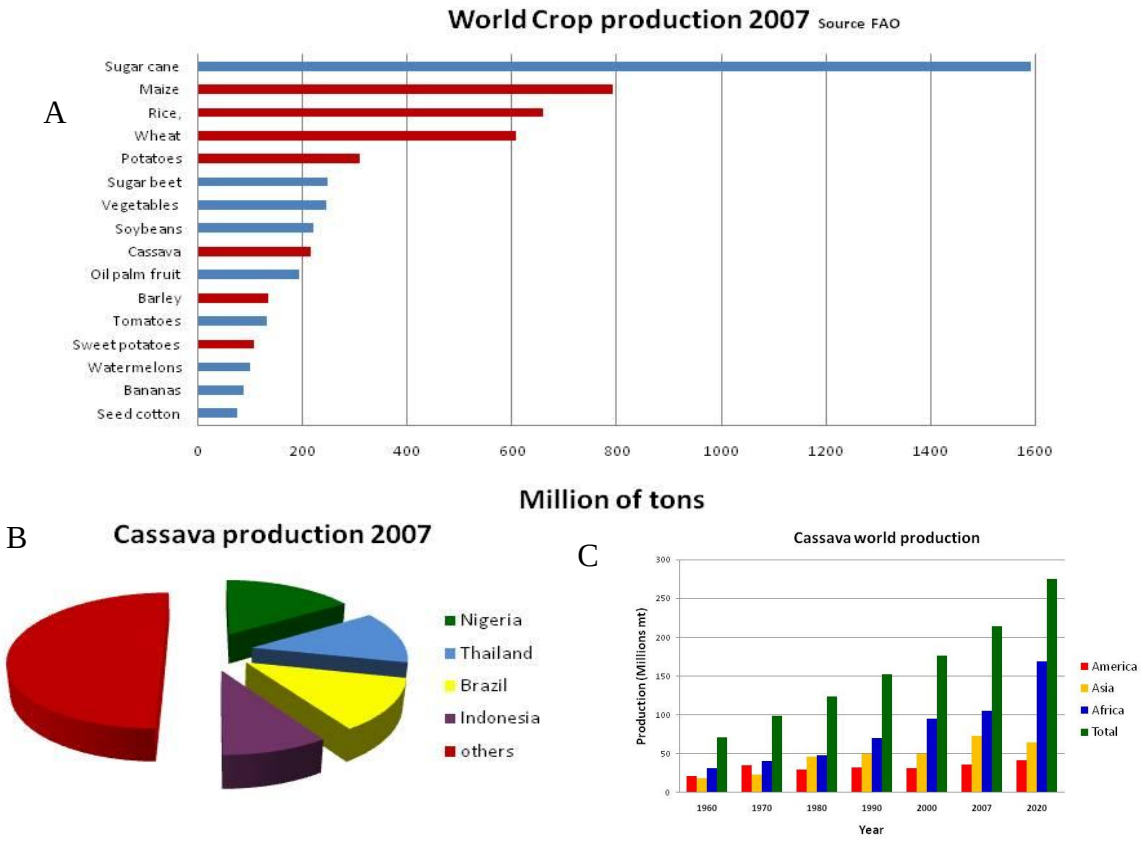


Figure 2.3 Cassava world production **A**. Comparison world main crops production (In red the staple foods) **B**. Main cassava country producers **C**. trends in cassava production and projection for 2020.Source Nyerhovmo 2004, (http://faostat.fao.org consulted in May 20, 2010).

Cassava is used to produce raw material for the business of starch, which has multiple applications in many industries. Cassava starch can substitute most of the applications in which maize, rice and wheat starch are used. Starch is used as sizing and dyeing in textiles industries, to increase brightness and weight of the cloth and in the pharmaceutical industries, starch serves as a filler material and bonding agent for making tablets (Nyerhovmo, 2004).

Cassava starch also has several other uses, such as an additive in cement to improve the setting time, and in oil wells to improve the viscosity of drilling muds. Starch is the main raw material in glue and adhesive industries, and it can be converted to modified sugar (maltotriose, maltose) and organic acids, fructose syrups. In addition, the use of cassava as source of ethanol for fuel is already being exploited, with promising results (Nyerhovmo, 2004).

2.3 Main constraints in cassava agriculture

Cassava is produced mostly by small farmers on marginal lands. Cassava farmers usually have scarce access to credit, markets and technology and constantly they are exposed to numerous biotic stress and production restrictions. According to a survey (Aerni, 2006) (Figure 2.4), the lack of clean planting material (clean stakes) and low yields (gap between the potential and the real per-hectare yield of cassava) are the most important problems in cassava cropping system.

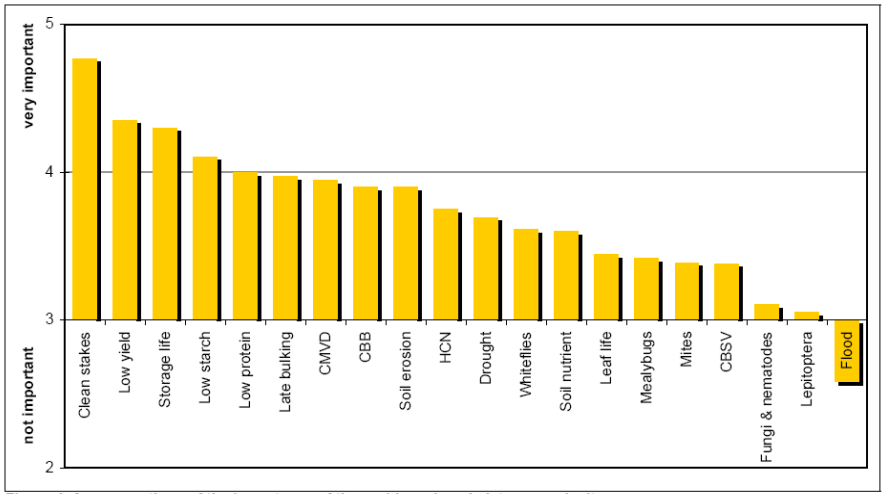


Figure 2.4 Average ratings of the importance of the problems in subsistence cassava agriculture. Source: Aerni, 2006.

These problems are followed by root quality problems (short storage life, low starch and low protein content, late bulking, and high HCN content), plant diseases (e.g., Cassava mosaic virus or cassava bacterial blight), abiotic stresses (soil erosion, drought, and low nutrient soil), and pest infestation (whiteflies, mealybugs, and mites).

2.3.1 Pests and diseases

The most important cassava's viral disease is African Cassava Mosaic Virus (ACMV), which can produce yield losses up to 45%. The virus is transmitted by a whitefly (*Bemisia tabaci*) vector and spread by infected stakes (Rodríguez, 2001). The most visible symptom of the disease is the characteristic leaf mosaic where young plants are more affected than old ones. Symptoms range from barely perceptible mosaic to extreme reduction of the leaf blades (Fauquet and Fargette, 1990).

Cassava bacterial blight (CBB) is caused by *Xanthonomonas axonopodis pv. Manihotis,* which leads to severe losses in susceptible varieties grown on poor soils. CBB displays a wide variety of symptoms, including angular leaf spots, blight, gum exudation, stem cankers, shoot wilt, etc (Rodríguez, 2001). CBB causes up to 70% reduction in yields of cassava tubers and planting materials. This disease is spread through infected planting material, wind, insects and the use of infected implements (Nassar, 2004).

Other microorganism mediated diseases include anthracnose (*Colletotrichum gloesporoides*) and twenty diseases caused by pathogenic fungi. The most important is root rot, caused by at least three species of *Phytophthora*, *P. drechsleri*, *P. erythoseptica* and *P. cryptogea*. Root destruction

can also be caused by *Diploidia manihotis*, *Armillaria spp*., *Fusarium spp*. and *Verticillum dahlia*. Another important disease is the frog skin disease (the causal organism unknown), which affects storage of carbohydrates in the roots. Phenotype of affected plants is characterized by the production of fewer and frequently deformed roots (Rodríguez, 2001).

The most important arthopods that affect cassava are the cassava mealybug (*Phenacoccus manihot*), and the cassava green mite (*Mononychellus tanajoa*). This mite is found principally on buds and leaves, and causing deformation of recently emerged leaves, and yellowish spots (Nassar, 2004). Cassava is also attacked by root knot nematodes (*Meloidogyne* spp) (Rodríguez, 2001).

2.3.2 Cyanogenesis

Cassava is a cyanogenic crop. All tissues except seeds contains potentially toxic levels of cyanogenic glycosides linamarin (95%) and lotaustralin (5%) (Siritunga and Sayre, 2003). Naturally acyanogenic cassava has never been observed (Nhassico *et al*., 2008). Cassava cultivars are classified as "bitter" or "sweet" based varying levels of cyanogenic glucosides (García and Dale, 1999). Cassava varieties containing >100 ppm cyanide are normally "bitter" (Nhassico *et al*., 2008). However, certain ecological stress factors, such as pest attacks, prolonged drought and low soil levels of phosphorus and potassium may cause roots to develop bitterness, coinciding with an increase in the levels of cyanogenic glucosides (Essers, 1995).

Cyanogenesis is initiated when the tissues suffer mechanical damage. Linamarin which is present in large amounts in the leaves and the peel of the roots (Nhassico *et al*., 2008), is hydrolyzed by

the linamarase to produce acetone cyanohydrin, which can be broken down into enzymatically by hydroxynitrile lyase to produce acetone and toxic hydrogen cyanide (Elias *et al*., 1997, cited by Rodríguez, 2001) or spontaneously at pH 5 or temperatures >35°C (White *et al*., 1998, cited by Rodríguez, 2001).

Cyanogenic glycosides are eliminated from the root by removal of the outer layer, soaking, boiling or drying before consumption (Rodríguez, 2001). A number of cyanide-associated health disorders have been attributed to eating poorly processed cassava, particularly by nutritionally compromised individuals (Rosling, 1974, cited by Siritunga and Sayre, 2003). Those include goitre, hyperthyroidism, tropical ataxic neuropathy and konzo (Rickard and Poulter, 1992, cited by Rodriguez, 2001).The severity of these disorders depends on the level and frequency of cyanogen exposure and the general health of the consumer (Siritunga and Sayre, 2003).

2.3.3 Low protein content

Cassava root provides a rich source of carbohydrates, but is low in vitamins and proteins. Depending on cultivars, the protein content in cassava storage root ranges from less than 1% to 5% of dry weight (Stupak *et al.,* 2006). The low protein level may have a very undesirable effect on millions of people who depend of cassava as a staple food, facing the risk of chronic protein deficiency (Ceballos *et al.*, 2006).The nutritional value cassava's root low protein amount is further reduced by low levels of the essential amino acids (lysine and leucine) and sulfur-containing amino acids (metionine and cysteine) (Stupak *et al*., 2006).

In contrast with the roots, the total protein content in leaves ranges from 23% to 35% of dry weight, but the levels of essential amino acids are low. The prolonged cooking of cassava leaves is essential to minimize the toxic concentrations of cyanide, however, essential and sulfur-containing amino acids are degraded during this processing (Stupak *et al.*, 2006).

2.3.4 Short storage life

After harvesting, cassava roots must be consumed or processed within 24–48 hours, or the tissues becomes unpalatable and unmarketable for consumption or industrial uses. This short storage life is one of the main constraints of cassava production, and is caused by a rapid post-harvest deterioration (Taylor *et al*., 2004).The post-harvest deterioration process in cassava occurs in two stages: primary or physiological post-harvest deterioration (PPD) and secondary or microbial deterioration (Noon and Booth, 1977; Plumbley and Richard, 1991). PPD is discussed in depth in section 2.5 and 2.6.

2.4 Cassava in the Caribbean and Puerto Rico

Latin America is home to cassava and all its wild relatives. But the region accounts for less than 20% of global output (FAO, 2000). Every tropical country of the region produces cassava, but it is most highly concentrated in four areas: Northern and Eastern coastal Brazil, Southern Brazil and Eastern Paraguay; North-Western South America (especially the Caribbean coast of Colombia); and in the Greater Antilles (Cuba, Haiti, and Dominican Republic). Although Central America has a comparatively small production area, Costa Rica, El Salvador and Nicaragua have

some of the highest production growth rates anywhere a six-fold increase between 1970 and 1996 (Hershey *et al*., 2004).

Cassava in the LatinAmericas is usually relegated to marginal environments, especially areas with uncertain rainfall, acidic soils, low native soil fertility, and difficult terrain. Similar to other farmer of the world, on average, growers apply few inputs, and yields are well below potential levels (Hershey *et al*., 2004).

In the Caribbean and North-eastern South America, roots are ground into a mash, which is partially dried to form a flat breads called *casabe*. In Northeast Brazil and throughout much of the Amazon basin, roots are grated and toasted to make cassava flour (*farinha*) (Hershey *et al*., 2004).

In Puerto Rico the cassava production reached 1,172 tons in 2007. The main producers were the municipalities of Isabela and Adjuntas and the cultivated area was approximately 80 ha (USDA, 2009). According to Goenaga *et al*. (2002), almost 90% of cassava in Puerto Rico is imported.

Goenaga *et al*. (2002), evaluated seven cassava introductions (CM 3064-4, CM 3311-3, CM 3380-7, CM 4484-2, SG 804-5, SM 494-2 and CM 523-7), and the local cultivar, Serralles. The yield data obtained in this study suggest that clones CM 3311-3 and CM 3380 may have the best potential for cassava production in Puerto Rico.

2.5 Cassava postharvest deterioration

Two types of deterioration in cassava roots have been identified: primary and secondary deterioration. Primary deterioration is usually the initial and major cause of loss of acceptability of roots; and is characterized by the presence of blue-black streaks in the vascular tissue, which later spread causing a more general brown discoloration. Because primary deterioration is considered an endogenous physiological process, it is also called physiological postharvest deterioration or PPD (Booth, 1975; Noon and Booth, 1977).

Secondary deterioration is due to a wide range of pathogenic microorganisms, and generally occurs when the roots have already become unacceptable because of primary deterioration (Booth, 1975) (Figure 2.5).

2.5.1 Socio-economic impact of postharvest deterioration

Post-harvest deterioration of cassava roots restricts the storage potential to only a few days, reducing the development of cassava as a commercial crop. PPD places a risks for the the reliability of raw material supplies for large-scale processors. In addition cassava farmers suffer economic losses due to direct physical damage, or reduction in root quality (Wenham, 1995; Taylor *et al*., 2004).

Fresh cassava roots are traditionally marketed without post-harvest treatment or protection. Harvested cassava is expected to reach the final consumer within a very short time before deterioration symptoms appear. The long distances between the field and the marketing place increase the impact of postharvest deterioration. Usually cassava farmers in marginal areas lack

adequate postharvest facilities and essential infrastructure such as roads, to avoid fresh cassava reaching the consumer with PPD symptoms (Wenham, 1995).

Little reliable information is available to compute the actual losses due to PPD. Furthermore, it is not clear at which stage of the market chain (producer-trader-processor or consumer) the losses occur (Wenham, 1995). Available data often does not differentiate between losses in fresh roots and loss of processed products. It is estimated that postharvest losses in cassava production in Latin America and the Caribbean and Asia reach 10 and 8% respectively, whereas in Africa they reach 29% (FAO, 2000).

The FAO reported that increasing the storage life of cassava roots to a minimum of two weeks could have a substantial effect in on cassava utilization and solve an estimated 90% of the deterioration constraints associated with current cassava marketing and utilization practices (Oirschot *et al*., 2000). To date, limited useful genetic variation to delay or reduce the post-harvest deterioration has been found, and the solution to this problem remains one of the most important objectives for cassava research (Ceballos *et al*., 2004).

2.5.2 Primary (physiological) postharvest deterioration

Physiological postharvest deterioration or PPD has been referred as ash-blue or black radial streaks in the xylem vessels, developed within 48 hours after cassava roots harvest (Plumbley and Richard, 1991). Subsequently, the deterioration spreads to the adjacent root storage

parenchyma, where stored starch undergoes structural changes (Pacheco, 1952; Drummond, 1953; Normanha and Pereira, 1963, cited by Plumbley and Richard, 1991).

Averre (1967, cited by Plumbley and Richard, 1991), introduced the term vascular streaking (VS) and vascular discoloration as alternative for describing PPD. Montaldo, (1973, cited by Ravi and Aked, 1996) observed that this vascular discoloration further increases to a more general brown to black color ring pattern near to the cortex in a period of seven days (Figure 2.5B).

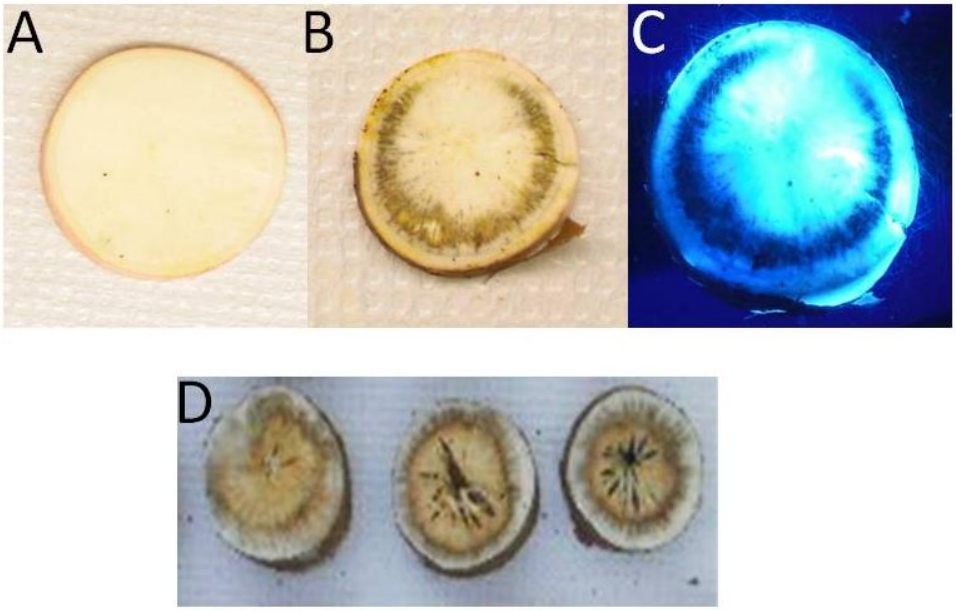


Figure 2.5 Postharvest deterioration in cassava roots cross sections **A.** No symptoms (1 day after harvest) **B**. Physiological postharvest deterioration (5days after harvest) **C.** Physiological postharvest deterioration (5days after harvest) under ultraviolet light. **D.** Secondary deterioration (15 days after harvest).

PPD or vascular streaking is divided into two types, which appear to occur independently (Kawano and Rojanaridpiched, 1983). The first type VS-I (vascular streaking-I) is characterized

by a light brown discoloration found as a ring around the inner part of the cortex, and is a physiological phenomenon independent of microbial activity (Data *et al*., 1982; Taniguchi *et al*., 1984). This first type tends to appear often when the roots are not damaged by the harvest process (Kawano and Rojanaridpiched, 1983).The second type VS-II (vascular streaking-II) is a blue black pigmentation of vessels which commonly appears on or adjacent to microbial rooted areas and is commonly observed in the initial stage of microbial deterioration. VS II appears often when the roots suffer severe damaged at harvest (Kawano and Rojanaridpiched, 1983; Taniguchi *et al*., 1984).

Pigmented material observed in PPD is the result of endogenous enzymatic action and was found to originate in the xylem parenchyma passing into the xylem vessel lumen through breaks in the pit membrane to form tyloses and occlusions consisting of lipids, carbohydrates and phenols. In addition, there are changes in the structure of the starch grains and blocking of xylem vessels (Ravi and Aked, 1996). At the later stage lignin-like material appears to develop on the xylem (Plumbley and Richard, 1991).

2.5.3 Secondary postharvest deterioration

Secondary cassava postharvest deterioration or root decay is the consequence of microbial activity that takes place when pathogens penetrate through wounds and bruises caused during roots harvesting and handling. This microbial activity occurs after primary deterioration, and the roots have already become unacceptable. Secondary deterioration is less important than primary deterioration, can produce similar pigmentation (but without any pattern) and lead eventually to a

general and complete root decay, five days or more after the harvest (Plumbley and Richard, 1991; Kawano and Rojanaridpiched, 1983; Wenham, 1995).

Secondary cassava postharvest deterioration is characterized by light brownish discoloration of the soft-rotten part and by blue-black pigmentation of the xylem vessels or extensive discoloration, light greyblack, of the non-rotten tissue adjacent to the rotten part (Tanaka *et al*.,1983) (Figure 2.5D).

Several kinds of microorganism have been isolated from decaying cassava root tissues. However, it is not clear what fungi play an important role in the decay (Taniguchi and Data, 1984).The number of different species of fungi and bacteria isolated from roots under post-harvest decay of cassava roots shows that secondary PPD involve more than a single initial organism. Two distinct specific types of rot have been described by Majumder, (1955; cited by Wenham 1995): a dry rot occurring under aerobic conditions and a soft rot which developes under anaerobic conditions and is caused by a complex of fungi: *Lasiodiplodia theobromae* (Pat.) Griff, *Aspergillus nigervan* Tieghem, *Aspergillus flavus* Link, *Cylindrocarpon candidum* (Link) *Trichoderma harizianum* Rifia, *A. niger* with *Cylindrium cladostrinum* (presumably *C. clandestrinium* Corda) ,and unidentified *Penicillium* and *Cladosporium spp* Wegmann, which are present on the later stages of decay (Wenham, 1995).

Noon and Booth (1977) tested different fungi and bacteria isolated from severely rotting cassava roots by inoculating in freshly harvested roots. In some cases inoculated roots developed

symptoms of vascular streaking, but there was no evidence that this was associated with the introduced organisms. In these cases the inoculated organisms could not be recovered from the advancing fronts of discoloration, although they could be recovered from the margins of the necrotic areas. In other cases rotting was caused by the inoculated pathogen, but no vascular streaking occurred.

2.5.4 Factors that affect cassava PPD

Mechanical damage which takes place during harvesting is a critical factor in the rapid occurrence of post-harvest deterioration of cassava roots. The activation and intensity of cassava PPD is closely related with mechanical damages (Booth, 1975; Aristizabal and Sánchez, 2007).

The first symptoms of PPD appear in areas where the peel has been damaged, removed or in the proximal and distal ends, which are zones susceptible to physical damage. PPD usually starts at the site of injury, and the pathogens responsible for rotting usually invade through these wounds, making internal tissues more exposed to oxidation and microbial attack. Others factor to be considered are root shape, root length, presence of peduncles, peel adherence, texture, soil compacting, and harvesting method (manual or mechanical) (Booth, 1975; Diamante ,1986; Aristizabal and Sánchez, 2007).

Comparative evaluations of PPD among different cassava varieties and within the same variety, has revealed a considerable variation in degree of development and severity of PPD (Plumbley and Richard 1991; Buschmann *et al*., 2000a; Aristizabal and Sánchez, 2007), implying that this response is under genetic control but also influenced by environmental factors Iglesias *et al.*,

(1994, cited by Beeching *et al.* 2002) These differences can provide researchers the opportunity to use the cassava genetic variability to improve the resistance to PPD (Buschmann *et al.*, 2000a).

In an experiment conducted in three different locations in Colombia using eight cassava clones, Kawano and Rojanaridpiched (1983) found a highly significant interaction among, location and season suggesting that PPD was sensitive to all these factors. Highly significant interaction between clone and location suggested that cultivar selection was highly important; however, the selection had to be done in each location.

Correlations between some traits and PPD have been made: a positive correlation between root dry matter content, high starch content (desirable agronomic traits) and degree of PPD have been suggested (Plumbley and Richard, 1991; Sánchez *et al*., 2006; Aristizabal and Sánchez, 2007), These findings are supported by Oirschot *et al*., (2000), who found a negative correlation between PPD and sugar/starch ratio, but contrast with Wheatley and Gomez (1985; cited by Ravi and Aked, 1996), who found no correlation between PPD and starch content.

Wheatley and Gómez (1985, cited by Ravi and Aked, 1996), found that the primary deterioration tended to decrease with plant age. These findings contrast with Hirose and Data (1984) who suggest that PPD development is not strongly correlated with root age after testing the PPD response of roots from 5 to 10 months old plant.

There is a high direct correlation between low susceptibility to PPD and cassava varieties with high carotenoid content in its roots. This low susceptibility may be due to the antioxidant properties of the carotenoids by quenching reactive oxygen species (ROS), which has been involved in oxidative stress found in PPD (Sánchez *et al*., 2006; Aristizabal and Sanchez, 2007; Chavez *et al*., 2007).

Some studies have been conducted to determine the potential of tolerance to PPD in cassava accessions cultivated in Puerto Rico. Gonzales *et al.,* (1991) evaluated PPD in four cassava accessions (Forastera, Llanera, Serrallés and Tremesiana) using sensorial and qualitative scales. After the evaluation they found the most severe level of PPD in Tremesiana contrasting with lowest deterioration in Llanera accession. In addition, Torres-Ramos (2001) evaluated the PPD in eight cassava accessions used in Puerto Rico (Serrallés, CM 3064, CM 3311, PI12902, Trinidad 1456, Jamaica 18, Abuelo and Chilena) with and without waxing treatment. The results showed less deterioration in Serralles and Trinidad 1456, compared to the others cultivars.

2.5.5 Approaches to overcome PPD

There are three main approaches to overcome the PPD problem in cassava: (i) the use of improved storage techniques; (ii) conventional breeding and (iii) genetic engineering to produce target changes in metabolism (Westby, 2000).

To date there is not a general technique to store and preserve cassava roots commercially (Aristizabal and Sánchez, 2007). Traditional marketing and storage systems have been adapted to the perishability of root crops.These adaptations include, processing very close to the production

areas to ensure a daily supply of raw material, processing into storable forms at farm level and the general practice of traders to deal with small quantities of roots (Weham, 1995; Westby, 2000).Traditional processing methods are well adapted to process cassava into a number of final products characterized by extended shelf-life (Miche, 1984, cited by Wenham, 1995), however they are often very time-consuming and laborious (Wenham, 1995).

2.5.5.1 Traditional storage methods

Traditional approaches to rapid postharvest deterioration have been developed by producers. A common way of avoiding loss is to leave the roots in the soil after the period of optimal root development, until they can be immediately consumed, processed or marketed. This sometimes can be even up to two years in the soil (Wenham, 1995; Ravi *et al.,* 1996).

This practice has disadvantages because large areas of land are used by the standing crop, unavailable for additional agriculture production. In this practice roots may increase in size, but they become more fibrous and woody, declining palatability, and increasing the cooking time. Also field storing has negative effects increasing the root's susceptibility to pathogenic attack, and reducing the extractable starch (Wenham, 1995; Ravi *et al.,* 1996).

Another traditional practice is pruning, which consist in to remove all leaves and stems of the cassava plant 30 cm high and leave the roots for two or more weeks before harvest. When roots are harvested they deteriorated more slowly than those with the un-prunned plants (Plumbley and Richard 1991; Tanaka *et al*., 1984).

There are other traditional practices involving storing cassava in field conditions such as in pits, clamps trenches, or boxes. There are reports that American indigenous kept the roots successfully buried in the ground, and trenches. The technique was studied by Booth (1977) but results were variable.

Balagopal and Pajadma (1985, cited; by Ravi *et al*., 1996) reported a low cost technology for extending the shelf life of fresh cassava roots using piths of sandy soils. This method prolonged shelf life for more than two months. However, the roots became very sweet and had poor cooking qualities, becoming useful only for cattle feed.

Later traditional methods (pit, trenches and boxes) are based in the curing process; this process has been used commonly for enhancing the storage life of other root crops. Curing relies on the fact that at relatively high temperatures (25 and 40ºC), and high relative humidity (HR) (80 to 85%), wounds produced by harvest are healed and subsequent deterioration is limited. At temperatures above 40ºC primary deterioration usually takes place before wound healing. When the roots were exposed to 95% RH the wounds rapidly become infected by pathogens, and at 75% RH roots dried out rapidly, especially around wounds (Booth, 1975; Ravi *et al*., 1996).

The use of traditional techniques are not widespread and adopted on a commercial scale as they are considered rather labor intensive, difficult manage and are not always entirely effective (Wenham, 1995; Ravi *et al*., 1996).

2.5.5.2 Modern storage methods

Cassava is a relatively low cost product which can not normally support the cost of more sophisticated storage techniques. Although these techniques can result in extending cassava root shelf-life, some of them have disadvantages in terms of the investment required, convenience, and availability of materials (Ravi *et al*., 1996; Oirschot *et al*., 2000). From the list of modern techniques, deep freezing and waxing are being applied commercially to a limited degree considering the conditions under which much of the world's cassava is grown (Ingram and Humphries, 1972, cited by Booth, 1975; Ravi *et al*., 1996).

PPD can be reduced by keeping cassava roots in polyethylene bags. This technique reduces the transpiration and respiration due to high RH inside the bag, preventing PPD up to 4 weeks. However the incidence of decay was greater when the roots were packed in thick polyethylene bags (0.003-0.05 mm) (Ravi *et al*., 1996). The use of polythene bags for long distance transport is now being widely adopted in South America but successful conservation depends on the quality of the roots (minimal damage), protection from sun light, treatment with fungicide, and packing within three hours after harvest (Ravi *et al*., 1996).

Another common way of limiting PPD is covering cassava roots with wax. In this method roots are dipped in ordinary paraffin wax at a temperature of 90-95ºC for a few seconds after treatment with fungicide. Using these techniques roots could be stored for up to 2 months (Ravi *et al*., 1996; Aristizabal and Sánchez, 2007).

Cassava roots can also be stored for 2 weeks at 0 to 4ºC without any internal deterioration, the most favorable temperature for storing fresh cassava is 3ºC but after 4 weeks microbial infection takes place and it is increased through subsequent storage time. However, even after 6.5 months of storage in this temperature range, the part of the flesh that had no decayed usually is in excellent conditions and suitable for human comsuption or processing (Ravi *et al*., 1996, Oirschot *et al.*, 2000). At temperatures above 4 ºC roots developed the same symptoms more rapidly and were rejected after 2 weeks of storage (Ravi *et al*., 1996). Alternatively, roots or more usually pieces of root can be stored frozen. Averre (1967, cited by Ravi *et al*., 1996), found that cassava roots could be stored satisfactorily under deep-freeze conditions in polyethylene bags. After thawing, the roots were quite palatable, although some spongyness was present, and could be kept for a further 4 days. This technique is already in use commercially in many Latin American countries such as Brazil, Colombia, Costa Rica and Puerto Rico (Ravi *et al*., 1996).

2.5.5.3 Plant breeding and genetic engineering

Breeding and genetic modification are long-term strategies to tackle PPD. Improved storage has more immediate impact, but the level of the improvement will be limited by the roots inherent perishability (Westby, 2000).

Conventional breeding potentially could produce cultivars with resistance to PPD by using recurrent selection methods. However, tremendous efforts would be required to incorporate the agronomical trait into different cassava cultivars without altering the favorable characteristics of the parent genotypes (Westby, 2000).

Furthermore, the high heterozygocity and the low natural fertility of cassava (Zhang, 2002), the lack of genetic variability for resistance to PPD (Ceballos *et al*., 2004) and the presence of an inverse relationship between PPD and cassava root dry mater (Estevao, 2007) complicates the use of breeding to alleviate postharvest deterioration in cassava.

PPD analysis of F_1 from crosses of susceptible and moderately resistant cassava clones, estimates heritability of 0.64 for PPD (64% of PPD variation is due to genetic variation). These findings suggest that inheritance of post-harvest root deterioration is, at least partially, controlled by additive factors and quantitative rather than qualitative (Kawano and Rojanaridpiched, 1983).

Cortes *et al.,* (1999) and Estevao (2007) linked quantitative trait loci (QTLs) influencing PPD of cassava to molecular markers finding ten and three putative QTLs, respectively, which explain between 5-13% of the phenotypic variance of the PPD. Both emphasized the necessity of implementing an objective quantitative and systematic phenotypic evaluation of PPD as the final phenotype is affected by several genes.

Conventional breeding has not been successful in generating germplasm with significantly improved storage qualities (Taylor *et al*., 2004). The only source of significantly delayed post-harvest physiological deterioration (PPD) has been the identification of an interspecific hybrid between cassava and *M. walkerae* (Blair *et al*., 2007) named CW 429-1, which after 15 days after harvest still displaying no visible sign of deterioration (Estevao, 2007). However in a recent PPD test, Morantes *et al.,* (2010) found a comparative high level of PPD for the interespecific hybrid CW 429-1.

Genetic modification to overcome PPD by using molecular techniques is considered most appropriate to solve the problem, since genetic engineering makes possible to transfer new traits to cassava varieties without altering other desired traits. However, there is a lack of concrete information available about the genes involved in the biochemical pathways associated with cassava PPD. Deeper studies in the understanding of the biochemistry, molecular biology, and genetics of physiological deterioration, need to be carried out in order to provide important new information on the genes involved in key pathways to develop potential strategies for PPD control (Westby, 2000; Taylor *et al*., 2004).

2.5.6 Physiology changes under PPD

In order to identify potential means by which PPD may be controlled, it is important to explain the mechanism and understand the processes of deterioration from a physiological, pathological and biochemical point of views (Hirose, 1986; Buschmann *et al*., 2000b). Cassava postharvest physiological deterioration is a complex process, which after several decades of study is not fully understood. The process is considered to be similar to a typical abiotic wounding stress response (Westby, 2000; Beeching *et al*., 2002).

Plant wounding induces the production of signaling components that initiate the wound response. The initial wounding releases signal molecules that trigger protective or defensive responses locally or systematically. Such signal molecules are produced by the wounding itself (cell wall fragments, lipid peroxidation products), released from inactive precursor or synthesized *de novo*

such as jasmonic acid, salicylic acid, ethylene, systemin or the reactive oxygen species (ROS) H_2O_2 (Beeching *et al*., 2002).

The main responses of the plant to these signals include (1) the production of enzymes and defensive secondary metabolites such as glucanases and chitinases, phytolaexins and antioxidants; (2) the synthesis of wound repair and sealing molecules (such as callose, lignin and suberin) and (3) the insolubilisation of hydroxyproline-rich glycoproteins (HRGPs) by H_2O_2 (Beeching *et al*., 2002).

These compounds repair and seal the wound site removing the source of signals and down modulate the response, returning the plant to basal conditions. The wound response is present in the cassava root, however the healing process, and the resultant down-modulation of the signals in cassava are inadequate or too slow compared with other plant species. As a result the production of wound response signals is not switched off, triggering a continual cascade of wound responses throughout the root which is observed as PPD. Compared with the detached root, wound repair does occur if the root remains attached to the plant (Richard, 1981; Plumbley and Richard, 1991; Reilly *et al*., 2004). Cassava root is a storage for photosynthates and, is not a propagule with reproductive function thus there is no biological need to repair wounds. Efficient wound repair of the detached cassava roots has been lost during evolution (Really *et al*., 2004).

The phenomenon of PPD is considered to be mediated by enzymes because the development of vascular streaking is inhibited by hot water treatment (53ºC for 45 min), by keeping the root

under water or under anaerobic conditions, and because an inhibition of PPD and fluorescence associated (Section 2.5.6.2) was observed when cassava roots were treated with cycloheximide, a protein synthesis inhibitor (Uritani *et al*., 1984a; Ravi and Aked, 1996) (Figure 2.6).

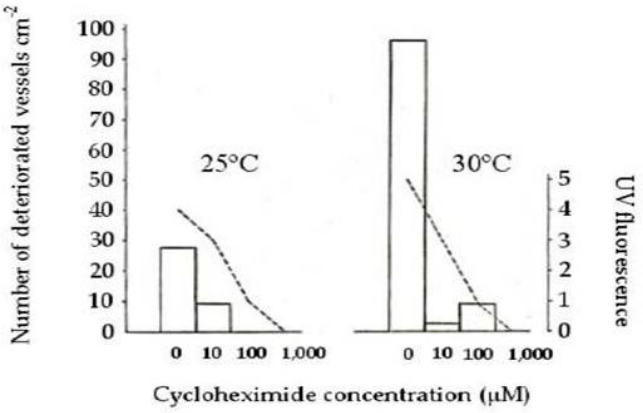


Figure 2.6 Effect of cyclohexamide treatment over cassava discs. The number of deteriorate vessels as a measure of PPD (Uritani *et al.*, 1984b).

2.5.6.1 Oxidative stress under PPD

From the beginning PPD was considered to be a product of an oxidative process. The findings of Noon and Both (1977) that anaerobic atmosphere and oxygen-depleted air inhibited the vascular streaking, while a bactericide and fungicide failed to inhibit the reaction suggested the involvement of oxidative reactions. In addition, decreases in the nonenzymatic antioxidants *β*-carotene and ascorbate have been reported during PPD (Iglesias *et al*., 1995, cited by Really *et al.,* 2004).

Peaks of ROS and increased activity of enzymes that modulate ROS have been detected during deterioration (Reilly *et al*., 2001). ROS refer to reactive molecules that result from the reduction of molecular di-oxygen. Under stress conditions, increased ROS formation often occurs through

perturbation of electron transport chains (photosystems, mitochondria), peroxisomes, endoplasmatic reticulum, and as result of photosynthesis excitation energy (Really *et al*., 2004). This 'oxidative burst' is defined as a rapid production of $^{\cdot}O_2$ and H_2O_2 in response to external stimuli. The ROS generated during the oxidative burst plays several complex and overlapping roles in facilitating plant defense. Essentially, these may be summarized as (1) cell wall strengthening, (2) induction of defense related genes, and (3) triggering of host cell death (Figure 2.7).

The concentration $^{\cdot}O_2$ and H_2O_2 are not toxic at cellular physiological concentrations, however, its toxicity arises because their roles as substrates in the iron catalyzed Haber-Weiss reaction. This reaction produces hydroxy radical ($OH^{\cdot}$), one the most reactive species known. Hydroxy radicals affect celuar homeostasis producing lipid peroxidation, protein denaturation and DNA damage (Really *et al*., 2004).

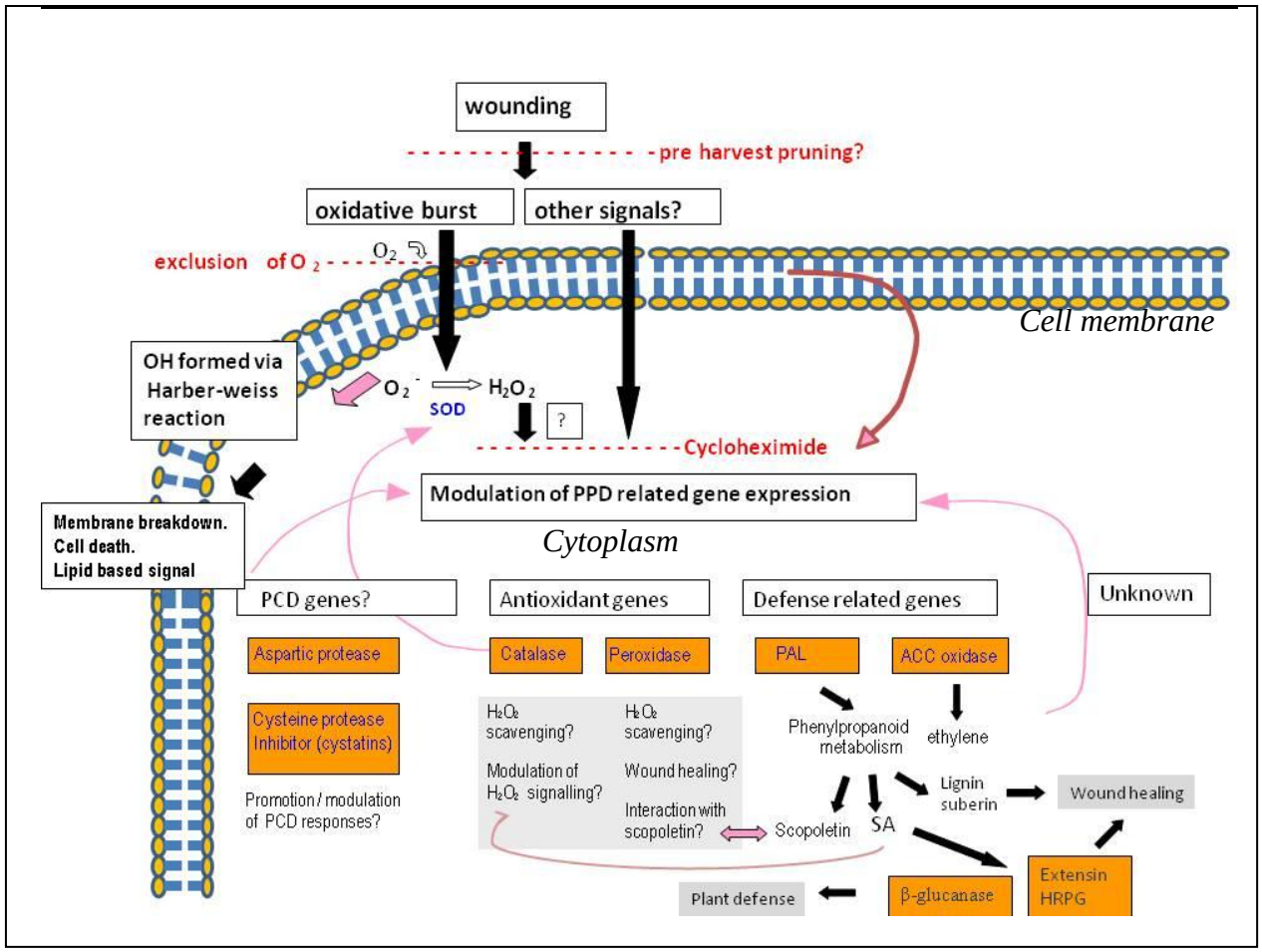


Figure 2.7 Model for PPD response based in Really *et al*., (2004) with some modifications. Possible effects of ROS (Reactive Oxygen Species) in signaling and cellular damage as well as the complex interactions with PPD modulation genes (in orange), and secondary metabolites are showed. *PCD* program cell death, *SA* Salicylic, Acid *PAL* Phenyl Alanine Lyase, *HRPG* hydroxyproline-rich glycoproteins.

In response to oxidative stress a number of enzymatic and non-enzymatic defenses have evolved to detoxify ROS and/or to prevent the formation of highly damaging and reactive forms, these enzymes include superoxide dismutase, catalases and peroxidases (Beechig *et al.*, 2002; Really *et al.*, 2004).

2.5.6.2 Accumulation of hydroxycoumarins

The initial visual symptoms of PPD are accompanied by a rapid accumulation of fluorescent compounds in the parenchyma of the cassava tissue. These compounds can be observable under ultraviolet light and have been identified as the hydroxycoumarins, which include scopolin (6 methoxy-7-hydroxy-coumaroyl-7β-D glucoside), scopoletin (6 methoxy-7-hydroxy coumarin) and esculin (6, 7 dihydroxycoumaroyl-6-β-D glucoside). The main phenolic components are catechin and gallocatechin. Other compounds that have been identified from cassava roots and that may play a role in PPD are the leucoanthocyanins, cyanidin, delphinidin and 22 diterpenoid compounds (Buschmann *et al*., 2000a). Hydroxycoumarins are produced in higher level in cassava root tissue block, contrasting with low levels detected in intact entire roots, and no detectable levels in intact entire roots from pruned plants. The high levels of hydroxycoumarins produced in tissue blocks may be due partly to wounding effect (Tanaka *et al*., 1984).

Scopoletin is not usually present or has very low levels in fresh roots. However, a few hours after harvest its concentration increases (Aristizabal and Sánchez, 2007). Wheatley and Schwabe (1985) found an increase of scopoletin during the first 24 to 48 h after wounding, using fluormetric measurements. Similar results were found by Buschmann *et al.,* (2000a) using HPLC. The first increase was followed by a decline, with a second smaller increase at 4 to 6 days (Figure 2.8). Uritani *et al*., (1983) found that scopoletin is produced at first, and subsequently scopolin and esculin are produced. The amount of scopoletin was maximal 20 hours after incubation, while the other two were maximal at around 40 hours.

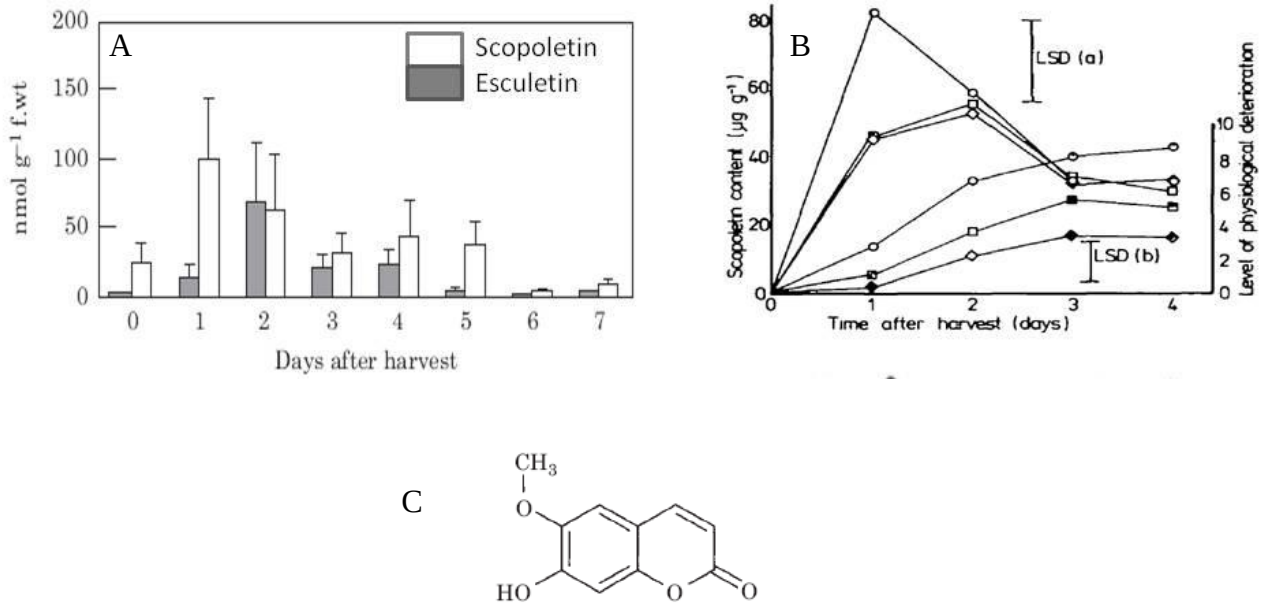


Figure 2.8 Quantification of scopoletin in cassava root slices (open bars) after 7 days of harvest using HPLC (Buschmann *et al*., 2000a). **B**. Quantification of scopoletin in cassava roots section (○)2-4 cm (□) 4-6 cm (◊)10-12 cm at 4 days of harvest using a fluorometer The level of PPD are showed (Wheatley and Schwabe, 1985). **C.** Chemical structure of Scopoletin.

Wheatle and Schwabe (1985) demonstrated that roots from plants whose tops were pruned off a few days prior to harvest were liable to deterioration but responded, vigorously to applied scopoletin; while roots stored in the absence of oxygen were no susceptible to exogenously applied scopoletin. This led them to suggest that pruning may be effective due to a reduction of internal scopoletin levels, while curing or oxygen depletion may involve loss of a scopoletin precursor or inactivation of the enzyme system.

These findings led to the assumption that scopoletin may be involved in PPD but the precise role is still unclear (Richard and Gahan, 1983, cited by Ravi and Aked, 1996). Scopoletin did not appear to diffuse into the xylem vessels and thereby contribute to occluding material. Wheatley

(1980, cited by Ravi and Aked, 1996) however, suggested that scopoletin was the substrate for deterioration rather than the inducer and it is oxidized to black pigments in the process.

The blue/black vascular streaking observed as PPD may result from peroxidase-mediated oxidation of scopoletin (Wheatley and Schwabe, 1985) All required components of the reaction, namely scopoletin, H_2O_2, and peroxidase activities, are localized near to the root xylem parenchyma vessels where vascular streaking symptoms occur (Really *et al*., 2004).

2.5.6.3 Respiration and PPD

Variation among cassava cultivars for the propensity to develop vascular streaking has been associated not only with varying levels of scopoletin, but also with differences in rates of wound respiration and wound ethylene production (Brench, 2003).

Roots as living organs of the plants continue to metabolize showing a rapid increase in respiratory rate after harvest (Westby, 2000). Experiments conducted by Hirose (1986) to examine the changes in respiration rate related to occurrence of PPD after harvest, showed that roots injured by removing cortex or periderm apparently have higher respiratory rates than intact roots. Respiratory rates of injured roots reached their peak on the first day of storage except those of the intact roots and 25% of periderm removed, in which the respiratory peak was delayed one day. Then the respiration rates decreased gradually until the 4^{th} day when they again increase, reaching their maximum on the 5^{th} or 6^{th} day (Figure 2.9).

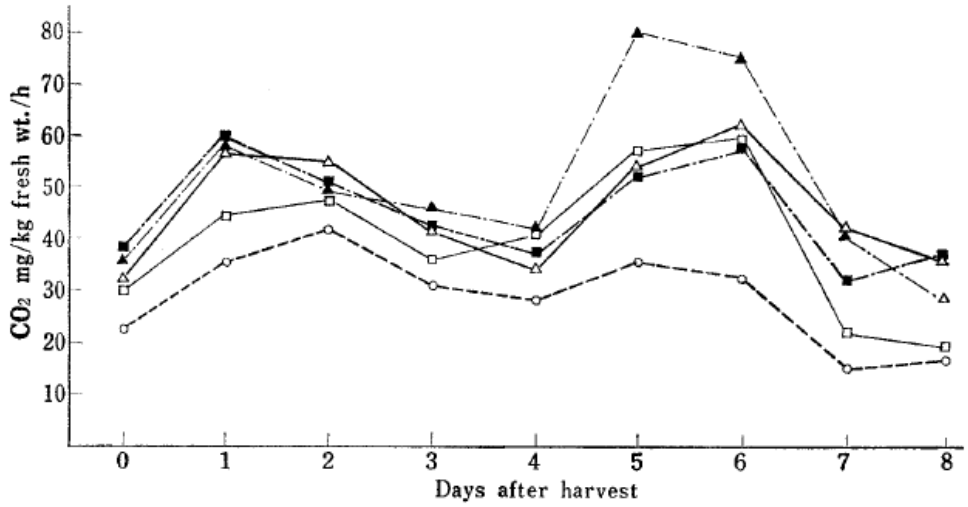


Figure 2.9 Respiratory rates of cassava pieces with different grades of injuries at 25ºC (Hirose *et al.*, 1984a) ○Intact root, □ 10 cm piece 25% periderm removed, ■10 cm piece 50% periderm removed, Δ 10 cm piece 25% cortex removed, ▲10 cm piece 50% cortex removed.

It was presumed that the first peak of the respiratory rate observed in cassava roots was due to the wound respiration induced by mechanical cutting and the second peak to biochemical changes induced by the development of PPD, since the rapid development of PPD on the 5th to 6th day coincided with the occurrence of maximum rate of respiration. On the 8th day, however the respiratory rate decreased to near the level at the time of harvest. Differences in respiratory rates particularly at one day after harvest were found among different cassava accessions under PPD (Hirose *et al*., 1984a).

Hirose *et al.*, (1984a) found initially that intact roots from pruned plants showed a lower respiration rate compared with unpruned plants at one day after harvest. The same behavior was observed when pruned and unpruned tissue blocks were compared one day after harvest. However, the respiration rate was always higher on the second day after harvest in the pruned plants. This difference in respiration rate observed only between intact roots and tissue blocks

was attributed to effective injury during preparation of tissue blocks, which have reduced the effect of pruning. In fact, tissue blocks from pruned plants deteriorated more rapidly than the intact tubers from unpruned plants without any injury (Ravi and Aked, 1996). However Hirose (1986) found that the respiratory rate of the roots taken from pruned plants was higher than the roots from unpruned plants. These results are contradictory with Hirose's previous work (Hirose *et al*., 1984a). Pruning treatment is one of most effective and low cost measures to reduce PPD but the mechanism involved is still unclear (Hirose *et al.*,1984b).

2.5.6.4 Metabolic compartmentalization

Uritani *et al.*, (1984a) differentiated the cassava root pith into three main parts: the A part (outermost parenchymatous tissue, 2 to 3 mm thick and adjacent to the peripheral cortex), the B part (the intervening tissue 7 to 8 mm thick between A and C parts), and the C part (the innermost tissue, 16 to 18 mm tick) (Figure 2.10).

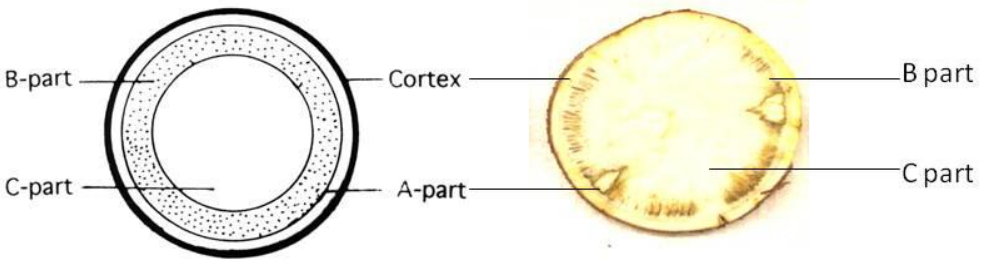


Figure 2.10 Cassava cross section showing the metabolic zones identified by Uritani *et al*. (1984a). Left: Illustrative diagram. Right: Cassava root cross section.

Uritani *et al*., (1984a) and Kojima *et al*., (1983) reported that the primary deterioration, involving brownish discoloration or vascular streaking, occurred in the intervening B part. The B part is

mainly composed of parenchymatous tissue and does not contain xylem vessels. Thus the parenchymatous cells in the B part may have specific characteristics different from those in the A and C parts. They also found that the moisture content in the B part was lower than in A and C parts, implying larger content of starch granules and a specific structure of the membrane system. They further reported the production of secondary metabolites, including coumarins and phenols and some enzymes involved with the production of these secondary metabolites in all three parts simultaneously, but often secondary metabolites were produce more strongly in the B part than the in the A and C parts. These results indicate that the stimulation of secondary metabolism is strongly associated with the occurrence of physiological deterioration (Uritani *et al*., 1983).

Tanaka *et al*., (1984) found that enzymatic activities (Phenyl alanine lyase, peroxidase, and acid invertase) and coumarin accumulation in pruned intact roots, was lower compared with unpruned intact roots. In the B-part (the site of physiological deterioration) of the unpruned roots, all enzymes showed the maximal activity compared with others parts. This increase in activity in the B part of unpruned intact roots seemed to be related directly with PPD, because enzymatic activity in all parts (A, B and C) of block tissue from pruned and unpruned plants was very similar. In block tissue the effect of wounding could be masking the PPD effect.

When the A, B, and C parts were separated from the tissue blocks and incubated individually, the biochemical changes were induced in all parts, but PPD take place only in the B part. This suggested that the biochemical changes in the B part are directly related with the occurrence of PPD and those in A and C part do not participate directly in the induction of PPD. These

observations further led them to speculate that the A and C part participate indirectly in the enhancing of the occurrence of PPD in the B part through biochemical and cytological changes (Uritani *et al.* 1983).

2.5.6.5 Ethylene and PPD

Phytohormons seems to be involved in cassava roots PPD. Ethylene is produced in cassava roots slices prior to the appearance of PPD symptoms, and might influence PPD by altering the respiratory pathway while also changing the activity of peroxidase enzyme (Plumbey *et al*., 1981, cited by Hirose, 1986). In addition, work with indoleacetic acid and napthaleneacetic acid indicated that auxins would enhance root deterioration (Plumbley and Richard, 1991).

Using transversal slices of cassava roots, Hirose (1986) found that ethylene production began after 15 to16 hours of incubation, then increased gradually and reached a maximum one day after incubation. Ethylene production decreased gradually and disappeared 44 hours after incubation, however differences in rate of ethylene accumulation were found among cassava accessions. When ethylene production in deteriorated root slices and root slices without deterioration was compared, it showed that the former produced four times as much ethylene as the latter. Curiously no clear difference in ethylene production was observed between the root tissue blocks taken from pruned plants and those taken from unpruned plants (Hirose *et al*., 1984b).

Ethylene production was compared in four histologically different parts of cassava roots (cortex, A, B, and C parts). Hirose *et al*., (1984b) found that each part produced ethylene at different rates,

and this varied among cassava accessions. Interestingly, the B part which normally developed deterioration, did not produce significantly more ethylene than the other parts.

Positive correlation between development of PPD and respiratory rate, and between development of PPD and ethylene production in the root or tissue blocks respectively is suggested, but no clear relationship was obtained between respiratory rate and ethylene, in spite of the fact that the ability of ethylene to stimulate respiration of storage plant tissues and fruits is well known. Thus more data should be accumulated to confirm the relationships among ethylene production, respiratory rate and postharvest deterioration of cassava roots (Hirose, 1986).

2.5.6.6 Others metabolic pathways involved

One or two days after the cassava root have been harvested, a rapid increase in total sugar content of roots was observed, accompanied by a decline in starch content and dry weight occurs. Because the roots from unpruned plants showed a significantly lower weight compared with those of pruned plants, it seems that the water loss accelerates PPD. The development of cracks due to dehydration, coupled with high sugar content later encourages the entry of microorganism and subsequent root decay (Booth, 1975; Maini and Balagopal, 1978; Ravi and Aked 1996).

Some authors report an accumulation of cyanogenic glucosides, a decrease in linamarase activity (a key enzyme in cyanogenic glucosides breakdown and release of HCN), and a slight decline in HCN content under PPD (Maini and Balagopal, 1978; Kojima *et al.*, 1983; Westby 2000). This reduction of linamarase activity and HCN is not uniform trought the cassava root; there is

evidence of a radial gradient from cortex to C part in terms of cyanide content and linamarase activity (Kojima *et al*., 1983).

Gloria and Uritani (1984) found that a decrease in β-carotene is correlated with severity of PPD especially in B and C parts; it may also be possible that either lipoxygenase or a kind of β-carotene-bleaching enzyme is produced in parallel with the appearance of PPD. It would be useful to study the mechanism, considering the loss of β-carotene observed in the C part, where PDD is not induced as well as the B part, the area of PDD occurrence.

Oxidation of polyphenols may play a significant role in PPD. Padmaja *et al.,* (1982) evaluated the total phenol and tiritable acidity changes, and polypenol oxidase in six cassava varieties. In all varieties a decrease in total phenols was observed during the second and third day of storage. This was followed by a slight increase on the fourth day of storage. There was a concomitant increase in polyphenol oxidase during the second day of occurrence of vascular deterioration.

Padmaja *et al*., (1982) hypothesized that browning or bluish browning color is caused by disorganization of the membrane system in the B part cells which release the substrates and enzymes. The B part membrane system disorganization leads to free oxidation of phenols such as (+)-catechin by polyphenol oxidase. The polyphenols may be enzymatically oxidized to highly reactive quinones, which may form colored complex on deposition in the vascular bundles with aminoacids and other micromolecules of cells. These studies indicate that polyphenol oxidase would be a key enzyme associated with vascular deterioration of cassava roots. However this

hypothesis was challenged by Richard and Gahan (1983, cited by Ravi and Aked 1996) who did not detect the production of quinone in cassava roots showing PPD.

The results of these studies suggest that tolerance to PPD can be found in different sources and they seem to be acting through different biochemical/genetic mechanisms. It is therefore justified to encourage other cassava scientists to systematically evaluate for additional sources of tolerance (Morantes *et al*., 2010).

2.6 Gene expression analysis of cassava roots

At the molecular level the development of PPD is evidently a complex phenomenon involving multiple components. By using a diverse array of experiments, such as cycloheximide inhibition of protein synthesis, in vivo labeling of proteins, directed and random sequencing of cDNA clones corresponding to genes that are expressed during PPD, together with cDNA-amplified fragment length polymorphism (cDNA-AFLP) and Northern hybridization experiments, clearly indicates PPD is an active rather than a degenerative process, involving changes in gene expression and protein synthesis (Really *et al*., 2004).

Several analyses of genes expressed during PPD show altered regulation of proteins and enzymes involved in signal transduction, ROS modulation, phytohormone synthesis, senescence and programmed cell death (PCD) responses, synthesis of anti-microbial, anti-oxidant or other defensive compounds, and the formation of other compounds that are involved in the synthesis of cell wall components, as well as proteins that are themselves components of the cell wall (Really *et al*., 2007; Buschmann *et al*., 2000a).

By using cDNA AFLP approach, Huang *et al*. (2001) isolated 70 transcripts derived fragments (TDFs) related with the regulation of gene expression during PPD. Based on the sequence homology of these TDFs, they catalogued 24% as metabolism, 22% stress/wounding, 12% signal transduction, 8% development, 6% programmed cell death and 28% unknown.

Really *et al*., (2007), using microarrays and a cDNA library of early and late cassava PPD, identified 72 genes whose expression changed significantly under PPD. In addition, a comparison between the expression in harvested storage roots and unwounded and wounded leaves led to the identification of a subset of 21 storage-root-wounding-specific transcripts potentially PPD specific. Components of these cDNAs and their corresponding genes could serve as potential tools in genetic constructs for exploring the nature of the PPD response and even to approach modulating that response in transgenic cassava.

2.7 Gene expression profiling and real time PCR

In gene expression profiling, the activity of several or thousands of genes is measured (as relative amount of mRNA expressed) under particular treatments or experimental conditions. Transcriptome analysis should make it possible to identify and dissect complex genetic networks and critical physiology processes, characterization of those gene networks in plants help to understand the molecular basis of plant physiological processes as weel as identify new targets for manipulating biochemical, physiological, and developmental processes (Alba *et al*., 2004).

Expression profiling has become a key tool to investigate how plants respond to environmental challenges. Plants can modify their gene expression patterns in response to environmental changes or biotic and abiotic stresses. In some cases these transcriptional changes leading to successful tolerance, while in other cases the trancriptional changes fail to adapt the plant to the new environment producing a susceptible phenotype. Expression profiling can help describe both tolerant and susceptible responses. These plant response profiles to external changes are expected to help identify genetic elements (regulator, genes) that will be useful in biotechnological approaches to improve stress tolerance (Hazen *et al*., 2003).

In order to study the function of a gene it is necessary to develop the ability to accurately quantify transcription levels of that gene, identifying temporal and spatial patterns (Bustin, 2000; Peirson *et al*., 2003). Real-time polymerase chain reaction or quantitative PCR (QPCR) has been dramatically changing the field of measuring gene expression. Real time PCR is a sensitivity technique of collecting data using PCR process as it occurs, allowing the amplification and quantification of a specific sequence with detection in real time of the PCR product accumulation. This is done using a variety of different fluorescent chemistries such as intercalating dyes (SYBR Green®) or alternatively fluorogenic probes that correlate the fluorescence intensity obtained with concentration of the PCR product (Wong and Medrano, 2005).

Real Time PCR reactions are characterized by the PCR cycle where the amplification target (DNA or cDNA) is first detected. This value is usually referred to as cycle threshold or Ct, the cycle at which fluorescence intensity detected is greater than background fluorescence.

Consequently, the greater the quantity of amplified PCR product in the starting material, the faster is the increase in fluorescent signal producing a lower Ct (Wong and Medrano, 2005). In addition, the final PCR product can be further characterized by subjecting temperature gradient (dissociation analysis) to determine at what temperature the double-stranded PCR product "melts" which is called the melting point. This melting point is a distinctive property which dependent on product length and nucleotide composition (Gachon *et al*., 2004).

The introduction of real-time PCR has allowed a faster, effiecient and high reproducible throughput gene expression quantification, with a wide dynamic range and high sensitivity. Real time PCR is fast becoming an important alternative for the quantification of gene expression and is often suggested for the validation of microarray data (Peirson *et al*., 2003). This preference is based in the questioned reliability of microarray experiments. Plant genomes show a high number of multigene families, which lead to cross-hybridization between cDNA of representative members of gene families as would occur in microarray experiments leading to false interpretations (Gachon *et al*., 2004). Also, artifacts in microarrays make it difficult to get reliable gene expression data or reach adequate statistical power with current microarray technology. Therefore, real-time PCR is considered as a supporting technique to more accurately validate and quantify the expression of interesting candidate genes identified by microarrays (Valasek and Repa, 2005).

A common strategy is to validate candidates using real-time PCR analysis after the identification of potentially interesting genes with microarray experiments. Real-time PCR also has the

advantage to move analysis further than confirmatory only studies. It can be used either to determine fine-tuned kinetics in gene expression becoming a reference to compare different methods of microarray analyses (Gachon *et al.,* 2004).

Real-time PCR data analysis methods are generally classified as absolute or relative approaches. Absolute quantitation uses serially diluted standards consisting of DNA, cDNA or plamid DNA at known concentrations to generate a standard curve. This standard curve produces a linear relationship between Ct and initial amounts of cDNA, allowing the determination of the concentration of unknown samples based on their Ct values assuming equal efficiencies in the PCR amplifications (Bustin, 2000).

On the other hand, in relative quantitation, the changes in gene expression are measured based on relative expression (normalization) of a target gene versus a reference gene or housekeeping gene which is not regulated by the experimental treatments or environmental conditions being tested. The results are expressed as a target/reference ratio and there are several mathematical models available to calculate it. Housekeeping genes are necessary for basic cell survival. The expression of these genes is considered to be stable under experimental treatments but numerous studies have already shown that presumed housekeeping genes are regulated and can sometimes vary under experimental conditions (Pfaffl, 2001).

The original mathematical model for relative quantification assumes ideal amplification efficiency (100%) for all genes and then to compare their Ct values directly. This method was

subsequently modified to include PCR efficiency obtained from standard curves. However, production of a standard curve is time consuming, requiring repeatable and reliable standards. Others have been using the kinetics of individual PCR reactions based in shape of the exponential curve of the raw PCR fluorescence data. However, there are some difficulties due to noise of PCR signal (Zhao and Fernald, 2005). Recently Zhao & Fernald (2005) developed a noise-resistant algorithm to calculate the efficiency and Ct from individual PCR reactions. This algorithm (Miner®) is independent of the real-time PCR platform and is characterized by the use a strictly objective criteria to determine the PCR efficiency.

2.8 Objectives and Hypothesis

2.8.1 Main objective

To contribute to the understanding of the physiological changes that take places in cassava roots under PPD by means of gene expression profiling.

2.8.2 Specific objectives

A. To evaluate the cassava root PPD tolerance of Puerto Rican cassava germplasm.

B. To validate a time course expression profile of a set of genes reported previously as PPD related for two cassava accessions with low and high levels of PPD by means of real time PCR.

C. To evaluate a time course expression profile of a set of genes involved in main metabolic pathways for two cassava genotypes with low and high PPD by means of real time PCR.

2.8.3 Hypotheses

Ho: There are no differences in gene expression profiling between two accesions with high and low PPD.

H_1: There are differences in gene expression profiling between two accesions with high and low PPD.

3 Physiological postharvest deterioration assessment of Puerto Rican cassava germplasm collection.

3.1 Summary

Roots from the Puerto Rican cassava germplasm collection, maintained in the field at Agriculture Research Station of the University of Puerto Rico Isabela, Puerto Rico, were evaluated for PPD tolerance. The objective was to identify two cassava accessions with contrasting responses to PPD. Two different analyses were made in an effort to quantify the susceptibility of PPD: 1) a visual inspection and 2) image analysis of root slices exposed to UV light.

3.2 Materials and Methods

3.2.1 Plant material

Puerto Rican cassava germplasm involves 25 different cassava accessions from breeding programs: PI12900, PI12902, PI 12903, CM 3064, CM 3311-3, CM 3380-7, CM 4484-2, CM 523-7, SM 494-1, 60444 (TMS), and MCol 2215, and landraces: Amarillo, Brava, Abuelo, Senon, Jamaica 18, Tremesiana, Forastera, Chilena, Seralles, Seda, Cubana, Trinidad, Valencia and Llanera. Cassava accessions were grown from stakes from January 5 2008 to November 6 2008, at the Isabela Agriculture Experimental Station of the University of Puerto Rico, located in the northeast coast of Puerto Rico 130 meters above sea level.

3.2.2 Cassava root phenotyping under PPD

Five intact roots were taken randomly from five different plants from each cassava accessions. In all cases, the entire root was removed carefully from soil rhizosphera avoiding any mechanical damage (scratch, cuts or root tip injury). Root peduncles were removed using a knife. The roots were stored for a period of five days exposed to air but protected from direct sun light and rain. During this time temperature and relative humidity were recorded every three hours using a hygrothermometer (Control Company, Friendswood, TX, USA).

Root length and maximum diameter were measured after five days. PPD data acquisitions were performed according to the following procedure (Figure 3.1).

i) Transverse cuts (root sections) of the roots were made at 15, 30, 45, 60, 75, and 90 % of the total root length using a knife (in total six sections). Each section was subjected to the following two methods of PPD analysis.

ii) *Method 1.* A score for postharvest deterioration based in the area of part B (Figure 2. 10) was assigned to each root section, according to the method described by Wheatley (1982). Basically a score of 1 is 10% of PPD, a score of 2 is 20% of PPD, and so on.

iii) *Method 2.* A transversal slice of ≈ 5 mm was cut from the proximal end of each root section and exposed to UV light at 260 nm using a VWR® bench top UV transilluminator. Fluorescence was captured by using a digital camera (Casio EX-S10).

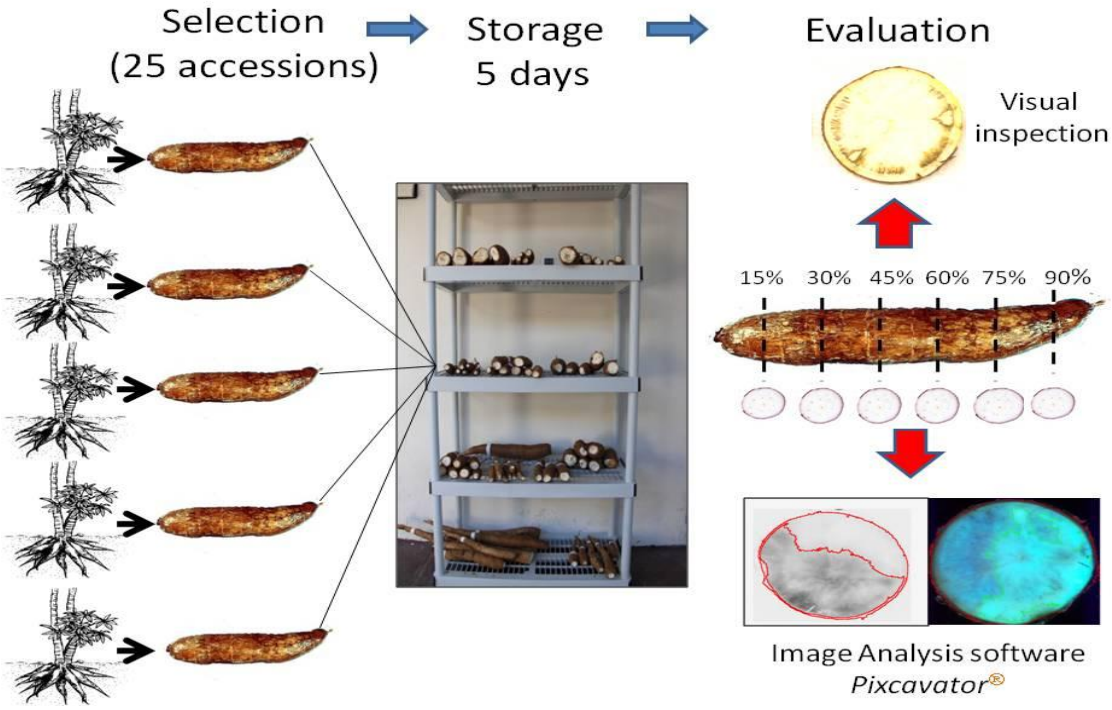


Figure 3.1 General strategies for PPD phenotyping data acquisition.

The PPD scoring for each root sections were averaged to obtain a percentage of deterioration for each root as a follows:

$$\%PPD = \frac{\sum_{i=1}^{n} PSi \cdot x100}{60} \qquad \textbf{Equation 1}$$

PSi: PPD scoring in root section

The digital pictures of the transverse section under UV light were analyzed by using Pixcavator Image Analysis Software v. 3.1 (Intelligent Perception Huntington, WV, USA) with the following settings: JPG format, green-blue channel, and a shrink factor 4. Contrast and growing rates were set until fitting non fluorescent area and total area of the root slide. The non

fluorescent area (showing the absence of hydroxycoumarins) was taken in account to calculate the percentage of fluorescence according with:

$$\%Fluorescence = \sum_{i=1}^{n} \left[1 - \left(\frac{NFAi}{TA}\right)\right] X100 \qquad \textbf{Equation 2}$$

Where:

NFAi: Non fluorescent area

TA: Total area.

3.2.3 Dry matter content assessment

Dry matter weight of each accession was measured in the field using the "specific gravity method" developed by Toro and Cañas (1979, cited, by Aristizabal and Sánchez 2007). Other roots, not used for the PPD analysis, were utilized for dry weight determination.

Approximately 3 kg of roots were measured using a scale to obtain dry weight on air (*DWA).* The same 3 kg were weighted by floating in water to obtain dry weight on water (*DWW).*

Specific gravity (SE) was then calculated by:

$$SE = \frac{DWA}{DWA - DWW} \qquad \textbf{Equation 3}$$

The percentage of dry mater is calculated by using the expression

$$\%\ Dry\ matter = (SE \times 158.26^{1}) - 142.05 \qquad \textbf{Equation 4}$$

1 These numbers are results of the International Center of Tropical Agriculture experience, calculating dry matter percentage in cassava roots from 10 to 12 months to SE values between 1.0200 to 1.1900

3.2.4 Statistical analysis

The statistical analysis to compare the PPD response of 25 cassava accessions was done using an analysis of variance (ANOVA) for completely randomized design (α=0.05). The dependent variables were the percentage of deterioration (%PPD, Equation 1) and percentage of fluorescence (Equation 2). Data adequacy for ANOVA comparisons was evaluated through the Shapiro-Wilks normality test, homoscedasticity was confirmed using graphs of residual vs predicted values (Appendix 2). Data transformations were made using Arcsin function. Significant differences were measured by F distribution. Multiple comparisons were made using Tukey's procedure.

The relationship between percentage of deterioration scores, percentage of fluorescence, root length, dry weight and maximal root diameter were assessed using linear regression (α=0.05) by taking the mean score of each cassava accession. All statistical analyses were carried out using InfoStat® *Software Estadístico* v. 2009 Grupo Infostat (Universidad Nacional de Córdoba, Argentina). Percentage of deterioration (% PPD) and percentage of fluorescence were used as dependent variables.

3.3 Results

Puerto Rican cassava germplasm collection was evaluated for PPD response. Cassava roots were evaluated five days after harvest using two methods: a visual inspection methodology based on Wheatley (1982) and by image analysis of the fluorescent accumulation area observable under UV light (Uritani *et al*., 1983). Additional morphological measurements were noted (root length,

maximum root diameter and dry weight) to investigate its potential relation with PPD. On average, selected roots were 27.6 cm length (range 19 -85 cm) with an average of maximum root diameter of 4.9 cm (range 11.5 -2.5 cm) and average for dry matter content of 35% (range 25% - 42%) (Table 3.1).

To determine the existence of a relationship between two methods of PPD evaluation, a linear regression analysis was performed. Linear regression using average values did not find a relationship between the two methodologies (r^2=0.007, p=0.81) (Appendix 1) (Figure 3.1, Figure 3.2).

Since entire roots were used, the effect of PPD was evaluated by averaging the PPD scores of the five slices per cassava accessions. Every slice was inspected by both methods. The proximal end sections showed highest scores of PPD compare with distal end sections using visual inspection. This trend was less evident using the fluorescent accumulation method (Figure 3.3).

Cassava Varieties	PPD visual (%)	PPD Fluorescent (%)	Max.Root Diameter (cm)	Root length (cm)	Dry mater (%)
SM 494-1	**1 ± 1.3[A]**	68 ± 6.8	3.9 ± 1.0	33.8 ± 8.8	0.31
Amarillo	**2 ±1.0[A]**	**89 ± 9.9[B]**	4.8 ± 1.0	26.8 ± 9.7	0.34
PI12900	**3 ± 0.4[A]**	45 ± 7.6	6.8 ± 0.7	32.2 ± 11.9	0.37
Brava	5 ± 3.4	34 ± 5.7	3.2 ± 0.5	28 ± 17.9	0.32
CM 3380-7	7 ± 3.4	76 ± 6.9	5.0 ± 1.8	30.6 ± 4.7	0.33
PI2902	9 ± 2.0	30 ± 3.6	6.9 ± 0.8	22.6 ± 2.5	0.33
Abuelo	13 ± 3.3	**85 ± 3.1 [B]**	6.5 ± 2.7	25 ± 4.6	0.36
Senon	13 ± 8.6	**83 ± 5.9 [B]**	6.7 ± 6.4	27.4 ± 7.6	0.40
Jamaica 18	13 ± 4.5	31 ± 4.0	5.8 ± 0.9	24.2 ± 4.6	0.39
Tremesiana	22 ±9.6	40 ± 12.5	4.1 ± 0.4	29.6 ± 8.8	0.40
PI12903	24 ± 8.4	33 ± 11.2	5.6 ± 0.6	58.2 ± 17.9	0.37
60444 (TMS)	24 ± 2.6	70 ± 9.9	4.7 ± 0.9	22.8 ± 7.2	0.29
Forastera	27 ± 6.1	**15 ± 4.7[A]**	4.6 ± 0.6	37.6 ± 17.5	0.43
Chilena	30 ±10.8	62 ± 8.0	5.2 ± 1.0	24.6 ± 5.1	0.31
Serralles	32 ± 5.5	27 ± 5.2	4.8 ± 0.3	22 ± 2.9	0.33
CM 4484-2	32 ±16.3	79 ± 5.3	6.4 ± 1.3	33 ± 14.7	0.34
Seda	33 ±5.1	39 ± 4.1	2.8 ± 0.5	23.4 ± 4.2	0.39
CM 3311-3	38 ± 6.1	66 ± 5.2	5.4 ± 1.6	22.4 ± 9.0	0.40
Cubana	51 ± 5.3	39 ± 7.1	4.0 ± 0.4	27.2 ± 9.8	0.37
CM 3064	57 ±17.7	64 ± 6.8	4.8 ± 0.5	24.2 ± 6.5	0.35
Trinidad	58 ± 17.3	33 ± 8.3	5.0 ± 1.6	28.2 ± 15.5	0.25
SM 523-7	61 ± 14.9	69 ± 3.2	3.5 ± 0.6	19.0 ± 8.8	0.37
Valencia	64 ±8.2	69 ± 8.9	4.3 ± 0.5	28.2 ± 5.1	0.32
Llanera	67 ± 13.2	45 ± 6.7	3.0 ± 0.6	20.4 ± 5.2	0.33
Mcol 2215	**70 ±7.0[B]**	64 ± 5.8	4.3 ± 0.8	20.2 ± 5.5	0.36

Table 3.1 Evaluation of 25 cassava accessions for Physiological Postharvest Deterioration (PPD) under two methodologies (PPD visual inspection and PPD florescent accumulation). Average of maximal root diameter, root length and dry matter are shown. [A] Cassava accessions with significant low PPD, [B] Cassava varieties with significant high PPD. ± Standard deviation.

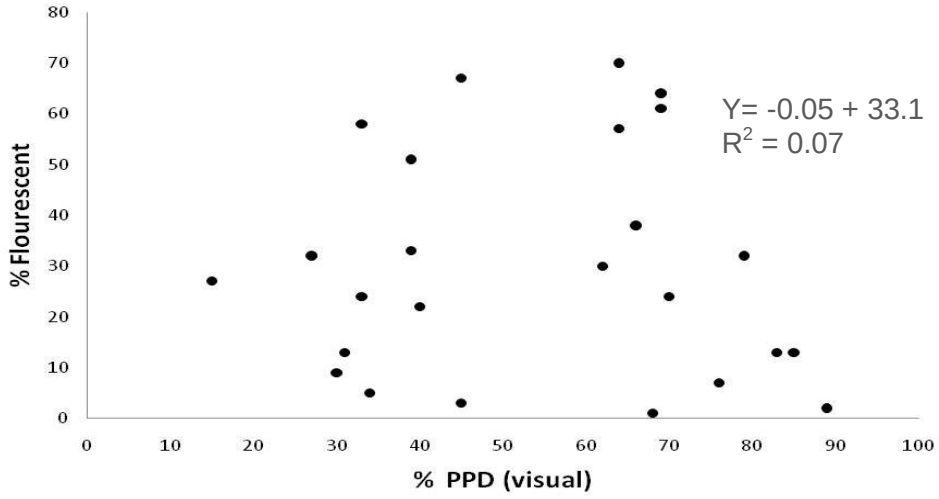


Figure 3.2 Association between PPD scores using florescence accumulation and PPD scores based in visual method (no transformed data) for 25 cassava accessions five days after harvest. The regression analysis was based on PPD data transformed by Arcsin fuction.

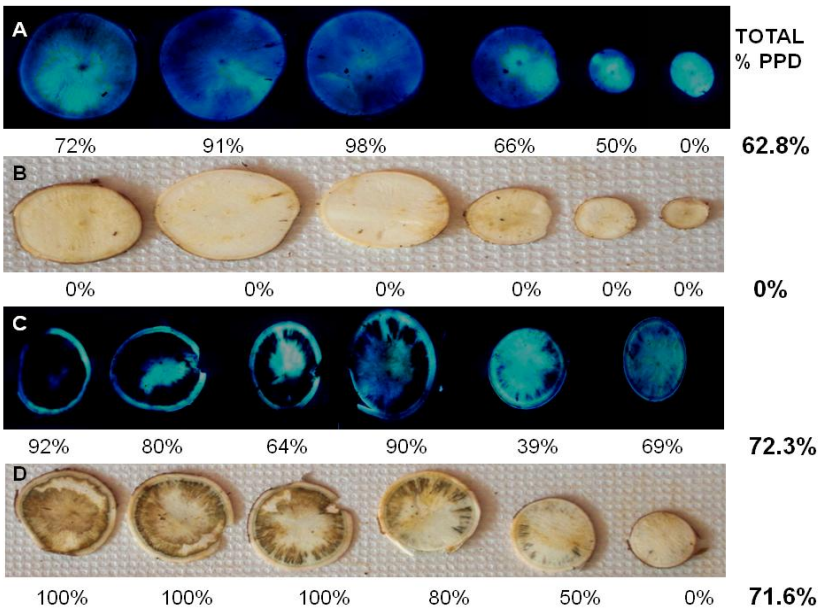


Figure 3.3 Score comparison between florescent accumulation and visual symptoms of PPD in two cassava accessions, one replication are showed. Fluorescent accumulation scores were obtained by means of image analysis software (Pixcavator v. 3.1). Visual scores based in methodology described by Wheatley *et al*. (1982). **A**. SM 494-1 fluorescent accumulation scores under ultraviolet light. **B**. SM 494-1 visual scores. **C**. Mcol 2215 fluorescent accumulation scores under ultraviolet light. **D**. Mcol 2215 visual scores

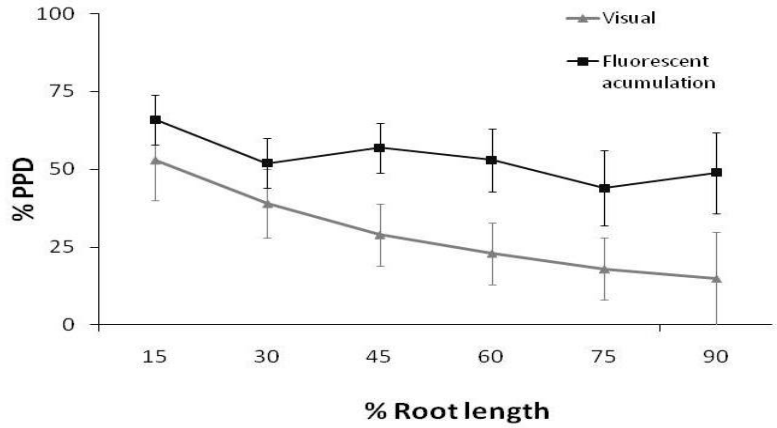


Figure 3.4 PPD scores average five days after harvest from 25 cassava accessions using entire roots. Average scores are shown in different sections based in the percentage of root length. PPD Scores (% PPD) were obtained by visual inspection and fluorescent accumulation methods. Bars represent standard error ($s / \sqrt{n}$).

There was no a linear relation between PPD (visual inspection method) and the root length, (r^2=0.09, p=0.13), main root diameter (r^2=0.18, p=0.06) and dry weight (r^2=0.01, p=0.61) as shown in figure 3.4, figure 3.5 and figure 3.6, respectively.

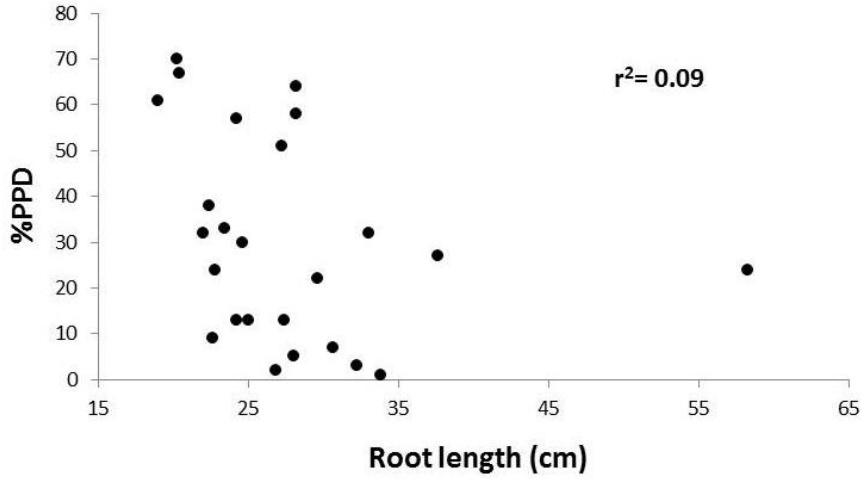


Figure3.5 Association between PPD scores based in visual method and root length for 25 cassava accessions five days after harvest.

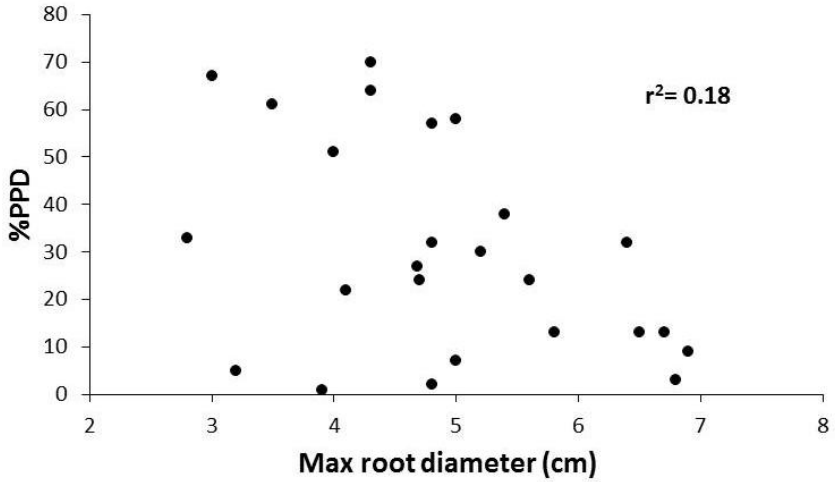


Figure 3.6 Association between PPD scores based in visual method and maximal root diameter for 25 cassava accessions five days after harvest.

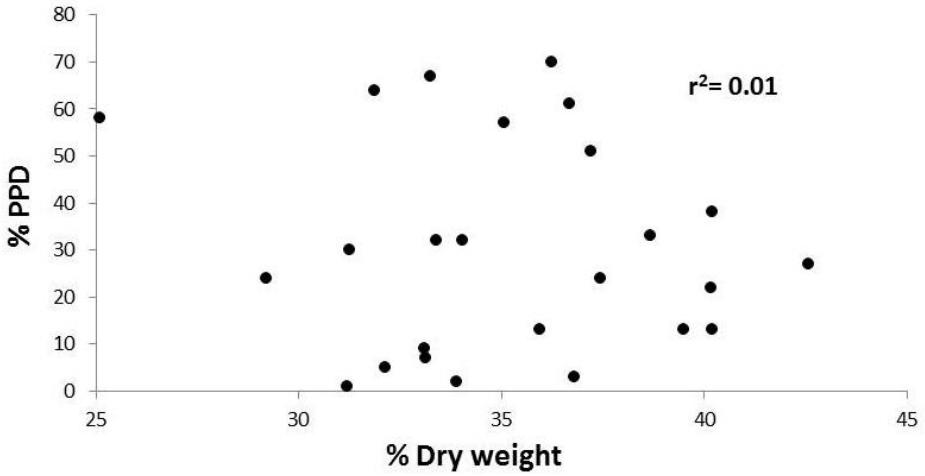


Figure 3.7 Association between PPD scores based in visual method and dry weight.

An analysis of variance (ANOVA) was made using scores from the visual inspection and fluorescent accumulation methods (five repeats per cassava accession), in order to identify the cassava varieties with significant low and high PPD. Significant differences were found ($p < 0.0001$) among cassava accessions. Multiple comparisons showed that accessions SM 494-1, Amarillo, and PI12900 under visual inspection and accessions Forastera under fluorescent

accumulation method obtained the lowest scores for PPD after five days of storing. Comparatively, accession Mcol 2215 under visual inspection and accessions Senon, Amarillo and Abuelo under fluorescent accumulation showed the highest scores for PPD after five days of storage (Table 3.1) (Appendix 2).

Temperature and relative humidity were measured during the five days (November 6 to November 11 2008) of PPD assessment experiment. During this storage the average temperature was 27.1 ºC (ranging from 24 ºC to 30.1 ºC) and the average relative humidity was 77.8% (ranging from 61 to 93%). Temperature and relative humidity were also measured during the time course experiment, performed during January 9 to January 13 2009, using cassava accessions with contrasting levels of PPD. During this period of storing the average temperature was 24.8 ºC (ranging from 20 ºC to 32ºC) and the average relative humidity was 85.3% (ranging from 47 to 97%) (Figure 3.8).

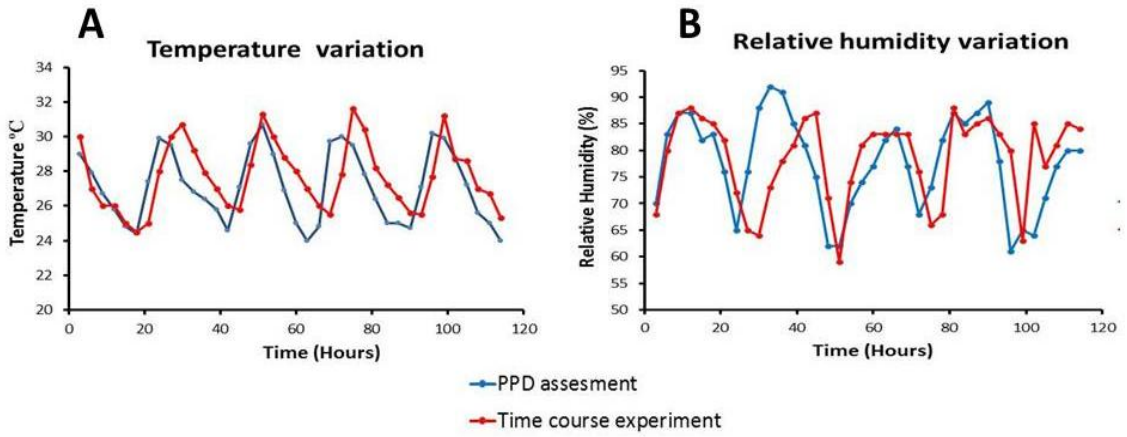


Figure 3.8 Variations in temperature (A) and relative humidity (B) during storage period for PPD physiological postharvest assesment for 25 cassava accessions (blue) and time course experiment using PPD contrasting accessions (red).

After analysis of the results obtained from the physiological test, the visual inspection method was chosen as the most reliable method to evaluate PPD and cassava accessions. SM-494-1(low PPD) and Mcol2215 (high PPD) were selected as cassava accessions with the most contrasting levels of PPD (Figure 3.8). These accessions were then used in the gene expression profiling experiment.

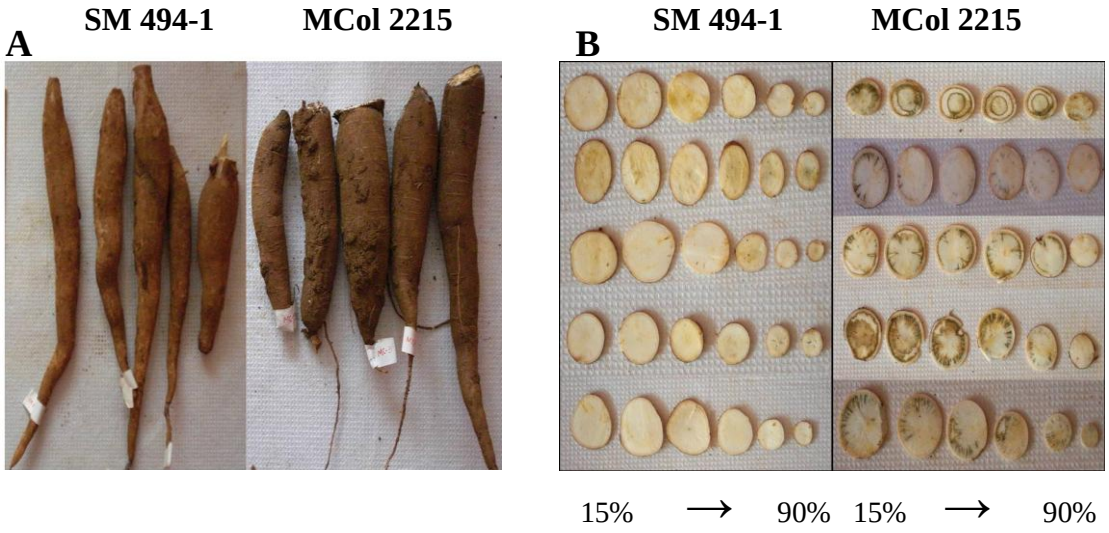


Figure 3.9 Selected cassava accessions with contrasting levels of deterioration. **A** General root morphology. The root length average for MCol 2215 was 33.8cm (± 8.8) The root length average for SM494-1was 20.2 cm (± 5.5). **B** Transversal cassava root sections from proximal to distal end (15 to 90 % length) after five days of storage. Average of PPD for MCol 2215 was 70% (±7), Average of PPD for SM494-1was 1% (±1.3).

3.4 Analysis of results

3.4.1 Experimental set up

Evaluation of cassava root under physiological postharvest deterioration (PPD) has been a key step in mapping QTLs associated to PPD tolerance (Cortes, 1999; Estevao, 2007) and gene expression (Han *et al*., 2001b).

Several strategies have been used to evaluate the susceptibility of cassava roots: the use of subjective visual scales in entire roots Booth (1972, cited by Pino 1979), subjective analysis of fluorescence in root transverse sections under UV lamp (Uritani *et al*., 1983), and the standard method use by CIAT, which employ block root tissue of ≈15 cm and PPD score based visual inspection of the ring pattern (zone B) formed during PPD (Wheatley, 1982).

In this research entire roots were used, which reduces the overall levels of PPD, unlike the original Wheatley (1982) method which removes the distal and proximal end of the roots accelerating the PPD process. The entire root strategy is more realistic to evaluate the tolerance, as it simulates the conditions in which farmer and processors keep the roots until processing (Morantes *et al*., 2010).

The average of PPD progression occurred from proximal to distal end of the root inversely proportional to root length (Figure 3.3), which suggests the need to use intact roots without significant mechanical damage produced during harvest. No correlation was found between root length, maximum and root diameter with PPD scores using visual inspection methods (α=0.05), suggesting that PPD is not influenced by those morphological traits.

In this study we found no correlation between dry matter and PPD, which contrasts with results obtained by Sánchez *et al*. (2006), who obtained a weak but positive correlation after, using 101 cassava accessions. These results agree with Morantes *et al.*, (2010), who evaluated 21 cassava accessions and found a negligible association between the two variables. It is necessary to note

that the methodology used for evaluating dry matter content in this research (specific gravity) was different to Sanchez and Morantes's method (oven-dried at 60°C for 24 hours).

Two additional procedures were used to set up the experimental conditions: the peduncle, if present, was removed from roots and temperature and humidity were measured during the experiment. According with CIAT (1991, cited by Torres-Ramos 2001), the presence of peduncle minimizes the exposure of root tissues to oxygen and thus roots with peduncles suffer less PPD. In order to avoid any bias among accessions all peduncles were removed, making all roots uniform.

PPD begins 24 to 48 hours after harvest at 20–30°C and 65–80% relative humidity (Morantes *et al*., 2010). Roots in this experiment were stored at an average temperature that favored PPD, but not curing. The average relative humidity measured during five days suggests that the store conditions did not favor the rapid infection of pathogens avoiding the early manifestation of secondary deterioration or rotting.

3.4.2 Cassava roots PPD assessment

The standard method of evaluation for PPD described by Wheatley (1982) has been used for several years. However, Cortes (1999) suggested the necessity to implement an objective, quantitative and systematic methodology for PPD phenotypic evaluation. Taking to account the direct relationship between PPD and hydroxycoumarin accumulation found by Wheatley and Schwabe (1985), and the possible relationship between susceptibility to PPD and the absolute

amount of scopoletin accumulation after 5 or 6 days (Buschmann *et al*., 2000a), digital pictures of slides obtained from cassava root sections (five days after harvest) and exposed to a UV lamp, were analyzed using Pixcavator image analysis software, to develop an objective and quantitative methodology.

No linear correlation was found between the two methodologies evaluated; only six varieties, Serralles, Seda, CM 3064, Mcol 2215, SM 523-7 and Valencia, showed a comparable percentage of PPD between both methodologies.

It is interesting to note that cassava variety Amarillo (with yellow color in its parenchyma cells) showed the maximum PPD under the fluorescence accumulation method but low levels of PPD under visual inspection. This can be explained by the presence of carotenoids which Sánchez *et al*., (2006) found to be highly correlated with parenchyma root color. The carotenoid content in cassava roots has been inversely associated to reduction or delaying PPD (Chávez *et al*., 2007). This shows that the accumulation of hydroxycoumarins itself cannot explain the susceptibility to PPD in all cassava accessions.

Results obtained are in total disagreement with the suggestion that the increase of fluorescence hydroxycoumarins between 4-6 days is related to PPD. Buschmann *et al*. (2000a), also found no clear correlation between high levels of scopoletin and susceptibility for several cassava accessions, while Orischt *et al*., (2000), who analyzed two cassava cultivars, also concluded that there was no straightforward causal relationship between them. Contrastingly, Estevao (2007)

found correlations values (r) between PPD and hydroxycoumarins (determined by qualitative visual scoring under UV lamp) of 0.45 and 0.47. However, these values are too low to suggest a strong significant correlation (Ott and Longnecker, 2001).

The reason for this behavior can be attributed to a decrease and stabilization of hydroxycoumarin content before main visible symptoms appear, in that sense all the transversal sections would be accumulating equivalent contents of hydroxycoumarins. This phenomenon was observed three or six days after harvest (Wheatley and Schwabe, 1985; Buschmann *et al*., 2000a). Evidence for this behavior can be found when the average of florescence accumulation and visual inspection at each percentage of length were compared. Under visual inspection there was progressive reduction of the percentage of PPD from proximal (15%) to distal (90%) sections, which is expected as the deterioration is controlled from proximal to distal end. However, this trend was less evident using the fluorescent method after five days (Figure 3.3).

Finally, the standard methodology based on Wheatley (1982) was used to evaluate the susceptibility to PPD for 25 cassava accessions. However, the use of entire roots and the percentage of root length to perform evaluations open other questions: how efficient was the harvest in avoiding mechanical damage and the development of deterioration at root distal end? Is the PPD correlated with root size or root diameter?

Analysis of variance found significant differences in the response to PPD for 25 cassava accessions. Previously, others authors also have tested some of the same accessions evaluated in

this research. Torres-Ramos (2001), using a colorimeter evaluated the cassava accessions Serralles, CM 3311, CM 3064, Trinidad, PI12902, Jamaica 18, Chilena and Abuelo, did not find any differences in PPD after four days. Gonzales *et al*., (1991) tested the cassava accessions Forastera, Llanera, Tremesiana and Serrallés using a subjective visual scale, and found that after 6 days of harvest Llanera was the most tolerant accession (PPD score of 67%) followed by Serralles (PPD score of 87%) while Forastera and Tremesiasa showed the highest levels of deterioration (100%). These results are in contradiction with the results obtained in this research. These differnces could be due to differences in methodology, timing or growing conditions (plants in our study were grown in Isabela Puerto Rico, while in the studies mentioned above the plants were grown in Corozal, Puerto Rico). Variations in phenotypic responses to PPD at different environments have been reported previously by Cortes *et al*. (1999).

Cassava accessions SM 494-1, for low PPD, and MCol 2215, for high PPD, were chosen for gene expression profiling. Even though there were no significant differences between PI12900 Amarillo and SM 494-1, the later was preferred. This was due to the accumulation of carotenoids in Amarillo and since the objective of this research was to find changes in gene expression and not in metabolites to explain the tolerance to PPD, this accession was not considered a good candidate. In addition, since Goenaga *et al*., (2002) gave the highest rating to SM 494-1 for marketable roots, tuber appearance after cooking and fiber content, SM 494-1 was chosen for this study due to its potential usefulness to Puerto Rico.

3.5 Conclusions

- No linear correlation between both methodologies was found. Visual inspection methodology was selected as the best method to assess PPD.

- No correlations were found between PPD scores (visual method) and some morphological traits (root length and maximal diameter), suggesting that these traits do not affect PPD.

- No correlation was found between PPD scores (visual method) and dry matter content, this relation is still unclear based on previous reports. However, the lack of correlation observed probably is the result of differences in the methods used.

- The experimental set up and the monitored temperature and relative humidity showed that environmental conditions favored the specific occurrence of PPD.

- Under visual inspection method cassava accessions SM 494-1 (PPD score 1%) and MCol 2215 (PPD score 70%) were selected as the most contrasting PPD cassava accessions.

4 Gene expression profiling of cassava accessions with contrasting PPD response

4.1 Summary

The selected cassava accessions with contrasting PPD responses MCol 2215 (high PPD) and SM 494-1(low PPD) were allowed to undergo PPD at different time points after harvest. Total RNA was extracted from root tissue samples of cortex and pith. RNA was treated with DNAse prior to cDNA synthesis. Real time PCR analysis was used to identify suitable housekeeping genes for gene expression normalization. In addition, this technique was used to evaluate the changes of gene expression of 21 genes belonging to different biological functional categories and potentially PPD specific. Multivariate exploratory and confirmatory statistical analysis were performed to identify and confirm coordinated gene expression patterns which were useful to develop a model to explain the tolerance observed.

4.2 Materials and methods

4.2.1 Plant Material

Five root systems from two cassava accessions with contrasting levels of PPD (MCol 2215 for high PPD and SM 494-1 for low PPD) selected in the cassava PPD assesment (Chapter 3), were removed carefully from the soil rhizosphera avoiding mechanical damage. Five roots of each root system were selected and distribute to produce five sample sets of five roots each. Sample sets were allowed to undergo PPD and were evaluated at different time points. Cassava roots were stored and exposed to air but protected from direct sun light and rain. Each time point

sample sets were processed for total RNA extraction (section 4.2.2) at 0 hours, 1 hour, 24 hours, 72 hours, and 120 hours (Figure 4.1). PPD evaluations were made according to cassava roots' phenotyping procedure (visual inspection) (section 3.2.2) at each time point.

4.2.2 Cassava root total RNA extraction

RNA extractions were performed over cortex and pith tissues from each root belonging to each time point sample set (Figure 4.1). Initially the periderm and cortex were removed from pith using a knife wiped with RNAseZAP® (Sigma Chemical Co., St. Louis, MO, USA). The periderm was then manually separated from the cortex.Cortex and pith were immediately grated independently to obtain small slices using a domestic grater in order to facilitate sample grinding. Cassava root slices were stored in 50 mL sterile tubes and immediately frozen in liquid nitrogen and kept at -80ºC until use.

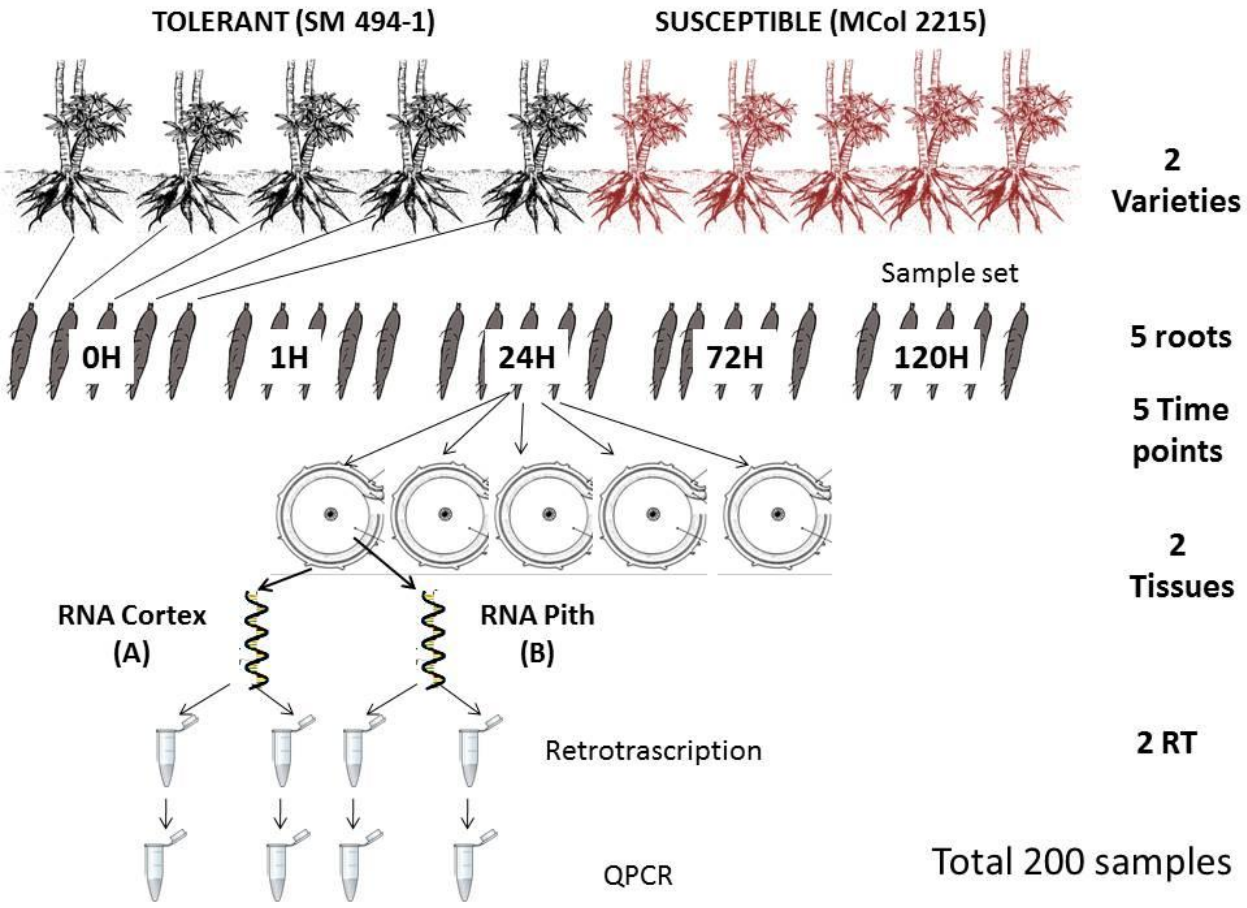


Figure 4.1 General experimental strategy for gene expression profiling. Five root systems (five plants) from two cassava accessions, MCol 2215 and SM 494-1 with contrasting PPD levels were selected. One root from each root system were selected to produce five time point sample sets (five root each) which were allowed to undergo PPD for different periods of time (0, 1, 24, 72, and 120 hours). Each root from each time point sample set was processed independently for RNA extraction using tissue from root cortex (A) and tissue from root pith (B). Two independent cDNA synthesis (RT) were perform using RNA extracted from each tissue. Each cDNA were used to perform a unique PCR reaction per transcript analyzed.

Total RNA of cassava root cortex and pith was isolated using the protocol developed by Eggermont *et al*., (1996), with modifications for cassava roots. Grated tissue was homogenized to a fine powder using a porcelain pestle and mortar previously chilled with liquid nitrogen. Aliquots of liquid nitrogen were added frequently during root grinding, in order to avoid sample thawing and consequent RNA degradation by action of RNAses.

Approximately 3g of powdered material were transferred to 50 mL sterile plastic tubes, previously chilled by liquid nitrogen. 12 mL of total RNA Cassava extraction buffer pre-warmed at 65ºC (Section 4.3) were added to the tubes. Tubes were vortexed vigorously for 1 minute and 5 mL of acid phenol chloroform:isoamyl alcohol (24:24:1) mix (Section 4.3) were added followed by vortexing for an additional minute.

The mixture was kept in ice, until centrifugation at 14200xg in 50 mL polypropilene centrifuge tubes (Nalgene Rochester, NY, USA) using a bench centrifuge 5810R (Eppendorf, Hamburg, Germany) for 5 minutes at 4ºC. The result upper phase was transferred to a new tube and extracted twice with 5 mL of chloroform:isoamyl alcohol (24:1) mix. Samples were vortexed vigorously for 1 minute and centrifuge 14200xg in at bench centrifuge 5810R (Eppendorf, Hamburg, Germany) for 3 minutes at 4ºC.

RNA Lithium chloride (8M) solution (Section 4.3) was added to the resultant upper phase until it reached a final concentration of 2M. Tubes were incubated at 4ºC overnight and centrifuged at 14200xg for 15 minutes at 4ºC in a bench centrifuge 5810R (Eppendorf, Hamburg, Germany). The supernatant was discarded; pellets were then washed with 70% ethanol in DEPC treated water. Pellets were dried for 5-10 minutes, resuspended in 50-100 μL of DEPC treated water (Section 4.3) and stored until use at -80ºC.

4.2.3 Assessing total RNA quality

To assess the quantity and quality of RNA samples, RNA aliquots were measured at $^{260}/_{280}$ nm and $^{260}/_{230}$ nm ratios by using NanoDrop®ND-1000 spectrophotometer (Nanodrop technologies Wilmington, DE, USA) and by evaluating the total RNA on agarose denaturing formaldehyde gels.

4.2.4 Electrophoresis of RNA on denaturing formaldehyde gels

Prior to electrophoresis, gel apparatus, combs and the gel tray were wiped with RNAseZAP® (Sigma Chemical Co., St. Louis, MO, USA) and rinsed with DEPC treated water. Denaturing formaldehyde gels at 1% were prepared combining (60 ml gel):

Agarose	0.6g
MOPS 5X (sec)	12 mL
DEPC treated water	42 mL

Agarose was boiled and left to cool (≈ 55ºC), then 6 mL of 37% formaldehyde were added and mixed. The gels were allowed to solidify in a fume hood.

RNA samples were prepared by mixing 1X volume of RNA (2-5µg) with 2X of RNA sample buffer (Section 4.3.8) up to a total volume of 10-30 µL. The mix was then heated for 65ºC for 5 minutes, and after cooling at room temperature, 2 µl of RNA loading buffer (Section 4.3) and 1 µL of ethidium bromide (200 µg/mL) were added.

Gels were pre-ran for 4-5V/ cm for 5 minutes in MOPS 1X buffer (Section 4.3.6) prior to loading the samples. After the samples were loaded the gels were ran at 4-5V/ cm until the bromophenol blue had migrated 10 cm. Nucleic acid bands were visualized and documented under UV light using BioDoct-IT™ Imaging System (Upland, CA, USA).

4.2.5 DNAse treatment

Once the cassava root total RNA quantity and quality were assessed, 1μg total RNA was treated with DNAse I amplification grade (Invitrogen Inc, Carlsbad, CA, USA) according with manufacturer's instructions. All components were mixed in ice in a 0.2 mL RNAse free PCR tube.

Total RNA	1 μg
10X reaction buffer	1 μL
DNAse I (1U/ μL)	1 μL
DEPC treated water	to 10 μL

Samples were incubated at 25ºC for 15 minutes in a Mastercycler® thermocycler (Eppendorf, Hamburg, Germany) and after addition of 1μl of 25mM EDTA to the mix the reactions were incubated at 65ºC for 10 minutes to inactivate the enzyme.

4.2.6 Checking the RNA for genomic DNA contamination

2μl of DNAse treated total RNA (≈ 180 ηg) were used to perform a PCR reaction using primers for rRNA18S in order to discard samples contaminated with residual genomic DNA. Distilled water was used as no template control (NTC) and genomic DNA from cassava as a positive control.

PCR reaction consisted of 1X PCR buffer (Applied Biological Materials, Richmond, BC, Canada), 2.5 mM of $MgCl_2$, 1 unit of taq polymerase (Applied Biological Materials, Richmond, BC, Canada), 0.2 mM of dNTP's (Promega, Madison, WI, USA), and 0.4 μM of each primer in a final volume of 25 μl. The cycling conditions were:

Step	Temp	Time
1	95ºC	5 min
2	95 ºC	15s
3	55 ºC	30s
4	72 ºC	30s
35 cycles step 2-4		
5	72 ºC	5 min

All PCR products were ran on 2% agarose gels (w/v) prepared in 1X TAE (Tris, Acetic Acid and EDTA) buffer (section 4.3) containing 0.05% (v/v) SafeView™ Nucleic Acid Stain (Applied Biological Materials, Richmond, BC, Canada).Electrophoresis was carried out at 4-8 V/cm and nucleic acid bands were visualized and documented under UV light using BioDoct-IT™ Imaging System (Upland, California, USA).

4.2.7 Primer design

Primers for all genes used were based on cassava cDNA sequences obtained from Genebank (www.ncbi.nlm.nih.gov), Cassava genome database Phytozome v.5.0 (University of California www.phytozome.net) and full length cDNA library data set of cassava developed by Sakurai *et al.* (2007).Sense and antisense primers were designed using Vector NTI v.11 software (Invitrogen Carlsbad, CA, USA) applying the following settings: amplicon length 100-250 bp, optimal Tm 60ºC, primer legth18-25 bases, maximum temperature difference of 3 ºC, and a GC

content from 20% to 60%. When it was possible primers were designed flanking an intron sequence.

Initially gradient temperature PCR was performed in a Mastercycler® thermocycler (Eppendorf, Hamburg, Germany) to confirm the optimal annealing temperature for each primer set. cDNA (5 µl of cDNA dilution 1:10) and genomic DNA (100 ηg) were use as templates and PCR was carried out by using 1X PCR buffer (Applied Biological Materials, Richmond, BC, Canada), 2.5 mM of $MgCl_2$, 1 unit taq polymerase (Applied Biological Materials, Richmond, BC, Canada), 0.2 mM of dNTP's (Promega, Madison, WI, USA) and 0.4 µM each primer or in a final reaction volume of 25 µL. The cycling conditions were:

Step	Temp	Time
1	95ºC	10 min
2	95 ºC	15s
3	55 ºC to 62 ºC	30s
4	72 ºC	30s
35 cycles step 2-4		
5	72 ºC	5 min

All PCR products were evaluated in 2% agarose gels (w/v) as described in section 4.2.5

4.2.8 Verification of amplified products

In order to verify the identity of amplicons, real time PCRs were performed over one pull combining all cDNA samples. PCR product sizes were checked on a 2% agarose gel and by dissociation analysis. PCR products were purified using Qiaquick PCR purification kit (Qiagen Inc, Valencia, CA) following the manufacturer's instructions. PCR products were sequenced at Nevada Genomics Center (University of Nevada, Nevada, USA).

4.2.9 Two-step real time PCR

cDNAs were obtained from each sample using approximately 0.8 μg of DNAse treated RNA (Figure 4.1). RNAs were retrotranscribed with 1 mM of Oligo $(dT)_{12-18}$,12.5 ηg/mL of random primers (Invitrogen, Carlsbad, CA, USA) and 1μL Omniscript RT® kit (Qiagen Inc, Valencia, CA), following the manufacturer's instructions. The mixture was incubated for 1 hour at 37ºC in an Eppendorf Mastercycler® thermocycler (Eppendorf, Hamburg, Germany). cDNA obtained was diluted in 1:10 in nuclease free water and stored at -80 ºC until further used.

Real time PCRs were performed in a Mastercycler® ep realplex^4 (Eppendorf, Hamburg, Germany) using SYBR® Green to monitor dsDNA synthesis. Reactions were prepared containing 5μL of 2X PerfeCta SYBR® Green Super Mix reagent (Quanta BioSciences Inc, Gaithersburg, MD, USA), 3 μL of cDNA and 300nM of each gene-specific primer in a final volume of 10μl.

The following thermal profile was used for all PCRs: 95°C for 10 min; 40 cycles of 95°C for 15sec, 60°C for 1min, 15sec for fluorescence detection at 60°C. At the end of this profile the PCR samples were subjected to an automatic dissociation analysis to confirm specificity of the amplicon.Data was analyzed using the Realplex software v 2.2.4 (Eppendorf, Hamburg, Germany). Baselines were calculated automatically and individually for every sample between cycles 3 and 15. All amplification plots were analyzed with automatic threshold based in noiseband (10 times the standard deviation above the noise of base line) in order to obtain the Ct

(threshold cycle) values. Dissociation analysis (melting curve) threshold was set manually in a range between 20%-33%.

Primer amplification efficiency for every sample was calculated individually by extracting raw florescence data in each cycle generated by Mastercycler® ep realplex^4 and by using Miner algoritm v 2.2 (www.miner.ewindup.info/version2). Settings were Max P value of 0.05, minimal efficiency 50% (0.5) maximal efficiency 100% (1.0).

4.2.10 Housekeeping reference genes validation

Five housekeeping genes were selected as candidates for best reference genes: ribosomal 18S (Genebank AB233568), ubiquitin-protein ligase, (Genebank DB923319), elongation factor 1-alpha, (Genebank AF041463), translation initiation factor 5A (Genebank AF266464) and tubulin β chain (Phytozome cassava 7734). Cassava sequences were obtained from Genebank (www.ncbi.nlm.nih.gov) or alternatively from Phytozome v.5.0 (www.phytozome.net) cDNAs (in total 60 cDNA samples) synthesized from RNAs extracted from cortex and pith tissues of three biological replicates (three cassava roots from each time point sample set) were chosen randomly to perform a Real time PCR as described in Section 4.2.9 in order to select the best reference gene.

The Ct values obtained were arranged in matrices per tissue and accesions (four 15x5 data matrices), with the genes as columns and time points as rows. Ct values were corrected for PCR efficiency (Equation 5 section 4.2.11.10). The four 15x5 data matrices were laminated in a 60x15

matrix, which was used to run NormFinder GeneEX® software (MultiD Analyses, Göteborg Sweden) and BestKeeper v.1 (Pfaffl *et al*., 2004) applications to select the best reference genes.

4.2.11 Real time PCR data statistical analysis

Ct values obtained from Mastercycler® ep realplex^4 were used for the analysis of time course gene expression profiling using GeneEX® software (MultiD Analyses, Göteborg, Sweden). Ct values were arranged in a matrix (200x 23) where rows represent all combinations of two cassava accessions (MCol 2215 and SM 494-1), two tissues (cortex and pith) five time points (0, 1, 24, 72 and 120 hours), five biological replications (five roots), two cDNA synthesis and one Real time PCR reaction. The columns of this matrix represent 21 target genes and 2 housekeeping reference genes. After data processing (section 4.2.11.1), this matrix was divided in four independent matrices representing each root tissue per cassava accession.

4.2.11.1 Data preprocesing

Samples that showed targets with low mRNA and never reached the threshold or obtained a Ct value with aberrant products (such as primer dimmers) after dissociation analysis were eliminated and replaced with limit of detection (LOD) + 1. This corresponds to assigning a concentration that is half of the LOD to the off-scale samples. Ct values were preprocessed as described by Ståhlberg *et al,* (2008):

The Ct values were corrected for PCR efficiency (E) using mean efficiency value calculated for each gene using Miner algorithm.

$$Ct_{E\equiv 100\%} = \frac{\log(1+E)}{\log(2)} \qquad \textbf{Equation 5}$$

The Ct values of target genes (TG) obtained were normalized with the average of Ct values of reference genes (RG).

$$Ct_{TG,norm} = Ct_{TG} - \frac{1}{n}\sum_{i=1}^{n} Ct_{RG} \qquad \textbf{Equation 6}$$

cDNA technical replications (RT samples) were then averaged:

$$Ct_{RT\_Average} = \frac{1}{n}\sum_{i=1}^{n} Ct_{RT\_repeats} \qquad \textbf{Equation 7}$$

Where:

n = Number of retrotranscription replicates or cDNA samples.

Biological samples (5 roots) were then averaged:

$$Ct_{Bio} = \frac{1}{n}\sum_{i=1}^{n} Ct_{BIO\_repeats} \qquad \textbf{Equation 8}$$

These procedures reduced the dimension of data matrix to from 200x23 to 5x21.The Normalized Ct values were converted to relative quantities (RQ)

$$RQ = 2^{CTaverage\text{-}Ct} \qquad \textbf{Equation 9}$$

Where $CT_{average}$ is the average of Ct values for all samples.

RQ values were transformed to fold changes (FC) with $\log_2$:

$$FC = \log_2 RQ$$ **Equation 10**

In order to give all genes equal weight (importance) for classification of expression profiles, the data was autoscaled (FC_{AS}) by subtracting the mean expression of every gene in each tissue per accession and dividing by the standard deviation

$$FC_{AS} = \frac{(FC - \overline{FC})}{SD}$$ **Equation 11**

Under this transformation the average expression of every gene in each tissue per accession is 0 and the standard deviation is 1.

4.2.11.2 Multivariate analysis

Autoscale fold changes were used to run multivariate analysis using Principal component analysis and hierarchical cluster with heat map, using GeneEX® software (MultiD Analyses, Göteborg Sweden). Data was analyzed using an augmented matrix created by catenation of four independent data matrices (section 4.2.11) in a 5x84 matrix, or alternatively the four raw data matrices were analyzed independently.

4.2.11.3 Confirmatory statistical analysis

In order to identify significant differences between varieties, time points, tissues and possible interactions among them, an analysis of variance using a completely randomized split plot design with mixed effects was carried out over each target gene using the GLM procedure of SAS® software (SAS Institute Inc, NC, USA). ANOVA was carried out using fold changes after preprocessing data with equations 5, 6 9 and 10 for each gene avoiding averaging and autoscaling (section 4.2.11.1). Data adequacy for ANOVA comparison was evaluated through Shapiro-Wilks normality test and scatter plot of residual vs predicted values to confirm homoscedasticity. Significant differences were measured by F distribution. Multiple comparisons were made using Fisher's Least Significant Difference (LSD) procedure.

The model for observations was:

$Y_{ijk} = \mu + \alpha_i + \delta_{ik} + \beta_j + \alpha\beta_{ij} + \varepsilon_{ijk} + \gamma_k + (\alpha\gamma)_{ik} + (\beta\gamma)_{jk} + (\alpha\beta\gamma)_{ijk} + \zeta_{ijkl}$ with n = 200

Where:

$Y_{ijk} = \mu + \alpha_i + \delta_{ik}$ is the observation in whole plot with α as effect of cassava variety, δ the random effect of roots.

$\beta_j + \alpha\beta_{ij} + \varepsilon_{ijk}$ is the observation in sub plot with β as a fixed effect of root tissue

$\gamma_k + (\alpha\gamma)_{ik} + (\beta\gamma)_{jk} + (\alpha\beta\gamma)_{ijk} + \zeta_{ijkl}$ is the observation in sub-sub plot with γ as a fixed effect of time.

$\delta_{ik} \sim N(0, \sigma^2_\delta)$, $\varepsilon_{ijk} \sim N(0, \sigma^2_\varepsilon)$, $\zeta_{ijkl} \sim N(0, \sigma^2_\zeta)$, are the random effects independent with Normal distribution and mean 0

4.3 Reagents and solutions

Acid Phenol:Chloroform:isoamyl alcohol 24:24:1

Acid Phenol:Chloroform:isoamyl alcohol (24:24:1) was prepared by mixing all components just before use and stored in a light tight container at 4ºC.

Acid phenol (Sigma P4682)

Chloroform (Fisher BP 1150)

Isoamyl alcohol (Fisher C606)

50X TAE electrophoresis buffer

Component	Per litre	
Tris Base	242g	Sigma T1378
Glacial acetic acid	57.2 mL	Fisher S70048
0.5M EDTA	100 mL	Sigma E4884

Adjust to 1 liter with deionized water

RNA extraction buffer

Component	Final concentration	
Sodium acetate (pH 6.1)*	100 mM	Sigma S2889
EDTA (pH 8.0)*	1 mM	Sigma E4884
SDS**	4% (w/v)	Sigma L9150

*****Prepared with DEPC treated water

** Can be added as pure reagent

DEPC treated water (0.01%)

Add 1 mL of 0.1% Diethylpyrocarbonate (DEPC) (Sigma D5758) to 1000 L of distilled H_2O. Mix overnight and at room temperature. Autoclave 1Hour at 122ºC, for 15 min at 15 PSI and cool to room temperature prior to use.

RNA Lithium Chloride Solution (8M)

Slowly dissolve LiCl (Sigma L4408) in DEPC treated water. LiCl is soluble to at least 8M and is an exothermic reaction.

5X MOPS

Component	Final concentration	
MOPS (pH 7.0)	0.2M	Sigma M3183
Sodium Acetate	0.05M	Sigma S2889
EDTA (pH 8.0)	0.05M	Sigma E4884

To prepare 2 liters of buffer, add 83.72g of MOPS (free acid) and 8.23g sodium acetate to 1.6L of DEPC-treated water and stir until completely dissolved. Add 20ml of DEPC-treated 0.5M EDTA and adjust the pH to 7.0 with10N NaOH. Bring the final volume to 2L with DEPC treated water.

RNA loading buffer

Component	Final concentration	
Glycerol	50%	EMD 4750
Bromophenol blue	0.4%	Sigma B0126
EDTA (pH 8.0)	1mM	Sigma E4884

Prepared using DEPC treated water and nuclease free glycerol

RNA sample buffer

Component	Volume	
Deionized formamide	10 mM	EMD 4750
37% formaldehyde	3.5 mL	Fisher T353
5X MOPS buffer	2.0 mL	

Dispense into single aliquots and store at –20°C. Stored for up to 6 months.

4.4 Results

4.4.1 Time course PPD response of contrasting cassava accessions

New roots from selected accessions with contrasting PPD were stored under the same conditions used in the physiological test carried out with 25 cassava accessions (section 3.2.1). No visible symptoms of deterioration were observed in both accessions at 0, 1, and 24 hours. At 72 and 120 hours susceptible accession MCol 2215 showed symptoms of deterioration (PPD score 17.3 % and 32% respectively) (Figure 4.2, Figure 4.3), while tolerant accession SM 494-1 showed null or very low symptoms of deterioration.

4.4.2 RNA extraction and quality assessment

Cortex and pith RNA was extracted from each root of every time point sets after harvest (0, 1, 24, 72 and 120 hours) using a protocol described by Eggermont *et al*., (1996) modified by an additional extraction step with chloroform: isoamyl alcohol (24:1 v/v)

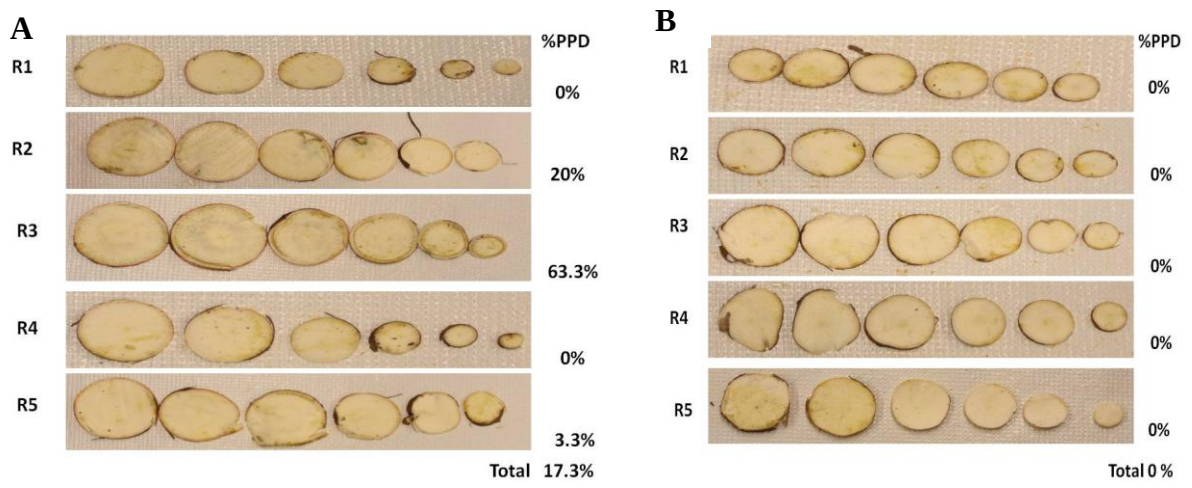


Figure 4.2 Transversal root sections from proximal to distal end (left to right) after three days of storage in contrasting PPD cassava accession **A**. MCol 2215 **B**. SM 494-1. **R** root sample. PPD score for each root and total average are showed.

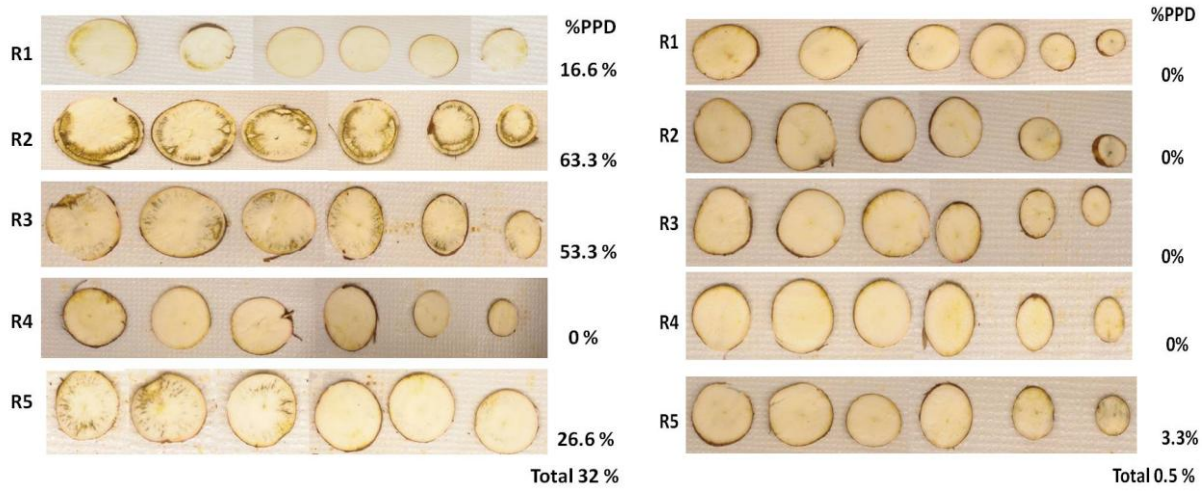


Figure 4.3 Transversal root sections from proximal to distal end after (left to right) five days of storage in contrasting PPD cassava accession **A**. MCol 2215 **B**. SM 494-1. **R** root sample. PPD score for each root and total average are shown.

This modification improved the RNA quality particularly for pith tissue in both selected accessions SM 494-1 and MCol 2215, where the ratios A_{260nm}/A_{280nm} and A_{260nm}/A_{230nm} were approximately 2.0. The quality of the cortex however was lower, with the same ratios being approximately 1.3. Overall, on average more quantity of RNA was obtained in cortex compare with pith (Figure 4.4).

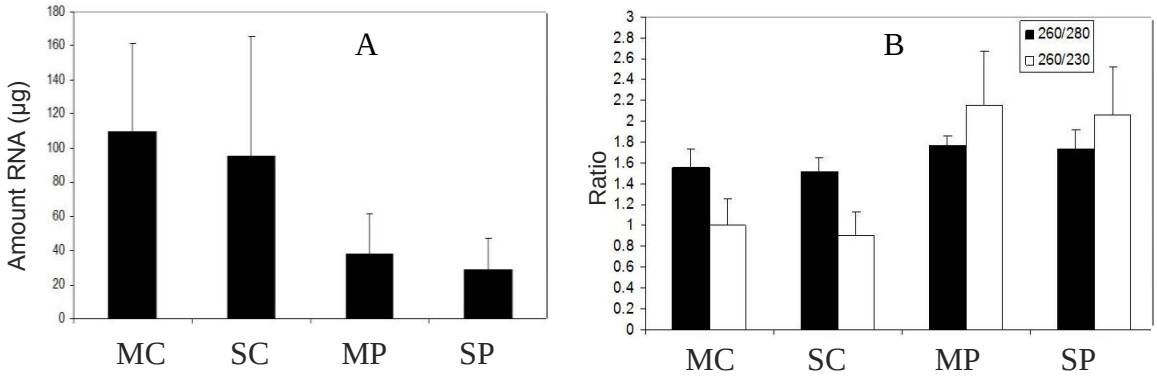


Figure 4.4 RNA quantity and quality assessment obtained in cortex and pith from contrasting PPD cassava accessions. **A** amount of RNA. **B** Ratios A_{260nm}/A_{280nm} and A_{260nm}/A_{230nm}. ***MC*** Mcol 2215 cortex ***SC***, SM 494-1 cortex, ***MP*** Mcol 2215 Pith, ***SP*** SM 494-1 Pith.

Formaldehyde denaturing agarose gels were used to assess the RNA quality (Figure 4.5). Formaldehyde gels prevents the appearance of secondary RNA structures formed via intramolecular base pairing, allowing RNA migration according to its size.

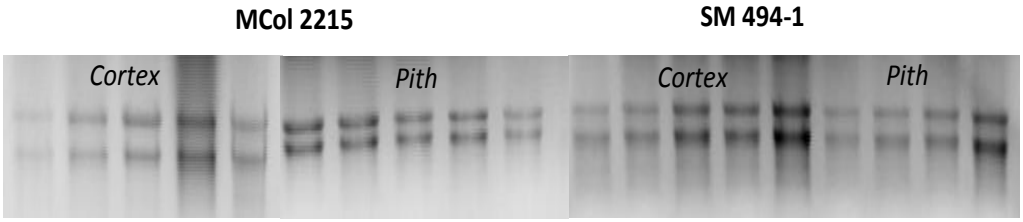


Figure 4.5 Inverted black and white picture of RNA denaturing formaldehyde gels of selected samples. RNAs extracted 1hour after harvest are shown. 2- 5 µg of total RNA was loaded per line

All RNAs were treated with DNase to avoid residual genomic DNA contamination present after RNA extraction. Elimination of this DNA was confirmed by PCR amplification using primers for ribosomal 18S gene (Echeverry, 2008) (Figure 4.6).

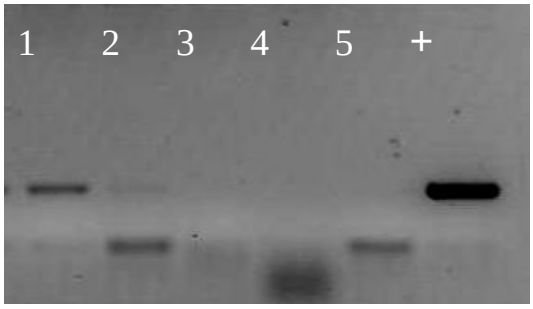


Figure 4.6 Inverted black and white agarose gel picture of PCR over RNA samples using specific primers for ribosomal 18S. Prior to PCR the RNA samples were treated with DNAse.1,2 DNA contamination 3,4 and 5 without DNA contamination, + Positive control (cassava genomic DNA).

4.4.3 Selection of target genes

In order to carry out gene expression profiling , 21 genes were selected, two genes were selected from transcripts derived fragments (TDFs) of cassava roots under PPD described by Huang *et al*., (2001), 13 genes were chosen from microarrays experiments (Really *et al*., 2007), 11 genes of this microarrays experiments were catalogued as potentially PPD specific. Six genes were selected from diverse metabolic pathways which can be involved in PPD response. Some of these are related with cyanide detoxification, changes in sucrose biosynthesis, oxidative stress and synthesis of jasmonate (allene oxidase), with the latter appearing as a candidate protein associated with PPD (Owitti *et al*., 2006) (Table 4.1).

Categories of selected genes are based in biological process, involving biosynthesis (6), plant defense and response to wounding (5), oxidative stress(3), program cell death (3), cyanide

detoxification (2), cell wall (1), water transport (1) and sulfur metabolism(1). Oxygen stress and programmed cell death are consider to be biochemical and physiological key process involved in cassava PPD (Huang *et al*., 2001; John Beeching pers. comm.) Since cyanide production in cassava roots can increase the production of ROS, the genes involved in cyanide detoxification would protect roots against PPD (Page, 2009; Dimuth Siritunga pers. comm.)

4.4.4 Primer design

Primers for real time PCR were designed for 100 to 250 bp amplicons by using Vector NTI software (Invitrogen, Carlsbad, CA, USA). Table 4.2 shows the primers used for gene expression profiling housekeeping validation and RNA quality assessment. Specificity of PCR products were confirmed by gel electrophoresis (Figure 4.7) and automatic dissociation analysis (melting curves) (Figure 4.9). The identity of amplified products was verified by nucleotide sequencing of the resulting amplicons (Appendix 3). Primer positions in the sequence are showed in Figure 4.8.

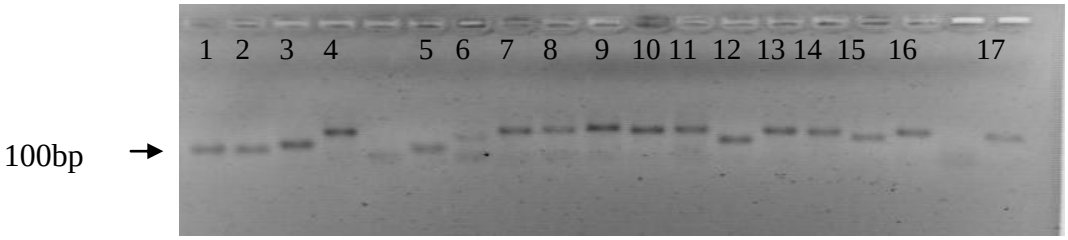


Figure 4.7 Inverted black and white gel picture of selected amplicons of target genes for expression profiling (for abbreviations see table 4.1). 1ALX, 2 AOX, 3APX, 4 BCA, 5 CAT, 6 CD1, 7 CD2, 8 CHT, 9 CDE, 10 CIP, 11 DAD, 12 EXT, 13 FSR, 14 HRP, 15 LPO, 16 PAL 17 PIP.

Gene name	Category	Source
Alternative oxidase	Oxidative stress	3
Secretory peroxidase	Oxidative stress	2
Catalase 1	Oxidative stress	2
Beta cyanoalaline synthase A	Cyanide detoxification	3
Beta cyanoalaline synthase B	Cyanide detoxification	3
Chitinase	Programmed cell dead	2
Cysteine protease	Programmed cell dead	2
Defense against cell Dead 1	Programmed cell dead	1
Sucrose phosphate synthase	Biosynthesis Sucrose	3
Cytochrome P450 CYP79D1	Biosynthesis cyanogenic glucosides	2
Cytochrome P450 CYP79D2	Biosynthesis cyanogenic glucosides	2
Cytochrome P450 CYP71E	Biosynthesis cyanogenic glucosides	2
Allene oxidase cyclase	Biosynthesis Jasmonate	3
1-aminocyclopropane-1-carboxylate synthase	Biosynthesis Ethylene	2
Lipooxigenase 1	Plant defense/response to wounding	3
Extensin	Plant defense/response to wounding	2
Phenylalanine ammonia-lyase	Plant defense/response to wounding	2
Pectinacetylesterase	Plant defense/response to wounding	1
Hidroxyproline rich protein	Cell wall	2
Plasma membrane intrinsic protein 1	Water/transport	2
Sulfite reductase	Sulfur metabolism	2
Tubuline β chain	Microtubule based process	4
Translation initiation factor 5A	Translation initiation	4
Ribosomal 18S	Translation	4
Ubiquitin-protein ligase	Protein ubiquitination	4
Elongation factor 1-alpha	Translation	4

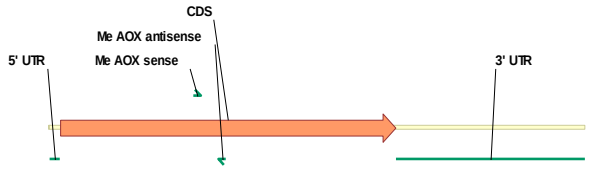
Table 4.1 Genes for gene expression profiling of cassava roots under PPD Sources: 1 Huang *et al.,* (2001), 2 Really *et al.,* (2007) 3Metabolic pathways selected and 4 Housekeeping genes for gene expression normalization.

Gene Name	Gene abbreviation	Primer sequence forward/reverse	Amplicon size (bp)	Tm	Gene Accesion	Average PCR efficiency
Tubulin β chain	TUB	5' -TGCCATGTTCCGTGGAAAGATG -3'	138	59.3	cassava 7734[1]	0.86 ± 0.06
		5' -GTGTCTGTGACATTCCACCTAGGGG-3'		59.5		
Translation initiation factor 5A	F5A	5'-AACACCAAGGACGATCTCAGGCTT-3'	106	59.1	AF266464[2]	0.85 ± 0.05
		5'-TTGCAGACATGACGCTCACTACGA-3'		59.4		
Ribosomal 18S**	18S	5'-ATGATAACTCGACGGATCGC-3	169	51.6	AB233568[2]	0.81 ± 0.05
		5'-CTTGGATGTGGTAGCCGTTT-3'		51.7		
Ubiquitin-protein ligase	UBI	5'-AGCAGCTCGCATGTTCAGTGAGAA-3'	120	59.9	DB923319[3]	0.84 ± 0.05
		5'-TATAGACTGCTGGGCTGGTGCTTT-3'		58.4		
Elongation factor 1-alpha	EF1	5'-TGAACCACCCTGGTCAGATTGGAA-3'	139	60.0	AF041463[2]	0.84 ± 0.07
		5'-AACTTGGGCTCCTTCTCAAGCTCT-3'		58.0		
1-aminocyclopropane-1-carboxylate synthase	ACO	5'-TTGATTTCAGTGAACTCAATGGGGA-3'	138	58.5	DN740375[3]	0.89 ± 0.05
		5'-CCAATTGCTTCACTTTCTCCATCAA-3'		58.3		
Secretory peroxidase	APX	5'-CAAGTGTTCAACACAAGCTGCCAAG-3'	126	59.7	AY973612[2]	0.87 ± 0.06
		5'-AAAATCCAAGTGTGTGTGCACCTGA-3'		59.4		
Allene oxidase cyclase	ALX	5'-AGCAACAAACTTTACACCGGAGACC-3'	107	58.7	cassava 46[1]	0.86 ± 0.04
		5'-GCTTCGTACCGGTCACCTTTCATT-3'		59.3		
Alternative oxidase	AOX	5'-AGCACCATGTGCCAAAGACATTTC-3'	102	59.0	cassava 1519[1]	0.86 ± 0.05
		5'-GGCACCCATACCGTCTCTGAAAA-3'		58.9		
Beta cyanoalaline synthase A	BCA	5'-GGAGTGTGTTGTCCACTGCGAGAG-3'	204	59.7	EU358583[2]	0.85 ± 0.06
		5'-AGGAGCTTCAGCAGCAGCGG-3'		59.3		
Beta cyanoalaline synthase B	BCB	5'-CCACTACTTGCGGTTGTTGCTTGT-3'	231	59.2	HQ257219[2]	0.85 ± 0.07
		5'-AAGCTGAGAAACTTCAGGCTTGATT-3'		56.1		
Catalase 1	CAT	5'-TCTTACTGGTCTAAGTGCGACGCG-3'	100	59.5	AF170272[2]	0.86 ± 0.06
		5'-CCACTGTGACCCATAGCCTCATCTT-3'		59.3		

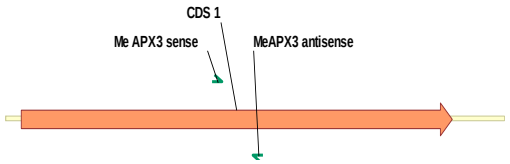
Cytochrome P450 CYP79D1	CD1	5'-ATCGGTTTACTTAACGCCACCTCCT-3' 5'-AGGAGGGAGTGGGAGTTTCTTGCTA-3'	159	59.3 59.6	AF140613[2]	0.85 ± 0.05
Cytochrome P450 CYP79D2	CD2	5'-CTGGACTTGAATTGTTCAGGGCAA-3' 5'-GGGAGTGGGAAGTTCTTGCTAGCTT-3'	207	59.1 59.3	AF140614[2]	0.86 ± 0.06
Cytochrome P450 CYP71E	CDE	5'-TTCATTGATTCTTTGACAGGAGCCC-3' 5'-ATTCCTCCCACAAAGACGTTCATG-3'	230	59.3 59.8	AY217351[2]	0.87 ± 0.07
Chitinase class IV	CHT	5'-CTAGACGCGAAGTTGCTGCCTTTT-3' 5'-TGTTGTCTTGACCAGCTGCTCCATA-3'	210	59.9 59.7	DN740377[3]	0.85 ± 0.07
Cysteine protease	CIP	5'-GCAGGGATTTCCAGTTTTACGCA-3' 5'-TCGCAATTCCACATATCCCTGTAGG-3'	210	59.1 59.9	AY973616	0.86 ± 0.07
Defense against dead 1	DAD	5'-CCAAGCTCTCTTCAGTTCACTTCGC-3' 5'-TGGATACGGAGACAAACAGCAAGG-3'	210	59.6 59.1	cassava 29680[1]	0.86 ± 0.06
Extensin	EXT	5'-TGGGGTCTCCAATGGCCTCTATC-3' 5'-TAAGATGGAGGGTGGACAGGCG-3'	148	60.0 59.8	cassava 483[1]	0.85 ± 0.07
Sulfite reductase	FSR	5'-TTTCCTCGCCTTGCTGAACCA-3' 5'-CACGGAAGGGCTCAAATTTCTTTC-3'	209	59.3 59.1	DT883576[3]	0.87 ± 0.08
Hidroxyproline rich protein	HRP	5'-TAGTACGGCGAAATCTTCTGGGATG-3' 5'-TGTCTTCAATTTCGCCGCCG-3'	202	59.4 60.3	AF239615[2]	0.87 ± 0.06
Lipooxigenase 1	LPO	5'-CCGTCTTGAGGAATTTCCTCCAAA-3' 5'-GCATCAGCACATCATGGTAATCCAA-3'	170	59.4 59.6	cassava 22662[1]	0.88 ± 0.05
Phenylalanine ammonia-lyase	PAL	5'-ATCGAACGCGAAATCAACTCTGTG-3' 5'-GAGATTTGAAGGCAACCCATTGTTG-3'	210	59.2 59.6	AY036011[2]	0.87 ± 0.04
Pectinacetylesterase	PEC	5'-AGCTCTGGATGATCGCCGGTT-3' 5'-CATAAGCCCATGCATTGGTTGGT-3'	127	59.5 59.5	DB941810[3]	0.87 ± 0.06

Plasma membrane intrinsic protein 1	PIP	5'-CAAGGATTATACTGAGCCACCACCG-3' 5'-CCAAAGGCCCAAGCAATCCC-3'	201	59.7 60.1	DN740371[3]	0.86 ± 0.06
Sucrose phosphate synthase	SPS	5'-ATCTGTATATTCGGTGGGGTGCAGA-3' 5'-CATTGCTGCCAACTCCCTTCAATAT-3'	132	59.5 59.3	cassava 8137[1]	0.87 ± 0.06

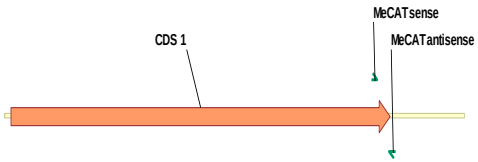
Table 4.2 Primer sequence list of target genes and reference genes that were used for gene expression profiling of cassava roots under PPD. **Primer set use as a candidate for reference gene validation and RNA assessment developed by Echeverry (2008). [1]Sequence obtained from Phytozome v.5.0 University of California (www.phytozome.net)[2], Genebank accessions[3], full length library Sakurai *et al*., 2007.



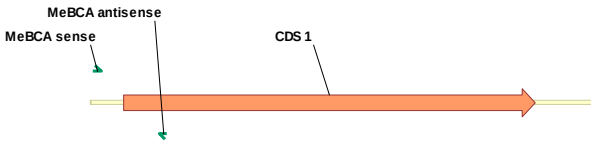
Alternative oxidase (AOX) 1677 bp



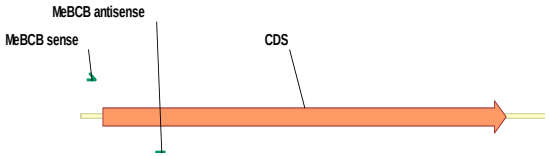
Secretory peroxidase (APX) 11234 bp



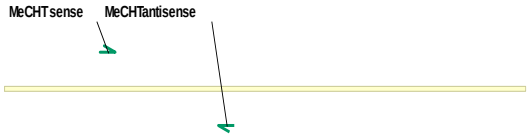
Catalase (CAT) 1792 bp



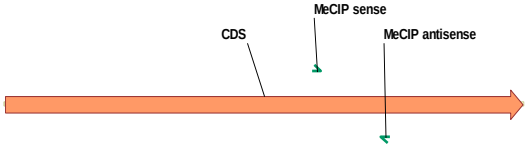
Beta cyanoalaline synthase A (BCA) 1497 bp



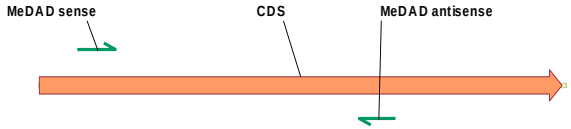
Beta cyanoalaline synthase B (BCB) 1352bp



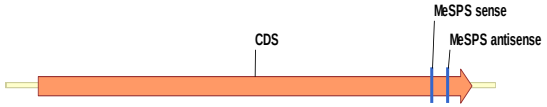
EST chitinase B (CHT) 1352bp



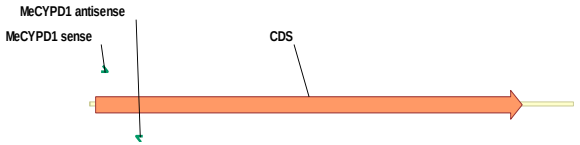
Cystein protease (CIP) 1404bp bp



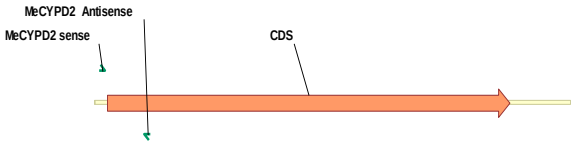
Defense against cell Dead (DAD) 349bp



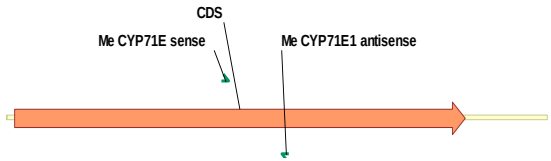
Sucrose phosphate synthase (SPS) 3332 bp



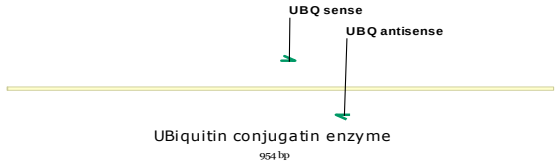
Cytochrome P450 CYP79D1 (CD1) 1845bp



Cytochrome P450 CYP79D2 (CD2) 1920 bp



Cytochrome P450 CYP71E (CDE) 1840 bp



EST Ubiquitin-protein ligase (UBI) 954 bp

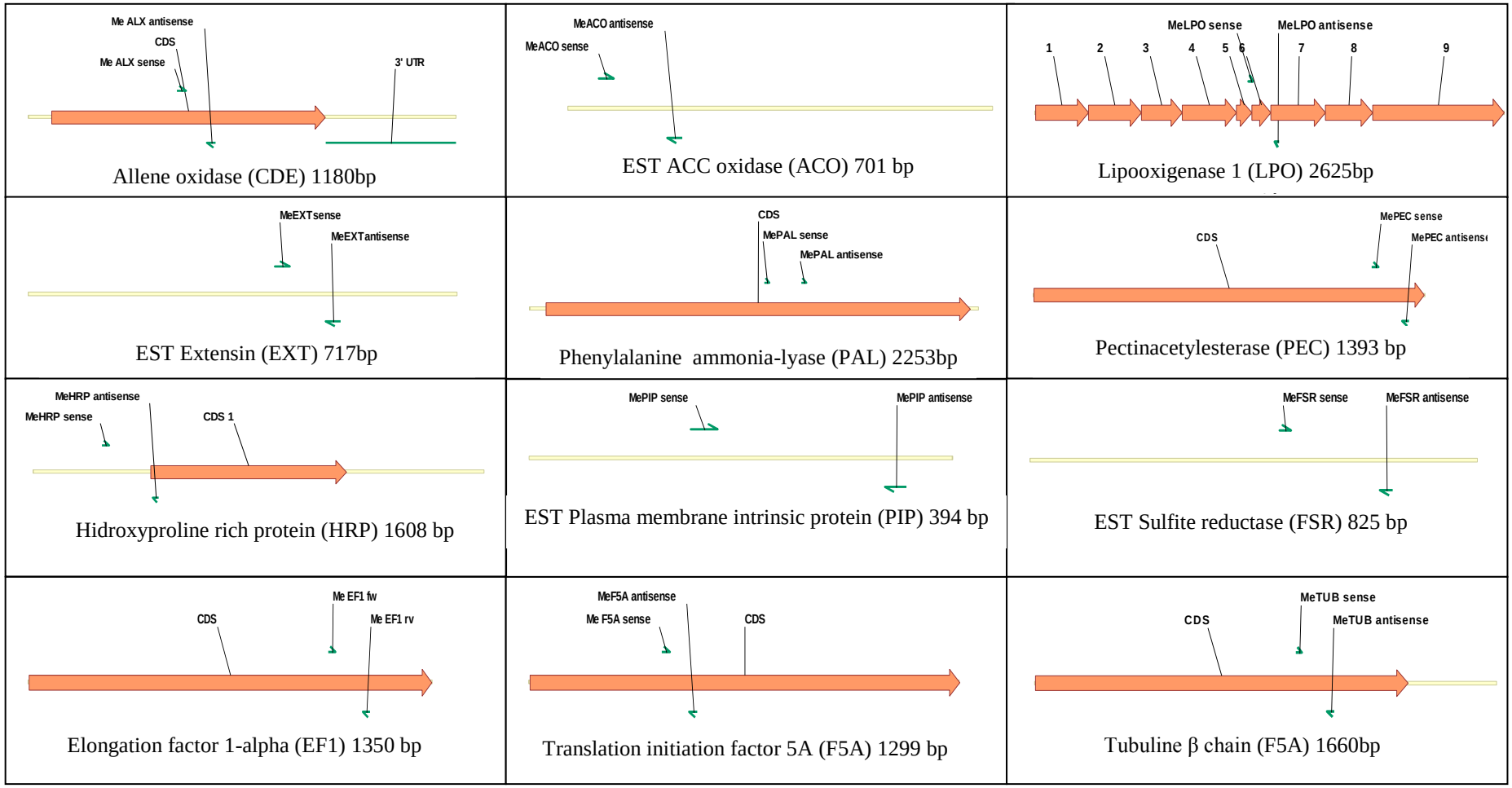


Figure 4.8 Primer set positions of target genes and housekeeping genes over sequences. ESTs (Express tag sequence) and annotate coding sequences (Drawings generate by Vector NTI graphic interphase).

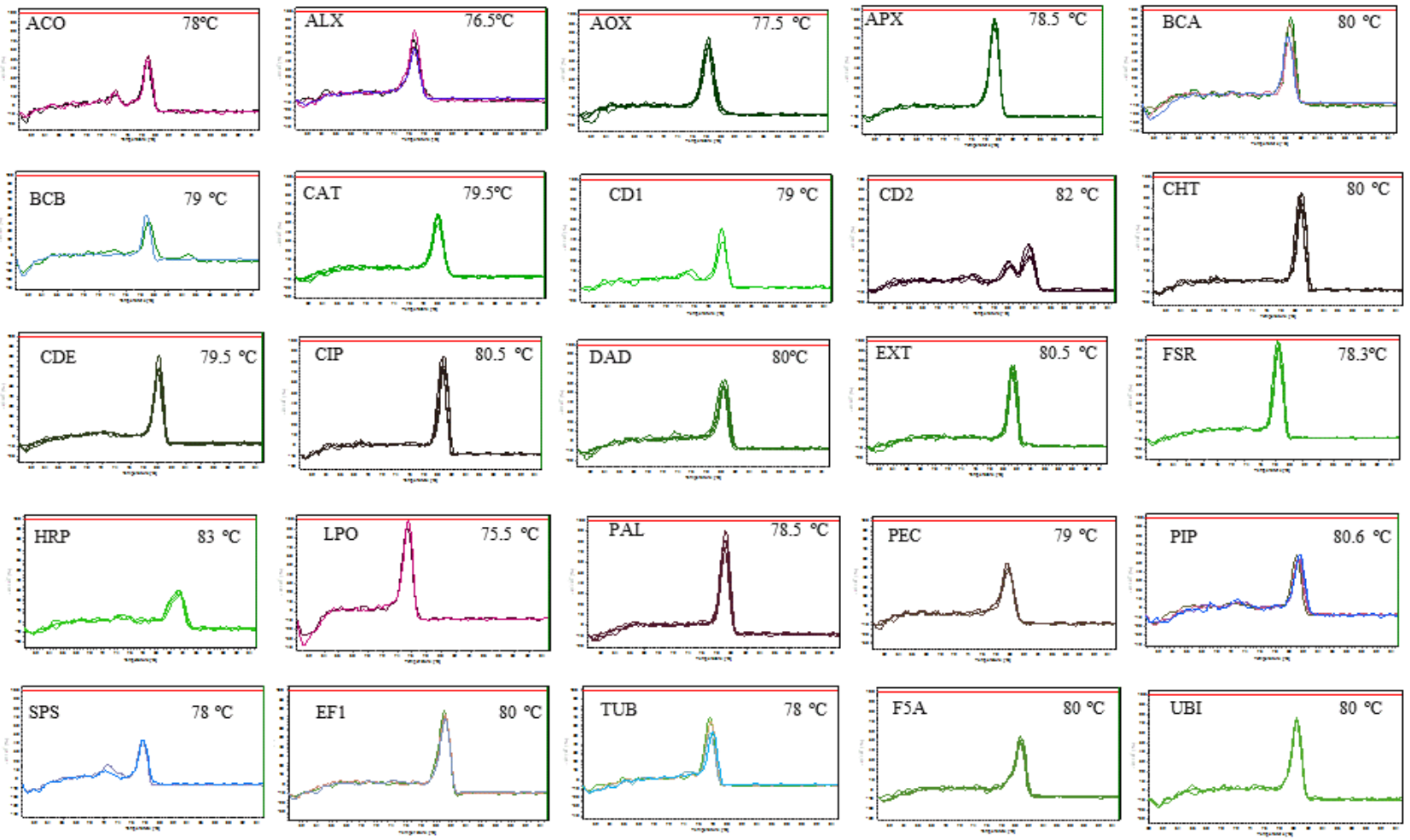


Figure 4.9 Dissociation analysis (melting curve) for 21 target genes and four reference genes. Gene abbreviation (top left) and melting temperature (top right) are noted in each panel (gene 18S is shown in Appendix 4)

4.4.5 Housekeeping gene validation

In order to evaluate the expression patterns of target genes in different tissues (cortex and pith) along different time points, it is crucial to normalize all the samples by the same set of reference genes. Potentially useful reference genes were selected from previous studies using real-time PCR or Northern blots. (Nicot *et al.*, 2005; Duarte Silveira *et al.*, 2009; Mallona *et al.*, 2010; Artico *et al.*, 2010; Quiang-Feng *et al.*, 2009).

The average Ct values obtained for all the genes studied did not differ between tissues or cassava accessions. The most highly expressed gene was 18S (low Ct values) and the lowest were F5A, UBI and TUB (high Ct values) (Figure 4.10 and 4.11). The range and distribution of the Ct values usually allows the visualization of the least variable genes among the samples (Duarte Silveira *et al*., 2009). However, it was not possible to identify the least variable gene using the range of distributions. In general, there was more variability in candidate genes for pith and cortex tissue of SM-494-1which showed more outliers compared with Mcol 2215.

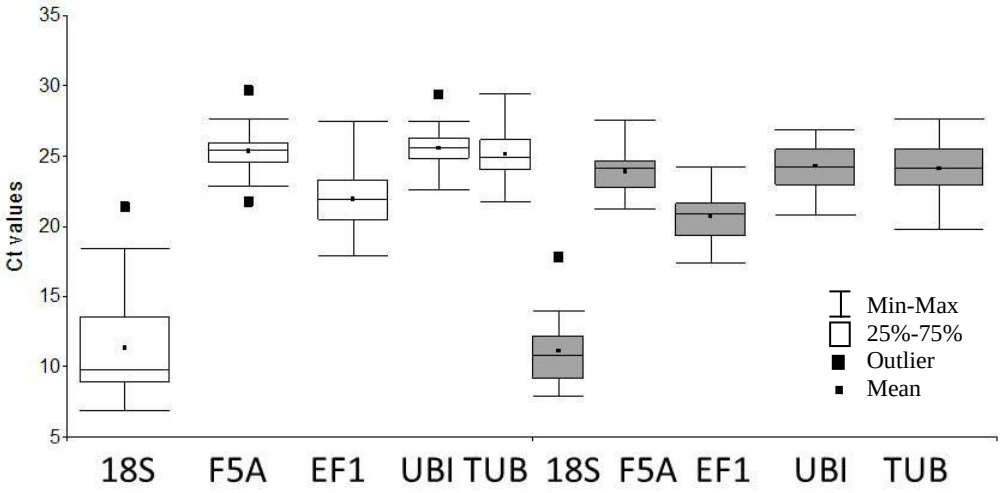


Figure 4.10 Ct variation of each candidate reference gene in root cortex (open boxes) and pith (gray boxes) for Mcol 2215 under PPD. The 25th and 75th quantiles and median are shown. Whiskers cover the range of no outlier observations. Black squares indicate outliers,

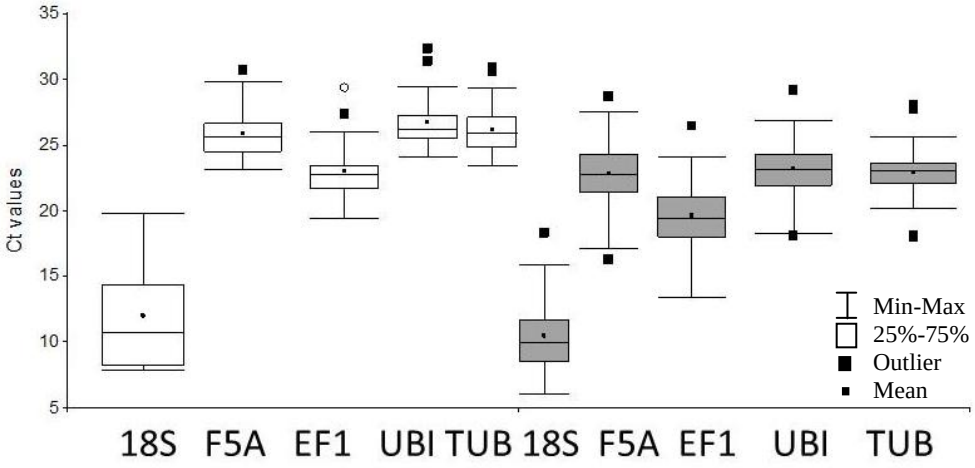


Figure 4.11 Ct variation of each candidate reference gene in root cortex (open boxes) and pith (gray boxes) for SM 494-1 under PPD. The 25th and 75th quantiles and median are shown. Whiskers cover the range of no outlier observations. Black squares indicate outliers,

Norm finder software and bestkeeper algorithm were used to evaluate the stability of candidate housekeeping genes. Initially all genes were tested by using all samples (2 cassava accessions, 3 roots, 2 tissues, at 0, 1, 24, 72 and 120 hours). By using Normfinder algorithm,

Ribosomal 18S showed the largest variability compared with other candidate genes. The best candidate gene was F5A (SD = 0.539) (Figure 4.12) However, the variability was still high ("high" is relative but standard deviations larger than 0.25 cycles are not attractive). The variability was reduced to an acceptable level when the average of all remaining four genes was used (accumulate SD = 0.285) (Figure 4.12B).

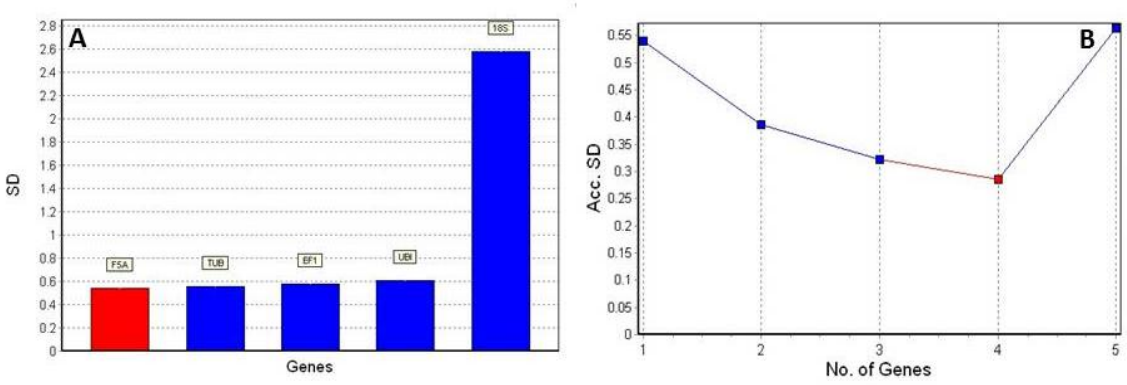


Figure 4.12 Norm finder analyses for five candidate reference genes using all sample set.**A**. Variation plot (red bar shown the best candidate gene). **B.** Accumulated standard deviation showing the best number of reference genes to normalize (in red). *SD* standard deviation, *Acc SD* accumulated standard deviation.

Since data from two different tissues was used, and metabolic compartmentalization has been proposed for cassava root, a further analysis was performed using two data sets: one for cortex tissue and another for pith tissue. This new analysis shows that the best gene for cortex tissue is tubuline (SD= 0.344) and using two genes reduces the variability to a minimum (Figure 4.13). Based on the accumulated standard deviation the best combination for cortex are tubuline and elongation factor 1-alpha (Accumulated standard deviation 0.244). Similar results were found by Bestkeeper with the highest correlation values for elongation factor 1-alpha (r= 0.92) and tubuline (r = 0.91)

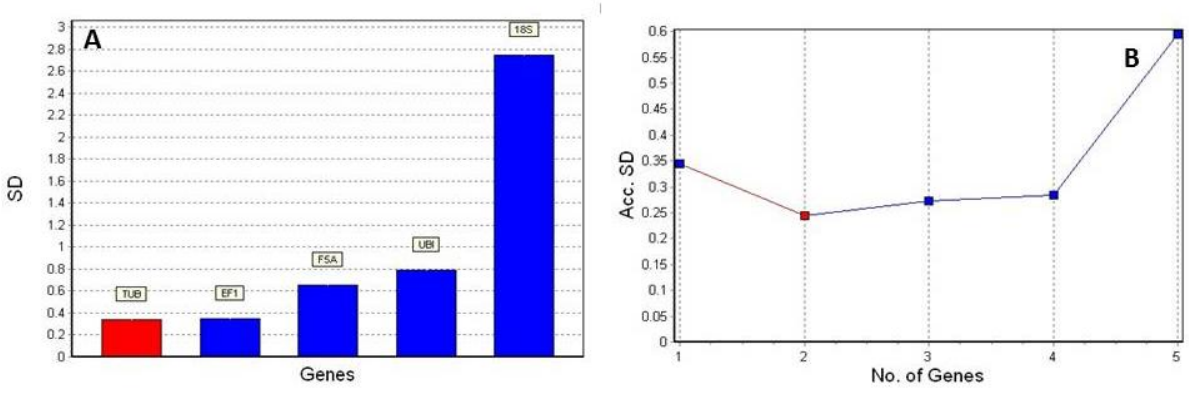


Figure 4.13 Norm finder analysis for five candidate reference genes in cassava root cortex tissue under PPD. **A**. Variation plot (red bar shown the best candidate gene) **B.** Accumulated standard deviation showing the best number of reference genes to normalize (in red). *SD* standard deviations, *Acc SD* accumulate standard deviation.

For the pith tissue the best gene was ubiquitin (SD= 0.28), and by using two genes the variability is reduced to a minimum (Figure 4.14). Based on the accumulate standard deviation the best combination for pith are ubiquitin and translation initiation factor 5A (Accumulate standard deviation 0.25). Similar results were found by Bestkeeper with the highest correlation value for ubiquitin (r= 0.93) and obtaining identical values for translation initiation factor 5A and elongation factor 1-alpha (r = 0.92)

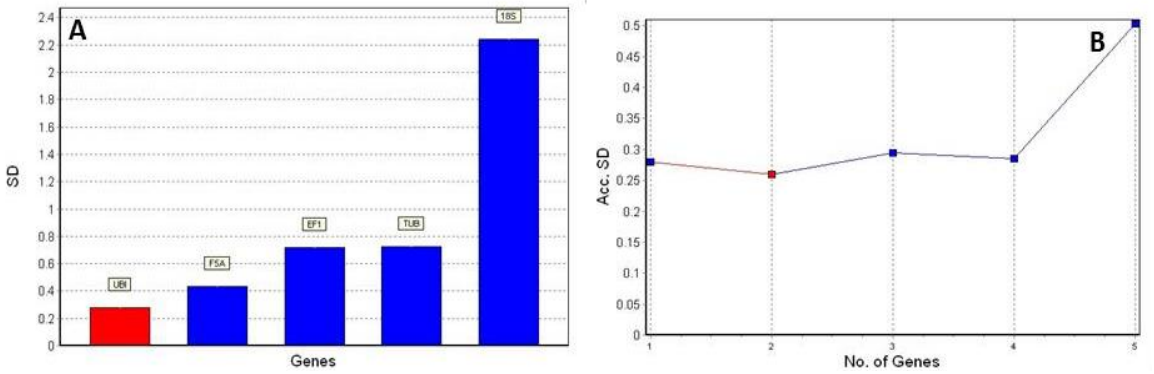


Figure 4.14 Norm finder analysis for four candidate reference genes in cassava root pith tissue under PPD. **A**. variation plot (in red the best candidate gene) **B.** Accumulated standard deviation showing the best number of reference genes to normalize (in red). *SD* standard deviations, *Acc SD* accumulate standard deviation.

4.4.6 Exploratory statistical study

In this study, 21 primers sets for PPD related genes and key metabolic pathways were used to compare their gene expression changes in two cassava accessions with contrasting PPD levels Mcol 2215 (high PPD) and SM 494-1(low PPD).Genes were tested in two different root tissues (cortex and pith) along five time points (0, 1, 24, 72, 120 hours) as was detailed in previous sections.

Reference gene validation showed the most suitable genes for each cassava root tissue. In the cortex tissue it was the average expression of tubuline and elongation factor 1-alpha, and in the case of pith tissue the best combination were the average expression of translation initiation factor 5A and ubiquitin.

Four raw data matrices (50x23= samples x genes) representing cortex and pith tissues from MCol 2215 and SM 494-1 were subject to data pre-treatment. Pre-treatment consist of

normalization with reference genes, average of technical replications (cDNAs) and average of biological samples (five roots from each time point set). Relative quantities were obtained with respect to mean expression of all genes, and then the data were transformed with $\log_2$ to obtain fold changes in gene expression.

Finally the data was autoscaled, this procedure is necessary since genes are expressed at different levels. Although most genes only have a few transcripts per cells, the high expressed genes with tenths of thousands of transcripts have much higher weight and completely dominate the results. Another undesirable effect is the magnitude of change, since low expressed genes may show a higher coefficient of variation than highly expressed genes. Those effects are removed by autoscaling (subtracting the mean expression of every gene to the corresponding gene thus further divided with the standard deviation) (Bergkvist *et al*., 2010).

Scatter plot can be used for direct data visualization of expression profiles; however, only two (2D) or three dimension (3D) can appropriately visualize one or several expression profiles, being useful to present expression trends as a function of time drug load or other metric variables (Figure 4.15). However, when there are many expression profiles it is difficult to distinguish trends in a scatter plot (Bergkvist *et al.,* 2010).

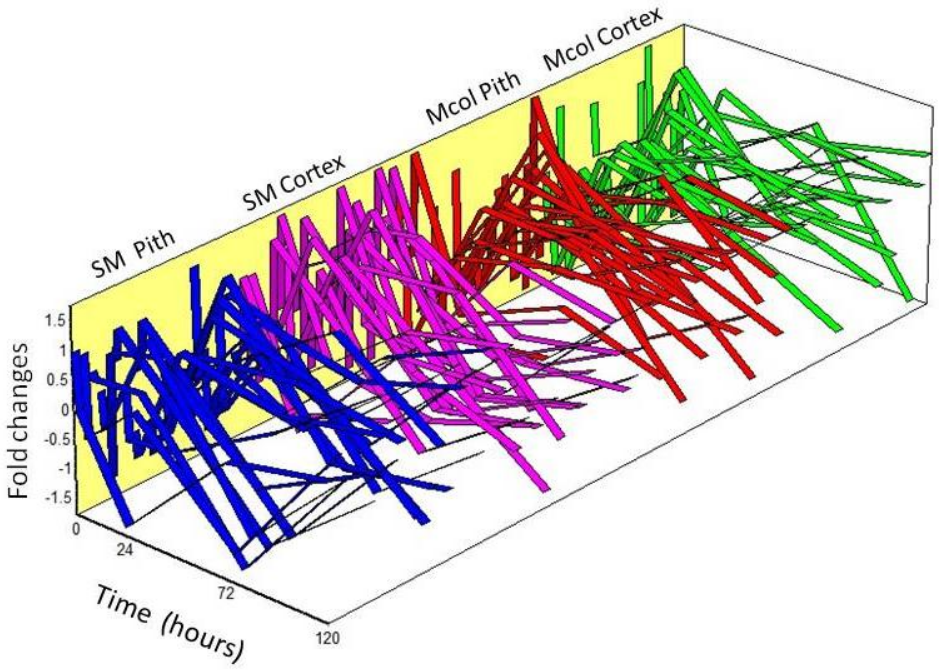


Figure 4.15 3D scatter plot of time course mean expression of 21 target genes in cassava root tissues under postharvest deterioration. Cortex and pith tissues from MCol 2215 (MCol) and SM 494-1 (SM) are shown. Data were autoscaled.

In order to overcome this bottleneck, an exploratory statistical study using several techniques was carried out to generate hypotheses that later were tested with a confirmatory statistical study. The four raw data matrices (from two accessions, and two tissues each) were used to build an augmented matrix and perform a multiway analysis with all sample sets together. Since the primary interests are the genes, these matrices were catenated in a 5x84 matrix.

Many mathematical tools are available for exploratory studies. In this research the augmented matrix was used to perform a principal component analysis (PCA), and a hierarchical clustering (a clustering visualization method).

Principal component analysis (PCA) is a potent tool to overcome the multidimensionality problem of showing several expression profiles in scatter plots. PCAs project the multi-dimensional expression data set in only two or three dimension plots making its visualization feasible. PCA allows the study of variables simultaneously reducing the dimensions without loss of important information; reflecting how the original variables are related and also how the samples are grouped (Ståhlberg *et. al*., 2008; Bergkvist *et al*., 2010).

In a PCA gene expression profiles are projected in a plot with a lower number of new variables, called principal components (PC's). The PC coordinates (also called scores) are extracted in consecutive order of importance, the first PC explains most of the data variance, and the successive PC's explain less. The variance from the experimental design conditions is expected to be systematic distributed, while error variance is expected to be randomly distributed. Since the last PC's explain a very low amount of varibility, they can be ignored because they include mainly noise or error variance. In this way, PCA can be a very efficient method to separate systematic effects from noise (Bergkvist *et al*., 2010).

The first three PC's obtained with augmented matrix explained 85.86% of the variability (PC1 42.08%, PC2 25 %, and PC3 18.78%). The most significant PC score vector reflects an increasing of the expression at 1 hour, while the PC2 reflects significant expression at 24 hours, and PC3 reflects increase at 72 hours (Figure 4.16).

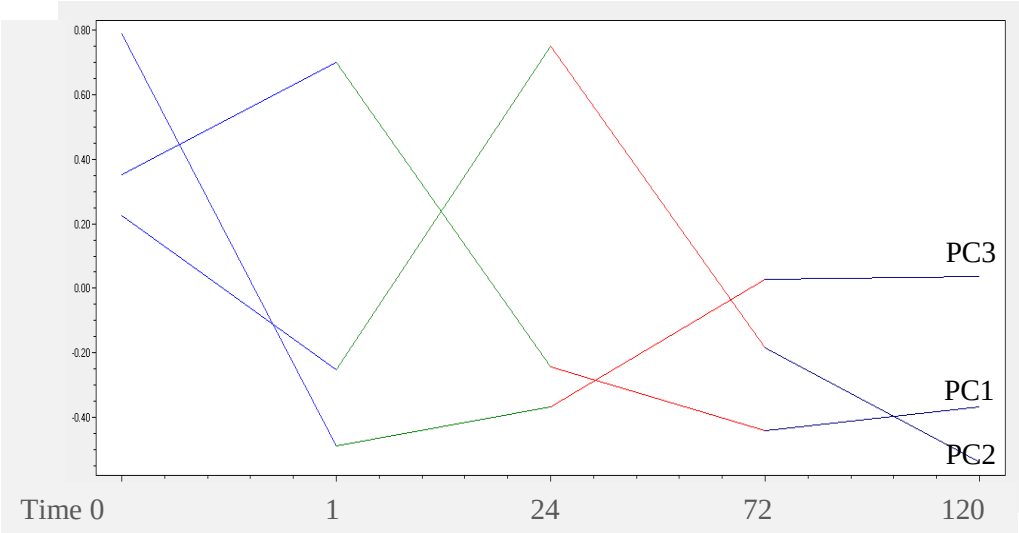


Figure 4.16 PC score vectors for augmented matrix built from cortex and pith matrices of Mcol2215 and SM494-1 expression of target genes

Figure 4.17 and 4.18 show the scatter plot for two and three main PCs derived from matrix augmented PCA analysis. The matrix augmented PCA analysis differentiated for each tissue is shown in Figure 4.19.

PCA helps to do general categorization of genes according with its position in the plot:

- PC1>>0, PC2 ≈0 Gene profiles with strong increase of expression at 1 hour.
- PC1<<0, Gene profiles with slightly increase (PC2<0) or strong decrease (PC2 >0) of expression at 1 hour.
- PC2>>0, PC1≈0 Gene profiles with strong increase of expression at 24 hours.
- PC2<<0, PC1≈0 Gene profiles with strong decrease of expression at 24 hours.
- PC3>0 Gene profiles with increase of expression at 72 hours (not shown in 2D plot. Figure 4.17).

- PC3<0 Gene profiles with decrease of expression at 72 hours (do not shown in 2D plot. Figure 4.17).

PC1 and PC2 distribute most of the gene profiles without clear clusters, however some clusters can be identified such as cluster 1 formed in PC1 <<0, PC2 ≈0. This cluster corresponds to genes with relative slightly increase or decrease in gene expression from 0 to 1 hour (Figure 4.17). It comprises gene profiles mainly from pith tissue of MCol 2215: APX, BCA, CIP, CD2, CDE, CHT, EXT, FSR, LPO, PAL and PIP. It also comprises profiles from cortex tissue of MCol 2215: APX, BCA, CIP, CDE, DAD, FSR, LPO, PAL and PIP. This cluster also includes some gene profiles for SM 494-1 only for pith tissue: APX, BCA, CIP, CAT and EXT.

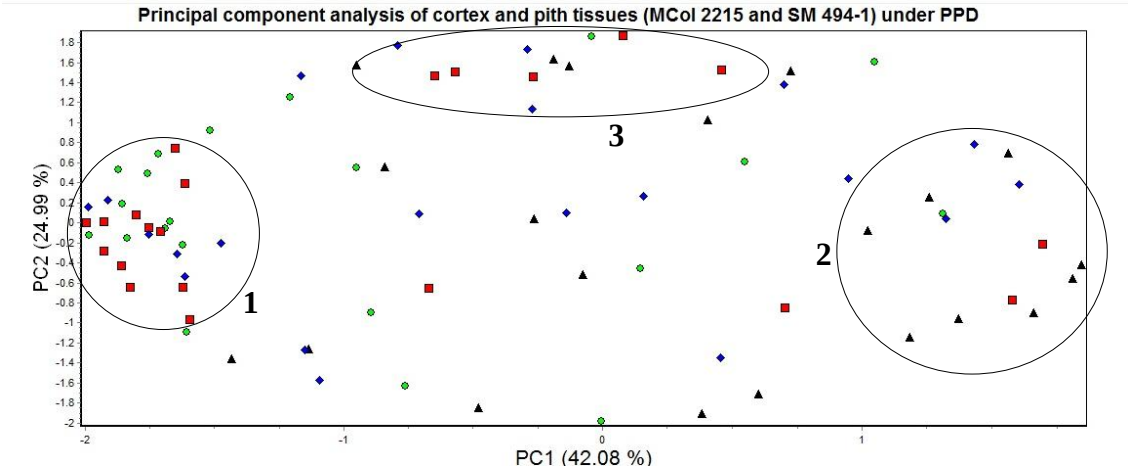


Figure 4.17 2D scatter plot for matrix augmented PCA analysis. Gene profiles of pith and cortex tissue from two cassava varieties with contrasting levels of PPD are shown. Green circles: MCol 2215-cortex, red squares MCol 2215-Pith, black triangle: SM 494-1-cortex, blue diamond: SM 494-1-pith. Clusters of interest are referenced.

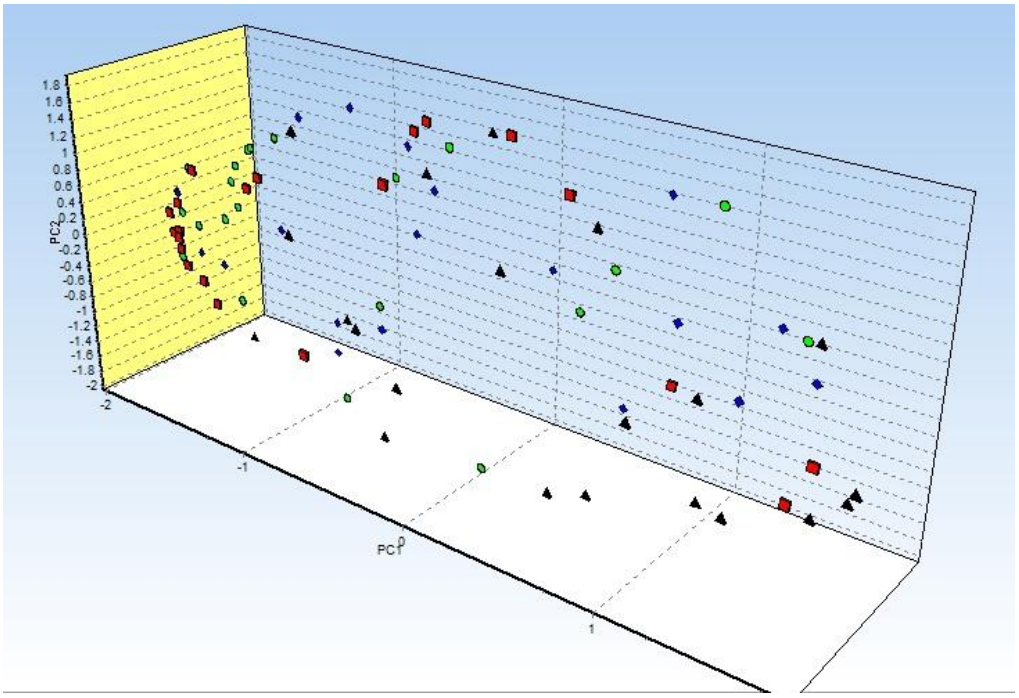


Figure 4.18 3D scatter plot for matrix augmented PCA analysis. Gene profiles of pit and cortex tissue from two cassava varieties with contrasting levels of PPD are shown. Green circles: MCol 2215-cortex, red squares MCol 2215-Pith, black triangle: SM 494-1-cortex, blue diamond: SM 494-1-pith.

A second cluster of gene profiles (cluster 2) for cortex tissue of SM 494 appears in PC1>>0 PC2 <0 with profiles for AOX, HRP, ACO, CD1 and BCB. These correspond to gene profiles with strong increase at 1 hour.

An interesting cluster of profiles (cluster 3) from pith tissue of MCol 2215 appears in PC1≈ 0 PC2 >>0, showing profiles for DAD, CD1, BCB, CAT and HRP. This coordinate corresponds to genes with strong increase of activity at 24 hours.

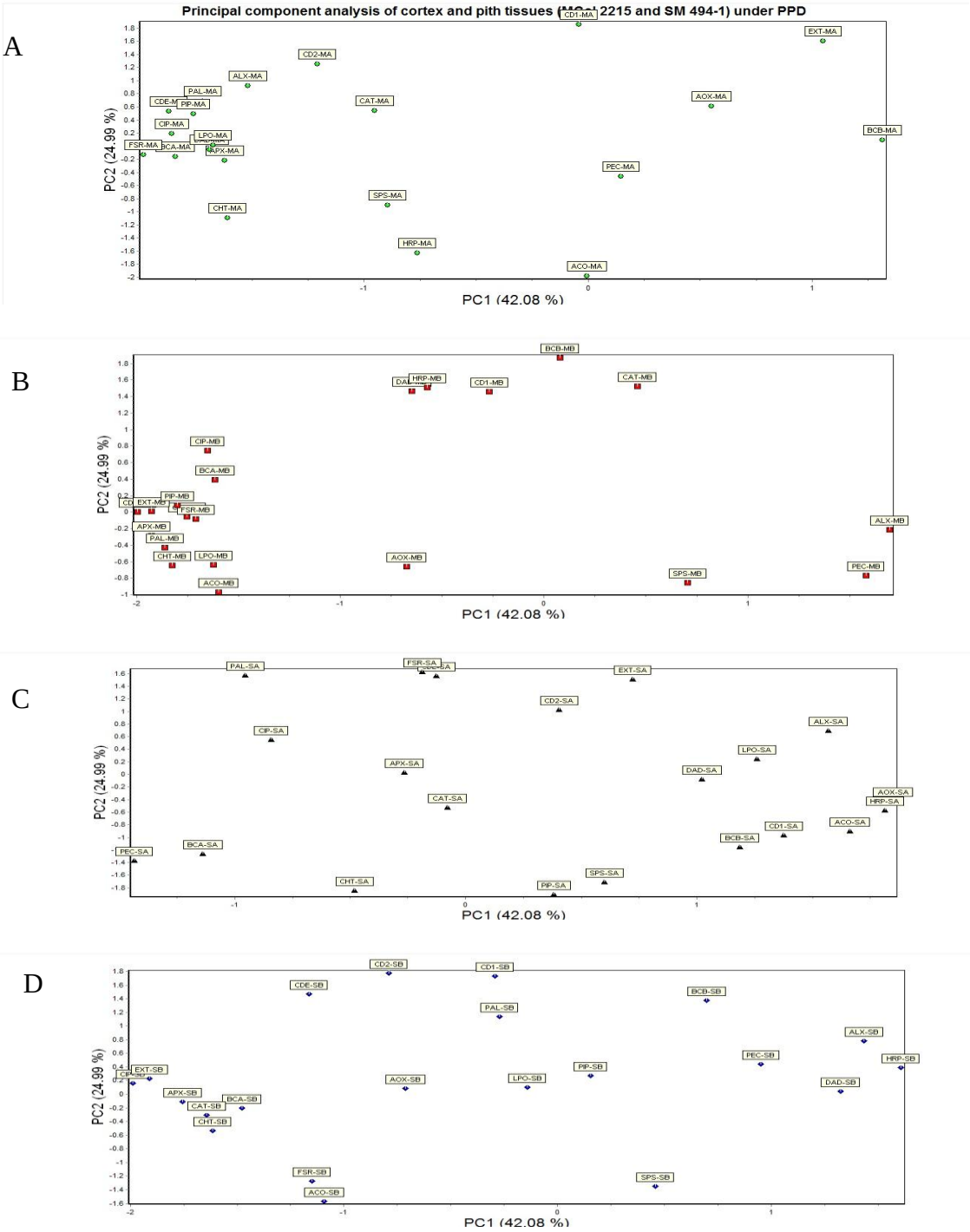


Figure 4.19 Independent 2D scatter plot for matrix augmented PCA analysis. Gene profiles of pit and cortex tissue from two cassava varieties with contrasting levels of PPD are shown. **A.** MCol 2215-cortex. **B.** MCol 2215-Pith, **C.** SM 494-1-cortex, **D.** SM 494-1-pith. Some gene profiles are not showed to easy visualization.

To obtain a more concise relationship among genes involved in cassava PPD, augmented matrix was analyzed by unsupervised clustering. The clustering performed by GeneEX® software is agglomerative clustering, also known as hierarchical type. Agglomerative clustering requires the selection of a measure of similarity among genes and a clustering algorithm (Bergkvist *et al*., 2010). In this research Euclidian distance was used as measure of similarity while the clustering algorithm selected was the average linkage Unweighted Pair Group Method using Arithmetic average (UPGMA). Different settings tested with augmented matrix showing similar outcomes.

The output of the agglomerative clustering method is a dendrogram (a figure similar to a tree) which displays the similarity distances among gene expression profiles clusters being formed as well as samples used. This information can be combined to produce a heatmap of the data set. Heatmap is a graphical representation of data; where the values of gene expression are represented as colors with a different intensity according to the pre-processed data, and coded in two dimensional mosaics (samples vs. gene targets). Heatmaps facilitate the interpretation of the cluster adding another dimension of information to the dendrogram (Bergkvist *et al*., 2010).

Clustering comparisons of data revealed interesting differences in profiles and coordinate expression between both contrasting PPD cassava accessions. Results obtained from heatmap and hierarchical clustering divided by type of tissue (cortex or pith) or by using augmented matrix defined 16 types of profiles (Figure 4.20, Figure 4.21).

According to the exploratory statistical study, accession MCol 2215 is characterized by the increase of the average expression of several genes at late stages (24 hours to 120 hours; profile type VII). Those genes include the transcripts ACO, APX, BCA, CD2, CHT, CDE, CIP, EXT, FSR, LPO, PAL and PIP, in pith tissue. The same profile was found in cortex tissue for transcripts APX, CDE, CHT, DAD, FSR and LPO. Also in the cortex tissue of MCol 2215 transcripts ALX, BCA, CIP, PAL and PIP were expressed at late stages but with slightly higher activity at 0 hour (type VI). One group of transcripts DAD, HRP, BCB showed high mean expression from 0 and 24 hours in pith tissue of MCol 2215 (type XV) (Figure 4.20, Figure 4.21).

According to the exploratory statistical study SM 494-1 accession is characterized by the increase mean expression of several genes at early stages. In pith tissue the transcripts AOX, BCB and PIP had early increase in gene expression at 0 hours with a drop in the expression at 1 hour (type V), or continuous expression until 24 hours (type IX; ALX, DAD, HRP, and PEC).The transcripts APX, CAT, CIP, EXT, CHT, and BCA in pith tissue however showed increase of mean expression at later stages (type VII). Cortex tissue of SM 494-1 had several genes with high expression at 1 hour (type IV; SPS, BCB, CD1, ACO, HRP, AOX), and some genes with increase mean expression at 1 hour and 24 hours (type X; LPO, DAD,) (Figure 4.20, Figure 4.21).

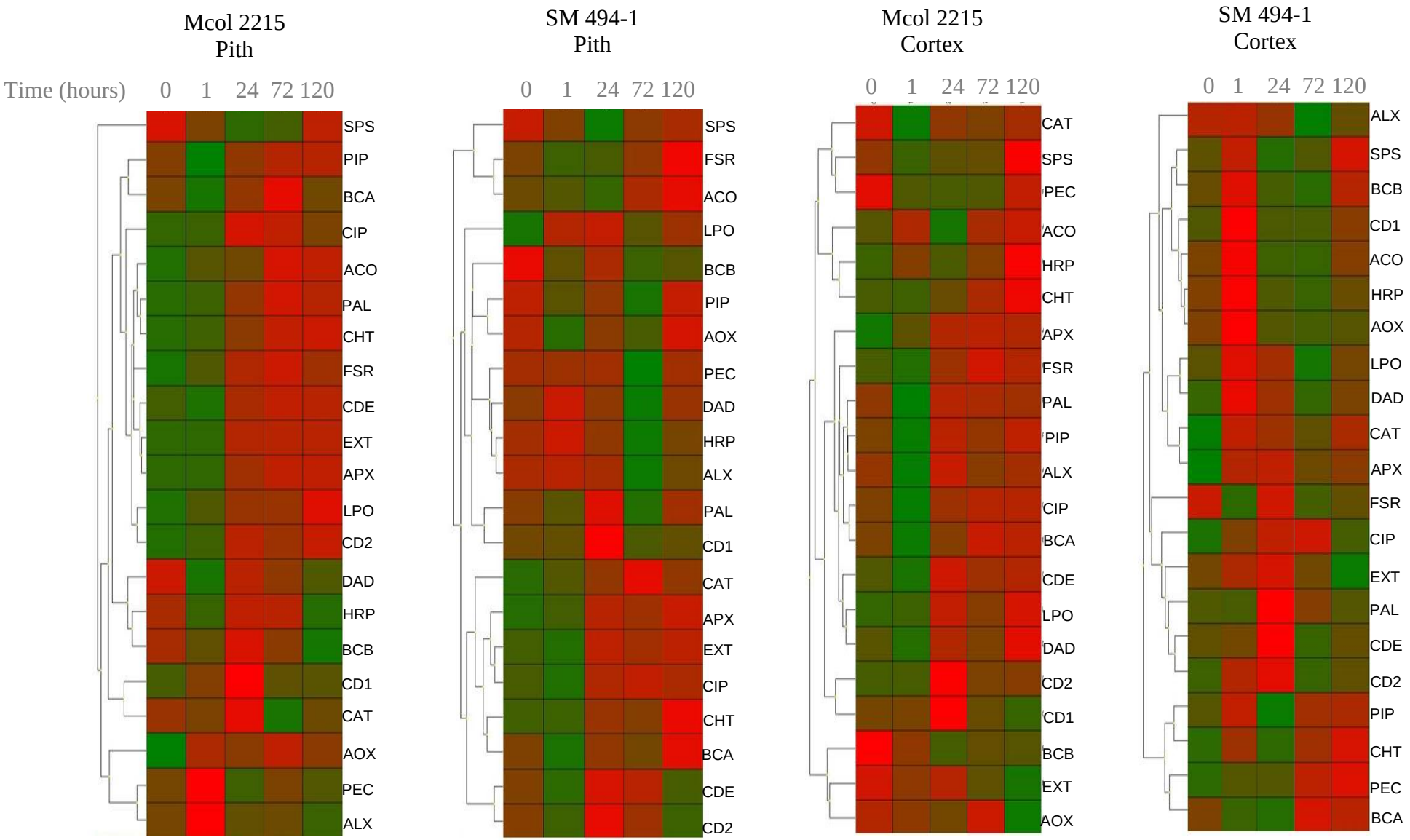


Figure 4.20 Independent gene expression hierarchical clustering and associated heatmap for pith and cortex tissue of contrasting cassava accessions under PPD

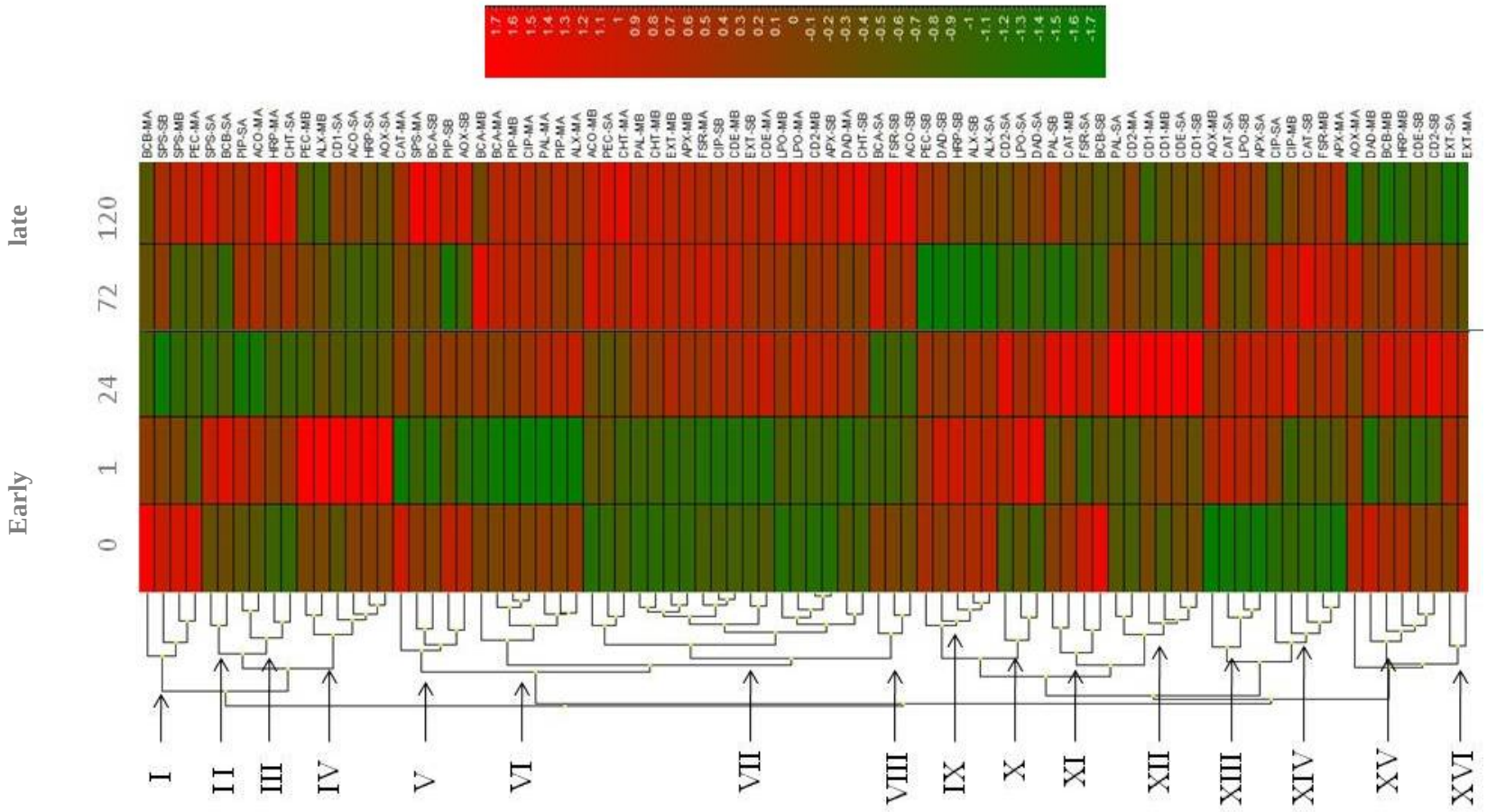


Figure 4.21 Gene expression profile groups (I to XVI) based in herachical clustering and heatmap obtained from augmented matrix. **Top** time line 0, 1, 24, 72, 120 hours **MA** Mcol 2215-cortex, **MB** Mcol 2215 pith, **SA** 494-1 cortex **SB** SM 494-1 pith.

The exploratory statistical analysis helped to produce a summary of gene classification which facilitated the generation of several hypotheses about the coordinate expression of genes in different tissues that were evaluated (Table 6.3).

	Mcol 2215		SM 494-1	
GENE	Pith	Cortex	Pith	Cortex
ACO	VII	III	VIII	IV
ALX	IV	VI	IX	IX
AOX	XIII	XV	V	IV
APX	VII	XIV	VII	XIII
BCA	VI	VI	V	VIII
BCB	XV	I	XI	II
CAT	XI	V	XIV	XIII
CD1	XII	XII	XII	IV
CD2	VII	XII	XV	X
CDE	VII	VII	XV	XII
CHT	VII	VII	VII	III
CIP	XIV	VI	VII	XIV
DAD	XV	VII	IX	X
EXT	VII	XVI	VII	XVI
FSR	XIV	VII	VIII	XI
HRP	XV	III	IX	IV
LPO	VII	VII	XIII	X
PAL	VII	VI	XI	XII
PEC	IV	I	IX	VII
PIP	VI	VI	V	III
SPS	I	V	I	II

Table 4.3 Summary of gene classification. Genes were classified into 16 groups after PCA and hierarchical analysis. Columns refer to root tissues (pith and cortex) evaluated in two cassava accessions. In red indicates genes of interest for pith tissue of MCol 2215, green indicates genes of interest for pith tissue of SM494-1, yellow indicates genes of interest for cortex tissue of SM494-1 and grey indicates genes with similar profile in pith tissue for both cassava accessions.

The hypotheses are:

1. There are not significant differences in the mean expression of genes APX, CD1, CHT, CIP, EXT and SPS between pith tissues.

2. There are significant differences in the mean expression between pith tissues of the genes ACO, PAL, FSR, CDE, LPO, and CD2 at 24, 72 and 120 hours.

3. There are significant differences in the mean expression between pith tissues of the genes BCB, DAD, HRP, and PEC at 0, 1 and 24 hours.

4. There are significant differences in the mean expression between cortex tissues of the genes ACO, AOX, BCB, CD1, HRP and SPS at 1hour.

5. There are significant differences between pith tissues for BCB, DAD, HRP and CAT at 24 hour

6. There are significant differences between pith tissues for LPO, BCB, PIP, AOX, PEC, DAD, HRP, ALX and PAL at 72 hours.

7. There are significant differences between cortex tissues for BCA, CIP, ALX, PAL, and PIP at 1 hour.

4.4.7 Confirmatory statistical study

To test these hypotheses an analysis of variance (ANOVA) was carried out using fold changes values obtained after data pre-treatment using equations 5, 6 9 and 10 for each gene (section 4.2.11.1) without averaging samples or technical replications and avoiding autoscaling . Significant differences in main effects and interactions are shown in Table 4.4.Results of separate multiple comparison analyses at each time points are shown in the next sections based in the categories described in Table 4.1.

Gene	Main Effects			Interactions			
	Accessions	*Time*	*Tissues*	*Accession x tissue*	*Accession x time*	*Tissue x time*	*Accession x tissue x time*
Alternative oxidase	0.047*	0.37	0.012*	0.05*	0.22	0.014*	0.0089**
Secretory peroxidase	0.49	0.001**	0.37	0.99	0.16	0.001*	0.77
Catalase 1	0.1	0.62	<0.001**	0.0008**	0.057	0.61	0.0087**
Beta cyanoalaline synthase A	0.83	<0.0001**	<0.001**	0.086	0.012*	0.023*	0.0001*
Beta cyanoalaline synthase B	0.87	<0.01**	<0.024	0.0028**	0.051	0.0001**	0.02*
Chitinase	0.76	<0.0003**	<0.0001**	0.87	0.032*	< 0.001**	0.0034*
Cysteine protease	0.94	<0.0003**	<0.0001**	0.8	0.21	0.66	< 0.01**
Defense against cell Dead 1	0.0049*	0.52	<0.0001**	0.58	0.19	0.6	0.64
Sucrose phosphate synthase	0.27	0.0067**	<0.0001**	0.35	0.34	0.25	0.4
Cytochrome P450 CYP79D1	0.68	<0.001**	0.724	0.16	0.057	<0.0001**	0.0001**
Cytochrome P450 CYP79D2	0.072	0.001**	0.0006**	0.0033**	0.035	<0.0001**	<0.0001**
Cytochrome P450 CYP71E	0.12	0.025*	<0.0001**	0.33	0.52	0.23	0.86
Allene oxidase cyclase	0.019*	0.0087*	<0.013*	<0.0001**	0.0026*	0.14	0.079
1-aminocyclopropane-1-carboxylate synthase	0.077	0.2	0.82	0.22	0.63	0.48	0.0001**
Lipooxigenase 1	0.4	0.0081*	<0.0001**	0.022*	0.045*	0.046*	0.83

Extensin	0.047*	0.0063*	0.32	0.28	0.89	0.6	<0.0001**
Phenylalanine ammonia-lyase	0.82	0.0095**	0.002**	0.014*	0.059	0.35	0.0152*
Pectinacetylesterase	0.6	0.038*	<0.0001**	0.007	0.059	0.021	0.31
Hydroxyproline rich protein	0.04	0.28	<0.0001**	0.27	0.011*	0.011*	0.15
Plasma membrane intrinsic protein	0.16	0.31	<0.0001**	0.022*	0.11	0.92	0.56
Sulfite reductase	0.08	0.057	<0.0001**	0.3	0.12	0.16	0.07

Table 4.4 Level of significance (p-value) for main effects (accessions, time and tissue) and its interactions after compare the gene expression of 21 genes in two cassava accessions with contrasting levels of PPD (Mcol 2215 high PPD, Sm 494- low PPD). * significant differences (α=0.05).**very significant differences

Oxidative stress genes

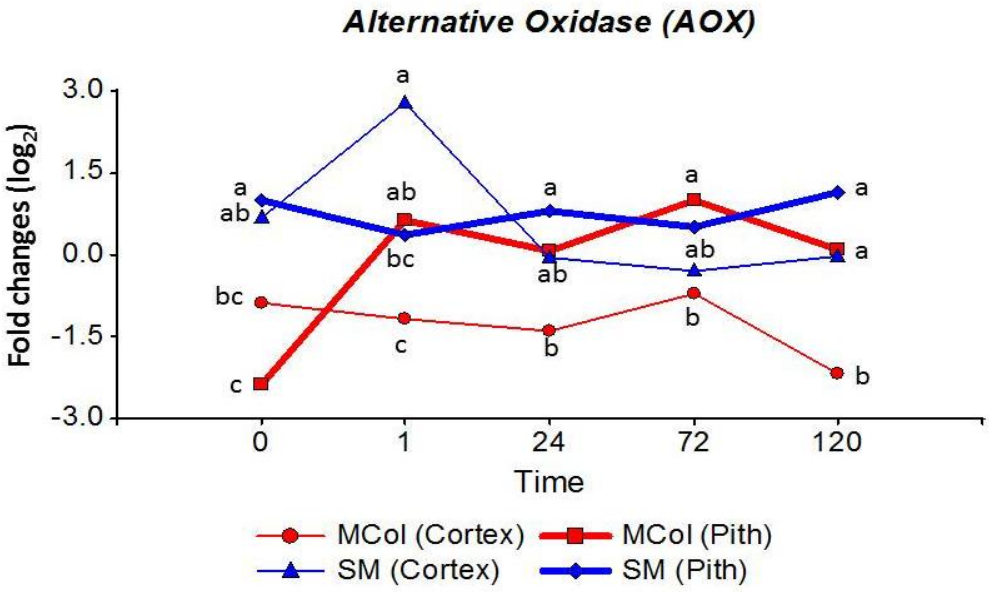


Figure 4.22 Gene expression profiles (fold changes vs time after harvest) for Alternative Oxidase. Red: MCol 2215 (susceptible accession), blue: SM 494-1(tolerant accession). Data were not autoscaled. Significant differences within each time are shown in lower case.

Gene expression profile for *Alternative Oxidase* (AOX) is shown in figure 4.22 LSD (α=0.05) multiple comparison demonstrated significant differences in mean expression for pith tissue in both accessions at 0 hours. Significant differences were found between cortex tissue in both accessions at 0, 1 and 120 hours (highest mean expression in cortex tissue of SM 494-1). By comparison, among all tissues significant differences were found at 0 hours (highest mean expression in pith and cortex tissue of SM 494-1), at 1 hour (highest mean expression in cortex tissue of SM 494-1 and lowest mean expression in cortex tissue of MCol 2215), 24, 72 and 120 hours (lowest mean expression in cortex tissue of MCol 2215).

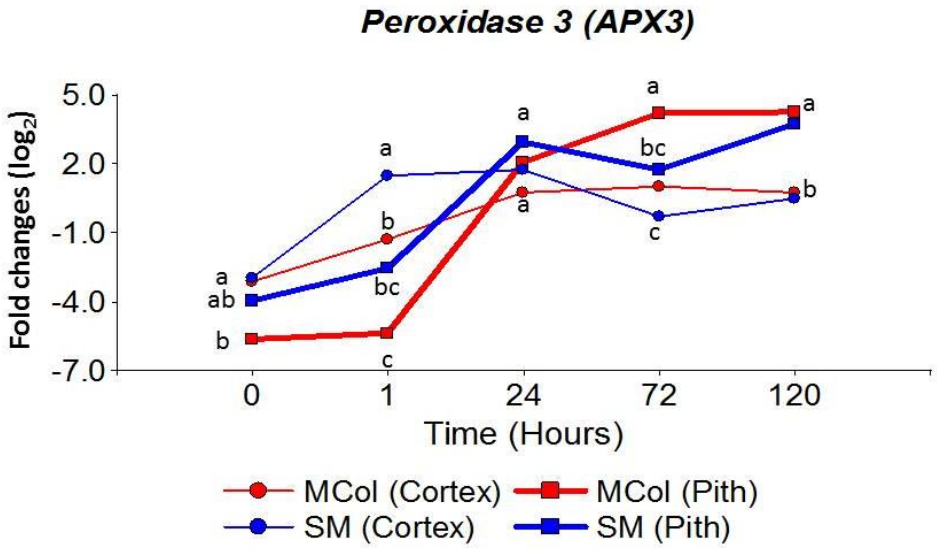


Figure 4.23 Gene expression profiles (fold changes vs time after harvest) for Peroxidase 3. Red: MCol 2215 (susceptible accession), blue: SM 494-1(tolerant accession) .Data were not autoscaled. Significant differences within each time are shown in lower case.

Gene expression profile for *Secretory peroxidase 3* (APX) is shown in Figure 4.23.

LSD (α= 0.05) multiple comparison demonstrated significant differences in mean expression of pith tissues for both accessions at 72 hours (highest mean expression in pith tissue of MCol 2215). In cortex tissues the profiles showed significant differences at 1 hour (highest mean expression in cortex tissue of SM 494-1). By comparison, among all tissues significant differences was observed at 0, and 1 hour (lowest mean expression in pith tissue of MCol 2215), 72 hours (highest mean expression in pith tissue of MCol 2215 and lowest mean expression in cortex tissue of SM 494-1) and 120 hours where significant differences were found between pith and cortex tissue (highest mean expression in pith tissue both accessions).

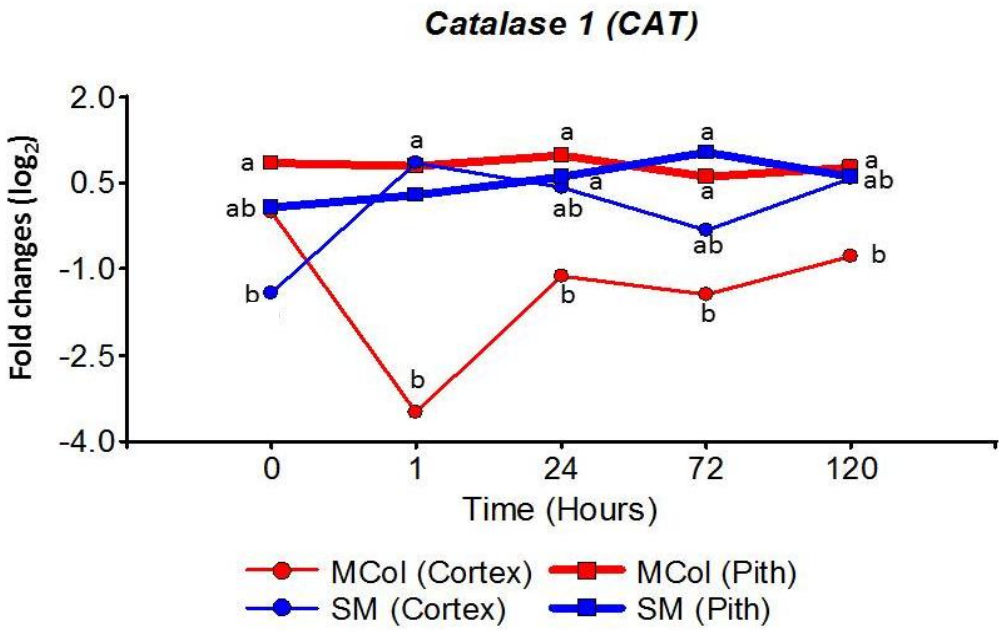


Figure 4.24 Gene expression profiles (fold changes vs time after harvest) for Catalase 1. Red: MCol 2215 (susceptible accession), blue: SM 494-1(tolerant accession). Data were not autoscaled. Significant differences within each time are shown in lower case.

Gene expression profile for *catalase 1* (CAT) is shown in Figure 4.24. LSD (α= 0.05) multiple comparisons did not find significant differences in mean expression of pith tissues for both accessions. In cortex tissue significant differences was found at 1hour (highest mean expression in cortex tissue of SM 494-1).

Genes of cyanide detoxification and biosynthesis of cyanogenic glucosides.

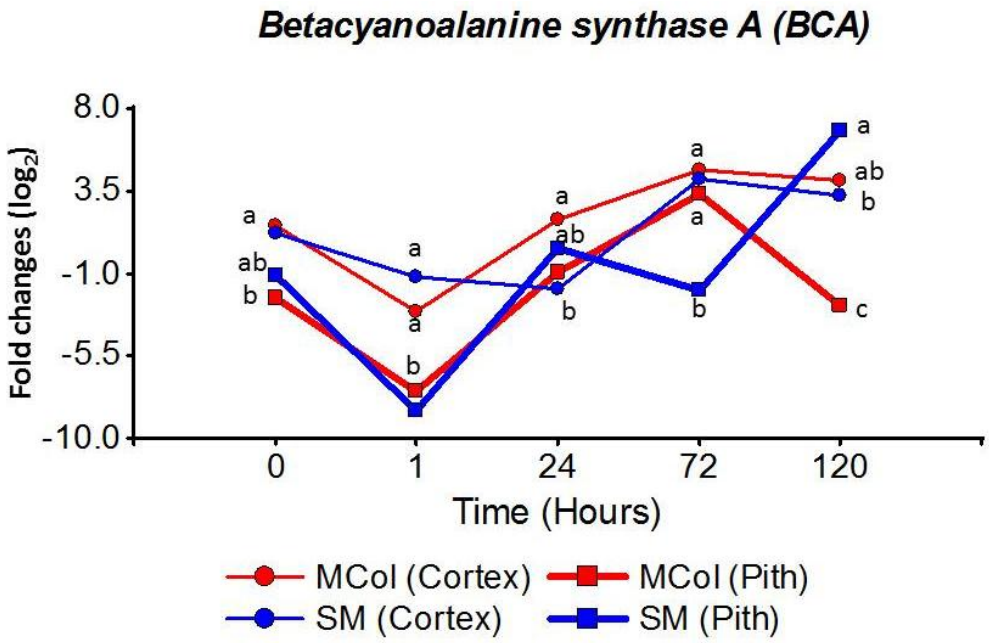


Figure 4.25 Gene expression profiles (fold changes vs time after harvest) for Beta cyanoalaline synthase.A. Red: MCol 2215 (susceptible accession), blue: SM 494-1(tolerant accession). Data were not autoscale. Significant differences within each time are shown in lower case

Gene expression profile for *Beta cyanoalaline ynthase A* (BCA) is shown in Figure 4.25. LSD (α=0.05) multiple comparison demonstrated significant differences in mean expression of pith tissues for both accessions at 72 hours (highest mean expression in pith tissue of MCol 2215) and 120 hours (highest mean expression in pith tissue of SM 494-1). In cortex tissue significant differences was found at 24 hours (highest mean expression in cortex of MCol 2215). By comparison among all tissues significant differences was found at 1 hour (highest mean expression in cortex tissue both accessions), 72 hours (lowest mean expression in pith tissue of SM 494-1), and 120 hours (highest mean expression in pith tissue SM 494-1 and lowest mean expression in pith tissue MCol 2215).

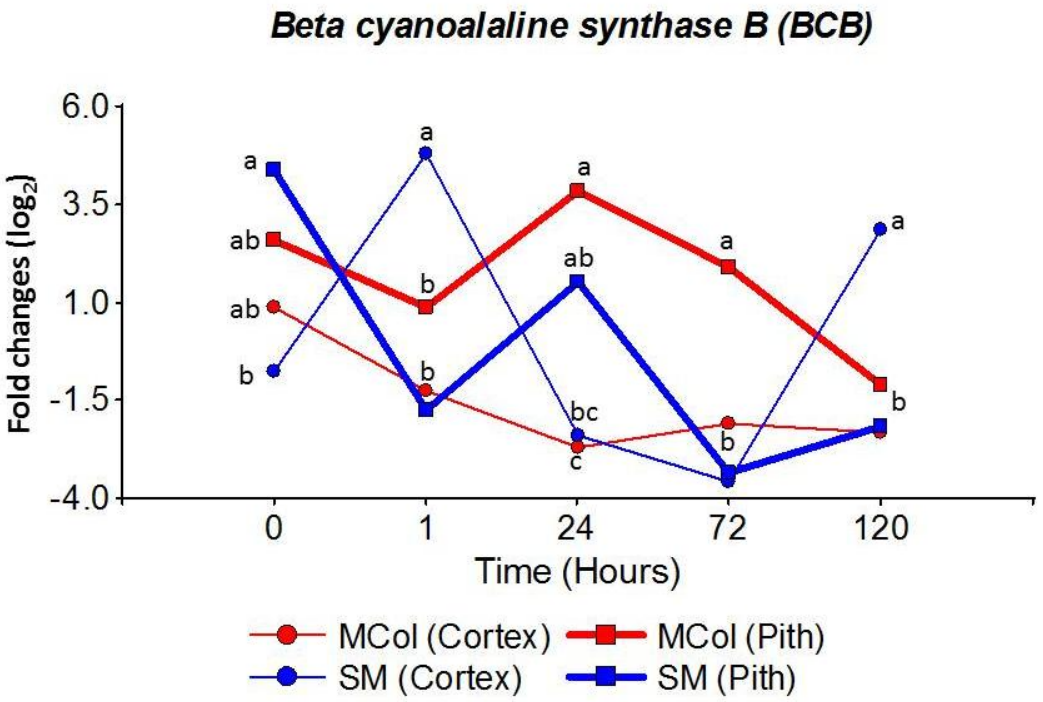


Figure 4.26 Gene expression profiles (fold changes vs time after harvest) for Beta cyanoalaline synthase B. Red: MCol 2215 (susceptible accession) blue: SM 494-1(tolerant accession). Data were not autoscaled. Significant differences within each time are shown in lower case.

Gene expression profile for *Beta cyanoalaline synthase B* (BCB) is shown in Figure 4.26. LSD (α= 0.05) multiple comparison demonstrated significant differences in mean expression of pith tissues for both accessions at 72 hours (highest mean expression in pith of MCol 2215). In cortex tissue significant differences were found at 1 hour and 120 hour (highest mean expression in cortex SM 494-1).By comparison, among all tissues there were significant differences at 0 hours (highest mean expression in pith tissue of SM 494-1and lowest mean expression in cortex tissue of SM 494-1), 1 hour (highest mean expression in cortex tissue of SM 494-1), 72 hours (highest expression in pith tissue of MCol 2215) and 120 hours (highest mean expression in cortex tissue SM 494-1).

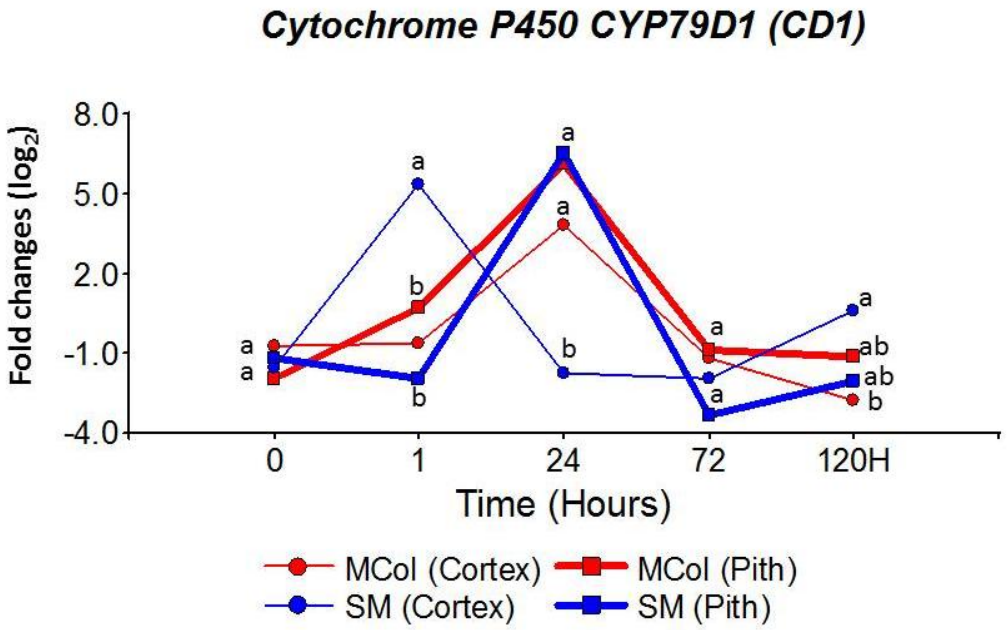


Figure 4.27 Gene expression profiles (fold changes vs time after harvest) for Cytochrome P450 CYP79D1 B. Red: MCol 2215 (susceptible accession), blue: SM 494-1(tolerant accession). Data were not autoscale. Significant differences within each time are shown in lower case.

Gene expression profile for *Cytochrome P450 CYP79D1* (CD1) is shown in figure 4.27.LSD (α= 0.05) multiple comparison did not found significant differences in mean expression of pith tissues for both accessions. In cortex tissue there was significant differences at 1 hour (highest mean expression in cortex tissue of SM 494-1), 24 hours (highest mean expression in cortex tissue of MCol 2215) and 120 hours (highest mean expression in cortex tissue of SM 494-1). By comparison, among all tissues there were significant differences at 1 hour (highest mean expression in cortex tissue of SM 494-1), 24 hours (lowest mean expression cortex tissue of SM 494-1) and 120 hours (highest mean expression in cortex tissue of SM 494-1).

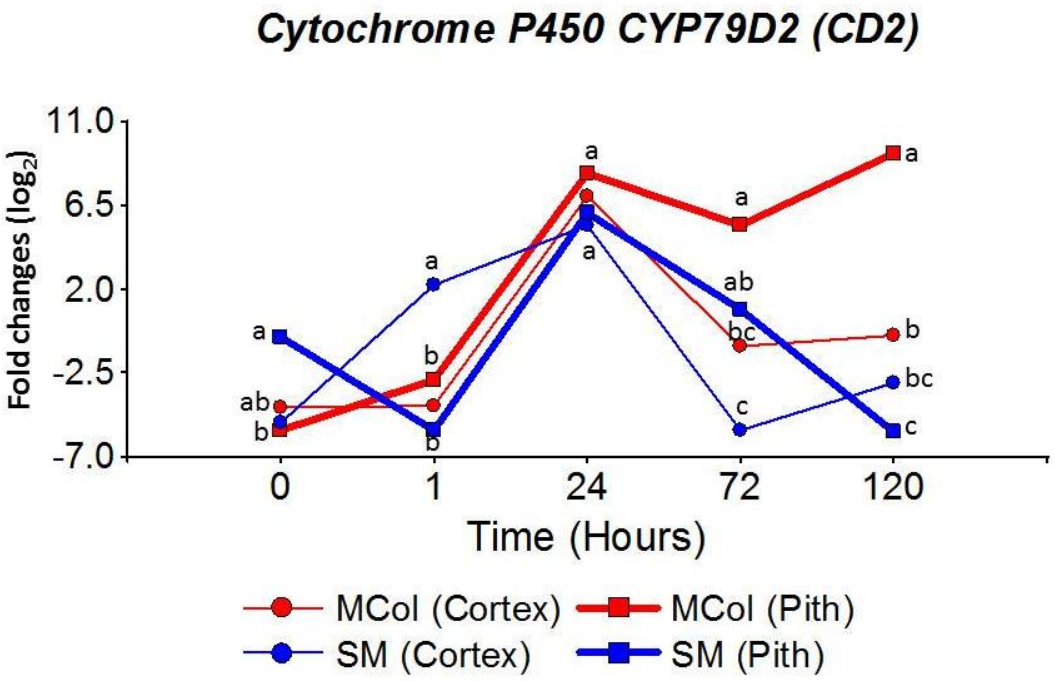


Figure 4.28 Gene expression profiles (fold changes vs time after harvest) for Cytochrome P450 CYP79D2. Red: MCol 2215 (susceptible accession), blue: SM 494-1(tolerant accession). Data were not autoscaled. Significant differences within each time are showed in lower case.

Gene expression profile for *Cytochrome P450 CYP79D2* (CD2) is shown in Figure 4.28. LSD (α= 0.05) multiple comparison found significant differences in mean expression of pith tissues at 0 hours (highest mean expression in pith tissue of SM 494-1) and 120 hours (highest mean expression in pith tissue of MCol 2215). In cortex tissue there was significant differences at 1 hour (highest mean expression in cortex tissue of SM 494-1). By comparison, among all tissues there was significant differences at 0 hours (highest mean expression in pith tissue of SM 494-1), at 1 hour (highest mean expression in cortex tissue of SM 494-1), 72 hours and 120 hours (highest mean expression in pith tissue of MCol 2215).

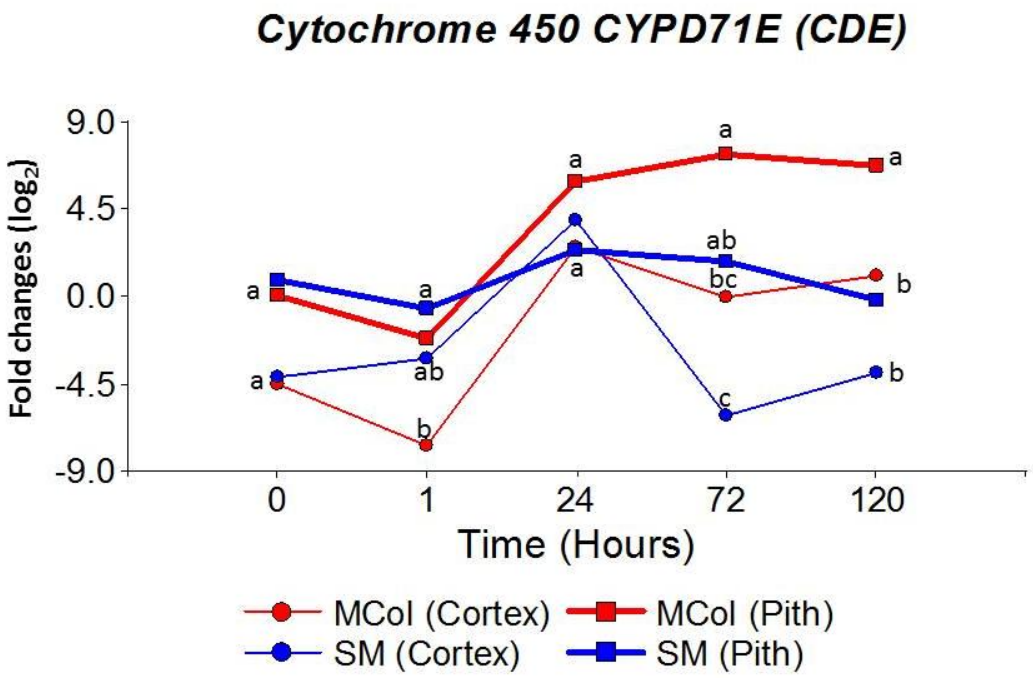


Figure 4.29 Gene expression profiles (fold changes vs time after harvest) for cytochrome P450 CYP71E. Red: MCol 2215 (susceptible accession), blue: SM 494-1(tolerant accession). Data were not autoscaled. Significant differences within each time are shown in lower case.

Gene expression profile for *Cytochrome P450 CYP71E* (CDE) are shown in figure 4.29 LSD (α= 0.05) multiple comparison found significant differences in mean expression of pith tissues only at 120 hours (highest mean expression in pith tissue of MCol 2215). In cortex tissue there was no significant differences in any time. By comparison among all tissues significant differences were found at 1 hour (highest mean expression in pith tissue of SM 494-1), at 72 hours and 120 hours (highest mean expression in pith tissue of MCol 2215).

Program cell death genes

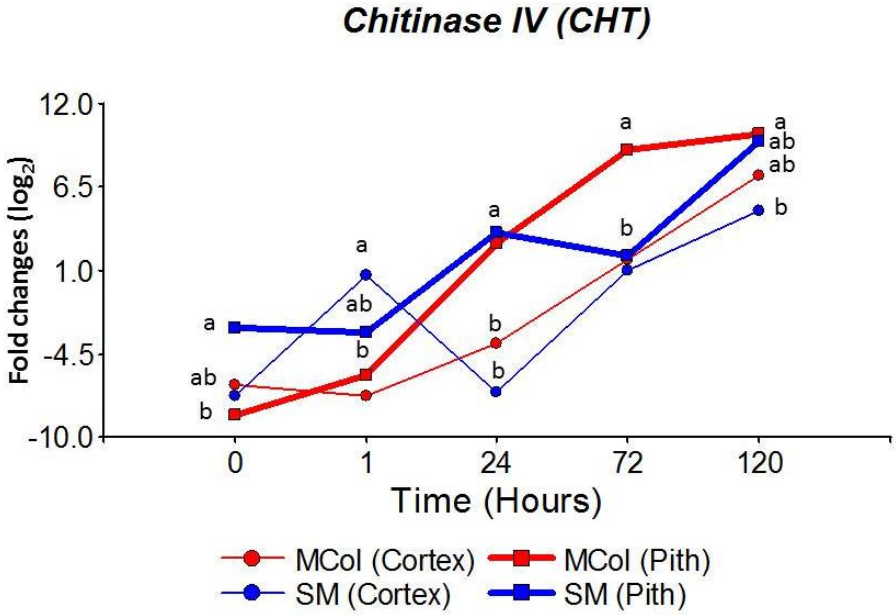


Figure 4.30 Gene expression profiles (fold changes vs time after harvest) for Chitinase. Red: MCol 2215 (susceptible accession), blue: SM 494-1(tolerant accession). Data were not autoscaled. Significant differences within each time are shown in lower case.

Gene expression profile for *Chitinase* (CHT) is shown in Figure 4.30. LSD (α= 0.05) multiple comparison found significant differences in mean expression of pith tissues at 0 hours (highest mean expression in pith tissue of SM 494-1) and at 72 hours (highest expression in pith tissue of MCol 2215). In cortex tissue there ware significant differences at 1 hour (high expression in cortex tissue of SM 494-1). By comparison, among all tissues significant differences were found in all time points: 0 hours (highest mean expression in pith tissue of SM 494-1), 1 hour (highest mean expression in cortex tissue of SM 494-1and lowest mean expression cortex tissue of MCol 2215), 24 hours (highest mean expression in pith tissue both accessions), at 72 hours (highest mean expression in pith tissue of MCol 2215) at

120 hours (highest mean expression in pith tissue both accessions and lowest mean expression of cortex tissue of SM 494-1).

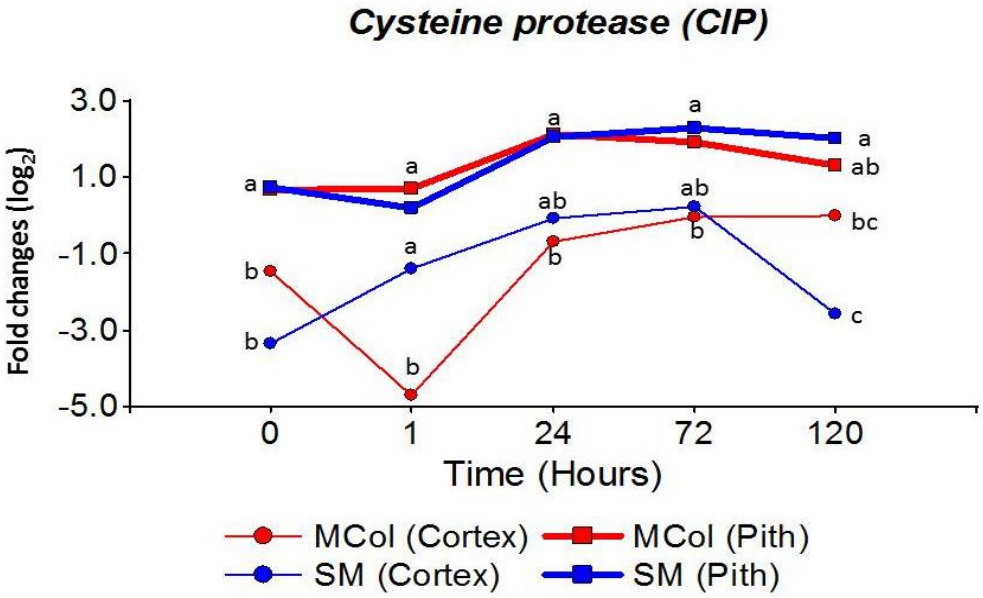


Figure 4.31 Gene expression profiles (fold changes vs time after harvest) for Cysteine protease. Red: MCol 2215 (susceptible accession), blue: SM 494-1(tolerant accession). Data were not autoscaled. Significant differences within each time are shown in lower case.

Gene expression profile for *Cysteine protease* (CIP) is showed in Figure 4.31 LSD (α= 0.05) multiple comparison did not find significant differences in mean expression of pith tissues at any time. In cortex tissue significant differences were found at 1 hour (highest mean expression in cortex tissue of SM 494-1) and at 120 hours (highest mean expression in cortex tissue of MCol 2215). By comparison, among all tissues, significant differences were found at 0 hours (lowest mean expression in cortex tissues both accessions), 1, 24, 72 and 120 hours (lowest mean expression in cortex tissue Mcol 2215).

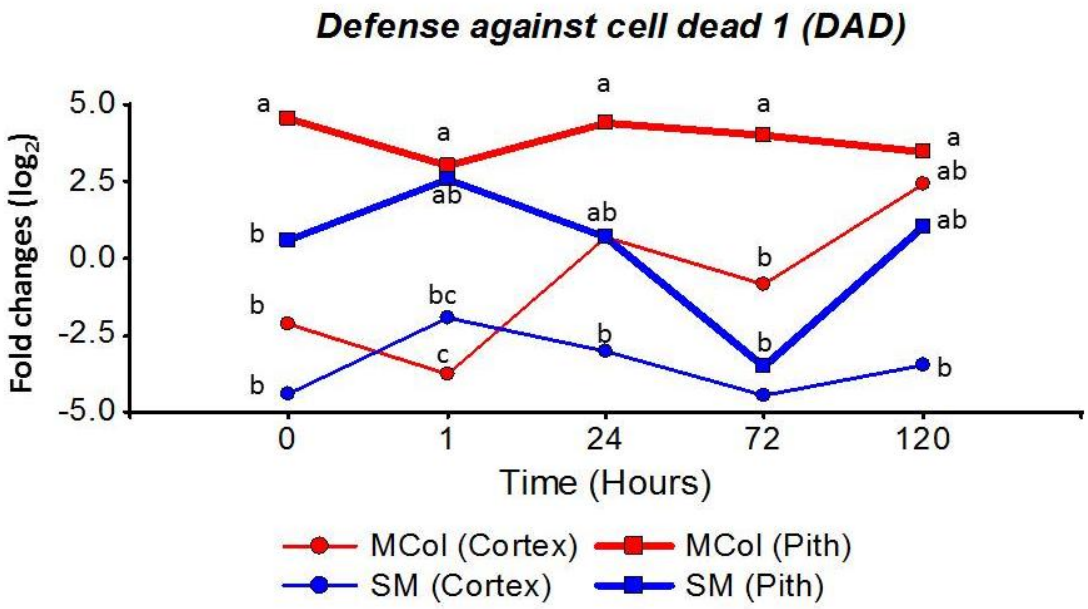


Figure 4.32 Gene expression profiles (fold changes vs time after harvest) for Defense against cell dead 1. Red: MCol 2215 (susceptible accession), blue: SM 494-1(tolerant accession). Data were not autoscaled. Significant differences within each time are showed in lower case

Gene expression profile for *Defense against cell Dead 1*(DAD) is shown in figure 4.32. LSD (α= 0.05) multiple comparison found significant differences in mean expression of pith tissues at 72 hours (highest mean expression in pith tissue of MCol 2215). In cortex tissue there was no significant differences in any time. By tissue comparison among all tissues significant differences were found at 0 hours between pith tissues and cortex tissue (highest mean expression in pith tissue of SM 494-1) at 1 hour (highest mean expression in pith tissue of Mcol 2215), at 24 and 72 hours (highest mean expression in pith tissue of MCol 2215), and finally significant differences were found at 120 hours (lowest mean expression in pith tissue of SM494-1).

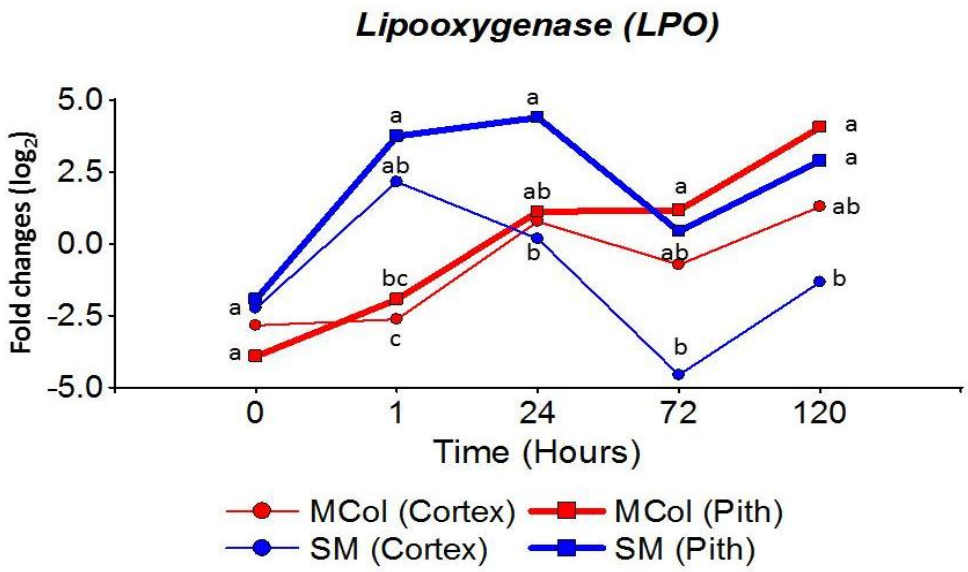


Figure 4.33 Gene expression profiles (fold changes vs time after harvest) for Lipooxigenase 1. Red: MCol 2215 (susceptible accession), blue: SM 494-1(tolerant accession). Data were not autoscaled. Significant differences within each time are shown in lower case.

Gene expression profile plot for *lipooxigenase 1* (LPO) is shown in Figure 4.33. LSD (α=0.05) multiple comparison found significant differences in mean expression of pith tissues at 1 hour (highest mean expression in pith tissue of SM 494-1). In cortex tissue there was significant differences at 1 hour (highest mean expression in cortex tissue of SM 494-1) By comparison, among all tissues significant differences were found at 1 hour (highest mean expression in pith tissue of SM 494-1 and lowest mean expression in cortex tissue of MCol 2215) at 24 hours (highest mean expression in pith tissue of SM 494 and lowest mean expression in cortex tissue of SM 494-1) at 72 hours (highest men expression in pith tissue of MCol 2215 and lowest mean expression cortex tissue of SM 494-1) and 120 hours (highest mean expression in pith tissue of MCol 2215 and lowest mean expression cortex tissue of SM494-1).

Biosyntheis and metabolism

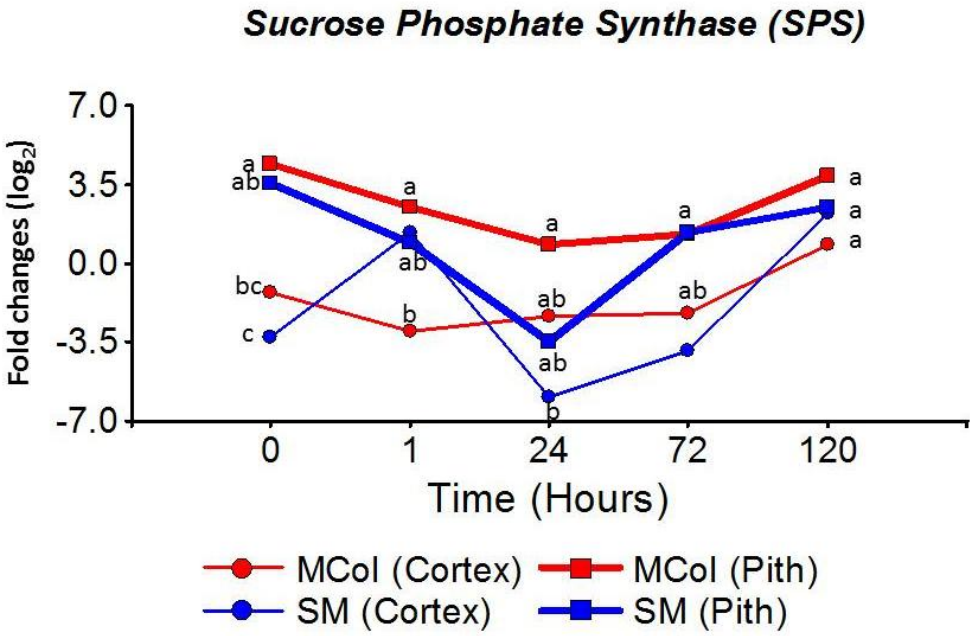


Figure 4.34 Gene expression profiles (fold changes vs time after harvest) for Sucrose phosphate synthase. Red: MCol 2215 (susceptible accession), blue: SM 494-1(tolerant accession). Data were not autoscaled. Significant differences within each time are showed in lower case.

Gene expression profile for *Sucrose phosphate synthase* (SPS) is shown in Figure 4.34. LSD (α= 0.05) multiple comparison did not found significant differences in mean expression neither pith tissues nor cortex tissue at any time. By comparison among all tissues significant differences were found at 0 hours (highest expression in pith tissue of MCol 2215), 1 hour (lowest mean expression in cortex tissue of MCol 2215), 24 hours (highest mean expression in pith tissue of MCol 2215; lowest mean expression in cortex tissue of SM 494-1)

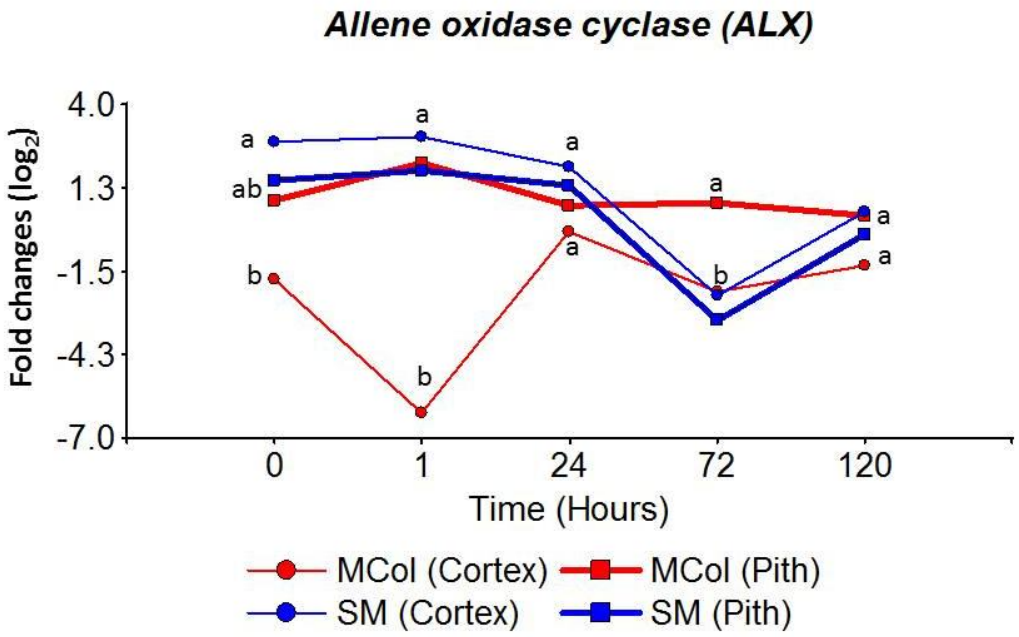


Figure 4.35 Gene expression profiles (fold changes vs time after harvest) for Allene oxidase cyclase. Red: MCol 2215 (susceptible accession), blue: SM 494-1(tolerant accession). Data were not autoscaled. Significant differences within each time are shown in lower case.

Gene expression profiles for *Allene oxidase cyclase* (ALX) is shown in Figure 4.35. LSD (α=0.05) multiple comparison found significant differences in mean expression of pith tissues at 72 hours (highest mean expression in pith tissue of MCol 2215). In cortex tissue significant differences were found at 0 and 1 hour (highest mean expression in cortex tissue of SM 494-1).By comparison, among all tissues significant differences were found at 0 hours (lowest mean expression in cortex tissue of MCol 2215) 1 hour (lowest mean expression in cortex tissue of MCol 2215) and 72 hours (highest mean expression in pith tissue of MCol 2215).

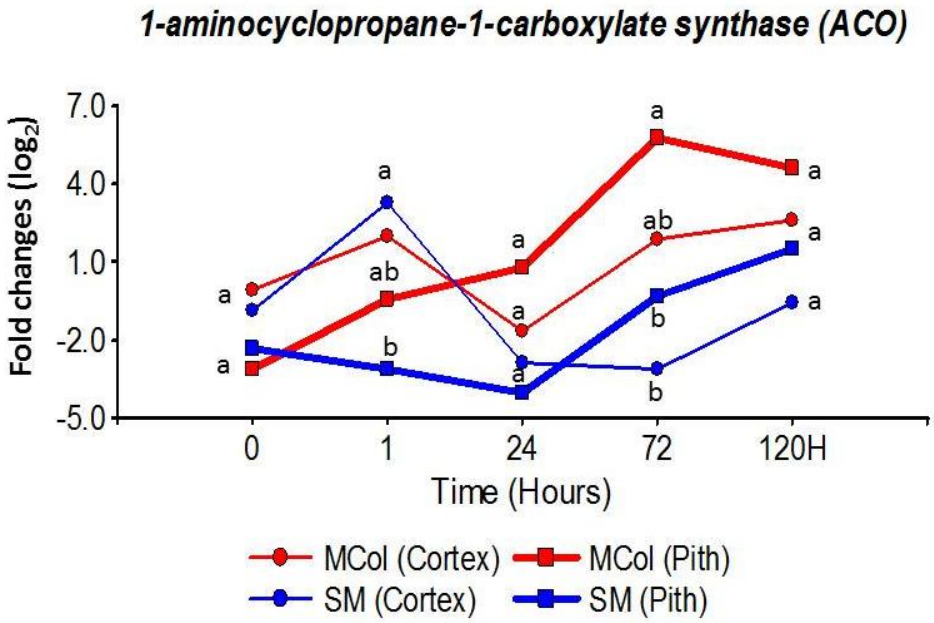


Figure 4.36 Gene expression profiles (fold changes vs time after harvest) for 1-aminocyclopropane-1-carboxylate synthase. Red: MCol 2215 (susceptible accession), blue: SM 494-1(tolerant accession). Data were not autoscaled. Significant differences within each time are shown in lower case.

Gene expression profiles for *1-aminocyclopropane-1-carboxylate synthase* (ACO) is shownin Figure 4.36. LSD (α= 0.05) multiple comparison found significant differences in mean expression of pith tissues at 72 hours (highest mean expression in pith tissue of MCol 2215). In cortex tissue there was not significant differences at any time. By comparison, among all tissues significant differences were found at 1 hour (lowest mean expression in pith tissue of SM 494-1) and at 72 hours (lowest mean expressions in pith and cortex tissue of SM 494-1).

Plant defense and wounding

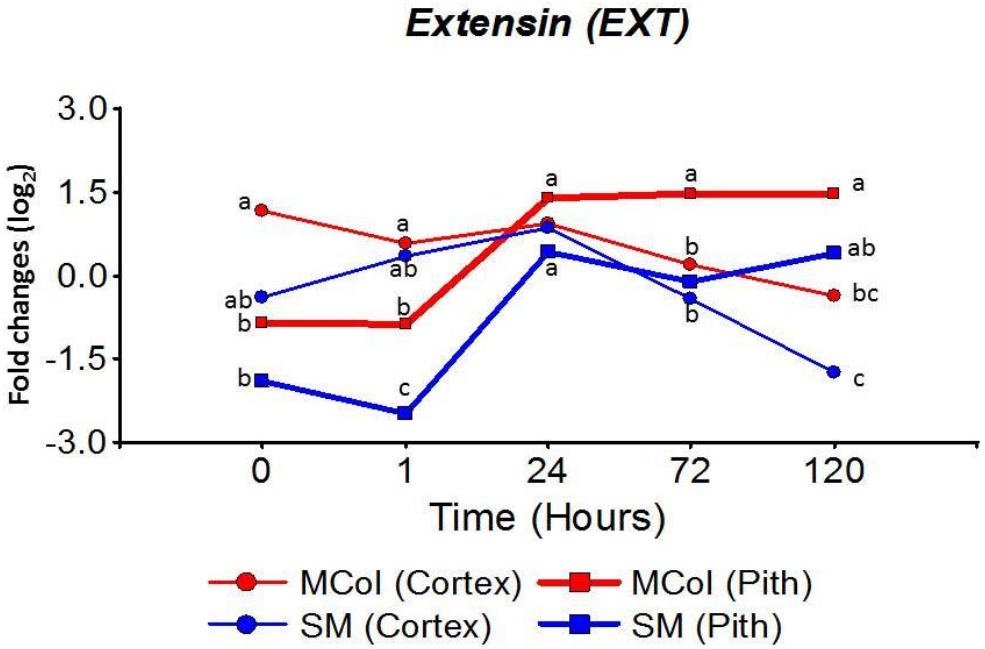


Figure 4.37 Gene expression profiles for (fold changes vs time after harvest) Extensin . Red: MCol 2215 (susceptible accession), blue SM 494-1(tolerant accession). Data were not autoscaled. Significant differences within each time are shown in lower case.

Gene expression profile for *Extensin* (EXT) is showed in Figure 4.37. LSD (α= 0.05) multiple comparison found significant differences in mean expression of pith tissues at 1 and 72 hours (highest mean expression in pith tissue of MCol 2215). In cortex tissues for both accessions there were not significant differences at any time. By comparison among all tissues significant differences were found at 0 hours (highest mean expression in cortex tissue of MCol 2215), 1 hour (lowest mean expression in pith tissue of SM 494-1), 72 hours (highest mean expression pith tissue of MCol 2215) and 120 hours (highest mean expression pith tissue of MCol 2215 and lowest mean expression in cortex tissue of SM 494-1).

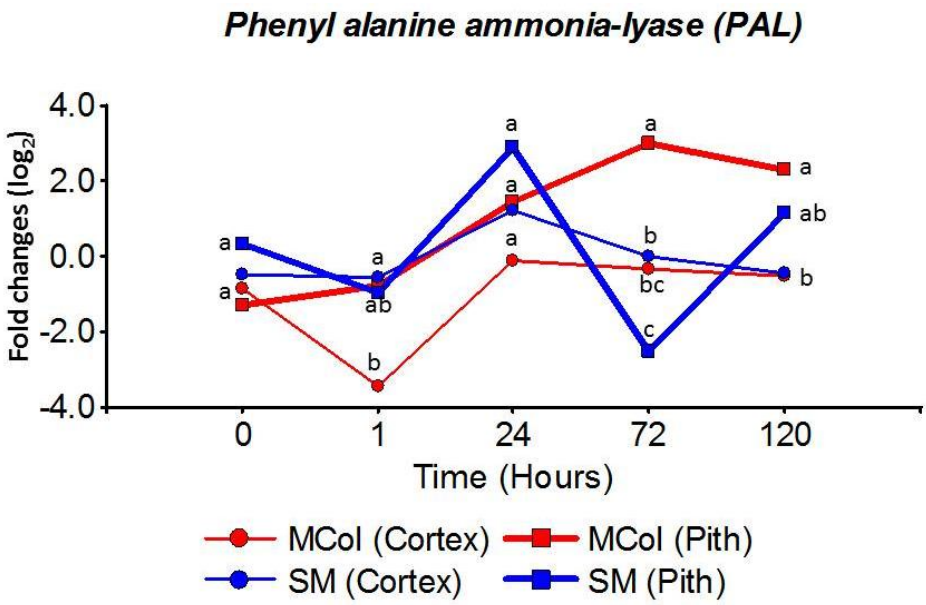


Figure 4.38 Gene expression profiles (fold changes vs time after harvest) for Phenylalanine ammonia-lyase. Red: MCol 2215 (susceptible accession), blue: SM 494-1(tolerant accession). Data were not autoscaled. Significant differences within each time are shown in lower case.

Gene expression profiles for *Phenylalanine ammonia-lyase* (PAL) is shown in fFgure 4.38. LSD (α= 0.05) multiple comparison found significant differences in mean expression of pith tissues at 72 hours (highest mean expression in pith tissue of MCol 2215). In cortex tissues significant differences was found at 1 hour (highest mean expression in cortex tissue of SM 494-1).By comparison, among all tissue significant differences were found at 1 hour (lowest mean expression in cortex tissue of MCol 2215), 72 hours (highest mean expression in pith tissue of MCol 2215, lowest mean expression in pith tissue of SM 494-1), and 120 hours (highest mean expression in pith tissue of MCol 2215 and lowest mean expression of cortex tissue both accessions).

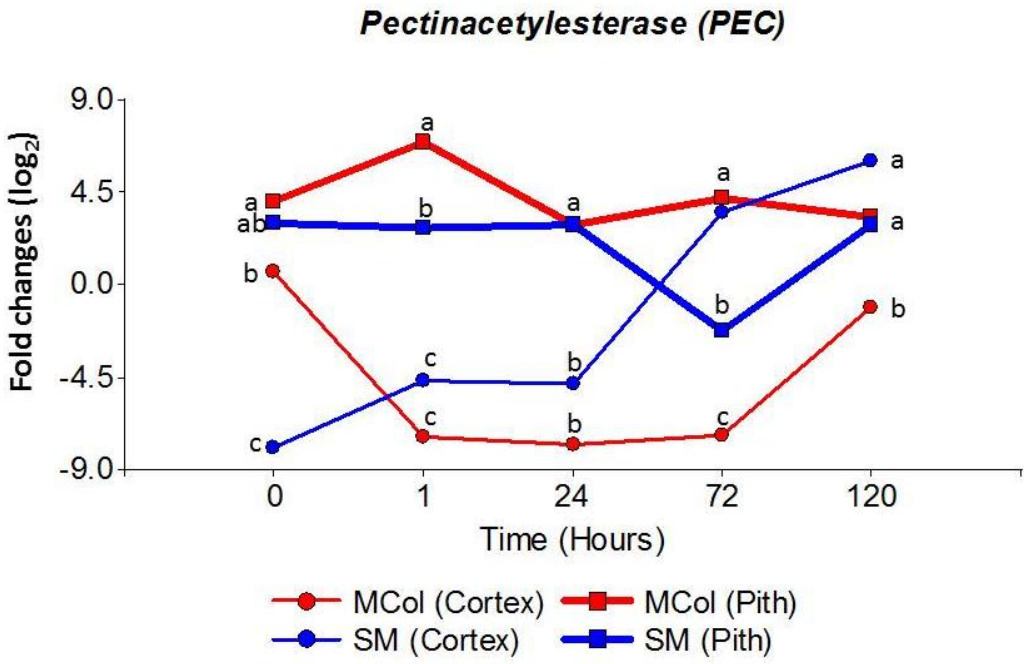


Figure 4.39 Gene expression profiles (fold changes vs time after harvest) for Pectinacetylesterase Red: MCol 2215 (susceptible accession), blue: SM 494-1(tolerant accession). Data were not autoscaled. Significant differences within each time are shown in lower case.

Gene expression profile for *Pectinacetylesterase* (PEC) is shown in Figure 4.39. LSD (α=0.05) multiple comparison found significant differences in mean expression of pith tissues at 1 and 72 hours (highest expression in pith tissue of MCol 2215). In cortex tissues significant differences were observed at 72 hours and 120hours (highest expression in cortex tissue of SM 494-1).By comparison, among all tissues significant differences were found at 0 hours (lowest mean expression in cortex of SM 494-1), 1 hour (highest mean expression in pith tissue of MCol 2215, lowest mean expression in cortex tissue of MCol 2215), 24, 72 hours (highest mean expression in pith tissue of MCol 2215), and 120 hours (highest mean expression in cortex tissue of SM 494-1and lowest mean expression in cortex tissue of MCol 2215).

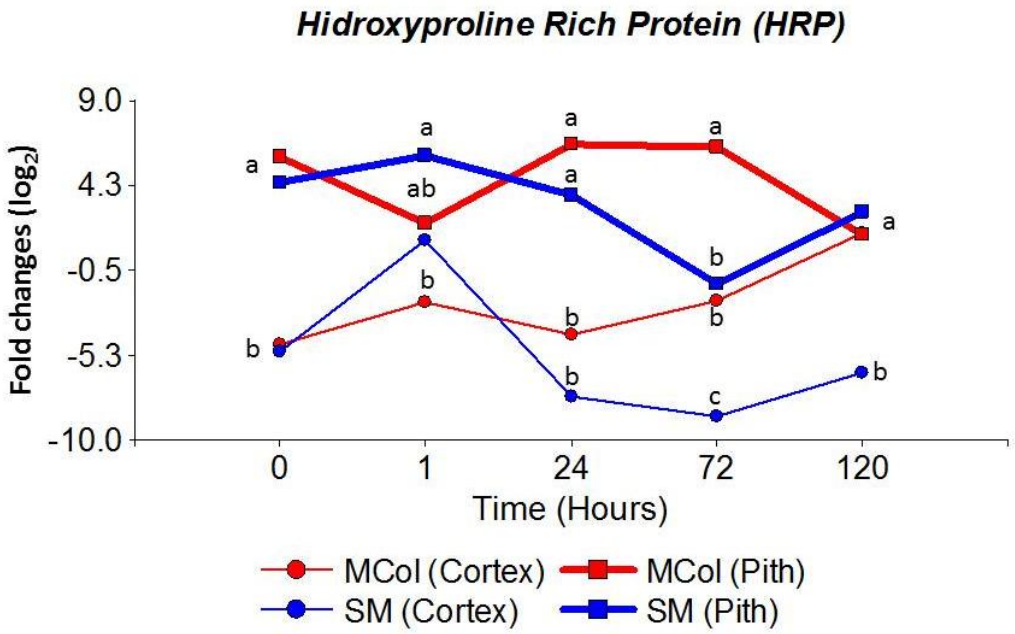


Figure 4.40 Gene expression profiles (fold changes vs time after harvest) for Hydroxyproline rich protein Red: MCol 2215, (susceptible accession), blue: SM 494-1(tolerant accession). Data were not autoscaled. Significant differences within each time are shown in lower case.

Gene expression profiles for *Hydroxyproline rich protein* (HRP) is showed in Figure 4.40. LSD (α= 0.05) multiple comparison found significant differences in mean expression of pith tissues at 72 hours (highest mean expression in pith tissue of MCol 2215). In cortex tissues significant differences were observed at 72 hours and 120hours (highest mean expression in cortex tissue of MCol 2215).By comparison, among all tissues significant differences were found at 0 hours (highest mean expression in pith tissue in both accessions), 1 hour (highest mean expression in pith tissue of SM 494-1, and lowest mena expression in cortex tissue of MCol 2215), 24 hours (highest mean expression in pith tissue in both accessions), 72 hours (highest mean expression in pith of MCol 2215 and lowest mean expression in cortex tissue of SM 494-1) and 120 hours (lowest mean expression in cortex tissue of SM 494-1).

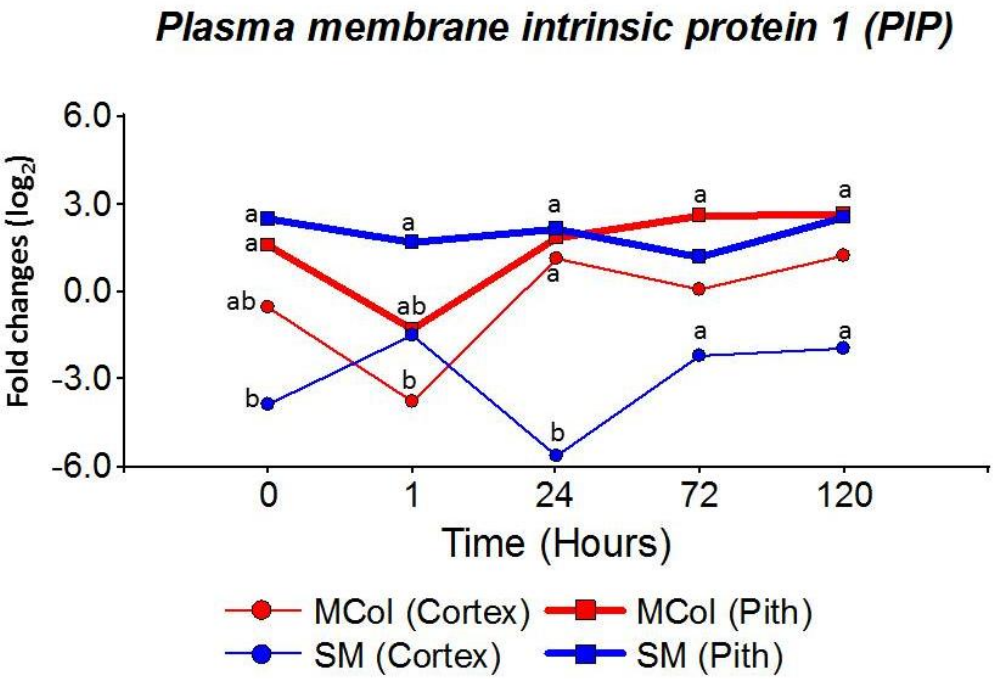


Figure 4.41 Gene expression profiles (fold changes vs time after harvest) for Plasma membrane intrinsic protein 1. Red: MCol 2215 (susceptible accession), blue: SM 494-1(tolerant accession). Data were not autoscaled. Significant differences within each time are shown in lower case.

Gene expression profile for *Plasma membrane intrinsic protein 1*(PIP) is shown in Figure 4.41. LSD (α= 0.05) multiple comparison did no find significant differences in mean expression of pith tissues at any time. In cortex tissues significant differences were observed at 24 hours (lowest mean expression in cortex tissue of SM 494-1). By comparison, among all tissues significant differences were found at 0 hours (highest mean expression in pith tissues both varieties, lowest mean expression in cortex tissue of SM 494-1), 1hour (highest expression in pith tissue of SM 494-1 and lowest mean expression in cortex tissue of MCol 2215), and 24 hours (lowest mean expression in cortex tissue of SM 494-1).

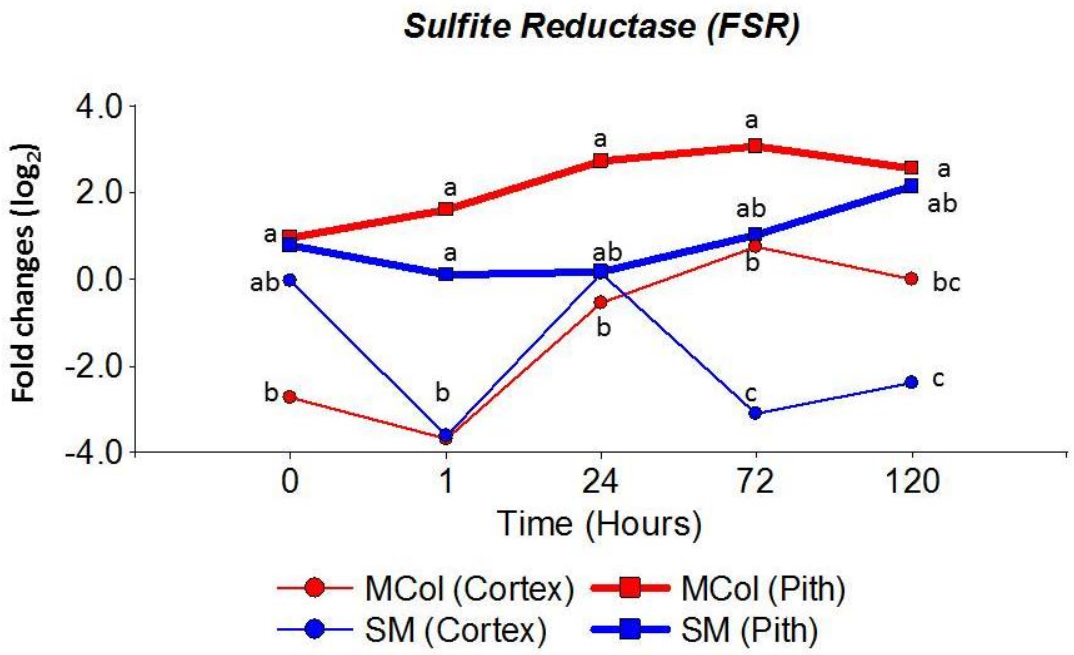


Figure 4.42 Gene expression profiles (fold changes vs time after harvest) for Ferodoxin Sulfite reductase. Red: MCol 2215 (susceptible accession), blue: SM 494-1(tolerant accession). Data were not autoscaled. Significant differences within each time are shown in lower case.

Gene expression profilet *for Ferodoxin Sulfite reductase* (FSR) is shown in Figure 4.42. LSD (α= 0.05) multiple comparison did no find significant differences in mean expression of pith tissues at any time. In cortex tissues significant differences were observed at 72 hours (highest mean expression in cortex tissue of MCol 2215). By comparison , among all tissues significant differences were found at 0 hours (lowest mean expression in cortex tissue of MCol 2215), at 1 hour (lowest mean expression in cortex tissue both accessions), 24 hours (highest mean expression in pith tissue of MCol 2215), 72 hours (highest mean expression in pith tissue of MCol 2215 and lowest mean expression in cortex tissue of SM 494-1), and 120 hours (highest mean expression in pith tissue MCol 2215 and lowest mean expression in cortex tissue of SM 494-1).

4.5 Analysis of results

4.5.1 RNA extraction

One requisite for gene expression analysis studies is the use of high quality RNA for cDNA synthesis. In this research, total RNA from pith and cortex tissue from cassava roots was extracted using a protocol modified by additional extraction with chloroform isoamyl alcohol 24:1. This additional step was necessary due to initial low A_{260nm}/A_{230nm} ratios obtained. Using the unmodified protocol, these low ratios can be attributed to phenol contamination which can affect the subsequent cDNA synthesis. The above mentioned modification was added.

The extraction with acid phenol removes proteins and DNA, while chloroform is added to facilitate the separation of aqueous and organic phases to carry out possible phenol contamination. However, it seemed that three extractions with chloroform are required for avoid residual phenol. This modification was very useful in pith tissues but the protocol needs to be improved for cortex tissue, where the radio $A_{260nm}/A_{230\ nm}$ was still low, probably due to the presence of polysaccharides that are in the cassava root phloem vessels. After the extraction, the formaldehyde denaturing agarose gels were another quality check point that guaranteed the integrity of the RNA. Samples with no clear sharp bands were re-extracted.

The protocol used for total RNA extraction usually, produce significant amounts of contamination with genomic DNA. DNAse was used to eliminate DNA contamination, due

to real time PCR's inability to discriminate between cDNA targets synthesized by reverse transcription and genomic DNA. A PCR was performed over all samples to detect if residual DNA was still present in the isolated total RNA samples.

4.5.2 Primer design

A primer set for each gene was designed using annotated gene sequences (Genbank and Phytozome databases) or ESTs referenced in microarray experiments (Really *et. al.*, 2007).The amplicon sizes were kept in a range from 100 to 231 bp since SYBR green reagent was used in real time PCR reactions. In the SYBR green system the fluorescent label of amplicons is proportional to its concentration and size. Larger products may strongly affect the efficiency of real time PCR and longer amplicons might incorporate additional SYBR green reagent reducing the Ct values and overestimating the gene expression (Colborn *et al*., 2008).

All annealing primer temperatures calculated with vector NTI software (Invitrogen Carlsbad, CA, USA) were near to 60ºC (except 18S, but this primer set works also at 60 ºC). Gradient temperature PCR of pooled cDNA showed that all genes work at the final annealing temperature profile used for real time PCR (60 ºC). This is a requirement to test all genes together in each individual run, thus avoiding interplate calibration.

Some primers were designed over UTR 5' region sequence (CD1, CD2, BCA, BCB, and HRP) (Figure 4.8). This strategy was necessary due to high similarity between sequences (CD1 vs CD2 80%, BCA vs BCB 88%). The UTR 5' region in these genes is most variable compared with the remaining coding sequence. In the case of HRP, one primer was designed over UTR 5' due to the tandem repeats of codons that code for prolines within the sequence, which makes it difficult to design suitable primers over coding sequences.

Amplification efficiency for all samples must be the same in order to allow sample comparisons. PCR efficiencies can be calculated from standard curves made from serial dilutions of plasmid DNA or pooled cDNA. However, the creation of repeatable and reliable standards is time consuming (Pfaffl, 2001) within each time. An alternative is to use Miner® software which uses an algorithm to calculate the efficiency and also calculates the Ct values for individual real time PCR reactions based on the kinetics of the PCR reaction (Zhao and Fernald, 2005).

The average efficiency for all primer sets was 86% (range 81% -89 %), and for data preprocessing, Ct values were corrected using the average efficiency of each primer set. This procedure has been recommended by Tichopad *et al*.(2004, cited by Zhao and Fernald, 2005).

4.5.3 Reference genes

In order to obtain accurate and reliable gene expression analysis it is imperative to choose suitable reference genes for normalization. Generally, housekeeping genes like actin, tubulin, rRNA 18S, and glyceraldehyde 3 Phospahte dehydrogenase are chosen as reference genes in real time PCR because their constant expression in living organisms (Huggett *et al*., 2005 cited by Quiang-Feng *et al*., 2010). Ideally those genes should not be influenced by experimental conditions. However, several studies have shown that housekeeping genes used in mRNA expression could vary with experimental conditions. Thus, it is recommended to use at least two or three reference genes to avoid large relative errors (Nicot *et al*., 2005 Artico *et al*., 2010).

Several algorithms such as Best keeper, geNorm, Norm Finder and qBase have been developed in order to identify the best reference genes for certain organism and experimental conditions (He *et al*., 2008; Duarte silveira *et al*., 2009; Quiang-Feng *et al*. 2010; Mallona, *et al.,* 2010). BestKeeper uses a pair-wise comparison for selecting the least variable gene using the geometric mean. Starting from raw Ct data, Bestkeeper produces a coefficient of correlation with the best reference gene to rank the candidate genes (Pfaffl *et al*., 2004). The NormFinder algorithm identifies the genes that display least variation, estimating both the intra- and intergroup variation, calculating a candidate gene stability value. The best reference gene exhibits minimal combined inter- and intragroup expression variation. The NormFinder method considers systematic differences between sample subgroups that are less

affected by candidate housekeeping co-regulation (Ståhlberg *et al*., 2008). Sometimes these approaches produce inconsistent results, but Normfinder was identified as a superior program compared with the others (He *et al*., 2008).

The number of potential candidate reference genes is virtually limitless, but practical considerations must be taken into account. Andersen *et al*., (2004) recommend evaluating a minimum of five genes. This minimum number of potential candidate reference genes (18S, F5A, UBI, TUB and EF1) was evaluated in this research. By pooling cortex and pith Ct data, the F5A gene was considered the most stable gene using the NormFinder algorithm. However, the high variability found suggested that selected the genes were not suitable or that that the most stables genes in cortex are not the most stable in pith tissue. After dividing the original data set in two sets (cortex and pith), it was evident that EF1 and TUB were most stable in cortex tissue while F5A and UBI were the best in pith tissue. By using Bestkeeper, similar results were obtained (the only variation was the same correlation value found beetwen EF1 and F5A in pith tissue). This discrepancy can be explained by the different principles employed by each approach: Bestkeeper uses a pair-wise comparison method that tends to select genes with similar expression profile taking into account their variability.

4.5.4 Exploratory statistical analysis

Exploratory statistical analysis using multivariate-multiway analysis tools (PCA, hierarchical clustering) was useful to analyze the complexity of the data set obtained in the cassava root

PPD experiment, and to generate hypotheses to be further tested by means of confirmatory statistical analysis.

Multivariate analysis tools used are based in different approach and mathematical models, but they are complementary. PCA helps to reveal patterns by calculating a set of abstract vectors (PCs). The number of PCs is lower than the number of variables, which makes the interpretation easy, eliminating most of the random variation. However, some biologically relevant information can be lost. Hierarchical clustering creates groups by inspecting subpopulations of data, but once the sample (gene profile) is entered in to cluster it cannot be removed again due to its hierarchical structure. Therefore, all information in the measurement is considered. Consequently, the final clusters may depend on slight differences between sample expression profiles.

PCA demonstrates high diversity in gene expression profiles for both accessions tested at different time points. PC scores revealed that a high percentage of variability is found in the range of 1 to 72 hours. PCA in 2D plot building with PC1 and PC2 scores helps to identify three regions of clusters (Figure 4.17). PC1 divides gene profiles into two regions. One region is integrated mainly by gene profiles present in cortex tissue of SM-494- 1, characterized by a strong increased of mean gene expression at 1 hour after harvest (cluster 2) while the other region (cluster 1) is integrated mainly by gene profiles present in both cortex and pith tissue of MCol 2215, which includes gene profiles with a strong decrease (PC1<<0,

PC2 >0, mainly in cortex tissue of Mcol 2215) or gene profiles that increase slightly at 1 hour (PC1<<0, PC2 <0, mainly in pith tissue of Mcol 2215). The third region (cluster 3, (PC1<0, PC2 >>0) corresponds to genes with a strong expression at 24 hours present in pith tissue of MCol 2215. It was difficult to assign a cluster for pith tissue of SM 494-1 using the first two PCs (PC1 and PC2) because some of their gene profiles were split between cluster 1 and near to cluster 2. However, a detailed analysis of PC3 was useful in showing a clear trend to obtain positive values (PC3>0) for several gene profiles belonging to pith tissue of SM 494-1 (data not shown). These values correspond to profiles with a strong decrease in expression at 72 hours.

Hierarchical clustering confirms the results obtained in PCA analysis, with the same groups of gene profiles that appear in the three clusters being clearly represented in tissue independent heat maps (Figure 4.20). In addition, independent heat maps identified clusters of gene profiles present in pith tissue of SM 494-1 with a strong decrease in mean gene expression at 72 hours.

Independent heat maps revealed that pith and cortex tissues of MCol 2215 accession are characterized by a cluster of genes with late activity (from 24 to 120 hours), contrasting with cortex and pith tissue of SM 494-1, which are characterized by a cluster of genes with high activity at early stages (from 0 to 24 hours).

By using hierarchical clustering of the augmented matrix (Figure 4.21), it was possible to categorize 16 expression groups. Comparison among the 16 profile categories in different tissues aided in proposing a set of hypotheses. These hypotheses tested whether genes which share similar profiles in both cassava accessions had no significant differences, and could be considered uninvolved in the tolerance observed. These hypotheses also test the existence of significant differences among tissues with different gene profiles confirming coordinate gene expression patterns and potential PPD tolerance related genes.

4.5.5 Confirmatory statistical analysis

Confirmatory statistical analysis using a split plot model (Table 4.4) showed that the main effect of *time* was significant in 66% of genes analyzed in this study, confirming the effect of PPD over time. Only AOX, CAT, ACO, EXT, HRP, DAD and FSR do not show significant differences across different time points. The main effect of *tissue* showed significant differences in 80% of the genes (only ACO, EXT, CAT CD1and APX did not shown differences) which confirms the diversity in gene expression profiles observed in exploratory statistical analysis. By contrast only 19% of genes showed significant differences between *accessions* (this is the difference in the mean expression of pith and cortex averaged in each accession).

Significant interaction among accession time and tissue was found in 52% of the genes, and the interaction between tissue and time was significant in 42% of the genes. Interactions

between accession and time and accession and tissue were found in 23% and 33% of genes, respectively. These interactions include both and orderly and disorderly interaction. In orderly interaction test about the main effects can be meaningful. However, in disorderly interactions a conclusion about main effects can be difficult and conclusions must be made separately at each factor level (for example, each time point). In addition it only makes sense to check the interaction between cortex and pith tissue of each accession separately.

PPD response variability could affect the results (by increasing the gene expression variability and reducing the significant differences in gene expression) since some roots in susceptible accession did not show comparative metabolic status, (i.e. one root of MCol 2215 did not show symptoms of PPD after five days).

The following hypotheses were proposed to confirm the coordinated expression of several genes. These hypotheses were tested by analyzing significant differences among tissues at each time point.

Hypothesis 1 tested a group of genes not involved in tolerance by having similar profiles in pith tissues of both accessions. Not significant differences were found for APX (type VII), CD1 (type XII), and SPS (type I), but CHT and EXT were discarded since there were significant differences at 1 and 72 hours, respectively. After analyzing the profile plots, CIP

and PIP showed no significant differences in each time point and thus can be considered as genes not involved in tolerance observed.

Hypothesis 2 tested a group of genes (ACO, PAL, FSR, CDE, LPO, and CD2) with an increase of mean expression from 24 hours to 120 hours in pith tissue of MCol 2215. No genes showed significant differences in any time points from 24 hours to 120 hours.

Hypothesis 3 tested a group of genes (APX, BCB, DAD, HRP, and PEC) with an increase of mean expression from 0 hours to 24 hours in pith tissue in SM 494-1. No genes showed significant differences in any time point from 0 to 24 hours compared with MCol 2215.

Hypothesis 4 tested a group of genes with an increase of mean expression at 1 hour in cortex tissue of SM 494-1 (cluster 2 PCA). After analyzing all profile plots, five genes (AOX, BCB, CD1, CHT, and LPO) showed significant differences in expression at 1 hour in cortex tissue of SM 494-1, compared with MCol 2215. HRP, however, did not show significant differences at 1 hour.

Hypothesis 5 tested the group of genes present in cluster 3 of PCA with a large increase of activity at 24 hours in pith tissue of MCol 2215. None of the genes proposed (DAD, HRP, and CAT) showed significant differences at 24 hours. However, after the analysis of all profile plots, the genes FSR and BCB showed significant differences at 24 hours in pith tissue of MCol 2215.

Hypothesis 6 tested a group of genes which showed a strong decrease at 72 hours in pith tissue of SM 494-1. Significant differences were found between MCol 2215 and SM 494-1 at 72 hours in genes BCB, PEC, DAD, HRP, ALX and PAL, though there was no significant decrease in LPO, PIP, or AOX. After analyzing all profile plots, the genes ACO, BCA, CD2, CDE, CHT, CIP and EXT showed significant differences at 72 hours between pith tissue of MCol 2215 and SM 494-1.

H*ypothesis 7* tested a group of genes which showed a strong decrease at 1 hour in cortex tissue of MCol 2215. Significant differences were found in CIP, ALX and PAL. Comparatively however, no significant differences were found in BCA or PIP. After analysis of all profile plots, CAT and CDE also showed a strong and significant decrease in the expression.

4.5.6 Gene expression profiling analysis

Oxidative stress genes

Alternative oxidase (AOX) (E.C.1-10-3) is an enzyme present in the electron transport chain, which provides an alternative route for electrons to reduce oxygen (Vanlerberghe and McIntosh, 1997). Alternative oxidase catalyzes cyanide-resistant reduction of oxygen to water without translocation of protons across the inner mitochondrial membrane, working as a non-energy-conserving component of the respiratory electron transfer chain (Jusczuck and Rychter, 2003). AOX is influenced by factors that reduce electron flow through the

cytochrome pathway of respiration. These factors include ROS, pathogen infections and cyanide (Yoshida *et al*., 2008). AOX may enhance an organism's ability to resist different types of stresses, by reducing the level of oxidative stress, and also by the initiation of stress adaptive cell programs (Maxwell *et al*., 1999; Arnholdt-Schmitt *et al*., 2006).

In pith tissue, significant AOX transcriptional activity was found in tolerant variety SM 494-1 at 0 hours. Interestingly, significant differences were also found in cortex tissue at early stages (0 and 1 hour). Experiments with transgenic tobacco suspension cell cultures, which constitutively express the antisense construct of AOX or alternatively over express the AOX gene, showed differences in AOX activity among transgenic lines and wild type cells, these differences were observed as soon as four hours under stress inductors (Maxwell *et al*.,1999; Vanlerberghe *et al*., 2002). In cassava roots, AOX can be involved in relatively faster scavenging of ROS generated by root wounding in early stages in SM 494-1, compared with MCol 2215. Significant differences were also observed in cortex tissue, with the expression of AOX being on average higher in tolerant accession SM494-1 at 0, 1 and 120 hours compared with MCol 2215. The pioneer work of Uritani *et al*., (1983) comparing the PPD response of cassava with and without cortex showed differential biochemical changes in A,B, and C parts (which form the pith tissue) and also in postharvest deterioration scores, supporting the idea that metabolic compartmentalization participate indirectly in PPD. Hence, metabolic differences in cortex tissue would affect metabolic changes in pith tissue.

Secretory Peroxidases (APX3) (E.C. 1-11-17) belong to a ubiquitous, diverse family of glycoproteins that catalyze oxidations using H_2O_2 as the electron acceptor. Peroxidases can be directly involved in defense mechanisms, acting as catalysts of organic and inorganic compounds using hydrogen peroxide (H_2O_2) (Gómez-Vázquez *et al*., 2004; Really *et al*., 2004). These substances include the polymerization of phenolic compounds to form lignin and suberin in the cell wall, which can act as cellular barriers (Pereira *et al*., 2003).

However, as the PPD response progresses, peroxidases have a negative effect of oxidation of scopoletin in the vicinity of the root xylem parenchyma vessels where PPD symptoms occur (Really *et al*., 2004). Tolerant accessions exhibit lower levels of peroxidase activity during the post-harvest period (Campos and Decarvalho, 1990, cited by Really *et al*., 2004).

The real time PCR gene expression profile of APX on tolerant accession SM 494-1 showed a significant high activity in cortex tissue at 1 hour, compared with the susceptible accession. This high activity would help to the improve process of suberization, blocking the entrance of oxygen; but later the activity of APX decreased avoiding the negative effects of scopoletin oxidation. This is based on the fact that in cassava roots H_2O_2 was initially localized in the cortical parenchyma, and the activity of peroxidase in freshly harvested roots was localized in epidermis and cortex with little activity in root parenchyma. In contrast, in deteriorated roots, showing visible symptoms of PPD, peroxidase activity was more extensive than in freshly harvested roots, having spread throughout the root parenchyma (Really *et al*., 2004).

After comparing pith tissue of MCol 2215 with SM 494-1, there was a statistical evidence to support a reduction of peroxidase activity in later stages, and as a consequence it explain the tolerance observed in SM 494-1. However, the mean expression trend showed a reduction of activity in cortex and pith tissue of SM 494-1 at 72 hours. Comparisons with microarray data, which used block tissue (instead of the entire root) of tolerant accession CM2177-2 (Really *et al*., 2007), suggest a strong activity at 24 hours followed by a reduction of gene activity at 48 hours and a slight increase at 72 hours. This behavior can be expected since the symptoms of PPD appear faster when block tissue is used.

Catalase 1(CAT) (EC 1.11.1.6) is one oxidoreductase found in virtually all aerobic organisms and serves to rapidly break down hydrogen peroxide (H_2O_2). It represents one of the primary defenses against oxidative stress. Catalase 1 is expressed predominantly in roots, with low levels in leaves (Really *et al*., 2001). Really *et al*., (2001) compared the transcription of Catalase 1 activity by Northern blotting between two PPD contrasting cassava accessions. Catalase showed an initial increase in both accessions; however, in the susceptible acession activity fell to barely detectable levels at day four, while in the in less susceptible accesion activity remained high until day six.

This kind of differential behavior was not observed when the gene profiles MCol 2215 and SM 494-1 were compared using real time PCR. The gene activity remained almost invariant in pith tissues; similar results were obtained in microarray using a tolerant accession (Really

et al., 2007). In contrast, the activity of catalase 1 was significantly lower from at 1 hour in cortex tissue of MCol 2215 compared with SM 494-1.

The tolerance observed could be due to the effect of high activity of CAT in cortex tissue of the tolerant accession, which would be boost, the enzymatic defense against oxidative stress. Another alternative to observed tolerance is based on the inconsistency between high transcriptional activity and low enzymatic activity of catalase, found in a susceptible accession by Really *et al*. (2001).These findings suggest a translational regulation and/or inactivation of protein. Such regulation of catalase activity has been observed in sweet potato roots and pumpkin cotyledons. This inactivation would be the product of several mechanisms, including binding of salicylic acid, free radicals attack and enzyme crosslinks by phenolics, including hidroxycoumarins produced during PPD (Really *et al*., 2001)

Cianide detoxification and byosyntesis of cianogenic glucosides

β-cyanoalanine synthases(BCA, BCB) (EC 4.4.1.9) are the most important enzymes in cyanide detoxification (Maruyama *et al*., 2001). Marrero-Degro *et al*., (2010) isolated two copies of this enzyme family which, although sharing a high percentage of similarity, have different activities. β-cyanoalanine synthase A (BCA, formerly called cystein synthase) use O-acetyl-serine and sulfide as substrates to produce cystein and is predominantly found in plastids and cytosol. β-cyanoalanine synthase B (BCB), on the other hand, has lost significantly its cystein synthase activity but has high higher β-cyanoalanine synthase activity

and thus is involved in cyanide detoxification. Cyanide detoxification is catalyzed by the incorporation of cyanide with L-cystein to form β-cyanoalanine with releasing of sulfide. Detoxification of cyanide prevents the inhibition of mitochondrial oxidase and thus β-cyanoalanine synthase is located in mitochondria (Warrilow and Hawkesford, 2002; Ganjewala *et al.,* 2010).

Gene expression profiles reveal that BCA and BCB have opposite behaviors: when the mean expression of BCA is increasing, the activity of BCB is progressively decreasing in both accessions. In BCA there was a decrease in the mean expression at 1 hour in pith and cortex tissues of both accessions with a more significant decrease in pith tissue. A significant decrease of mean expression at 72 hours followed by a significant increase at 120 hours was observed in the pith tissue of tolerant accession SM 494-1. The increase of mean expression in BCA has as consequence an increase in the production of cysteine. This cystein serves as acceptor for toxic cyanide and as precursor for the synthesis of various sulfur-containing metabolites, including gluthatione (GSH), which has been proposed as a regulator of gene expression, and most importantly in the context of oxidative stress resistance, where it acts as a scavenger of reactive oxygen species (ROS) (Wirtz and Hell, 2006).

The results obtained in BCB can be understood as a connection with the activity of linamarase (a cell wall enzyme that facilitates the de-glycosilation of cyanogenic glucosides and posterior cyanide release). Kojima *et al*. (1983) found a reduction of linamarase activity

in cassava roots under PPD. Linamarase activity would be higher in susceptible accession MCol 2215, producing more cyanide which can not be detoxified, increasing the ROS levels. The opposite would occur in tolerant accession SM 494-1 However transcriptional activity of linamarase needs to be measured to confirm this as tolerance mechanism.

In cortex tissue, however, significantly high expression of BCA and BCB was found in tolerant accession SM 494-1 at 1 hour and 120 hours. Since this tissue accumulates more cyanide, it could be possible that the differential expression contributes to the reduction of the toxic effects of cyanide in cortex tissue.

Cytochrome P450 CYP79D, (CD1) *CYP79D2* (CD2) and *CYP71E* (CDE) belong to a small family of cytochrome P450 that catalyzes the first step in the synthesis of cyanogenic glucosides. In cassava, CD1 and CD2 are multifunctional enzymes that catalyze the conversion of primarily valine to its respective oximes, and subsequently CDE converts the oxime into nitrile. Linamarin (the main cyanogenic glucoside) is subsequently produced by the addition of glucose to this oxime, catalized by an UDP-glucosyl transferase (Siritunga and Sayre, 2004).

Microarray profiles of cassava roots under PPD with a tolerant accession showed a strong activity at 24 hours, and subsequent reduction of mean expression in CD1, CD2 and CDE (Really *et al*., 2007). This is consistent with the profiles obtained for CD1, CD2 and CDE in

pith tissue of SM 494-1, but it contrasts with the profile of MCol2215 in CD2 and CDE, which showed a significant increase in the mean expression after 24 hours.

The only significant difference in cortex tissue was found in the mean expression of CD1 at 1 hour (highest mean expression in SM 494-1), 24 hours (lowest mean expression in cortex tissue of SM 494-1) and 120 hours (highest mean expression in cortex tissue of SM 494-1). Furthermore, the differential activity of CD2 and CDE suggests that the production of cyanogenic glucosides is higher in the susceptible accession MCol 2215 during PPD. Therefore, it can be hypothesized that MCol 2215 accumulates more cyanide with its consequent toxic effects by ROS production in mitochondria.

Programmed cell death (PCD)

Chitinase class IV (CHT) (E.C. 3.2.1.14) catalyzes the hydrolisis of β-1, 4 linkages of N-acetyl-D glucosamide polymers of chitin, a major component of the cell walls. In plants, chitinase is rapidly accumulated in response to pathogen attacks, physical wounding and exogenous ethylene (Park *et al.,* 1991).

In tolerant accession SM 494-1, significant differences in chitinase gene expression were observed in pith tissues at 1 hour (with significant high mean expression) and 72 hours (with significant low mean expression). This profile agrees with the CHT profile in microarray

experiments (Really *et al.*, 2007), which showed a high increase at 24 hours followed by a slight reduction of mean expression at 48 hours. In cortex tissue significant differences were observed at 1 hour, where tolerant accession SM 494-1 shows the highest expression.

Faster response of CHT would be related with the tolerance observed in SM 494-1, due to the fact that over expression of CHT has been associated with tolerance to abiotic stress such as high concentrations of salt and heavy metals, which produce oxidative damage by enhancing ROS production (Dana *et al*., 2006).

Cystein protease (CIP) (E.C. 3.4.22) is a proteolytic enzyme involved in hydrolysis of proteins, and it is suggested that it can also act as a signal transductor and /or effector of programmed cell death. This enzyme has been associated with senescence and stress response (drought, cold, wounding, and ethylene treatment) (Really *et al*., 2007). Arabidopsis with constitutively expressed cystein protease exhibited high tolerance to salt and drought compared with non transgenic plants (Chen *et al*., 2010).

The profiles in pith tissue of MCol 2215 and SM 494-1 were identical with no significant differences (increase of the expression at 24 hours and constant expression until 120 hours), unlike the microarray experiments, which showed increase of activity at 24 hours and a decrease of mean expression after 48 hours (Really *et al*., 2007). A significant difference was

found at 1 hour in cortex tissue in tolerant accession SM 494-1, with high expression at 1 hour. This gene has minimal impact in the explanation for tolerance observed in pith tissue.

Defense Against Cell Dead 1 (DAD) (E.C. 2.4.119) is considered a conserved evolutionary inhibitor of programmed cell death in animals and plants. By using cDNA AFLP in susceptible accession MCol 22, Huang *et al*. (2001) found that the expression profile of DAD1 displays a strong increase of the expression at 24 hours, and a constant expression until 96 hours.

Interestingly, the mean expression in the tolerant accession SM 494-1was significantly lower compared with susceptible accession MCol 2215. These differences were found at 72 hours in pith tissue and 72 and 120 hours in cortex tissue. Since the cassava accession used by Huang *et al*. (2001) is susceptible it can be inferred that the reduction of DAD 1 activity after 24 hours in SM 494-1 is related to its tolerance.

Biosynthesis

Sucrose phosphate synthase (SPS) (E.C. 2.4.1.14) is a key regulatory enzyme located in cytoplasm, involved in the partitioning between sucrose and starch in plants, SPS is involved in the major pathway for sucrose synthesis (Lee *et al*., 2003). Since Oirschot *et al*. (2000) suggested that a high ratio of sugar/starch is inversely correlated with PPD; it was expected

to find differences in expression of SPS in cassava accessions. However, no significant differences in mean gene expression were observed between pith tissues.

The pith tissue contains 85% of the root's weight and accumulates the main portion of starch. Since significant changes were not observed in this tissue, SPS is a candidate to have minor effect in tolerance observed in SM 494-1. It would be interesting to measure the gene expression changes that take place in the secondary pathway for sucrose synthesis, which involves sucrose synthase.

Allene oxidase cyclase (ALX) (E.C. 5.3.99.6) is an enzyme involved in jasmonate synthesis, catalyzing the intermediary produced by lypooxigenase (LPO) in the jasmonate pathway (Wasternack, 2007). Jasmonate has a variety of biological functions, such as growth inhibitor and promoter of senescence activities (Hofmman *et al*., 2006). Jasmonate synthesis is generally viewed as a chloroplast associated process. However, four genes coding for allene oxidase cyclase have been recently identified in *Arabidopsis thaliana*, suggesting the possibility of dual localization in mitochondria (Stenzel *et al*., 2003).

The profile of the tolerant accession was characterized by a strong decreased of activity at 72 hours in both cortex and pith tissues. In contrast, MCol 2215 showed high expression at that time. Significant differences were also observed at 1 hour in cortex tissue of MCol 2215 which showed a decrease of mean expression activity at 1 hour. Allene oxidase cyclase in the

tolerant accession would affect the expression of genes involved in PPD, based on the functions of jasmonate, which has been proposed to modulate the wound-induced expression of several genes (Bell *et al*., 1995).

1-aminocyclopropane-1-carboxylate oxidase (ACO) (E.C. 1.14.17.4) is the final enzyme catalyzing the formation of ethylene by the oxidative fragmentation from 1-aminocyclopropane-1-carboxylic acid (ACC) (Really *et al*., 2007). It is also considered to be the rate limiting step in ethylene production (Taiz and Zeiger, 2002).

Ethylene is a gaseous plant hormone, involved in a wide range of responses in plants, including fruit ripening, stress response (abscission, senescence, wound healing and disease resistance) and activation of defenses against potential pathogens. Ethylene also induces the accumulation of hydroxyporline-rich glycoproteins in cell walls and the stimulation of lignifications as well as induction of lytic enzymes (Park *et al.,* 1991;Taiz and Zeiger, 2002).

According to Hirose (1986) the production of ethylene in cassava roots under PPD begins at 15 hours and then decreases gradually, with higher production of ethylene in PPD susceptible roots compared with tolerant roots. The gene expression profile obtained with real time PCR in pith tissue found that the mean expression of ACO was significant higher in susceptible accession MCol 2215 at 72 hours. The comparison with the microarray gene profile using a PPD tolerant accession (Really *et al*., 2007) showed a high expression of ACC at 12 and 24

hours and then a reduction of mean expression. This profile is similar to the ACO profile observed in tolerant accession SM 494-1 by using real time PCR.

In cortex tissue, significant expression was observed at 1 hour in both accessions. The results obtained agreed with previous results of Hirose (1986), suggesting that the early production of ethylene observed in cassava roots takes place in cortex tissue.

Plant defense and response to wounding

Lipoxygenase (LPO) (E.C. 1.13.11.12) is a non-heme iron containing dioxygenase widely distributed in plants and animals, LPO catalyzes the hydroperoxidation of polyunsaturated fatty acids and is the first step in the synthesis of fatty acid metabolites in plants. LPO initiates the synthesis of oxylipins, a group of compounds with diverse functions in cell metabolism (Porta and Rocha-Sosa, 2002). The functions of oxylipins include growth regulation, synthesis of antimicrobial compounds, plant response to pathogen infection, wound stress and lipid based signal molecules and synthesis of jasmonates (Bell *et al.*, 1995; Rosahl, 1996; Akram *et al.*, 2008).

LPO gene expression is regulated by different effectors such as source/sink status, jasmonates, and different stresses. LPO isolated from different plant species showed differential organ specific expression (Akram *et al*., 2008). The early phase of H_2O_2-induced programmed cell death in plants is characterized by enhancement of LPO activity,

attributable to up regulation of gene expression at the transcriptional or post-transcriptional level (Maccarone *et al*., 2000). LPO plays an important role in generating peroxidative damage in membrane-lipids which is a critical step in programmed cell death (Zhao *et al*., 2010).

The expression profile obtained by real time PCR was similar when cortex and pith in each accession were compared. However, significant differences were found at 1 hour (highest mean expression in pith tissue of SM 494-1) and 72 hours (significant reduction in mean expression at in cortex tissue of SM 494-1).

The early response observed at 1 hour can produce some early induction of program cell death; however its activity can be decreased avoiding excessive peroxidative damage and the execution of program cell death. These results agree with the experiments of Maccarone *et al*. (2000) who used pulses of H_2O_2 over lentils. This experiment showed that LPO activity increased reaching a maximum six hours after H_2O_2 pulse and later declined. Also, this experiment suggested that lipooxygenase inhibitors result effective in protection against cell death if they are added before LPO activity but become infective when added later.

Extensins (EXT) are a family of hydroxyproline rich proteins (HRGP) and constitute the major protein components in cell walls of dicot plants (Ahn *et al.*, 1996). Extensins are mainly expressed in roots and, a less amount, in stems. Their expression is induced by

various stresses such as wounding, hormone treatment, cold, hypoxia, and pathogen infections. (Hirsinger *et al*., 1999).Once synthesized, extensin monomers rapidly become insolubilised in the cell wall in a reaction involving H_2O_2 mediated by a cell wall peroxidase, thus sealing and strengthening the cell wall (Really *et al*., 2007).

Gómez-Vásquez *et al.,* (2001) suggest that large extensin gene family is expressed during the PPD response in cassava roots. However, the timing or localization of the expression and insolubilisation of these extensins family is not appropriate, making the wounding repair inadequate.

Using real time PCR, significant differences were observed between 1 hour and 72 hours in pith tissues (highest expression in MCol 2215). These results contrast with microarrays experiments results using a PPD tolerant cassava accession (Really *et al*., 2007), where the mean expression of EXT was up-regulated at 24 hours with a second peak of activity at 72 hours. Cortex tissues showed similar profile and there were no significant differences. Since this gene is connected with peroxidase activity this profile is expected, but does not explain the tolerance observed.

Phenylalanine ammonia-lyase (PAL) (E.C. 4.3.1.5) catalyses the first committed step of the phenylpropanoid metabolism pathway, which leads to the synthesis of compounds that have diverse functions in plants including defense, cell wall strengthening and repair (e.g. lignin

and suberin), antimicrobial activity (e.g. furanocoumarin, phytoalexins), and as signaling compounds such as salicylic acid (Hammerschmidt, 1999, cited by Gómez-Vázquez *et al*., 2004). The resulting phenolic compounds are often converted into more reactive species by phenol oxidases and peroxidases. PAL genes can be regulated developmentally, induced by wounding, by low temperatures, pathogen attack and by other stress conditions.

Significant differences in pith tissue using real time PCR were found at 72 hours (highest expression in MCol 2215) and in cortex tissue at 1 hour (highest expression in SM 494-1). The tolerance observed in SM 494-1could be a product of a metabolic strategy for reduction of PAL activity after 24 hours. This reduction of PAL activity has also been observed in cassava roots under pruning treatment three days after harvest (Tanaka *et al*., 1983).

Pectins are major components of the middle lamella of plant cells. Pectins are composed of D-galacturonic acid polymers with interspersed methylgalacturonic acid residues, which can be esterified by acetyl groups. The complexity of the pectin network is modulated by the level of pectin acetylation (McNeil *et al.,* 1984, cited by Vercauteren *et al.,* 2002).

Pectinacetylesterase, (PEC) (E.C. 3.1.1) catalyzes the deacetylation of esterified pectin diminishing the pectin backbone hydrophobicity, increasing its solubility in water (Dea and Madden, 1986, cited Vercauteren *et al*., 2002).

Significant differences in gene expression using real time PCR were found at 1 hour and at 72 hours in pith tissue. The highest expression was found in susceptible accession MCol 2215. The profile of pith tissue of MCol 2215 is similar to the PEC expression profile obtained by using cDNA AFLP in cassava roots using the susceptible accession MCol 22 under PPD (Huang *et al*., 2001), which showed a progressive increase reaching its maximum at 72 hours. Interestingly, in cortex tissues of SM 494-1, the expression of PEC was increased at 72 hours and at 120 hours.

Cell wall

Hydroxyproline rich glycoproteins (HRP) are abundant proteins in the plant cell wall. They strengthen cell walls and control cell wall extension by insolubilization of the molecules through intermolecular cross links. HRP are induced by wounding and are involved in the wound healing process preventing desiccation and penetration of pathogens (Han *et al*., 2001a; Han *et al*., 2001b).

Real time PCR expression profile showed a significant decrease in the mean expression of HRP at 72 hours in pith tissue of tolerant accession SM 494-1. Similar results were obtained with the gene expression profile using microarrays (Really *et al*., 2007), which showed a strong reduction after 24 hours in a PPD tolerant cassava accession.

Han *et al.* (2001b) evaluated the expression of HRP during postharvest deterioration in accession MDOM 5, which showed initial visible symptoms of PPD within 24 hours of harvest. Gene expression of HRP was detectable in MDOM 5 by Northern blotting after three days followed by a slightly reduction over the next two days. These results agree with the expression profile observed in MCol 2215. Similar results were obtained in cortex tissue tolerant accession SM-494-1; it showed a significant strong reduction at 72 and at 120 hours. The tolerance observed can be attributed to a deficit in the expression timing of HRP which compared with SM 494-1can be delayed in MCol 2215.

Water transport and sulfur metabolism

Water can pass through plant cellular membranes by simple diffusion as well as helped by water transmembrane channels called aquaporins. One kind of aquaporins is the *Plasma membrane intrinsic protein*. PIPs can by divided in two major groups, PIP1 and PIP2. Compared with PIP1, PIP2 has a shorter N terminal extension and longer C terminal end containing putative phosporilation sites (Fetter *et al*., 2004). In addition aquaporins can transport other low molecular weight compounds such as CO_2, silicon, boron, ammonia and H_2O_2 (Katsuhara and Hanba, 2008).

Gene expression profile using real time PCR did not show significant differences in pith tissue between MCol 2215 and SM 494-1 and the expression kept relatively constant in all time points. These results contrast with a strong increase at 24 hours observed in microarray experiments (Really *et al*., 2007) profile followed by a slightly increase after 72 hours. In the

cortex tissue significant differences were observed at 24 hours with a strong reduction of mean expression in tolerant accession SM 494-1.

Bienert *et al*. (2006) conducted a study using yeast as heterologous expression system expressing several plant aquaporins. They found that aquaporins can increase the capacity for H_2O_2 diffusion in yeasts cells. *Arabidopsis* plants with down regulated plasma membrane intrinsic protein using RNA interference technology result in plants with early senescence and death. In those plants, water status seemed to be unaffected. However, genes related with redox control, defense, carbon metabolism and signaling were affected. Finally, PIP1 is highly expressed in cells surrounding developing vascular tissues in maize roots. High levels of ROS including H_2O_2 have been detected in the same tissues and have been implicated in cell wall loosening and elongation growth. These findings suggest a role of PIP in regulation the level of H_2O_2 by memebrane permeability (Bienert *et al*., 2006). In tolerant cassava accession an early induction or maintenance of this gene in pith and cortex would be responsible for tolerance observed. However, there was no statistical evidence to support this.

Sulphite reductase (FSR) (E.C. 1.8.7.1) is a key enzyme in sulfur metabolism; this enzyme catalyzes the reduction of sulfite SO_3^{2-} to sulfide S^{2-}, process that occurs in the plastid (Taiz and Zeiger, 2002).

In addition to its catalytic function, FSR plays another role in plant plastids. It has been reported that FSR from pea and maize have DNA-binding properties, suggesting that FSR is essential for proper compacting of nucleoids in plastids, which influences the transcriptional activity of chloroplast DNA. It is possible that FSR could act as a sensor of the redox state of the chloroplast, which is connected with regulation of chloroplast genes by the redox state (Lewandowska and Sirko, 2008).

Multiple comparison did no find significant differences in mean expression o FSR in pith tissues at any time.These results contrast with microarray gene expression profile (Really *et al*., 2007) which showed an increase in the mean expression at 24 hours and important reduction of expression at 48 hours. In the cortex tissue significant differences were found at 72 hours, where tolerant accession SM 494-1showed a reduction in mean expression compared with susceptible accession.

In cassava roots, FSR can be involved in regulation of genes involved in ROS scavenging. Sojikul *et al*., (2010) recently have suggested that FSR would be involved in sulfur containing proteins biosynthesis (gluthatione) or detoxifying the cyanogenic glucoside content through aspartate biosynthesis in cassava roots. It seems that these strategies are not present in tolerant accession SM 494-1.

Model for tolerance to PPD

The tolerance displayed by SM 494-1 can be understood considering three main aspects: 1) the dual effect of H_2O_2, 2) the effect of metabolic compartmentalization and 3) the effect of ethylene. The initial consequence of inevitable wounding during cassava root harvest is the accumulation of H_2O_2 in the cortex tissue by oxidative burst. This oxidative burst actives cell wall proteins (PEC, EXT, HRP with apparent minimum effects in the tolerance observed), up regulates phenylpropanoid pathway genes (PAL) providing ligning precursor to seal the wound and plant defense compounds, and oxidative burst triggers the peroxidase (APX) to limit the effect of H_2O_2 accumulation (significant higher at 1 hour in cortex tissue of tolerant accession).

In addition, peroxide also induces the production of ethylene (as is shown in the gene expression profile of ACO). Ethylene can activate several PPD related genes, but the significant high response at 1 hour displayed in cortex tissue of tolerant accession SM 494-1, contrast with the coordinate lower or even reduced response in susceptible accession MCol 2215 observed in the expression patterns of ALX, AOX, APX, BCB, CAT, CD1, CD2, CAT, CHT, CIP, LPO and PAL. This significant increase in the activity would be responsible for tolerance observed by activating an early response in cortex tissue of SM 494-1.

Eventually, some genes reach a maximum activity at 24 hours as a response to wounding (though with no significant differences). This increase of activity is followed by a recovery

phase after wounding which attemps to return the tissues to normal physiology (Zhou and Thornburg, 1999). However, in susceptible accession MCol 2215 this return to normality would be affected by significant increase in gene expression after 24 hours, particularly ACO which raises the ethylene production with negative effects in pith tissue resulting in PPD symptoms.

After 24hours, the pith tissue of the tolerant accession virtually shut down the transcription of several genes: ACO, ALX AOX, BCA, BCB, CHT, DAD, EXT, HRP PAL PEC and LPO. This strategy resembles the differences found in pruning treated cassava roots vs untreated roots (Tanaka *et al*., 1984). The roots of pruned plants (PPD tolerant) displayed a reduced enzymatic activity, reduced accumulation of hydroxycoumarins, and probably a reduction in ethylene.

4.6 Conclusions and recommendations

- The plant material (cassava roots under PPD) used for gene expression profiling showed differences in PPD scores compared with previous physiological test (particularly in MCol 2115 accession). This can be a result of slight differences in environmental conditions between both experiments and the high innate variability in PPD found in cassava roots.

- This is the first report of gene expression profiling for 21 genes in cassava roots using real time PCR to study a physiological phenomenon.

- Exploratory statistical analysis was useful in showing the global picture of gene expression profile for each tissue (and indirectly the cassava accession) under PPD. It also helped to identify trends and possible coordinated expressions. However, the results have to be analyzed carefully since some of the hypotheses proposed were unproven by confirmatory statistical analysis.

- Results obtained suggest that APX, SPS, CIP and PIP do not play a major role in the tolerance observed in SM 494-1, since their gene profiles are quite similar in both accessions and confirmatory results did not show significant differences. However, metabolically APX is directly linked with EXT and the reduction in mean expression observed in tolerant accession at 72 hours would be actually significant. The absence of significant differences would be based on the lack of uniformity in metabolic status showen in samples of susceptible accession. CIP and PIP showed large differences compared with microarray profile, supporting the idea of diversity in strategies for PPD tolerance in cassava roots.

- Results suggest that tolerance is explained by spatiotemporal changes in gene expression by the increase of gene expression of several genes at one hour in cortex tissue followed by a reduction of gene expression in pith tissue after 24 hours.

- The cyanogensis that takes place upon mechanical damage of cassava produces ROS which triggers PPD. However, after analyzing gene patterns of the pathway there is still no clear relationship between biosynthesis and detoxification of cyanogenic glucosides pathway and PPD. Gene profiles of CD2 and CDE suggest an accumulation of cyanogenic glucosides in pith tissue of susceptible accession MCol 2215 after 72hours. However, the activity of BCB (involved in cyanide detoxification) was reduced after 24 hours in pith tissues of both accessions (but is particulary low in tolerant accession SM 494), despite of the increase of activity of BCA which produces a supply of cysteine to perform the detoxification process. Key information to solve this riddle is to compare the linamarase gene activity among tissues which shows the availability to produce free cyanide in deteriorated tissue.

- The tolerance to PPD observed is a product of the reduction of the effects of inadequate wound repair and inadequate regulation of gene expression. This involves a dual activity of H_2O_2 (served as messenger to wound sealing in cortex tissue, but it has detrimental effects in pith tissue) and differential changes in plant hormone production (ethylene, jasmonate). In order to support the model for tolerance it is

suggested to compare changes in gene expression in roots of pruned cassava roots vs roots from unpruned plants and different parts (A, B and C) of the pith tissue during PPD. The changes in gene expression in other tolerant and susceptible cassava accessions shoul be compare as well.

- Comparison average changes in gene expression obtained using microarrays (Really *et al*., 2007), show similar expression profiles for APX, CD1, CD2, CDE, CAT, CHT, ACO, HRP, but contrasts with FSR, CIP, PIP and CHT. In addition, similar expression profiles were observed after the comparation of gene expression profiles of DAD and PEC with cDNA AFLP results (Huang *et al*., 2001). In order to obtain a clear picture other time points (i.e. 48 hours and 96 hours) should be evaluated.

- Several genes were differentially affected beetwen accessions with contrasting PPD accessions five days after harvest. These genes belong to different functional categories (oxidative stress, cyanide detoxification, plant defense, biosynthesis and cell wall). Also differnces were observed in the profiles among all tissues, supporting the idea that PPD is a multigenic trait. With the information obtained, a biotechnological approach to reduce PPD will require promoter and regulatory gene analysis.

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APPENDICES

APPENDIX 1.

Table of analysis of variance (liner regression Visual inspection vs Fluorescent accumulation)

	df	*SS*	*MS*	*F*	p-value
Regression	1	0.002991683	0.002992	0.062944186	0.804129404
Residual	23	1.093170171	0.047529		
Total	24	1.096161854			

APPENDIX 2

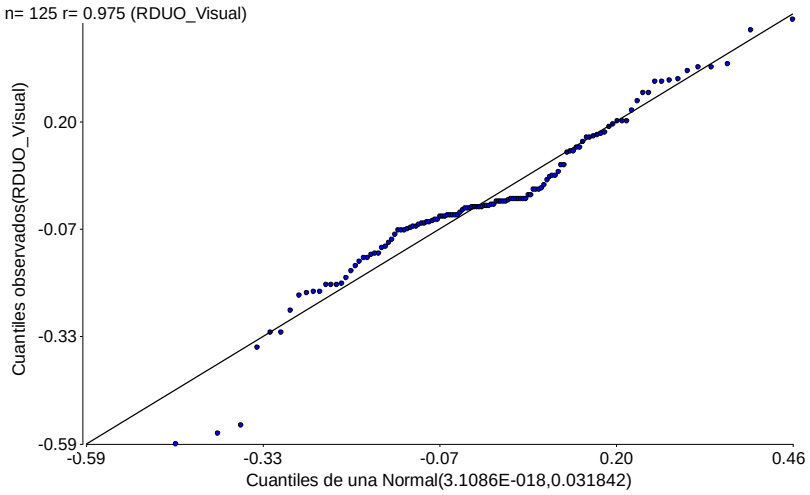
2.1 Table of analysis of variance Complete Random Design PPD scores visual inspection

S.V.	SC	df	MS	F	p-value
Model	6.03	24	0.25	6.37	<0.0001
Accessions	6.03	24	0.25	6.37	<0.0001
Error	3.95	100	0.04		
Total	9.98	124			

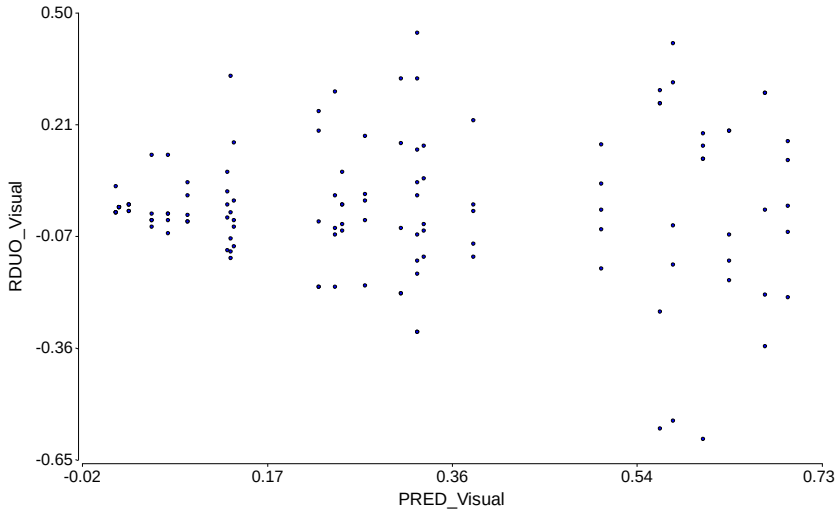
2.2 Shapiro-Wilks test (Normal distribution)

Variable	n	Mean	SD..	W*	p (one way)
RDUO_Visual	125	0.00	0.18	0.95	0.0011

2.3 QQ Plot Normality test



2.4 Homoscedasticity test (Scatter plot of residuals vs predicted).



2.5 Multiple comparison tests

Test:Tukey Alfa=0.05 DMS=0.47436

Error: 0.0395 gf: 100

Cassava accession	Mean	n					
SM 494-1	0.01	5	A				
Amarillo	0.02	5	A				
PI12900	0.03	5	A				
Brava	0.05	5	A	B			
CM 3380	0.07	5	A	B			
PI2902	0.09	5	A	B			
Abuelo	0.13	5	A	B	C		
Senon	0.13	5	A	B	C		
Jamaica 18	0.13	5	A	B	C		
Tremesiana	0.22	5	A	B	C	D	
PI12903	0.24	5	A	B	C	D	E
Tms	0.24	5	A	B	C	D	E
Forastera	0.27	5	A	B	C	D	E
Chilena	0.30	5	A	B	C	D	E
Serralles	0.32	5	A	B	C	D	E
CM 4484	0.32	5	A	B	C	D	E
Seda	0.33	5	A	B	C	D	E
CM 3311	0.38	5	A	B	C	D	E
Cubana	0.51	5		B	C	D	E
CM 3064	0.57	5			C	D	E
Trinidad	0.58	5			C	D	E
SM 523	0.61	5				D	E
Valencia	0.64	5				D	E
Llanera	0.67	5				D	E
Mcol 2215	0.70	5					E

Distinct Letters indicate significant differences (p<= 0.05)

APPENDIX 3

Alignment sequences of reference genes
(Original sequence on top, PCR sequence on bottom)

Tubuline (TUB)

```
Score = 158 bits (85), Expect = 3e-44
Identities = 95/99 (95%), Gaps = 4/99 (4%)

TGAGCAGATGATCAATGTCCAAAACAAGAACTCATCATACTTTGTTGAGTGGATTCCAAA
||| ||||||| | |||||| ||||||||||||||||||||||||||||||||||||||
TGA-CAGATGA-C-ATGTCC-AAACAAGAACTCATCATACTTTGTTGAGTGGATTCCAAA

TAATGTGAAATCAAGTGTCTGTGACATTCCACCTAGGGG
||||||||||||||||||||||||||||||||||||||
TAATGTGAAATCAAGTGTCTGTGACATTCCACCTAGGGG
```

Translation initiation factor 5A(F5A)

```
Score=110 bits (59),  Expect = 5e-30
Identities = 66/69 (96%), Gaps = 2/69 (2%)

ATCTGCTCAGCCAGATTAAAGATGGGTTTGCTGAGGGGAAGGACCTCGTAGTGAGCGTCA
|||||||| | |||||| ||||||||||||||||||||||||||||||||||||||||||
ATCTGCTC-GTCAGATT-AAGATGGGTTTGCTGAGGGGAAGGACCTCGTAGTGAGCGTCA

TGTCTGCAA
|||||||||
TGTCTGCAA
```

Ubiquitin(UBI)

```
Score = 131 bits (144), Expect = 3e-36
Identities = 76/77 (98%), Gaps = 1/77 (1%)

AGAGTGCGTGAAATAGTGGAGCAGAGCTGGACTGCCGACTAAAAACCCAACAAAAAGCAC
|||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
AGAGTGCGTGAA-TAGTGGAGCAGAGCTGGACTGCCGACTAAAAACCCAACAAAAAGCAC

CAGCCCAGCAGTCTATA
|||||||||||||||||
CAGCCCAGCAGTCTATA
```

Elongation Factor 1α(EF1)

```
Score =251 bits (278),Expect = 4e-72
Identities = 141/142 (99%), Gaps = 0/142 (0%)

GTAGGTCTGGCAAAGAGCTTGAGAAGGAGCCCAAGTTTTTGAAAAATGGTGATGCTGGGT
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
GTAGGTCTGGCAAAGAGCTTGAGAAGGAGCCCAAGTTTTTGAAAAATGGTGATGCTGGGT

TCGTGAAGATGATTCCGACCAAGCCCATGGTTGTGGAGACTTTCTCTGCGTACCCTCCAC
|||||||||||||||||||||||||||||||||||||||||||| ||||||||||||
TCGTGAAGATGATTCCGACCAAGCCCATGGTTGTGGAGACTTTCTCTGGGTACCCTCCAC

TGGGTAGATTCGCTGTTAGGGA
|||||||||||||||||||||
TGGGTAGATTCGCTGTTAGGGA
```

Alignment sequences of target genes
(original sequence on top)

Alternative oxidase (AOX)

```
Score = 96.9 bits (106),Expect = 4e-26
Identities = 60/62 (96%), Gaps = 2/62 (3%)

CAGGACAGTGAAACTGCTTCGACTCCCTACGGATATATTTTTTCAGAGACGGTATGGGTGCC
||||| |||||| ||||||||||||||||||||||||||||||||||||||||||||||||
CAGGA-AGTGAA-CTGCTTCGACTCCCTACGGATATATTTTTTCAGAGACGGTATGGGTGCC
```

Secretory peroxidase (APX3)

```
Score =141 bits (156),Expect = 2e-39
Identities = 85/87 (97%), Gaps = 2/87 (2%)

TAGACCAGCTCAATTCTATGTTCGCTTCACTTGGCCTTACCCAAACTGACATGATTGCTT
||||| |||||| ||||||||||||||||||||||||||||||||||||||||||||||
TAGAC-AGCTCA-TTCTATGTTCGCTTCACTTGGCCTTACCCAAACTGACATGATTGCTT

TATCAGGTGCACACACACTTGGATTTT
||||||||||||||||||||||||||
TATCAGGTGCACACACACTTGGATTTT
```

Catalase 1(CAT)

```
Score = 87.9 bits (47),Expect = 1e-23
Identities = 55/58 (94%), Gaps = 3/58 (5%)

TGGCTTCTCGTCTCAACGTGAGGCCAAATATATGAAGATGAGGCTATGGGTCACAGTG
|||| ||||||||| |||||||| ||||||||||||||||||||||||||||||||||
TGGC-TCTCGTCTC-ACGTGAGG-CAAATATATGAAGATGAGGCTATGGGTCACAGTG
```

Beta cyanoalaline synthase A (BCA)

```
Score =243 bits (131),Expect = 2e-69
Identities = 131/131 (100%), Gaps = 0/131 (0%)

TTCTAGCTTTCATCAATGGCTACTCTTACATCAATGGCTACTCTTAGGAACTTGTTCACG
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
TTCTAGCTTTCATCAATGGCTACTCTTACATCAATGGCTACTCTTAGGAACTTGTTCACG

AAAAAATCTTTTGCCTCAAACGAGCTTGCTATGCGGAGGTTCTTCACACCGCAGGCCGCTGCTGCTGAAGC
|||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AAAAAATCTTTTGCCTCAAACGAGCTTGCTATGCGGAGGTTCTTCACACCGCAGGCCGCTGCTGCTGAAGC
```

Beta cyanoalaline synthase B (BCB)

```
Score =226 bits (122),Expect= 2e-64
Identities = 122/122 (100%), Gaps = 0/122 (0%)

CAACAATCTTCCTATAAGGAGGTTTTTCTCTTCCGAAGCTGCGCTTGAATCTCCTTCATT
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CAACAATCTTCCTATAAGGAGGTTTTTCTCTTCCGAAGCTGCGCTTGAATCTCCTTCATT

TGCTCACAAAATCAGGGATCTGCCCAACAATCGTCCTCAAGCTAAAATCAAGCCTGAAGTTT
|||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
TGCTCACAAAATCAGGGATCTGCCCAACAATCGTCCTCAAGCTAAAATCAAGCCTGAAGTTT
```

Chitinase (CHT)

```
Score =239 bits (129),Expect = 2e-68
Identities = 129/129 (100%), Gaps = 0/129 (0%)

CCTGCTACTGAAAACTACTGCGACACAAACTTCCCACAGTATCCATGCACCCCAGGGAAG
|||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CCTGCTACTGAAAACTACTGCGACACAAACTTCCCACAGTATCCATGCACCCCAGGGAAG

AGATACTTTGGGCGAGGTCCAATTCAACTATCATGGAACTACAACTATGGAGCAGCTGGTCAAGACAAC
|||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AGATACTTTGGGCGAGGTCCAATTCAACTATCATGGAACTACAACTATGGAGCAGCTGGTCAAGACAAC
```

Cysteine protease (CIP)

```
Score =145 bits (78),Expect = 3e-40
Identities = 78/78 (100%), Gaps = 0/78 (0%)

GGGGAAAAGCTGGGGAGAAAATGGATATCTCAGGATGGCGCGAAGTATTAATAGCCCTAC
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
GGGGAAAAGCTGGGGAGAAAATGGATATCTCAGGATGGCGCGAAGTATTAATAGCCCTAC

AGGGATATGTGGAATTGC
||||||||||||||||||
AGGGATATGTGGAATTGC
```

Defense against Dead (DAD)

```
Score =176 bits (194),Expect = 2e-49
Identities = 106/112 (94%), Gaps = 0/112 (0%)


AGCTCTCTTCAGTTCACTTCGCTCTGCTTATGCCGCCACTCCAACTAGCCTTAAGATCAT
|||||||||||||||||||||| |||||||||| |||||||| ||||||||||||||||
AGCTCTCTTCAGTTCACTTCGCGCTGCTTATGCTGCCACTCCTACTAGCCTTAAGATCAT

CGATCTCTATGTGGGTTTTGCGGTTTTCACCGCTCTGATTCAGGTAGTTTAC
|||||| || |||||||||||||||||||| ||||||||||||||||||||
CGATCTGTACGTGGGTTTTGCGGTTTTCACTGCTCTGATTCAGGTAGTTTAC
```

Sucrose phosphate synthase (SPS)

```
Score =125 bits (138),Expect = 1e-34
Identities = 73/74 (98%), Gaps = 1/74 (1%)

GGTGATACAGATTACGAAGGC-TTACTTGGTGGAATTCACAAGTCAGTTATATTGAAGGG
|||||||||||||||||||| |||||||||||||||||||||||||||||||||||||
GGTGATACAGATTACGAAGGCCTTACTTGGTGGAATTCACAAGTCAGTTATATTGAAGGG

AGTTGGCAGCAATG
|||||||||||||
AGTTGGCAGCAATG
```

Cytochrome P450 CYP79D1 (CD1)

```
Score =158 bits (85),Expect= 3e-44
Identities = 85/85 (100%), Gaps = 0/85 (0%)

CCTCTTTATTTCCATTGTTAGTACTATTGTAAAACTTCAAAAGAGTGCTGCTAACAAGGA
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CCTCTTTATTTCCATTGTTAGTACTATTGTAAAACTTCAAAAGAGTGCTGCTAACAAGGA

AGGTAGCAAGAAACTCCCACTCCCT
|||||||||||||||||||||||||
AGGTAGCAAGAAACTCCCACTCCCT
```

Cytochrome P450 CYP79D2 (CD2)

```
Score =248 bits (134),Expect = 4e-71
Identities = 136/137 (99%), Gaps = 0/137 (0%)

CGGCCTCCTTCGCCTCCACGTCCTCCATGAACAATACTGCCAAAATCCTCCTTATCACCC
|||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CGGCCTCCTTCGCCTCCACGTCCTCCATGAACAATACTGCCAAAATCCTCCTTATCACCC

TCTTCATTTCCATTGTCAGTACTGTTATAAAACTTCAAAAAAGGGCATCCTACAAGAAAG
|||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||
TCTTCATTTCCATTGTCAGTACTGTTATAAAACTTCAAAAAAGGGCTTCCTACAAGAAAG

CTAGCAAGAACTTCCCA
|||||||||||||||||
CTAGCAAGAACTTCCCA
```

Cytochrome P450 CYP71E (CDE).

```
Score =293 bits (324),  Expect = 2e-84
Identities = 179/186 (96%), Gaps = 3/186 (1%)

TTCATTGATTCTTTGACAGGAGCCCTTGCCAAGAGGCAGCGAACATTTACAGACGTTGAT
|||||||||||| ||||||||||||||  |||||||||||||||||||||||||||||||
TTCATTGATTCTATGACAGGAGCCCTTATCAAGAGGCAGCGAACATTTACAGACGTTGAT

CGTTACTTCGAGAAAGTTATTGAACAGCATCTTGATCCTAACAGGCCTAAACCAGAAACT
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CGTTACTTCGAGAAAGTTATTGAACAGCATCTTGATCCTAACAGGCCTAAACCAGAAACT

GAAGACATTGTTGATGTCTTGATTGGGTTGATGAAGGACGAGAGTACCTCTTTCAAAATCACCAAG
|||||||||||||||||||||||||||||||||||||||||||| |||| ||||||| || ||||
GAAGACATTGTTGATGTCTTGATTGGGTTGATGAAGGACGAGAGT-CCTC-TTCAAAA-CATCAAG
```

Allene oxidase (ALX).

```
Score =114 bits (126),Expect = 1e-31
Identities = 63/63 (100%), Gaps = 0/63 (0%)

AACAGCAGGAATATGTGTATTGATCGAAAACAAACCGGAAATGAAAGGTGACCGGTACGAAGC
|||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AACAGCAGGAATATGTGTATTGATCGAAAACAAACCGGAAATGAAAGGTGACCGGTACGAAGC
```

1-aminocyclopropane-1-carboxylate oxidase (ACO)

```
Score =141 bits (156),Expect = 2e-39
Identities = 90/94 (95%), Gaps = 3/94 (3%)

GGACTTCTGCATGAAGCCTGCGAGAAATGGGGATTCTTTCAGGTTGAGAACCATGGAATT
|| |||||||||||  |||||||||||  |||||||  |||||||||||||||||||||||
GGGCTTCTGCATGA-GCCTGCGAGAA-TGGGGAT-CTTTCAGGTTGAGAACCATGGAATT

GATAAGAGTTTGATGGAGAAAGTGAAGCAATTGG
|||||||||||||||||||||||||||||||||
GATAAGAGTTTGATGGAGAAAGTGAAGCAATTGG
```

Lipooxygenase(LPO)

```
Score =199 bits (220),Expect = 1e-56
Identities = 110/110 (100%), Gaps = 0/110 (0%)

CAGTTCAATAACAAAAGAGCATATAGAGAATAAATTACATGGAATGATTATAGAAGAGGCAATAGAGAAGAACA
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CAGTTCAATAACAAAAGAGCATATAGAGAATAAATTACATGGAATGATTATAGAAGAGGCAATAGAGAAGAACA

AGTTATTCATATTGGATTACCATGATGTGCTGATGC
|||||||||||||||||||||||||||||||||||
AGTTATTCATATTGGATTACCATGATGTGCTGATGC
```

***Extensin (EXT*)**

```
Score =128 bits (69),  Expect = 1e-34
Identities = 69/69 (100%), Gaps = 0/69 (0%)

CAAACTACCCTTACCACTCTCCTTCACCTCCAAAGAAATCCCCACTACCGCCTGTCCACC
|||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CAAACTACCCTTACCACTCTCCTTCACCTCCAAAGAAATCCCCACTACCGCCTGTCCACC

CTCCATCTT
|||||||||
CTCCATCTT
```

Phenyl alanine lyase (PAL)

```
Score =246 bits (272),Expect= 2e-70
Identities = 136/136 (100%), Gaps = 0/136 (0%)

GGAGGCAATTTCCAGGGGACCCCAATTGGTGTTTCAATGGATAACACTCGTTTAGCCATT
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
GGAGGCAATTTCCAGGGGACCCCAATTGGTGTTTCAATGGATAACACTCGTTTAGCCATT

GCTTCAATTGGTAAACTCATGTTTGCTCAATTCTCTGAGCTTGTTAATGATTTTTACAAC
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
GCTTCAATTGGTAAACTCATGTTTGCTCAATTCTCTGAGCTTGTTAATGATTTTTACAAC

AATGGGTTGCCTTCAA
||||||||||||||||
AATGGGTTGCCTTCAA
```

Pectinacetylesterase (PEC)

```
Score =122 bits (66),Expect = 1e-33
Identities = 82/89 (92%), Gaps = 3/89 (3%)

CATAAGCCCATGCATTGGTTGGTTAATGCCAGACTTGTTATCCTTGTGTTTTCTGGCTAG
||||||  ||||||||||||||||||||||||||||||||| ||||||||||||||||
CATAAG-GCATGCATTGGTTGGTTAATGCCAGACTTGTTATCCGTGTGTTTTCTGGCTAG

ACAATCTTCACAGCCATCAAGATCACTCT
|||  |||||||||  ||||||||  ||  |||
ACA-TCTTCACAGACATCAAGA-CATTCT
```

Hidroxyproline rich protein (HRP)

```
Score =230 bits (124),Expect = 1e-65
Identities = 124/124 (100%), Gaps = 0/124 (0%)

CTTTCCCTCTATGGCTCATCTCCATAACACTCATGCCAGATCCGCGCCAGTTGTAGAGCT
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CTTTCCCTCTATGGCTCATCTCCATAACACTCATGCCAGATCCGCGCCAGTTGTAGAGCT

CCGCCTGGGCTTTCTTGAGGACGTTGGCAGGTAAGATGGCAGGACCGGCGGCGAAATTGAAGAC
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CCGCCTGGGCTTTCTTGAGGACGTTGGCAGGTAAGATGGCAGGACCGGCGGCGAAATTGAAGAC
```

Aquaporin PIP transporter (PIP)

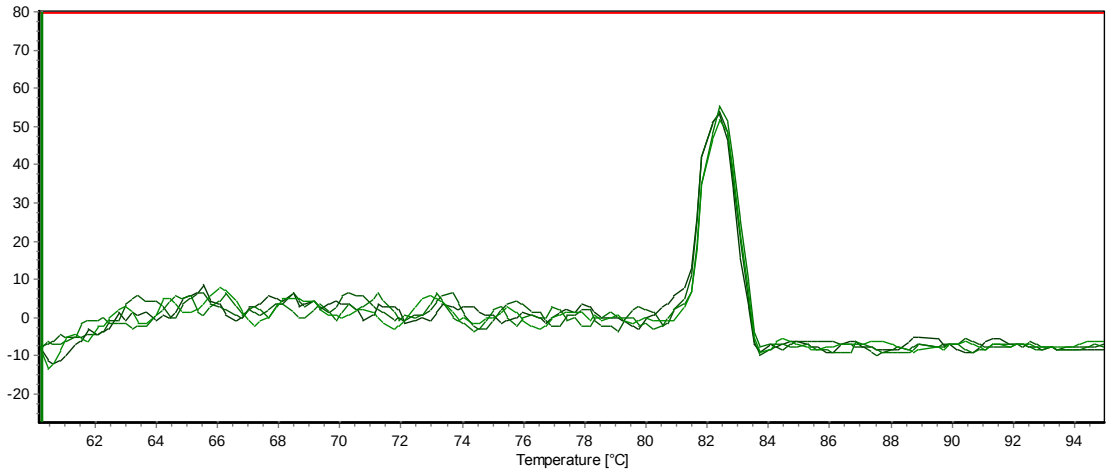
```
Score =  154 bits (170),  Expect = 6e-43
Identities = 101/110 (91%), Gaps = 1/110 (0%)

CAAGGATTATACTGAGCCACCACCGGCTCCGCTGTTTGAGCCGTCTGAGCTCACTTCTTG
|||||||||||||||||||||||||| ||||| || ||||| || ||||||||||||||
CAAGGATTATACTGAGCCACCACCGGCGCCGCT-TTCGAGCCATCGGAGCTCACTTCTTG

GTCCTTTTACAGAGCTGGTATTGCAGAGTTTATTGCCACTTTCTTGTTCT
|||||||||||||||||| |||||||| || || ||||||||||||||||
GTCCTTTTACAGAGCTGGAATTGCAGAATTCATAGCCACTTTCTTGTTCT
```

Ferodoxin sulfate reductase (FSR)

```
Score =221 bits (244),  Expect = 6e-63
Identities = 126/127 (99%), Gaps = 1/127 (0%)

CGAGAAAATGGGAGAAGAGATGATCGTAAGTACAGTAGAATGAAATATTTGATTAGCTCC
|||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CGAGAAAATGGGAGAAGAGATGATCGTAAGTACAGTAGAATGAAATATTTGATTAGCTCC

TGGGGGGATTGAGAAGTTCAGAACTGTGGTTGAGCAATACTATGGAAAGAAATTTGAGCC
| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
T-GGGGGATTGAGAAGTTCAGAACTGTGGTTGAGCAATACTATGGAAAGAAATTTGAGCC

CTTCCGT
|||||||
CTTCCGT
```

APPENDIX 4

Ribosomal 18S Dissociation analysis (melting curve)



Threshold: 80%