QUANTIFICATION OF THE TRANSCRIPTIONAL ACTIVITY OF GENES ASSOCIATED WITH CYANOGENESIS IN CASSAVA (*Manihot esculenta* Crantz)

By

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Abstract

Cassava has cyanogenic glycosides in its roots and leaves. These compounds function in plants as protective agents against herbivores as well as the translocable form of reduced nitrogen. The genes involved in the cyanogenic pathway [CYP79D1, CYP79D2, linamarase, α-hydroxynitrile lyase (HNL) and β-cyanoalanine synthase (β-CAS)] have been identified, though very little is known regarding their transcriptional regulation. Our work aimed to identify the differential expression of these genes using conventional PCR and Real Time PCR in cassava plants of different cultivars grown *in vitro* under different environment conditions. Four experiments were designed to accomplish this objective: 1. Semiquantitative comparison of the transcriptional activity in leaves and roots of two cassava cultivars using conventional PCR; 2. Quantitative comparison of the transcriptional activity in leaves and roots of three cassava cultivars using Real-Time PCR; 3. Quantitative comparison of the transcriptional activity in leaves and roots of two cassava cultivars under reduced nitrogen stress using Real-Time PCR; 4. Quantitative comparison of the transcriptional activity in leaves and roots of two cassava cultivars under temperature stress using Real-Time PCR.

Statistical analyses demonstrated a higher expression of the genes in leaves relative to the roots, consistent with the fact that cyanogens are synthesized predominantly in the leaves and subsequently transported to the roots where they are converted to proteins. In the comparison of the expression among three cultivars, the results suggest that differences in cyanide content in roots could be explained by the coordinated cyanogenic glycoside synthesis in leaves and the linamarase catabolic step in the roots. The comparison under reduced nitrogen stress shows the impact of this environmental condition over the expression pattern of these genes. Thus, it is reported that CYP79D1/D2 genes increase their transcriptional activity in inverse relation to reduced nitrogen concentration in roots after one day of stress; while the β-CAS gene responds in direct relation to reduced nitrogen concentrations in leaves and roots primarily after 10 days of stress. The results obtained from the temperature stress experiment illustrate an influence of temperature on β-CAS gene expression, though this result was not considered a direct effect of temperature stress over the general cyanogenic glycoside pathway in cassava. Finally, multivariate analysis of the expression of those genes suggest a correlation between the expression of linamarse and HNL genes (involved in the breakdown of linamarin) and among CYP79D1/D2 and β-CAS. This fact is corroborated by the responses of CYP79D1/D2 and β-CAS genes under reduced nitrogen stress, as well as the antecedents that illustrate the linamarase and HNL protein are located separately in the cell wall. This is the first study that evaluate the transcriptional activity of the genes (known to date) involved in the cyanogenic glycoside metabolism in an integral way. The results show the complexity of cyanogenic glycosides metabolism in cassava.

Resumen

La yuca contiene glicósidos cianogénicos en sus raíces y hojas. Estos compuestos funcionan en las plantas como agentes protectores contra herbívoros, así como fuente de nitrógeno reducido translocable. Los genes involucrados en el metabolismo de estos compuestos [CYP79D1, CYP79D2, linamarasa, alpha-hydroxynitril liasa (HNL) y β-cianolalanina sintasa (β-CAS)] han sido identificados, aunque poco es conocido respecto a su regulación transcripcional. Nuestro trabajo se enfocó en conocer la expresión diferencial de estos genes usando *PCR* convencional y *PCR* en Tiempo Real en plantas *in vitro* de yuca de diferentes cultivares bajo diferentes condiciones ambientales. Cuatro experimentos fueron diseñados para llevar a cabo este objetivo: 1. Comparación semicuantitativa de la actividad transcripcional en hojas y raíces de dos cultivares de yuca usando *PCR* convencional; 2. Comparación cuantitativa de la actividad transcripcional en hojas y raíces de tres cultivares de yuca usando *PCR* en Tiempo Real; 3. Comparación cuantitativa de la actividad transcripcional en hojas y raíces de dos cultivares de yuca bajo estrés de nitrógeno reducido usando *PCR* en Tiempo Real; 4. Comparación cuantitativa de la actividad transcripcional en hojas y raíces de dos cultivares de yuca bajo estrés de temperatura usando *PCR* en Tiempo Real.

Los análisis estadísticos mostraron una alta expresión de los genes en hojas en relación a las raices, lo cual es consistente con el hecho de que los glicósidos cianogénicos son sintetizados principalmente en las hojas y luego transportados a las raíces donde son convertidos a proteínas. En la comparación de la expresión entre tres cultivares, los resultados sugieren que las diferencias en contenido de cianuro en las raíces pueden ser explicadas por la coordinación entre la síntesis de glicósidos cianogénicos en las hojas y la acción catalítica de la linamarasa en las raíces. Las comparaciones bajo estrés de nitrógeno reducido muestran el impacto de esta condición ambiental sobre el patrón de expresión de estos genes. De esta manera, se reporta que los genes CYP79D1/D2 aumentan su actividad transcripcional en raíces en el primer día de estrés en inversa relación a la concentración de nitrógeno reducido; mientras que la expresión del gen β-CAS en hojas responde en directa relación a la concentración de nitrógeno reducido a los 10 días de estrés principalmente. Los resultados obtenidos del experimento de estrés de temperatura muestran un impacto sobre la expresión del gen β-CAS, aunque este resultado no es considerado como un efecto directo de la temperatura sobre el metabolismo de los glicósidos cianogénicos en la yuca. Finalmente los análisis multivariados de la expresión de estos genes sugieren una correlación en la expresión de la linamarasa y el gen HNL (los cuales están implicados en la degradación de la linamarina) y entre CYP79D1/D2 y β-CAS. Este hecho es corroborado por la respuesta de CYP79D1/D2 y β-CAS bajo el estrés de nitrógeno, así como por los antecedentes que ilustran que la linamarasa y la proteína de HNL están localizadas de manera separada en la pared celular. Este es el primer estudio que evalua la actividad transcripcional de los genes (conocidos a la fecha) envueltos en el metabolismo de los glicósidos cianogénicos de manera integral. Los resultados muestran la complejidad del metabolismo de los glicósidos cianogénicos en la yuca.

Dedication

To God, my Parents, Brother and Wife.

En el Campo di Fiore, en el trastevere romano lo encontré, dándoles migajas a las palomas. Le pregunté: ¿Usted es el que yo creo? y me dijo: Yo soy el que tú quieras. Le pregunté: ¿Usted es el maestro? Y me dijo: No, maestro es el que te puso delante de mí y a mí delante de ti. Yo soy Arthur Rubinstein.

Anécdotas de Facundo Cabral

Toda teoría es gris, querido amigo, y verde es el dorado árbol de la vida.

Johann Wolfgang von Goethe.

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Mayagüez made a change in my life, where God's blessings allowed me to grow as a human being and as a scientist. Thus, I want to begin expressing my acknowledgements to God for all His love.

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CHAPTER ONE
INTRODUCTION AND OBJECTIVES

1.1 Introduction

Cassava is the fourth most important food crop in the world after rice, wheat and corn (Ceballos, 2000). It is the main food source of more than 500 million people in addition to other applications in industry and as animal feed (El-Sharkawy, 2004). At present the global area dedicated to cassava cultivation is about 13 million hectares, more than 70% being in Asia and Africa, and the rest in Latin America and the Caribbean (El-Sharkawy, 2004). The most important agronomic properties of cassava include high potential yield, drought tolerance, ability to grow in poor soils, and food security (El-Sharkawy, 2004; Mejia de Tafur, 2000). These qualities have made cassava an important food crop in the tropics, particularly for subsistence farmers in Africa (Siritunga and Sayre, 2004), who are prompt to suffer droughts and famine. However cassava has cyanogenic glycosides in its roots and leaves, which function in plants as protective agents against herbivores as well as the translocable form of reduced nitrogen (McMahon et al., 1995; Siritunga and Sayre, 2004). Cyanogenic glycosides release cyanide in the human body if consumed; so the cassava roots must be processed before it can be used as food (Rosling, 1994)

The metabolism of cyanogenic glycosides in cassava has been extensively studied and genes involved in their synthesis (CYP79D1 and CYP79D2) (Andersen et al., 2000), in their catabolism under plant disruption (Linamarase and HNL) (Hughes et al., 1992; Hughes et al., 1994; White *et al*., 1998) and recently a gene involved in the HCN re-assimilation [β-cianolalanine sinthase (β-CAS)] [Gen Bank accession No*:* EU 350583] has been identified. However, there still remains the possibility of identifying other genes

that are involved in the cyanogenic glycoside metabolism in intact plants that will allow us to understand the function of those compounds in more detail (Selmar, 1994).

Cyanogenesis in cassava is not always identical in all cultivars. In fact, it is a complex and variable process that involves different genes and proteins, the environment and the individual history, generating a spectrum of cultivars with different contents of cyanogenic glycosides (Cardoso et al., 1999; Santana et al., 2002). Thus, a better understanding of the metabolism of cyanogenic glycoside will require a detailed knowledge of the genes involved, and their transcriptional and postranscriptional regulation under determined environments and developmental stages.

The regulation of all genes involved in cyanogenic pathway has not been studied simultaneously. Previous research has focused on a particular gene under specific conditions. Thus, the present research is the first study that attempts to evaluate the transcriptional activity of the genes (known to date) involved in this metabolism in a coordinated way. We will then be able to contribute to the comprehension of the differences in cyanogenic glycosides quantities between tissues, cultivars and under specific environmental conditions. To achieve our objective we used a modern molecular technique, Real-Time PCR. In conclusion, the results of this project have allowed us to understand in more detail the expression of genes implicated in the metabolism of cyanogenic glycosides in cassava, as well as contribute new knowledge for possible strategies directed towards reducing cyanogenic compounds in cassava.

1.2 General Objective

To quantify the transcriptional activity of genes associated with the cyanogenic glycosides metabolism in cassava (*Manihot esculenta* Crantz) under standard *in-vitro* conditions and under specific stress *in-vitro* conditions.

CHAPTER TWO:
LITERATURE REVIEW

2.1 Cassava and Cyanogenesis

Cassava (*Manihot esculenta* Crantz) is a plant that traditionally is known for its roots, which are used as food in different countries of Africa, Asia, South America and the Caribbean. It is consumed by 500 million people worldwide (Cock, 1982) and according to the FAO (Food and Agriculture Organization) in 2004 there were 18,547,650 ha of cassava harvested, with a production of 203,162,926 tons (http://faostat.fao.org, revised 5th of August of 2008).

In cassava there is a biochemical and physiological process known as cyanogenesis, which is the ability of living organisms to release hydrogen cyanide (HCN). Although this phenomenon has been recognized in over 3,000 species of higher plants, there are only approximately 300 plants species in which the source of HCN, such as cyanogenic glycosides, cyanolipids and co-product of ethylene biosynthesis, have been identified (Poulton, 1990). In the specific case of cassava the cyanide precursors are the cyanogenic glycosides. A glycoside is a molecule whose structure has a sugar (glycone) and another nonsugar component (aglycone). Therefore the cyanogenic glycosides are a kind of glycoside, whose aglycone has one cyanide group. There are approximately 25 cyanogenic glycosides known, most of them found in the edible plants: amygladin (almonds), dhurrin (sorghum), linamarin (cassava, lima beans) and lotaustralin (cassava, lima beans) (Food Standards Australia New Zealand, 2005).

In the human body, the cyanogenic glycosides from cassava release cyanide by generalized β-glycosidases at elevated temperatures (greater than 35^0C) and pH (greater than 5.0) (McMahon et al., 1995). For this reason, cassava foods must be

processed to remove cyanogens prior to consumption, using process such as pounding, maceration, cooking and/or fermentation (Siritunga and Sayre, 2004). Hence if cassava is poorly processed it may be consumed with residual amounts of cyanogenic glycosides (Rosling, 1992). The human body has the ability to detoxify low amounts of cyanide via the enzyme rhodanase. This enzyme converts cyanide to the less toxic isothiocynate which then is excreted through the urine (Rosling, 1994). Nevertherless the synthesis of isothiocynate requires cysteine which may be limiting for nutritionally compromised individuals thus acerbating the effect of cyanide poisoning (Siritunga and Sayre, 2004; Rosling, 1994).

The principal role of cyanogenic glycoside in plants is associated with protection against herbivores which generate a disruption on plant tissues (plant disruption) (Poulton, 1990; Wynne-Edwards, 2001; Gebrehiwot and Beuselinck, 2001). For example, in birdsfoot trefoil (*Lotus corniculatus* L) acyanogenic plants suffer disproportionate levels of herbivory (Gebrehiwot and Beuselinck, 2001) while in some tropical environments, where insect pressure is high, as much as 4% of woody plants are cyanogenic and concentrate HCN precursors in reproductive parts (Thomsen and Brimer, 1997). In cassava the presence of cyanogenic glycosides has been associated with herbivores and pest defense, thus, for example, the large amounts of cyanogens produced in leaves are a deterrent for the grasshopper, *Zonocerus variegates*; meanwhile the cyanide present in roots is a deterrent for the burrowing bug, *Cyrtomenus bergi* (Belloti and Riis, 1994).

2.2 Cyanogenic Glycosides in Cassava: Synthesis, Catabolism, Transport and Re-Assimilation

Traditionally, the cyanogenesis in plants has been explained as a herbivore defense mechanism. Accordingly, the cyanogenesis is studied as a catabolic process in which the cyanogenic glycosides releases free HCN when the plant is disrupted. However, in an attempt to completely understand the biochemical process scientists also have focused on the synthesis of cyanogenic glycosides (Andersen et al., 2000; McMahon et al., 1995). Recently, researches have focused on the study of the cyanogenic glycosides when the cassava plant has no disruption (when the plant is intact). This has led to evidence suggesting that cyanogenic glycosides are a main source of reduced nitrogen in plants and are generally translocated from leaves to roots (Selmar, 1994; Jørgensen et al., 2005; McMahon et al., 1995; Siritunga and Sayre, 2003 and 2004; Santana et al., 2002). However, this hypothesis implies that the plant also releases free HCN without the presence of one herbivore or disruption; therefore cassava needs a mechanism of detoxification or re-assimilation of HCN (Selmar, 1994, Siritunga and Sayre, 2004) to prevent auto-toxicity.

In the following sections, the principal reaction sets in which cyanogenic glycosides and free HCN are involved in will be discussed: 1. synthesis of cyanogenic glycosides; 2. catabolism of cyanogenic glycosides (with plant disruption and without plant disruption[1]); and 3. re-assimilation of HCN in intact plants.

[1] Although plant disruption could imply herbivore activity on the plant or some other kind of environmental damage, we use this concept in relation to the herbivores attacks, which is more related with the cyanogenic glycosides role.

2.2.1 Synthesis of Cyanogenic Glycosides

All cassava tissues, with the exception of seeds, contain the cyanogenic glycosides linamarin and lotaustralin. Leaves have the highest cyanogenic glycoside levels (5.0 g linamarin/kg fresh weight), whereas roots have approximately 20-fold lower linamarin levels (White et al., 1998). Both molecules are structurally related, however lotaustralin has an extra methyl group in the aglicone; moreover, the presence of linamarin in cassava is superior (>90% total cyanogen) than lotaustralin present (<10% total cyanogen) (Conn, 1979).

The linamarin and lotaustralin synthesis in cassava has been studied in detail in the last decade, although some aspects still need more research. The metabolic pathway starts with the conversion of L-valine and L-isoleucin to oximes: [(z)-2-methylpropanal oxime and (z)-2-methylbutanal oxime respectively]. This step is synthesized by the enzymes CYP79D1 and CYP79D2. Subsequently, a putative second enzyme (CYP71E) converts the oxime to the respective nitrile. The final step in the synthesis of cyanogenic glycosides is the addition of glucose to acetone cyanohydrin catalyzed by UDP-glucosyl transferase (See Figure 1 and Figure 3). Previous research suggests that the UDP-glucosyltransferase is presumably vacuolar enzyme whereas the cytochromes involved in the initial biosynthesis steps are localized in the tonoplast (McMahon et al., 1995).

CYP means CYtochrome Pigment 450 and in the literature P450 and CYP450 have been used synonymously. According to the database from Doctor Nelson (http://drnelson.utmem.edu/CytochromeP450.html; checked on the August 31st, 2007) there are a total of 7703 CYP450 proteins in 866 families, of which 2675 proteins in 94 families are in plants. Because of this large number of DNA sequences encoding cytochrome

P450s, the scientific community has established classification and nomination rules for them. For example, *CYP79A1* means the family 79, subfamily A and gene number 1.

In cassava, one of the cytochrome families of interest is CYP79. The proteins belonging to this family catalyze the conversion of an aliphatic or aromatic amino acid or a chain-elongated methionine homologue to the corresponding oxime. In plants they are involved in the production of secondary metabolites that work as defense against herbivores and pathogens, such as cyanogenic glycosides and glucosinolates (Mikkelsen et al., 2003). Within this family, the most important subfamilies in relation with cyanogenic glycosides synthesis are the subfamilies A, D and E; and within the subfamily D, there are two paralogous *CYP79D1* and *CYP79D2* genes, which are 85% identical. Andersen et al. (2000) showed that both cytochromes have affinity for L-valine and L-isoleucine. However, in the case of cassava leaves the L-valine conversion is larger than the L-isoleucine conversion which is consistent with the high levels of linamarin present in cassava leaves.

The other CYP gene involved in cassava cyanogenic glycoside synthesis is *CYP71E1*. The gene family of *CYP71* is the largest set of P450s in plants showing large clusters of duplicated genes (Nelson et al., 2004). *CYP71E1* gene was found in *Sorghum bicolor* (sorghum) catalyzing the conversion from z-oxime to cyanohydrin in the biosynthesis of dhurrin (Jones et al., 2000). The similarities between the linamarin synthesis pathway in cassava and the dhurrin synthesis pathway in sorghum to the belief that cassava has the same kind of cytochrome which is involved in the conversion of 2-methylpropanol oxime to acetone cyanohydrin (Zhang et al., 2003; Siritunga and Sayre, 2007). At

present, only one cDNA has been found in cassava with high similarity with this gene (Zhang et al., 2003) but no biochemical characterization has been reported yet.

The last enzyme in the linamarin synthesis is UGT (Uridine diphosphate Glucosyl Transferase). This enzyme catalyzes the transfer of glucosyl groups from one compound to another. This enzyme also was found in the synthesis of cyanogenic glycoside dhurrin in sorghum (Jones et al., 1999), which changes by *O*-glucosylation the (*S*)-*p*-hydroxymandelonitrile to the cyanogenic glycoside dhurrin. In the case of cassava case, six different kinds of UGT were found in cassava which are related to multiple secondary plant products (Hughes and Hughes, 1994).

Though the genes have been identified and the biochemistry of the cyanogen synthetic pathway has been studied, there are no studies about the regulation of the activity of these genes or consequent proteins. The only implication in this regard is the demonstration that leaves and roots can synthesize cyanogenic glycosides (although the amount in roots is very low compared to leaves), leading to the conclusion that both tissue has *CYP79* transcriptional activity (McMahon and Sayre, 1995).

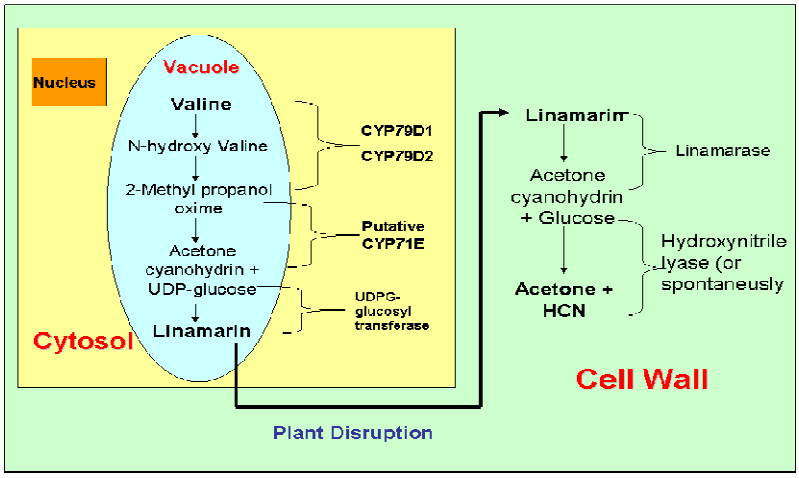


Figure 1: Linamarin synthesis and catabolism in cassava plant under disruption
The linamarin is synthesized in the vacuole and once the plant is disrupted, linamarin comes in contact with the enzymes (linamarase and HNL) stored in the cell wall, which in turn begins the linamarin catabolism.

2.2.2 Cyanogenic Glycosides Breakdown

2.2.2.1 Cyanogenic Glycosides Breakdown in Plants under Disruption.

Traditionally, the breakdown of cyanogenic glycosides refers to the catabolism of linamarin and/or lotaustralin and release of HCN when the plant is disrupted (White et al., 1998). The enzymes committed in this set of reactions are Linamarase and Hydroxynitrile Lyase (HNL), which have been studied in detail because of their impact in releasing HCN, thus detoxifying the food for human consumption. When the plant is damaged, linamarin from the vacuole is released which then is hydrolyzed by linamarase and HNL to produce cyanide. Linamarase and HNL are located in the cell wall and/or laticifers-cells (McMahon et al., 1995). This result in the compartmentalization of the cyanogen synthesis reactions and catabolism reactions in cassava cells (McMahon et al., 1995) (see Figure 1).

Cassava linamarase is a β-glucosidase belonging to the glycoside hydrolase family number 1 (β-glucosidase, EC 3.2.1.21). This family involves enzymes with a number of known activities such as chemical defense against herbivores and pathogens, lignin biosynthesis, and plant growth and development (Sadder et al., 2007). Specifically in cassava, linamarase hydrolyzes linamarin to produce an unstable intermediate, acetone cyanohydrins, from which HCN is released (Figure 1 and Figure 3). Linamarase is an enzyme of 65 kDa molecular weight that has three different isoforms (isoelectric points 2.9, 3.3, and 4.3) (Hughes et al., 1992; Mkpong et al., 1990). Those isoforms differ in their tissue specific localization which means one post-translational system involved in regulation of linamarase activity. Studies have shown that this post-translational system could be glycosilation, which affects the enzymatic property (McMahon et al., 1995). In

addition, through sequencing of a linamarase cDNA clone, Hughes et al. (1992) identified a protein of 532 residues, having a predicted weight of 65 kDa. This clone sequenced showed that cassava protein has 43% amino acid sequence identity with clover linamarase, but is most similar to the β-glucosidase of *Agrobacterium tumefaciens.*

In 2002, Santana et al. developed a study where they reported patterns of linamarase expression in cassava. They found that linamarase activity is influenced by the cultivar, the tissue and the plant growth stage. They did not find transcriptional activity of linamarase in roots, although the enzyme was active in this tissue. Using this result they supported the hypothesis proposed by Pancoro and Hughes (1992), that the linamarase enzyme is being transported from shoots to roots through laticefer cells[2]. The same study found that in low-cyanide cultivar, young leaves showed high linamarase activity during the early plant growing steps, whereas in root peel, the activity increased during the growth cycle. Conversely, in the high cyanide cultivar the leaf linamarase activity was higher in 11 months-old plants whereas the root peels linamarase activity decrease during the growth cycle.

The other enzyme involved in cyanogenic glycosides breakdown when the plant is disrupted is the hydroxynitrile lyases (HNL) (EC 4.1.2.39). This enzyme catalyzes the cleavage of cyanohydrins to hydrocyanic acid plus the corresponding aldehyde or ketone. In cassava, it does the breakdown of acetone cyanohydrin to cyanide and

[2] This hypothesis from Pancoro and Hughes, (2002) is different to the hypothesis "Linustatin pathway" (proposed by Selmar, 1994) in which the linamarin is the molecule that is transformed to linustatin, which is transported from the shoots to the roots.

acetone (Figure 1 and Figure 3). This reaction can occur spontaneously at temperatures greater than 35^0C or at pH greater than 5.0 (White et al., 1998). This enzyme has one subunit with a molecular weight of 28.5 kDa and is classified inside of the α/β hydrolase family (Gruber et al., 2004).

According to Hughes et al. (1998), the cassava HNL has different gene copies, forming a gene family constituted by the *HNL 4, HNL 10* and *HNL 24* loci. These genes have difference sequences and different expression patterns in tissues, showing the complexities of cassava cyanogenesis regulation. White et al. (1998) and Hughes et al. (1998) found that *HNL10* is expressed in high levels in leaves and low amounts in roots (according to Northern blot analysis) which explain the accumulation of acetone cyanohydrin in cassava roots. Interestingly, HNL4 gen is expressed at high levels in roots and at lower levels in cotyledons and young leaves whereas the expression pattern of *HNL 24* has not been studied (Hughes et al., 1998).

2.2.2.2 Cyanogenic Glycosides Breakdown in Intact Plants

The present evidence shows that in an intact plant the enzymatic degradation of linamarin by linamarase does not happen. However it does not mean that an intact plant does not produce HCN. Selmar (1994) proposed the hypothesis named the “Linustatin Pathway” (see figure 2), which is one extrapolation of the results and evidence found in *H. brasilensis*. According to this explanation, the linamarin produced in cassava leaves is transported to the roots through of an apoplastic route. This transport from the leaves requires a special molecular configuration of the linamarin, which otherwise could lead to hydrolysis by the linamarase presents in the cell wall. The answer to this requirement

is the glycosilation of linamarin which results in linustatin, a diglucoside, which linamarase cannot hydrolyze. Thus, cyanogens can be translocated via the phloem to the roots. Once in the roots, the linustatin can be deglucosilated in two different ways known as “sequential” and “simultaneous” cleavage. In the sequential cleavage the linustatin is converted back to linamarin and possibly stored, but in the simultaneous reaction the linustatin is converted in acetone-cyanohydrin, the precursor of free HCN. However, although Selmar (1994) explains that these events occur in the roots, the studies developed by Elias et al. (1997B) in cassava leaves show the presence of at least one enzyme involved in re-assimilation of HCN (see 2.2.3 section). This leads us to believe that “sequential” and “simultaneous” reactions occur in leaves as well (see figure 2).

It must be noted that the linustatin has not been detected in cassava in quantities sufficient to facilitate cyanogen transport by the linustatin pathway (McMahon et al., 1995). The high content of latex in cassava does not allow the direct analysis of phloem sap with the aim to find this diglucoside (Selmar, 1994). However we have different indirect evidence that let to conjecture that the translocation of linamarin from leaves to roots occurs: 1. Ramanujam and Indira (1984) reported that when girdling in the stems near to the base of cassava plants is performed, linamarin accumulated above the girdling site, leading to a 13-fold increase ; 2. Makame et al. (1987) working with grafts between roots of high-cyanogenic cultivars and shoots of low- and high-cultivars confirmed the predominant influence of scion (leaf or shoot) as a source of HCN that is accumulated in roots; 3. the results from cassava transgenic plants by Siritunga and Sayre (2003) and Jørgensen et al., (2005), in which CYP79D1/D2 genes were silenced

in leaves, demonstrated that both leaf and roots levels of linamarin were reduced, but much more drastically in the roots (for more detail about this experiment see 6.1).

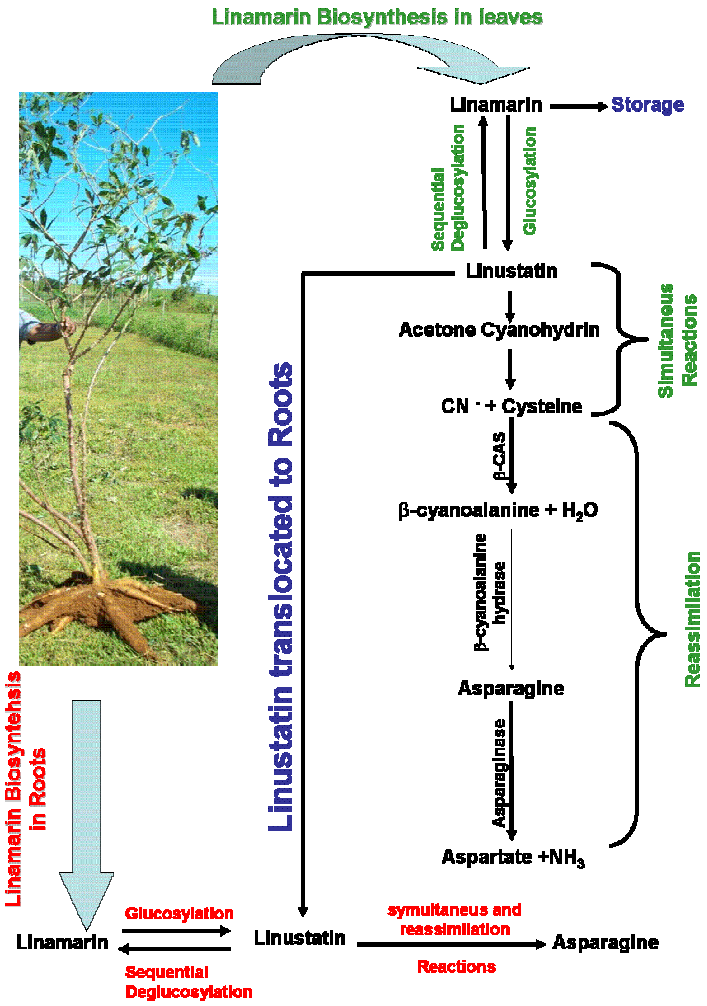


Figure 2: **Linustatin pathway and re-assimilation reactions of intact cassava plant**
The Linamarin is synthesized in high amounts in cassava leaves and low amounts in roots. The linamarin present in the leaves could be stored, used as amino acid source or translocated to the roots as linustatin. In the roots the translocated linustatin and the synthesized linamarin are mainly used as amino acid and reduced nitrogen source for the roots.
Picture: Dimuth Siritunga, Ph.D. University of Puerto Rico at Mayagüez.

2.2.3 Re-assimilation reactions

We will refer to the subsequent reactions after the linustatin simultaneous cleavage, where non-cyanogenic compounds are produced in an intact plant as the re-assimilation pathway. As previously explained in the “simultaneous cleavage”, linustatin in the roots

is first converted to acetone-cyanohydrin and then to HCN. In plants, like in other living organisms, HCN is a toxic compound that interferes with the electron transport chain of the respiratory pathway by inhibiting cytochrome oxidase. Thus HCN detoxification is of paramount necessity (Selmar, 1994; Siritunga and Sayre 2004). In cassava this detoxification process is performed by the enzyme β-CAS (E.C 4.4.1.9) (Selmar, 1989, Selmar et al., 1994, Siritunga and Sayre, 2004), which belongs to the family of lyases, specifically to the class of carbon-sulfur lyases. This enzyme catalyzes the formation of the non-protein amino acid β-cyano-Ala (β-cyanoalanine) from cysteine and cyanide and is present in a broad spectrum of species including bacteria, plants and insects (revised by Hatzfel et al., 2000).

Cassava β-CAS protein was partially isolated and characterized by Elias et al. (1997B) from leaves, rind and tubers. Interestingly in this study the β-CAS activity was significantly larger in roots than in leaves and rind, results that contrast with the tissue specific activity of linamarase (Santana et al., 2000) and HNL (White et al*.,* 1998). Molecular characterization of β-CAS gene was done recently by Marrero and Siritunga (Gen Bank accession No*:* EU 350583). However, at least at this time, the cassava specific cellular localization has not been defined yet, although it is believed that β-CAS is present in the mitochondria and cytosol (Dimuth Siritunga, personal communication), as reported for the potato β-CAS where two copies have been detected: cytosolic β-CAS and mitochondrial β-CAS (Maruyama et al., 2001).

Once β-cyanoalanine is produced by β-CAS, it is metabolized into asparagine with the enzyme β-cyanoalnine hydrase. Thereafter asparagine could be used as a plant amino acid or metabolized to aspartate and NH_3 through of asparaginase enzyme. The

enzymatic activity of β-cyanoalnine hydrase and asparaginase has been detected in leaves, rinds and cassava tubers by Elias et al. (1997A), however, they found that in edible tubers cyanoglucoside catabolism results in the accumulation of asparagine while in other tissues, it is further metabolized to aspartatic acid and ammonia (reduced nitrogen) (see figure 2). Although the enzyme activity of those proteins (β-cyanoalnine hydrase and asparaginase) in cassava has been evaluated, the isolation and molecular characterization has not been performed yet.

The entire cassava cyanogenic metabolism is summarized in the Figure 3. The initial steps lead to the synthesis of cyanogenic glycosides which then can be catabolyzed under plant disruption to HCN or re-assimilation to aspartate in intact plants.

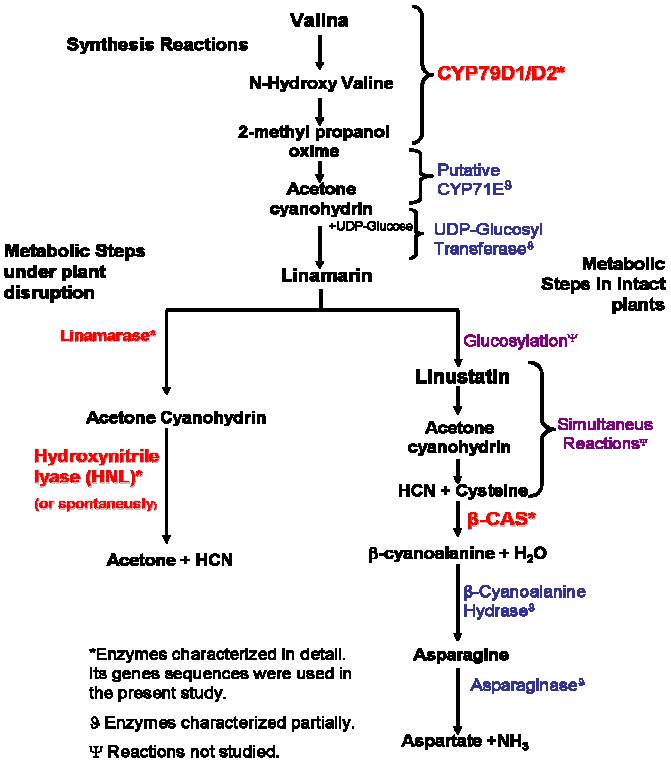


Figure 3: Cyanogenic glycosides metabolic pathways.
This summarizes all the reactions involved in the metabolism of cyanogenic glycosides in cassava.

2.3 Cassava Cyanogenic Glycosides from Functional Genomic Perspective

Many research have shown that cyanogenic glycosides synthesis, catabolism or re-assimilation regulation is a complex process that involves post-transcriptional factors (Andersen et al., 2001, Hughes et al., 1998, McMahon et al., 1995), environmental (Siritunga and Sayre 2003 and 2004) and developmental circumstances (Santana et al 2002). The initial step in finding comprehensive information regarding this complex process is to study the transcriptional activity of the genes involved. Furthermore, by controlling variables such as cultivar differences and environment we would be able to gather important information on the transcriptional control of these genes.

The number of transcriptional studies in plants has increased with the development of functional genomics and the completion of *Arabidopsis* genome sequencing. According to Hieter and Boguski (1997), functional genomics refers to the development and application of global experimental approaches to assess gene function. However under this scheme, the researcher aims to study a larger number of genes, generating transcriptional gene profiles of cells, tissues or any sample under determinate conditions.

In the development of functional genomics, two techniques have played an important role: Microarrays and Real-Time PCR. Microarrays is a technique that allows the analysis of transcriptional activity of a large number of known and unknown genes in a sample under determined conditions (Schena et al., 1995). Meanwhile, Real-Time PCR is a technique that allows the quantification of the transcriptional activity of genes

previously known. Thus, microarrays have allowed the processing of gene expression profiles of thousands of genes although with low quantification accuracy, whereas Real-Time PCR has allowed the quantification of gene expression of fewer genes with more accuracy. However in the last years, with the development of specialyzed software like GenEx® (see section 2.4.3) and with the help of multivariable analysis, the analysis of larger number of genes through Real-Time PCR has been possible. This has allowed a more precise and accurate profiling of gene expression of complex biochemical pathways when many genes are involved (Kubista et al., 2006; Ståhlberg et al., 2008).

In cassava, microarrays have been used to identify genes involved in post-harvest physiological deterioration (Reilly et al., 2007) or to understand the response of genes for *Xanthomonas axonopodis* pv. *manihotis* infection (Lopez et al., 2005). Meanwhile Real-Time PCR has been used to quantify the transcriptional activity of some genes involved in carotene synthesis (Arango, 2005) or for transgene quantification (Beltran et al., 2008). In the field of cyanogenic glycosides, the present study is the first to use Real Time PCR for the analysis of genes involved and is the first to analyze the transcriptional activity of all the genes as a complete set.

The next section will describe the technique used in the present research: Real-Time PCR.

2.4 Real Time PCR

One of the more common objectives in molecular genetics is to determine and/or quantify the presence of transcriptional activity of several genes in samples, tissues or organs. To achieve this objective in practice the researchers isolate total RNA and subsequently identify and/or quantify the target molecule through a specific technique. For years this purpose was achieved through the utilization of techniques such as Northern blot hybridizations, RNA *in situ* hybridizations, and RNase Protection Assays, which are commonly characterized as being highly complex, labor intensive and having comparatively low sensitivity (Bustin, 2000). Nevertheless, the transcriptional studies improved with the combination of Reverse Transcriptase (cDNA synthesis) and the PCR (Polymerase Chain Reaction) (Heid et al., 1996*)* methodologies, which are more sensitive.

The cDNA synthesis and PCR amplification could be performed in one step (in the same tube) or in two steps (first the cDNA synthesis, followed by the PCR reaction in another tube). The use of two steps is more common, and helps to avoid problems related with cDNA synthesis efficiency (Wong and Medrano, 2005). However, independent of the cDNA method used, two strategies have been developed with the objective of quantifying and comparing amounts of mRNA among samples: semiquantitative PCR and competitive PCR. In the semiquantitative PCR method a housekeeping gene is used as a reference. Here, the same cDNA amount is used to amplify the target gene and the housekeeping gene in separate reactions. The results are observed in agarose gel electrophoresis leading to the qualitative comparison of the band intensities from the target gene and the housekeeping gene. This methodology was used by Siritunga and Sayre (2002) and Santana et al. (2002) with the analysis of CYP79D1/D2 genes and

linamarase using SBEII (Starch branching enzyme) and GADPH (Glyceraldehyde diphosphate dehydrogenase) as housekeeping genes, respectively. On the other hand, in competitive PCR, a known amount of a DNA fragment (competitor) is added to the sample. This competitor must contain sequences for the same primers used to amplify the target. When the target DNA and competitor are amplified together, both templates will compete for the same set of primers. The ratio of the amounts of the two amplified products reflects the ratio of the amounts of the target DNA and competitor (Takara, 1999).

However accurate mRNA quantitation with the combination of transcriptase and PCR is difficult with normal PCR because each reaction has their own kinetic (see section 2.4.1). In theory we want a direct relationship between the total nucleic acid concentration used as template and the band intensity analyzed in the agarose gel after the PCR amplification. However the amount of amplified product at the end of the reaction does not necessarily reflect the amount of template initially present (Takara, 1999) because the PCR components (primers, dNTPS, Taq) interact differently in each reaction throughout the cycles. Only in the first cycles of a PCR we can observe a direct relationship between the original amount of template and the product amplified (see section 2.4.1). Thus, with the objective to study these cycles, Higushi *et al*. (1993) developed Real-Time PCR as a technique of collecting data throughout the PCR process as it occurs. This combined the amplification and detection into a single step using fluorescence (fluorochromes like SYBR Green, see section 2.4.1) in the reaction and a special thermocycler with fluorescence detection video camera incorporated. In general, Real-Time PCR works like conventional PCR, but the presence of fluorochromes and the accomplishment of quantitative analysis in the cycles of PCR

without agarose gel electrophoresis give numerical information that can be evaluated using specialyzed software packages.

Real-time PCR assays are 10,000- to 100,000-fold more sensitive than RNase protection assays, 1000-fold more sensitive than dot blot hybridization, and can even detect a single copy of a specific transcript. Real-Time PCR can also discriminate between messenger RNAs (mRNAs) with almost identical sequences, requires much less RNA template than other methods of gene expression analysis, and can be relatively high-throughput given the proper equipment (Wong and Medrano, 2005). For this reason, Real-Time PCR is one of the enabling technologies of the genomic age and has become the method of choice for the detection of mRNA (Bustin, 2000). The major disadvantage to Real-Time PCR is that it requires expensive equipment and reagents. In addition, due to its extremely high sensitivity, sound experimental design and an in-depth understanding of normalization techniques are imperative for accurate conclusions (Wong and Medrano, 2005).

2.4.1 PCR Kinetics

Real-Time PCR can utilize a great variety of fluorochromes but the most popular is SYBR® Green I. This is a dye that is intercalated only in double stranded DNA which, when excited, emits fluorescence. Thus, when the PCR product is being exponentially produced, an increase in the fluorescence through each cycle of the reaction can be detected (Bustin, 2000)[3]. The Figure 4 shows an amplification curves obtained with a

[3] Generally, the SYBR® Green is used together with a passive dye that provides an internal fluorescence reference to which the reporter dye (SYBR Green) signal can be normalized during data analysis. The most popular passive reference dye is Rox ®, which corrects fluorescence fluctuations caused by changes in concentration or in volume (Applied Biosystem, 2005).

Real-Time PCR machine using SYBR green as the fluorochrome. From this figure we can observe that the PCR have four phases: 1. phase of background where the amount of double stranded DNA with incorporate dye is negligible and produce a fluorescence signal close to the background levels; 2. phase with exponential amplification where the target amount is for the first time above of the background. At this point the reaction is very specific and exact doubling of the product is accumulated; 3. Linear phase where the reaction components are being consumed and the reagents are depleted; 4. Plateau or end point where the reaction is stopped and no more products are being made (Pfaffl, 2004). In this way, considering our objective of identifying and accurately quantifying target molecules from the total RNA sample we have to acquire information from the exponential phase because in this step the amount of amplified target is almost directly proportional to the original sample amount.

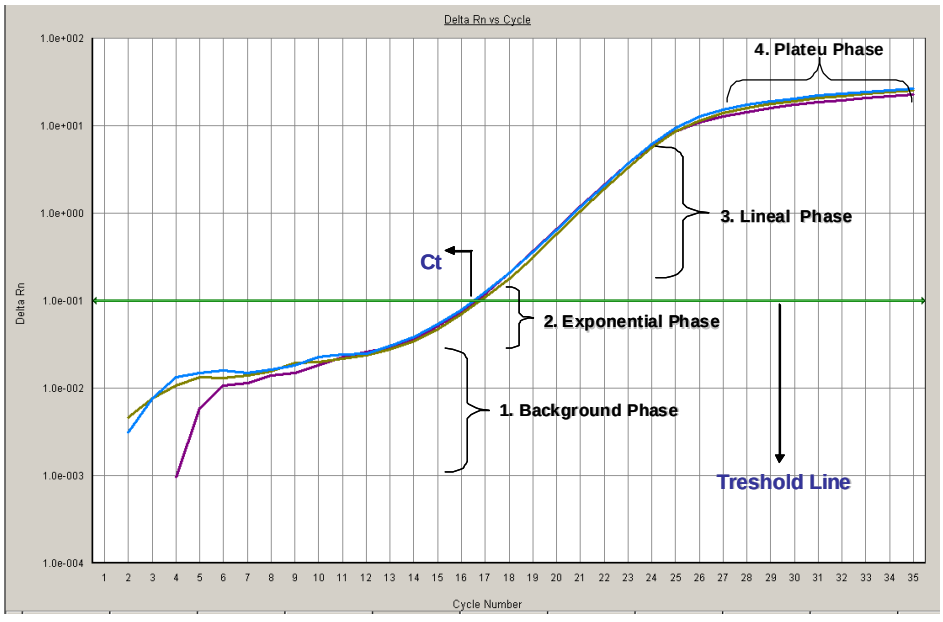


Figure 4: Phases in Real Time PCR amplification.
Three curves from technical replicates are shown. The four phases are illustrated, together with the threshold line and Ct.

Thus, in one PCR reaction the rate of target molecules produced change with the cycles and it is only in the exponential phase that the maximum rate of molecules are produced since the PCR efficiency is maximum. PCR Efficiency refers to the measurement of the relative increment of product in one PCR step (Raeymakers, 2000). Hence, according to Schnell and Mendoza (1997) the product formed after *n* successive cycles, *T*n, should ideally result in the geometric accumulation if the efficiency is maximum such as:

$$T_n = 2^n T_0. \qquad \textbf{\textit{Equation 1}}$$

Where T_0 is the initial target nucleic acid amount.

However, in practice, it is well understood that the reaction efficiency is not the same because it can change according to the kinds of primers, nucleic acid and reactives used. For this reason we can established an equation as:

$$T_n = (1+ E)^n \, . \, T_0 \qquad \textbf{\textit{Equation 2}}$$

Where *E* is the PCR amplification efficiency.

Several methodologies have been used for efficiency calculation, however, the more traditional strategy is to use a standard curve (Wong and Medrano, 2005) (see section 3.4.14). Generally in Real-Time PCR it is always recommended that in the subsequent analysis (especially in relative quantification strategy, see 2.4.2.2 section) to calculate the primer efficiency with the objective of making a PCR efficiency correction (Pfaff, 2004).

2.4.2 Data Acquisition and Quantification Strategies in Real-Time PCR

In Real-Time PCR we are looking for a time point (or fractional PCR cycle) where the target amplification is first detected. This value is in the exponential phase and is usually referred to as Cycle Threshold (Ct) or Crossing Point (Cp): the time at which fluorescence intensity is greater than background fluorescence (Wong and Medrano, 2005) (see figure 3). Thus, a high quantity of target nucleic acid in the starting material means a faster and significant increase in fluorescence yielding a lower Ct value. For Ct determination various acquisition methodologies are possible: The Threshold Cycle Method, The Fit Point Method and The Second Derivate Maximum Method (see Rasmussen, 2001, for more details of theses methodologies). However, the most traditional methodology used is the Threshold Method, which allows the determination of the Ct in the exponential phase making the Ct a common threshold to compare samples. Thus, in the intersection among the threshold and the fluorescence reaction the Ct value is obtained (see figure 3). It has been established that the threshold is usually 10 times the standard deviation of the base line (Pfaffl, 2004) but in the practice it does not matter very much where the threshold is set as long as it is the same for all the reactions that is being compared (Rasmussen, 2001).

The Ct is the raw data from a Real-Time PCR run. Nevertheless, the objective of a Real-Time PCR reaction is to quantify the amount of a target molecule in a sample, therefore it needs strategies that allow us to move from the values obtained at Ct to the actual target amount present. For this, two quantification strategies have been developed: the absolute and relative quantification (Pfaffl, 2004). As its name implies, the absolute quantification gives absolute values of the activity of a target gene in a sample, while the

relative quantification gives values of transcriptional activity in a sample in relation to another sample or an average. Although each strategy has its own characteristics, both work on the same mathematical principle derived from *equation 2* now expressed under Real-Time PCR concepts:

$$T_0 = T_n/(1+ E)^{ct} \qquad \textbf{\textit{Equation 3}}$$

Thus, in a transcriptional study we are looking for T_0 and Real-Time PCR helps to identify T_n and the efficiency depending of the acquisition methodologies and quantification strategy used (for more mathematical details and the different models used see Livak and Schmittgen, 2001; Rutledge and Coté, 2004; Pfaff et al., 2002; Gentle et al., 2001; Liu and Saint, 2002).

2.4.2.1 Absolute quantification

Absolute quantification uses serially diluted standards (recombinant plasmid DNA, genomic DNA, retrotranscriptase PCR product and commercially synthesized large oligonucleotides) of known concentrations to generate a standard curve from which the amount of a targeted gene present in an experimental sample is estimated. The standard curve produces a linear relationship between Ct and initial amounts of total DNA, RNA or cDNA allowing the determination of the unknown concentration based on their Ct values (Wong and Medrano, 2005). However this method assumes all standards and samples have approximatly equal amplification efficiencies and this is not always true (Souzae et al., 1996). Additionally, stability and reproducibility in kinetic RT-PCR

depend on the type of standard used and depend strongly on “good laboratory practices” (Revised by Pfaffl, 2004).

2.4.2.2 Relative quantification.

Relative quantification relates the PCR signal of the target transcript as a treatment group to that of another sample which is an unrelated control (Livak and Schmittgen, 2001). This strategy is adequate for investigating physiological changes in gene expression levels (Pfaffl, 2004); however this kind of comparison requires normalization to compensate for differences in the amount of biological material in the tested samples (Kubista et al., 2006).

Different normalization strategies have been developed in relative quantification, such as to normalize to total RNA amount, to ribosomal RNA (rRNA), to externally added RNA standard or internal references genes like housekeeping genes. However the latter is the more popular one (Kubista et al., 2006). The researcher normalizes the target gene to a housekeeping gene, which must be expressed at a constant level among different tissues of an organism, at all stages of development, and should be unaffected by the experimental treatments (Bustin, 2000). However finding a perfect housekeeping gene is difficult because all the genes are regulated under some conditions (Bustin, 2000; Kubista et al., 2006). For this purpose software like GeNorm has been developed to find the optimal reference genes for each experiment (Kubista et al., 2006).

There are several mathematical models to calculate the mean of normalized gene expression from relative quantification assays: Relative quantification using standard

curve[4], comparative $C_t(2^{-\Delta\Delta ct})$ method, Pfaff method (also named REST method), Q-Gene method, Gentle et al. method, Liu and Saint method, DART-PCR method (all of them are described in Wong and Medrano 2005) and recently the GenEx method (Kubista et al., 2004; Kubista and Sindelka, 2007) which is discuss in more detail in the section 2.4.3. This is the strategy used in the present study. Once the results from relative quantification are produced, it is analyzed statistically following the corresponding experimental design and statistical considerations.

2.4.3 GenEx® Methodology

GenEx ® is a program developed by ***Multid Company***, Sweden, for the analysis of real-time PCR expression profiling data. This means that this software was designed especially for the analysis of complex biological processes where a large number of genes are involved. Thus, GenEx® makes simultaneous analysis of Real-Time PCR data from a large number of genes, using multivariable analysis tools such as PCA (Principal Component Analysis), SOM (Self Organized Maps) and Cluster Analysis. However the software can also be used for the analysis of one gene or few samples using univariable t-test.

The Real-Time PCR results are introduced into GenEx through specific data matrices, which depend on the experimental design. Thus, usually the data are organized in columns for each gene and rows for each experimental sample, indicanding biological repetitions and other technical repetitions (see below). GenEx ® generates quantities relatives to a Ct average or to a specific Ct from one sample selected arbitrary.

[4] Although this methodology uses standard curve for the calculation of RNA present in each sample, the results are expressed relative to a single calibrator sample.

Nevertheless, prior to this relativization, a data-preprocessing protocol is necessary; in which the Ct from Real-Time PCR is transformed with the objective of finding accurate results. This pre-processing and generation of relative quantities is explained in the GenEx Manual (http://www.multid.se/genex/web_manual/GenEx423_manual.pdf) and by Kubista and Sindelka (2007):

Correct for Off-scale Measurements: Usually in samples with low mRNA, the corresponding Ct value is without meaning, generating off-scale measurements. In this samples Ct values are not produced or false Ct values are generated due to the formation of aberrant products, such as primer dimer. In this case the data need to be "cleaned" from the matrix of data and replaced with other Ct values, which is the Limit of Detection (LOD) + 1 for a truly positive sample. This corresponds to assigning a concentration that is half of LOD to the off-scale samples.

Efficiency Correction: Previously, the efficiency for each primer in the study must be calculated. GenEx makes the efficiency correction according to:

$$Ct_{E=100\%} = Ct_E \frac{Log\ (1 + E)}{Log\ (2)}$$

Where:
E = Efficiency

Equation 4

Variation between Runs: This is an optional correction when all samples cannot be run in one plate. In this way an identical sample named interplate calibrator (IC) is put in all runs.

$$Ct_{plate\ norm} = Ct_r - Ct_r^{IC} + \frac{1}{\mathbf{m}} \sum_{t=1}^{m} CT_{IC}$$

Where:
m = Number of runs.

Equation 5

Sample Amount: This is an optional correction when we have samples based on different starting amounts (generally RNA).

$$Ct_{conc=1} = Ct_{conc} - log_2 (conc)$$

Equation 6

QPCR Repeats: Because one of the principal problems of the Real Time PCR is the reproducibility (Pfaff, 2004), it is always recommended to make technical replicates (repetitions of the same reaction) which are averaged by GenEx®

$$Ct_{QPCR\_average} = \frac{1}{n} \sum_{i=1}^{n} CT_{QPCR\_repeats}$$

Where:
n = Number of technical repelicates.

Equation 7

Reference Genes (RG): The results from the target gene are normalized with one or several housekeeping genes, also named reference gene.

$$Ct_{,\,norm} = Ct - \frac{1}{n}\sum_{i=1}^{n} CT_{RG}$$

Where:
n = Number of Reference Genes

Equation 8

Retrotranscriptase Technical Repetition (RT). It has been demonstrated that one of the reason for the low Real-Time reproducibility is the variation in cDNA synthesis (Ståhlberg et al., 2004). For this reason the use of replicates in the experiment is recommended. However this is an optional procedure for the analysis.

$$CT_{RT\_average} = \frac{1}{n}\sum_{i=1}^{n} CT_{RT\_repeats}$$

Where:
n = Number of retrotranscriptase repelicates

Equation 9

Relative Quantities (RQ)

As previously explained, GenEx is based on relative quantification. GenEx offers different ways of relativization: 1. Relativization to the Ct mean from the samples compared. 2. Relativization to the maximum Ct from the samples compared. 3. Relativization to the minimum Ct from the samples compared. 4. Relativization to a special sample chosen by the user.

$$RQ = 2^{CT\ ref\ -\ CT}$$

Where:
Ct ref = Ct min
Ct Max
Ct mean
Ct sample

Equation 10

Log Scale

Generally the Real-Time PCR data has a Normal Distribution if transformed to Log Scale (Fold Differences = FD). This transformation is needed for the subsequent statistical analysis.

$$FD = log_2(RQ)$$

Equation 11

Auto-scaled Data (AS)

This is an option when multivariate analysis will be used. With this transformation all the genes has the same weight.

$$FD_{AS} = (FD - \overline{FD}) / SD \qquad \textit{Equation 12}$$

Where:

$\overline{FD}$ = *Mean of data transformed to Fold Differences*
SD: Standard Desviation of data transformed to Fold Differences

It is important to highlight that GenEx® is a flexible software and thus some of the above mentioned pre-processing steps could be used in a different order depending on the study. Once the Ct data from a sample are pre-processed with GenEx, the subsequent statistical comparison among samples can be done depending on the experimental design.

CHAPTER THREE
MATERIALS AND METHODS

3.1 Plant Material

The plants used in this study are maintained and propagated in the Plant Biotechnology Laboratory of the University of Puerto Rico at Mayagüez. Three cassava cultivars were used: 1. the sweet cassava cultivar (low cyanide amount) Mcol 2215 also named Venezolana, very popular among North Colombian farmers. This is a high starch cultivar susceptible to pest and insects (http://www.ciat.cgiar.org/yuca/pdf/nuevas_variedades_yuca.pdf). 2. The bitter-sweet cultivar 60444 also named Nig 11. This is an African cultivar which seems to be protected from attack of cassava hornworms (Chavarriaga et al., 2003). 3. The bitter cultivar Mtai 16 whose name refers to Thailand, where the cultivar was introduced. Table 1 summarizes the cyanogen characteristics of those cultivars.

Table 1. Cyanogenic Characteristic of the cassava cultivars used in this study.
(Source, Teresa Sanchez and Paul Chavarriaga., CIAT. Personal Communication)

Cultivar	Category	Total cyanogens of 8-month old cassava roots dry base (ppm)
Mcol 2215	Sweet	142
60444	Bitter-Sweet	182
Mtai 16	Bitter	569

3.2 Tissue culture

All the experiments developed in this study were done with *in vitro* plants**.** All plants were grown in 4E liquid or solid media, using programmed incubators to 12h/day photoperiod (5 µmol photons $m^{-2}s^{-1}$) at 28^0C.

3.2.1 Culture Media for Growth and Maintenance of *in vitro* Plants

The growth media used were changed according to the objectives of the specific experiment. The pH was adjusted between 5.7 and 5.8 using KOH before adding agar (0.25% w/v) if required. Media was made up to 1L and sterilized by autoclaving at 15 psi, 15 minutes at 121^{o}C. The different cassava growth media used were:

4E Medium (Liquid or Solid) (Murashighe and Skoog, 1962)

	per Litre:
MS salts (Murashige & Skoog 1962)	4.3 g/L
Thyamine	1 ppm
M-inositol	100 ppm
Sucrose	2%
6-benzilaminopurina (BAP)	0.04 ppm
naftalenacetic acid (NAA)	0.025 ppm
gibberellin (GA_3)	0.05 ppm
$CuSO_4$	2 µM

4E Liquid Medium with 40 mM nitrate and 20 mM reduced nitrogen (Standard Condition).

	Per Liter
MS salts without NH_4NO_3 but with NO_3 from Caisson Laboratories	2.68 g/L
NH_4NO_3	40 mM
KNO_3	20 mM
Thyamine	1 ppm
M-inositol	100 ppm
Sucrose	2%
6-benzilaminopurina (BAP)	0.04 ppm
Naftalenacetic acid (NAA)	0.025 ppm
Gibberellin (GA_3)	0.05 ppm
$CuSO_4$	2 µM

4E Liquid Medium with 50 mM nitrate and 10 mM reduced nitrogen (intermediate NH_4 Condition)

	Per Liter
MS salts without NH_4NO_3 but with NO_3 from Caisson Laboratories	2.68 g/L
NH_4NO_3	10 mM
KNO_3	20 mM
Thyamine	1 ppm
M-inositol	100 ppm
Sucrose	2%
6-benzilaminopurina (BAP)	0.04 ppm
Naftalenacetic acid (NAA)	0.025 ppm
Gibberellin (GA_3)	0.05 ppm
$CuSO_4$	2 µM

4E Liquid Medium with 60mM nitrate without reduced nitrogen (without NH_3)

	Per Liter
MS salts without NH_4NO_3 but with NO_3 from Caisson Laboratories	2.68 g/L
KNO_3	40 mM
Thyamine	1 ppm
M-inositol	100 ppm
Sucrose	2%
6-benzilaminopurina (BAP)	0.04 ppm
naftalenacetic acid (NAA)	0.025 ppm
gibberellin (GA_3)	0.05 ppm
$CuSO_4$	2 µM

3.3 Molecular Methods

3.3.1 Primer Design

The genes studied were CYP79D1/D2, linamarase, HNL and β-CAS as well as 18S rRNA (housekeeping gene). Figure 3 illustrates the specific metabolic steps of the genes involved in the cyanogenic glycoside pathway of cassava. The primer set CYP79D is a

consensus primer complementary to CYP79D1 and CYP79D2 genes (see appendix A). This is due to the fact that both genes are 85% identical and both encode for cassava P450 involved in the same linamarin synthesis step (Andersen et al. 2000). Furthermore, Jørgensen et al. (2005) has shown that the localization of the transcript is similar for both. In the case of linamarase analysis, two kinds of linamarase primers were used.

Each primer was designed according to Table 2 using *Primer Quest*^{SM} software from IDT (Integrated DNA Technologies, Inc) and was checked for their compliance to the recommendations of the ABI guidelines for Real-Time PCR Primers and amplicons (Real-Time PCR handbook (http://www.uic.edu/depts/rrc/cgf/realtime/primer.html). Subsequently, the primers generated and the original GenBank sequences were introduced into a BLAST program verifying non homologies among the primers and other reported cassava sequences.

Table 2. Genes studied and their respective primer sequences utilized

Gene		Primer Sequence	Gen Bank Accesion Number	Size Amplicon (BP)
CYP79D1/D2	Forward	5'- CTCCATGGCCACTCATCGGAAA -3'	AF140613	86
	Reverse	5'- TGTCCTTCATGAGTTGGTGAATCC -3'	AF140614	
Linamarase (A)	Forward	5'-CTCAAGCACTGCAGGACAAA-3'	S35175	158
	Reverse	5'-ATCATGGGCAAATCCAACAT-3		
Linamarase (B)	Forward	5'-CCTAACAAGGTATCCCAAGAAATCG-3'	S35175	111
	Reverse	5'-CCA ACC TTC CTT GAA TCC TTT GAT G-3'		
HNL	Forward	5'-GCTTTTGGAGTCGTTTCCTG-3'	Z 29091	228
	Reverse	5'-CCGTAACCTTTTTCGGTGAA-3'		
β-CAS	Forward	5'-TCCACTGCGAGAGAGAGCTTAGAG-3'	EU350583	99
	Reverse	5'-GCCATTGATGTAAGAGTAGCCATTG-3'		
18S rRNA	Forward	5'-ATGATAACTCGACGGATCGC-3	AB233568	169
	Reverse	5'-CTTGGATGTGGTAGCCGTTT-3'		

3.3.2 DNA Isolation

DNA isolation was completed according to Dellaporta et al. (1983), with the following modifications: Leaves from *in-vitro* plants were collected and immediately frozen in liquid nitrogen. The tissues were stored at -80^{0}C until their use. The material was ground thoroughly with a mortar and pestle in liquid nitrogen. About 0.3 g of the resulting powder was mixed with 1 ml of pre-heated (65^{0}C) Isolation Buffer (see appendix B). The samples were incubated at 65ºC in a water bath for 45 min with mixing every 5 min. This was followed by the addition of 400 µL of chilled 5M potassium acetate and the incubation of the sample on ice for 30 minutes. The samples were centrifuged twice, once at 1500 rcf rpm for 10 minutes followed by 9300 rcf for 5 minutes; in each case the supernatant was saved. To the resulting supernatant 2X volumes of chilled isopropanol and 1/10 of volume of sodium acetate were added and incubated at -20^{0}C for 12 hours. The samples were centrifuged to 13400 rpm for 5 min and the pellets were washed with 70% ethanol. After the evaporation of the ethanol the sample was re-suspended in 100 µL off TE (Tris-EDTA) with RNase which was activated by incubation at 37^{0}C for 30min. The DNA sample quality was analyzed in an agarose gel and a 1:100 dilution of a sample was quantified in GENESYS 10 UV spectrophotometer by O.D measurements at 260 nm. The samples were quantified in GEANESYS 10 UV spectrophotometer (Thermo, Fisher Scientific INC, Madison, WS, USA) at 260 nM and stored at -20^{0}C.

3.3.3 RNA Isolation

Leaves and roots were collected from *in vitro* cultures and immediately frozen in liquid nitrogen. The tissues were stored at -80^{0}C until their use. The RNA isolations were

made following RNeasy® Mini Kit protocol from Qiagen (Qiagen®, Valencia, CA, USA) and DNA contaminant was eliminated using RNAse-Free DNAse from Qiagen (Qiagen®, Valencia, CA, USA). The sample quality was analyzed in an agarose gel (0.8%). The RNA was quantified (see section 3.3.6) and finally protected with 1 unit of RNasin® Ribonuclease inhibitor (Promega, USA). The samples were quantified in GEANESYS 10 UV spectrophotometer (Thermo, Fisher Scientific INC, Madison, WS, USA) at 260 nM and stored at -80^{0}C.

3.3.4 Electrophoresis of Nucleic Acids on agarose Gels

Agarose gels were prepared in TAE (Tris, Acetic Acid and EDTA) buffer containing ethidium bromide at a final concentration of 0.5µg/mL. The agarose concentration (w/v) changed according with the specific objective: gels for DNA and RNA visualization were made at 0.8%; gels for PCR amplicon visualization were made at 1.5- 2%. Electrophoresis was carried out 4-8 V/cm for 1-2 hour. Nucleic acid bands were visualized and documented under UV light using BioDoct-IT^{TM} (UVP, Upland, California USA) Imaging System. In the specific case of RNA gels, the TAE buffer was made from DEPC treated water (Diethilpirocarbonated water) and the electrophoresis chambers was cleaned with RNase AWAY^{TM} from Invitrogen ® (San Diego, California, USA)

3.3.5 cDNA Syntesis

The cDNA synthesis was completed using a two step transcriptional analysis. One µg of total RNA was used for the cDNA synthesis following the manufacturers instructions for Omniscript RT ® kit (Qiagen Inc, Valencia, CA) using 1 µM of Oligo $(dT)_{12\text{-}18}$ and 12.5

ng/μL of Random Primers. The mixture was incubated for 2 hours at 37^0C in an Eppendorf Mastercycler® thermocycler. The samples were quantified in GEANESYS 10 UV spectrophotometer (Thermo, Fisher Scientific INC, Madison, WS, USA) at 260 nM and stored at -80^0C

3.3.6 Conventional PCR

Conventional PCRs were prepared with 1 μg of cDNA using specific primers for each gene (see Table 2). The reaction conditions and amplification programs are described in Table 3. All the reactions were run in an Eppendorf Mastercycler® thermocycler (Eppendorf, Hamburg, Germany)

3.3.7 Isolation of PCR fragments

Products from conventional PCR of the different genes were run in low-melting agarose gels and the DNA bands were cut with the help of a scalpel and purified with QIAEX II from QIAGEN® (Qiagen®, Valencia, CA, USA) following recommendations from the manufacter.

Table 3: Conventional PCR conditions. All the reaction was completed at 25 µL.

Gene	PCR Reaction Condition [Final Concentration]		Cycling Parameters
CYP79D1/D2	Buffer 10X	[1X]	1. 96^{0}C 5min
	$MgCl_2$	[2.5mM]	2. 96^{0}C 1min
	Primer F	[0.4µM]	3. 60^{0}C 45sg
	Primer R	[0.4µM]	4. 72^{0}C 45sg
	dNTPS	[0.2mM]	30 cycles of steps 2-4
	Taq	1Unit	5. 72^{0}C 5min
Linamarase (A)	Buffer 10X	[1X]	1. 96^{0}C 5min
	$MgCl_2$	[2.5mM]	2. 96^{0}C 1min
	Primer F	[0.4µM]	3. 60^{0}C 5min
	Primer R	[0.4µM]	4. 72^{0}C 5min
	dNTPS	[0.2mM]	35 cycles of steps 2-4
	Taq	1Unit	5. 72^{0}C 5min
Linamarase (B)	Buffer 10X	[1X]	1. 96^{0}C 5min
	$MgCl_2$	[2.5mM]	2. 96^{0}C 1min
	Primer F	[0.4µM]	3. 56^{0}C 1min
	Primer R	[0.4µM]	4. 72^{0}C 1min
	dNTPS	[0.2mM]	35 cycles of steps 2-4
	Taq	1Unit	5. 72^{0}C 5min
HNL	Buffer 10X	[1X]	1. 96^{0}C 5min
	$MgCl_2$	[2.5mM]	2. 96^{0}C 1min
	Primer F	[0.2µM]	3. 58^{0}C 1min
	Primer R	[0.2µM]	4. 72^{0}C 1min
	dNTPS	[0.2mM]	35 cycles of steps 2-4
	Taq	1Unit	5. 72^{0}C 5min
β-CAS	Buffer 10X	[1X]	1. 96^{0}C 5min
	$MgCl_2$	[2.5mM]	2. 96^{0}C 1min
	Primer F	[0.4µM]	3. 56^{0}C 1min
	Primer R	[0.4µM]	4. 72^{0}C 1min
	dNTPS	[0.2mM]	35 cycles of steps 2-4
	Taq	1Unit	5. 72^{0}C 5min
18S rRNA	Buffer 10X	[1X]	1. 96^{0}C 5min
	$MgCl_2$	[2.5mM]	2. 96^{0}C 1min
	Primer F	[0.2µM]	3. 55^{0}C 5min
	Primer R	[0.2µM]	4. 72^{0}C 1min
	dNTPS	[0.2mM]	30 cycles of steps 2-4
	Taq	1Unit	5. 72^{0}C 5min

3.3.8 Cloning of PCR Product

Products from conventional PCR of less than 110 bp (CYP79D1/D2, Linamarase B and β-CAS) were cloned in the pCR 2.1 vector using the TA Cloning® kit of Invitrogen™ (Carlsband, CA, USA). The resultanting constructs were transformed in *E.coli* DH5α

cells and transformants were detected in LB agar supplemented with 100 µg/ml of ampicilin.

3.3.9 Plasmid Purification

The colonies of transformants were grown overnight in liquid LB media (see appendix B) at 37^0C. Bacterial cells were harvested by centrifugation at 16100 rcf for 3 min at room temperature. The bacterial pellet was used for plasmid purification using QIAprep® from Qiagen (Valencia, CA, USA).

3.3.10 Sequencing

PCR products (from Linamarase A, HNL and 18S rRNA) and cloned amplicons (CYP79D1/D2, Linamarase B and β-CAS) were sequenced by Nevada Genomics Center (University of Nevada). The PCR products were sequenced with gene specific primers while the plasmids were sequenced with the vector specific primers: T7 primer (5'-TAATACGACTCACTATAGGG-3') and M13 primer (5'-GAGCGGATAACAATTTCACACAG-3')

3.3.11 Test for the absence of genomic DNA contamination in RNA samples used for cDNA amplification.

All the cDNAs were evaluated with HNL primers using conventional PCR for the presence of genomic DNA. HNL primers were designed flanking an intron, thus cDNA without DNA contamination only amplified the exon region. The comparisons were performed on an agarose gel.

3.3.12 Real-Time PCR

Real-Time PCR reactions were prepared with aproximatelly 200 ng (normally the synthesized cDNA had to be diluted 1:8 and 2 uL of the dilution used) of cDNA using primers described in table 2. The reaction was completed using *DyNAmo SYBR® Green qPCR kit* (FINNZYMES Keilaranta. Finland) following the instruction manual and ROX® (INVITROGEN Carlsband, CA, USA) as internal reference. Three technical repetitions (repetition of the same sample) for each sample were made according to the reaction conditions and programs of Table 4. All the reactions were run in the 7300 Real-Time PCR Thermocycler (Applied Biosystems Foster City, CA, USA) which automatically makes the dissociation curve.

3.3.13 Primer Efficiency Calculation Through of Standard Curve

The efficiencies of each primer were calculated for subsequent transcriptional relative quantification. With an arbitrary amount of the corresponding PCR product isolated (section 3.4.9) or plasmid (section 3.4.11), 10-fold serial dilutions were made (five dilutions). Those dilutions were used as templates in Real-Time PCR reactions using three technical repetitions. The Ct from the samples were inverse to the DNA amount allowing the establishment of a standard curvewhich was generated by SDS Software (version 1.3.1) incorporated to the 7300 Real-Time PCR Thermocycler. The slope of the curve was used for the efficiency calculation according to equation 13:

$$E = (10^{(-1/slope)}) - 1 \qquad \textbf{\textit{Equation 13}}$$

Table 4: Real Time PCR conditions. All the reaction was completed at 20 µL.

Gene	Reaction Real Time Conditions [FinalConcentration]		Real-Time PCR Program
CYP79D1/D2	Master Mix 2x	[1X]	1. 94^{0}C 5min
	$MgCl_2$	[0.63mM]	2. 94^{0}C 20 sec
	Primer F	[0.05µM]	3. 58^{0}C 1min:15sg lecture
	Primer R	[0.05µM]	Repetition (2-3) x 35
	Rox	[1X]	4. Automatic Dissociation Curve
Linamarase (A)	Master Mix 2x	[1X]	1. 94^{0}C 5min
	Primer F	[0.1µM]	2. 94^{0}C 20 sec
	Primer R	[0.1µM]	3. 62^{0}C 1min lecture
	Rox	[1X]	Repetition (2-3) x 35
			4. Automatic Dissociation Curve
Linamarase (B)	Master Mix 2x	[1X]	1. 94^{0}C 5min
	$MgCl_2$	[0.63mM]	2. 94^{0}C 15 sec
	BSA	[0.4mg/ml]	3. 59^{0}C 45 sec lecture
	Primer F	[0.1µM]	Repetition (2-3) x 35
	Primer R	[0.1µM]	4. Automatic Dissociation Curve
	Rox	[1X]	
HNL	Master Mix 2x	[1X]	1. 94^{0}C 5min
	Primer F	[0.1µM]	2. 94^{0}C 15 sec
	Primer R	[0.1µM]	3. 62^{0}C 1min lecture
	Rox	[1X]	Repetition (2-3) x 35
			4. Automatic Dissociation Curve
β-CAS	Master Mix 2x	[1X]	1. 94^{0}C 5min
	Primer F	[0.05µM]	2. 94^{0}C 15 sec
	Primer R	[0.05µM]	3. 62^{0}C 1min lecture
	Rox	[1X]	Repetition (2-3) x 35
			4. Automatic Dissociation Curve
18S rRNA	Master Mix 2x	[1X]	1. 94^{0}C 5min
	Primer F	[0.15µM]	2. 94^{0}C 15 sec
	Primer R	[0.15µM]	3. 53^{0}C 35 sec lecture
	Rox	[1X]	Repetition (2-3) x 30
			4. Automatic Dissociation Curve

3.3.14 Limit of Detection (LOD) in Real Time PCR

The Limit of Detection was calculated for the genes with low transcriptional activity, where off-scale measurements are produced (see section 2.4.3). The PCR product or the plasmid quantified in the spectrophotometer (260 nM) was serially diluted using a dilution factor of 10. With the objective of simulating the conditions in a cDNA sample,

Real-Time PCR reactions were made using as template those serial dilutions plus 200 ng of cDNA which previously was demonstrated to have no-transcriptional activity of the gene being evaluated for LOD.

The LOD was reported in relation to copies/µl. Thus, the concentrations of PCR product or plasmid were transformed using the equation 14:

$$\frac{6 \times 10^{23}\ [\text{copies/mol}] \times \text{concentration}\ [\text{g/µl}]}{MW\ [\text{g/mol}]} = \text{amounts}\ [\text{copies/µl}]$$

MW = Size of plasmid or PCR fragment (bp) x 660 Daltons/bp

1 mol= $6x10^{23}$ molecules (= copies).

Equation 14

3.3.15 Artificial Sample Construction

Due to the flexivility of GenEx® software, multiple kinds of comparisson could be completed depending of the experimental design and the objectives of the research. For example, we can be interested in the trancriptional activity comparison of a gene among several samples, in this way, the data usually is relativized to the maximum Ct, minimum Ct or to a average Ct (equation 10). However sometimes we can be interested in the comparison among several genes in a sample or several samples. In this case, the relativization (equation 10) must to be completed in relation to a artificial sample containing equal amount of all amplicons (from the genes to compare) (personal recommendations from Dr. Mikael Kubista (TATAA Biocenter, Prague, Czch Republic). Those Relative Quantities are subsequently transformed to Fold Differences (Equation

11), optionally Autoscaled (Equation 12) and finally used for bar plot pictures or multivariable comparisons (Cluster Analysis, Principal Component Analysis and others).

In the present study, an artificial sample was made for the comparison among the genes. This sample was a mixture at the same concentration of all PCR products from the different genes isolated as described in section 3.3.7. This mixture was made from 40ng of the 5 genes amplified thus simulating the 200 ng of cDNA used in the biological samples.

3.4 Data Acquisition and Data Pre-Treatment

The quality of amplification from each Real-Time PCR reaction was assessed through the dissociation curves generated for the SDS Software (version 1.3.1) incorporated to the 7300 Real-Time PCR Thermocycler. According to Dr. Mikael Kubista recommendations and in order to correct for off-scale measurements, samples with dimmer primers or without amplification were eliminated from the analysis or replaced with the LOD following the criteria:

Criterion 1: If the three technical repetitions amplified clearly, no data was removed or replaced.

Criterion 2: If one technical repetition amplified with some problem it was eliminated from the data. The reported Ct for the biological sample was the average from the two remaining technical repetitions.

Criterion 3: If two technical repetitions were not reliable, these were eliminated from the data and the Ct for the remaining replicate was reported for the corresponding biological sample.

Criterion 4; if none of the three technical repetitions amplified then it was considered that the sample has very low transcriptional activity and the data is replaced with the LOD.

The Ct data were collected using the Manual Threshold Method posting the threshold in the logarithmic phase of all the samples run in a PCR. The data of all the genes were joined in different matrices depending of the kind of comparisons being conducted in the specific experiments. All the matrices were the n x 5 type where the *5* means the number of genes studied (CYP79D1/D2, linamarase, HNL, β-CAS and 18S) organized as columns and *n* means the number the comparisons done depending on the experiment and its corresponding biological replicates (3) and technical replicates (3). For example, in the comparison between 2 tissues in one variety using three biological replicates and three technical replicates the matrix is 18 x 5. Thus the following matrices were developed:

- Matrices for tissue comparison for each variety
- Matrices for cultivar comparison in each tissue
- Matrices for gene comparison and cluster analysis.
- Matrices for treatments comparison.
- Matrices for cluster analysis

Afterward the Ct matrices were introduced to GenEx Light Software for the data-preprocessing. This consisted of the following steps:

- Efficiency Correction using *equation 4*.
- qPCR repetition average using *equation 7.*
- Housekeeping correction using *equation 8.*
- Relative quantities with Ct Average from each gene or with the Ct for each gene from the artificial sample using equation *10*
- Log Scale using *equation 11*

3.5 Experimental Designs and Statistical Analysis

The data processed in GenEx from the different experiments cited in this thesis were used for the statistical analysis according to the corresponding experimental design. The data quality for ANOVA comparison was evaluated through Shapiro-Wilks Normality Test and Levene Test for Variance Homogeneity using InfoStat® *Software Estadistico* (Universidad Nacional de Córdoba, Argentina).

The following comparisons and statistical tests were completed:

- **Comparison between Tissues:** The data processed in GenEx Software from Matrices for tissue comparison for each variety were statistically compared through paired t-tests.

- **Comparison among Cultivars and treatments:** The data processed in GenEx Software from Matrices for cultivar comparison in each tissue cultivar and for treatment comparison, were statistically analyzed using ANOVA for Completely Ramdomized Design and the mean differences were assessed with contrasts.
- **Cluster Analysis:** Multivariable analyses for gene classification and sample classification for non-standardized data were done with Cluster analysis. The Euclidean distance and unweighted centroid method were used in this analysis.

The statistical significance used was of 10% (chapter five and chapter six) or 5% (chapter seven) depending of the experiment. The confidence intervals for each mean and for the mean difference were made using $1-\alpha = 90\%$ or $1-\alpha = 95\%$ (depending of the experiment) as the confidence level.

CHAPTER FOURTH
STUDIES ON THE TRANSCRIPTIONAL ACTIVITY OF GENES INVOLVED IN CYANOGENIC GLYCOSIDES PATHWAYS THROUGH SEMIQUANTITATIVE PCR

4.1 Literature Review

Although cassava cyanogenesis has been well studied in the past there is no complete transcriptional expression profile of all of the genes (at least the genes identified) involved in cyanogenic glycoside metabolism. Each transcriptional study has been done only for one gene under determinate conditions using northern blot hybridizations, reverse transcriptase conventional PCR and *in situ* PCR. White et al*.* (1998) found using northern blot analysis that the HNL gene had high transcriptional activity in leaves and reduced activity in roots and stems. Santana et al*.* (2002) focused their research on the linamarase gene leading to the use of reverse transcriptase conventional PCR that led to the finding that the linamarase gene is expressed in leaves but not in roots. With the same technology, Siritunga and Sayre (2003) detected the expression of CYP79D1 and CYP79D2 genes in leaves and roots when transgenic plants were evaluated. Jørgensen et al. (2005) found through *in situ* PCR that the expression of CYP79D1 and CYP79D2 genes take place in tissues of unfolded leaves and around laticifers cell. Table 5 is a summary of these studies. Thus, an integral and semiquantitative comparison among the genes related to cyanogenic glycosides pathways using controlled conditions is necessary. This will help to normalize the previous studies developed with different methodologies and different cultivars. In addition, the results obtained through this strategy will be used as a starting point for subsequent studies using a sensitive technique such as Real-Time PCR.

Table 5: Previous transcriptional studies with the genes involved in cyanogenic glycosides pathways

Gene	Referece	Cultivar Studied	Technique	Detection			
				Leaves	Petiole	Stems	Roots
CYP79D1/D2	Siritunga and Sayre, 2003	Mcol2215 (Low-cyanide cultivar)	Retrotrasncriptase PCR Semiquantitative	Yes	Not attempted	Not attempted	Yes
	Jørgensen *et al.*, 2005	Mcol22 (Low-cyanide cultivar)	In Tube *in Situ* Retrotranscription PCR on Tissue Sections	Yes	Yes	Not attempted	Not attempted
Linamarase	Santana *et al*., 2002	V47(High-cyanide) V56 (Low-cyanide cultivar)	Retrotrasncriptase PCR Semiquantitative	Yes	Not attempted	Not attempted	Not Detection
HNL	White *et al*., 1998	Not specified	Northern blot	Yes	Not attempted	Yes	Yes
β-CAS	No references yet	Not attempted	Not attempted	Not attempted	Not attempted	Not attempted	Not attempted

4.2 Specific Objective

To evaluate under *in vitro* conditions the transcriptional activity of genes involved in cyanogenic glycosides pathway ((CYP79D1/D2, linamarase, HNL and β-CAS) using semiquantitative reverse transcriptase PCR.

4.3 Biological Hypotheses

Four hypotheses were proposed according to previous studies.

1. The CYP79D1/D2 genes have transcriptional activity higher in leaves than roots of cassava.
2. The linamarase gene has transcriptional activity in leaves but not in roots of cassava.
3. The HNL gene has transcriptional activity higher in leaves than in roots of cassava
4. The β-CAS gene has transcriptional activity higher in roots than in leaves of cassava

4.4 Specific Methodology

Five explants from the cultivars Mcol 2215 and 60444 were propagated in magenta boxes (one magenta for cultivar) and grown for two months. Leaves and roots present in each magenta box were used for RNA isolation, followed by synthesis of cDNA. The cDNA was then used for conventional PCR of genes CYP79D1/D2, Linamarase (primers A and B), HNL, β-CAS and 18S rRNA. DNA from leaves of Mcol 2215 was isolated and used as a positive control.

4.5 Results and Discussion

Figure 5 shows the agarose gel from each reverse transcriptase PCR, and Table 6 summarizes these results. The corresponding amplicons were sequenced and the results are shown in the appendix C.

In general the comparison of transcriptional activity among tissues, the results agree with the previous enzymatic activity studies conducted and with the hypothesis proposed. All the genes with the exception of β-CAS have more activity in leaves than in roots (Elias et al., 1997; McMahon et al., 1995; Mkpongo et al., 1990; White et al., 1998). However, these results show some differences from the previous transcriptional studies conducted as summarized in table 5 and with the hypothesis number 2.

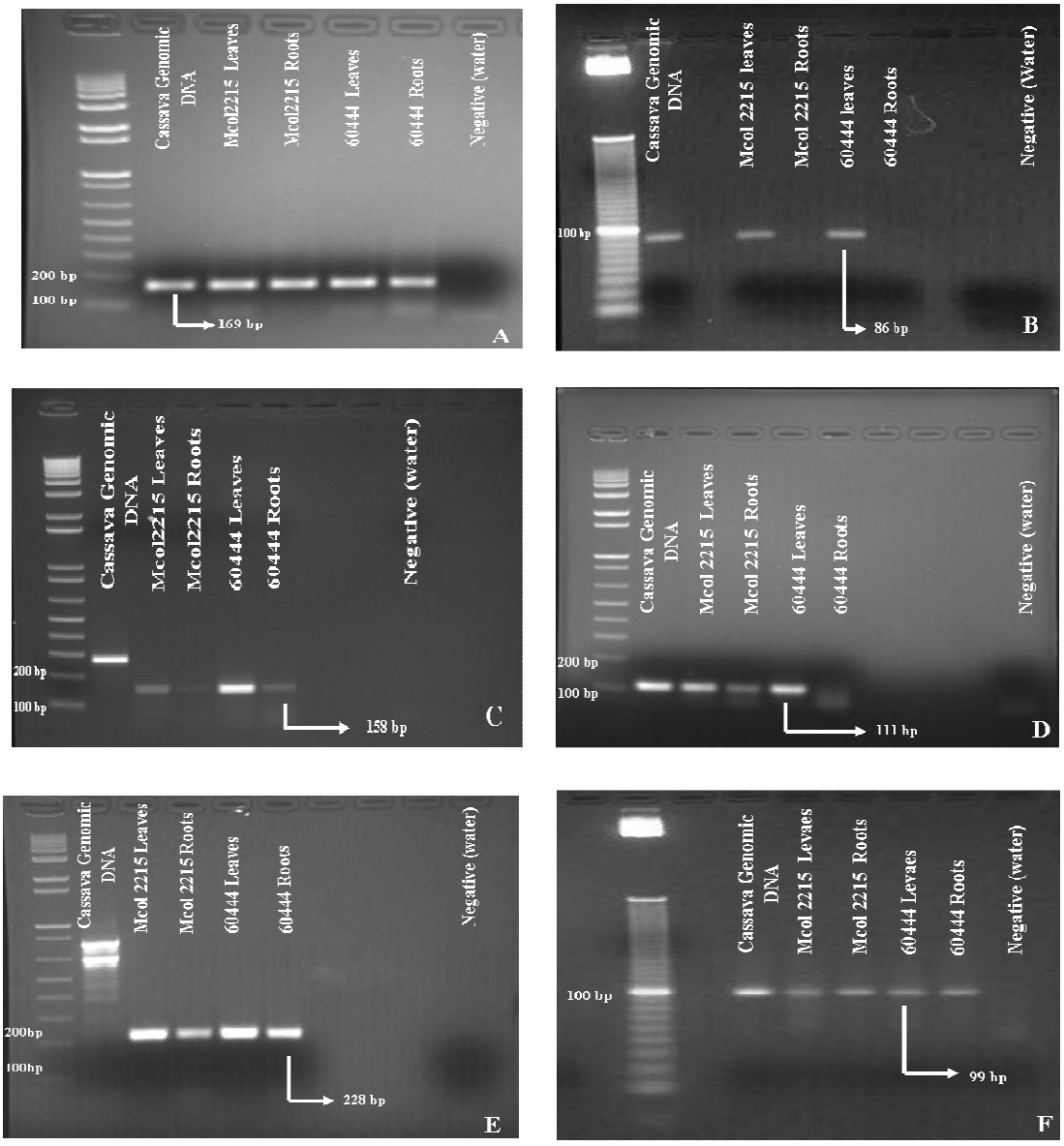


Figure 5: Semiquantitative reverse transcriptase PCR of genes involved in cyanogenic glycosides pathways in leaves and roots of cassava cultivars Mcol 2215 and 60444.

The 18S rRNA gene was used as housekeeping gene. Cassava genomic DNA was used as an amplification control and as well as for testing for DNA contamination in cDNA samples, using the primers linamarase A gene and HNL gene. Panel A, agarose gel 1.5% for 18s rRNA. Panel B, agarose gel 2% for CYP79D1/D2. Panel C, agarose gel 1.5% for Linamarase gene primers A. Panel D, agarose gel 1.5% for Linamarase primers B. Panel E, agarose gel 1.5% for HNL gene. Panel F, agarose gel 2% for β-CAS gene. Two molecular marker were used: 1 Kb Plus DNA ladder (INVITROGEN San Diego, CA, USA) in agarose gels for 18S, linamrase primers A, linamarse primer B and HNL genes; TrackIt 10 bp DNA ladder (INVITROGEN San Diego, CA, USA)

Table 6. Summary of transcriptional activity detection of genes involved in cyanogenic gycosides pathways through conventional PCR.

	Detection			
	Mcol 2215		60444	
Gene	Leaves	Roots	Leaves	Roots
CYP79D1/D2	Yes	Not	Yes	Not
Linamarase	Yes	Yes	Yes	Yes
HNL	Yes	Yes	Yes	Yes
B-CAS	Yes	Yes	Yes	Yes
18S rRNA	Yes	Yes	Yes	Yes

In the case of CYP79D1/D2 the detection in leaves was as predicted. However this is not the case for roots where Siritunga and Sayre (2003) detected transcriptional activity. But there are differences between that study and ours: different plant age (two months vs. four months), different primer set (they studied *CYP79D1* and *CYP79D2* independently while we used a consensus sequence), different RNA amount for cDNA synthesis (1µg vs. 6µg), and DNase treatment (according with Dimuth Siritunga personal communication, this kind of treatment of the RNA was not done in the previous study). It is possible that some of these issues could be the reason of the differences. However even though our results on the expression pattern of *CYP79D1/D2* suggest that the roots do not synthesize linamararin, we believe that the conventional retrotanscriptase PCR is not sensitive enough to detect the transcription of this gene. Therefore, more evaluations with a sensitive technique like Real-Time PCR are necessary.

The results obtained for the transcriptional activity of linamarase are also different as those shown in table 5 and with the hypothesis 2, since we detected transcription in roots (Figure 5 C and D). The previous studies by Santana et al. (2002) reported no transcriptional linamarase activity in roots, supporting the Pancoro and Hughes (1992), hypothesis that implies that linamarase is transported to the roots from the leaves. Our

results refute this hypothesis and show that the roots can synthesize linamarase although at very low levels compared to the leaves. Again the Santana et al. (1992) and the present study have differences in their respective methodologies: 1. cultivars used (Mcol 2215, 60444 Vs V47 and V56); 2. age of the plant (2 months vs. 9 months); 3. cDNA synthesis (they worked one-step synthesis PCR, whereas we worked two-step synthesis). Thus, it is possible that the linamarase transcriptional activity could not be detected with conventional PCR is some cultivars or under some conditions (for example in our case, in the figure 5 C*,* linamarase was not detected in 60444 roots).

In HNL the results are consistent with the transcriptional studies developed by White et al. (1998) and with hypothesis 3. However, it is important to note that these authors detected HNL transcriptional activity with northern Blot, a technique less sensitive than conventional PCR, and also found that HNL does not have protein activity in cassava roots. The semiquantitative comparison of gene expression of all the genes in figure 5 shows that HNL in roots has more activity that any of the other studied genes in roots. This suggests that the HNL activity is regulated postranscriptionally in the roots as the HNL gene is transcriptionally active in this tissue but no protein activity has been detected.

In the case of β-CAS there are no previous results to compare; our hypothesis 4 was proposed according to the enzymatic studies developed by Elias et al. (1997). The result obtained shows that the gene is expressed at the same levels in all the tissues, which is in agreement with Elias et al. (1997) who demonstrated that the enzyme is distributed in all tissues with the tuber exhibiting the highest activity.

4.6 Conclusions

- The semiquantitative comparison of leaves and roots indicates that in both cultivars, the leaves have more transcriptional activity that the roots with the exception of β-CAS.
- Cassava leaves show transcriptional activity of all the genes involved in the cyanogenic glycoside pathways. The semiquantitative comparisons indicate that the HNL gene has the highest transcriptional activity in this tissue.
- Trancriptional activity of CYP79D1/D2 in cassava roots was not detected contrasting with the previous results obatained by Siritunga and Sayre (2004). In the same way, we detected transcriptional activity of linamarase in cassava roots contrasting with Santana et al. (2002) results. The implementation of a more sensitive technique like Real-Time PCR will help clarify these contrasting results.

CHAPTER FIVE:
QUANTIFICATION OF TRANSCRIPTIONAL ACTIVITY OF GENES INVOLVED IN THE CYANOGENIC GLYCOSIDES PATHWAYS IN THREE CASSAVA CULTIVARS USING REAL-TIME PCR

5.1 Literature Review

The high degree of interspecific hybridization in cassava is associated with the high number of morphological and agronomical characteristics, which allows the classification of cassava into groups (Alves, 2002). Cyanide content is one of these cultivar descriptors. Cassava cultivars can be classified into two groups based on the linamarin content of their roots. Group 1 is named “low cyanide” and has ≤50 mg of linamarin per kilogram of fresh weight whereas the group 2 is named “high cyanide” and has ≥ 100 mg of linamarin per kilogram of fresh weight (Mkpong et al., 1990). Although several environmental factors such as drought and soil composition have been associated with cyanogen levels (Grace, 1977) the physiological basis for cultivar-dependent differences in root linamarin content remains one of the more controversial aspect of cyanogenesis in cassava (McMahon et al., 1995).

In an attempt to explain this issue, researchers have considered the linustatin pathway hypothesis as one explanation. According with this, the steady state pool size of linamarin in roots could be regulated in two ways: by linustatin conversion and transport or through the steady state level of linamarin or linamarase activity in leaves. However since linustatin has not been detected yet in cassava, scientist have been focused on studying the second alternative (McMahon et al., 1995). Mkpongo et al., (1990) showed that the linamarin content and linamarase activity of leaves were approximately 18-times higher than in roots in the low cyanide cultivar. Nevertheless, the comparison between a high cyanide cultivar and a low cyanide cultivar showed that the linamarin content of leaves from both varieties was nearly identical. Moreover, they found that the average linamarase activity in leaves was only slightly higher in high cyanide cultivar. Hence,

they concluded that the varietal differences in root linamarin content cannot be attributed to the differences in the catabolism of linamarin in leaves.

Santana et al. (2002) also investigated the differences in cyanide quantities among cassava cultivars. They reported that all the tissues of high cyanogenic cassava have more cyanide than a low cyanide cultivar, indicating a higher rate of synthesis and accumulation of linamarin in the high cyanogenic cultivars. Comparatively they found that in low cyanide cultivars the expression of linamarase was higher than in high cyanide cultivars for most of the tissues. However they indicated that each cultivar has a differential pattern of linamarase activity during the growth cycle. The low-cyanide cassava showed very high expression of linamarase enzymes in young leaves of 3 and 8-months-old plants, whereas the root peel activity increased throughout the cycle. Conversely, the high cyanide cultivar showed a decrease in the root peel linamarase activity during growth cycle, but in 11-month-old plants (time in the growth cycle when the starch stored in the roots starts to be utilized for new growth) the linamarase activity in expanded leaves increased, and 4 months later there was a peak of linamarase activity in root peel of this cultivars. Interestingly, Santana et al. (2002) found differences in linamarase cell localization among the two varieties. Using inmunolocalization they showed the presence of linamarase in laticifers of petioles and roots of both cultivars. However in the high-cyanide cultivar the enzyme was located in the cell wall and in the cytosol, whereas in the low-cyanide cultivar linamarase was located in modified vacuoles of laticifer cells and cytosol of parenchyma cells.

The results of Mkpongo et al. (1990) and Santana et al. (2002) suggest the complexity of the cultivar based cyanide differences in cassava. The genotype, development and

environment are important issues that modulate the differences among cultivars. Nevertheless these research projects have focused mainly in the linamarase synthesis and catabolism, but no studies have been conducted to compare the transcriptional activity between cultivars of the genes involved in the cassava cyanogenic pathway. Such research would help complement the previous physiological studies and would give new explanations for the understanding of the biological basis for the differences of cyanogens in cultivars.

5.2 Specific Objective

To compare the transcriptional activity of genes involved in cyanogenic glycoside pathway in leaves and roots among sweet (Mcol 2215, 142 ppm of HCN for dry base), intermediate (60444, 182 ppm of HCN for dry base), and bitter (Mtai 16, 569 ppm of HCN for dry base), cassava cultivars.

5.3 Biological Hyphoteses

Four hypotheses were proposed according to previous studies.

1. The genes CYP79D1/D2, Linamarase and HNL are transcribed higher in leaves than roots whereas the β-CAS gene is transcribed higher in roots than in leaves.
2. The transcriptional activity in leaves as well roots of genes involved in cyanogenic synthesis (CYP79D1/D2) is higher in bitter cultivars than in sweet cultivars.
3. The transcriptional activity in leaves as well roots of genes involved in catabolic steps (linamarase and HNL) are higher in sweet cultivars that in bitter cultivars

4. The transcriptional activity in leaves as well roots of genes involved in re-assimilation steps (β-CAS) is higher in the sweet variety that in bitter cultivars.

5.4 Specific Methodology

The cassava cultivars, Mcol 2215 (142 ppm cyanide dry base), 60444 (182 ppm cyanide dry base), and Mtai 16 (569 ppm cyanide dry base) were used in this study. For each cultivar three magenta boxes with five explants each were used to grow the plants for 2 months in 5 mL of 4E liquid media. Afterwards, leaves and roots were harvested from each magenta box and frozen in liquid nitrogen. These were the three biological replicates for each cultivar..

The samples collected were used subsequently for RNA isolations, cDNA synthesis and its corresponding nucleic acid spectrophotometrical quantifications. Real-Time PCR was done with the primers CYP79D1/D2, Linamarase B, HNL, β-CAS and 18S rRNA using three technical repetitions for each amplification. The data were collected according to the recommendation described in section 3.4 and classified in different matrices: for general gene comparison (one matrix 57x5; all the Ct for the three cultivars in both tissues plus the Ct for artificial sample); for tissue comparison of each variety (three matrixes 18x5) and for cultivar comparison in each tissue (two matrixes 27x5). Each matrix was introduced into GenEx for the corresponding data pre-processing described in the section 3.4, normalized with the corresponding primer efficiency described in appendix D, using the LOD for off-scale data in CYP79D1/D2 and Linamarase (see appendix E). For classification (cluster classification), the data processed with GenEx from the general gene comparison Matrix (matrix 57x5) were reorganized to obtain gene

classifications for each cultivar. The data from leaves and roots were analyzed as one vector; this means that they were not distinguished. Thus, there were three 6x4 matrices for gene classification, where 6 is the samples (leaves + roots) and 4 is the genes tested; whereas for the cultivar classification there was one 18x4 matrix where 18 represents the samples and 4 the genes.

The biological hypotheses were used in the statistical test as alternative hypotheses (see 5.3).Thus, for tissue comparison (hypothesis 1) the statistical test were made using paired t test with one tail at 10% significance level. The statistical comparison among cultivars (hypothesis 2, 3, 4) was done using ANOVA for Completely Randomized Design comparing through contrasts at 10% significance level.

5.5 Result and Discussion

5.5.1 Detection of Transcriptional Activity using Dissociation Curves

The background information illustrated in chapter 4 showed that the detection of transcriptional activity in roots for CYP79D1/D2 and linamarase contrasted with previous studies. As a result it was proposed that the analysis be conducted with more accurate technologies like Real-Time PCR and with more repetitions. This was implemented in the work presented here in chapter 5, finding a clear detection of all the genes in leaves. But in the case of roots, intermittent detection (variation in the detection over technical replicates) for CYP79D1/D2 and linamarase occurred even though HNL and β-CAS were clearly detected (figure 6). In order to improve the quality of detection, the PCR amplification was again conducted using more cDNA but did not result in a clear improvement (data not shown). Thus, our conclusion is that all the genes studied have

transcriptional activity in leaves and roots of three cassava cultivars, although CYP79D1/D2 and linamarase have very low mRNA in roots, sometimes complicating their detection.

These problematic results for the analysis of CYP79D1/D2 and linamarase in the roots (figure 6) is labeled as missing data and off-scale measurements which are common in Real-Time PCR studies when the samples have very low transcriptional activity. According to a personal communication from Dr. Mikael Kubista, there are two possible reasons for a missing data: 1. Failed reaction, when the sample almost certainly contains target molecules but the reaction failed for technical reasons; 2. Off scale reading, when the sample contains too few target molecules to be detected resulting in a primer-dimer signal. This problem is generally handled with technical replicates. Thus, according to Dr. Mikael Kubista, all genes/samples with at least one successful measurement from the technical repetition could be used in the analysis (criterion 3 developed by us). Thus, if there is no amplification in the technical replicates the conclusion is that the sample contains very little of that particular mRNA and the data must to be correct with the LOD (criteria 4 developed by us). This is essentially the highest CT + 1 obtained for a positive sample, and represents the 50% of the amount that can be possibly detected (see section 2.4.3).

The intermittent detection of CYP79D1/D2 with this technology contrasts with our previous conventional result where this gene could not be detected. The reason for this results lies in the fact that the Real-Time PCR followed by dissociation curve is more sensitive that the conventional PCR with more dynamic range (Dagher *et al*., 2004; Dorak, 2006). However the result does not clarify the discrepancy with the Siritunga and

Sayre (2003) paper where the gene was clearly detected. The explanation could be the differences between the methodologies used for the analysis (section 4.5).

In the case of linamarase the intermittent expression was also seen in conventional PCR. The most interesting point in linamarase detection is the contrast with Santana et al. (2002) since our result shows transcriptional activity of this gene in roots. As previously mentioned in chapter 4, Santana et al. (2002) reported the absence of transcriptional activity in the roots and for this reason supported Pancoro and Hughes (1992) hypothesis that imply that linamarase travel from the leaves to roots. But our results suggest that cassava roots could process their own linamarin or the linamarin translocated from the leaves following the catabolic or the re-assimilation pathways.

5.5.2 Comparison among Genes

This is the first study that shows a comparison on the levels of expression among the genes involved in the cyanogenic glycoside synthesis in leaves and roots of three cassava cultivars. Through a 59x5 matrix relativized with an artificial sample, we obtained a general gene expression profile represented in figure 7. Although this approach allows us to appreciate the main differences between tissues and among cultivars in each gene, more accurate comparisons among these factors were developed through a specific matrix (see details in sections 5.5.3 and 5.5.4). Figure 7 show that HNL and linamarase have higher transcriptional activity levels in leaves and lower levels in roots. A similar pattern was observed in CYP79D1/D2 but their transcriptional activity in leaves as well as in roots was less than linamarase and HNL. Furthermore β-CAS transcriptional levels are similar in leaves and roots. In roots the

levels of β-CAS and HNL transcriptional activity was similar in all 3 cultivars tested in this study.

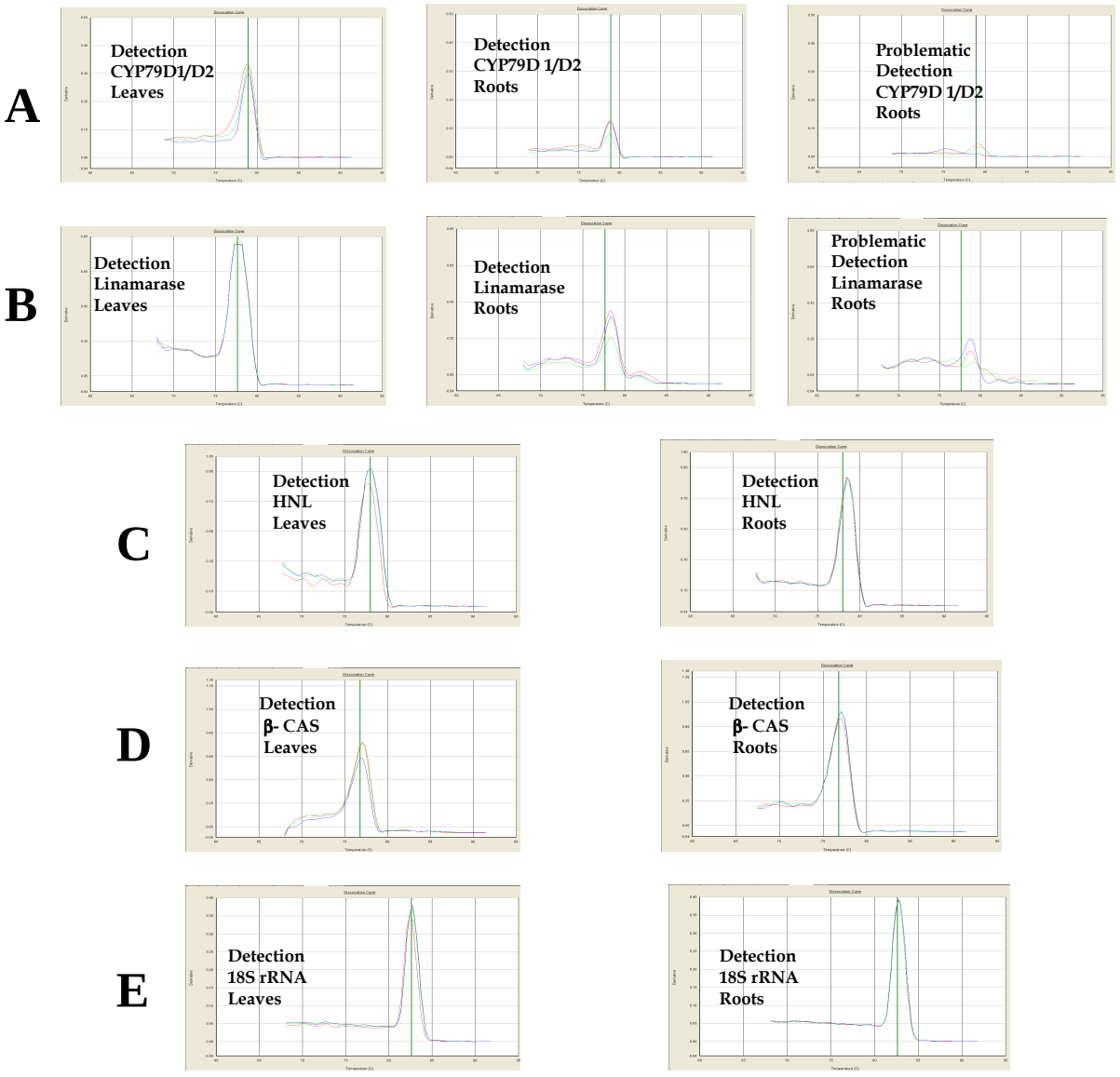


Figure 6: Melting Curves in Mcol 2215.
Detection of transcriptional activity in leaves and roots of the genes CYP79D1/D2, linamarase, HNL and β-CAS through melting-curve analysis. Panel A, CYP79D1/D2 detections. Panel B, linamarase detections. Panel C, HNL detection. Panel D, β-CAS detections. Panel E, 18s rRNA detections. The problematic detetection (intermitent detection) in CYP79D1/D2 and linamarase in cassava roots is also illustrated. Each box shows curves for three technical replicates.

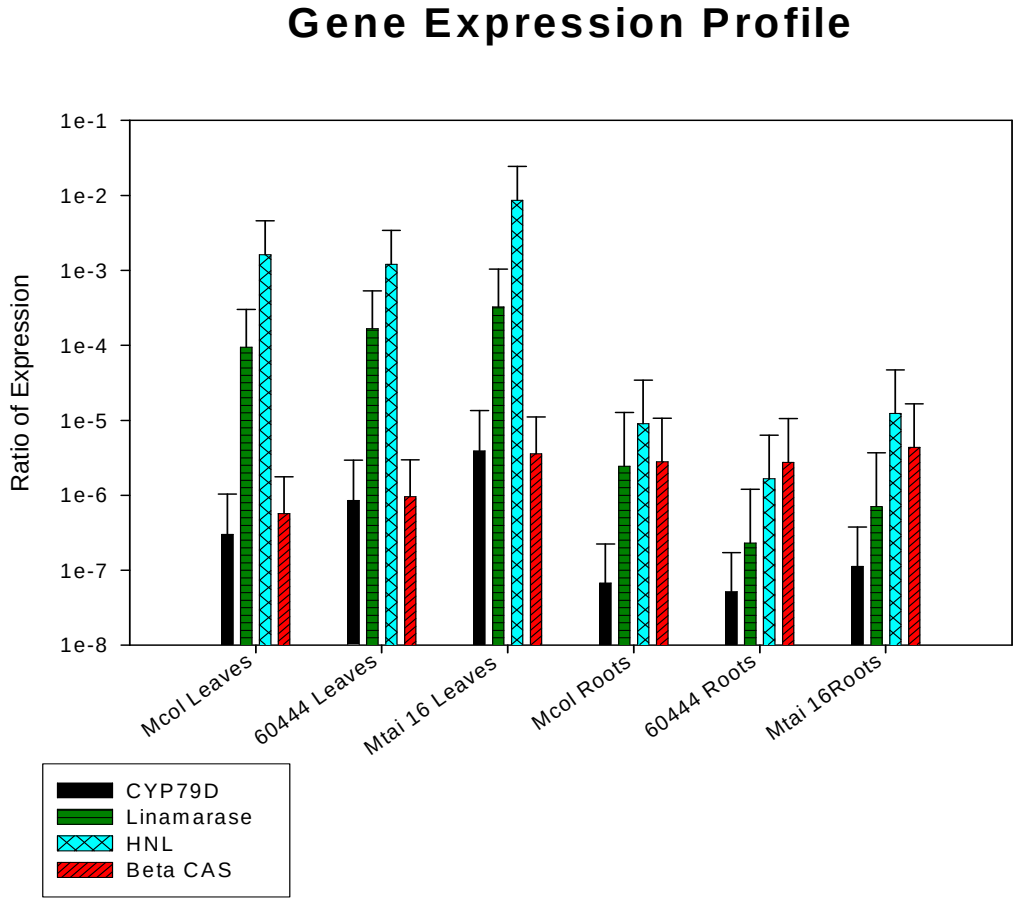


Figure 7: Gene Expression profiles of 4 genes involved in cyanogenic glycosidespathway in leaves and roots of three cassava cultivars.
The leaves have the higher transcriptional activity for the genes CYP79D1/D2, linamarase and HNL as compared to the roots, whereas β-CAS gene has higher transcriptional activity in roots than in leaves or at least the same level in both tissues. The error bar is the upper value of the Confidence Interval.

5.5.3 Comparison Between tissues in each variety

To compare tissues among cultivars, three 18x5 matrixces were used. The results of these comparisons are illustrated in figure 8 and table 7. The corresponding descriptive statistic and paired t-tests are in appendix F.

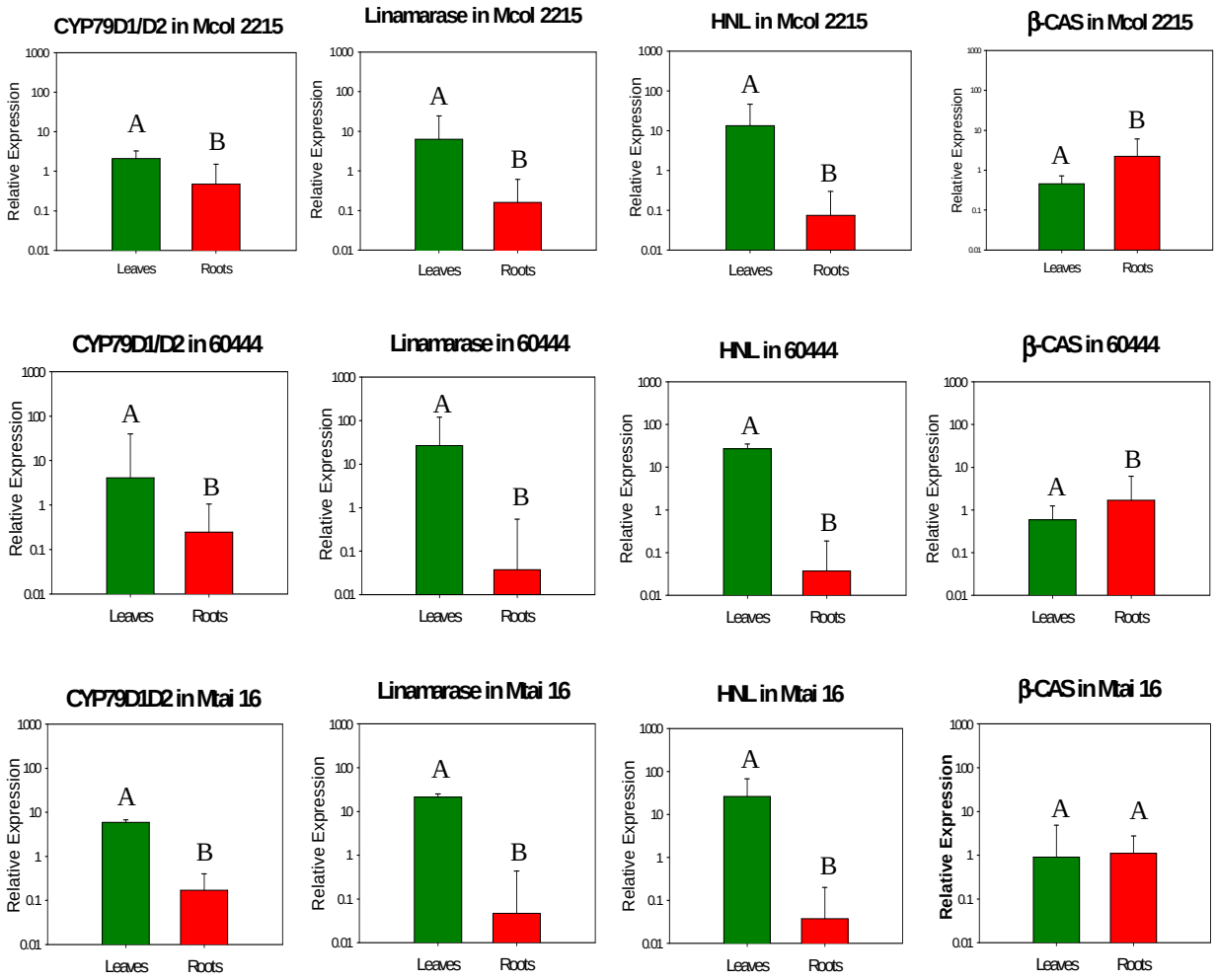


Figure 8: Comparison between leaves and roots of the transcriptional activity of the genes CYP79D1/D2, linamarase, HNL and β-CAS in three cassava cultivars.
The genes CYP79D1/D2, linamarase and HNL has highest transcriptional activity in leaves in relation to roots; meanwhile β-CAS has more transcriptional activity in roots or at least the same level in both tissues. The bar error is the upper value of 90% the Confidence Interval. A different letter above the bars indicates significant differences ($p <= 0.10$).

In general, we can conclude that the Real-Time PCR results agree with previous research work, the hypothesis number 1 (section 5.3) and the semiquantitative results of chapter 4 all the genes with exception of β-CAS have more transcriptional activity in leaves that in roots (figure 8 and table 7). However this is the first report that shows quantitative and statistically significant differencial expression between each tissue in the three cassava cultivars (table 7).

Table 7: **Ratios of expression between leaves and roots and their Confidence Interval for the means differences.**

Cultivar	Gene	Comparison	Ratio	Two sided C. I for means differences (α = 10%)	P-value (one side)
Mcol 2215	CYP79D1/D2	Leaves/Roots	4.44	(1.28; 15.45)	0.0364* (Right)
Mcol 2215	Linamarase	Leaves/Roots	38.86	(2.7; 556.41)	0.0284* (Right)
Mcol 2215	HNL	Leaves/Roots	178.52	(58.49; 548.75)	0.0027* (Right)
Mcol 2215	Beta-Cas	Roots/Leaves	4.89	(1.15, 20.83)	0.0428* (Right)
60444	CYP79D1/D2	Leaves/Roots	16.44	(1.26; 215.27)	0.0431*(Left)
60444	Linamarase	Leaves/Roots	724.01	(106.89; 4904.87)	0.0049* (Right)
60444	HNL	Leaves/Roots	719.29	(177.29; 1176.23)	0.0026* (Right)
60444	Beta-Cas	Roots/Leaves	2.86	(0.86; 9.58)	0.0624* (Left)
Mthai 16	CYP79D1/D2	Leaves/Roots	34.78	(14.03; 86.22)	0.0038* (Right)
Mthai 16	Linamarase	Leaves/Roots	458.26	(41.93; 5007.9)	0.0087* (Right)
Mthai 16	HNL	Leaves/Roots	699.41	(112.98; 4359.6)	0.0045* (Right)
Mthai 16	Beta-Cas	Leaves/Roots	1.12	(0.52; 2.86)	0.2883 (Left)

Each Ratio is interpreted as: For Cultivar (*Mcol 2215/60444/Mtai16*), the mean amount of RNAm from (*CYP79D1/D2/ Linamarase/HNL/β-CAS*) gene is (*Ratio Value*) times larger in (*Fraction Numerator in Comparison Cells*) that in (*Fraction denominator in Comparison Cells*) (according to the 90% two-sided confidence interval for mean differences, it is at least (*Lower C.I Value*) and not more than (*upper C.I Value*)**.** The ratio (*is/ is not*) significantly different from 1 using α = 0.10 (analysis done using paired t-tests for log transformed data).
*Indicates significant difference. The corresponding descriptive statistics and statistical comparisons are in appendix F.

β-CAS is the gene with the lowest expression ratio (*leaves:roots*) (table 7) in the three cultivars; this is due to its transcriptional activity which is almost similar in both tissues (figure 7 and figure 8). This particularity of β-CAS contrasts with the other genes which have higher activity in leaves and low mRNA production in roots. CYP79D1/D2 is the gene with the second lowest ratios, but its expression is quite distinct between both tissues (figure 7 and figure 8) to the point that it was difficult to detect in roots (figure 6). However, the leaf amount is low which explains the low ratios. Linamarase behavior was similar to CYP79D1/D2 because it was sometimes difficult to detect in roots (figure 6) but the mRNA production in leaves was high explaining the high ratios. Finally HNL had the highest ratios which are explained by the very high activity found in leaves (figure 7 and figure 8)

According to our results the synthesis of cyanogenic glycosides, catabolism and re-assimilation happens in both tissues. The occurrence of higher transcriptional activity in the synthesis pathway in leaves is in agreement with the translocation hypothesis (Selmar, 1994) in which the cyanogenic glycosides are synthesized in leaves and transported to roots as mobile source of nitrogen (Siritunga and Sayre 2004). Furthermore the high presence of linamarase and HNL transcripts in leaves could be associated with herbivore defense mechanisms (Belloti and Riis, 1994). In addition, higher levels of β-CAS expression in the roots shows that possibly this tissue produce free HCN in intact plants, which needs to be re-assimilated to prevent a build-up of HCN to toxic levels in the cells.

5.5.4 Comparison among Cultivars

As previously mentioned, the basis for a comprehensive understanding of the differences in the levels of cyanogenic glycosides among cassava cultivars is one of the most controversial issues in cassava biology (McMahon et al., 1995). Our transcriptional activity results agree with two of the original hypotheses: 1. In leaves the transcriptional activity rate of genes involved in the synthesis steps are higher in the bitter cultivar (Mtai 16) than in the two others (between Mcol 2215 and 60444 statistical differences were not found); 2. The behavior of linamarase in roots partially agrees with the hypothesis for catabolic genes, because the transcriptional activity in Mcol 2215 was higher than in the other two, although the only statistical differences were found between Mcol 2215 and 60444 (Figure 9, Figure 10, Table 8, Table 9). It must also be noted that the differences found in this cultivar comparison are low in relation to the difference found between

tissues [the statistical power could be affected by the sample size (n = 3) and the high standard deviation present].

The cyanide cultivar differences are explained by the cyanogenic glycosides amounts present in the roots. However considering the verified hypothesis and the similarity in CYP79D1/D2 expression in roots among the three cultivars (figure 10 and table 9) we suggest that the difference in the cyanogenic glucosides in the roots are due to the combinatorial effect of their synthesis in the leaves and the linamarase activity in roots due to the translocation of linamarin. This hypothesis agrees with all the previous studies that demonstrate that the cyanogenic glycosides are synthesized in leaves and translocated to roots (Ramanujam and Indira, 1984; Makame et al., 1987; Siritunga and Sayre, 2003 and Jørgensen et al., 2005). It also agrees with the previous studies conducted on linamarase by Santana et al. (2002) where they found that linamarase activity from the root peel of a low cyanide cultivar is about 10 times higher than in the root peel of a high cyanide cultivar.

The linamarase, HNL and β-CAS gene expression among leaves of the three cultivars showed an opposite pattern to the hypotheses suggested in the methodology (figure 9, table 8). For this reason we believe that the behaviors of those genes in leaves do not explain the cultivars differences. It is important to point out that similar results were obtained for Mkpongo et al. (1990) when comparing the protein activity of linamarase in cassava leaves. They concluded that the cultivar differences in root linamarin content cannot be attributed to the differences in the catabolism of linamarin in leaves.

Nevertheless aother interesting pattern was found in leaves. The transcriptional activity of all the genes in the leaves is always higher in the bitter Mtai16 cultivar when compared to the other two while the transcriptional activity of all the genes were similar between Mcol 2215 and 60444. This implies that although the synthesis, catabolism by disruption and the re-assimilation are spatially different and temporally separated events, they are related in some way in the leaves. In addition this pattern is correlated with the total HCN for dry base in each cultivar, where the amount of HCN between Mcol 2215 and 60444 were more similar (142 ppm and 182 ppm respectivelly) than Mtai 16 amount (569 ppm). Thus, we suggest that a bitter cassava is more dangerous for one herbivore not only due the high levels of linamarin presents but for the high level of catabolic enzymes presents that help to release HCN faster when the foliar tissues are ingested by the animal.

In the roots, with the linamarase exception previously discussed, we only found statistical differences in HNL expression levels of Mtai 16 compared to the other two cultivars (figure 10 and table 9). However it must be noted that previous studies conducted by White et al. (1998) reported non-HNL enzymatic activity in cassava roots. As a result, as previously discussed (section 4.5), although HNL has a high transcriptional activity, the protein activity could be dependent of other post-transcriptional factor. Thus, we suggest that the HNL transcriptional activity in roots does not explain directly the differences found between cultivars.

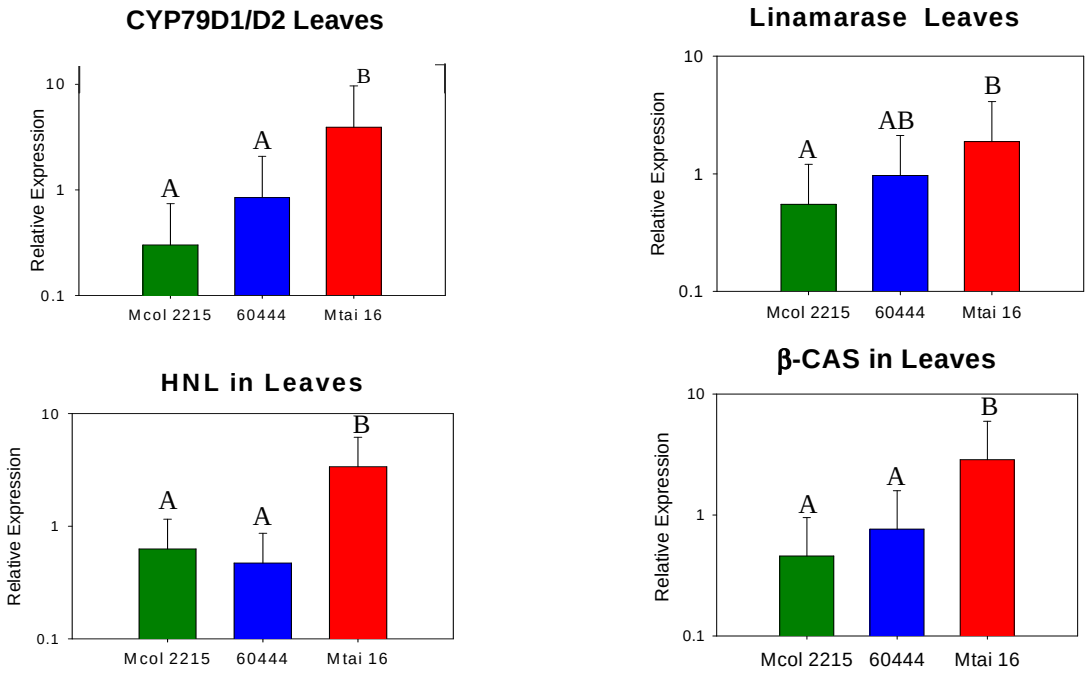


Figure 9: Expression pattern of the genes CYP79D1/D2, linamarase, HNL and β-CAS in leaves of three cassava cultivars.

The cultivars used were Mcol 2215 (142 ppm of HCN), 60444 (182 ppm of HCN) and Mtai 16 (569 ppm of HCN). Only the pattern illustrated in CYP79D1/D2 gene could explain the cyanide cultivar differences considering the metabolic activity of each enzyme. The bar error is the upper value of 90% the Confidence Interval. A different letter above the bars indicates significant differences (p <= 0.10).

Table 8: Ratios of expression in leaves among cultivars and their confidence interval for the means differences

Gene	Comparison	Means Differences: Ratio	Two sides C. I for means differences (α = 10%)	Contrast. P-value
CYP79D1/D2	Mthai16/Mcol 2215	13	(3.66; 46.21)	0.0077*
CYP79D1/D2	Mthai 16/TMS 60444	4.63	(1.31;16.56)	0.0571*
CYP79D1/D2	TMS 60444/Mcol 2215	2.82	(0.80; 10.05)	0.1636
Linamarase	Mthai16/Mcol 2215	3.41	(1.13; 10.26)	0.0737*
Linamarase	Mthai 16/TMS 60444	1.95	(0.65; 5.86)	0.2857
Linamarase	TMS 60444/Mcol 2215	1.75	(0.58; 5.28)	0.3594
HNL	Mthai16/Mcol 2215	5.35	(2.27; 12.64)	0.0092*
HNL	Mthai 16/TMS 60444	7.16	(3.03; 16.91)	0.0044*
HNL	Mcol 2215/TMS 60444	1.34	(0.56; 3.16)	0.5332
β-CAS	Mthai16/Mcol 2215	6.27	(2.23; 17.63)	0.0134*
β –CAS	Mthai 16/TMS 60444	3.76	(1.34; 10.56)	0.0473*
β –CAS	TMS 60444/Mcol 2215	1.67	(3.66; 46.21)	0.3676

Each Ratio is interpreted as: For Leaves, the mean amount of mRNA from (*CYP79D1/D2/ Linamarase/HNL/β-CAS*) gene is (*Ratio Value*) times larger in (*Fraction Numerator in Comparison Cells*) that in (*Fraction denominator in Comparison Cells*) (according to the 90% two-sided confidence interval for mean differences, it is at least (*Lower C.I Value*) non more than (*upper C.I Value*)**.** The ratio (*is/ is not*) significantly different from 1 using α = 0.10 (analysis done using contrast for log transformed data).*Indicate significant difference. The corresponding descriptive statistics and statistical comparisons are in appendix F.

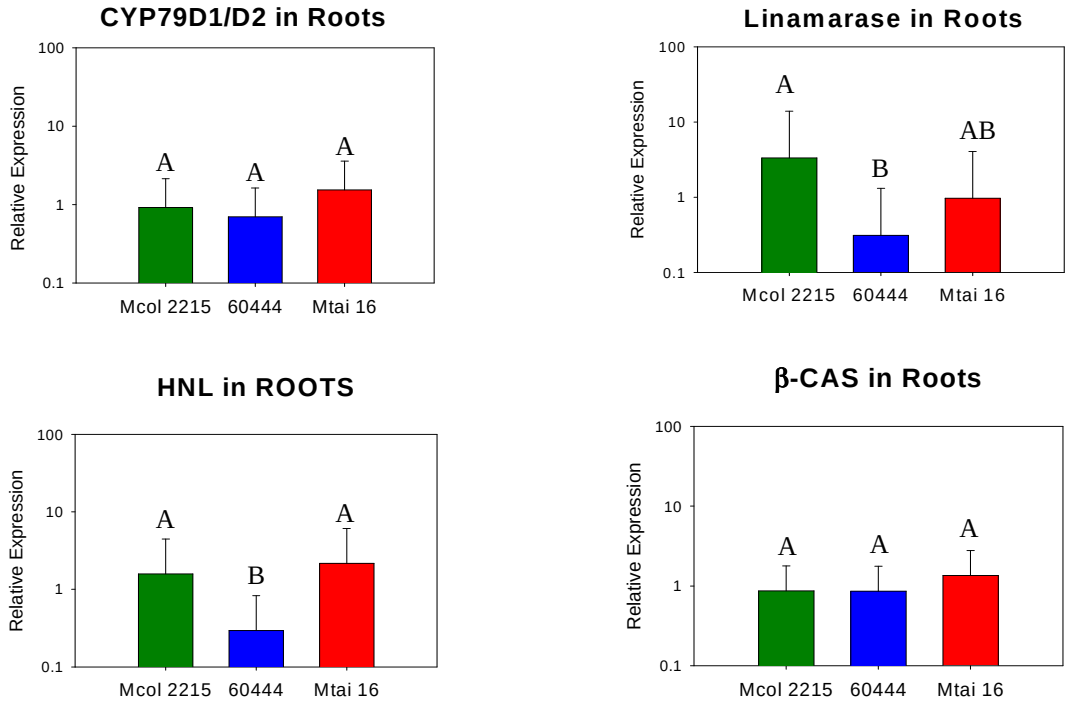


Figure 10: Expression pattern of the genes CYP79D1/D2, linamarase, HNL and β-CAS in roots of three cassava cultivars.

The cultivars used were Mcol 2215 (142 ppm of HCN), 60444 (182 ppm of HCN) and Mtai 16 (569 ppm of HCN). Only the pattern illustrated in linamarase gene could explain partially the cyanide cultivar differences considering the metabolic activity of each enzyme. The bar error is the upper value of 90% the confidence interval. A different letter above the bars indicates significant differences ($p <= 0.10$).

Table 9: Expression Ratios expression in roots among cultivars and their confidence interval for the means differences.

Gene	Comparison	Means Differences: Ratio	Two sides C. I for means differences (α = 10%)	Contrast. p-value
CYP79D1/D2	Mtai16/Mcol 2215	1.67	(1.16; 5.50)	0.4367
CYP79D1/D2	Mtai 16/TMS 60444	2.18	(0.66; 7.21)	0.2488
CYP79D1/D2	Mcol 2215/TMS 60444	1.31	(0.39; 4.31)	0.6727
Linamarase	Mcol 2215/Mtai 16	3.44	(0.45; 26.17)	0.2830
Linamarase	Mtai16/TMS60444	3.1	(0.40; 23.59)	0.3235
Linamarase	Mcol 2215/TMS 60444	11.1	(1.40; 81)	0.0650*
HNL	Mtai16/Mcol 2215	1.36	(0.31; 5.90)	0.6936
HNL	Mtai 16/TMS 60444	7.36	(0.58; 31.78)	0.0381*
HNL	Mcol 2215/TMS 60444	5.55	(1.24; 23.26)	0.0668*
β-CAS	Mtai16/Mcol 2215	1.56	(0.55; 4.35)	0.4326
β-CAS	Mtai 16/TMS 60444	1.57	(0.56; 4.38)	0.4210
β-CAS	TMS 60444/Mcol 2215	1	(0.36; 2.80)	0.9827

Each Ratio is interpreted as: For Roots, the mean amount of mRNA from (*CYP79D/ Linamarase/HNL/β-CAS*) gene is (*Ratio Value*) times larger in (*Fraction Numerator in Comparison Cells*) that in (*Fraction denominator in Comparison Cells*) (according to the 90% two-sided confidence interval for mean differences, it is at least (*Lower C.I Value*) and not more than (*upper C.I Value*)**.** The ratio (*is/ is not*) significantly different from 1 using α = 0.10 (analysis done using contrast for log transformed data). *Indicate significant difference. The corresponding descriptive statistics and statistical comparisons are in appendix F.

5.5.5 A multivariable approach

In order to find a pattern of co-regulation among the genes and cultivar classification, multivariable analysis was performed with clustar analysis. Comparisons were conducted for each cultivar for the expression in leaves and the expression in roots independently. However, no clear pattern was found. Nevertheless when the comparisons were conducted together for leaves and roots per cultivar, we found an association gene pattern that again suggests the coordinate action between leaves and roots. It also supported the cyanogen similarities between Mcol 2215 and 60444.

In this way in Mcol 2215 and 60444, the cluster results for gene comparisons (figure 11) suggest co-regulation between linamarase/HNL (catabolic cluster) and between CYP79/β-CAS (synthesis and re-assimilation cluster). This result match with the spatial separation in the cell wall of the HNL protein and linamarase protein (figure 1), which could imply that those enzymes are co-regulated because they are involved in the metabolism under plant disruption; meanwhile CYP79D1/D2 and β-CAS are related to the nitrogen turnover in intact plants. Also is important to consider that linamarase and HNL are classified together in Mcol 2215 and 60444 but not in Mtai 16. This differential result could be explained by the higher HNL level in the leaves (Figure 7) that has resulted in this manner of organization. Besides this differential result in Mtai 16 also could be explained by cyanide levels present in each cultivar, where Mcol 2215 and 6044 are more closed (table 1).

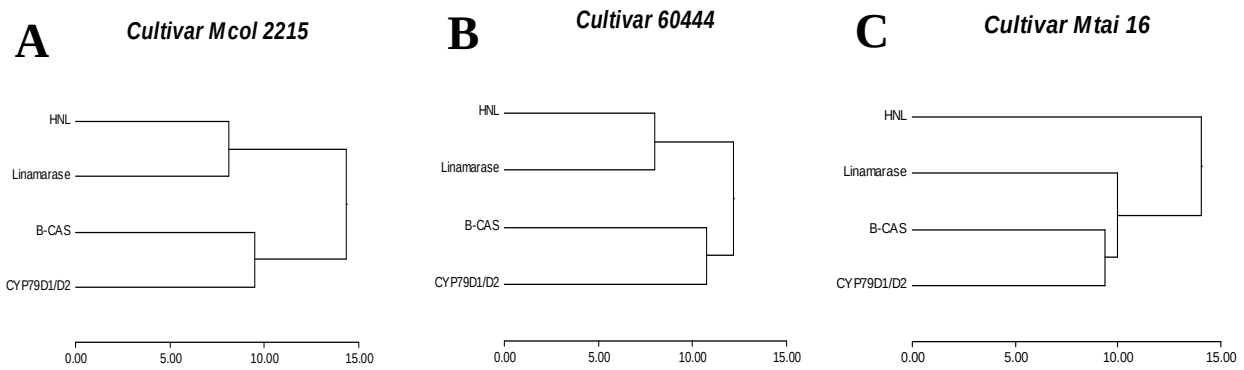


Figure 11: Dendograms from cluster classification of the genes involved in cyanogenic glycoside metabolism in three cassava cultivars.
The data from leaves and roots were analyzed together. Two clusters were generated in Mcol 2215 and 60444: 1. Synthesis/Re-assimilation cluster (CYP79D/Linamarse). 2. Catabolic cluster (linamarase/HNL). Meanwhile in Mtai 16 only the Synthesis/Re-assimilation cluster was observed.. In Mtai 16, HNL could be classified separately due to the higher level of transcriptional activity in this cultivar. On the other hand, this result agrees with the cyanide levels in the three cultivars: Mcol 2215 (142 ppm of HCN), 60444 (182 ppm of HCN) and Mtai 16 (569 ppm of HCN).

Cluster classification of cultivars also showed the clustering of Mcol2215 and 60444 into one group which was distant from Mtai16 (Figure 12). This again is in agreement with the cyanogen levels of the 3 varieties, where Mcol2215 and 60444 have very similar levels. This result also supports the explanation for the higher level of transcriptional activity of all the genes in Mtai 16 exposed in the section 5.5.4.

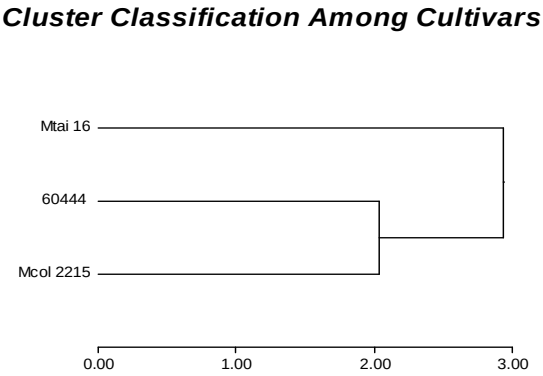


Figure 12: Dendograms from cluster analysis of three cassava cultivars with different cyanide.
The data from leaves and roots were analyzed together**.** The cluster generated match with the cyanide similarities among the three cultivars [Mcol 2215 (142 ppm of HCN), 60444 (182 ppm of HCN) and Mtai 16 (569 ppm of HCN)].

5.6 Conclusions

- All the genes studied have transcriptional activity in both tissues and in all three cultivars. However the levels of CYP79D1/D2 and linamarase in roots are very low which limits their detection.
- Cassava leaves in relation to roots have more transcriptional activity of the genes CYP79D1/D2, linamarase and HNL whereas β-CAS is more expressed in roots (or at least at the same level as in the leaves).
- The gene with the highest ratio differences among tissue was HNL and the lowest was β-CAS.
- The cultivar Mtai 16 has the highest transcriptional activity for all the genes. This could be associated with protection against herbivores.
- The root linamarin differences in cassava cultivars could be explained by the coordinated action of the cyanogenic synthesis in the leaves and the linamarase catabolism in roots.
- Multivariable analysis with cluster shows that in Mcol 2215 and 60444 the genes have the same pattern of activity generating definite clusters for synthesis, for re-assimilation and for catabolism. Meanwhile this pattern was not clear in Mtai 16.

CHAPTER SIX:
QUANTIFICATION OF TRANSCRIPTIONAL ACTIVITY OF GENES INVOLVED IN THE CYANOGENIC GLYCOSIDES PATHWAYS IN TWO CASSAVA CULTIVARS UNDER *IN-VITRO* NITROGEN STRESS

6.1 Literature Review

Plants assimilate nitrogen as nitrate ion or as reduced nitrogen (Ammonium; NH_4^+), which is obtained from the nitrogen cycle in the atmosphere (Taiz & Zeiger, 2002). However, nitrate cannot simply be used to synthesize organic molecules but has to be previously reduced to ammonia. This reaction is executed in two steps by the enzymes nitrate reductase and nitrite reductase. Thus, nitrate is converted into nitrite by nitrate reductase, followed by reduction from nitrite to ammonia ion (NH_4^+) by nitrite reductase. Nevertheless, despite the importance in the plant, ammonia and ammonium are toxic. Therefore, they should be converted quickly into non-toxic compounds like glutamine, asparagine, arginine, allantion and betain (Duchefa, 2003). Glutamine synthetase and glutamate synthase are the key enzymes for this detoxification. In this way, the reduced nitrogen from nitrogen assimilation is used as a precursor of other compounds whose function is the supply of organic bonds for the synthesis of amino acids and proteins (Duchefa, 2003).

The nitrogen incorporated by the plant could be stored as secondary metabolites such as alkaloids and cyanogenic glycosides, which traditionally have been considered as compounds against herbivores or as nitrogen storage compounds in intact plants (see section 2.1, 2.2 and 2.3). To establish the precise function of cyanogenic glycosides has been the focus of some research in other cyanogenic plants. Thus, Fourslound and Jonsson (1997) tried to clarify the role of those compounds in barley (*Hordeum vulgare*) cultivating plants at different levels of nitrogen supply. Their results showed that the relative amount of nitrogen in cyanogenic glycosides in nitrogen-limited plants was three times higher than in control plants. At the same time, the nitrogen limitation affected

plant growth when the total amount of cyanogenic glycosides decreased (day 22). Thus, they concluded that cyanogenic glycosides could be used as a nitrogen storage form. However, when studying the activity of β-glucosidase and CAS, they did not show any obvious correlation, neither with the content of cyanogenic glycosides nor with the nitrogen levels of barley.

Similar studies were developed by Busk and Møller (2002) in sorghum where, unlike Fourslound and Jonsson (1997) who removed external nitrogen, they added nitrogen fertilizer (nitrate) to older plants. Thus, when the exogenous nitrogen was added, the activity of CYP79A1 and CYP71E1 increased as well as dhurrin content. Furthermore, the corresponding mRNA levels matched with the enzymatic activity pattern, indicating that their activity in plants is controlled by direct transcriptional mechanisms and not by post-transcriptional factors. They concluded that the biosynthetic enzymes are the major determinant of cyanide potential in sorghum compared with the rate of cyanogenic glycoside degradation.

In cassava several studies suggest the double role of cyanogenic glycoside (Ramanujam and Indira, 1984; Makame et al., 1987; Belloti & Riis, 1994) but it has been through transgenic cassava plants that key evidence has been provided for the importance of those molecules as as a source of nitrogen. Siritunga and Sayre (2003) conducted studies with the aim of reducing the linamarin content in cassava through the transgenic silencing of CYP79D1/D2 genes. They expressed these genes in an antisense orientation in transgenic cassava under the control of the leaf specific Cab1 promoter, demonstrating that both leaf and root levels of linamarin are reduced in transgenic plants, but much more drastically (99% reduction) in roots. Those plants were

grown on *in-vitro* medium, to which traditionally reduced nitrogen is added. Krikorian (1991) showed that in plant tissue culture several explants grow better with reduced nitrogen incorporated into the medium. However, Siritunga and Sayre (2004) reported that the plants exhibited a normal growth pattern when grown in MS salts containing both nitrate (40 mM) and reduced nitrogen (20 mM NH_3) but failed to produce roots when grown on MS media in which the ammonia was replaced with nitrate (60 mM). In addition, these plants died when transferred to potting soil lacking reduced nitrogen (Siritunga and Sayre, 2004). These results suggested that linamarin may be transported from leaves to roots in young plants. To confirm this hypothesis they reported a similar cassava transformation with the 5´end of CYP79D1 and CYP79D2 genes in reverse orientation under the control of the tuber specific potato patatin promoter. CYP79D1/D2 transcript levels were reduced to non-detectable levels in transgenic roots but the roots had normal levels of cyanogenic glucosides. These plants grew normally when grown in modified MS media lacking ammonia or in plotting soil. This shows that cyanide synthesized in the leaves is a mobile source of nitrogen to the roots and the production of cyanide thus might be influenced by the levels of reduced nitrogen available in the roots.

A similar experiment was developed by Jørgensen *et al.* (2005) transforming CYP79D1/D2 siRNA constructs controlled by the CaMV 35S promoter. Their results showed plants with a 92% reduction in cyanogenic glucosides content in tubers and acyanogenic (<1% of wild type) leaves were obtained. However although their conclusions demonstrated the importance of linamarin synthesis in leaves for the cyanide potential in roots, they reported transgenic plants growing well in soil. Thus, although the contribution of linamarin metabolism to root nitrogen balance remains

unknown, the evidence suggests that linamarin transport from leaves and its subsequent metabolism in roots play a substantial role in root nitrogen metabolism (Siritunga and Sayre, 2007).

6.2 Specific Objective

To compare the transcriptional activity of genes involved in cyanogenic glycosides pathways in leaves and roots, between *in vitro* grown Mcol2215 (sweet variety) and 60444 (bitter-sweet variety) under reduced nitrogen stress conditions.

6.3 Biological Hypotheses

Considering the antecedents in cassava and other plants regarding cyanogenic glycosides pathway regulation three hypotheses are proposed:

1. The gene CYP79D1/D2 increases its transcriptional activity under reduced nitrogen stress in leaves and roots.
2. The gene β-CAS increases its transcriptional activity under reduced nitrogen stress in both tissues.
3. The genes linamarase and HNL will not change their transcriptional activity.

The hypotheses one and two are proposed because it is expected that with a decrease in reduced nitrogen concentration the plant will use the cyanogenic glycoside as an alternative source of nitrogen, increasing the transcriptional activity of CYP79D1/D2 and β-CAS; This last hypothesis is according to Fourslound and Jonsson (1997), whose

study demonstrated no changes in β-glucosidase activity in barley. Besides according to literature review these genes work in cassava plants under tissue disruption (see section 2.2.2.1)

6.4 Specific Methodology

The cassava cultivars Mcol 2215 (142 ppm cyanogens) and 60444 (182 ppm cyanogens) were used in this study. For each cultivar eighteen magenta boxes with five explants each were used to grow the plants for 5.5 months in 5 mL of 4E liquid media in incubator at 28^0C. After this, the 4E liquid media was discarded and the plants were washed with autoclaved deionized water. Then, the 18 magentas boxes per cultivar were separated into three groups of 6 magenta boxes each. For each group, 5mL of a specific liquid media with different reduced nitrogen concentrations was added. The first group received 4E liquid media containing 40 mM nitrate and 20 mM reduced nitrogen (control); the second group received 4E liquid media containing 50 mM nitrate and 10 mM reduced nitrogen (intermediate NH_3 condition) and the third group received 4E liquid media containing 60mM nitrate without reduced nitrogen (without NH_3). In all conditions the total amount of nitrogen remained constant. Of the six magenta boxes per group, three were in each treatment for one day, and other three for 10 days. Thus the number of replicate was three (n = 3), and a treatment consisted of combination of four factors (Cultivar: Mcol 2215 or 60444; Tissue: leaves or roots, media with different reduced nitrogen concentrations: standard, Intermediate and without condition; and time in stress condition: 1 day and 10 days) (See Figure 13).

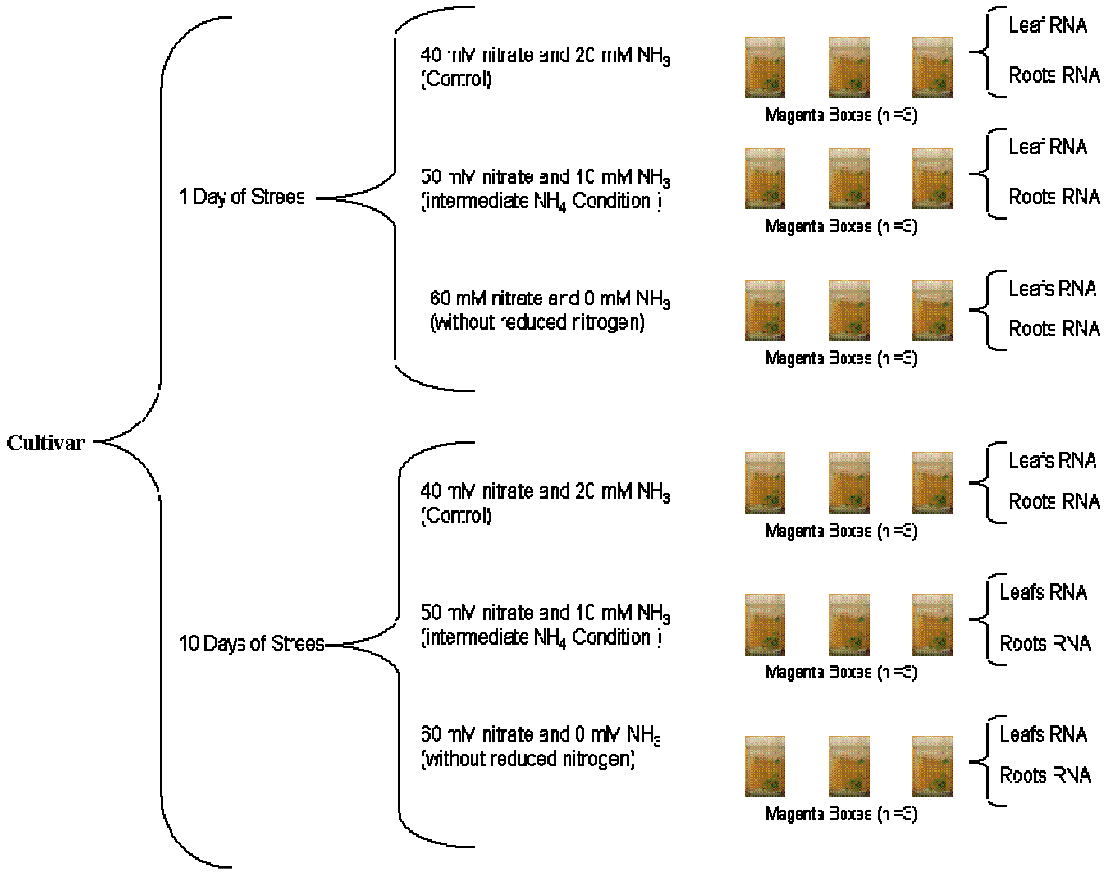


Figure 13 Scheme used in the Nitrogen Test experiment.
This strategy was used in each cultivar using for each specific treatment (specific NH_4 concentration in specific day) three samples (n = 3)

The samples collected were used subsequently for RNA isolations from leaves and roots followed by cDNA synthesis and its corresponding nucleic acid spectrophotometrical quantifications. Real-Time PCR was conducted with the primers CYP79D1/D2, Linamarase B, HNL, β-CAS and 18S rRNA using three technical repetitions for each amplification. The data were collected according to the recommendation described in the section 3.4 and classified in 8 matrixes (27x5) for treatment comparisons. Each matrix consisted in the Ct data from the three specific liquid media with different reduced nitrogen concentrations using three biological replicates with three technical repetitions. It is must be noted that each matrix was specific for one tissue in a specific cultivar at a determined stress time. Each matrix was

introduced into GenEx for the corresponding data pre-processing described in the section 3.4, normalizing with the corresponding primer efficiency described in appendix D and using the LOD for cut-off data in CYP79D1/D2 and Linamarase (see appendix E). Finally cluster classification was done with the objective of testing if the changes in co-regulation patterns among leaves and roots were present under the different experimental conditions. The data from leaves and roots were analyzed as one vector (this means that they were not distinguished). Thus, there were twelve matrices (6x4) for gene classification, where 6 represents the samples (leaves + roots) and 4 represents the genes tested.

The statistical comparisons among reduced nitrogen conditions (in specific cultivar, tissue and days of stress) were done using ANOVA for Completely Randomized Design and through contrast at 10% significance level.

6.5 Result and Discussion

6.5.1 Phenotypical Description

The plants were monitored daily with the objective of finding any phenotypical difference between the control plants and treated plants. However, no differences were found during the experimental time.

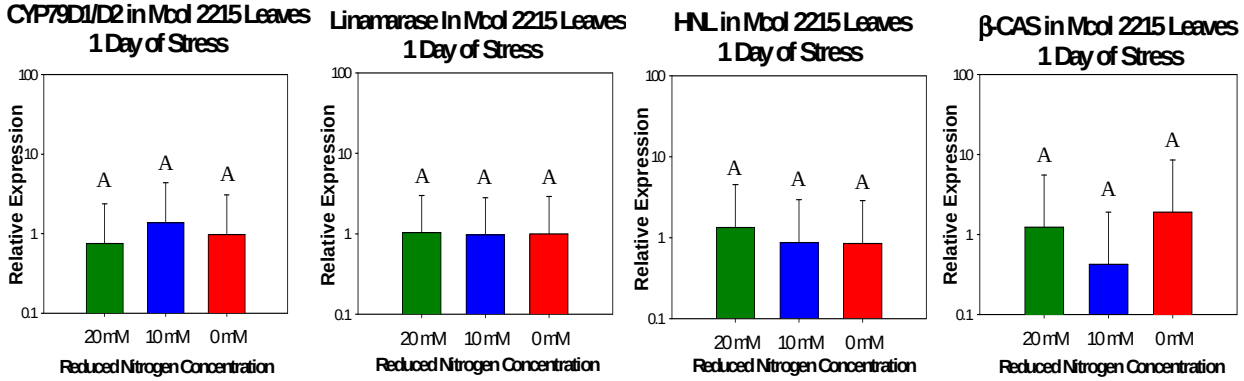
6.5.2 Comparison of transcriptional Activity in Mcol 2215

The Mcol 2215 plants with 1 day of stress showed only a significant increase in CYP79D1/D2 gene and linamarase gene activity in roots (figure 14 and table 10). This result shows an independent and quick response in roots that suggests the importance of cyanogenic glycosides synthesis in this tissue.

The CYP79D1/D2 result in roots agrees with previous studies conducted in other plants (Busk and Møller, 2002), in which this gene family is regulated by nitrate enriched environments. Thus, according with our result it is possible that in cassava these genes are also regulated at the transcriptional level, although other test (like linamarin quantification) is required for validating this. On the other hand, our linamarase result in roots is novel considering the studies in barley of Fourslound and Jonsson (1997) where no special changes in β-glucosidase activity were detected. However it must be noted that the present results are at the transcriptional level and the involvement of post-transcriptional factor in linamarase regulation in cassava is unknown.

No changes were observed in HNL or β–CAS. In the specific case of HNL it is important to remember that perhaps post-transcriptional factors are involved in root regulation (see sections 5.5.3) but in the case of β–CAS, the results agree with Fourslound and Jonsson (1997). Thus, for this specific condition hypothesis 1 was verified only in roots as we observed the increase of CYP79D1/D2; hypothesis 2 was not verified because no changes were detected in β-CAS. Meanwhile the third hypothesis was not verified because changes in linamarase expression in roots were not detected.

A. Leaves



B. Roots

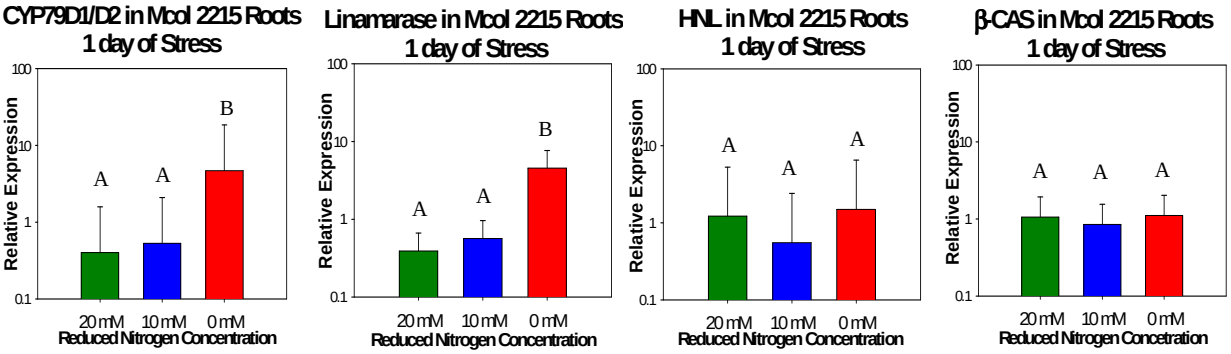


Figure 14: Genes Expression Profiles in Mcol 2215 plants after one day of reduced nitrogen stress. Panel A: Result in Leaves; Panel B: Result in Roots. Three treatments were used: Control (20 mM of reduced nitrogen), Intermediate condition (10 mM of reduced nitrogen), Without condition (total reduced nitrogen depleted). Results are presented for leaves and roots for CYP79D1/D2, linamarase, HNL and β-CAS. No differences were found for any genes in leaves. In roots the genes CYP79D1/D2 and linamarase increased their transcriptional activity in direct relation to reduce nitrogen depletion; nevertheless only the expression pattern illustraded for CYP79D1/D2 is according with the hyphotesis waited. The genes linamarase and HNL in roots did not change their transcriptional activity under nitrogen stress in relation to control. The bar error is the upper value of 90% the confidence interval.. A different letter above the bars indicates significant differences ($p <= 0.10$).

The Cultivar Mcol 2215 after ten days of reduced nitrogen stress did not show the same pattern found in the plants under stress for one day (figure 15 and table 11). In CYP79D1/D2 gene and linamarase gene in roots did not show the same increase reported for one day of stress, meanwhile a significant and special pattern was found in the transcriptional activity of β-CAS in leaves. This gene reduced its transcriptional activity in direct relation with the reduction of NH_4^+, which could imply that amino acid

synthesis from linamarin in leaves is also reduced. Therefore the two first hypotheses were not verified.

Table 10. **Expression Ratios and Confidence intervals in Mcol 2215 plants under one day of reduced nitrogen Stress**

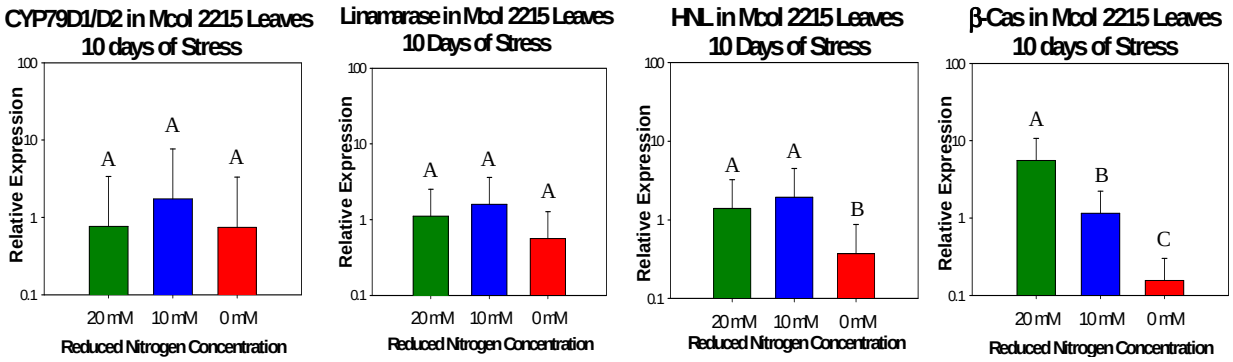
Tissue	Gene	Comparison	Means Differences: Ratio	Two sides C. I for means differences (α = 10%)	Contrast. p-value
Leaves	CYP79D1/D2	Intermediate/Standard	1.84	(0.36; 9.38)	0.4915
Leaves	CYP79D1/D2	Intermediate/Without	1.41	(0.28; 7.16)	0.6878
Leaves	CYP79D1/D2	Without/Standard	1.3	(1.19; 95.67)	0.7667
Leaves	Linamarase	Standard/Intermediate	1.07	(0.23; 4.82)	0.9285
Leaves	Linamarase	Standard/Without	1	(0.23; 4.66)	0.965
Leaves	Linamarase	Withou/Intermediate	1.04	(0.21; 4.65)	0.9634
Leaves	HNL	Standard/Without	1.57	(0.28; 8.75)	0.6318
Leaves	HNL	Standard/Intermediate	1.53	(0.27; 8.51)	0.9808
Leaves	HNL	Intermediate/Without	1.03	(0.18; 5.74)	0.6485
Leaves	Beta-Cas	Without/Standard	1.55	(0.48; 4.92)	0.705
Leaves	Beta-Cas	Withou/Intermediate	4.45	(1.40; 14.22)	0.219
Leaves	Beta-Cas	Standard/Intermediate	2.89	(0.90; 9.2)	0.367
Roots	CYP79D1/D2	Without/Standard	11.55	(1.66; 80.44)	0.0502*
Roots	CYP79D1/D2	Without/Intermediate	8.75	(1.26; 60.96)	0.0736*
Roots	CYP79D1/D2	Intermediate/Standard	1.31	(0.20; 9.19)	0.7894
Roots	Linamarase	Without/Standard	11.55	(5.50; 24.25)	0.0007*
Roots	Linamarase	Without/Intermediate	8	(3.81; 16.80)	0.0017*
Roots	Linamarase	Intermediate/Standard	1.44	(0.69; 3.03)	0.3709
Roots	HNL	Without/Standard	1.23	(0.15; 9.85)	0.8525
Roots	HNL	Withou/Intermediate	1.44	(0.34; 21.70)	0.3877
Roots	HNL	Standard/Intermediate	2.2	(0.28; 17.63)	0.4889
Roots	Beta-Cas	Without/Standard	1.05	(0.44; 1.05)	0.9167
Roots	Beta-Cas	without/Intermediate	1.3	(0.55; 3.07)	0.5778
Roots	Beta-Cas	Standard/Intermediate	1.23	(0.52; 2.93)	0.6487

Each Ratio is interpreted as: In Mcol 2215 (leaves/Roots) under 1 day of stress, the mean amount of RNAm from (*CYP79D1/D2/ Linamarase/HNL/β-CAS*) gene is (*Ratio Value*) times larger in (*Fraction Numerator in Comparison Cells*) that in (*Fraction denominator in Comparison Cells*) (according to the 90% two-sided confidence interval for mean differences, it is at least (*Lower C.I Value*) and not more than (*upper C.I Value*)**.** The ratio (*is/ is not*) significantly different from 1 using α = 0.10 (analysis done using contrast for log transformed data).* Indicates significant differences with a significance of 10%. The corresponding descriptive statistics and statistical comparisons are in appendix G.

On the other hand, under the same 10 day of stress a similar pattern of expression was observed among linamarase and HNL in leaves as well as roots (figure 15): under Intermediate condition the activity of both genes increased in comparison to standard condition and without reduced nitrogen condition. A possible explanation for this

behavior is not clear, considering that both genes are involved in cyanogenesis under disruption; however this result evidences (again) the co-regulation among these genes illustrated in the section 5.5.5. For this reason under this specific condition the hypothesis number three was not verified.

A. Leaves



B. Roots

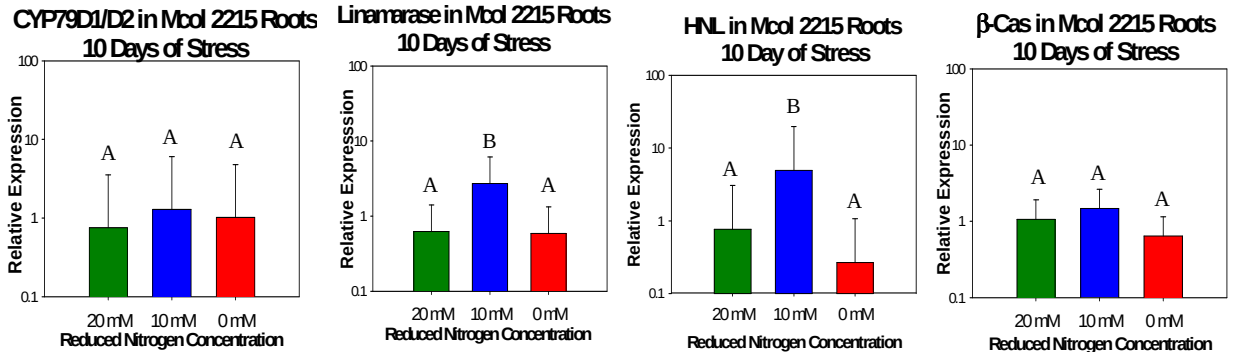


Figure 15: Genes Expression Profiles in Mcol 2215 plants after ten days of reduced nitrogen stress.
Panel A: Result in Leaves; Panel B: Result in Roots Three treatments were used: Control (20 mM of reduced nitrogen), Intermediate condition (10 mM of reduced nitrogen), Without condition (total reduced nitrogen depleted). Results are presented for leaves and roots for CYP79D, linamarase, HNL and β-CAS. In leaves only difference found was among the treatments in β-CAS. This gene decreases its transcriptional activity in relation to reduce nitrogen depletion; this behavior is not according with the hypothesis waited. In roots the genes HNL and linamarse increased their transcriptional activity in the intemdial conditios and the reason for this gene pattern is not clear. The genes CYP79D1/D2 and β-CAS linamarase did not change their transcriptional activity under nitrogen stress in relation to control. The bar error is the upper value of 90% the confidence interval. A different letter above the bars indicates significant differences ($p <= 0.10$).

Table 11: Expression Ratios and Confidence intervals in Mcol 2215 plants after ten days of reduced nitrogen Stress

Cultivar	Tissue	Gene	Comparison	Means Differences: Ratio	Two sides C. I for means differences (α = 10%)	Contrast. p-value
Mcol 2215	Leaves	CYP79D1/D2	Intermediate/Standard	2.28	(0.28; 18.64)	0.4762
Mcol 2215	Leaves	CYP79D1/D2	Intermediate/Without	2.33	(0.28; 19.03)	0.4647
Mcol 2215	Leaves	CYP79D1/D2	Standard/Without	1.02	(0.13; 8.33)	0.984
Mcol 2215	Leaves	Linamarase	Standard/Without	1.97	(0.62; 6.23)	0.2977
Mcol 2215	Leaves	Linamarase	Standard/Intermediate	1.44	(0.45; 4.56)	0.5613
Mcol 2215	Leaves	Linamarase	Intermediate/Without	2.84	(0.90; 9)	0.1298
Mcol 2215	Leaves	HNL	Standard/Without	3.76	(0.57; 6.23)	0.0759*
Mcol 2215	Leaves	HNL	Intermediate/Standard	1.39	(0.42; 4.60)	0.6207
Mcol 2215	Leaves	HNL	Intermediate/Without	5.21	(1.57; 17.27)	0.0373*
Mcol 2215	Leaves	β-CAS	Standard/Without	35.51	(14.03; 89.88)	0.0003*
Mcol 2215	Leaves	β-CAS	Standard/Intermediate	4.79	(1.89; 12.12)	0.0166*
Mcol 2215	Leaves	β-CAS	Intermediate/Without	7.41	(2.93; 18.77)	0.0058*
Mcol 2215	Roots	CYP79D1/D2	Without/ Standard	1.34	(0.15; 11.96)	0.7957
Mcol 2215	Roots	CYP79D1/D2	Intermediate/Standard	1.7	(0.26; 20.25)	0.6501
Mcol 2215	Roots	CYP79D1/D2	Intermediate/Without	1.26	(0.14; 11.24)	0.8432
Mcol 2215	Roots	Linamarase	Standard/Without	1.06	(0.29; 3.61)	0.9363
Mcol 2215	Roots	Linamarase	Intermediate/Without	1.97	(0.54; 7.11)	0.0604*
Mcol 2215	Roots	Linamarase	Intermediate/Standard	2.12	(1.20; 15.67)	0.0677*
Mcol 2215	Roots	HNL	Standard/Without	2.85	(1; 51.62)	0.62
Mcol 2215	Roots	HNL	Intermediate/Standard	4.65	(0.90; 46.20)	0.0248*
Mcol 2215	Roots	HNL	Intermediate/Without	18.38	(2.57; 131.60)	0.0129*
Mcol 2215	Roots	β-CAS	Standard/Without	1.66	(0.72; 3.81)	0.2838
Mcol 2215	Roots	β-CAS	Intermediate/Standard	1.38	(0.60; 3.18)	0.4746
Mcol 2215	Roots	β-CAS	Intermediate/Without	2.3	(1; 5.28)	0.1005

Each Ratio is interpreted as: In Mcol 2215 (leaves/Roots) under 10 day of stress, the mean amount of RNAm from (*CYP79D1/D2/ Linamarase/HNL/β-CAS*) gene is (*Ratio Value*) times larger in (*Fraction Numerator in Comparison Cells*) that in (*Fraction denominator in Comparison Cells*) (according to the 90% two-sided confidence interval for mean differences, it is at least (*Lower C.I Value*) and not more than (*upper.C.I Value*)**.** The ratio (*is/ is not*) significantly different from 1 using α = 0.10 (analysis done using contrast for log transformed data).
* Indicates significant differences with a significance of 10%. The corresponding descriptive statistics and statistical comparisons are in appendix G.

6.5.3 Comparison of transcriptional Activity in 60444

The data from cultivar 60444 illustrated in figure 15 and table 13 show a high variability in comparison with the Mcol 2215 results (this is also appreciated in the tables of appendix G). This condition limits a satisfactory data interpretation. Besides, it must be

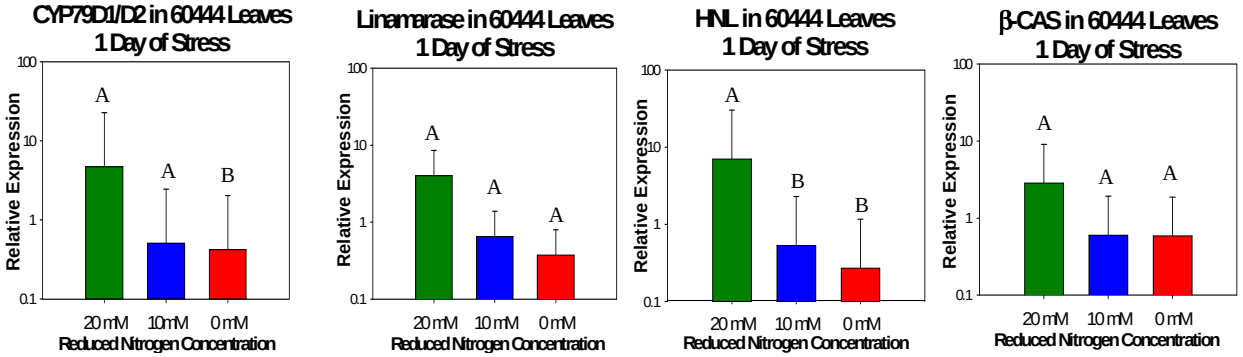
noted that in the data from leaves after one day of stress under the standard condition the 18S rRNA had an unusual behavior: the Ct were higher relative to the other two conditions (data not shown). The reason for this event is unknown and evidences the need for other housekeeping genes in future experiments of gene regulation in cassava.

In cultivar 60444 leaves after 1 day of stress, all the genes reduced the transcriptional activity compared to the standard condition (figure 16). Even though siginificant differences were observed in CYP79D1/D2 and HNL we believe it maybe due to the unusual behavior of the 18S rRNA (high Ct) and thus cannot be used for any further conclusions regarding the activity patterns of the genes unfortunately.

In the roots of cultivar 60444 after 1 day of stress, the CYP79D1/D2 genes increased its transcriptional activity under reduced nitrogen stress condition, although only a borderline difference was found among standard condition and without nitrogen condition (perhaps due to the data variability and sample size; figure 16 and table 12). Therefore, it seems that the hypothesis number 1 is partially verified although data noise prevented finding statistical differences. It is important to remember that this gene expression pattern was similar to that found in Mcol 2215 after 1 day of stress, although with significant differences (Figure 14 table 10). Meanwhile β-CAS in roots showed a reduction in transcriptional activity in the stress condition. This pattern was not found in roots of Mcol 2215 under the same condition. Hence the hypothesis number 3 was not verified.

Linamarase and HNL in roots did not have significant differences and no pattern was appreciated. For this reason hypothesis number 3 was verified in roots.

A. Leaves



B. Roots

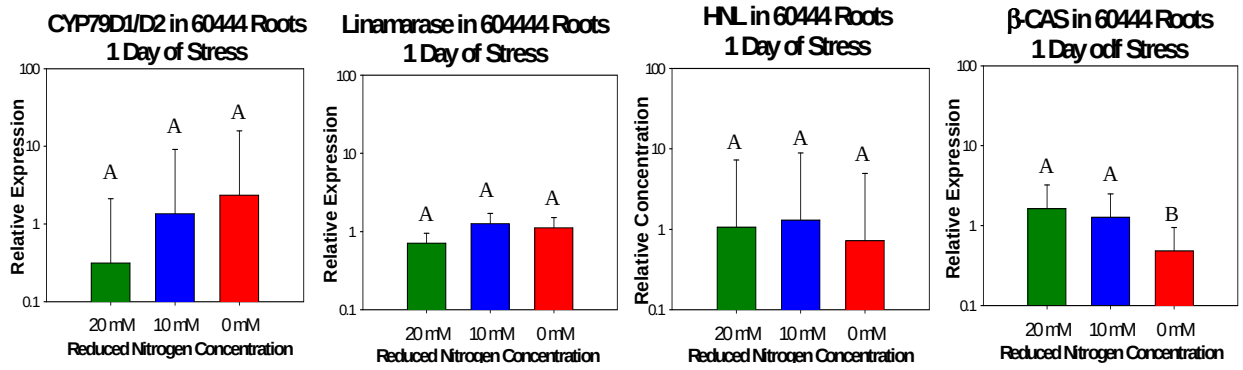


Figure 16: Genes Expression Profile in 60444 plants after one day of reduced nitrogen stress.
Panel A: Result in Leaves; Panel B: Result in Roots. Three treatments were used: Control (20 mM of reduced nitrogen), Intermediate condition (10 mM of reduced nitrogen), Without condition (total reduced nitrogen depleted). Results are presented for leaves and roots for CYP79D1/D2, linamarase, HNL and β-CAS. In leaves there was a decrease of transcriptional activity in relation to reduce nitrogen depletion, however, this result is due to changes in 18s rRNA normalizer. Thus, the results in this tissue under this specific condition are not considered. In roots there is an increase in the transcriptional activity of CYP79D1/D2 in relation to reduce nitrogen depletion; meanwhile, β-CAS gene decreases its transcriptional activity in relation to reduce nitrogen depletion. The genes linamarase and HNL in roots did not change their transcriptional activity under nitrogen stress in relation to control. The bar error is the upper value of 90% the confidence interval. A different letter above the bars indicates significant differences ($p <= 0.10$).

The results in cultivar 60444 after ten days of stress in leaves show an incerment in the transcriptional activity of HNL (figure 17 and table 13). This event is not clear considering that this gene works when the plant is disrupted. Thus hypothesis number 3 was not verified. Meanwhile the behavior of β-CAS in this tissue showed the same pattern of transcriptional activity decrease as Mcol 2215 leaves after 10 days and 60444 roots after 1 day. However, only borderline differences were found among the standard

condition and without condition (table 13). Hence hypothesis number 2 was not verified. It must be noted that CYP79D and linamarase did not show significant differences.

Table 12: Expression Ratios and Confidence intervals in cultivar 60444 after one day of reduced nitrogen Stress

Tissue	Gene	Comparison	Means Differences: Ratio	Two sides C. I for means differences (α = 10%)	Contrast. p-value
Leaves	CYP79D1/D2	Standard/Without	4039.6	(437.5; 37380)	0.0803*
Leaves	CYP79D1/D2	Standard/Intermediate	3691.52	(398.93; 34159.52)	0.1005
Leaves	CYP79D1/D2	Intermediate/Without	1.1	(0.12; 10.12)	0.8765
Leaves	Linamarase	Standard/Without	10.7	(0.85; 133.44)	0.1183
Leaves	Linamarase	Standard/Intermediate	6.15	(0.49; 76.64)	0.2125
Leaves	Linamarase	Intermediate/Without	1.86	(0.15; 23.26)	0.6844
Leaves	HNL	Standard/Intermediate	13.27	(1.68; 104.70)	0.0512*
Leaves	HNL	Standard/Without	26	(3.29; 205)	0.022 *
Leaves	HNL	Intermediate/Without	1.96	(4.02; 15.45)	0.5471
Leaves	β-CAS	Standard/Without	4.85	(0.94; 25.11)	0.1112
Leaves	β-CAS	Standard/Intermediate	4.76	(0.92; 24.59)	0.115
Leaves	β-CAS	Intermediate/Without	1.02	(0.20; 5.28)	0.9813
Roots	CYP79D1/D2	Without/Standard	7.46	(0.5; 111.43)	0.1983
Roots	CYP79D1/D2	without/Intermediate	1.73	(0.11; 25.81)	0.7088
Roots	CYP79D1/D2	Intermediate/Standard	2.11	(0.29; 64.44)	0.3322
Roots	Linamarase	Without/Standard	1.58	(0.47; 5.35)	0.4957
Roots	Linamarase	Intermediate/Standard	1.79	(0.53; 6.06)	0.3930
Roots	Linamarase	Intermediate/Without	1.13	(0.33; 3.84)	0.8518
Roots	HNL	Standard/Without	1.47	(0.097; 22.32)	0.7935
Roots	HNL	Intermediate/Standard	1.22	(0.08; 18.50)	0.8879
Roots	HNL	Intermediate/Without	1.8	(0.12; 27.28)	0.6885
Roots	β-CAS	Standard/Without	3.4	(1.30; 8.82)	0.0479*
Roots	β-CAS	Standard/Intermediate	1.28	(0.49; 3.34)	0.6313
Roots	β-CAS	Intermediate/Without	2.64	(1.01; 6.87)	0.0959*

Each Ratio is interpreted as: In 60444 (leaves/Roots) under 1 day of stress, the mean amount of RNAm from (*CYP79D1/D2/ Linamarase/HNL/β-CAS*) gene is (*Ratio Value*) times larger in (*Fraction Numerator in Comparison Cells*) that in (*Fraction denominator in Comparison Cells*) (according to the 90% two-sided confidence interval for mean differences, it is at least (*Lower C.I Value*) and not more than (*upper.C.I Value*)**.** The ratio (*is/ is not*) significantly different from 1 using α = 0.10 (analysis done using contrast for log transformed data).* Indicates significant differences. The corresponding descriptive statistics and statistical comparisons are in appendix G.

In roots of cultivar 60444 after ten days of stress no significant differences were found in the expreesion of CYP79D1/D2 among the three nitrogen conditions (figure 17, table 13). This result is similar to the result shown in Mcol 2215 for this gene. Thus, it is

concluded that in both cultivars the gene CYP79D1/D2 is activated under reduced nitrogen depletion in the first 24 hours, but this activation is not detected 10 days after. Therefore we consider that the hypothesis 1 under this condition is not verified in roots. Finally, regarding the expression of HNL and Linamarase in roots after ten days of stress, it also was characterized for a non clear pattern of expression and for the absence of significant differences (figure 17, table 13). In this case the hypothesis 3 were not verified.

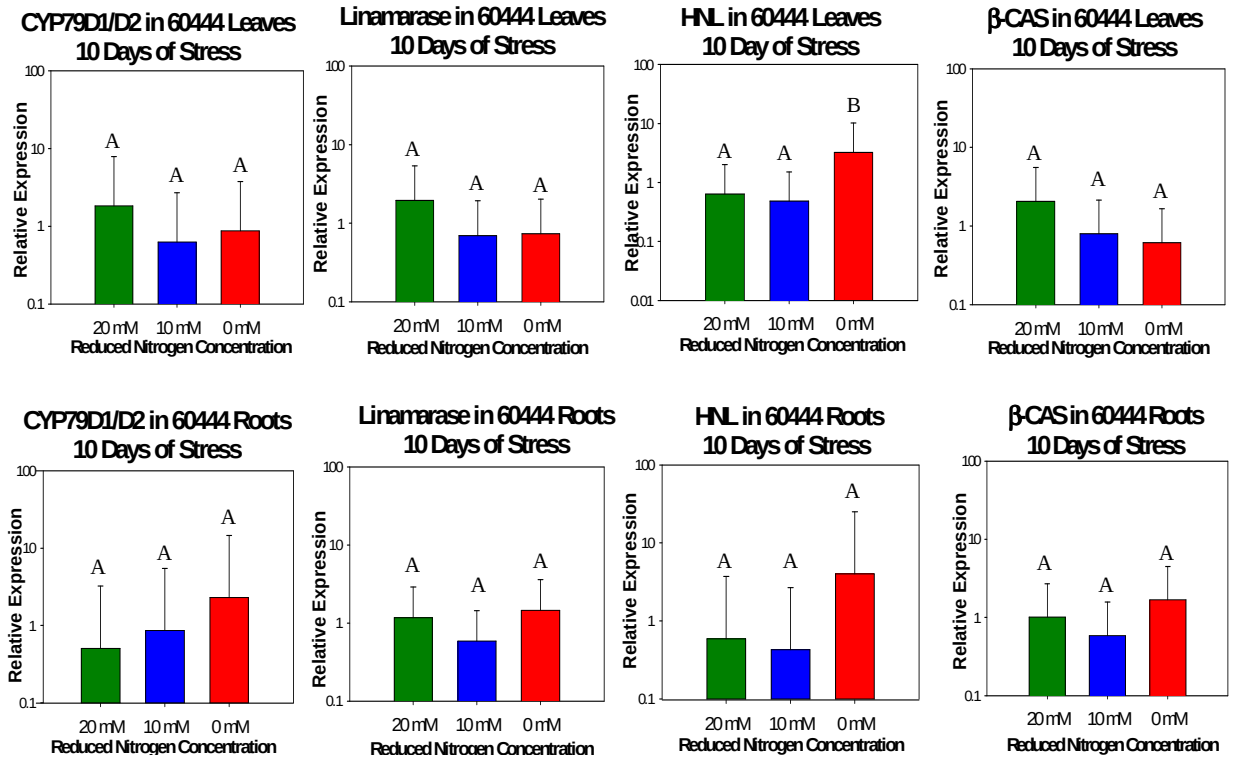


Figure 17: Genes Expression Profile in 60444 plants under ten day of reduced nitrogen stress.
Panel A: Result in Leaves; Panel B: Result in Roots. Three treatments were used: Control (20 mM of reduced nitrogen), Intermediate condition (10 mM of reduced nitrogen), Without condition (total reduced nitrogen depleted). Results are presented for leaves and roots for CYP79D1/D2, linamarase, HNL and β-CAS. In leaves there was an increment in the transcriptional activity of HNL in relation to reduce nitrogen depletion. This result is not in agreementaccording with the hypothesis proposed in the study. Comparatively, in this tissue β-CAS reduced its transcriptional activity in relation to reduce nitrogen depletion; this result is not waited according with the hypothesis. In roots although there is an increase in the transcriptional activity of CYP79D1/D2 in relation to reduce nitrogen depletion, it this is not signicant. Similarly, the other genes studied in this tissue did did not change significantly their transcriptional activity under nitrogen stress in relation to control. The bar error is the upper value of 90% the confidence interval. A different letter above the bars indicates significant differences ($p <= 0.10$).

Table 13: Expression Ratios and Confidence intervals in cultivar 60444 after ten day of reduced nitrogen Stress

Tissue	Gene	Comparison	Means Difference: Ratio	Two sides C. I for means differences (α = 10%)	Contrast. p-value
Leaves	CYP79D1/D2	Standard/Without	2.08	0.27; 16.33	0.5136
Leaves	CYP79D1/D2	Standard/Intermediate	2.93	0.37; 22.94	0.352
Leaves	CYP79D1/D2	without/Intermediate	1.4	0.18; 11	0.7636
Leaves	Linamarase	Standard/Without	2.64	0.63; 11.08	0.2397
Leaves	Linamarase	Standard/Intermediate	2.79	0.66; 11.71	0.2159
Leaves	Linamarase	without/Intermediate	1.06	0.25; 4.44	0.9401
Leaves	HNL	Without/Standard	5.1	1; 25.63	0.0973*
Leaves	HNL	Standard/Intermediate	1.32	0.26; 6.63	0.7507
Leaves	HNL	without/Intermediate	6.73	1.34; 33.82	0.0615*
Leaves	β-CAS	Standard/Without	3.36	0.84; 13.55	0.143
Leaves	β-CAS	Standard/Intermediate	2.58	1.52; 10.41	0.2363
Leaves	β-CAS	Intermediate/Without	1.3	0.32; 5.24	0.7248
Roots	CYP79D1/D2	Without/Standard	4.53	0.33; 62.25	0.304
Roots	CYP79D1/D2	without/Intermediate	2.67	0.19; 36.75	0.4924
Roots	CYP79D1/D2	Intermediate/Standard	1.7	0.12; 23.26	0.7079
Roots	Linamarase	Without/Standard	1.24	0.097; 15.78	0.8762
Roots	Linamarase	Standard/Intermediate	2	0.31; 50.91	0.613
Roots	Linamarase	Without/Intermediate	2.48	0.19; 31.56	0.5126
Roots	HNL	Without/Standard	2.76	2.53; 18.13	0.2024
Roots	HNL	Standard/Intermediate	1.38	0.52; 3.71	0.8154
Roots	HNL	Without/Intermediate	9.38	3.51; 25.11	0.145
Roots	β-CAS	Without/Standard	1.66	0.41; 6.63	0.5046
Roots	β-CAS	Standard/Intermediate	1.73	0.43; 6.91	0.4715
Roots	β-CAS	Without/Intermediate	2.87	0.72; 11.47	0.1899

Expression Ratios and Confidence intervals in cultivar 60444 under one day of stress.
Each Ratio is interpreted as: In 60444 (leaves/Roots) under 10 day of stress, the mean amount of RNAm from (*CYP79D1/D2/ Linamarase/HNL/β-CAS*) gene is (*Ratio Value*) times larger in (*Fraction Numerator in Comparison Cells*) that in (*Fraction denominator in Comparison Cells*) (according to the 90% two-sided confidence interval for mean differences, it is at least (*Lower C.I Value*) and not more than (*upper C.I Value*)**.** The ratio (*is/ is not*) significantly different from 1 using α = 0.10 (analysis done using contrast for log transformed data).
* Indicates significant Differences. The corresponding descriptive statistics and statistical comparisons are in appendix G

6.5.4 Cluster Analysis for Gene Classification

The results of cluster classification show two clusters: 1. CYP79D1/D2 and β-CAS and 2. Linamarase and HNL (Figure 18). These results agree with previous results illustrated

in section 5.5.5, where is suggested that the cluster between CYP79D1/D2 and β-CAS is related with metabolism under plant disruption; meanwhile the cluster between linamarase and HNL is related to the nitrogen turnover in intact plants. It is important to consider that in the present chapter the genes CYP79D1/D2 and β-CAS had a direct or inverse answer to the reduced nitrogen concentration, which could be in relation with this cluster result (see section 6.5.5).

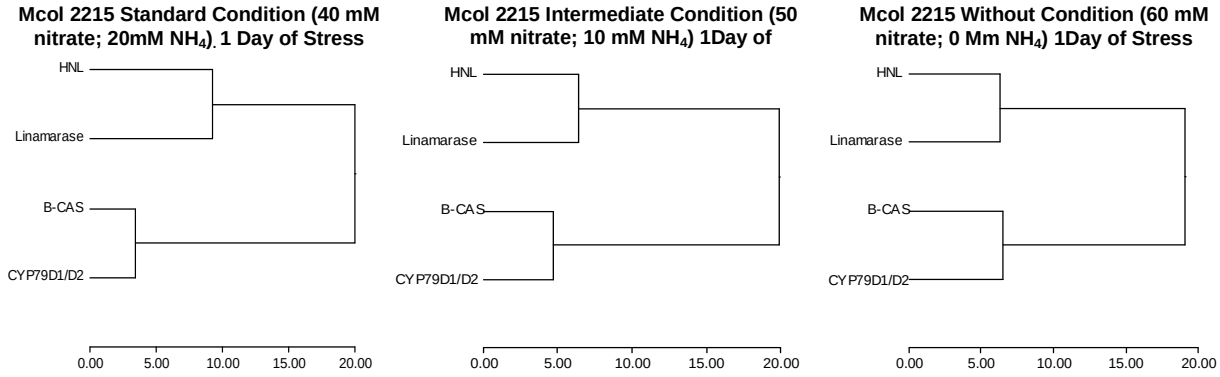


Figure 18: Clusters of gene classification in Mcol 2215 plants under 1 day of stress.
The data from leaves and roots were analyzed together. The same classification pattern was found in the 10 days of stress condition in Mcol and 60444. The cluser generated are according with the spatial separation in the cell wall of the HNL protein and linamarase protein, which suggest that those enzymes are more related in the metabolism under plant disruption; meanwhile CYP79D1/D2 and β-CAS are related to the reduced nitrogen metabolism

6.5.5 Suggestions

Although a first approach to our data seems inconclusive through the statistical comparisons, it is important to highlight that Real Time PCR data are known for their high variability (Pfaff 2004). Thus, is possible to explain the borderline differences in some comparisons with the sample size used (n=3) and for the high variance, especially in the data from 60444.

Consistent with the fact that cyanogenic glycosides are not normally broken down in the intact plant because the glycosides and degradative enzymes are spatially separated, we considered in the hypothesis that the plants under reduced nitrogen stress respond by changing the transcriptional activity of CYP79D1/D2 and β-CAS to regulate their nitrogen levels. Although linamarase and HNL could be impacted by the stress, it is not believed that these genes control the nitrogen turnover in the plant. These assumptions are related with the cluster classifications illustrated in section 6.5.4 (where CYP79D1/D2 and β-CAS belong to one cluster different from linamarase/HNL cluster) and with past research findings. Thus, according with this, the results obtained showed an interesting pattern where the expression of CYP79D1/D2 and β-CAS is directly or inversely related with the concentration of reduced nitrogen:

- In roots of both Mcol 2215 and 60444[5] after one day of stress the expression of CYP79D1/D2 increases in an inverse relationship with the reduced nitrogen concentration.
- In leaves of both Mcol 2215 and 60444[6] after 10 days of stress and in roots of 60444 under 1 day of stress there was a decrease of transcriptional activity of β-CAS in a direct relationship with the reduced nitrogen concentration

Meanwhile in the genes linamarase and HNL, although some comparisons showed significant differences, an expression pattern related to the reduced nitrogen concentration was not found:

[5] It is important remember that in 60444 statistical differences were not found (border line differences could be proposed, following Table 12 and 13), however it was appreciated the same trend in the means founded in Mcol 2215. It is suggested that the ausence of statistical differences in 60444 is because the data variability is high.

- In roots of Mcol 2215 after one day of stress there was an increase of the transcriptional activity of linamarase gene under total reduced nitrogen depletion.
- In the leaves and roots of Mcol 2215 there was an increase in the activity of linamarase and HNL in Intermediate condition and a decrese under 10 days of stress
- In leaves of cultivar 60444 after 10 days of stress was found an increase in the activity of HNL gene under nitrogen depletion.

Thus, focusing in the gene expression patterns of CYP79D1/D2 and β-CAS, we can conclude that in cassava roots there is a trend to increase the cyanogenic glycoside synthesis (more CYP79D1/D2 transcription) in a inverse relationship with reduced nitrogen supply in the first 24 hours of stress; even though these cyanogenic glycosides will not be used for high rates of amino acid synthesis, considering the trends in β-CAS in roots to remain constant or to decrease. In the subsequent 10 days, the CYP79D1/D2 activity in roots under stress conditions is similar to the control, however the activity of β-CAS in leaves decrease with reduce nitrogen depletion, which could imply low rates in the amino acid synthesis.

Possible explanation for these behaviors requires considering the special characteristics of our experiment. Thus, although we tested the plants under reduced nitrogen stress, we can not disregard that the total nitrogen levels in the media remain constant independent of the reduced nitrogen concentration (the reduced nitrogen was changed for nitrate). Thus it is possible that the increase in CYP79D1/D2 activity is due to the nitrate increments and not to the NH_3 depletion, like what was reported by Busk and

Møller (2002) in sorghum plants, which increased the activity of CYP79A1 with more nitrate (fertilizer). However in our study as well as in Busk and Møller (2002) a physiological explanation for this event is not clear.

The reduction in β-CAS activity could also be explained through the nitrate present in the media. Therefore, it is possible that the plants prefer the nitrate for amino acid synthesis (through nitrate reduction to NH_3 using nitrate reductase and nitrite reductase and subsequent NH_3 use for glutamate, glutamine, aspartate and asparagine synthesis) instead of cyanogenic glycosides (through re-assimilations steps which release asparagine, aspartate and NH_3, see figure 2). Thus, with this alternative pathway, the levels of NH_3 in our experimental plants could increase or a least compensate for the levels not present in the media. Therefore, one alternative experiment could be to test the genes in cassava plants grown in an *in vitro* medium without any nitrogen source (without reduced nitrogen and nitrate). Another experiment that could be performed is to evaluate the activity of nitrate reductase and nitrite reductase in cassava roots and leaves. Even though enzyme activity can be performed, it is not possible to evaluate the transcriptional activity of nitrate reductase and nitrite reductase since the sequences of these have not been isolated in cassava.

Finally, it must be noted that although both cultivars had a reduction in β-CAS activity in the leaves at 10 days of stress, cultivar 60444 also had a reduction of this activity in roots at 1 day of stress. The physiological explanation for this behavior could be the same offered in the last paragraph; nevertheless this event highlights that responses to stress could be dependent on the genotype (cultivar).

6.6 Conclusions

- The change of reduced nitrogen for nitrate affected the transcriptional activity of the genes involved in cyanogenic glycoside synthesis in cassava.
- Although the data were characterized for a high variability and a small sample size (n=3), the roots of both cultivars after 1 day of stress showed a reponse in CYP79D1/D2 gene, which is inversly related with reduced nitrogen concentration. Likewise the leaves of both cultivars under 10 days of stress showed a reponse in β-CAS directly related with reduce nitrogen concentration.
- The roots of 60444 after 1 day of stress also presented a response in β-CAS directly related with reduce nitrogen concentration. It is suggested that this response is exclusive of this cultivar.
- Although linamarase and HNL were affected in some conditions, a clear pattern of response was not observed. This is suppoted by the facts that these genes appear to be co-regulated; they work in plants only after disruption and for this reason they probably do not influence the nitrogen turnover in the plant.
- It is suggested that the increase in the CYP79D1/D2 activity is due to the increment in nitrate concentration in the media. However a clear physiological explanation is not offered.
- It is suggested that the plant compensates the NH_3 depletion through conventional nitrate assimilation instead of cyanogenic glycoside re-assimilation.

CHAPTER SEVEN:
QUANTIFICATION OF TRANSCRIPTIONAL ACTIVITY OF GENES INVOLVED IN THE CYANOGENIC GLYCOSIDES PATHWAYS IN TWO CASSAVA CULTIVARS UNDER *IN-VITRO* TEMPERATURE STRESS

7.1 Literature Review

Although the cyanogenesis is a plant characteristic controlled by different genes, it is also affected by environmental factors (see chapter 6) or developmental factors (Santana et al., 2002). Thus, abiotic and biotic factors have been related to the level of cyanogens in plants. For example in *Lotus spp*.**,** a plant with cyanogenic glycosides linamarin and lotaustarin, it has been shown to have high concentration of hydrogen cyanide occurence in the summer and spring while low levels occur in the winter (Gebrehiwot and Beuselinck, 2001). Vickery et al. (1987) showed in white clover (*Trifolium repens)* that the HCN concentration was reduced by high light intensity, high temperature and phosphorus application.

In cassava, there is evidence that shows a correlation between low rainfall and an increase in the cyanogen content of cassava roots (Bokanga et al., 1994). Besides in time of drought the number incidences of acute cyanogen poisoning for cassava increases in Africa. Cardoso et al. (1999) showed that the concentration of cyanogens in cassava were lower in the harvest months (August to October) of 1996, 1997 and 1999 if there was normal or high rainfall during the previous rainy season. The very high concentrations found in November 1998 and July 1999 was probably due to the fact that the roots from which the flour was produced were grown during a period of low rainfall.

Temperature is an environmental factor associated with drought. It affects the cyanide potential in other plants. Thus, Stochmal and Oleszek (1997) found in white clover that during the periods when temperature is below 15 ℃ and vegetative growth is very slow high levels of cyanogens occurred. Meanwhile cyanogen production decreased when

environmental conditions for rapid re-growth improved and grazing created little hazard for the plant. However the conclusions of this study contrast with the results found by Yu et al. (2005) who studied the effect of temperature on the rate of cyanide metabolism in two woody plants, weeping willow (*Salix babylonica* L.) and Chinese elder (*Sambucus chinensis* Lindl.) with the objective of testing their capacity for soil detoxification (phytoremediation). The plants in cyanide solution with elevated temperatures removed the cyanide faster (>24^{o}C) although the cyanide metabolism rate of the weeping willow was more susceptible to changes in temperature than that of the Chinese elder. Thus, environmental factors like temperature affect the cyanogen level in plants in different ways generating complex interactions between environmental factors, genetic makeup and developmental history in each individual.

7.2 Specific Objective

To compare the transcriptional activity of genes involved in cyanogenic glycosides pathways in leaves and roots, between *in vitro* grown Mcol2215 (sweet variety) and 60444 (bitter-sweet variety), under temperature stress conditions.

7.3 Biological Hypothesis

The hypotheses consisted that all the genes (CYP79D1/D2, Linamarase and HNL and β-CAS) change their transcriptional activity in leaves and roots under oscillation temperature stress. This hypothesis is general because no previous study in cassava about this issue has been described.

7.4 Specific Methodology

The cassava cultivars Mcol 2215 (142 ppm cyanogens dry base) and 60444 (182 ppm cyanogens dry base) were used in this study. For each cultivar 15 magenta boxes with 5 explants each were used to grow the plants for 3 months and 18 days in 4E solid media in an incubator at 28^0C. Afterwards the plants were subjected to the temperature test. Five plants from each variety were put in the same temperature (28^0C) (controls) for 8 days; meanwhile 10 plants of each variety were subjected to temperatures oscillation stress (figure 19). This condition consisted in exposing the plants for 12 hours at 35^0C (light) and for 12 hours at 28^0C (darkness) during 8 days. On the eighth day, 5 plants after the 12 hours 35^0C light regime were collected and frozen; and the remaining 5 plants were collected after the 12 hours 28^0C darkness regime.

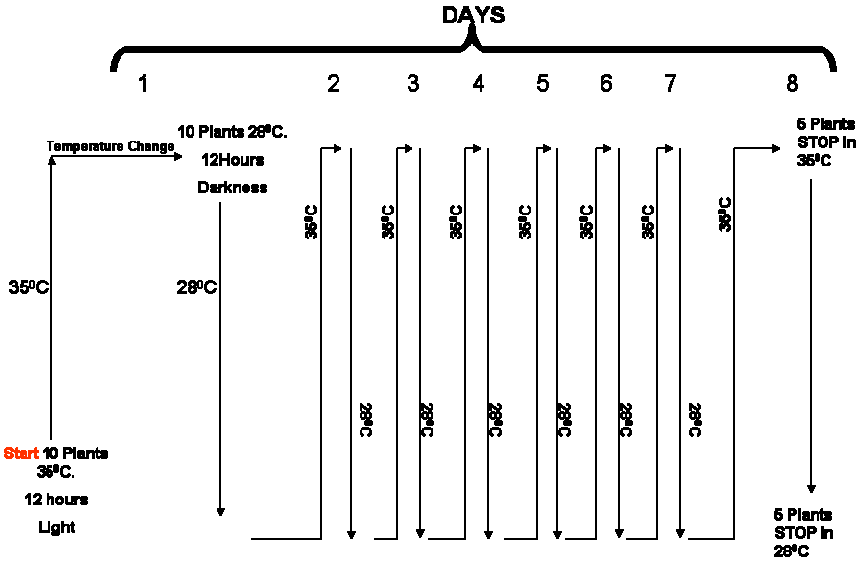


Figure 19: Scheme of stress temperature oscillation.
Ten plants per cultivar were used. On the eight day 5 plants were collected at 35^0C meanwhile the other 5 plants were collected in the end of 28^0C

The samples collected were used subsequently for RNA isolations from leaves and roots, cDNA synthesis and their respective nucleic acid spectrophotometrical quantifications. Real-Time PCR were conducted with the primers CYP79D1/D2,

Linamarase B, HNL, β-CAS and 18S rRNA using three technical repetitions for each amplification. The data were collected according to the recommendations described in section 3.4 and classified in 4 matrices (45x5) for treatment comparison.

Each matrix consisted in the Ct data from the three specific temperature conditions (control at 28^0C constant, and samples from the oscillation temperature stress) using three biological replicates with three technical repetitions. It must be noted that each matrix was specific for one tissue and cultivar. Each matrix was entred into GenEx for the corresponding data pre-processing as described in section 3.4, normalized for primer efficiency as described in appendix D and used the LOD for cut-off data in CYP79D1/D2 and linamarase (see appendix E).

The statistical comparison among cultivars was done using ANOVA for Completely Randomized Design and contrasts at 5% significance levels (n = 5).

7.5 Result and Discussion.

7.5.1 Phenotypical Description.

The plants were monitored daily to evaluate phenotypical differences among control plants and treated plants. However, no phenotypic differences were observed during the experimental time.

7.5.2 Comparison of transcriptional Activity in Mcol 2215

In cultivar Mcol 2215 there were no significant differences in the transcriptional activity of the CYP79D1/D2 gene, linamarase gene and HNL gene in leaves or roots. Thus, with the present data, there was no evidence that suggest that the cyanogenic glycoside amount is influenced by changes in temperature. Only changes in the expression of β-CAS in leaves were found (figure 20 and table 14). There was an increase in the activity in the plants under oscillation temperature stress stopped at 28^0C; and a slight decrease in the transcriptional activity of the plants stopped at 35^0C.

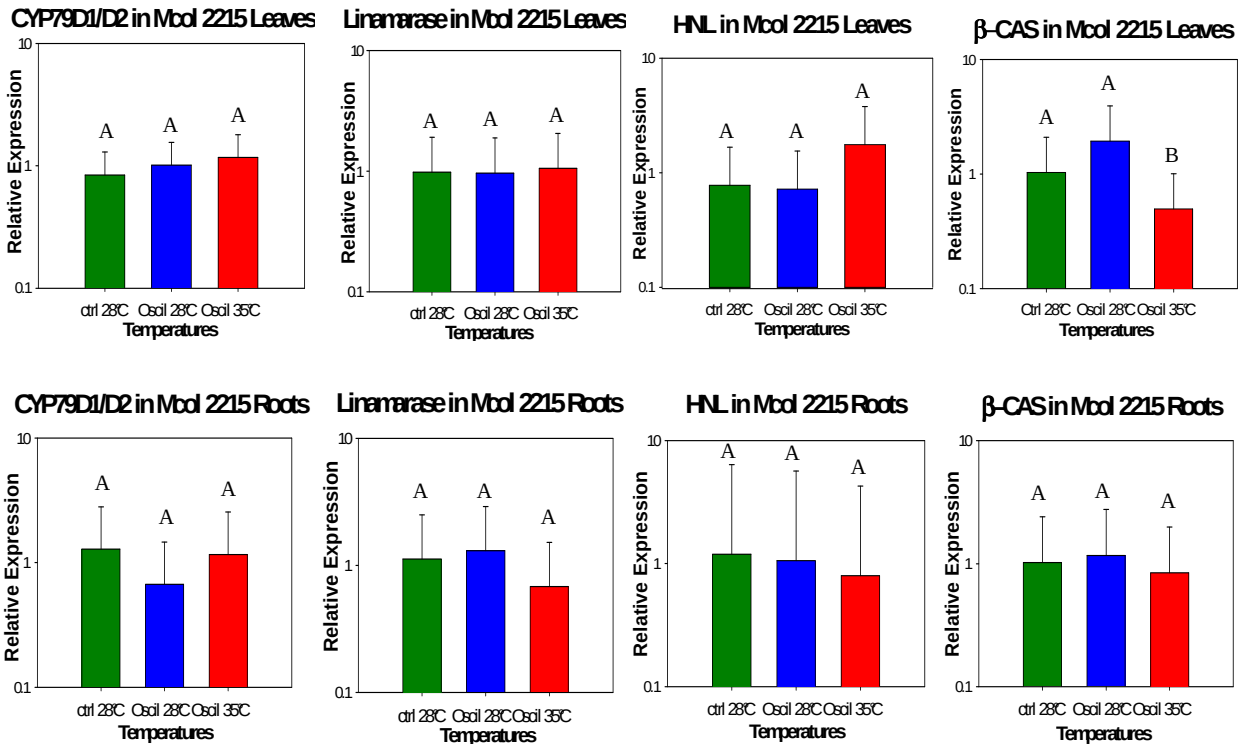


Figure 20: Genes Expression Profile in Mcol 2215 plants under oscilation temperature stress.
Ctrl = Control; Oscil= Oscilation. Three treatments were used: Control, plants under constant temperature (28^0C); Oscilation (28^0C), plants under changes of temperature (28^0C 12 hours – 35^0C 12 hours) stopped the experiment to 28^0C; Oscilation (35^0C), plants under changes of temperature (28^0C 12 hours – 35^0C 12 hours) stopped the experiment to 35^0C. Results are presented for leaves and roots for CYP79D1/D2, linamarase, HNL and β-CAS. No significant changes were detected in all the genes in both tisue with the exception of β-CAS gene in leaves where a slight increase in the transcriptional activity was found in oscilation 28^0C. This result does not seem corelate with changes in the regulation of cyanogenic glycosides metabolism. The bar error is the upper value of 90% the confidence interval. A different letter above the bars indicates significant differences ($p <= 0.10$).

Table 14: Expression Ratios and Confidence intervals in cultivar Mcol 2215 under temperature oscilation stress

Tissue	Gene	Comparison	Means Differences: Ratio	Two sides C. I for means differences (α = 10%)	Contrast. p-value
Leaves	CYP79D1/D2	35^0C-28^0C	1.16	0.63; 2.12	0.6089
Leaves	CYP79D1/D2	35^0C-ST Temp	1.39	0.76; 2.55	0.256
Leaves	CYP79D1/D2	28^0C-ST Temp	1.21	0.66; 2.21	0.5171
Leaves	Linamarase	35^0C-28^0C	1.09	0.42; 2.82	0.8319
Leaves	Linamarase	35^0C-ST Temp	1.08	0.42; 2.78	0.8546
Leaves	Linamarase	ST Tempe- 28^0C	1.01	0.39; 2.61	0.9768
Leaves	HNL	35^0C-28^0C	2.45	0.83; 7.20	0.0972
Leaves	HNL	35^0C-ST Temp	2.27	0.77; 6.68	0.1256
Leaves	HNL	ST Temp- 28^0C	1.08	0.37; 3.18	0.8814
Leaves	β-CAS	28^0C - 35^0C	3.92	1.46; 10.62	0.0112*
Leaves	β-CAS	28^0C -ST Tem	1.88	0.69; 5.09	0.1949
Leaves	β-CAS	ST Temp- 35^0C	2.08	0.77; 5.65	0.1313
Roots	CYP79D1/D2	ST Temp- 28^0C	1.92	0.63; 5.80	0.2265
Roots	CYP79D1/D2	ST Temp- 35^0C	1.10	0.36; 3.33	0.8597
Roots	CYP79D1/D2	35^0C-28^0C	1.74	0.58; 5.26	0.2953
Roots	Linamarase	28^0C - 35^0C	1.91	0.61; 5.90	0.2405
Roots	Linamarase	28^0C -ST Temp	1.16	0.37; 3.58	0.7928
Roots	Linamarase	ST Tempe- 35^0C	1.65	0.53; 5.10	0.353
Roots	HNL	ST Temp- 35^0C	1.49	0.14; 16.1	0.7179
Roots	HNL	ST Temp- 28^0C	1.13	0.10; 12.11	0.9177
Roots	HNL	28^0C - 35^0C	1.33	0.12; 14.30	0.7959
Roots	β-CAS	28^0C - 35^0C	1.39	0.41; 4.67	0.5719
Roots	β-CAS	28^0C -ST Temp	1.14	0.34; 3.85	0.8233
Roots	β-CAS	ST Temp- 35^0C	1.21	0.36; 4.1	0.7303

Expression Ratios and Confidence intervals for mean difference in cultivar Mcol 2215 under temperature stress. Each Ratio is interpreted as: In Mcol 2215 (leaves/Roots) the mean amount of RNAm from (*CYP79D1/D2/ Linamarase/HNL/β-CAS*) gene is (*Ratio Value*) times larger in (*Fraction Numerator in Comparison Cells*) that in (*Fraction denominator in Comparison Cells*) (according to the 95% two-sided confidence interval for mean differences, it is at least (*Lower C.I Value*) and not more than (*upper C.I Value*)**.** The ratio (*is/ is not*) significantly different from 1 using α = 0.05 (analysis done using contrast for log transformed data).
*Indicates significant differences. The corresponding descriptive statistics and statistical comparisons are in appendix H.

7.5.3 Comparison of transcriptional Activity in Cultivar 60444

Similar to cultivar Mcol 2215, in cultivar 60444 there was no significant differences found in the transcriptional activity of the CYP79D1/D2, linamarase and HNL genes in

leaves or roots (figure 21 and table 15). Thus, in the same way, the evidence suggests that in this cultivar the cyanogenic glucoside synthesis is not influenced by changes in temperature. However, similar to cultivar Mcol 2215 the same pattern of expression on β-CAS in leaves was found: In plants stopped at 28^0C an increase in the activity was evidenced, meanwhile in the plants stopped at 35^0C a decrease in the transcriptional activity was detected. Furthermore in 60444, β-CAS in roots showed an increase in the transcriptional activity in both temperatures of oscillation stress condition (table 15).

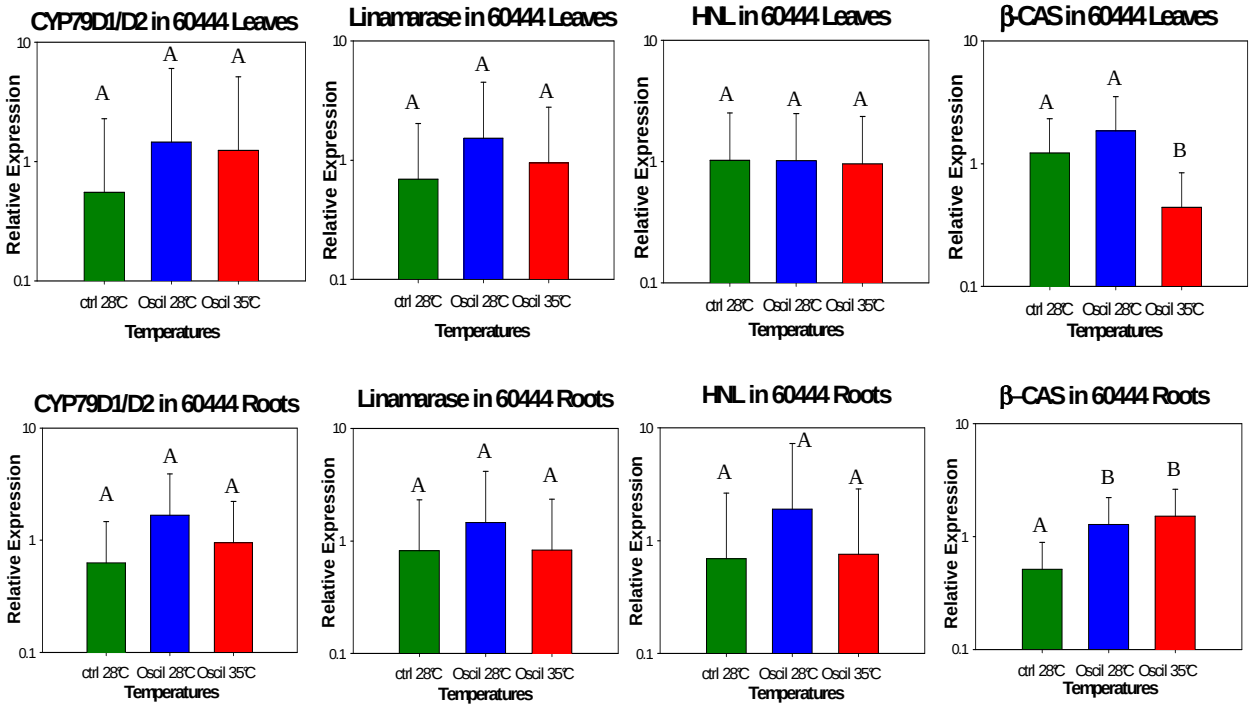


Figure 21:. Genes Expression Profile in 60444 plants under oscilation temperature stress
Ctrl = Control; Oscil= Oscilation. Three treatments were used: Control, plants under constant temperature (28^0C); Oscilation (28^0C), plants under changes of temperature (28^0C 12 hours – 35^0C 12 hours) stopped the experiment to 28^0C; Oscilation (35^0C), plants under changes of temperature (28^0C 12 hours – 35^0C 12 hours) stopped the experiment to 35^0C. Results are presented for leaves and roots for CYP79D1/D2, linamarase, HNL and β-CAS. Only β-CAS shows significant changes amog the treatments in the transcriptional activity in leaves and roots; however this result does not seem to correlate with changes in the regulation of cyanogenic glycosides metabolism. The bar error is the upper value of 90% the confidence interval. A different letter above the bars indicates significant differences ($p <= 0.10$).

Table 15: Expression Ratios and Confidence intervals in cultivar 60444 under temperature oscilation stress

Tissue	Gene	Comparison	Means Differences: Ratio	Two sides C. I for means differences (α = 5%)	Contrast. p-value
Leaves	CYP79D1/D2	28^0C - 35^0C	1.17	(0.22; 6.09)	0.8639
Leaves	CYP79D1/D2	28^0C -ST Temp	2.64	(0.50; 13.73)	0.3145
Leaves	CYP79D1/D2	35^0C-ST Temp	2.25	(0.43; 11.69)	0.399
Leaves	Linamarase	28^0C - 35^0C	1.61	(0.46; 5.62)	0.5052
Leaves	Linamarase	28^0C -ST Temp	1.53	(0.44; 5.31)	0.2801
Leaves	Linamarase	35^0C-ST Temp	1.37	(0.39; 4.76)	0.6648
Leaves	HNL	ST Temp- 28^0C	1.01	(0.36; 2.85)	0.9876
Leaves	HNL	ST Temp- 35^0C	1.06	(0.37; 3.00)	0.9159
Leaves	HNL	28^0C - 35^0C	1.06	(0.37; 2.99)	0.9282
Leaves	β-CAS	28^0C - 35^0C	4.20	(2.00; 8.81)	0.0047*
Leaves	β-CAS	28^0C -ST Temp	1.52	(0.72; 3.18)	0.3397
Leaves	β-CAS	ST Temp- 35^0C	2.77	(1.32; 5.81)	0.0299*
Roots	CYP79D1/D2	28^0C - 35^0C	1.75	(0.66; 4.68)	0.3287
Roots	CYP79D1/D2	28^0C -ST Temp	2.66	(0.99; 7.09)	0.1006
Roots	CYP79D1/D2	35^0C-ST Temp	1.52	(0.57; 4.04)	0.4616
Roots	Linamarase	28^0C - 35^0C	1.77	(0.71; 4.41)	0.4154
Roots	Linamarase	28^0C -ST Temp	1.79	(0.71; 4.47)	0.4059
Roots	Linamarase	35^0C-ST Temp	1.01	(0.41; 2.53)	0.9862
Roots	HNL	28^0C - 35^0C	2.51	(0.54; 11.76)	0.3088
Roots	HNL	28^0C -ST Temp	2.75	(0.58; 12.87)	0.2634
Roots	HNL	35^0C-ST Temp	1.09	(0.24; 5.12)	0.9138
Roots	β-CAS	35^0C-28^0C	1.41	(0.75; 2.67)	0.6437
Roots	β-CAS	35^0C-ST Temp	2.55	(1.35; 4.81)	0.0104*
Roots	β-CAS	28^0C -ST Temp	1.80	(0.96; 3.40)	0.0251*

Expression Ratios and Confidence intervals for mean difference in cultivar 60444 under Temperature stress. Each Ratio is interpreted as: In 60444 (leaves/Roots) the mean amount of RNAm from (*CYP79D1/D2/ Linamarase/HNL/β-CAS*) gene is (*Ratio Value*) times larger in (*Fraction Numerator in Comparison Cells*) that in (*Fraction denominator in Comparison Cells*) (according to the 95% two-sided confidence interval for mean differences, it is at least (*Lower C.I Value*) and not more than (*upper.C.I Value*)**.** The ratio (*is/ is not*) significantly different from 1 using α = 0.05 (analysis done using contrast for log transformed data).
* Indicates significant differences. The corresponding descriptive statistics and statistical comparisons are in appendix H.

7.5.4 Cluster Analysis

The results of the cluster classification show two clusters: 1. CYP79D1/D2 and β-Cas and 2. Linamarase and HNL (figure 22). These results agree with previous results illustrated the section 5.5.5 and 6.5.4, where it is suggested that the cluster between

CYP79D1/D2 and β-CAS is related with metabolism under plant disruption; meanwhile the cluster between linamarase and HNL is related to the nitrogen turnover in intact plants

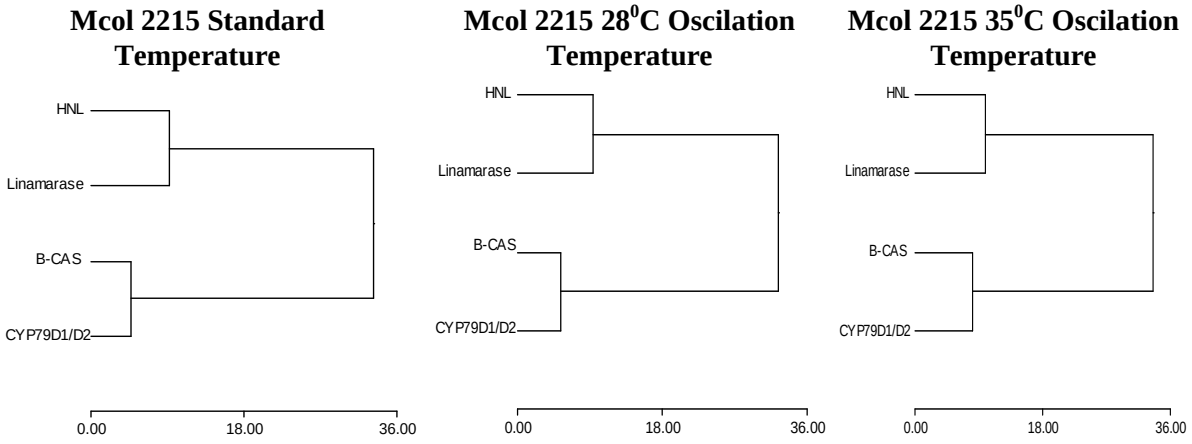


Figure 22: Clusters of gene classification in Mcol 2215 plants under temperature stress.
The data from leaves and roots were analyzed together. The same classification pattern was found in cultivar 60444 . The cluser generated are according with the spatial separation in the cell wall of the HNL protein and linamarase protein, which suggest that those enzymes are more related in the metabolism under plant disruption; meanwhile CYP79D1/D2 and β-CAS are related to the reduced nitrogen metabolism

7.5.5 Suggestions

Differences were found in both cultivars in the expression of β-CAS in leaves, evidencing the same gene expression pattern among the means compared: under oscilation temperature stress at 35^0C the plant reduced its capacity for detoxify cyanogenic glycosides in the leaves in comparison to 28^0C (Figures 20 and 21). This result could explain the increase in the level of caynogens in the roots through the linustatin pathway. However, we can suggest this hypothesis only in Mcol 2215 because the levels of β-CAS remain constant in its roots (Figure 20); meanwhile in 60444 the trancriptional activity of this gene in roots increased at 35^0C, which imply an efficient

detoxification of cyanogens in this tissue. Thus, an explanation for these events is not clear considering that in both cultivars and in both tissues significant differences were not found in the expression of the other genes. Threfore a clear influence of temperature over the cyanide amount in cassava roots is not completly clear through this study. The increase in cyanide in times of high temperatures and drought reported in the literature of cassava plants (Cardoso et al.1999) could be related with water deficiencies and not with temperature changes.

7.6 Conclusions

- The temperature does not affect the transcriptional activity of the genes involved in cyanogenic glycoside synthesis or the genes involved in catabolic activity in plants under stress.
- The oscillation temperature affect the transcriptional activity of β-CAS gene in leaves of Mcol 2215 cultivars and 60444.The transcriptional activity of these genes also is affected in roots of cultivar 60444 under oscillation Temperaturtes.
- The effect of temperature over the transcriptional activity of β-CAS observed here perhaps does not have any relationship with the cyanogens amount present in the roots. Considering, the fact that the other genes (CYP79D1/D2, linamarase and HNL) were not affected. It is suggested that the changes in this genes could be related with other metabolic pathways.

CHAPTER EIGHT
CONCLUSIONS AND SUGGESTIONS

For years, the metabolism of cyanogenic glycosides in cassava has been researched due to the agronomical importance of this crop. The first studies assigned these compounds as herbivore deterrents. However, more recently other researches concluded that cyanogenic glycosides also work as a nitrogen storage source. These studies showed that these molecules are synthesized in leaves and translocated to roots, where apparently the plant uses for amino acid synthesis. However, to date many aspects like gene regulation and details about the transport from leaves to roots remain unknown. Therefore the present research showed for the first time a systematic and quantitative analysis of one of the issues less studied in cassava cyanogenesis biology: the transcriptional activity of the genes involved in the metabolism of cyanogenic glycosides. For this purpose, the gene expression profile was obtained for CYP79D1/D2, linamarase, HNL and β-CAS genes in leaves and roots of different cassava cultivars using conventional PCR and Real-Time PCR.

The results in conventional PCR let us establish a comparison with previous studies and conclude that all the genes are transcribed in the leaves and roots of cassava, although some times in roots CYP79D1/D2 and linamarase were difficult to detect due to the low transcriptional activity. In our first experiment using Real-Time PCR, it was established a general gene profile in three cassava cultivars with different cyanide amounts in their roots: Mcol 2215 (sweet cultivar); cultivar 60444 (bitter-sweet cultivar) and Mtai 16 (bitter cultivar). There we reported that the genes CYP79D1/D2, linamarase and HNL have higher transcriptional activity in leaves than in roots. Nevertheless, β-CAS gene tends to remain constant among the two tissues or increase the transcriptional activity in roots relative to leaves. Also a comparison among cultivars revealed that the cultivar differences could be explained through a coordinate interaction between the cyanogenic

glycoside synthesis in leaves and their catabolism by linamarase in the roots. Cluster analysis illustrated that according to cyanogenic glucoside pathway, cultivars Mcol 2215 and 60444 are more closely related than cultivar Mtai 16.

The second experiment was developed to test the influence of reduced nitrogen stress over the transcriptional activity of these genes in Mcol 2215 and 60444 cultivars under specific *in vitro* conditions. Here we found a response in the expression of CYP79D1/D2 gene in roots at 1 day of stress inversely related to the reduced nitrogen concentration. Similarly there was a response in the expression of β-CAS gene in leaves at 10 days of stress stress directly related to the reduced nitrogen concentration. A similar pattern of expression was found in β-CAS gene in roots of cultivar 60444 after 1 day of stress. Comparatively, though linamarase and HNL also were impacted by the reduced nitrogen stress, no clear pattern of regulation was found. Although the results show that the genes involved in cyanogenenic glycoside metabolism are influenced by nitrogen stress, more research is necessary to clarify the possible physiological reasons for these responses. Thus, it is suggested that an experiment with tissue culture medium without any nitrogen source (without reduced nitrogen and nitrate) or experiments in which the trancriptional activity of genes as nitrate reductase and nitrite reductase be conducted.

On the other hand, it must be noted that in our nitrogen stress experiment there were statistical inference problems due to the sample size and the large variability in the data. In addition there were specific problems in using 18S rRNA as the only gene for the normalization. For this reason it is recommended that future transcriptional studies in cassava with Real-Time PCR select more housekeeping genes and increase the sample size. Furthermore, through programs such as GeNorm (incorporated in GenEx

Software) a correct housekeeping selection may be conducted (like tubulins, GADPH and others).

In the third experiment we tested the influence of the temperature over the transcriptional activity of the genes involved in cyanogenic glycosides pathways. Although there was no impact on the genes involved in the synthesis and catabolism of this molecules, changes over the transcriptional activity of β-CAS gene were found. Due to the absence of a clear behavior of β-CAS in both cultivars, and for the absence of changes in the transcriptional activity of the other genes, we suggest that this result in β-CAS gene could be related to other pathways.

Based on results from gene cluster analysis, the responses to nitrogen stress and on previous studies that reported the spatial proteins separation in the cell, allow us to suggest that the gene pair CYP79D1/D2 and β-CAS and the gene pair linamarase and HNL are co-regulated. Thus, each gene cluster seems to be involved in a specific physiological function: 1. Synthesis-Reassimilation of cyanide and 2. Release of cyanide upon the disruption of the plant cell. However on certain occassions these gene clusters can interact, such as in the cyanide differences among cultivars where, the coordinated activity of CYP79D1/D2 in leaves and the linamarase activity in roots explain the differences in roots cyanide levels in different cultivars. Furthermore, it should not be forgotten that the present results are only focused on transcriptional activity on *in vitro plants*; thus, the results generated under environmental stress must be compared with other analyses such as linamarin quantification and/or protein analysis; and also contrasted with similar studies developed with plants in fields or green houses, where more environmental factors could affect the cyanide amount in cassava roots.

Finally, we conclude that the cyanide content, one of the cassava agronomical descriptors, is a complex process that involves the interaction among genes, tissues, environment and the plant's developmental stage. It is impossible to explain this agronomical trait only through genetic reduction where the genes are always expressed independent of the environment or through an environment reduction where the external condition determines if the genes are expressed or not. Besides, previous reports such as by Santana et al. (2002) shows that the plant development also affects the gene expression as well as the protein activity. Figure 23 presents the overall scheme for the factors (genes, tissue, development and environment) ans interactions that govern cyanogen potential in cassava, where gene activity is only one of the pieces of a complex puzzle, in which many pieces remain to be found.

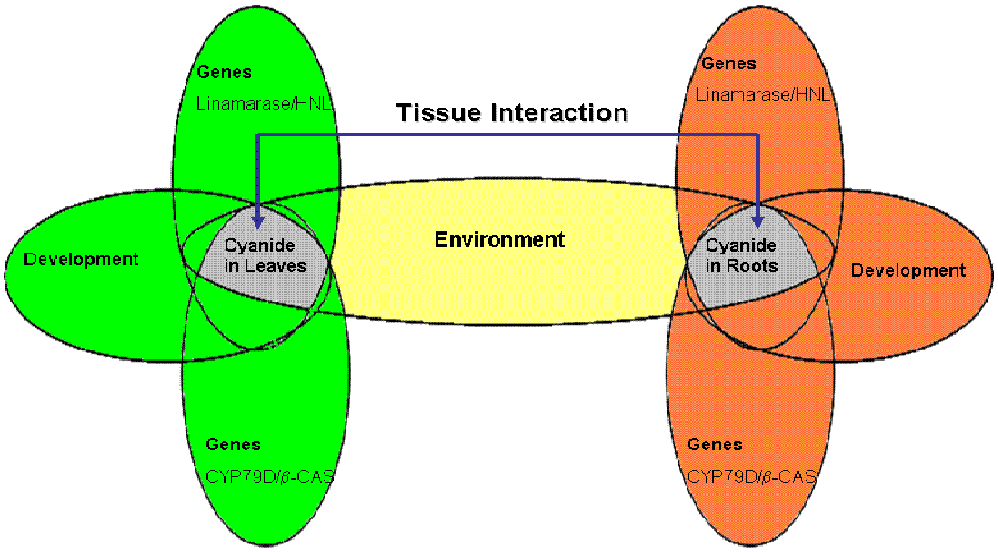


Figure 23: Factors that govern the cyanogen potential in cassava leaves and roots and their interactions.

The cyanide content in cassava is a complex trait that is governed by the genes and their interactions, the plant development, the environment and the "communication" among leaves and roots (considering that cyanogenic glycosides are produced in both tissues and the same time translocated from the leaves to roots). The figure represents the factors that influence the cyanide levels in the leaves with green color, while the factor that influenced the cyanide levels in roots are represented with orange. The genes involved in cyanogenic glycoside metabolism are represented in two independent circles due to a suggested co-regulation: linamarase-HNL cluster and CYP79D1/D2-β-CAS cluster.

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APPENDICES

Appendix A: Consensus primers for CYP79D1 and CYP79D2 genes.

The sequences were aligned with Clustal software using the sequences reported in GenBank under accessions number AF140613 (CYP79D1) and AF140614 (CYP79D2)

```
CYP79D2         GGTCTTGGTCATAGCCCTGGACTTGAATTGTTCAGGGCAACACCAATATGGCCATGAACG 60
CYP79D1         ----------------------------GTTCAGGGCA-TATCAATATGGCCATGAACG 30
                                            **********  * *****************

CYP79D2         TCTCCACCACCGC------AACCACCACGGCCTCCTTCGCCTCCACGTCCTCCATGAACA 114
CYP79D1         TCTCCACCACCATCGGTTTACTTAACGCCACCTCCTTCGCCTCCTCCTCCTCCAT---CA 87
                ***********        *   * * *  ************** * ********   **

CYP79D2         ATACTGCCAAAATCCTCCTTATCACCCTCTTCATTTCCATTGTCAGTACTGTTATAAAAC 174
CYP79D1         ACACGGTCAAGATCTTGTTCGTCACCCTCTTTATTTCCATTGTTAGTACTATTGTAAAAC 147
                * ** * *** *** *  *  ********** *********** ****** ** ******

CYP79D2         TTCAAAAAAGGGCATCCTACAAGAAAGCTAGCAAGAACTTCCCACTCCCTCCTGGTCCGA 234
CYP79D1         TTCAAAAGAGTGCTGCTAACAAGGAAGGTAGCAAGAAACTCCCACTCCCTCCTGGCCCTA 207
                ******* ** **  *  ***** *** *********  ***************** ** *

CYP79D2         CTCCATGGCCACTCATCGGAAACATCCCTGAAATGATCCGGTACAGACCGACGTTTCGTT 294
CYP79D1         CTCCATGGCCACTCATCGGAAACATCCCGGAAATGATCCGGTACAGACCCACGTTTCGGT 267
                ************************** ******************* ******** *

CYP79D2         GGATTCACCAACTCATGAAGGACATGAACACCGATATTTGTCTGATCCGTTTCGGAAAAA 354
CYP79D1         GGATTCACCAACTCATGAAGGACATGAACACTGATATTTGTCTCATTCGTTTTGGAAGAA 327
                ****************************** *********** ** ***** **** **

CYP79D2         CTAACGTTGTTCCTATTAGCTGCCCTGTCATTGCTCGTGAAATCCTGAAAAAGCACGATG 414
CYP79D1         CTAACTTTGTTCCTATAAGCTGTCCTGTTCTTGCTCGTGAAATACTAAAAAAGAATGACG 387
                ***** ********** ***** *****  ************* ** ****** * ** *
```

Appendix B: Other Buffers and solutions used

DNA Isolation Buffer

Reagent	Final Concentration
TrisHCl pH 8.0	100mM
EDTA	50mM
NaCl	500mM
SDS	1.25%
Sodium Bisulfite	3.8 gr/L

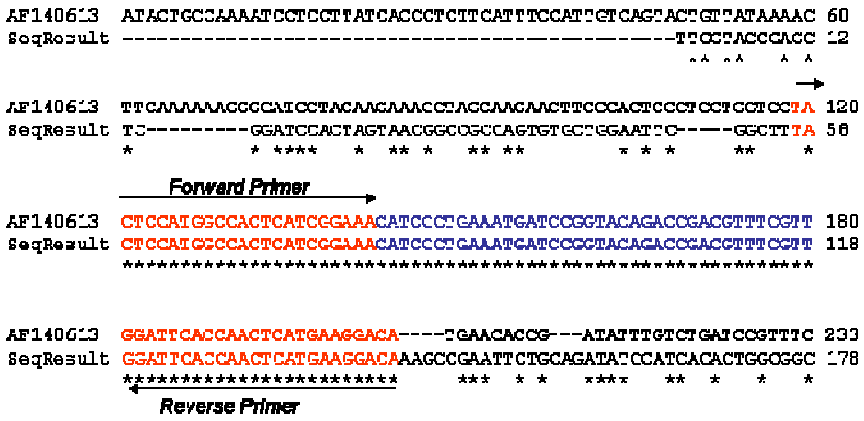
LB Media for Growth and Maintenance of Bacteria	Per Liter
Bacto Tryptone	10 g
Bacto Yeast Extract	5g
NaCl	10g

The pH was adjusted to 7.0 before adding agar (1.5%m/v) if required

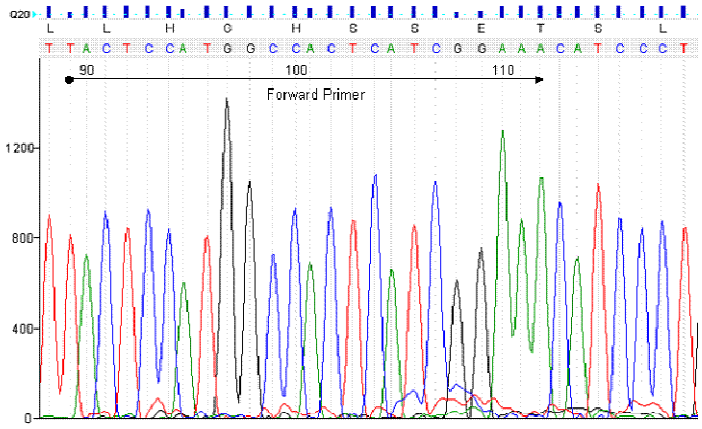
Appendix C: Sequencing Results.

1. CYP79D1/D2 Gene. Amplicon size: 86 pb. The result was aligned with Clustal software using the sequence reported in GenBank under accessions number AF140613

A. Clustal Alignment



B. Pherogram Fragment with Forward Primer

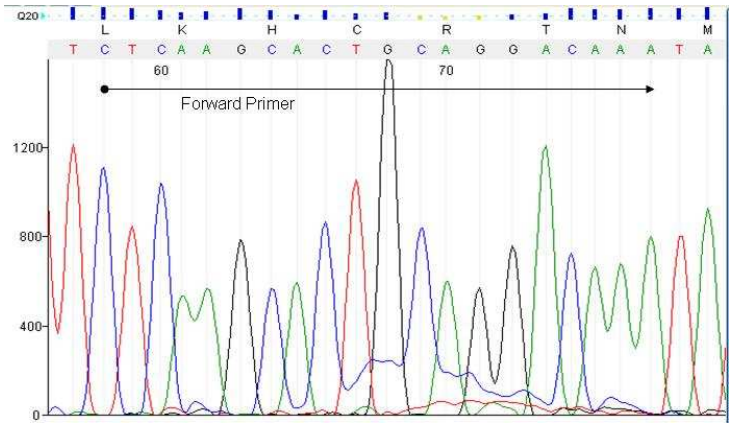


2. Linamarase Gene Primers A. Amplicon size: 158 pb. The result was aligned with Clustal software using the sequence reported in GenBank under accessions number S35175

A. Clustal Alignment

```
S35175          ATGATGTTATCAATGAAATTATAAGCAATGGACTAGAGCCTTTTGTTACTATTTTTCATT 430
SeqResult                                                     TCTGCAGA 8
                                                              * * **
                          Forward Primer
S35175          GGGATACTCCTCAAGCACTGCAGGACAAATATGGGGCTTCTTAAGCCGTGATATTGTGT 540
SeqResult       ATTCCCCTTCTCAACCACTGCACCACAAATATCCCCCTTCTTAACCCGTGATATTCTGT 63
                      ** ************************************************

S35175          ACGATCATCTCCAATATGCAGATCTTCTCTTTGAAAGATTCGGTGATCGAGTGAAACCGT 600
SeqResult       ACGATCATCTCCAATATGCAGATCTTCTCTTTGAAAGATTCGGTGATCGAGTGAAACGCT 128
                ************************************************  *

S35175          GGATGACTTTTAATGAACCATCAGCATATGTTGGATTTGCCCATGATGA-TGGAGTTTTT 659
SeqResult       GGATGACTTTTAATGAACCATCAGCATATGTTGGATTTGCCCATGATAAGCCGAATTCCA 188
                ****************************************** *   ** **
                                          Reverse Primer
```

B. Pherogram Fragment with Forward Primer

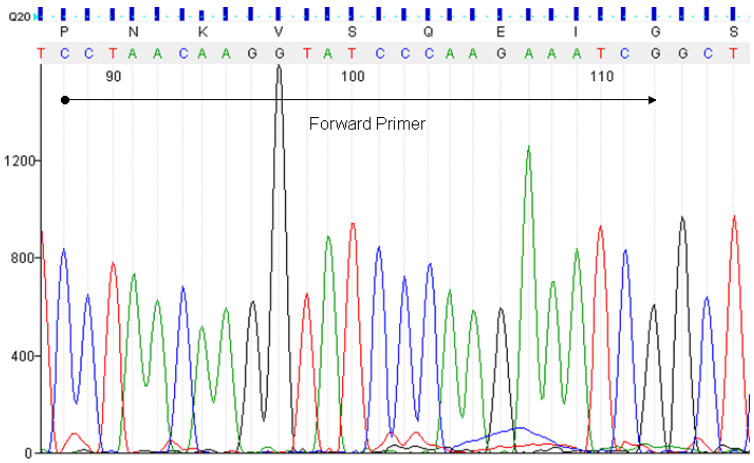


3. Linamarase Gene Primers B. Amplicon size: 111 pb. The result was aligned with Clustal software using the sequence reported in GenBank under accessions number S35175

A. Clustal Alignment

```
S35175          CATATTTAGACAACTTCGAATGGAATATTGGTTATACATCAAGATTTGGGTTGTACTATG  120
SeqResult       ------------GCTTGGTACCGAGC-TCGG--ATCCACTAGTAAC-GGCCGCCAGTGTG   44
                            *** * *  **   * **  ** **  *  *   **     * * **

                                      Forward Primer
S35175           TAGACTACAAAAATAACCTAACAAGGTATCCCAAGAAATCGGCTCATTGGTTCACAAAA  179
SeqResult       CTGGAATTCGC---CTTCCTAACAAGGTATCCCAAGAAATCGGCTCATTGGTTCACAAAA  101
                * ** * *        ****************************************

S35175          TTCCTGAATATATCGGTTAATGCAAATAATATCCATGAGCTTACATCAAAGGATTCAAGC  239
SeqResult       TTCCTGAATATATCGGTTAATGCAAATAATATCCATGAGCTTACATCAAAGGATTCAAGC  161
                ************************************************************
                                                    Reverse Primer
S35175          AAGGTTGG----CAAATTCTATGTGATGTAG--------------------------  266
SeqResult       AAGGTTGGAAGCCGAATTCTTGCA-GATATCCATCACACTGGCGGCCGCTCGAGCATGCAT  220
                ********    * ******    *** *
                ←
```

B. Pherogram Fragment with Forward Primer

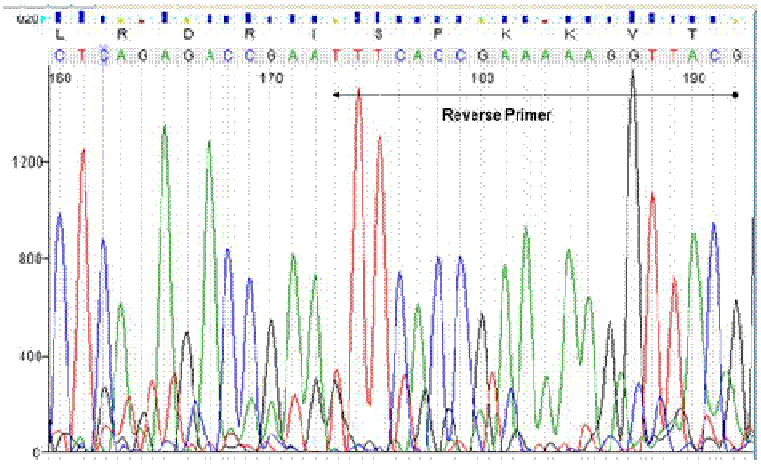


4.HNL Gene. Amplicon size: 228 pb. The result was aligned with Clustal software using the sequence reported in GenBank under accessions number Z29091

A. Clustal Alignment

```
                                                Forward Primer
                                                ------------->
Z29091          TAGCCCATCTTACACTGTGGAAAAGCTTTTGGAGTCGTTTCCTGACTGGAGAGACACAGA 420
SeqResult       ----------------TGGAAAAGCTTTTGGAGTCGTTTCCTGACTGGAGAGACACAGA 43
                                ************************************

Z29091          GTATTTTACGTTCACTAATATCACTGGAGAGACAATTACAACAATGAAGCTGGGCTTCGT 480
SeqResult       GTATTTTACGTTCACTAATATCACTGGAGAGACAATTACAACAATGAAGCTGGGCTTCGT 103
                ************************************************************

Z29091          ACTTCTGACGGAAAATTTATTACCAAATGCACTGATGGGGAATATGAACTGGCAAAAAT 540
SeqResult       ACTTCTGACGGAAAATTTATTACCAAATGCACTGATGGGGAATATGAACTGGCAAAAAT 163
                ************************************************************

Z29091          GGTAATGAGGAAGGGATCACTGTTTCAAAATGTTTTGGCTCAGAGACCGAAGTTCACCGA 600
SeqResult       GGTAATGAGGAAGGGATCACTGTTTCAAAATGTTTTGGCTCAGAGACCGAAGTTCACCGA 223
                ************************************************<-----------

Z29091          AAAAGGTTACGGATCAATTAAGAAAGTTTATATTTGGACCGATCAAGACAAAATATTTTT 660
SeqResult       AAAAGGTTACGGATCAATTAAGAAAGTTTA---------------------------- 253
                ****************************
                <-------------
                Reverse Primer
```

B. Pherogram Fragment with Reverse Primer

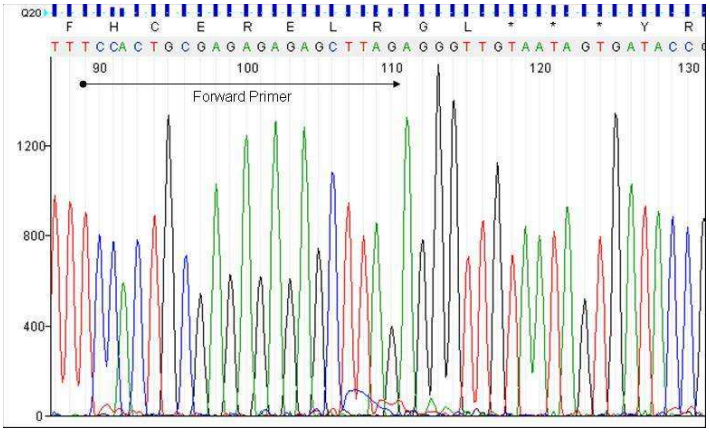


5. **β-CAS Gene, Amplicon Size 99 pb.** The result was aligned with Clustal software using the sequence reported in GenBank under accessions number EU350583

A. Clustal Alignment

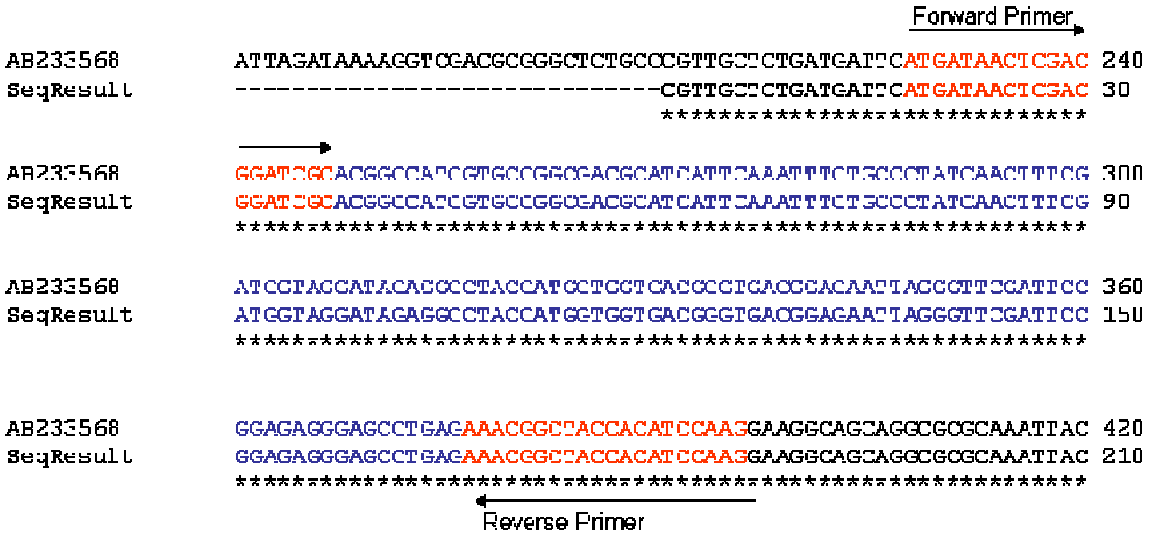
```
EU 350583      CCTTCCTACCCACCTCCCATCCACTACTAACCCCCCCCACTGTCCTCCAATTCCCCTTCT 60
SeqResult      ------------------------------ACCTGTA-CACACTGGAGTGTG--TTGT 25
                                              **   *     **-** * *  ****
                    Forward Primer
EU 350583      CCACTGCGAGAGAGAGCTTAGAGGGTTGTAATAGTGATACCGTGGGCGAGGAAAAGCTTC 120
SeqResult      CCACTGCGAGAGAGAGCTTAGAGGGTTGTAATAGTGATTCCGTGGGCGAGGAAAAGCTTC 05
               ************************************ ********************

EU 350583      TAGCTTTCATCAATGGCTACTCTTACATCAATGGCAAGCCGAATTCTGCAGATATCCATC 180
SeqResult      TAGCTTTCATCAATGGCTACTCTTACATCAATGGCTA------CTCTTAGGAACTTGTTC 139
               ************************************ *       ***   **  *   **
                          Reverse Primer
```

B. Pherogram Fragment with Forward Primer

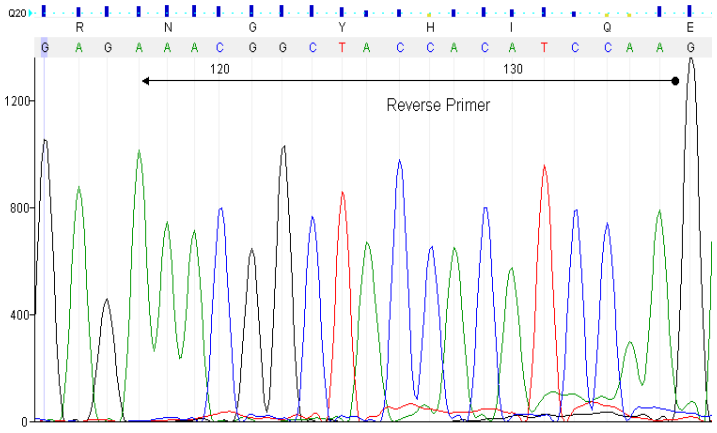


6. **18S Gene, Amplicon Size 169 pb.** The result was aligned with Clustal software using the sequence reported in GenBank under accessions number AB233568

A. Clustal Alignment

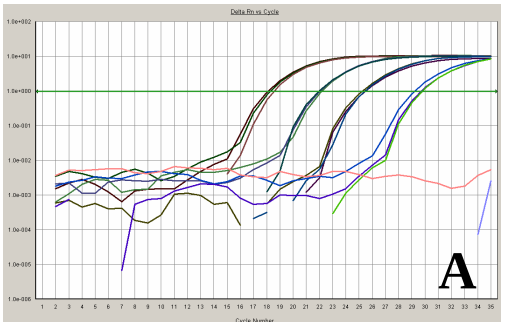


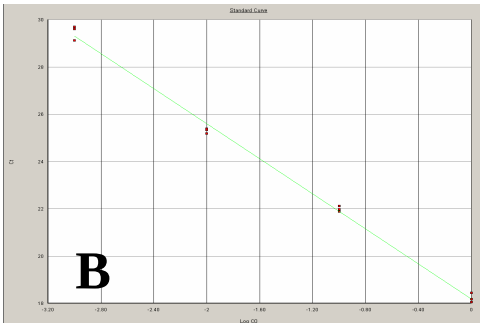
B. Pherogram Fragment with Reverse Primer

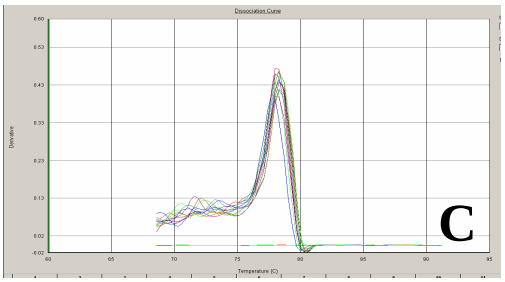


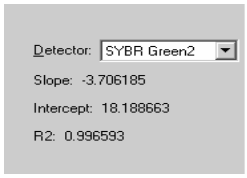
Appendix D: Primers Efficience Calculations

CYP79D1/D2 Gene









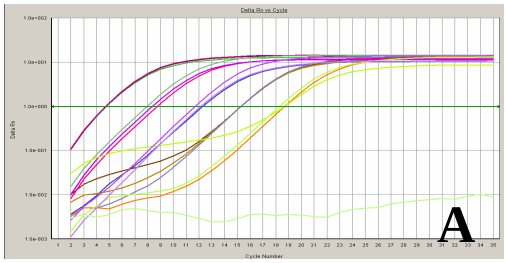
D

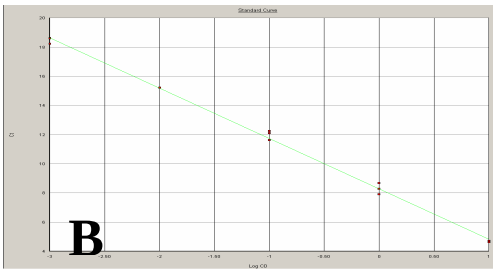
$E = (10^{(-1/slope)}) - 1$

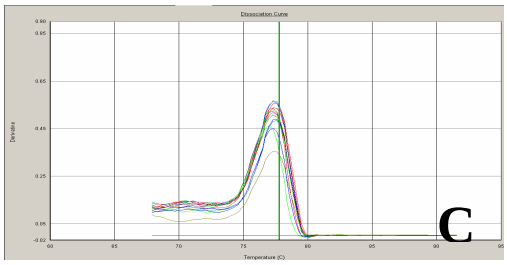
$E = 0.86$

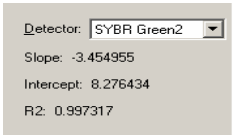
Panel A. Amplification Plot ; Panel B. Standard Curve ; Panel C. Melting Curve Plot ; Panel D. Slope from Standard curve

Linamarase Gene Primers A









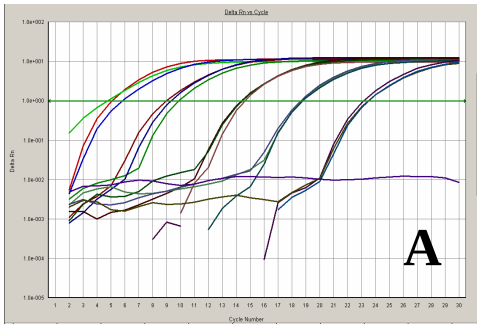
D

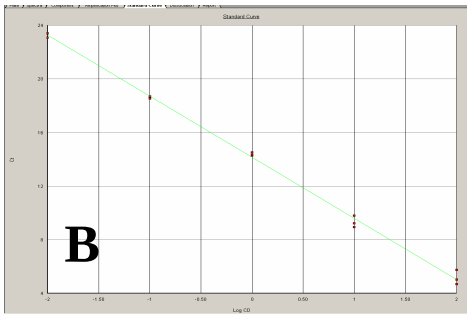
$E = (10^{(-1/slope)}) - 1$

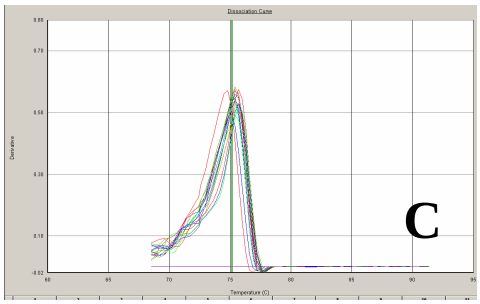
$E = 0.95$

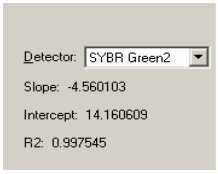
Panel A. Amplification Plot ; Panel B. Standard Curve ; Panel C. Melting Curve Plot ; Panel D. Slope from Standard curve

Linamarase Gene Primers B







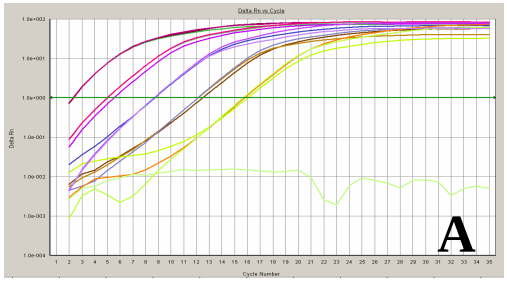


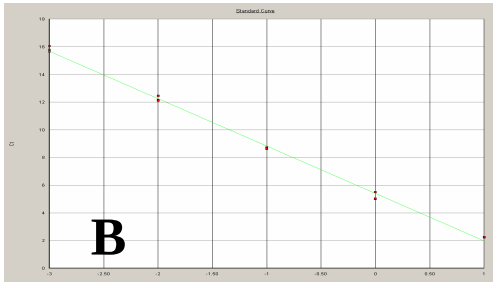
D $E = (10^{(-1/slope)}) - 1$

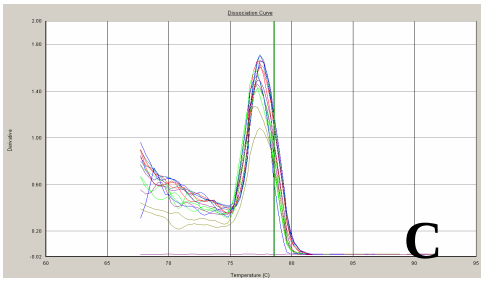
$E = 0.66$

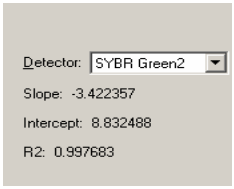
Panel A. Amplification Plot ; Panel B. Standard Curve ; Panel C. Melting Curve Plot ; Panel D. Slope from Standard curve

HNL Gene







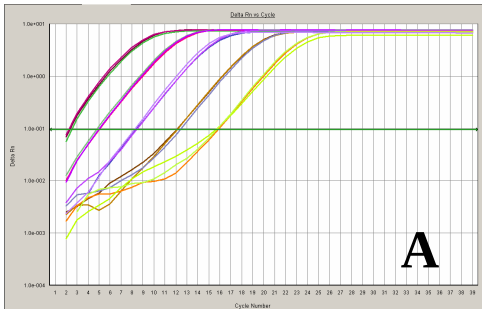


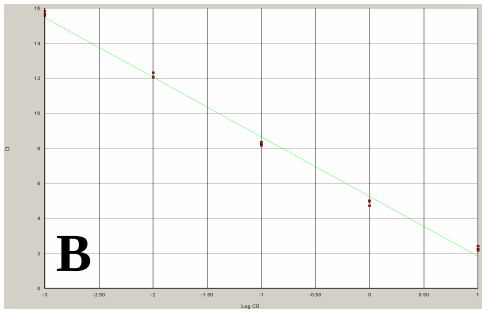
$E = (10^{(-1/slope)}) - 1$

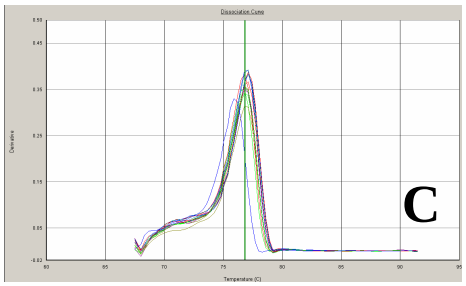
D $E = 0.96$

Panel A. Amplification Plot ; Panel B. Standard Curve ; Panel C. Melting Curve Plot ; Panel D. Slope from Standard curve

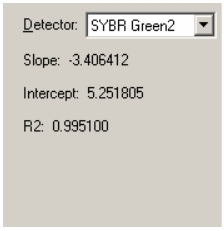
β-CAS







D

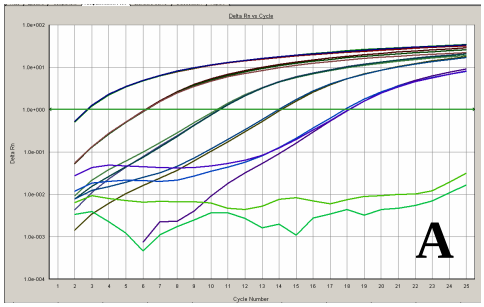


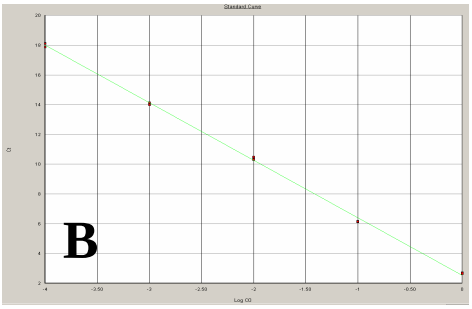
$E = (10^{(-1/slope)}) - 1$

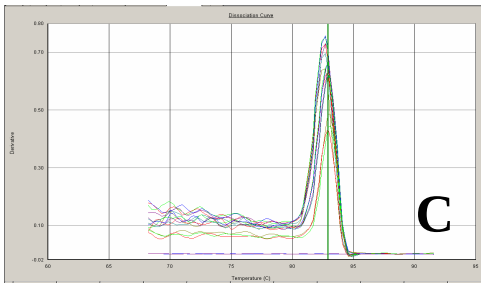
E = 0.97

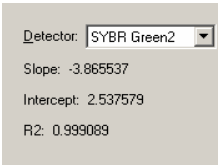
Panel A. Amplification Plot ; Panel B. Standard Curve ; Panel C. Melting Curve Plot ; Panel D. Slope from Standard curve

18S rRNA







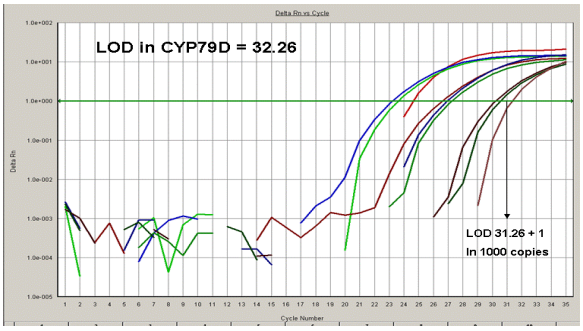


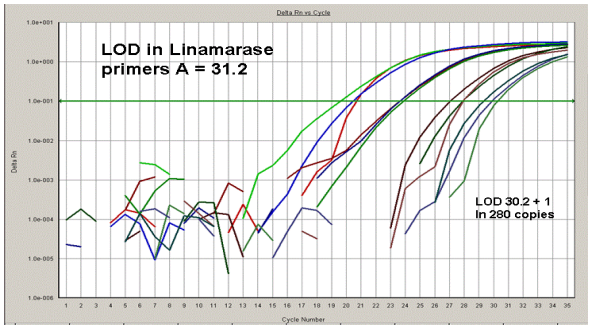
D $E = (10^{(-1/slope)}) - 1$

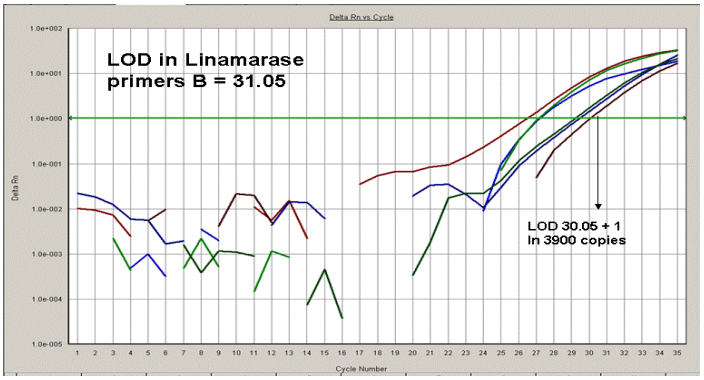
E = 0.81

Panel A. Amplification Plot ; Panel B. Standard Curve ; Panel C. Melting Curve Plot ; Panel D. Slope from Standard curve

Appendix E. Limit of Detection for CYP79D1/D2, linamarase A and linamarase B.







Appendix F. Statistical Tables and Statistical Analysis for Chapter Five

Descriptive Statistics for comparison among genes in three cassava cultivars. The Ct from each cultivar was organized in a 59 x 5 Matrix following the subsequent data processing. Here we show the descriptive statistics for each gene comparison in given gene and tissue. The means and confidence intervals were re-transformed to original scale meanwhile the other descriptors are in Log_2 Scale.

Cultivar	Tissue	Gene	n	Mean	Standard Deviation	Standard Error	Confidence Interval
Mcol 2215	Leaves	CYP79D1/D2	3	$2.99x10^{-07}$	0.37	0.22	($1.22x10^{-07}$; $7.35x10^{-07}$)
Mcol 2215	Leaves	Linamarase	3	$9.46x10^{-05}$	1.17	0.68	($4.32x10^{-05}$; 0.000206)
Mcol 2215	Leaves	HNL	3	0.001	1.06	0.61	(0.000876; 0.002952)
Mcol 2215	Leaves	B-CAS	3	$5.71x10^{-07}$	0.4	0.23	($2.75x10^{-07}$; $1.18x10^{-06}$)
60444	Leaves	CYP79D1/D2	3	$8.48x10^{-07}$	1.96	1.13	($3.46x10^{-7}$; $2.08x10^{-06}$)
60444	Leaves	Linamarase	3	0.0001	1.28	0.74	($7.63x10^{-05}$; 0.000364)
60444	Leaves	HNL	3	0.0012	0.22	0.13	(0.000655; 0.002207)
60444	Leaves	beta	3	$9.60x10^{-07}$	0.63	0.36	($4.63x10^{-07}$; $1.99x10^{-06}$)
Mtai 16	Leaves	CYP79D1/D2	3	$3.92x10^{-06}$	0.11	0.06	($1.6x10^{-06}$; 9.6110^{-06})
Mtai 16	Leaves	Linamarase	3	0.0003	0.14	0.08	(0.000148; 0.000709)
Mtai 16	Leaves	HNL	3	0.0086	0.81	0.47	(0.00469; 0.01581)
Mtai 16	Leaves	B-CAS	3	$3.58x10^{-06}$	1.44	0.83	($1.73x10^{-06}$; $7.43x10^{-06}$)
Mcol 2215	Roots	CYP79D1/D2	3	$6.75x10^{-08}$	0.99	0.57	$2.9x10^{-08}$; $1.57x10^{-07}$)
Mcol 2215	Roots	Linamarase	3	$2.43x10^{-06}$	1.14	0.66	($5.77x10^{-07}$; $1.02x10^{-05}$)
Mcol 2215	Roots	HNL	3	$9.01x10^{-06}$	1.18	0.68	($3.19x10^{-06}$; $2.54x10^{-05}$)
Mcol 2215	Roots	B-CAS	3	$2.79x10^{-06}$	0.86	0.5	($9.9x10^{-07}$; $7.88x10^{-06}$)
TMS 60444	Roots	CYP79D1/D2	3	$5.15x10^{-08}$	1.42	0.82	($2.21x10^{-08}$; $1.2x10^{-07}$)
TMS 60444	Roots	Linamarase	3	$2.3x10^{-07}$	2.3	1.33	($5.47x10^{-08}$; $9.7x10^{-07}$)
TMS 60444	Roots	HNL	3	$1.66x10^{-06}$	1.38	0.8	($5.88x10^{-07}$; $4.69x10^{-06}$)
TMS 60444	Roots	B-CAS	3	$2.75x10^{-06}$	1.11	0.64	($9.76x10^{-07}$; $7.77x10^{-06}$)
Mtai 16	Roots	CYP79D1/D2	3	$1.12x10^{-07}$	0.74	0.43	($4.85x10^{-08}$; $2.62x10^{-07}$)
Mtai 16	Roots	Linamarase	3	$7.07x10^{-07}$	1.91	1.1	($1.68x10^{-07}$; 2.98 $x10^{-06}$)
Mtai 16	Roots	HNL	3	$1.23x10^{-05}$	1.43	0.82	($4.36x10^{-06}$; $3.47x10^{-05}$)
Mtai 16	Roots	B-CAS	3	$4.35x10^{-06}$	0.78	0.45	$1.54x10^{-06}$; $1.23x10^{-05}$)

Descriptive Statistics for tissue comparison in each cassava cultivars. The Ct from each cultivar was organized in a 18 x 5 Matrix following the subsequent data processing. Here we show the descriptive statistics for each tissue comparison in given gene and cultivar. The means and confidence intervals were re-transformed to original scale meanwhile the other descriptors are in Log_2 Scale.

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Gene	Cultivar	Tissue	n	Mean	Standard Deviation	Standard Error	Confidence Interval (90%)
CYP79D1/D2	Mcol 2215	Leaves	3	2.11	0.37	0.22	(1.37; 3.26)
CYP79D1/D2	Mcol 2215	Roots	3	0.47	0.99	0.57	(0.14; 1.50)
Linamarase	Mcol 2215	Leaves	3	6.23	1.17	0.68	(1.58; 24.46)
Linamarase	Mcol 2215	Roots	3	0.16	1.14	0.66	(0.04; 0.61)
HNL	Mcol 2215	Leaves	3	13.36	1.06	0.61	(3.87; 46.11)
HNL	Mcol 2215	Roots	3	0.07	1.18	0.68	(0.02; 0.30)
B-CAS	Mcol 2215	Leaves	3	0.45	0.4	0.23	(0.28; 0.72)
B-CAS	Mcol 2215	Roots	3	2.20	0.86	0.5	(0.81; 6.02)
CYP79D1/D2	60444	Leaves	3	4.06	1.96	1.13	(0.41; 40.06)
CYP79D1/D2	60444	Roots	3	0.25	1.42	0.82	(0.06; 1.05)
Linamarase	60444	Leaves	3	26.91	1.28	0.74	(6.03; 120.08)
Linamarase	60444	Roots	3	0.04	2.3	1.33	(0.00; 0.55)
HNL	60444	Leaves	3	26.91	0.22	0.13	(20.81; 34.80)
HNL	60444	Roots	3	0.04	1.38	0.8	(0.00; 0.18)
B-CAS	60444	Leaves	3	0.59	0.63	0.36	(0.28; 1.24)
B-CAS	60444	Roots	3	1.69	1.11	0.64	(0.46; 6.20)
CYP79D1/D2	Mthai 16	Leaves	3	5.90	0.11	0.06	(5.19; 6.71)
CYP79D1/D2	Mthai 16	Roots	3	0.17	0.74	0.43	(0.07; 0.40)
Linamarase	Mthai 16	Leaves	3	21.41	0.14	0.08	(18.17; 25.21)
Linamarase	Mthai 16	Roots	3	0.05	1.91	1.1	(0.00; 0.43)
HNL	Mthai 16	Leaves	3	26.54	0.81	0.47	(10.30; 68.39)
HNL	Mthai 16	Roots	3	0.04	1.43	0.82	(0.01; 0.20)
B-CAS	Mthai 16	Leaves	3	0.91	1.44	0.83	(0.17; 4.88)
B-CAS	Mthai 16	Roots	3	1.10	0.78	0.45	(0.44; 2.74)

t-Paired Test for tissue comparison in three cassava cultivars

Gene	Cultivar	Comparison	Alternative Hypothesis	Mean Difference (Ratio)	S.D (difference)	C.I Mean Difference (90%)	T	p(One side)
CYP79D1/D2	Mcol 2215	Leaves:Roots	Leaves>Roots	4.44	1.07	(1.28; 15.45)	3.5	0.0364R
Linamarase	Mcol 2215	Leaves:Roots	Leaves>Roots	38.85	2.28	(2.7; 556.41)	4.02	0.0284R
HNL	Mcol 2215	Leaves:Roots	Leaves>Roots	178.53	0.96	(58.49; 548.75)	13.54	0.0027R
B-CAS	Mcol 2215	Roots: Leaves	Roots> Leaves	0.20	1.24	(1.15, 20.83)	-3.19	0.0428R
CYP79D1/D2	60444	Leaves:Roots	Leaves>Roots	16.45	2.2	(1.26; 215.27)	3.18	0.0431L
Linamarase	60444	Leaves:Roots	Leaves>Roots	724.08	1.64	(106.89; 4904.87)	10.06	0.0049R
HNL	60444	Leaves:Roots	Leaves>Roots	719.08	1.2	(177.29; 1176.23)	13.71	0.0026R
B-CAS	60444	Roots: Leaves	Roots> Leaves	0.35	1.03	(0.86; 9.58)	-2.56	0.0624L
CYP79D1/D2	Mthai 16	Leaves:Roots	Leaves>Roots	34.78	0.78	(14.03; 86.22)	11.43	0.0038R
Linamarase	Mthai 16	Leaves:Roots	Leaves>Roots	458.25	2.05	(41.93; 5007.9)	7.48	0.0087R
HNL	Mthai 16	Leaves:Roots	Leaves>Roots	699.41	1.56	(112.98; 4359.6)	10.47	0.0045R
B-CAS	Mthai 16	Roots: Leaves	Roots> Leaves	0.82	0.72	(0.52; 2.86)	-0.66	0.2883L

Descriptive Statistics for tissue comparison among three cassava cultivars

The Ct from each cultivar was organized in a 27 x 5 Matrix following the subsequent data processing. Here we show the descriptive statistics for each tissue comparison in gave gene and cultivar. The means and confidence intervals were re-transformed to original scale meanwhile the other descriptors are in Log_2 Scale. .

Comparison among Leaves of Three Cassava Cultivars

Tissue	Gene	Cultivar	n	Mean	S.D	Standard Error	Confidence Interval (90%)
Leaves	CYP79D1/D2	Mcol 2215	3	0.301452	0.37	0.22	(0.13; 0.74)
Leaves	CYP79D1/D2	60444	3	0.8467453	1.96	1.13	(0.34; 2.07)
Leaves	CYP79D1/D2	Mtai 16	3	3.9176812	0.11	0.06	(1.59; 9.60)
Leaves	Linamarase	Mcol 2215	3	0.5509526	1.17	0.68	(0.25; 1.20)
Leaves	Linamarase	60444	3	0.9659363	1.28	0.74	(0.44; 2.11)
Leaves	Linamarase	Mtai 16	3	1.8790455	0.14	0.08	(0.86; 4.11)
Leaves	HNL	Mcol 2215	3	0.6285067	1.06	0.61	(0.34; 1.53)
Leaves	HNL	60444	3	0.4697614	0.22	0.13	(0.26; 0.86)
Leaves	HNL	Mtai 16	3	3.3635857	0.81	0.47	(1.83; 6.17)
Leaves	B-CAS	Mcol 2215	3	0.4569157	0.4	0.23	(0.22; 0.95)
Leaves	B-CAS	60444	3	0.7631296	0.63	0.36	(0.36; 1.58)
Leaves	B-CAS	Mtai 16	3	2.8679105	1.44	0.83	(1.38; 5.95)

Comparison among Roots of Three Cassava Cultivars

Tissue	Gene	Cultivar	n	Mean	S.D	Standard Error	Confidence Interval (90%)
Roots	CYP79D1/D2	Mcol 2215	3	0.920188	0.99	0.57	(0.40; 2.14)
Roots	CYP79D1/D2	60444	3	0.702222	0.42	0.82	(0.30; 1.63)
Roots	CYP79D1/D2	Mtai 16	3	1.536875	0.74	0.43	(0.66; 3.58)
Roots	Linamarase	Mcol 2215	3	3.317278	1.14	0.66	(0.79; 13.97)
Roots	Linamarase	60444	3	0.312083	2.3	1.33	(0.07; 1.31)
Roots	Linamarase	Mtai 16	3	0.965936	1.91	1.1	(0.23; 4.07)
Roots	HNL	Mcol 2215	3	1.580083	1.18	0.68	(0.56; 4.46)
Roots	HNL	60444	3	0.293209	1.38	0.8	(0.10; 0.83)
Roots	HNL	Mtai 16	3	2.158456	1.43	0.82	(0.76; 6.09)
Roots	B-CAS	Mcol 2215	3	0.864537	0.86	0.50	(0.42; 1.79)
Roots	B-CAS	60444	3	0.858565	1.11	0.64	(0.42; 1.77)
Roots	B-CAS	Mtai 16	3	1.347234	0.78	0.45	(0.65; 2.78)

Analyses of variance (ANOVA) and contrast for CYP79D1/D2 expression ratios in leaves of three cassava cultivars

S.V.	SS	df	MS	F	p-value
Model	20.86	2	10.43	7.85	0.0212
Cultivar	20.86	2	10.43	7.85	0.0212
Error	7.98	6	1.33		
Total	28.83	8			

Contrasts

Cultivar	SS	df	MS	F	p-value
Mtai16 Vs Mcol 2215	20.60	1	20.60	5.50	0.0077
60444 Vs Mcol 2215	3.35	1	3.35	2.52	0.1636
Mtai Vs 60444	7.34	1	7.34	5.52	0.0571
Total	20.86	2	10.43	7.85	0.0212

Analyses of variance (ANOVA) and contrasts for Linamarase expression ratios in leaves of three cassava cultivars

S.V.	SS	df	MS	F	p-value
Model	4.74	2	2.37	2.35	0.1766
Cultivar	4.74	2	2.37	2.35	0.1766
Error	6.06	6	1.01		
Total	10.80	8			

Contrasts

Cultivar	SS	df	MS	F	p-value
Mtai16 Vs Mcol 2215	4.73	1	4.73	4.68	0.0737
60444 Vs Mcol 2215	0.99	1	0.99	0.98	0.3594
Mtai Vs 60444	1.39	1	1.39	1.37	0.2857
Total	4.74	2	2.37	2.35	0.1766

Analyses of Variance (ANOVA) and Contrasts For HNL expression ratios in leaves of three cassava cultivars

S.V.	SS	df	MS	F	p-value
Model	14.11	2	7.06	11.48	0.0089
Cultivar	14.11	2	7.06	11.48	0.0089
Error	3.69	6	0.61		
Total	17.80	8			

Contrasts

Cultivar	SS	df	MS	F	p-value
Mtai16 Vs Mcol 2215	8.78	1	8.78	14.28	0.0092
60444 Vs Mcol 2215	0.27	1	0.27	0.44	0.5332
Mtai Vs 60444	12.12	1	12.12	19.71	0.0044
Total	14.11	2	7.06	11.48	0.0089

Analyses of variance (ANOVA) and contrast for β-CAS expression ratios in leaves of three cassava cultivars

The Ct from each cultivar was organized in a 5 x 27 Matrix following the subsequent data processing

S.V.	SS	df	MS	F	p-value
Model	11.19	2	5.60	6.37	0.0328
Cultivar	11.19	2	5.60	6.37	0.0328
Error	5.27	6	0.88		
Total	16.46	8			

Contrasts

Cultivar	SS	df	MS	F	p-value
Mtai16 Vs Mcol 2215	10.52	1	10.52	11.98	0.0134
60444 Vs Mcol 2215	0.83	1	0.83	0.95	0.3676
Mtai Vs 60444	5.43	1	5.43	6.19	0.0473
Total	11.19	2	5.60	6.37	0.0328

Analyses of variance (ANOVA) and contrasts for CYP79D1/D2 expression ratios in roots of three cassava cultivars

S.V.	SS	df	MS	F	p-value
Model	1.99	2	0.99	0.84	0.4767
Cultivar	1.99	2	0.99	0.84	0.4767
Error	7.09	6	1.18		
Total	9.08	8			

Contrasts

Cultivar	SS	df	MS	F	p-value
Mtai16 Vs Mcol 2215	0.82	1	0.82	0.69	0.4367
60444 Vs Mcol 2215	0.23	1	0.23	0.20	0.6727
Mtai Vs 60444	1.93	1	1.93	1.63	0.2488
Total	1.99	2	0.99	0.84	0.4767

Analyses of variance (ANOVA) and contrasts for Linamarase expression ratios in roots of three cassava cultivars

S.V.	SS	df	MS	F	p-value
Model	17.39	2	8.69	2.54	0.1585
Cultivar	17.39	2	8.69	2.54	0.1585
Error	20.51	6	3.42		
Total	37.89	8			

Contrasts

Cultivar	SS	df	MS	F	p-value
Mtai16 Vs Mcol 2215	4.75	1	4.75	1.39	0.2830
60444 Vs Mcol 2215	17.37	1	17.37	5.08	0.0650
Mtai Vs 60444	3.95	1	3.95	1.16	0.3235
Total	17.39	2	8.69	2.54	0.1585

Analyses of variance (ANOVA) and contrast for HNL expression ratios in Roots of three cassava cultivars

S.V.	SS	df	MS	F	p-value
Model	14.44	2	7.22	4.06	0.0767
Cultivar	14.44	2	7.22	4.06	0.0767
Error	10.67	6	1.78		
Total	25.11	8			

Contrasts

Cultivar	SS	df	MS	F	p-value
Mtai16 Vs Mcol 2215	0.30	1	0.30	0.17	0.6936
60444 Vs Mcol 2215	8.89	1	8.89	5.00	0.0668
Mtai Vs 60444	12.48	1	12.48	7.02	0.0381
Total	14.44	2	7.22	4.06	0.0767

Analyses of variance (ANOVA) and contrasts for β-CAS expression ratios in Roots of three cassava cultivars

S.V.	SS	df	MS	F	p-value
Model	0.84	2	0.42	0.48	0.6382
Cultivar	0.84	2	0.42	0.48	0.6382
Error	5.20	6	0.87		
Total	6.04	8			

Contrasts

Cultivar	SS	df	MS	F	p-value
Mtai16 Vs Mcol 2215	0.61	1	0.61	0.71	0.4326
60444 Vs Mcol 2215	4.4E-04	1	4.4E-04	5.1E-04	0.9827
Mtai Vs 60444	0.65	1	0.65	0.75	0.4210
Total	0.84	2	0.42	0.48	0.6382

Appendix G. Statistical Tables and Statistical Analysis for Chapter Six

Descriptive Statistics for tissue comparison among Reduced Nitrogen Concentration. The Ct from each cultivar was organized in a 27 x 5 Matrix following the subsequent data processing. Here we show the descriptive statistics for each Reduced Nitrogen Condition in given, Time of Stress, cultivar, Gene and Tissue. The means and confidence intervals were re-transformed to original scale meanwhile the other descriptors are in Log_2 Scale.

Comparison among Leaves in Three Reduced Nitrogen Concentrations in Mcol 2215 1 after Day of Stress

Gene	Reduced Nitrogen Condition	Tissue	n	Mean	Standard Deviation	Standard Error	Confidence Interval (90%)
CYP79D1/D2	Mcol Standard	Leaves	3	0.75	1.89	1.09	(0.24; 2.36)
CYP79D1/D2	Mcol Intermediate	Leaves	3	1.38	1.33	0.77	(0.44; 4.36)
CYP79D1/D2	Mcol Without	Leaves	3	0.97	1.09	0.63	(0.30; 3.07)
Linamarase	Mcol Standard	Leaves	3	1.04	0.92	0.53	(0.36; 3)
Linamarase	Mcol Intermediate	Leaves	3	0.97	1.93	1.11	(0.33; 2.80)
Linamarase	Mcol Without	Leaves	3	1	1.02	0.59	(0.35; 2.90)
HNL	Mcol Standard	Leaves	3	1.34	1.49	0.86	(0.40; 4.51)
HNL	Mcol Intermediate	Leaves	3	0.88	1.78	1.03	(0.26; 2.95)
HNL	Mcol Without	Leaves	3	0.85	1.4	0.81	(0.25; 2.87)
β-CAS	Mcol Standard	Leaves	3	1.23	0.6	0.35	(0.27; 5.53)
β-CAS	Mcol Intermediate	Leaves	3	0.43	3.09	1.78	(0.09; 1.91)
β-CAS	Mcol Without	Leaves	3	1.91	1.14	0.66	(0.42; 8.56)

Comparison among Roots in Three Reduced Nitrogen Concentrations in Mcol 2215 after 1 Day of Stress

Gene	Reduced Nitrogen Condition	Tissue	n	Mean	Standard Deviation	Standard Error	Confidence Interval (90%)
CYP79D1/D2	Mcol Standard	Roots	3	0.40	2.12	1.22	(0.10; 1.59)
CYP79D1/D2	Mcol Intermediate	Roots	3	0.53	0.51	0.29	(0.13; 2.10)
CYP79D1/D2	Mcol Without	Roots	3	4.66	2.15	1.24	(1.18; 18.40)
Linamarase	Mcol Standard	Roots	3	0.39	0.71	0.41	(0.23; 0.66)
Linamarase	Mcol Intermediate	Roots	3	0.57	0.87	0.5	(0.33; 0.96)
Linamarase	Mcol Without	Roots	3	4.53	0.35	0.2	(2.67; 7.68)
HNL	Mcol Standard	Roots	3	1.21	1.94	1.12	(0.28; 5.28)
HNL	Mcol Intermediate	Roots	3	0.55	2.04	1.18	(0.13; 2.4
HNL	Mcol Without	Roots	3	1.49	1.68	0.97	(0.34; 6.51)
β-CAS	Mcol Standard	Roots	3	1.06	0.33	0.19	(0.58; 1.94)
β-CAS	Mcol Intermediate	Roots	3	0.85	0.38	0.22	(0.46; 1.56)
β-CAS	Mcol Without	Roots	3	1.11	1.25	0.72	(0.60; 2.03)

Comparison among Leaves in Three Reduced Nitrogen Concentrations in Mcol 2215 after 10 Day of Stress

Gene	Reduced Nitrogen Condition	Tissue	n	Mean	Standard Deviation	Standard Error	Confidence Interval (90%)
CYP79D1/D2	Mcol Standard	Leaves	3	0.76313	2.43	1.4	(0.17; 3.38)
CYP79D1/D2	Mcol Intermediate	Leaves	3	1.741101	1.03	0.6	(0.39; 7.72)
CYP79D1/D2	Mcol Without	Leaves	3	0.747425	2	1.16	(0.17; 3.31)
Linamarase	Mcol Standard	Leaves	3	1.109569	0.85	0.49	(0.50; 2.51)
Linamarase	Mcol Intermediate	Leaves	3	1.60214	1.59	0.92	(0.71; 3.62)
Linamarase	Mcol Without	Leaves	3	0.562529	0.25	0.14	(0.25; 1.27)
HNL	Mcol Standard	Leaves	3	1.394744	0.25	0.15	(0.59; 3.25)
HNL	Mcol Intermediate	Leaves	3	1.931873	1.6	0.93	(0.82; 4.51)
HNL	Mcol Without	Leaves	3	0.371131	0.97	0.56	(0.16; 0.87)
β-CAS	Mcol Standard	Leaves	3	5.540438	0.63	0.37	(2.88; 10.67)
β-CAS	Mcol Intermediate	Leaves	3	1.156688	1.06	0.61	(0.60; 2.22)
β-CAS	Mcol Without	Leaves	3	0.156041	0.78	0.45	(0.08; 0.30)

Comparison among Roots in Three Reduced Nitrogen Concentrations in Mcol 2215 after 10 Day of Stress

Gene	Reduced Nitrogen Condition	Tissue	n	Mean	Standard Deviation	Standard Error	Confidence Interval (90%)
CYP79D1/D2	Mcol Standard	Roots	3	0.757858	1.61	0.93	(0.16; 3.55)
CYP79D1/D2	Mcol Intermediate	Roots	3	1.292353	1.97	1.14	(0.28; 6.04)
CYP79D1/D2	Mcol Without	Roots	3	1.021012	2.3	1.33	(0.22; 4.78)
Linamarase	Mcol Standard	Roots	3	0.624165	0.35	0.2	(0.28; 1.41)
Linamarase	Mcol Intermediate	Roots	3	2.713209	1.97	1.14	(1.2; 6.13)
Linamarase	Mcol Without	Roots	3	0.590496	0.29	0.16	(0.26; 1.33)
HNL	Mcol Standard	Roots	3	0.76313	1.57	0.9	(0.19; 3.07)
HNL	Mcol Intermediate	Roots	3	4.924578	0.81	0.47	(1.22; 19.80)
HNL	Mcol Without	Roots	3	0.267943	1.33	0.77	(0.07; 1.07)
β-CAS	Mcol Standard	Roots	3	1.06437	0.91	0.53	(0.60; 1.91)
β-CAS	Mcol Intermediate	Roots	3	1.474269	0.39	0.23	(0.81; 2.65)
β-CAS	Mcol Without	Roots	3	0.641713	0.86	0.49	(0.36; 1.15)

Comparison among Leaves in Three Reduced Nitrogen Concentrations in 60444 after 1Day of Stress

Gene	Reduced Nitrogen Condition	Tissue	n	Mean	Standard Deviation	Standard Error	Confidence Interval (90%)
CYP79D1/D2	TMS Standard	Leaves	3	4.69	3.4	1.96	(0.97; 22.7)
CYP79D1/D2	TMS Intermediate	Leaves	3	0.51	0.76	0.44	(0.11; 2.45)
CYP79D1/D2	TMS Without	Leaves	3	0.42	0.4	0.23	(0.09; 2.03)
Linamarase	TMS Standard	Leaves	3	4.03	3.62	2.09	(1.90; 8.56)
Linamarase	TMS Intermediate	Leaves	3	0.66	1.37	0.79	(0.31; 1.39)
Linamarase	TMS Without	Leaves	3	0.38	0.93	0.54	(0.17; 0.80)
HNL	TMS Standard	Leaves	3	7.01	2.76	1.59	(1.62; 30.22)
HNL	TMS Intermediate	Leaves	3	0.53	0.93	0.54	(0.12; 2.28)
HNL	TMS Without	Leaves	3	0.27	1.45	0.84	(0.06; 1.16)
β-CAS	TMS Standard	Leaves	3	2.85	2.51	1.45	(0.89; 9.11)
β-CAS	TMS Intermediate	Leaves	3	0.60	0.56	0.32	(0.18; 1.92)
β-CAS	TMS Without	Leaves	3	0.59	0.33	0.19	(0.18; 1.88)

Comparison among Roots in Three Reduced Nitrogen Concentrations in 60444 after 1Day of Stress

Gene	Reduced Nitrogen Condition	Tissue	n	Mean	Standard Deviation	Standard Error	Confidence Interval (90%)
CYP79D1/D2	TMS Standard	Roots	3	0.31	1.89	1.09	(0.04; 2.13)
CYP79D1/D2	TMS Intermediate	Roots	3	1.36	1.59	0.92	(0.20; 9.16)
CYP79D1/D2	TMS Without	Roots	3	2.35	3.46	2	(0.35; 15.83)
Linamarase	TMS Standard	Roots	3	0.71	1.5	0.87	(0.52; 0.95)
Linamarase	TMS Intermediate	Roots	3	1.27	1.17	0.68	(0.93; 1.71)
Linamarase	TMS Without	Roots	3	1.12	0.25	0.14	(0.82; 1.51)
HNL	TMS Standard	Roots	3	1.06	3.23	1.87	0.15; 7.29)
HNL	TMS Intermediate	Roots	3	1.3	1.53	0.88	(0.19; 8.91)
HNL	TMS Without	Roots	3	0.72	2.36	1.36	(0.10; 4.94)
β-CAS	TMS Standard	Roots	3	1.64	1.14	0.66	(0.83; 3.22)
β-CAS	TMS Intermediate	Roots	3	1.27	0.16	0.09	(0.65; 2.51)
β-CAS	TMS Without	Roots	3	0.48	0.97	0.56	(0.25; 0.95)

Comparison among Leaves in Three Reduced Nitrogen Concentrations in 60444 after 10 Day of Stress

Gene	Reduced Nitrogen Condition	Tissue	n	Mean	Standard Deviation	Standard Error	Confidence Interval (90%)
CYP79D1/D2	TMS Standard	Leaves	3	1.827663	2.3	1.33	(0.45; 7.84)
CYP79D1/D2	TMS Intermediate	Leaves	3	0.624165	1.93	1.11	(0.15; 2.68)
CYP79D1/D2	TMS Without	Leaves	3	0.876606	1.24	0.71	(0.20; 3.76)
Linamarase	TMS Standard	Leaves	3	1.94531	1.72	0.99	(0.70; 5.38)
Linamarase	TMS Intermediate	Leaves	3	0.697372	1.08	0.62	(0.25; 1.93)
Linamarase	TMS Without	Leaves	3	0.737135	1	0.58	(0.27; 2.03)
HNL	TMS Standard	Leaves	3	0.63728	2.41	1.39	(0.20; 2)
HNL	TMS Intermediate	Leaves	3	0.482968	0.55	0.32	(0.15; 1.51)
HNL	TMS Without	Leaves	3	3.24901	0.58	0.34	(1.03; 10.19)
β-CAS	TMS Standard	Leaves	3	2.056228	1.83	1.05	(0.76; 5.53)
β-CAS	TMS Intermediate	Leaves	3	0.795536	0.78	0.45	(0.29; 2.14)
β-CAS	TMS Without	Leaves	3	0.61132	0.95	0.55	(0.22; 1.65)

Comparison among Roots in Three Reduced Nitrogen Concentrations in 60444 after 10Day of Stress

Gene	Reduced Nitrogen Condition	Tissue	n	Mean	Standard Deviation	Standard Error	Confidence Interval (90%)
CYP79D1/D2	TMS Standard	Roots	3	0.50698	2.31	1.33	(0.08; 3.23)
CYP79D1/D2	TMS Intermediate	Roots	3	0.858565	2.28	1.32	(0.13; 5.47)
CYP79D1/D2	TMS Without	Roots	3	2.297397	2.55	1.47	(0.36; 14.66)
Linamarase	TMS Standard	Roots	3	1.172835	2.06	1.19	(0.47; 2.9)
Linamarase	TMS Intermediate	Roots	3	0.586417	2.3	1.33	(0.24; 1.45)
Linamarase	TMS Without	Roots	3	1.453973	2.55	1.47	(0.58; 3.6)
HNL	TMS Standard	Roots	3	0.590496	1.31	0.76	(0.09; 3.71)
HNL	TMS Intermediate	Roots	3	0.426317	1.15	0.67	(0.07; 2.68)
HNL	TMS Without	Roots	3	4	3.7	2.14	(0.64; 25.15)
B-CAS	TMS Standard	Roots	3	1.013959	1.14	0.66	(0.37; 2.711)
B-CAS	TMS Intermediate	Roots	3	0.586417	0.61	0.35	(0.22; 1.57)
B-CAS	TMS Without	Roots	3	1.681793	1.77	1.02	(0.63; 4.5)

Analyses of variance (ANOVA) and contrast for CYP79D1/D2 expression ratios in leaves of Mcol 2215 in reduced nitrogen stress (three concentrations) after 1 day

S.V.	SS	df	MS	F	p-value
Model	1.18	2	0.59	0.27	0.7719
Treatments	1.18	2	0.59	0.27	0.7719
Error	13.08	6	2.18		
Total	14.25	8			

Contrasts

Treatments	SS	df	MS	F	p-value
Interm Vs Standard	1.17	1	1.17	0.54	0.4915
Interm Vs Without	0.39	1	0.39	0.18	0.6878
Standard Vs Without	0.21	1	0.21	0.10	0.7667

Analyses of variance (ANOVA) and contrast for Linamarase expression ratios in leaves of Mcol 2215 in reduced nitrogen stress (three concentrations) after 1 day

S.V.	SS	df	MS	F	p-value
Model	0.02	2	0.01	4.4E-03	0.9956
Treatments	0.02	2	0.01	4.4E-03	0.9956
Error	11.22	6	1.87		
Total	11.23	8			

Contrasts

Treatments	SS	df	MS	F	p-value
Interm Vs Standard	0.02	1	0.02	0.01	0.9285
Interm Vs Without	4.3E-03	1	4.3E-03	2.3E-03	0.9634
Standard Vs Without	3.9E-03	1	3.9E-03	2.1E-03	0.9650
Total	0.02	2	0.01	4.4E-03	0.9956

Analyses of variance (ANOVA) and contrast for HNL expression ratios in leaves of Mcol 2215 in reduced nitrogen stress (three concentrations) for 1 day

S.V.	SS	df	MS	F	p-value
Model	0.79	2	0.39	0.16	0.8542
Treatments	0.79	2	0.39	0.16	0.8542
Error	14.65	6	2.44		
Total	15.44	8			

Contrasts

Treatments	SS	df	MS	F	p-value
Interm Vs Standard	0.56	1	0.56	0.23	0.6485
Interm Vs Without	1.5E-03	1	1.5E-03	6.3E-04	0.9808
Standard Vs Without	0.62	1	0.62	0.25	0.6318
Total	0.79	2	0.39	0.16	0.8542

Analyses of variance (ANOVA) and contrast for β-CAS expression ratios in leaves of Mcol 2215 in reduced nitrogen stress (three concentrations) after 1 day

S.V.	SS	df	MS	F	p-value
Model	7.45	2	3.73	1.00	0.4226
Treatments	7.45	2	3.73	1.00	0.4226
Error	22.41	6	3.73		
Total	29.86	8			

Contrasts

Treatments	SS	df	MS	F	p-value
Interm Vs Standard	3.55	1	3.55	0.95	0.3670
Interm Vs Without	7.03	1	7.03	1.88	0.2190
Standard Vs Without	0.59	1	0.59	0.16	0.7050

Analyses of variance (ANOVA) and contrast for CYP79D1/D2 expression ratios in roots of Mcol 2215 in reduced nitrogen stress (three concentrations) after 1 day

S.V.	SS	df	MS	F	p-value
Model	22.36	2	11.18	3.58	0.0948
Treatments	22.36	2	11.18	3.58	0.0948
Error	18.75	6	3.12		
Total	41.11	8			

Contrasts

Treatments	SS	df	MS	F	p-value
Interm Vs Standard	0.24	1	0.24	0.08	0.7894
Interm Vs Without	14.64	1	14.64	4.69	0.0736
Standard Vs Without	18.66	1	18.66	5.97	0.0502

Analyses of variance (ANOVA) and contrast for linamarase expression ratios in roots of Mcol 2215 in reduced nitrogen stress (three concentrations) after 1 day

S.V.	SS	df	MS	F	p-value
Model	21.77	2	10.89	23.51	0.0014
Treatments	21.77	2	10.89	23.51	0.0014
Error	2.78	6	0.46		
Total	24.55	8			

Contrasts

Treatments	SS	df	MS	F	p-value
Interm Vs Standard	0.43	1	0.43	0.93	0.3709
Interm Vs Without	13.48	1	13.48	29.11	0.0017
Standard Vs Without	18.74	1	18.74	40.48	0.0007

Analyses of variance (ANOVA) and contrast for HNL expression ratios in roots of Mcol 2215 in reduced nitrogen stress (three concentrations) after 1 day

S.V.	SS	df	MS	F	p-value
Model	3.46	2	1.73	0.48	0.6392
Treatment	3.46	2	1.73	0.48	0.6392
Error	21.51	6	3.58		
Total	24.97	8			

Contrasts

Treatments	SS	df	MS	F	p-value
Interm Vs Standard	1.95	1	1.95	0.54	0.4889
Interm Vs Without	3.11	1	3.11	0.87	0.3877
Standard Vs Without	0.14	1	0.14	0.04	0.8525

Analyses of variance (ANOVA) and contrast for β-CAS expression ratios in roots of Mcol 2215 in reduced nitrogen stress (three concentrations) after 1 day

S.V.	SS	df	MS	F	p-value
Model	0.24	2	0.12	0.20	0.8271
Treatments	0.24	2	0.12	0.20	0.8271
Error	3.64	6	0.61		
Total	3.88	8			

Contrasts

Treatments	SS	df	MS	F	p-value
Interm Vs Standard	0.14	1	0.14	0.23	0.6487
Interm Vs Without	0.21	1	0.21	0.35	0.5778
Standard Vs Without	0.01	1	0.01	0.01	0.9167

Analyses of variance (ANOVA) and contrast for CYP79D1/D2 expression ratios in leaves of Mcol 2215 in reduced nitrogen stress (three concentrations) after 10 day

S.V.	SS	df	MS	F	p-value
Model	2.90	2	1.45	0.40	0.6896
Treatments	2.90	2	1.45	0.40	0.6896
Error	22.00	6	3.67		
Total	24.90	8			

Contrasts

Treatments	SS	df	MS	F	p-value
Interm Vs Standard	2.12	1	2.12	0.58	0.4762
Interm Vs Without	2.23	1	2.23	0.61	0.4647
Standard Vs Without	1.6E-03	1	1.6E-03	4.4E-04	0.9840

Analyses of variance (ANOVA) and contrast for linamarase expression ratios in leaves of Mcol 2215 in reduced nitrogen stress (three concentrations) after 10 day

S.V.	SS	df	MS	F	p-value
Model	3.50	2	1.75	1.59	0.2800
Treatments	3.50	2	1.75	1.59	0.2800
Error	6.61	6	1.10		
Total	0.11	8			

Contrasts

Leaves Mcol 10 dia	SS	df	MS	F	p-value
Interm Vs Standard	0.42	1	0.42	0.38	0.5613
Interm Vs Without	3.39	1	3.39	3.08	0.1298
Standard Vs Without	1.43	1	1.43	1.30	0.2977

Analyses of variance (ANOVA) and contrast for HNL expression ratios in leaves of Mcol 2215 in reduced nitrogen stress (three concentrations) after 10 day

S.V.	SS	df	MS	F	p-value
Model	9.52	2	4.76	3.99	0.0792
Treatments	9.52	2	4.76	3.99	0.0792
Error	7.16	6	1.19		
Total	16.68	8			

Contrasts

Leaves Mcol 10 dia	SS	df	MS	F	p-value
Interm Vs Standard	0.32	1	0.32	0.27	0.6207
Interm Vs Without	8.47	1	8.47	7.10	0.0373
Standard Vs Without	5.48	1	5.48	4.59	0.0759

Analyses of variance (ANOVA) and contrast for β-CAS expression ratios in leaves of Mcol 2215 in reduced nitrogen stress (three concentrations) after 10 day

S.V.	SS	df	MS	F	p-value
Model	39.90	2	19.95	28.11	0.0009
Treatments	39.90	2	19.95	28.11	0.0009
Error	4.26	6	0.71		
Total	44.16	8			

Contrasts

Leaves Mcol 10 dia	SS	df	MS	F	p-value
Interm Vs Standard	7.69	1	7.69	10.84	0.0166
Interm Vs Without	12.45	1	12.45	17.54	0.0058
Standard Vs Without	39.71	1	39.71	55.96	0.0003

Analyses of variance (ANOVA) and contrast for CYP79D1/D2 expression ratios in roots of Mcol 2215 in reduced nitrogen stress (three concentrations) after 10 day

S.V.	SS	df	MS	F	p-value
Model	0.90	2	0.45	0.11	0.8937
Treatments	0.90	2	0.45	0.11	0.8937
Error	23.63	6	3.94		
Total	24.53	8			

Contrasts

Treatments	SS	df	MS	F	p-value
Interm Vs Standard	0.90	1	0.90	0.23	0.6501
Interm Vs Without	0.17	1	0.17	0.04	0.8432
Standard Vs Without	0.29	1	0.29	0.07	0.7957

Analyses of variance (ANOVA) and contrast for Linamarase expression ratios in roots of Mcol 2215 in reduced nitrogen stress (three concentrations) after 10 day

S.V.	SS	df	MS	F	p-value
Model	9.34	2	4.67	3.43	0.1016
Treatments	9.34	2	4.67	3.43	0.1016
Error	8.17	6	1.36		
Total	17.51	8			

Contrasts

Treatments	SS	df	MS	F	p-value
Interm Vs Standard	6.74	1	6.74	4.95	0.0677
Interm Vs Without	7.26	1	7.26	5.33	0.0604
Standard Vs Without	0.01	1	0.01	0.01	0.9363

Analyses of variance (ANOVA) and contrast for HNL expression ratios in roots of Mcol 2215 in reduced nitrogen stress (three concentrations) after 10 day

S.V.	SS	df	MS	F	p-value
Model	45.64	2	22.82	7.12	0.0260
Treatments	45.64	2	22.82	7.12	0.0260
Error	19.23	6	3.20		
Total	64.86	8			

Contrasts

Treatments	SS	df	MS	F	p-value
Interm Vs Standard	28.37	1	28.37	8.85	0.0248
Interm Vs Without	39.21	1	39.21	12.24	0.0129
Standard Vs Without	0.87	1	0.87	0.27	0.6200

Analyses of variance (ANOVA) and contrast for β-CAS expression ratios in roots of Mcol 2215 in reduced nitrogen stress (three concentrations) after 10 day

S.V.	SS	df	MS	F	p-value
Model	2.19	2	1.09	1.91	0.2281
Treatments	2.19	2	1.09	1.91	0.2281
Error	3.44	6	0.57		
Total	5.62	8			

Contrasts

Treatments	SS	df	MS	F	p-value
Interm Vs Standard	0.33	1	0.33	0.58	0.4746
Interm Vs Without	2.15	1	2.15	3.76	0.1005
Standard Vs Without	0.79	1	0.79	1.39	0.2838

Analyses of variance (ANOVA) and contrast for CYP79D1/D2 expression ratios in leaves of 60444 in reduced nitrogen stress (three concentrations) after 1 day

S.V.	SS	df	MS	F	p-value
Model	22.41	2	11.21	2.74	0.1431
Treatments	22.41	2	11.21	2.74	0.1431
Error	24.58	6	4.10		
Total	46.99	8			

Contrasts

Treatments	SS	df	MS	F	p-value
Interm Vs Standard	15.41	1	15.41	3.76	0.1005
Interm Vs Without	0.11	1	0.11	0.03	0.8765
Standard Vs Without	18.10	1	18.10	4.42	0.0803

Analyses of variance (ANOVA) and contrast for Linamarase expression ratios in leaves of 60444 in reduced nitrogen stress (three concentrations) after 1 day

S.V.	SS	df	MS	F	p-value
Model	19.16	2	9.58	1.82	0.2418
Treatments	19.16	2	9.58	1.82	0.2418
Error	31.66	6	5.28		
Total	50.82	8			

Contrasts

Treatments	SS	df	MS	F	p-value
Interm Vs Standard	10.27	1	10.27	1.95	0.2125
Interm Vs Without	0.96	1	0.96	0.18	0.6844
Standard Vs Without	17.51	1	17.51	3.32	0.1183

Analyses of variance (ANOVA) and contrast for HNL expression ratios in leaves of 60444 in reduced nitrogen stress (three concentrations) after 1 day

S.V.	SS	df	MS	F	p-value
Model	36.96	2	18.48	5.24	0.0483
Treatments	36.96	2	18.48	5.24	0.0483
Error	21.16	6	3.53		
Total	58.12	8			

Contrasts

Sample Name TMS 6044	SS	df	MS	F	p-value
Interm Vs Standard	20.82	1	20.82	5.90	0.0512
Interm Vs Without	1.43	1	1.43	0.41	0.5471
Standard Vs Without	33.19	1	33.19	9.41	0.0220

Analyses of variance (ANOVA) and contrast for β-CAS expression ratios in leaves of 60444 in reduced nitrogen stress (three concentrations) after 1 day

S.V.	SS	df	MS	F	p-value
Model	10.27	2	5.14	2.29	0.1821
Treatments	10.27	2	5.14	2.29	0.1821
Error	13.44	6	2.24		
Total	23.72	8			

Contrasts

Treatments	SS	df	MS	F	p-value
Interm Vs Standard	7.60	1	7.60	3.39	0.1150
Interm Vs Without	1.3E-03	1	1.3E-03	6.0E-04	0.9813
Standard Vs Without	7.81	1	7.81	3.48	0.1112

Analyses of variance (ANOVA) and contrast for CYP79D1/D2 expression ratios in roots of 60444 in reduced nitrogen stress (three concentrations) after 1 day

S.V.	SS	df	MS	F	p-value
Model	13.50	2	6.75	1.12	0.3864
Treatments	13.50	2	6.75	1.12	0.3864
Error	36.19	6	6.03		
Total	49.69	8			

Contrasts

Treatments	SS	df	MS	F	p-value
Interm Vs Standard	6.71	1	6.71	1.11	0.3322
Interm Vs Without	0.93	1	0.93	0.15	0.7088
Standard Vs Without	12.62	1	12.62	2.09	0.1983

Analyses of variance (ANOVA) and contrast for Linamarase expression ratios in roots of 60444 in reduced nitrogen stress (three concentrations) after 1 day

S.V.	SS	df	MS	F	p-value
Model	1.16	2	0.58	0.47	0.6462
Treatments	1.16	2	0.58	0.47	0.6462
Error	7.38	6	1.23		
Total	8.54	8			

Contrasts

Treatments	SS	df	MS	F	p-value
Interm Vs Standard	1.04	1	1.04	0.85	0.3930
Interm Vs Without	0.05	1	0.05	0.04	0.8518
Standard Vs Without	0.65	1	0.65	0.53	0.4957

Analyses of variance (ANOVA) and contrast for HNL expression ratios in roots of 60444 in reduced nitrogen stress (three concentrations) after 1 day

S.V.	SS	df	MS	F	p-value
Model	1.12	2	0.56	0.09	0.9141
Treatment	1.12	2	0.56	0.09	0.9141
Error	36.72	6	6.12		
Total	37.84	8			

Contrasts

Treatments	SS	df	MS	F	p-value
Interm Vs Standard	0.13	1	0.13	0.02	0.8879
Interm Vs Without	1.08	1	1.08	0.18	0.6885
Standard Vs Without	0.46	1	0.46	0.07	0.7935

Analyses of variance (ANOVA) and contrast for β-CAS expression ratios in roots of 60444 in reduced nitrogen stress (three concentrations) after 1 day

S.V.	SS	df	MS	F	p-value
Model	5.20	2	2.60	3.43	0.1014
Treatments	5.20	2	2.60	3.43	0.1014
Error	4.55	6	0.76		
Total	9.75	8			

Contrasts

Treatments	SS	df	MS	F	p-value
Interm Vs Standard	0.19	1	0.19	0.26	0.6313
Interm Vs Without	2.95	1	2.95	3.90	0.0959
Standard Vs Without	4.66	1	4.66	6.15	0.0479

Analyses of variance (ANOVA) and contrast for CYP79D1/D2 expression ratios in leaves of 60444 in reduced nitrogen stress (three concentrations) after 10 day

S.V.	SS	df	MS	F	p-value
Model	3.74	2	1.87	0.53	0.6123
Treatments	3.74	2	1.87	0.53	0.6123
Error	21.07	6	3.51		
Total	24.82	8			

Contrasts

Leaves TMS	SS	df	MS	F	p-value
Interm Vs Standard	3.57	1	3.57	1.02	0.3520
Interm Vs Without	0.35	1	0.35	0.10	0.7636
Standard Vs Without	1.69	1	1.69	0.48	0.5136

Analyses of variance (ANOVA) and contrast for Linamarase expression ratios in leaves of 60444 in reduced nitrogen stress (three concentrations) after 10 day

S.V.	SS	df	MS	F	p-value
Model	4.13	2	2.06	1.21	0.3625
Leaves10	4.13	2	2.06	1.21	0.3625
Error	10.25	6	1.71		
Total	14.38	8			

Contrasts

Leaves10	SS	df	MS	F	p-value
Interm Vs Standard	3.27	1	3.27	1.91	0.2159
Interm Vs Without	0.01	1	0.01	0.01	0.9401
Standard Vs Without	2.91	1	2.91	1.70	0.2397

Analyses of variance (ANOVA) and contrast for HNL expression ratios in leaves of 60444 in reduced nitrogen stress (three concentrations) after 10 day

S.V.	SS	df	MS	F	p-value
Model	13.28	2	6.64	3.08	0.1203
Treatments	13.28	2	6.64	3.08	0.1203
Error	12.95	6	2.16		
Total	26.23	8			

Contrasts

Treatment	SS	df	MS	F	p-value
Interm Vs Standard	0.24	1	0.24	0.11	0.7507
Interm Vs Without	11.37	1	11.37	5.27	0.0615
Standard Vs Without	8.31	1	8.31	3.85	0.0973

Analyses of variance (ANOVA) and contrast for β-CAS expression ratios in leaves of 60444 in reduced nitrogen stress (three concentrations) after 10 day

S.V.	SS	df	MS	F	p-value
Model	5.07	2	2.53	1.57	0.2832
Treatments	5.07	2	2.53	1.57	0.2832
Error	9.69	6	1.62		
Total	14.76	8			

Contrasts

Treatments	SS	df	MS	F	p-value
Interm Vs Standard	2.80	1	2.80	1.73	0.2363
Interm Vs Without	0.22	1	0.22	0.14	0.7248
Standard Vs Without	4.58	1	4.58	2.84	0.1430

Analyses of variance (ANOVA) and contrast for CYP79D1/D2 expression ratios in Roots of 60444 in reduced nitrogen stress (three concentrations) after 10 day

S.V.	SS	df	MS	F	p-value
Model	7.39	2	3.69	0.65	0.5550
Treatments	7.39	2	3.69	0.65	0.5550
Error	34.07	6	5.68		
Total	41.46	8			

Contrasts

Treatments	SS	df	MS	F	p-value
Interm Vs Standard	0.88	1	0.88	0.15	0.7079
Interm Vs Without	3.03	1	3.03	0.53	0.4924
Standard Vs Without	7.17	1	7.17	1.26	0.3040

Analyses of variance (ANOVA) and contrast for Linamarase expression ratios in Roots of 60444 in reduced nitrogen stress (three concentrations) after 10 day

S.V.	SS	df	MS	F	p-value
Model	2.83	2	1.41	0.26	0.7758
Treatments	2.83	2	1.41	0.26	0.7758
Error	32.01	6	5.34		
Total	34.84	8			

Contrasts

Treatments	SS	df	MS	F	p-value
Interm Vs Standard	1.52	1	1.52	0.28	0.6130
Interm Vs Without	2.58	1	2.58	0.48	0.5126
Standard Vs Without	0.14	1	0.14	0.03	0.8762

Analyses of variance (ANOVA) and contrast for HNL expression ratios in Roots of 60444 in reduced nitrogen stress (three concentrations) after 10 day

S.V.	SS	df	MS	F	p-value
Model	18.31	2	9.16	1.64	0.2707
Treatments	18.31	2	9.16	1.64	0.2707
Error	33.55	6	5.59		
Total	51.86	8			

Contrasts

Treatments	SS	df	MS	F	p-value
Interm Vs Standard	0.33	1	0.33	0.06	0.8154
Interm Vs Without	15.68	1	15.68	2.81	0.1450
Standard Vs Without	11.45	1	11.45	2.05	0.2024

Analyses of variance (ANOVA) and contrast for β-CAS expression ratios in Roots of 60444 in reduced nitrogen stress (three concentrations) after 10 day

S.V.	SS	df	MS	F	p-value
Model	3.49	2	1.74	1.09	0.3939
Treatments	3.49	2	1.74	1.09	0.3939
Error	9.57	6	1.60		
Total	13.06	8			

Contrasts

Treatments	SS	df	MS	F	p-value
Interm Vs Standard	0.94	1	0.94	0.59	0.4715
Interm Vs Without	3.48	1	3.48	2.18	0.1899
Standard Vs Without	0.80	1	0.80	0.50	0.5046

Appendix H. Statistical Tables and Statistical Analysis for Chapter Seven

Descriptive Statistics for tissue comparison among Temperatures

The Ct from each cultivar was organized in a 45 x 5 Matrix following the subsequent data processing. Here we show the descriptive statistics for each tissue in gave cultivar, temperature stress and gene. The means and confidence intervals were re-transformed to original scale meanwhile the other descriptors are in Log_2 Scale.

Comparison among Leaves in Mcol 2215. Temperature Experiment

Gene	Temperature	Tissue	n	Mean	Standard Deviation	Standard Error	Confidence Interval (95%)
CYP79D1/D2	Standard	Leaves	5	0.84	0.47	0.21	0.59; 1.19
CYP79D1/D2	28^0C	Leaves	5	1.01	0.88	0.39	0.71; 1.44
CYP79D1/D2	35^0C	Leaves	5	1.17	0.46	0.21	0.82; 1.66
Linamarase	Standard	Leaves	5	0.98	1.39	0.62	0.57; 1.70
Linamarase	28^0C	Leaves	5	0.97	0.94	0.42	0.55; 1.67
Linamarase	35^0C	Leaves	5	1.06	0.37	0.17	0.61; 1.83
HNL	Standard	Leaves	5	0.78	1.45	0.65	0.41; 1.46
HNL	28^0C	Leaves	5	0.72	0.9	0.4	0.39; 1.35
HNL	35^0C	Leaves	5	1.77	0.96	0.43	0.94; 3.30
β-CAS	Standard	Leaves	5	1.04	0.76	0.34	0.58; 1.84
β-CAS	28^0C	Leaves	5	1.95	1.09	0.49	1.02; 3.46
β-CAS	35^0C	Leaves	5	0.50	1.22	0.55	0.27; 0.88

Comparison among Roots in Mcol 2215. Temperature Experiment

Gene	Temperature	Tissue	n	Mean	Standard Deviation	Standard Error	Confidence Interval (95%)
CYP79D1/D2	Standard	Roots	5	1.28	0.94	0.42	0.68; 2.43
CYP79D1/D2	28^0C	Roots	5	0.67	1.22	0.55	0.35; 1.27
CYP79D1/D2	35^0C	Roots	5	1.16	1.29	0.57	0.61; 2.21
Linamarase	Standard	Roots	5	1.13	0.32	0.14	0.58; 2.16
Linamarase	28^0C	Roots	5	1.3	1.41	0.63	0.68; 2.50
Linamarase	35^0C	Roots	5	0.68	1.46	0.65	0.35; 1.31
HNL	Standard	Roots	5	1.19	1.75	0.78	0.30; 4.70
HNL	28^0C	Roots	5	1.06	2.93	1.31	0.26; 4.18
HNL	35^0C	Roots	5	0.8	2.64	1.18	0.20; 3.14
β-CAS	Standard	Roots	5	1.02	0.99	0.44	0.51; 2.06
β-CAS	28^0C	Roots	5	1.16	1.11	0.5	0.58; 2.35
β-CAS	35^0C	Roots	5	0.84	1.63	0.73	0.42; 1.70

Comparison among Leaves in 60444. Temperature Experiment

Gene	Temperature	Tissue	n	Mean	Standard Deviation	Standard Error	Confidence Interval (95%)
CYP79D1/D2	Standard	Leaves	5	0.55	1.65	0.74	0.17; 1.77
CYP79D1/D2	28^0C	Leaves	5	1.45	1.96	0.88	0.45; 4.66
CYP79D1/D2	35^0C	Leaves	5	1.24	2.6	1.16	0.38; 3.97
Linamarase	Standard	Leaves	5	0.69	1.68	0.75	0.29;1.67
Linamarase	28^0C	Leaves	5	1.53	1.16	0.52	0.63; 3.69
Linamarase	35^0C	Leaves	5	0.95	1.86	0.83	0.39; 2.29
HNL	Standard	Leaves	5	1.02	0.68	0.3	0.49; 2.13
HNL	28^0C	Leaves	5	1.01	1.11	0.5	0.48; 2.11
HNL	35^0C	Leaves	5	0.96	1.9	0.85	0.46; 2
β-CAS	Standard	Leaves	5	1.22	0.58	0.26	0.72; 2.06
β-CAS	28^0C	Leaves	5	1.85	1.08	0.48	1.10; 3.12
β-CAS	35^0C	Leaves	5	0.44	1.09	0.49	0.26; 0.74

Comparison among Roots in 60444. Temperature Experiment

Gene	Temperature	Tissue	n	Mean	Standard Deviation	Standard Error	Confidence Interval (95%)
CYP79D1/D2	Standard	Roots	5	0.63	1.39	0.62	0.31; 1.26
CYP79D1/D2	28^0C	Roots	5	1.67	1.56	0.7	0.83; 3.34
CYP79D1/D2	35^0C	Roots	5	0.95	0.61	0.27	0.48; 1.90
Linamarase	Standard	Roots	5	0.82	2.08	0.93	0.35; 1.91
Linamarase	28^0C	Roots	5	1.46	1.56	0.7	0.62; 3.42
Linamarase	35^0C	Roots	5	0.83	0.6	0.27	0.35; 1.94
HNL	Standard	Roots	5	0.69	1.19	0.53	0.23; 2.06
HNL	28^0C	Roots	5	1.91	2.88	1.29	0.64; 5.67
HNL	35^0C	Roots	5	0.76	1.41	0.63	0.25; 2.25
B-CAS	Standard	Roots	5	0.51	0.56	0.25	0.32; 0.80
B-CAS	28^0C	Roots	5	1.28	0.71	0.32	0.82; 2.01
B-CAS	35^0C	Roots	5	1.52	1.08	0.48	0.97; 2.37

Analyses of variance (ANOVA) and contrast for CYP79D1/D2 expression ratios in Leaves of Mcol 2215 in temperature oscillation stress

S.V.	SS	df	MS	F	p-value
Model	0.58	2	0.29	0.71	0.5090
Treatments	0.58	2	0.29	0.71	0.5090
Error	4.85	12	0.40		
Total	5.43	14			

Contrasts

Treatments	SS	df	MS	F	p-value
28^0C Vs 35^0C	0.11	1	0.11	0.28	0.6089
28^0C Vs Standard	0.18	1	0.18	0.45	0.5171
35^0C Vs Standard	0.57	1	0.57	1.42	0.2560

Analyses of variance (ANOVA) and contrast for Linamarase expression ratios in Leaves of Mcol 2215 in temperature oscillation stress

S.V.	SS	df	MS	F	p-value
Model	0.05	2	0.03	0.03	0.9728
Treatment	0.05	2	0.03	0.03	0.9728
Error	11.77	12	0.98		
Total	11.83	14			

Contrasts

Sample Name	SS	df	MS	F	p-value
28^0C Vs 35^0C	0.05	1	0.05	0.05	0.8319
28^0C Vs Standard	8.7E-04	1	8.7E-04	8.8E-04	0.9768
35^0C Vs Standard	0.03	1	0.03	0.04	0.8546

Analyses of variance (ANOVA) and contrast for HNL expression ratios in Leaves of Mcol 2215 in temperature oscillation stress

S.V.	SS	df	MS	F	p-value
Model	5.09	2	2.54	1.99	0.1793
Treatment	5.09	2	2.54	1.99	0.1793
Error	15.34	12	1.28		
Total	20.42	14			

Contrasts

Sample Name	SS	df	MS	F	p-value
28^0C Vs 35^0C	4.14	1	4.14	3.24	0.0972
28^0C Vs Standard	0.03	1	0.03	0.02	0.8814
35^0C Vs Standard	3.47	1	3.47	2.71	0.1256

Analyses of variance (ANOVA) and contrast for β-CAS expression ratios in Leaves of Mcol 2215 in temperature oscillation stress

S.V.	SS	df	MS	F	p-value
Model	9.76	2	4.88	4.49	0.0351
Treatment	9.76	2	4.88	4.49	0.0351
Error	13.05	12	1.09		
Total	22.81	14			

Contrasts

Sample Name	SS	df	MS	F	p-value
28^0C Vs 35^0C	9.74	1	9.74	8.95	0.0112
28^0C Vs Standard	2.05	1	2.05	1.88	0.1949
35^0C Vs Standard	2.85	1	2.85	2.62	0.1313

Analyses of variance (ANOVA) and contrast for CYP79D1/D2 expression ratios in roots of Mcol 2215 in temperature oscillation stress

S.V.	SS	df	MS	F	p-value
Model	2.55	2	1.28	0.95	0.4134
Treatment	2.55	2	1.28	0.95	0.4134
Error	16.11	12	1.34		
Total	18.66	14			

Contrasts

Treatment	SS	df	MS	F	p-value
28^0C Vs 35^0C	1.61	1	1.61	1.20	0.2953
28^0C Vs Standard	2.18	1	2.18	1.63	0.2265
35^0C Vs Standard	0.04	1	0.04	0.03	0.8597

Analyses of variance (ANOVA) and contrast for Linamarase expression ratios in roots of Mcol 2215 in temperature oscillation stress

S.V.	SS	df	MS	F	p-value
Model	2.37	2	1.18	0.84	0.4542
Treatment	2.37	2	1.18	0.84	0.4542
Error	16.85	12	1.40		
Total	19.22	14			

Contrasts

Treatments	SS	df	MS	F	p-value
28^0C Vs 35^0C	2.14	1	2.14	1.52	0.2405
28^0C Vs Standard	0.10	1	0.10	0.07	0.7928
35^0C Vs Standard	1.31	1	1.31	0.93	0.3530

Analyses of variance (ANOVA) and contrast for HNL expression ratios in roots of Mcol 2215 in temperature oscillation stress

S.V.	SS	df	MS	F	p-value
Model	0.90	2	0.45	0.07	0.9303
Treatment	0.90	2	0.45	0.07	0.9303
Error	74.33	12	6.19		
Total	5.23	14			

Contrasts

Treatments	SS	df	MS	F	p-value
28^0C Vs 35^0C	0.43	1	0.43	0.07	0.7959
28^0C Vs Standard	0.07	1	0.07	0.01	0.9177
35^0C Vs Standard	0.85	1	0.85	0.14	0.7179

Analyses of variance (ANOVA) and contrast for β-CAS expression ratios in roots of Mcol 2215 in temperature oscillation stress

S.V.	SS	df	MS	F	p-value
Model	0.56	2	0.28	0.17	0.8445
Treatment	0.56	2	0.28	0.17	0.8445
Error	19.45	12	1.62		
Total	20.01	14			

Contrasts

Treatments	SS	df	MS	F	p-value
28^0C Vs 35^0C	0.55	1	0.55	0.34	0.5719
28^0C Vs Standard	0.08	1	0.08	0.05	0.8233
35^0C Vs Standard	0.20	1	0.20	0.12	0.7303

Analyses of variance (ANOVA) and contrast for CYP79D1/D2 expression ratios in Leaves of 60444 in temperature oscillation stress

S.V.	SS	df	MS	F	p-value
Model	5.63	2	2.82	0.63	0.5481
Treatment	5.63	2	2.82	0.63	0.5481
Error	53.43	12	4.45		
Total	59.06	14			

Contrasts

Treatments	SS	df	MS	F	p-value
28^0C Vs 35^0C	0.14	1	0.14	0.03	0.8639
28^0C Vs Standard	4.91	1	4.91	1.10	0.3145
35^0C Vs Standard	3.41	1	3.41	0.76	0.3990

Analyses of variance (ANOVA) and contrast for Linamarase expression ratios in Leaves of 60444 in temperature oscillation stress

S.V.	SS	df	MS	F	p-value
Model	3.31	2	1.66	0.65	0.5397
Treatment	3.31	2	1.66	0.65	0.5397
Error	30.59	12	2.55		
Total	33.90	14			

Contrasts

Treatments	SS	df	MS	F	p-value
28^0C Vs 35^0C	1.20	1	1.20	0.47	0.5052
28^0C Vs Standard	3.26	1	3.26	1.28	0.2801
35^0C Vs Standard	0.50	1	0.50	0.20	0.6648

Analyses of variance (ANOVA) and contrast for HNL expression ratios in Leaves of 60444 in temperature oscillation stress

S.V.	SS	df	MS	F	p-value
Model	0.02	2	0.01	0.01	0.9932
Treatments	0.02	2	0.01	0.01	0.9932
Error	21.26	12	1.77		
Total	21.29	14			

Contrasts

Tretments	SS	df	MS	F	p-value
28^0C Vs 35^0C	0.02	1	0.02	0.01	0.9282
28^0C Vs Standard	4.4E-04	1	4.4E-04	2.5E-04	0.9876
35^0C Vs Standard	0.02	1	0.02	0.01	0.9159

Analyses of variance (ANOVA) and contrast for β-CAS expression ratios in Leaves of 60444 in temperature oscillation stress

S.V.	SS	df	MS	F	p-value
Model	11.40	2	5.70	6.34	0.0132
Treatments	11.40	2	5.70	6.34	0.0132
Error	10.80	12	0.90		
Total	22.20	14			

Contrasts

Sample Name	SS	df	MS	F	p-value
28^0C Vs 35^0C	0.75	1	10.75	11.95	0.0047
28^0C Vs Standard	0.89	1	0.89	0.99	0.3397
35^0C Vs Standard	5.46	1	5.46	6.07	0.0299

Analyses of variance (ANOVA) and contrast for CYP79D1/D2 expression ratios in roots of 60444 in temperature oscillation stress

S.V.	SS	df	MS	F	p-value
Model	5.03	2	2.52	1.59	0.2434
Sample Name	5.03	2	2.52	1.59	0.2434
Error	18.96	12	1.58		
Total	24.00	14			

Contrasts

Sample Name	SS	df	MS	F	p-value
28^0C Vs 35^0C	1.64	1	1.64	1.04	0.3287
28^0C Vs Standard	5.00	1	5.00	3.16	0.1006
35^0C Vs Standard	0.91	1	0.91	0.58	0.4616

Analyses of variance (ANOVA) and contrast for linamarase expression ratios in roots of 60444 in temperature oscillation stress

S.V.	SS	df	MS	F	p-value
Model	2.30	2	1.15	0.48	0.6274
Treatments	2.30	2	1.15	0.48	0.6274
Error	28.48	12	2.37		
Total	30.78	14			

Contrasts

Sample Name	SS	df	MS	F	p-value
28^0C Vs 35^0C	1.69	1	1.69	0.71	0.4154
28^0C Vs Standard	1.76	1	1.76	0.74	0.4059
35^0C Vs Standard	7.4E-04	1	7.4E-04	3.1E-04	0.9862

Analyses of variance (ANOVA) and contrast for HNL expression ratios in roots of 60444 in temperature oscillation stress

S.V.	SS	df	MS	F	p-value
Model	6.55	2	3.28	0.84	0.4558
Sample Name	6.55	2	3.28	0.84	0.4558
Error	46.83	12	3.90		
Total	53.38	14			

Contrasts

Sample Name	SS	df	MS	F	p-value
28^0C Vs 35^0C	4.41	1	4.41	1.13	0.3088
28^0C Vs Standard	5.37	1	5.37	1.38	0.2634
35^0C Vs Standard	0.05	1	0.05	0.01	0.9138

Analyses of variance (ANOVA) and contrast for β-CAS expression ratios in roots of 60444 in temperature oscillation stress

S.V.	SS	df	MS	F	p-value
Model	7.01	2	3.50	5.32	0.0222
Treatment	7.01	2	3.50	5.32	0.0222
Error	7.90	12	0.66		
Total	14.91	14			

Contrastes

Sample Name	SS	df	MS	F	p-value
28^0C Vs 35^0C	0.15	1	0.15	0.23	0.6437
28^0C Vs Standard	4.31	1	4.31	6.54	0.0251
35^0C Vs Standard	6.05	1	6.05	9.19	0.0104