UNIVERSIDADE FEDERAL DE SANTA CATARINA
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PROGRAMA DE PÓS-GRADUAÇÃO EM RECURSOS
GENÉTICOS VEGETAIS

Virgílio Gavicho Uarrota

ANÁLISES METABOLÔMICAS, ENZIMÁTICAS E HISTOLÓGICAS DE RAÍZES DE MANDIOCA (*Manihot esculenta* Crantz), DURANTE A DETERIORAÇÃO FISIOLÓGICA EM PÓS-COLHEITA

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Tese submetida ao Programa de Pós-Graduação em Recursos Genéticos Vegetais da Universidade Federal de Santa Catarina para a obtenção do Grau de Doutor em Ciências, especialidade em Recursos Genéticos Vegetais
Orientador: Prof. Dr. Marcelo Maraschin

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Análises metabolômicas, enzimáticas e histológicas de raízes de mandioca (*Manihot esculenta* Crantz) durante a deterioração fisiológica pós-colheita

por

Virgílio Gavicho Uarrota

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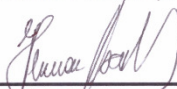
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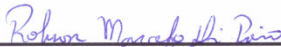
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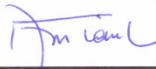
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Aos meus amigos e colegas de Moçambique, e a todos, que este trabalho sirva de fonte de inspiração.

The "food of the poor" has become a multipurpose crop that responds to the priorities of developing countries, to trends in the global economy and to the challenge of climate change.

Governments need to encourage smallholders' participation in a sustainable cassava development agenda, and support research and extension approaches that "let farmers decide".

(Howeler et al. 2013)

O "alimento dos pobres" se tornou numa cultura de múltiplos propósitos que responde às prioridades de desenvolvimento, tendências na economia global e aos desafios sobre mudanças climáticas.

Governos precisam encorajar a participação de pequenos agricultores na agenda sobre o desenvolvimento sustentável da mandioca, e dar suporte às pesquisas e abordagens de extensão "que facilitam aos agricultores na tomada de decisões".

(Tradução de Virgílio Gavicho Uarrota, 2015)

RESUMO

O presente trabalho teve como objetivo investigar as alterações no metabolismo secundário, na atividade enzimática e na histologia de raízes de quatro cultivares de mandioca (*Manihot esculenta* Crantz) durante a deterioração fisiológica em pós-colheita (PPD). Os resultados mostraram que durante os estágios iniciais de armazenamento das raízes, metabólitos secundários são formados (e.g., compostos fenólicos totais, antocianinas, flavonoides, escopoletina); glicosídeos cianogênicos (ácido cianídrico total, linamarina, acetona cianoidrina) são incrementados até 72 h de armazemento; a atividade de sistemas enzimáticos antioxidantes é ativada (e.g., catalase, superóxido dismutase, cobre/zinco dismutase, manganês dismutase, polifenol oxidase, ascorbato e guaiacol peroxidases, proteínas, linamarase); sistemas não enzimáticos antioxidantes (e.g., peróxido de hidrogênio, tocoferol, ácido ascórbico) açúcares solúveis, polissacarídeos ácidos e ácidos orgânicos foram observados se acumular durante o armazenamento nas cultivares.

A principal hidroxicumarina observada foi a escopoletina. As cultivares mais tolerantes à deterioração (Branco/IAC576-70) mostraram níveis mais elevados deste metabólito, comparativamente às susceptíveis (Oriental/Sangão). Os conteúdos de escopoletina mostraram-se crescentes durante a deterioração, fato que nos permite concluir que a escopoletina poder estar envolvida na redução da deterioração no estágio inicial deste processo. A ruptura dos compartimentos celulares derivada das lesões tissulares por ocasião da colheita permitiu o contacto da linamarase com o seu substrato linamarina, fato constatado pelo incremento dos teores de ácido cianídrico até 3-5 dias de deterioração, seguido de degradação. A atividade da linamarase foi elevada nestes estágios, mostrando-se, contudo inativa nas fases avançadas de PPD. Acúmulos de polissacarídeos ácidos e neutros (e.g. celulose) foram constatados nas paredes celulares durante a evolução do processo de deterioração, um fato eventualmente associado a respostas de defesa vegetal à infecção por microrganismos, ou ainda a espécies reativas de oxigênio formadas na deterioração fisiológica. A hidrólise dos grânulos de amido foi detectada, subsidiando a hipótese de catabolização daquele polissacarídeo e o acúmulo do produto metabólico derivado, conforme observado durante o processo de deterioração.

Técnicas estatísticas multivariadas (métodos supervisionados e não supervisionados) e modelos de predição permitiram discriminar as

amostras de acordo com as variáveis metabólicas estudadas, revelando quais variáveis bioquímicas influenciaram mais intensamente a deterioração fisiológica (PPD). Modelos preditivos mostraram ser as atividades da guaiacol peroxidase, ascorbato peroxidase, superóxido dismutase e catalase e os conteúdos totais de proteínas, carotenoides e peróxido de hidrogênio as variáveis mais relevantes para determinar o atraso da PPD. O presente estudo também permitiu identificar maior tolerância à PPD da cultivar Branco, enquanto a suscetibilidade mostrou-se maior na cultivar Oriental. Por fim, considerando-se que a PPD pode ser influenciada por uma série de fatores bióticos e abióticos, um maior detalhamento dos estudos e uma integração de dados a nível genômico, proteômico, transcriptômico e metabolômico corroborará com a geração de um quadro de melhor entendimento dos processos bioquímicos relacionados à PPD da mandioca, com consequências positivas nos contextos agronômico e sócio-econômico afins.

Palavras-chave: Mandioca; Deterioração fisiológica; Metabólitos, Glicosídeos Cianogênicos; Antioxidantes; Hidroxicumarinas; Ácidos orgânicos; Açúcares; Polissacarídeos; Metabolômica; Histoquímica; Quimiometria.

ABSTRACT

This study aimed to investigate the changes in the secondary metabolism, enzyme activity and histology of four cultivars of cassava roots (*Manihot esculenta* Crantz) during postharvest physiological deterioration. The results showed that during the early stages of storage of roots, secondary metabolites are formed (e.g., phenolic compounds, anthocyanins, flavonoids, scopoletin); cyanogenic glucosides (total cyanide, linamarin, acetone cyanohydrin) are increased up to 72 h of storage; antioxidant enzyme systems are activated (e.g., catalase, superoxide dismutase, copper/zinc superoxide, manganese superoxide, polyphenol oxidase, guaiacol and ascobate peroxidase, proteins, linamarase); non-enzymatic antioxidant systems (e.g., hydrogen peroxide, tocopherol, ascorbic acid), soluble sugars, organic acids, and acidic polysaccharides were observed to accumulate during storage.

The main hydroxycoumarin observed was scopoletin. The most tolerant cultivars (Branco / IAC576-70) showed higher levels of that metabolite compared to those susceptible (Oriental / Sangão). The scopoletin content increased during the deterioration (PPD), a fact that allows us to conclude that scopoletin might be involved in the reduction of deterioration at an early stage of the process. Disruption of cellular compartments derived from tissue damage at harvest allowed the contact of linamarase with your substrate limanarin, a fact confirmed by the increase of cyanide contents 3 to 5 days of storage, followed by the degradation. The activity of linamarase was high in these stages, however in the advanced stages of PPD, a decreasing was observed. Accumulation of acidic polysaccharides were found in the cell walls during the course of deterioration, a fact possibly associated with plant defense responses to infection by microorganisms, or the reactive oxygen species formed in the physiological deterioration. Hydrolysis of starch granules was detected, supporting the hypothesis of that polysaccharide catabolism with the accumulation of their derivatives, as observed during the process of deterioration.

Multivariate statistical techniques (supervised and unsupervised methods) and prediction models discriminated the samples in accordance with their metabolic variables, revealing which biochemical variables influenced more intensively in postharvest physiological deterioration (PPD). Predictive models proved the activities of guaiacol peroxidase, ascorbate peroxidase, superoxide dismutase and catalase, total proteins,

carotenoids and hydrogen peroxide as the most relevant variables. This study also identified Branco cultivars as the most tolerant, while Oriental cultivar showed to be the most susceptible. Finally, considering that PPD can be influenced by a number of factors, more detailed studies and data integration with genomics, proteomic, transcriptomic, and metabolomic are crucial for a better understanding of the biochemical processes related to PPD in cassava, which can have positive effects on future agronomic and socio-economic contexts.

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1 INTRODUÇÃO

A mandioca (*Manihot esculenta* Crantz) é um arbusto perene cultivado há cerca de 9000 anos, principalmente devido às suas raízes amiláceas, sendo uma das culturas agrícolas mais antigas (HOWLER et al. 2013). Apesar de alguns estudos indicarem vários centros de origem da mandioca, a espécie cultivada parece ser originária do sudeste da Amazônia brasileira (ALLEM, 2002; OLSEN & SCHAAL, 1999; HERSHEY, 1987; NASSAR, 1978). Na era pré-colombiana, a mandioca foi cultivada em diversas partes da América do sul, Mesoamérica e nas ilhas do Caribe. Logo após a conquista do continente sul-americano pelos espanhóis e portugueses, a cultura foi levada do Brasil à costa atlântica da África e no século XIX já era cultivada na costa leste de África e sudeste da Ásia. O cultivo da mandioca se expandiu no século XX, quando esta emergiu como uma importante cultura alimentar na África Sub-Sahariana, Índia, Indonésia e Filipinas (HOWLER et al. 2013).

Sendo uma cultura sensível a geadas e por ter um ciclo de aproximadamente 1 ano, a mandioca é cultivada quase exclusivamente em regiões tropicais e subtropicais. A cultura possui qualidades que a tornam atrativa aos pequenos agricultores que dispõem de solos pobres em nutrientes aos seus cultivos, adaptando-se também a regiões com pouca ou imprevisível precipitação pluviométrica. Por ser de propagação vegetativa, o material de plantio se torna de baixo custo e facilmente disponível. A planta é altamente tolerante a solos ácidos e associa-se simbioticamente a fungos do solo, permitindo-lhe maior eficiência à absorção de fósforo e micronutrientes. Como forma de prevenção ao ataque de herbívoros, dois glicosídeos cianogênicos são encontrados em seus tecidos foliares, os quais após metabolização no trato digestivo do herbívoro geram ácido cianídrico, um composto altamente tóxico. Por ser eficiente no uso de água e nutrientes do solo e tolerante ao ataque esporádico de pragas, gera aos agricultores com poucos insumos a perspectiva de produções razoáveis, fato não observado com outras culturas, e.g., trigo e arroz (FAO, 1997).

As raízes da mandioca possuem 70% de água, sua matéria seca é rica em carboidratos (250 a 300 Kg por tonelada de raízes frescas), amido, sendo uma fonte de energia de alto rendimento comparativamente aos cereais. Possuem alto teor de vitaminas C, tiamina, riboflavina e niacina e raízes amarelas geralmente apresentam elevados níveis de carotenos. Dependendo da variedade, as raízes podem conter altos níveis de glicosídeos cianogênicos, especialmente nas camadas exteriores. Suas folhas contêm 25% de proteínas (em peso seco) e tanto folhas e raízes podem ser consumidas por populações humanas. O amido das raízes é usado em muitas indústrias, e.g., alimentar, farmacêutica, têxtil, compensados, papel e adesivos, bem como matéria-prima para produção de etanol. Todos esses atributos fazem da mandioca uma das espécies mais confiáveis à segurança alimentar mundial (HOWLER et al. 2013).

Globalmente, a mandioca é a segunda raiz mais importante depois da batata e a quinta cultura mais importante em termos de consumo calórico humano (ROSENTHAL et al., 2012). No entanto, a subsistência de populações dependentes desta espécie e seu uso comercial são afetados pelo seu curto tempo de armazenamento, devido ao rápido processo de deterioração fisiológica em pós-colheita (PPD, do inglês, "*postharvest physiological deterioration*"). Este processo torna a raiz impalatável dentro de 72 horas após a colheita e parece ser um problema não conhecido em outras culturas (LYER et al. 2010). Ativada pelo dano mecânico (inevitável durante o processo de colheita), a PPD se desenvolve a partir do local da lesão radicular, eventualmente causando uma descoloração generalizada do parênquima vascular daquele órgão, acompanhada por um odor desagradável. Wenham (1995) estimou perdas devido à PPD da ordem de 5 a 25% do valor total dos cultivos. Mais recentemente, Zidenga (2012) estimou que o aumento do tempo de armazenamento daquela biomassa em algumas semanas contribuiria com redução de perdas financeiras em cerca de US$ $2,9.10^9$ a Nigéria, num período de 20 anos.

Trabalhos prévios sobre a PPD usualmente referem-se à perecibilidade e ao consequente reduzido período de armazenamento das raízes de mandioca (RICKARD & COURSEY, 1981), não fornecendo indicações sobre a natureza, ou mesmo os sintomas do processo de deterioração envolvido. Adicionalmente, mencionam a necrose tissular, dando a impressão de que a deterioração resulta essencialmente de uma infecção microbiológica. No entanto, a PPD é um processo fisiológico ativo caracterizado por mudanças na expressão gênica, síntese de proteínas e

acúmulo de metabólitos secundários (REILLY et al. 2007; UARROTA et al. 2014).

Estudos moleculares e bioquímicos sobre a PPD têm associado o importante efeito de espécies reativas de oxigênio (ROS, do inglês, "reactive oxygen species") ao desencadeamento do processo, bem como a alteração do perfil metabólico associado a compostos de baixo peso molecular, por exemplo. Genes específicos sobre a PPD têm sido identificados e caracterizados em sua expressão (REILLY et al. 2007), bem como metabólitos secundários, particularmente as hidroxicumarinas que são acumuladas no processo (GNONLONFIN et al., 2012; BAYOUMI et al., 2010; BLAGBROUGH et al., 2010). No entanto, assume-se que pouco se sabe ainda sobre as alterações bioquímicas envolvidas na deterioração fisiológica de raízes de mandioca após a colheita.

Em função do exposto e tendo em conta a importância da cultura, o objetivo principal deste estudo foi identificar alterações bioquímicas, enzimáticas e histológicas em raízes de quatro cultivares de mandioca durante a deterioração fisiológica em pós-colheita. Pesquisas relacionadas à PPD abrirão novas perspectivas à engenharia de novas cultivares de mandioca com tempo de armazenamento prolongado usando os biomarcadores relacionados com o atraso da PPD, contribuindo para a ampliação dos usos industriais da cultura e à segurança alimentar mundial.

2 OBJETIVOS

2.1 Objetivo Geral

Investigar as alterações no metabolismo secundário, na atividade enzimática e na histologia de raízes de quatro cultivares de mandioca (*Manihot esculenta* Crantz) durante a deterioração fisiológica em pós-colheita.

2.2 Objetivos Específicos

a) Determinar o conteúdo total de compostos fenólicos, flavonóides, carotenóides e antocianinas por espectrofotometria UV-visível (*UV-vis*) em amostras de raízes frescas e durante a deterioração fisiológica em pós-colheita (PPD);

b) Determinar o teor de glicosídeos cianogênicos (ácido cianídrico total, linamarina e acetona cianoidrina) e a atividade enzimática da linamarase*;*

c) Quantificar as atividades de sistemas enzimáticos (superóxido dismutase, manganês e cobre-zinco dismutase, catalase, ascorbato peroxidase, guaiacol peroxidase, polifenol oxidase) e não enzimáticos (ácido ascórbico, proteínas, tocoferol e peróxido de hidrogênio) com efeito antioxidante em raízes de mandioca frescas e durante a PPD;

d) Determinar o perfil metabólico de ácidos orgânicos, açúcares solúveis e hidroxicumarinas em amostras de mandioca fresca e durante a PPD;

e) Detectar alterações morfo-anatômicas em amostras de raízes de mandioca via microscopia de luz e técnicas histoquímicas, associadas a polissacarídeos ácidos, neutros e proteínas;

f) Estabelecer o perfil metabólico de amostras de raízes de mandioca frescas e durante a PPD, via espectroscopia vibracional de infravermelho médio (ATR-FTIR);

g) Construir modelos estatísticos preditivos e descritivos, de classificação e de reconhecimento de padrões, a partir dos dados de perfis metabólicos e enzimáticos, via técnicas de estatística multivariada não supervisionada e supervisionada.

3 REVISÃO BIBLIOGRÁFICA

3.1 Origem e domesticação da Mandioca

Todas as 98 espécies do gênero *Manihot* são originárias dos neotrópicos, a partir de onde a mandioca foi disseminada para outras regiões do mundo (CEBALLOS & DE LA CRUZ, 2012). A origem da mandioca cultivada permanece ainda incerta, no entanto, três questões relevantes levantadas por Allem (2002) devem ser consideradas: A origem botânica (parentais selvagens que eventualmente deram origem a *M. esculenta*), origem geográfica (área onde os progenitores evoluíram no passado geológico) e origem agrícola (região de domesticação inicial dos ancestrais selvagens). A hipótese prevalecente é de que a espécie cultivada é originária da América do Sul (ALLEM, 2002).

Até 1982, havia certa tendência na literatura de assumir que a mandioca não tinha ancestrais conhecidos (ALLEM, 1994; 2002). No entanto, quando populações selvagens com características morfológicas indistinguíveis à espécie cultivada foram encontradas no estado de Goiás (Região Central do Brasil), uma nova era surgiu, pois permitiu fazer-se uma relação de tais populações com a origem botânica da mandioca cultivada. Um reconhecimento formal surgiu, mostrando que 3 subespécies, (*Manihot esculenta* Crantz ssp. *flabellifolia* (Pohl); *Manihot esculenta* Crantz ssp. *peruviana* (Mueller) e *Manihot pruinosa*) poderiam ser parentais selvagens mais próximos da espécie cultivada. Estudos têm mostrado que a subespécie *flabellifolia* e a espécie *pruinosa* descendem do mesmo estoque genético ("*commom primeval stock*"). No que se refere à origem geográfica, o centro primário de diversidade pode ser o bioma cerrado. A hipótese atual considera que dado a maior diversidade biológica da espécie se encontrar no Distrito Federal e regiões vizinhas de Goiás, o estoque genético que deu origem a *M. pruinosa* e à ssp. *flabellifolia* pode ter surgido na savana centro-brasileiro, diferenciando-se em duas espécies que mais tarde colonizaram a Amazônia. Quanto à origem agrícola, considera-se que as três áreas possíveis de domesticação inicial são os estados de Rondônia, Mato Grosso e Goiás. Tal domesticação pode ter ocorrido nas periferias da floresta amazônica, uma vez que os ancestrais selvagens não se distribuem dentro desta floresta (ALLEM, 2002).

3.2 Taxonomia e morfologia da mandioca

A mandioca pertence à família Euphorbiaceae, composta por cerca de 7200 espécies, e caracterizada pelo desenvolvimento notável de vasos lactíferos formados por células secretoras de látex. A arquitetura da planta varia enormemente nesta família, desde o tipo arbóreo como na borracha (*Hevea brasiliensis*) até arbustos de importância econômica como no rícino (*Ricinus communis*). O gênero mais significante é *Manihot*, ao qual a mandioca pertence (CEBALLOS & DE LA CRUZ, 2012; CEBALLOS et al. 2010). Na America Latina a espécie é conhecida por yuca (do espanhol) ou mandioca (português). No Brasil, a mandioca doce (aipim) é distinguida da amarga (mandioca). Outros nomes incluem manioc, manioca, tapioca e mhogo.

Cerca de 50% das Euforbiáceas são poliplóides, no entanto, a planta é diplóide funcional (2n=2x=36). É uma planta monóica, com sistema radicular hábil em armazenar quantidades importantes de amido, razão de seu valor econômico (CEBALLOS & DE LA CRUZ, 2012). Apesar de a propagação comercial ser feita por estacas, a reprodução sexual, elemento chave no melhoramento da espécie, é comum e fácil de ser obtida (CEBALLOS et al. 2012; CEBALLOS et al. 2010).

3.3 Produção de mandioca e sua importância econômica mundial

Tanto definida como uma cultura impossível de intensificação, a mandioca cresceu dramaticamente na agricultura mundial. O "*alimento de pobres*" se tornou mais recentemente uma cultura de múltiplos propósitos que responde às prioridades de desenvolvimento de diversos países, às tendências na economia global e aos desafios sobre mudanças climáticas e segurança alimentar (HOWLER et al. 2013). Referenciando-se ao início deste século, a produção mundial cresceu numa estimativa de cerca de 100 milhões de toneladas, impulsionada pela Ásia (demanda de mandioca seca e amido ao consumo animal e industrial) e África (expansão de mercados urbanos). No entanto, existe ainda uma grande margem de expansão à produção, especialmente considerando o fato de que sob condições ótimas de cultivo, o rendimento da mandioca pode alcançar 80 t/ha, comparativamente ao valor médio mundial corrente de 12,8 t/ha (HOWLER et al. 2013).

A mandioca (*Manihot esculenta* Crantz) é a maior raiz tropical produzida na África, Ásia, America Latina e Oceania, alimentando mais de 800

milhões de pessoas diariamente. A raiz, maior porção comestível da planta, é uma importante fonte de energia à dieta humana e compreende mais de 80% de amido (ISAMAH et al. 2003, ISAMAH, 2004, LYER et al. 2010). Historicamente, a mandioca tem desempenhado papel importante na segurança alimentar como uma cultura de reserva à fome. No leste e sul da África onde o milho é preferido, mas a seca é recorrente, a mandioca, mais tolerante aos *deficits* hídricos em relação aquele cereal, tem sido cultivada quando outras culturas falham. De forma similar, a mandioca fornece segurança alimentar adicional quando conflitos armados destroem outras culturas, por permanecer viável no solo até um período de 36 meses (ROSENTHAL & ORT, 2012). Apesar de ser a mandioca uma cultura vital à subsistência de um significativo número de agricultores familiares, sua importância também tem crescido na escala regional e global. Para além do seu papel na segurança alimentar, tem sido utilizada como fonte de biomassa à produção de bioconbustível na China, Tailândia e Brasil, por exemplo (ZHANG et al. 2003; DAY et al. 2006; NGUYEN et al. 2007; ZIDENGA, 2012; ZIDENGA et al. 2012). Globalmente, a mandioca é a segunda raiz mais importante depois da batata e a quinta cultura mais importante em termos gerais no que se refere ao consumo calórico humano (ROSENTHAL et al. 2012).

A figura 1 mostra o mapa de produção média de mandioca no mundo, no período 2009-2013. De 1980 a 2011, a área global de produção desta espécie aumentou cerca de 44%, (13,6 $\rightarrow$ 19,6 milhões de hectares), constituindo o maior crescimento das cinco culturas mais relevantes (milho, arroz, trigo, batata e mandioca). Nesse mesmo período, a produção da mandioca mais que dobrou, i.e., 124.10^6 t para 252.10^6 t (HOWLER et al. 2013).
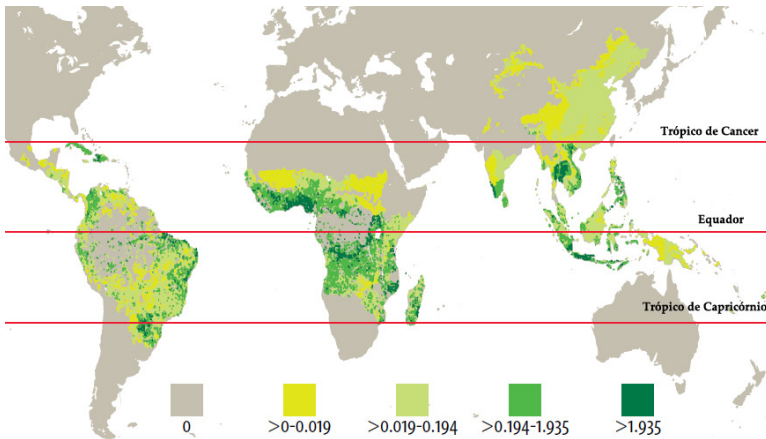
Figura 1: Áreas de produção de mandioca (ha/km^2) no mundo no período de 2009 a 2013. Adaptado de Monfreda et al (2008).

Nos últimos cinco anos, configuram-se como os maiores produtores mundiais de mandioca a Nigéria (480.10^6 t), Tailândia (268.10^6 t), Brasil (238.10^6 t), Indonésia (236.10^6 t) e Republica Democrática de Congo (155.10^6 t – Fig. 2). Em termos continentais, da África provém a maior produção de mandioca (55,3%), seguida da Ásia (32,1%) e America Latina (12,5% - Fig. 3). Considerando as tendências de produção nos principais países, incremento insignificante tem sido observado, exceto a Nigéria e Tailândia, onde um crescimento acelerado nos últimos cinco anos tem ocorrido (Fig. 4 - FAOSTAT, 2014).
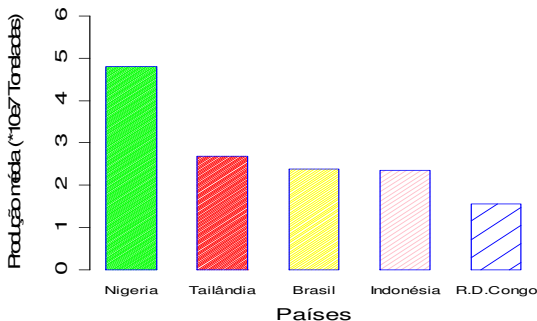
Figura 2. Maiores produtores mundiais de mandioca no período 2009-2013. Gráfico produzido usando dados oficiais da FAO, divisão de estatística (FAOSTAT, 2014).
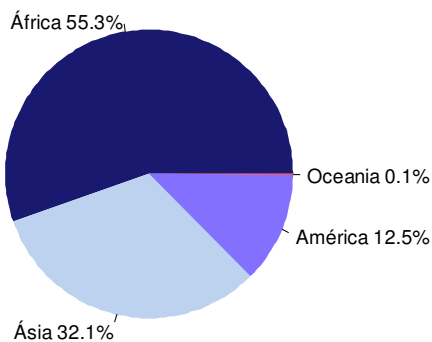
Figura 3. Contribuição relativa (%) dos continentes à produção de mandioca mundial. Gráfico produzido usando software R a partir dos dados oficiais da FAO, divisão de estatística (FAOSTAT, 2014)
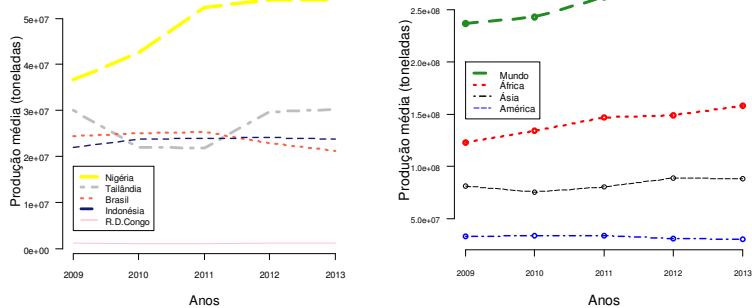
Figura 4: Tendências de produção média de mandioca (2009-2013) nos principais países (gráfico à esquerda) e nas regiões produtoras no mundo (gráfico à direita). Gráficos produzidos a partir dos dados oficiais da FAO, divisão de estatística (FAOSTAT, 2014).

Na África sub-sahariana, a mandioca é cultivada em pequenas propriedades por agricultores que quase não usam insumos externos à produção. É usualmente associada a outras culturas como o milho, arroz

e legumes, sendo essencialmente uma cultura alimentar. Cerca de 90% das raízes produzidas destinam-se ao consumo humano e 10% são semi-processadas ao consumo animal. O maior ganho na produção de mandioca na África desde 2000 foi observado na região oeste, com incremento de 60% (i.e., 47.10^6 t $\rightarrow$ 76.10^6 t). O aumento se deveu ao reconhecimento do potencial industrial da espécie que poderá diversificar o rendimento dos agricultores e a gerar emprego (HOWLER et al. 2013).

Na Ásia, o crescimento se deu basicamente ao cultivo intensivo, uma vez que a área cultivada no período 1980-2011 manteve-se constante (~ $3,9.10^6$ ha), ainda que o rendimento médio tenha crescido de 11,8 t/ha para 19,2 t/ha no mesmo período. A mandioca é produzida para atender a demanda de produção de chips e de amido para uso indústrial. Em 1980, a Tailândia passou a industrializar a mandioca, exportando "pellets" secos à Europa para consumo animal, tendo alcançado o valor de US$ $1,5.10^9$, em 2010. Mais recentemente, o uso da biomassa de mandioca tem sido destinado a uma nova área na Ásia, i.e., a produção de biocombustíveis. Países como a China, Japão e República da Coréia buscam reduzir sua dependência na importação de óleo e emissão de gases de estufa, desenvolvendo esforços para produção em larga escala da mandioca à geração de bioetanol (HOWLER et al. 2013).

Na America Latina, a área produzida cresceu apenas 1% no período 1980-2011, ainda que incrementos de 15% na produção tenham ocorrido, devido a ganhos no rendimento dos cultivos. De igual forma a outras regiões tropicais, a mandioca nas Américas é usualmente cultivada nas áreas marginais onde as chuvas são incertas, em solos ácidos e com baixa fertilidade. A produção é dominada pelo Brasil, com 75% do total ($24,4.10^6$ t - 2011), seguido de Paraguai ($2,4.10^6$ t), Colômbia ($2,2.10^6$ t) e Peru ($1,1.10^6$ t). Embora o consumo urbano da mandioca como alimento nesta região tenha decrescido nos últimos 50 anos, na Colômbia e no nordeste brasileiro a mandioca contínua ser um alimento de primeira necessidade (HOWLER et al. 2013). A FAO estima que regionalmente, 50% da produção de mandioca é usada ao consumo humano e o restante ao consumo animal. O consumo da mandioca é promovido no Brasil através de políticas direcionadas à substituição de cereais importados pela farinha de mandioca. O governo autorizou a mistura de 10% da farinha de mandioca à farinha de trigo na produção de pães, uma iniciativa que busca absorver metade da produção de mandioca do país (HOWLER et al. 2013).

3.4 Deterioração fisiológica das raízes e seu impacto na produção da mandioca

3.4.1 Estudos prévios, tipos de deterioração e conceitualização

As raízes da mandioca são altamente perecíveis quando comparadas com raízes de outras culturas temperadas e tropicais (e.g., Tubérculos e rizomas). Tal pode estar associado ao fato de que, diferente dos órgãos de reserva de outras culturas, as raízes da mandioca não possuem dormência endógena, não possuem nenhuma função na propagação e não possuem botões primordiais por onde a brotação pode ocorrer (COURSEY & BOOTH, 1977; PASSAM & NOON, 1977). Em geral, devido à deterioração fisiológica e patológica as raízes da mandioca não podem ser armazenadas em condições satisfatórias por mais de três dias (WENHAM, 1995).

Estudos sobre a deterioração da mandioca fresca foram conduzidos nos anos 1970 pelo Centro Internacional de Agricultura Tropical (CIAT) e o Instituto de Recursos Naturais (NRI). Pesquisas levaram ao desenvolvimento de sistemas simples de armazenamento baseados no tratamento de raízes em sacos de polietileno e tratamento químico como o tiabendazole para prevenir o início da deterioração (WHEATLEY, 1989).
Tradicionalmente, o problema de deterioração tem sido ultrapassado, postergando-se a colheita até o necessário. As desvantagens deste sistema são: *i.)* as áreas são mantidas ocupadas, impedindo o uso a outros cultivos, *ii.)* as raízes perdem parte do seu amido, *iii.)* a palatibilidade diminui à medida que a raiz se torna mais fibrosa (RICKARD & COURSEY, 1981) e *iv.)* aumento do tempo de cozimento (WHEATLEY & GOMEZ, 1985). A reconhecida perecibilidade em pós-colheita das raízes de mandioca dificulta o armazenamento de grandes volumes de raízes frescas e restringe a área de fornecimento daquela biomassa. Em muitos países, especialmente na America Latina, consumidores urbanos têm substituido a mandioca por outras fontes de carboidratos tais como o arroz e o trigo. As vantagens produtivas, no entanto, justificam seu cultivo como um alimento urbano.

Publicações que mais reportam a respeito da PPD são usualmente pouco informativas, referindo à alta perecibilidade das raízes de mandioca em curtos períodos de armazenamento (RICKARD & COURSEY, 1981), porém sem endereçar a natureza ou os sintomas do processo. O número

de espécies fúngicas e bacterianas isoladas de raízes em diversas condições de armazenamento demonstram que a deterioração é um processo complexo, geralmente envolvendo um conjunto de espécies de microrganismos (EKUNDAYO & DANIEL, 1973; WEGMANN, 1970; BURTON, 1970). Booth (1976) isolou varias espécies de fungos e bactérias saprófitas, porém foi incapaz de identificar aquela responsável pelo processo, concluindo que os estágios iniciais da deterioração, caracterizados pela descoloração (mudança de cor) dos vasos xilemáticos, não eram inerentemente o resultado do ataque de patógenos; antes os estágios tardios do processo resultavam da ação de vários saprófitas. Posteriormente, Noon & Booth (1977) demonstraram que o estriamento vascular era um processo fisiológico. Tais resultados foram substanciados por estudos citoquímicos detalhados e corroborados por Rickard et al. (1979), os quais não identificaram sinais de infecção microbiológica durante os estágios iniciais da descoloração vascular. Na base de suas observações, Booth (1976) fez uma clara distinção dos tipos de PPD em raízes de mandioca, dividindo-os em deterioração primária, um processo fisiológico (Fig. 5a) e deterioração secundária (Fig. 5b), sendo esta comumente derivada de atividade microbiana.
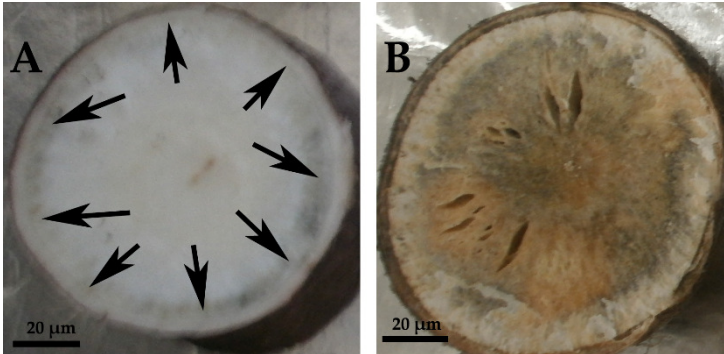
Figura 5. Imagens ilustrativas dos tipos de deterioração fisiológica em pós-colheita de raízes de mandioca. (**A**) Deterioração primária ou fisiológica. As setas indicam áreas azul-escuras características, associadas à degeneração dos vasos condutores, em amostras de raízes aos 3 dias de armazenamento. (**B**) Imagem típica da deterioração secundária de amostra de raiz ao 5º dia de armazenamento.

A deterioração primária, i.e., a deterioração fisiológica, é a primeira e a maior causa da perda de aceitabilidade da raiz. A deterioração secundária ocorre quando patógenos penetram através de ferimentos ou contusões causadas durante a colheita ou manuseio (WENHAM, 1995).

3.4.2 Deterioração primária ou fisiológica em pós-colheita na mandioca

Em muitos casos, a deterioração fisiológica se desenvolve a partir dos locais do tecido danificado durante a colheita, manuseio ou transporte, sendo inicialmente detectada pela pigmentação azul-escuro dos tecidos vasculares, a qual é referida por "estriamento vascular" (Figura 5a). A este sintoma segue a descoloração (mudança de cor) generalizada do parênquima de reserva. De acordo com Bowles (1990), a injúria tissular resulta em uma cascata de respostas de defesa e subseqüente oclusão da área lesionada, gerando uma barreira protetiva caracterizada pela formação da periderme. Respostas bioquímicas geralmente envolvem a ativação enzimática (glucanase e quitinases), a síntese de inibidores de proteases e a produção de glicoproteínas ricas em hidroxiprolina. Enzimas associadas à via dos fenilpropanóides, e.g., fenilalanina amônia-liase (PAL) e chalcona sintase, são produzidas, resultando na síntese de compostos fenólicos que atuam diretamente no processo de defesa ao estresse fisiológico.

Investigações citoquímicas em amostras de tecidos radiculares de mandioca danificados e armazenados sob condições de alta umidade relativa do ar (> 80%) revelaram a formação de depósitos incolores e coloridos nas camadas celulares superficiais, acúmulos de compostos fenólicos, lipídios, carboidratos e lignina e aumento de atividade da polifenol oxidase e peroxidase. As análises citoquímicas não foram suficientes para identificar a natureza química do(s) material(is) depositado(s) nas superfícies celulares danificadas. Contudo, o teste para detecção de compostos fenólicos identificou a presença de catequinas e proantocianidinas, enquanto a lignificação foi substanciada por técnicas de microscopia eletrônica.

Os principais compostos fenólicos associados com o desenvolvimento da deterioração fisiológica foram caracterizados e incluem a escopoletina, escopolina, esculina, proantocianidinas, catequina e galocatequina (RICKARD, 1981; 1985; TANAKA et al. 1983;WHEATLEY, 1982; WHEATLEY & SCHWABE, 1985). O teor máximo de escopoletina (principal responsável pela fluorescência do parênquima) foi observado após 24 h de injúria e previamente à detecção visual dos sintomas de deterioração (WHEATLEY & SCHWABE, 1985). Subsequentemente, aumentos continuados de flavonois e proantocianidinas foram identificados ao longo do processo deteriorativo. De fato, a rota biossintética dos fenilpropanóides está envolvida na resposta aos danos, incluindo a produção de isoflavonóides, flavonóides e lignina

(HAHLBROCK & SCHEEL, 1989). A PAL, uma enzima reguladora da via dos fenilpropanóides, tem sua atividade elevada logo após a lesão, i.e., 2 a 3 minutos após o dano (RICKARD, 1982; 1985; TANAKA et al. 1983; LORENZO et al. 1987; FRITZEMEIER et al. 1987).

3.4.3 Importância sócio-econômica da deterioração pós-colheita na mandioca

Embora a rápida deterioração pós-colheita da mandioca seja considerada um fator sócio-econômico importante, há pouca informação sobre perdas causadas pela deterioração. Estudos realizados pelo CIAT na Ásia, em colaboração com parceiros locais, indicam perdas no nível mais geral e não apontam suas causas. Os dados coletados indicam, por exemplo, perdas na ordem de 10-12% na Índia e 5,3% na Indonésia (CIAT, 1987). Estimativas mais recentes da FAO para China apontam para valores na ordem de 3%, 15-30% para Gana e 27% para Costa de Marfim. De modo geral, os dados em diversos estudos não indicam em que fase da cadeia de comercialização as perdas ocorrem, tampouco as diferenciam quanto à deterioração em pós-colheita daquelas ocorridas no processamento (WENHAM, 1995; WESTBY et al. 2002).

Numa avaliação sistemática mundial recente, a FAO sugere que perdas em raízes tuberosas estão na ordem de 30 a 60%. No caso da mandioca para a África, as perdas em 2002 foram estimadas em 19.10^6 t do total produzido no continente, i.e., 101.10^6 t. A magnitude das perdas difere significativamente entre países e mesmo ao longo dos estágios da cadeia comercial num mesmo país, uma vez que depende de como a mandioca é produzida, processada, consumida e o nível de coordenação entre diferentes atores da cadeia (NAZIRI et al. 2014). Entretanto, no caso dos agricultores de subsistência, as perdas pós-colheita são negligenciáveis uma vez que, quando o propósito é consumo familiar, os agricultores colhem apenas a quantidade necessária para preparar suas refeições. Além disto, quando o produto se deteriora é usado para consumo animal (HALL, 1998).

Tomando como exemplo a mandioca na África sub-saharina, Westby et al. (2005) apontaram vários tipos de perdas:
   a) Físicas, da mandioca seca (*chips*), na ordem de 3 a 15%;
   b) Financeiras, ao manusear a mandioca não fresca, devido aos descontos nos preços, os quais podem atingir 90% do valor total, para raízes com mais de 3 dias após a colheita;

c) Mudança no uso. Se as raízes colhidas não são comercializadas em 2 dias, são processadas (secas), reduzindo seu valor;

d) Potencial, devido à não colheita em momento de ótimo fisiológico;

As perdas referidas acima podem ocorrer em diversos estágios da cadeia comercial, como ilustrado na figura 6. Em Gana, por exemplo, o efeito combinado do impacto econômico nas perdas da mandioca em termos monetários foi estimado em até US\$ 500.10^6 por ano (NRI, 2014).
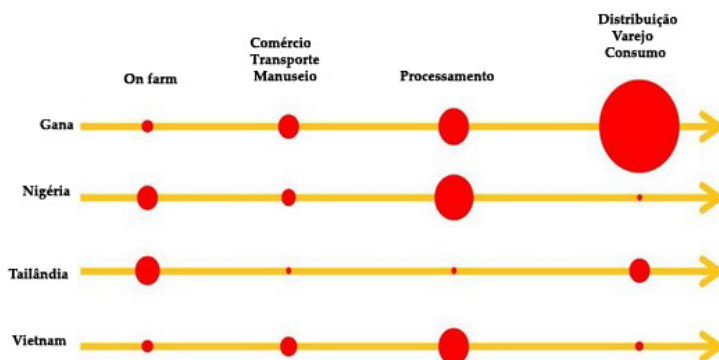
Figura 6. Perdas físicas de raízes de mandioca ao longo dos estágios da cadeia comercial em Gana, Nigéria, Tailândia e Vietnam. O tamanho do círculo indica de forma proporcional a grandeza da perda no estágio da cadeia referenciada. Imagem adaptada do Instituto de Recursos Naturais (NRI, 2014).

3.5 Metabolômica

3.5.1 Conceito e importância

A rede metabólica nas plantas é mais extensa do que em outros organismos. Para além dos metabólitos primários, as plantas também sintetizam uma vasta gama de metabólitos secundários (AHARONI & GALILI, 2011). Apesar da definição entre metabólitos primários (MPs) e secundários (MSs) não ser inteiramente clara, MPs são compostos produzidas por todas as espécies vegetais e outros organismos, ao passo que MSs são substâncias geralmente essenciais à sobrevivência, ou aquelas produzidas exclusivamente por algumas espécies de plantas e que são estruturalmente muito diversos (PICHERSKY & GANG, 2000). Os MSs desempenham importante papel na manutenção da performance ecológica da planta, atuando na proteção da planta contra microrganismos

(fungos e bactérias) e infecções virais, herbivoria, radiação ultravioleta (UV), atração de polinizadores e frugívoros (dispersores de sementes). Os principais grupos de MSs vegetais são os compostos fenólicos, terpenos e os alcalóides. Estas três grandes classes são produzidas a partir de diferentes rotas dos MPs que incluem a via glicolítica, a via dos ácidos tricarboxílicos (TCA), a via das pentoses fosfato e os aminoácidos (AHARONI & GALILI, 2011).

Kumar et al. (2014) definem metabólito como uma substância de massa molecular < 1500 Da e metaboloma como sendo uma coleção quantitativa de todos os metabólitos presentes em uma célula, num estado fisiológico particular, refletindo o fenótipo metabólico celular naquele momento. A metabolômica fornece informações sobre interações do genoma, proteoma e o ambiente externo, assim como reflete o conjunto total dos processos metabólicos (anabolismo, catabolismo, transporte e acúmulo) e eventos de transdução de sinais e regulação associados (KUMAR et al. 2014; COX et al. 2014).

Plataformas metabolômicas primariamente se agrupam em 5 categorias (cromatografia gasosa (GC) x espectroscopia de massa (MS), cromatografia liquida (LC) x MS, ressonância magnética nuclear (NMR) e aplicações integradas, tais como a combinação da metabolômica com a estatística multivariada e ou técnicas computacionais de análise de dados – Quimiometria – COX et al. 2014). Outras técnicas também usadas na análise metabolômica incluem a espectroscopia vibracional de infravermelho médio (FTIR), a espectroscopia Raman e a eletroforese capilar. Duas abordagens existem à interpretação de dados metabolômicos: metabolômica não direcionada e direcionada ao alvo ("untargeted and targeted metabolomics"). O primeiro caso (não direcionada) constitui uma completa análise de todos os metabólitos presentes na amostra, incluindo os compostos desconhecidos. Esta abordagem pode ser acoplada a técnicas avançadas de quimiometria. Como desvantagens mencionam-se o tempo necessário para processar a vasta gama de dados gerados e obstáculos na identificação e caracterização de pequenas moléculas. A metabolômica direcionada é um processo quantitativo para caracterização de grupos de compostos alvo e conhecidos (KUMAR et al. 2014).

Atualmente, estima-se em cerca de 200.000 o número total de metabólitos encontrados em plantas. Este grande grupo de compostos, estruturalmente diversos, dinâmicos temporalmente e espacialmente, constitui um grande

desafio às técnicas analíticas modernas (MISRA et al. 2014) e à capacidade de processamento e análise do mega (i.e., *omics*) conjunto de dados. A integração de diferentes dados com características estruturais diferentes e a extração de informações relevantes ao metabolismo celular é inquestionavelmente um elemento crucial à metabolômica de plantas (YI et al. 2014). Ademais, a integração de dados metabolômicos com aqueles de outras ciências ômicas (e.g. genômica, transcriptômica e proteômica) poderá ser usada à construção de redes moleculares, as quais colaborarão ao melhor entendimento dos complexos processos bioquímicos vegetais (YIN & XU, 2014).

3.5.2 Metabólitos secundários

3.5.2.1 Compostos fenólicos

As plantas desenvolveram mecanismos de defesa para sua proteção a várias perturbações. Para além das barreiras constitutivas, plantas adquirem tolerância e resistência a vários fatores (a)bióticos, devido a sua capacidade de ativar mecanismos de defesa, tais como respostas de hipersensibilidade, fortalecimento da parede celular, seqüestro de espécies oxidativas e produção de metabólitos secundários (CONRATH et al. 2002). Os compostos fenólicos são substâncias caracterizadas por conter um grupo fenol, isto é, um grupo hidroxila ligado a um anel aromático. Constituem um grupo de compostos altamente heterogêneos e são sintetizados por duas rotas, i.e., a via do ácido chiquímico e via do ácido malônico. Devido a sua diversidade química, estes metabólitos apresentam uma grande variedade de funções nos vegetais, agindo como compostos de defesa contra herbívoros e patógenos, atração de polinizadores ou dispersores de frutos, proteção à radiação ultravioleta, ou ainda reduzindo o crescimento de plantas adjacentes (alelopatia e aleloquímica– TAIZ & ZEIGER, 2009; BAIS et al. 2004). A atividade química dos compostos fenólicos em termos de suas propriedades redutoras prediz seu potencial em atuar na eliminação de radicais livres, i.e., antioxidantes. A atividade de um antioxidante é função do(a): *i)* potencial de redução, *ii)* capacidade de estabilizar elétron não pareado, *iii)* reatividade com outros antioxidantes e *iv)* potencial de transição entre metal e agente quelante. Os compostos fenólicos possuem uma estrutura química ideal para atuar na eliminação de radicais livres (RICE-EVANS et al. 1997).

3.5.2.2 Flavonoides

Os flavonoides constituem uma vasta classe de compostos presentes em muitas plantas e geralmente ocorrendo na forma glicosilada. Variações estruturais subdividem este grupo em seis famílias: flavonol, flavonas, flavanonas, chalconas, antocianinas e isoflavonas. A glicosilação reduz a reatividade dos flavonoides perante os radicais livres e aumenta sua solubilização em água (RICE-EVANS et al. 1997).

De acordo com Shirley (1996), os flavonoides são uma classe importante de metabólitos secundários extensivamente estudados e com atividade na interação planta-microrganismos, proteção contra a luz ultravioleta, fertilidade do pólen, havendo também evidencia de seu papel na alelopatia (interação planta-planta). Estes compostos possuem reconhecida atividade antioxidante e são sinalizadores a microrganismos benéficos na rizosfera. Os isoflavonoides, por exemplo, (grupo limitado primariamente a leguminosas) possuem atividade estrogênica e antitumoral e em conjunto, os flavonoides têm recebido grande atenção como nutracêuticos (ZHAO & DIXON, 2009; DIXON & STEELE, 1999).

Muitos genes para biossíntese de flavonoides são ativados durante a exposição a estresses bióticos e abióticos tais como: dano, seca, toxicidade de metais e falta de nutrientes. O denominador comum nessas condições parece ser a produção de espécies reativas ao oxigênio (ROS), tais como o ânion superóxido (O^-_2), peróxido de hidrogênio (H_2O_2), radical hidroxil (OH^-) e oxigênio singleto ($^1O_2$). O estresse oxidativo pode danificar componentes celulares tais como o ADN, lipídios, proteínas e açúcares. Para minimizar os danos relacionados ao estresse, a homeostase de ROS em plantas é um processo complexo e os flavonóides foram relatados atuar nesse processo (HERNÁNDEZ et al. 2008). Para Hernández et al. (2008), flavonoides têm capacidades antioxidantes superiores ao ascorbato (vitamina C) e tocoferol (vitamina E) devido a sua forte capacidade de doar elétrons ou prótons.

3.5.2.3 Carotenoides

Carotenoides são metabólitos com importantes funções fotossintéticas: fotoproteção (seqüestro de oxigênio singleto), molécula acessória à coleta de radiação solar (absorção nos comprimentos de onda entre 400 a 550 nm e transferência da energia à clorofila) e função estrutural (estabilizam

complexos fotossintéticos pigmento-proteína). A cor dos carotenóides se deve principalmente ao número de ligações duplas conjugadas (GARCIA-ASUA et al. 1998).

Os carotenoides compreendem um grupo de pigmentos que ocorrem naturalmente e estruturalmente são terpenos derivados da condensação de unidades de isopentenil pirofosfato. São compostos lipofílicos sintetizados e seqüestrados nos plastídeos. Possuem função antioxidante, seqüestrando radicais carboxila e oxigênio singleto. Carotenoides com grupo β-ionona não substituído são precursores de vitamina A. O uso industrial dos carotenoides envolve sua aplicação como suplemento nutricional, para propósitos farmacêuticos, corantes alimentares e consumo animal (SANDMANN, 2001).

Vishnevetsky et al. (1999) relatam que os carotenoides possuem atividade de atração de polinizadores. Tais metabólitos também servem de precursores de hormônios vegetais como o ácido abscísico. A biosíntese de carotenoides é regulada durante o ciclo da planta com mudanças dinâmicas na germinação, fotomorfogênese, frutificação e em resposta a estímulos ambientais externos (CAZZONELLI & POGSON, 2010).

3.5.3 Hidroxicumarinas na mandioca

3.5.3.1 Propriedades físico-químicas das cumarinas e biossíntese

As cumarinas têm a benzo-α-pirona seu precursor comum. Sua origem biogenética os caracteriza como compostos derivados de fenilpropano devido ao seu esqueleto básico C_6-C_3. De acordo com a sua estrutura química são classificadas em cumarinas com estrutura básica benzo-α-pirona, isocumarinas, furanocumarinas e piranocumarinas (WAKSMUNDZKA & HAWRYL, 2008). As cumarinas ocorrem em plantas como moléculas livres e/ou combinadas (glicosiladas). Muitas cumarinas na forma aglicona possuem substituintes como hidroxil, metoxil e alquil na posição 7 e, algumas vezes, nas posições 5, 6 e 8 (Figura 7). Sua solubilidade depende da presença de grupos hidroxila e glicosídeos. Os derivados glicosilados são solúveis em água e em solventes polares como metanol e etanol. Furanocumarinas e piranocumarinas são solúveis em meio apolar, e.g., solventes como diclorometano, éter de petróleo e clorofórmio. Cumarimas revelam uma fluorescência à luz UV, fato que as torna fáceis de identificação (WAKSMUNDZKA & HAWRYL, 2008).
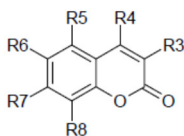
Figura 7. Estrutura química básica de cumarina simples encontrada na mandioca. Escopoletina: $R_3=R_4=R_5=R_8=H$, $R_7=OH$, $R_6=OCH_3$ (Fonte: WAKSMUNDZKA & HAWRYL, 2008).

As cumarinas, especialmente as furanocumarinas e pirocumarinas possuem função protetiva ao vegetal e são biosintetizados "*de novo*" em condições de estresse como fitoalexinas. Fitoalexinas são sintetizados por plantas em resposta a vários fatores bióticos (bactérias, vírus, fungos e larvas) e abióticos (metais, herbicidas, detergentes). Também tem influência sobre a fisiologia das células vegetais. Em baixas concentrações exibem atividade sinérgica com substâncias promotoras do crescimento vegetal. Contrariamente, em altas concentrações inibem o crescimento das plantas por atuarem como antagonistas de hormônios de crescimento. Em altas concentrações, cumarinas como o psoraleno e o 8-metoxipsoraleno são substâncias mutagênicas, inibindo a germinação da semente. A atividade biológica de cumarinas é diversa e depende da sua estrutura química. Algumas hidroxi e metoxicumarinas têm a habilidade de absorver luz UV e são usadas como aditivos em protetores solares. Cumarinas como xantotoxina, inperatorina, escopoletina, esculina, esculetina e escoparona possuem propriedades citotóxicas (WAKSMUNDZKA & HAWRYL, 2008). A escopoletina foi relatada apresentar ação antimicrobiana contra uma variedade de organismos fungícos e bacterianos (RODRIGUEZ et al. 2000).

3.5.3.2 Escopoletina

A formação da escopoletina, assim como outras hidroxicumarinas como escopolina, esculina e esculetina (Figura 8) ocorre logo após a ruptura dos tecidos radiculares por ocasião da colheita da mandioca, havendo uma forte evidência de que este metabólito contribua à deterioração daquela biomassa (WHEATLEY et al. 1985; HUANG et al. 2001).

Esculetina   Esculina

Escopoletina   Escopolina

Figura 8. Detalhes da estrutura química de hidroxicumarinas identificadas em raízes de mandioca.

A escopoletina (7-hidroxi-6-metoxicumarina) é uma hidroxicumarina encontrada em muitas espécies vegetais, incluindo a mandioca, batata doce, girassol, algodão e tabaco (BAYOMI et al. 2008; KAI et al. 2008). Estudos têm relacionado a escopoletina com respostas de defesa da planta. Por exemplo, em tabaco, acúmulos de escopoletina foram correlacionados com a resistência adquirida ao vírus do mosaico (COSTET et al. 2002).

3.5.4 Glicosídeos cianogênicos (HCNglic)

3.5.4.1 Ocorrência, biossíntese e sua importância na mandioca

A mandioca contém dois glicosídeos cianogênicos (Figura 9): linamarina e lotaustralina em todas as partes da planta, exceto nas sementes (KANNANGARA et al. 2011). A presença destes compostos causa problemas à alimentação de populações em áreas rurais menos favorecidas, onde a mandioca é um alimento essencial. A ingestão de biomassa de mandioca não processada com altos níveis de HCNglic combinado com uma dieta deficiente em aminoácidos contendo enxofre pode levar à intoxicação crônica, uma vez que esses aminoácidos são necessários à detoxificação de cianetos. Um efeito típico da intoxicação aguda por HCNglic é a inativação da citrocromo oxidase mitocondrial, bloqueando a respiração (JORGENSEN et al. 2011). HCNglic têm aminoácidos protéicos (isoleucina, valina, leucina, tirosina e fenilalanina) e não protéicos (ciclopentenil glicina) como precursores – JORGENSEN et al. 2011; BJARNHOLT & MOLLER, 2008; BJARNHOLT et al. 2008) e oximas (aldoxima ou ainda cetoxima - RR'C=NOH) e cianoidrina (α-hidroxinitrila) como intermediários. A glicosilação de uma cianoidrina leva à formação de um HCNglic.

Prunasina: R₁=H, R₂=benzeno, R₃=H
Durina: R₁=H, R₂=4-hidroxifenil, R₃=H
Linamarina: R₁=CH₃, R₂=CH₃, R₃=H
Linustatina: R₁=CH₃, R₂=CH₃, R₃=glucose

Figura 9. Detalhes da estrutura química básica de glicosídeos cianogênicos comuns em plantas. Extraído de Neilson et al. (2013).

HCNglic são compostos estáveis, no entanto, quando a ligação β-glicosídica é rompida pela ação de uma β-glicosidase, a cianoidrina resultante se dissocia para formar ácido cianídrico, um processo chamado cianogênese (Figura 10).
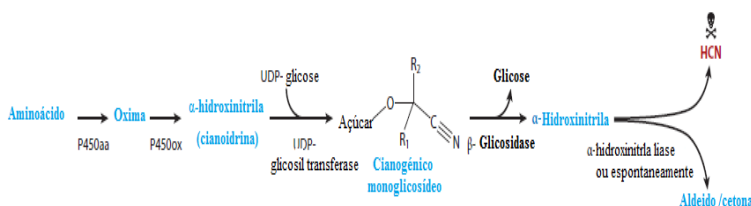
Figura 10. Biossintese e bioativação de HCNglic a partir de precursores aminoácidos, numa série de reações catalisadas por complexos citocromos P450 ligados à membrana plasmática e uma glicosiltransferase, tendo como intermediários uma oxima e a cianoidrina. A cianogênese ocorre quando a ligação éter é rompida por uma glucosidase, formando uma hidroxinitrila instável que dissocia em HCN e uma cetona, seja espontaneamente a pH elevado, ou via catálise por uma hidroxinitrilase. **aa** = aminoáido, **ox** = oxima. Adaptado de Gleadow & Moller, 2014.

De forma específica e ilustrativa, a rota biossintética de HCNglic em mandioca é ilustrada na figura 11.
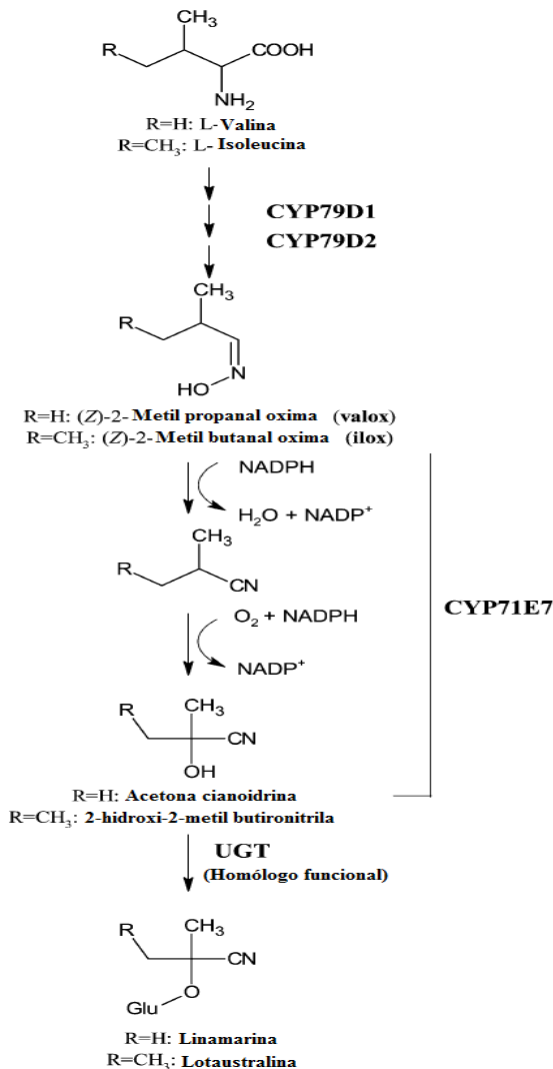
Figura 11. Biossíntese dos HCNglic linamarina e lotaustralina a partir dos aminoácidos proteicos isoleucina e valina em mandioca, com destaque às reações catalisadas pelos complexos citocromo P450. Extraído de Jorgensen et al. 2011.

A exposição aguda ao HCN pode levar à morte de humanos e animais, por ser aquele ácido um potente inibidor da atividade de metaloenzimas, principalmente a citocromo c oxidase, a enzima terminal na cadeia de transporte de elétrons do processo respiratório. A hidrólise de HCNglic provoca um aumento na liberação de compostos contendo grupo carbonil, o que aumenta a toxicidade do HCN. O intermediário oxima pode também ser tóxico, especialmente para fungos (GLEADOW & MOLLER, 2014).

HCNglic e suas enzimas catabólicas específicas estão armazenadas em compartimentos celulares distintos, prevenindo assim a autotoxicidade. No entanto, quando trazidos em contacto após a disrupção celular (herbívoria, congelamento, maceração, etc) GLEADOW & MOLLER, 2014), verifica-se a cianogênese e suas eventuais consequências fisiológicas e ecológicas. Por exemplo, HCNglic são deterrentes generalistas de herbívoros e em algumas espécies regulam os processos germinativos e de brotação. Podem também servir de transporte de carbono e nitrogênio e modular o estresse oxidativo (GLEADOW & MOLLER, 2014; NELSON et al. 2013). Além disto, os HCNglic são classificados como fitoantecipinas, sendo considerados como um sistema de defesa constitutivo.

3.6 Mecanismos enzimáticos e não enzimáticos à detoxificação de espécies reativas de oxigênio (ROS)

A evolução dos processos metabólicos aeróbicos tais como a fotossíntese e a respiração inevitavelmente levam à produção de ROS na mitocôndria, cloroplastos e peroxissomos. Por exemplo, o peróxido de hidrogênio e o íon superóxido são continuamente produzidos na cadeia transportadora de elétrons na fotossíntese e simultaneamente removidos dos cloroplastos por redução ou assimilação (APEL & HIRT, 2004). Uma característica comum dos vários tipos de ROS é a sua capacidade de causar dano oxidativo a proteínas, ADN e lipídios. Estas propriedades citotóxicas das ROS explicam a evolução de mecanismos complexos de detoxificação de natureza enzimática e não enzimática em plantas. Sob condições fisiológicas de estresse, ROS são seqüestrados por sistemas antioxidantes confinados em diversos compartimentos celulares. As plantas também produzem ROS quando expostas a mudanças ambientais bruscas, ativando várias oxidases e peroxidases como estratégia de defesa.

Sob condições de homeostase, as plantas produzem ROS como moléculas sinalizadoras para controlar processos como a apoptose (morte celular

programada) e regulação sistêmica. (MITTLER et al. 2011; MITTLER, 2002). Para tal, desenvolveram um sofisticado mecanismo para manter sob controle as concentrações de ROS (APEL & HIRT, 2004), os quais são de natureza enzimática e não enzimática.

Mecanismos não enzimáticos incluem o sistema de redox celular ascorbato e glutationa, assim como o tocoferol, flavonoides, alcaloides e carotenoides. Por sua vez, mecanismos enzimáticos em plantas incluem a superóxido dismutase (SOD), ascorbato peroxidase (APX), glutationa e guaiacol peroxidases (GPX) e a catalase (CAT). SOD atua como primeira linha de defesa, dismutando o íon superóxido a peróxido de hidrogênio. APX, GPX e CAT subsequentemente detoxificam o peróxido de hidrogênio. Isoformas destas enzimas existem nos cloroplastos, mitocôndria e peroxissomos, assim como no apoplasto e citosol. O estresse oxidativo celular é função da quantidade de ROS, no entanto, o balanço de SOD, APX e CAT é crucial à supressão do efeito tóxico daquelas espécies reativas e do H_2O_2 (APEL & HIRT, 2004; MITTLER et al. 2004).

3.7 Visão geral e sintética do metabolismo de carboidratos em vegetais

Os açúcares não apenas fornecem energia à maquinaria celular, como também atuam na sinalização celular (DOIDY et al. 2012). Os açúcares modulam processos vitais que são também controlados por hormônios durante o crescimento e desenvolvimento da planta, incluindo a germinação, o desenvolvimento de plântulas, a diferenciação radicular e foliar, a transição floral, a maturação do fruto, a embriogênese, a senescência e as respostas à luz e ao estresse de patógenos (LEÓN & SHEEN, 2003; MOORE & SHEEN, 1999). Análises moleculares revelam que a glucose, por exemplo, apresenta efeito regulatório da síntese do ácido abscísico e de genes de sinalização que antagonizam o efeito do etileno durante o desenvolvimento de plântulas (LEÓN & SHEEN, 2003). Para Paul et al. (2001), uma boa performance fotossintética pode ser alcançada modificando os mecanismos de sinalização de açúcares que controlam a expressão de genes associados à capacidade fotossintética e ao balanço fonte-dreno. Em plantas superiores, a fixação de dióxido de carbono ocorre predominantemente nas células do mesófilo foliar, estruturas essas consideradas fontes de carbono ou exportadores de carbono. Células heterotróficas de raízes, flores e órgãos de reserva (e.g.) requerem o fornecimento de açúcares, sendo, portanto reconhecidas como estruturas drenos de carbono, ou

importadores de carbono. Para que as células drenos tenham um aporte adequado de açúcares ao crescimento e desenvolvimento, um mecanismo de transporte eficiente deve existir (PAUL et al. 2001). Em muitas espécies vegetais o carbono assimilado é transportado na forma de sacarose. O catabolismo da sacarose dá início a sua utilização nos sítios dreno, sendo desencadeado por duas enzimas com propriedades distintas: invertase e sacarose sintase (STURM & TANG, 1999). Invertase é uma hidrolase que cliva a ligação *O*-glicosídica gerando glucose e frutose. Ao contrário, sacarose sintase é uma glicosiltransferase que, na presença de uridina difosfato (UDP – substrato enzimático) converte sacarose em UDP-glucose e frutose. Isoformas de invertases com propriedades bioquímicas distintas podem se acumular no citoplasma (invertase neutra ou alcalina), no vacúolo (invertase vacuolar) e no espaço extracelular (invertase da parede celular – STURM & TANG, 1999).

Para Blamire (2004), açúcares simples podem se ligar a proteínas e lipídios, modificando a *performance* de suas funções biológicas como enzimas, moléculas sinalizadoras e componentes de membranas. A adição de um ou mais açúcares a um substrato torna-o mais solúvel em meio aquoso, regulando seu tranporte no sistema vascular vegetal. Glucose e outros monossacarídeos são bastante hidrofílicos e plantas que armazenam grandes quantidades de glucose podem enfrentar problema com a osmose devido ao movimento incessante da água de fora para dentro da célula. A pressão osmótica será tão grande, tal que, mesmo com suas paredes celulares protetoras, as células podem ter problemas no funcionamento. Uma das alternativas à resolução deste problema é converter monossacarídeos em polissacarídeos (e.g., celulose e amido). Este último constitui a maior reserva de energia que pode ser mobilizada rapidamente pelos vegetais quando necessário. Muitas plantas como a mandioca e a batata, por exemplo, apresentam regiões especializadas ao armazenamento de amido, i.e., as células parenquimatosas que processam o amido e o empacotam para uso de longo termo (BLAMIRE, 2004).

3.8 Visão geral e sintética do metabolismo de ácidos orgânicos em vegetais

Ácidos orgânicos são compostos de carbono que possuem pelo menos um grupo carboxila (RYAN et al. 2001). O metabolismo de ácidos orgânicos é de fundamental importância ao nível celular para várias rotas bioquímicas, incluindo a produção de energia e a formação de precursores

à biossíntese de aminoácidos, regulação osmótica e balanço de excesso de cátions (LÓPEZ-BUCIO et al. 2000), por exemplo. Uma característica marcante nos tecidos vegetais é de que o conteúdo total de ácidos orgânicos é mais elevado comparativamente a outros organismos e explica sua importância como intermediários fotossintéticos. A composição e o conteúdo destes compostos dependem, entre outros fatores, da espécie, da idade da planta e do tipo de tecido (LÓPEZ-BUCIO et al. 2000).

Os ácidos orgânicos são principalmente produzidos na mitocôndria através do ciclo dos acidos tricarboxílicos (TCA), ou ciclo de Krebs, e em pequena extensão nos glioxissomos, sendo preferencialmente armazenados nos vacúolos e em pequenas quantidades na mitocôndria. Também se encontram direta ou indiretamente envolvidos em vários processos incluindo assimilação de carbono e nitrogênio, regulação do pH citosólico e fornecimento de energia a bactérias simbióticas (RYAN et al. 2001; LÓPEZ-BUCIO et al. 2000).

Durante o período de luz, os ácidos orgânicos acumulados são descarboxilados para produzir dióxido de carbono (LÓPEZ-BUCIO et al. 2000). A biossíntese, acúmulo, transporte e exsudação radicular (e.g., ácido cítrico em milho) dos ácidos orgânicos são afetados por fatores (a)bióticos. Durante o período de crescimento vegetativo as taxas de redução de nitratos, síntese de carboxilatos e aminoácidos são elevadas, havendo a produção de íons alcalinos tóxicos. De forma a manter estável o pH celular naquelas condições, ácidos orgânicos são produzidos, atuando como sistemas tampões. Ácidos orgânicos também estão relacionados ao processo de absorção de nitrato, fósforo e ferro e espécies cultivadas em solos alcalinos, onde cálcio é abundante, reduzem o excesso deste nutriente livre complexando-o com ácidos orgânicos (LÓPEZ-BUCIO et al. 2000).

Ácidos orgânicos também participam na detoxificação de alumínio, um evento importante em solos ácidos usualmente encontrados em regiões de cultivo de mandioca. Um dos mecanismos de tolerância mais eficaz é a exclusão de alumínio pelo ápice radicular, ligado a ácidos orgânicos, e.g., cítrico, oxálico e málico (MA et al. 2001; MA, 2000). Os ácidos orgânicos também se encontram associados ao mecanismo de tolerância ao excesso de bicarbonato e a deficiência de zinco (ROSE et al. 2011).

3.9 Quimiometria e análise estatística multivariada

3.9.1 Conceito e importância em estudos metabolômicos

A quimiometria ou quimio-informática, segundo Varmuza & Filzmoser (2008), pode ser definida como uma disciplina que usa métodos estatísticos e matemáticos para delinear ou selecionar procedimentos ou experimentos ótimos, fornecendo máxima quantidade de informação derivada da análise de dados químicos. De modo geral, a quimiometria utilizada técnicas de análise estatística multivariada para resolução de diversos tipos de problemas, tais como:
a) Determinação da concentração de compostos a partir de misturas complexas (incluindo dados de infravermelho);
b) Classificação da origem de amostras (analíticos ou espectroscópicos);
c) Predição de propriedades ou atividades de compostos químicos (dados de estrutura química);
d) Reconhecimento de ausência e presença de subestruturas a partir da estrutura química de um composto orgânico desconhecido;
e) Avaliação do estado de um processo na tecnologia química.

A estatística multivariada é uma extensão da estatística univariada. A estatística univariada investiga cada variável separadamente, ou relaciona uma variável independente com uma variável dependente. No entanto, reações (bio)químicas e processos biológicos são multivariados por natureza, implicando em que para uma boa caracterização de um dado evento de interesse, muitas variáveis sejam consideradas, propiciando aumento de qualidade na análise de dados (VARMUZA & FILMOSER, 2008).

É interessante compreender os porquês especialistas em química reconhecem a importância deste campo de conhecimento. Os contínuos avanços na instrumentação analítica e no processamento de dados têm acelerado a geração de informações. Contudo, nossas habilidades interpretativas não têm avançado na mesma velocidade, de modo a possibilitar a total exploração das informações contidas no mega-conjunto de dados (*omics*) que vem sendo produzido. Neste contexto, a quimiometria tem se desenvolvido visando facilitar a transferência de técnicas estatísticas à resolução de problemas químicos. A quimiometria

continua sendo uma disciplina da química, porque o poder interpretativo das técnicas estatísticas só pode ser atingido quando um contexto químico é inserido (BROWN, 1995; MEGLEN, 1988).

O grande sucesso da quimiometria é a sua aplicação na indústria. Métodos quimiométricos como a calibração multivariada estão sendo usados, desde o monitoramento da produção de cerveja até ao controle de qualidade nas formulações farmacêuticas, por exemplo. De fato, a quimiometria tem obtido sucesso na calibração multivariada, na modelagem de processos (bio)tecnológicos, no reconhecimento de padrões e na classificação e discriminação de amostras (WOLD & SJOSTROM, 1998).

3.9.2 Técnicas de estatística multivariada correntemente usadas em quimiometria

Os métodos de estatística multivariada mais usados em quimiometria são: análise de componentes principais (PCA), análise de agrupamento hierárquica ou *cluster* (HCA), análise parcial de quadrados mínimos (PLS), análise discriminante linear (LDA), classificação por vizinhos mais próximos (k-NN), regressão de componentes principais (PCR), redes neurais artificiais (ANN), árvores de decisão (*random forests* e *decision trees*) e máquinas de vetor suporte (SVM). PCA e HCA fazem parte de métodos não supervisionados, aonde os grupos de interesse, *a priori*, não são especificados ou conhecidos na análise. As demais técnicas denominam-se supervisionadas, ou técnicas de aprendizado máquina "*Machine learning*" (VARMUZA & FILZMOSER, 2009; WALLACE & HIGGINS, 2007).

3.9.2.1 Análise de componentes principais (PCA)

A PCA pode ser considerada como um método mãe de todos outros métodos de análise multivariada e seu principal objetivo é a redução da dimensão dos dados sem perda de informação. A PCA transforma variáveis altamente correlacionadas em variáveis latentes não correlacionadas (componentes ou distância entre objetos no espaço dimensional), permitindo a separação e extração de informação relevante que caracteriza um processo químico-tecnológico (VARMUZA & FILZMOSER, 2009; MEGLEN, 1988).

3.9.2.2 Análise de agrupamento hierárquica (HCA)

A HCA busca identificar grupos concentrados (*clusters*) dos objetos, sem prévia informação da relação entre os constituintes dos grupos. Assim, a HCA permite identificar grupos contendo objetos similares, sem assumir que cada objeto pertença a um grupo. Os objetos dentro do grupo são supostamente similares, enquanto aqueles de diferentes grupos dissimilares. Os resultados dependem da medida de distância usada, do algoritmo e dos parâmetros usados.

O uso de técnicas não supervisionadas é recomendável como um ponto de partida na avaliação de dados, gerando "insights" relevantes quanto à estrutura de dados (grupos, e/ou *outliers*), importantes no processo seguinte de desenvolvimento de modelos de classificação e/ou calibração (VARMUZA & FILZMOSER, 2009). Em geral, os resultados de PCA e HCA são complementares e quando utilizados em "tandem" constituem uma ferramenta interessante à construção de modelos de reconhecimento confiáveis (MEGLEN, 1988).

3.9.2.3 Análise parcial de quadrados mínimos (PLS)

A PLS é um algoritmo versátil que pode ser usado para predizer variáveis discretas ou contínuas, assim como categóricas. Os modelos de classificação usando PLS usualmente encontram-se associados à análise discriminante (DA) e designa-se PLS-DA. PLS-DA é um método clássico de PLS onde a variável dependente *y* é categórica e representa classe de amostras. Usando a informação da classe, PLS-DA tende a melhorar a separação entre dois grupos de amostras. Ela é comumente usada à classificação e seleção de biomarcadores (SZYMANSKA et al. 2011). PLS-DA possui uma abordagem de reduzir a dimensão de dados acoplada a modelos de regressão. Diferente dos outros métodos, as variáveis latentes encontradas por este método têm em mente a variável resposta (BOULEISTEX & STRIMMER, 2006). No caso de muitas variáveis respostas, todavia, a construção de modelos precisos se torna mais complicada, devido à dificuldade de encontrar variáveis latentes suficientes e confiáveis a explicar muitas variáveis respostas. PLS permite analisar dados colineares (correlacionados) com múltiplas variáveis *x* e simultaneamente modelar muitas variáveis respostas (WOLD et al. 2001).

3.9.2.4 Análise discriminante linear

A extração de boas informações é crucial ao reconhecimento de padrões. A LDA foi proposta por Fisher para resolver problemas binários e, mais tarde, estendida a problemas multi-classes. A LDA busca encontrar um subespaço que minimiza a distância intra-classes e maximiza a distância entre classes simultaneamente (YAO et al. 2014; ZHAO et al. 2014). A análise discriminante, segundo Teknomo (2014), é uma técnica estatística que classifica objetos em grupos mutuamente exclusivos e exaustivos baseada em características medidas de objetos. Em geral, o objeto é colocado em certo grupo, baseado nas observações desse mesmo objeto. O propósito fundamental é:

a) Quais características melhor determinam certo grupo de objetos (seleção de características "feature selection");
b) Qual o modelo de classificação que melhor separa os grupos (classificação).

A análise discriminante se diferencia da HCA, porque a HCA é um método não supervisionado, a categoria do objeto é desconhecida, a classificação baseia-se na distância entre os objetivos e se conhecem as características das variáveis independentes que descrevem a classificação do objeto. No entanto, não há um treinamento do modelo para verificar se a classificação está correta ou não (TEKNOMO, 2014). Na análise discriminante, os grupos de objetos são conhecidos, assim como há um treinamento do modelo. Conhece-se o modelo de classificação (linear, quadrático, etc) e objetiva-se definir os parâmetros do modelo que melhor explicam a separação dos grupos (TEKNOMO, 2014).

Por exemplo, quando se busca saber se uma cultivar de mandioca recentemente lançada por uma empresa agrícola deteriorará ou não em pós-colheita, baseando-se em várias medições, e.g., metabólitos secundários, enzimas, glicosídeos cianogênicos, açúcares, etc., ter-se-á o objeto sendo a cultivar e a classe categórica (ou grupo) "deteriora" e "não deteriora". As variáveis medidas que descrevem o objeto são ditas independentes, enquanto a variável dependente é categórica (escala nominal). Assumindo que os grupos são linearmente separáveis, pode-se usar a LDA e os grupos são separados por uma combinação linear das características que descrevem os objetos. Se tivermos duas variáveis, a separação será em um plano e havendo mais de três variáveis independentes, a separação ocorrerá em um hiperplano (TEKNOMO, 2014).

3.9.2.5 Árvores de decisão (DTs)

Árvores de decisão (DTs) fazem parte dos métodos não paramétricos de aprendizado de máquina usados à classificação ou regressão. O objetivo é criar um modelo que prediz o valor da variável (dependente) alvo a partir de um aprendizado de simples decisões inferidas das características dos dados. O método pode ser usado para predizer tanto uma ou diversas variáveis dependentes (DEBELJAK & DZEROSKI, 2011). Elas podem ser usadas como modelos à decisão de problemas de forma seqüenciada, sob uma condição de incertezas. Elas descrevem graficamente as decisões a serem tomadas, os eventos que podem ocorrer, e os resultados associados à combinação de decisões e eventos. As probabilidades são atribuídas aos eventos e os valores são determinados para cada resultado. O objetivo é determinar a melhor decisão.

As árvores de decisão são compostas por nós (ponto onde a escolha deve ser feita), ramos (representam umas das possíveis alternativas, ou ação disponível naquele ponto), folhas e valores terminais. As alternativas nos ramos são mutuamente exclusivas e coletivamente exaustivas (todas as alternativas possíveis devem ser incluídas). Um evento num nó representa um ponto de incerteza, ou ponto onde o tomador de decisões aprende sobre a ocorrência do evento. A cada evento é atribuída uma probabilidade e o nó terminal representa o resultado final da combinação de decisões e eventos (DEBELJAK & DZEROSKI, 2011).

DTs constituem estruturas hierárquicas onde cada nó interno contém o teste sobre um atributo, cada ramo correspondendo o resultado de cada teste e a folha dando o valor da predição naquela classe de variáveis. Dependendo do tipo de variáveis dependentes, DTs podem ser do tipo classificação (variáveis discretas) e regressão (variáveis contínuas - DEBELJAK & DZEROSKI, 2011; LOH, 2011). Algumas vantagens das DTs são: Simples de entender e interpretar, facil visualização das árvores e requerem pouco preparo de dados, ao passo que outros métodos requerem normalização de dados. No entanto, as árvores de decisão não suportam valores perdidos no conjunto de dados e, além disto, como desvantagens as árvores complexas não generalizam muito bem os dados e conceitos difíceis de aprender podem não gerar bons modelos (SCIKIT, 2014).

3.9.2.6 Máquinas de vetor suporte (SVM)

As SVM têm sido reconhecidas como um dos algoritmos de aprendizado de máquina mais efetivos em diversos problemas de reconhecimento de padrões, fornecendo usualmente bons modelos de classificação, comparativamente às redes neurais e árvores de decisão (CHEN et al. 2011). A SVM representa uma extensão de modelos não lineares e pode ser usada para separar classes que não são discriminadas por outros classificadores lineares. Este método utiliza as variáveis originais dos objetos em combinações lineares. O conceito básico é procurar o melhor espaço que discrimina duas classes, maximizando as margens das classes mais próximas (MEYER, 2012). O uso de classificadores não lineares fornece ao método uma habilidade de modelar separações complicadas. No entanto, como não existe uma ferramenta teórica para predizer o melhor classificador, o experimentar vários classificadores constitui a melhor forma para se encontrar o melhor modelo (IVANCIUC, 2007; KARATZOGLOU et al. 2006).

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Parte II
Metabolomics combined with chemometric tools (PCA, HCA, PLS-DA and SVM) for screening cassava (*Manihot esculenta* Crantz) roots during postharvest physiological deterioration[1]

1. INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is widely cultivated for its starchy storage roots and is a staple food and animal feed in tropical and sub-tropical areas. It is also considered to be an important source of modified starches and bioethanol in China and other Southeast Asian countries. Nevertheless, as a tropical root crop, cassava is categorized as a post-harvest deterioration sensitive species (AN et al. 2012). It is a key subsistence crop, and its industrial uses are steadily growing. In spite of its economic and social relevance, relatively little investment has been made for research on cassava (CEBALLOS et al. 2012).
Cassava roots deterioration is related to two separate processes: one being physiological (also termed primary deterioration) and the other microbiological or secondary deterioration. Physiological deterioration is usually the initial cause of loss of acceptability of roots and is shown by blue-black streaks in the root vascular tissue, which later spread, causing a more general brown discoloration, unsatisfactory cooking qualities and adverse tastes. Primary deterioration also involves changes in oxidative enzyme activities which generate phenols including catechins and leucoanthocyanidins, which in later stages polymerize to form condensed tannins. Microbiological deterioration is due to pathogenic rots, fermentation and/or softening of the roots and generally occurs when the roots have already become unacceptable because of physiological deterioration (SÁNCHEZ et al. 2013; GARCIA et al. 2013; ZIDENGA, 2011; WHEATLEY & SCHWABE, 1984; MAINI & BALAGOPAL, 1978; BOOTH, 1975).

Postharvest physiological deterioration (PPD) often begins rapidly within 24 hours. Because of PPD, cassava roots need to be consumed shortly after harvesting. The short postharvest storage life of cassava is a characteristic that limits the marketability of the roots (CHÁVEZ et al., 2005). Cortés et al. (2002) evaluated the inheritance of PPD in cassava with the aim to identify and localize those regions of the cassava genome

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that control PPD. They concluded that the biochemical processes involved in the rapid deterioration of cassava are essentially wound healing responses, which are well known in many plant species (CORTÉS et al. 2002).

However, phytochemical analysis of compounds of low molecular weight (<500Da) of roots, as well as those produced during deterioration is a matter of interest, with few reports in the literature to improve the understanding of the process of PPD. Metabolomic approaches enable the parallel assessment of the levels of a broad range of metabolites and have been documented to have great value in both phenotyping and diagnostic analyses in plants (FERNIE & SCHAUER, 2008). These tools have recently been turned to the evaluation of the natural variance apparent in metabolite composition (FERNIE & SCHAUER, 2008). A combination of metabolomic techniques with chemometric tools provides a fast, easy, and reliable method for chemotaxonomy characterization (SCHULZ & BARANSKA, 2007), for instance.

Aiming to identify changes in the chemical and enzymatic composition of four cassava genotype samples during post-harvest deterioration and possibly seeking for related biochemical markers, metabolomics and chemometric tools, e.g., multivariate analysis using algorithms implemented in the R language, were used, as well as enzymatic assays related to the oxidative stress process. Supervised and unsupervised methods of data analysis were used to discriminate cassava samples during the post-harvest physiological deterioration (fresh samples (non-stored), 3, 5, 8, and 11 days postharvest).

2. MATERIALS AND METHODS

2.1 Selection of cassava cultivars and on farm trials

Cassava cultivars were provided by Santa Catarina State Agricultural Research and Rural Extension Agency (EPAGRI), experimental station of Urussanga and produced over the growing season in 2011/2012. Four cultivars were selected for this study as follows: SCS 253 Sangão (hereafter designed as SAN); Branco (hereafter designed as BRA); IAC576-70 –"*Instituto Agronômico de Campinas*", hereafter designed as (IAC), and Oriental (hereafter designed as ORI). The cultivars were selected as they are widely used by small farmers, with morpho-agronomic characteristics and lacking more research for better applicability. On farm trials were conducted at the Ressacada

Experimental Farm (Plant Science Center, Federal University of Santa Catarina, Florianópolis, SC, Brazil -27^0 35'48" S, 48^0 32' 57" W), starting in September 2011. The experimental design was done in randomized blocks (DBCC), with 4 blocks (6.3 x 15m^2/block) spaced at 1m. Each block consisted of four plots (12 x 1.2 m^2/plot), spaced at 0.5 m. Cassava cuttings (stakes) with 15 cm in length were used, which were planted upright, spaced 1 x 1m. Each plot was considered a treatment. For laboratory analysis, each cultivar in each plot was mixed as only one sample. All crop management was mechanized and cultivation was done manually. All soil analyzes were done previously. According to the soil analysis, corrections of bases were made applying calcium before cultivation.

2.2 Post-harvest physiological deterioration (PPD)

Samples of cassava roots 12 month-old were collected and all roots within each plot were mixed for analysis of fresh samples, as well as for induction of physiological deterioration in the laboratory under controlled conditions. Induction of PPD was performed for 11 days. Immediately after harvest, the roots were washed, proximal and distal parts of the root were removed and cross sections were made (0.5-1 cm) over the remaining root and stored at room temperature (66-76% humidity, $\pm 25^0$C). Monitoring the development of PPD and associated metabolic disturbances were performed daily after induction of PPD (See supplementary Fig.1 for root images during PPD). Fresh samples (non-stored) and those with 3, 5, 8, and 11 days post-harvest were collected, dried in an oven (35-40^0C), milled with a coffee grinder (Coffee grinder Model DGC-20N series), and stored (-18^0C) for further analysis. For enzymatic analysis, fresh samples were collected and stored (-80^0C) until analysis.

2.3 Postharvest physiological deterioration scoring (PPD scoring)

Five independent evaluations of PPD were carried out at laboratory conditions. A random sample of 3 sliced roots from each plant variety was scored (from 1 -10% to 10 -100%) in each point of the PPD and imaged with a digital camera (OLYMPUS FE-4020, 14 megapixel, China). The mean PPD score for each root was calculated by averaging the scores for the 3 transversal sections and five evaluations. Roots showing symptoms of microbial rotting (very different from those related to PPD) or affected by insects were discarded.

2.4. Mid-Infrared Vibrational Fourier transformed Spectroscopy (ATR-FTIR)

Infrared spectroscopy allows the analysis of physiological changes during post-harvest physiological deterioration. Flour samples of fresh roots and those with 3, 5, and 8 days after harvest were collected for analysis. An IFS-55 (Model Opus v. 5.0, Bruker Biospin, Germany) spectrometer with a DTGS detector equipped with a golden gate single reflection diamond attenuated total reflectance (ATR) accessory (45^0 incidence angle) was used. A background spectrum of the clean crystal was acquired and samples (100 mg) were spread and measured directly after pressing them on the crystal. The spectra were recorded at the transmittance mode over a spectral window from 4000 to 400 cm^{-1} at the resolution of 4 cm^{-1}. Five replicate spectra (128 co-added scans before Fourier transform) were collected for each sample, in a total of 80 spectra. In the pre-processing (using R software), the spectra were normalized, baseline-corrected (see supplementary figure 3A and B) in the region of interest by drawing a straight line before resolution enhancement (k factor of 1.7) using Fourier self deconvolution (WILSON & BELSTON, 1988; RUBENS et al. 1999). The assumed line shape was Lorentzian with a half width of 19 cm^{-1} (MATHLOUTHI & KOENIG, 1987 and recently improved by COPIKOVÁ et al. 2006).

2.5 Extraction of phenolic compounds

The dried and powdered cassava material (1 g per batch) was mixed with 10 mL of ethanol 80% and extracted using water bath at 55^0C, during 30 minutes. The mixture was centrifuged (4000 rpm/5 min), filtered on Whatman No. 2 filter paper, ethanol was removed using rotatory evaporator at 65^0C and dried extract diluted to 3 mL with ethanol (ENGIDA et al. 2013).

2.5.1 Determination of total phenolic content

The total phenolic contents of the cassava extracts during PPD was determined by Folin-Ciocalteu reagent (FCR) method. For a 2.0 mL total volume, 200µL of extract were first mixed with 100 µL of FCR reagent after adding 1.40 mL distilled water and the contents were kept at room temperature for 10 minutes. Later, 300 µL of Na_2CO_3 aqueous solution (20%) were added and incubated for 1 hour. The absorbance was measured at 765 nm using a spectrophotometer UV-visible (Spectrumlab

D180). Total phenolics content was expressed as µg of gallic acid equivalents/g of dry extract (µg GAE/g) using a standard curve (0-1000 µg/mL) of gallic acid (FOLIN & CIOCALTEU, 1927).

2.5.2 DPPH radical scavenging activity assay

The free radical scavenging activity of plant extracts (g/mL) at day 3 of PPD, where superior phenolic contents were detected, was determined by using the DPPH assay according to the procedure described by Blois (1958) and reviewed by Kedare & Singh (2011). 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) 0.002% methanolic solution (m/v) was prepared in a volumetric flask covered with aluminum foil. 1 mL of plant extract (g/mL) in methanol was mixed with 1 mL solution of DPPH (0.002%). For blank solution, the extract was substituted by methanol. Reduction of DPPH by antioxidants was monitored at 520 nm using a spectrophotometer (Spectrumlab D180). Anti-radical activity was based on the measurement of the reducing ability of plant extract toward DPPH radical. Scavenging effect was calculated as described in the formula below, where A_0 is the absorbance of the control reaction and A_t is the absorbance in the presence of the extract sample.

$$\left[[\%A] = \frac{(A_0 - A_t)}{A_0} * 100 \right] \quad \text{(Eq. A1)}$$

2.6 Determination of total flavonoid content

Total flavonoid content of plant extract was determined using aluminum chloride colorimetric method (WOISKY & SALATINO, 1998 and revised by CHANG et al. 2002) and standard solutions (0-1000 µg/mL of quercetin in 80% methanol). For that, 1 mL extract solution was mixed with 0.5 mL 95% ethanol (v/v), 0.1 mL 1M potassium acetate, 0.1 mL aluminum chloride solution (10% $AlCl_3$), and 0.8 mL distilled water to a total volume of 2.5 mL. The mixture was well mixed and incubated at room temperature for 30 minutes versus reagent blank containing water instead of sample. Quercetin was used as the standard (y =0.0006x-0.0995, r^2=0.80) for the quantification of total flavonoid. Results were expressed as milligrams of quercetin equivalent per gram of dry weight (mg QE/g). Data was reported as arithmetic mean ± standard deviation (SD) for three replications.

2.7 Determination of total monomeric anthocyanins

The extraction and purification were performed according to the established method (LEE et al. 2005) taking into account the pH of the reaction medium. Briefly, from 1 g of flour sample, 5 mL of methanol acidified with 1N HCl (85:15 v/v) were added and pH adjusted to 1. The solution was centrifuged (4000 rpm/15 minutes), the supernatant collected and dried in a rotatory evaporator (55^0C). The dried extract was reconstituted with 2 mL of methanol and filtered (0.45 µm). Two dilutions were made, one to pH 1.0 buffer by using 3M potassium chloride and other to pH 4.5 using 3M sodium acetate buffer. Samples were diluted 10-fold to a final volume of 2 mL and the absorbance read after 30 minutes of incubation at 520 and 700nm (Spectrumlab D180 spectrophotometer). The concentration (mg/L) was expressed as equivalent of cyanidin-3-glucoside according to the following formula:

$$\left[\frac{A * PM * DF * 10^3}{\varepsilon * \iota} \right] \text{(Eq. A2)}$$

Where A is the absorbance determined according to the equation

$$\left[\left(A_{520nm} - A_{700nm} \right) \text{pH1.0} - \left(A_{520nm} - A_{700nm} \right) \text{pH4.5} \right] \quad \text{(Eq. A3)}$$

PM - molecular weight of cyanidin-3-glucoside (449.2 g/mol), DF-dilution factor (0.2 mL sample diluted to 2 mL, DF = 10), ι – cuvette width (cm), ε-extinction coefficient (26900 L/ mol.cm) of cyanidin-3-glucoside, and 10^3 - conversion factor g to mg (HOSSEINIAN et al. 2008; LEE et al. 2005)

2.8 Determination of total carotenoids

Carotenoid content was determined according to the described method (CEBALLOS et al. 2012). Briefly, 1g of flour samples was added to 2 mL of cold acetone. After 10 minutes, 2 mL of petroleum ether were added and mixed using ultraturrax for 1 minute. Samples were then centrifuged (3000 rpm/10 minutes), supernatant collected, 2 mL of sodium chloride 0.1M were added, the solution centrifuged again (3000 rpm/7minutes), dried in rotatory evaporator (55^0C), and the dried extract dissolved with 3 mL of petroleum ether. Absorbance was read at 450 nm in a spectrophotometer using the absorption coefficient of β-carotene in petroleum ether (2592 L/ mol.cm) and calculated as represented in the equation 4.

$$\left[(\mu g / g) = \frac{A * V\left(mL\right) * 10^4}{A_{1cm}^{1\%} * P\left(g\right)} \right] \text{ (Eq. A4)}$$

Where: A -absorbance, V-total volume and P- the weight of the sample

2.9 Changes in glucoside cyanides during PPD

2.9.1 Simple picrate and buffer filter papers

Prior to cyanide analysis, picrate and buffer papers were prepared according to Bradbury et al. (1998), linamarin isolated from leaves as reported by Haque & Bradbury (2004), and linamarase by Yeoh et al. (1997). The picrate paper was prepared by dipping a sheet of Whatman 3mm filter paper (3cm x 1cm) in a picrate solution with a concentration of 0.5% (w/v) of picric acid dissolved with stirring and warming in a 2.5% (w/v) sodium carbonate solution. After that, the paper was allowed to air dry (BRADBURY et al. 1999).
The filter paper was prepared by loading it with 50 µL of 1M phosphate buffer at pH 8.0 and after air drying, 60 µL of linamarase solution containing 1% (w/v) gelatin and 5% (w/v) of polyvinylpyrrolidone-10 (PVP) was added and this was allowed to air dry (BRADBURY et al. 1999).

2.9.2 Linamarin isolation from cassava leaves

In other to monitor cyanide contents in cassava samples, a simple method for preparation of an acid-stabilized solution of linamarin, suitable for preparation of standard linamarin filter paper discs were chosen as previously reported by Haque & Bradbury (2004).
A 5 g sample of very young cassava leaves was cut up with scissors and immediately ground in a glass pestle and mortar with 5 mL 0.1M HCl. Five mL of 0.1M HCl were added with further grinding and the pasty solution was filtered. The pink-colored, cloudy solution was then centrifuged and the clear supernatant liquid (about 7mL) removed with a Pasteur pipette. This solution, which also contained linamarase (inactivated in the 0.1M HCl) and linamarin, was stored frozen in a deep freeze cabinet at -20^{0}C until the analyses (HAQUE & BRADBURY, 2004).

2.9.2.1 Linamarin assay from cassava leaves and roots

The linamarin solution was assayed in triplicate by adding 100 µL of the pink solution and 0.5 mL water to a small plastic bottle, followed by a 2.1cm diameter filter paper disc previously loaded with phosphate buffer 0.1M at pH 6 (3 mL) and 3 mL of linamarase. A picrate paper was placed in the bottle, which was closed with a screw cap and left at 30^0C overnight. The brownish picrate paper was removed from the bottle and immersed in 5.0 mL water for 30 min and the absorbance of the solution measured at 510 nm (Spectrumlab D180 spectrophotometer).   The cyanide content, in ppm, was obtained by multiplying the absorbance (A) by 396 – See equation 6 (BRADBURY et al. 1999).

$$\left[ppm = A*396 \right] \text{ (Eq. A5)}$$

Where 396 is the gradient factor observed in the normal picrate method. Linamarin content (ppm) from cassava roots was determined as the difference accordingly to equation 6 (Bradbury, 2009). *Tcy* represents total cyanide and *AcCN* is acetone cyanohydrin.

$$\left[\frac{mgHCN}{Kg} = \left(Tcy - AcCN \right) \right] \text{ (Eq. A6)}$$

2.9.3 Linamarase isolation from cassava leaves

Because the cost of commercial linamarase is prohibitive, an acid hydrolisis method was adopted. Linamarase was isolated with a simple and rapid method developed by Yeoh et al. (1997). Briefly, about 2g of fresh, fully expanded young leaves were homogenized in a mortar with 10 mL 100mM Na-citrate buffer at pH 6.0 containing 10g/L of PVP, followed by the addition of 12 mL of 2M $(NH_4)_2SO_4$. The homogenate was allowed to stand for 30 minutes at room temperature and then filtered with Whatman No.1 filter paper.

2.9.3.1 Linamarase assay

Linamarase assay was carried out by using 1.5 mL of the homogenate previously prepared (*item 2.9.3*), 0.5 mL of 5mM linamarin in 50mM of Na-citrate, pH 6.0 at 37^0C (Yeoh et al., 1997). After 15 minutes, the reaction was stopped by boiling the reaction mixture for 2 minutes and the glucose released was measured using glucose oxidase method using glucose-oxidase kit (Glucose-PAP, LAB TEST diagonostica). Briefly, 3

mL kit reagent was added of 0.3 mL of the sample, followed by mixing and incubation at 37^0C during 15 minutes and absorbance read at 520 nm (Spectrumlab D180 spectrophotometer). The glucose released in (mg/dL) was quantified accordingly (Eq. 7) and converted to mmol/L.

$$\left[Glu\cos e\left(\frac{mmol}{L}\right) = \frac{A_{sample}}{A_{s\tan dard}} * \frac{100}{18} \right] \quad \text{(Eq. A7)}$$

Where A is the absorbance; dL-deciliter; L-liter; mg-milligram; $mmol$-millimoles.

2.9.4 Total cyanide and acetone cyanohydrin in cassava roots during PPD

To determine total cyanide, the method reported by Bradbury (2009) with some modifications was used. Briefly, 1g flour samples during PPD were weighed out into plastic bottles; 10 mL 1M phosphate buffer pH 7.0 and buffer paper were added. A picrate paper was also added; the bottle was closed with a lid and was left 16h at 30^0C. The picrate paper was removed, eluted with 0.5 mL of water, incubated during 30 min, and absorbance measured at 510 nm against a blank in a spectrophotometer and converted to ppm using equation 5. Acetone cyanohydrin was determined on the same flour samples as described for total cyanide, but by adding also 0.5 mL of 0.1M Hcl. Calculations were performed using the equation A8, where A is the observed absorbance and 45.7 is the gradient factor.

$$\left[ppm = A * 45.7 \right] \quad \text{(Eq. A8)}$$

2.10 Enzyme activities during PPD

2.10.1 Catalase activity (CAT)

For the measurement of enzyme activity, flour samples (1g) from different days of PPD (0, 3, 5, 8, and 11) were homogenized in 5 mL of 10mM potassium phosphate buffer (pH 7.0) containing 4% (w/v) PVP (Mr 25 000). The homogenate was centrifuged (4000 rpm/30 min) and the supernatant used as enzyme extract (An et al., 2012). CAT activity was measured directly by the decomposition of H_2O_2 at 240 nm in a spectrophotometer ($y = 2.1247x+0.1807$, $r^2 = 0.97$) and expressed in units (U) per milligram (U.mg^{-1}, 1U = 1mM of H_2O_2 reduced per minutes x milligrams of protein) (Aebi, 1983). The reaction mixture contained 1mL of 50mM potassium phosphate buffer (pH 7.0), 1mL of 10mM H_2O_2, and

1mL of the extract. Protein was measured following the procedure of Bradford (1976).

2.10.2 Determination of hydrogen peroxide (H₂O₂) content

Hydrogen peroxide was determined according to Velikova et al. (2000). 1g flour sample was homogenized in ice bath with 5mL of 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged (4000 rpm for 5 minutes), the supernatant collected (1mL) and added of 50 mM of 1 mL potassium phosphate buffer (pH 7.0) and 2mL of 1M KI. The reaction mixture was read at 390 nm in a spectrophotometer and the content of hydrogen peroxide calculated through a standard curve (y = 2.1247x+0.1807, $r^2 = 0.97$).

2.10.3 Total superoxide dismutase, copper/zinc (Cu/ZnSOD) and manganese superoxide dismutase (MnSOD) (SOD family of enzymes) activities during PPD in cassava roots

SOD family enzymes analysis was carried out according to Fridovich (1995). Briefly, 1g flour sample was homogenized with 10 mL of 50mM potassium phosphate buffer (pH 7.0), centrifuged (4000rpm/30min) and the supernatant containing the crude enzyme extract for assay recovered. For total superoxide dismutase enzyme (Total SOD), 1 mL of 0.05 M sodium carbonate buffer (pH 10.2) was added to 1mL of enzyme extract and 0.5 mL of 4.10^{-4}M of epinephrine. The rate of epinephrine auto-oxidation was observed by monitoring spectrophotometrically the absorbance in samples in a starting point of reaction and 2.5 minutes later. SOD was expressed in units per dry basis (U/g of dry weight – Eq. A9), where 1 $U.g^{-1}$ of SOD is the amount of enzyme required for 50% inhibition of the oxidation of epinephrine to adenochrome at 480 nm, per minute.

The MnSOD was assayed using the same method as above, except with the addition of sodium cyanide (NaCN), an inorganic compound with high affinity for metals to inhibit Cu/ZnSOD activity. The enzyme activity of Cu/ZnSOD was then determined as difference of total SOD and MnSOD.

$$\left[(U/g) = \frac{(\%inhibition)}{y} * \frac{1}{50} * 1000 \right] \quad \text{(Eq. A9)}$$

Where y is mg of tissue per mL of reaction medium

2.11 Exploratory Data analysis using chemometric tools

The spectral profiles of ATR-FTIR and other metabolic profiling data were processed using multivariate statistical techniques. For such analyzes, classification, clustering, and regression methods were used, namely principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA), hierarchical clustering analysis (HA), and support vector machines (SVM). Chemometric analyzes were implemented in the R language (version 3.0.1), using the packages *ChemoSpec, Chemometrics, mixOmics, e1071,* and *pls.* All statistical analyzes were performed using the same software and data represented as mean ± standard deviation of three repetitions (n=3). One-way ANOVA and TukeyHSD tests were applied to the data set when adequate.

3. RESULTS AND DISCUSSIONS

3.1 PPD scoring, secondary metabolites in cassava roots, and DPPH radical scavenging activity

For PPD scoring, three transversal slices for each cultivar and PPD stage (3, 5, 8 and 11 days of storage after harvest) and five independent evaluations were made. A score ranging from 1 to 10 is assigned to each slice, corresponding to the percentage of the cut surface showing discoloration (from 1 = 10% until 10 = 100%). The mean PPD score for the five evaluations is calculated by averaging the scores for the three transversal sections. Roots showing symptoms of microbial rotting (very different from those related to PPD) or affected by insects were discarded. Results of PPD scoring of the four genotypes studied revealed the cultivar ORI as the most susceptible, while BRA was found to be the most tolerant (Fig. 1). One-way ANOVA revealed differences in deterioration rates in all cultivars ($p < 0.05$). For BRA, significant differences ($p < 0.05$) among fresh samples and those at stages 8 and 11 days of PPD were detected. Similarly, differences in deterioration rates at different times of PPD (8 and 11 days post-harvest) have also been found for IAC, ORI, and SAN genotypes. Its important to say that comparing different cultivars at each point of PPD scoring, we did not found statistical differences ($p < 0.05$) but visual evaluation according to vascular discoloration clearly showed rapid discoloration in ORI when compared to BRA cultivar (Fig. 2)
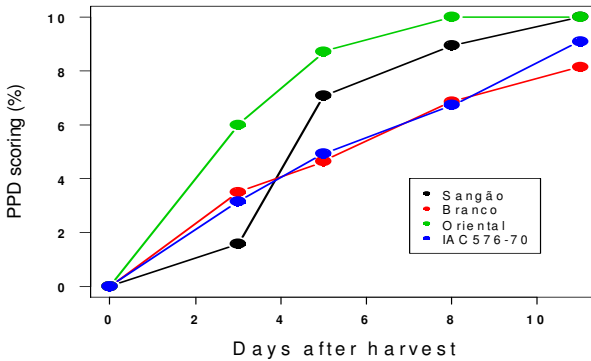
Figure 1. Postharvest physiological deterioration (PPD) scoring of the cassava cultivars studied, from 0 (without deterioration) to 10 (100%) of deterioration evaluated in five stages of PPD. i.e., 0, 3, 5, 8, and 11 days. Scores represent means of five independent experiments of PPD scoring and 3 sliced roots for each cultivar and evaluation.
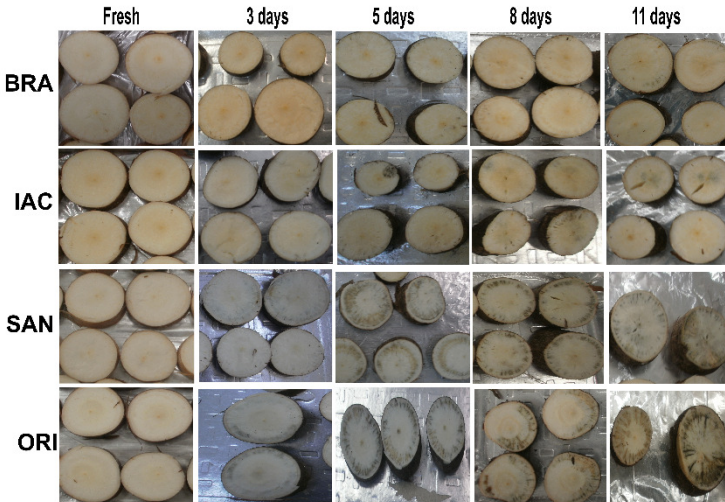
Figure 2. Cassava roots during postharvest physiological deterioration. SAN, cassava roots of Sangão cultivar at different stages of PPD (fresh, 3, 5, 8 and 11 days of PPD). ORI, cassava roots of Oriental cultivar. BRA- cassava roots of Branco cultivar and IAC, for IAC576-70 cultivar.

Secondary metabolites (carotenoids, phenolics, flavonoids, and anthocyanins) are found in many species of the plant kingdom and are well recognized as potential antioxidants. Physiological deterioration has been related to changes in enzyme activities, which generate phenols and leucoanthocyanins (RICKARD, 1981). Carotenoids have been related in the literature to delayed or reduced postharvest deterioration in cassava roots (MORANTE et al. 2010; SANCHEZ et al. 2006). The biochemical changes in carotenoids detected during the storage of fresh cassava roots up to 11 days are summarized in the table 1 part I. Spectrophotometric analysis of cassava root extracts demonstrated that, in fresh samples, the largest amount of carotenoids was detected for IAC, followed by SAN and ORI cultivars. No significant differences ($p < 0.05$) in carotenoid contents for fresh samples and those at stage 3 of PPD were found in all cultivars, as further increases were noticed in the root pieces following injury until 3 to 5 days post-harvest. In the ORI cultivar, increases in those pigments were found to occur only until day 3 of PPD (Table 1 part I), but SAN and BRA genotypes showed higher amounts until days 5 and 8 days, respectively. Besides, significant differences ($p < 0.05$) were observed at stages 5 and 8 for ORI, IAC, and SAN. At stage 11, only SAN differed from ORI regarding the carotenoid concentration.

For the flavonoids, in fresh samples, the largest amount was found to occur for ORI, followed by SAN and BRA cultivars and significant differences ($p < 0.05$) were observed among all the studied cultivars. The concentration of these secondary metabolites was found to be considerably higher in the tolerant (BRA) cultivar (3 days post-harvest) and continued increasing until 5 days in the other cultivars (SAN and IAC- Table 1 part-I). Similarly, at stages 5 and 8 of PPD, discrepancies were found only between BRA and SAN cultivars. At the end of the experimental period, i.e. stage 11, flavonoid contents differed between SAN-ORI and IAC-BRA. Few studies have been reported on flavonoid contents during PPD. Buschmann et al. (2000a) reported increases in flavonoid during the first 1-2 days post-harvest. After 4-6 days there was some accumulation and after 7 days there was a rapid decline. Tanaka et al. (1983); Uritani et al. (1984a); Uritani et al. (1984b) also reported increases in flavonoid contents during PPD. Our results are in accordance with those previously reported.

Table 1. Part I: Changes in secondary metabolites and cyanogenic glucosides during PPD. Values are represented as means of three repetitions in (µg/g) for carotenoids, flavonoids, anthocyanins, phenolics and in units (mg/Kg) for cyanogenic glucosides. Statistical differences are represented with different letters in the column. Comparisons are related only for each group of compound reported.

Group of compound	Days after harvest	Sangão	Oriental	IAC576-70	Branco
Carotenoids (µg/g)	Fresh samples	3.90b	3.02b	4.34a	1.52c
	3	4.63b	5.08a	4.12a	4.22b
	5	6.71a	4.85ab	4.38a	5.00b
	8	5.63b	3.66b	3.67a	5.28a
	11	7.27a	3.13b	4.75a	4.90ab
Flavonoids (µg/g)	Fresh samples	578.61d	772.50b	398.06d	509.17c
	3	959.17c	1299.17a	1373.61b	2288.06a
	5	1408.06a	781.39b	1670.28a	1583.61b
	8	1317.50ab	1054.72ab	1018.06c	1170.28b
	11	1230.83b	1365.28a	1386.94b	1445.28b
Phenolics (µg/g)	Fresh samples	44.94b	64.06c	70.05c	63.99c
	3	353.03a	546.76a	513.61a	538.22a
	5	112.26b	169.65b	134.42b	151.98b
	8	154.70b	183.33b	202.75b	229.73b
	11	112.44b	117.22bc	137.36b	177.47b
Anthocyanins (µg/g)	Fresh samples	7.12c	5.01c	13.19b	6.35c
	3	8.13c	14.25a	5.84c	5.62c
	5	16.25b	5.51c	0.50d	8.96c
	8	19.04a	5.84c	6,79c	11.80b
	11	6.46c	10.74b	22,15a	42.92a
Total cyanide (mg/Kg)	Fresh samples	56.87c	33.04d	24.21d	22.80e
	3	84.65b	56.21c	60.05c	80.22a
	5	63.16c	38.16d	22.18d	55.37b
	8	60.57c	64.45b	53.38b	39.03c
	11	93.79a	79.32a	66.62a	29.17d
	Fresh samples	3.64d	8.68a	10.05b	9.64a

	3	5.38b	5.88e	10.02b	6.55e
Acetone cyanohydrin	5	6.96a	6.35d	8.91c	8.58b
(mg/Kg)	8	6.00b	8.38b	4.39d	6.90d
	11	4.84bc	7.01c	10.92a	7.98c
	Fresh samples	53.23c	24.36d	14.15c	13.16e
	3	79.27b	50.33b	50.03b	73.67a
Linamarin	5	56.20c	31.81c	13.27c	46.80b
(mg/Kg)	8	54.57c	56.07b	48.99b	32.13c
	11	88.95a	72.31a	55.69a	21.19d

*(continuation of table 1 part I)

The total phenolic compounds analysis revealed the largest amount in IAC followed by ORI, BRA, and SAN cultivars in fresh samples. A further increase in the phenolics was observed in all cultivars until 3 days after harvest (Table 1 part-I (Table 1-I)). In fresh samples, and those with 5 days of PPD, significant differences ($p < 0.05$) were observed among ORI, BRA, IAC, and SAN, at stage 11, however, only BRA and SAN genotypes differed. ORI showed larger levels of phenolics at stages 5 and 8. Previous studies by Uritani et al. (1984a and b), Buschmann et al. (2000a), Blagbrough et al. (2010) also confirmed similar trends in phenolic compounds during PPD.

IAC fresh samples showed to contain the largest amount of anthocyanins, followed by SAN, BRA, and ORI cultivars. No meaningful differences ($p < 0.05$) were detected for the fresh samples and those at stage 3 of PPD. Contrarily, samples at stages 5, 8, and 11 of PPD showed to differ ($p < 0.05$) in their contents of those secondary metabolites. Taking together, the results seems to indicate that a typical genotype-dependent effect occur for the studied feature. Rickard (1981) previously reported small increases in anthocyanins during PPD.

Changes in flavonoid contents along with phenolics, anthocyanins, and carotenoids ones indicate that the latter changes are, at least partly, due to de *novo* synthesis of these compounds and not only to qualitative changes. We found a positive correlation for flavonoids and anthocyanins with PPD ($y=0.003x+1.09$, $r^2=0.38$ – flavonoids and $y=0.1x+3.4$, $r^2 =0.27$ - anthocyanins), while the total phenolic and carotenoid amounts negatively correlated to PPD ($y=-0.01x+8.01$, $r^2=-0.53$ – total phenolics and $y=-0.28x+6.96$, $r^2=-0.12$ – carotenoids, Fig. 3A-D). Despite of the

low correlations found for those variables, the results presented indicate that the complexity of the changes occurring in cassava roots in response to injury commences as a non-specific response to wounding, during harvest or root slicing as we know that upon injury, a set of biochemical events takes place in a closely orchestrated cascade to repair the damage. The results reinforce previous indications that relate PPD delay to carotenoid levels and presents new insight relating also phenolics to PPD delay. Increases in flavonoids may be related to the wound healing responses. The results herein shown indicate that biochemical changes in cassava are induced by the combined effects of the mechanical injury and duration of storage, as previously described by Uritani et al. (1984a). Increases in phenolics, flavonoids, and anthocyanins until 144 hours were also found by Rickard (1981) in cassava samples, e.g., flavonoids ranging from 400 to 2250 ppm, phenolics from 50 to 550 ppm, anthocyanins from 1 to 42 ppm, and 1.2 to 7.2 ppm for carotenoids.
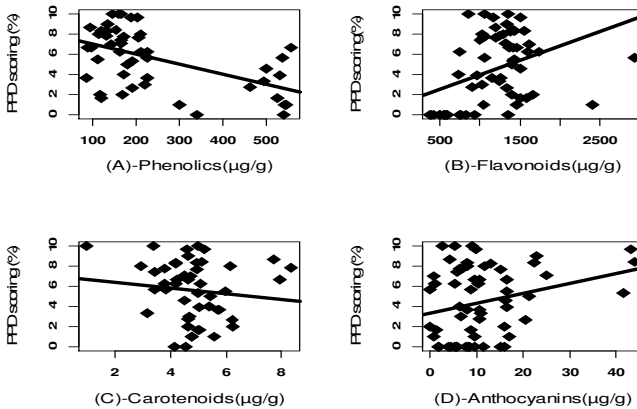
Figure 3. Correlations between PPD and the secondary metabolites studied. A - Association with phenolic content (μg/g, $y = -0.01x + 8.01$, $r^2 = -0.53$); B - Association with flavonoid content (μg/g, $y = 0.003x + 1.09$, $r^2 = 0.38$); C - Association with carotenoid content (μg/g, $y = -0.28x + 6.96$, $r^2 = -0.12$); and D- Association with anthocyanin content (μg/g, $y = 0.1x + 3.4$, $r^2 = 0.27$). Data presented here are representative of all cultivars studied.

Since abundant literature indicates that phenolic are important antioxidants in plant foods, the antioxidant activity assay was also made for cassava extracts with 3 days after harvest, since those samples showed major phenolic contents for all the cultivars. In this study, DPPH was quantified and correlated to PPD. DPPH radical scavenging activity

ranged from 4.92 to 41.36% with the highest activity being found in cassava extracts of ORI and IAC cultivars (Fig. 4). A lower DPPH activity was detected in the tolerant cultivar BRA extract, indicating a lower concentration of phenolic compounds of this genotype to quench reactive species.
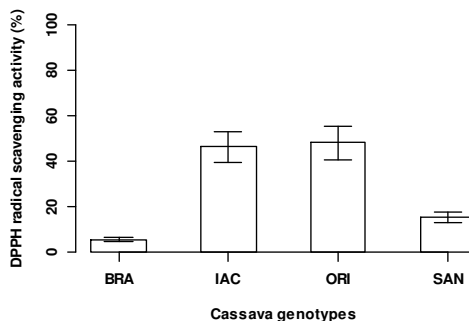
Figure 4. DPPH radical scavenging capacity of cassava root extracts at stage 3 of PPD (3 days after harvest). Data represent means ± SD (n=3).

3.2 Changes in glucoside cyanides during PPD

Cassava roots contain cyanogenic glucosides (CGs) that are phytoanticipins widely distributed in the plant kingdom. Also known as β-glucosides or α-hydroxinitriles, they are derived from aliphatic protein amino acids (L-valine, L-isoleucine, and L-leucine), from aromatic amino acids (L-phenylalanine, L-tyrosine), and from aliphatic non-protein amino acid cyclopentenyl-glycine (ZAGROBELNY et al. 2004). Our results on CGs during PPD are summarized in the table 1-I. Large variations in cyanide content and acetone cyanohydrin was found among cultivars during the 11 days of storage. When cassava roots were stored under room temperature, physiological and microbial deterioration occurred. In fresh roots, the cultivar SAN showed the highest amount of cyanide (56.87 ppm), while BRA showed the lowest (22.80 ppm) one. As shown in the table 1, cyanide content increased in all studied samples at stage 3 of PPD and decreased until stage 8. At advanced stages of PPD, cassava samples showed high levels of cyanide. The results showed a marked tendency towards increased cyanide content in response to storage, physiological, and microbial deterioration. This variation may be explained taking into consideration the catabolism of CGs initiated through enzymatic hydrolysis by a β-glucosidase to afford the

corresponding α-hydroxinitrile, which at pH values above 6 spontaneously dissociates into a sugar, a keto compound, and HCN. HCN can be detoxified by two main reactions: conversion into asparagine and into thiocyanate (ZAGROBELNY et al. 2004). More than one pathway can be utilized for cyanide biodegradation and synthesis, in some organisms like bacteria and fungi, because cyanide plays a principal role in the evolution of life and remains an important source of nitrogen for microorganisms (EBBS, 2004). This can also explain the decrease of cyanide 3 days post-harvest and its increase in the last stage of PPD, which surely is associated to microbial deterioration. Similar results in cyanide were found by Iwatsuki et al. (1984). However, Maini & Balagopal (1978) found an oscillation of cyanide contents, decreasing until 3 days and increasing until 7 days of PPD. They attributed those results to the increase of sugar contents in the tubers. One-way ANOVA and TukeyHSD test ($p<0.05$) revealed the existence of significant differences for that variable among the cultivars, at all stages of PPD.

Acetone cyanohydrin (AcCN) is completely broken down to cyanide in weakly alkaline conditions. AcCN concentrations ranged from 3.64ppm (SAN) to 10.92ppm (IAC), with the highest value detected for the latter, followed by BRA and ORI. In SAN cultivar samples, an increase of acetone cyanohydrin was observed until stage 5 and then a decrease (Table 1-I). BRA and ORI cultivars showed a decrease until stage 3, as for IAC such behavior was found after stage 3 of PPD (Table 1-I). Reports on AcCN during PPD are scarce in the literature. Bradbury et al., (1999) found values ranging from 0 to 7 mg/Kg of AcCN in cassava flours and from 0 to 14.4 mg/Kg in fresh dried cassava flours (BRADBURY, 2009). In general, AcCN increased during PPD, but any eventual correlation with PPD has not been established so far.

Linamarin contents and linamarase activity of the root samples during PPD are summarized in Table 1(part I and II respectively) and for leaf samples in Table 2. Results of leaf samples demonstrated high levels of linamarin (IAC, 1030.26mg HCN/Kg) and linamarase (SAN, 3.02mmol/L) for those cultivars. Lower values of linamarin and linamarase in leaves were observed for SAN and BRA, respectively. In fresh root samples, SAN showed the highest level of linamarin (53.23ppm) and BRA the lowest one during PPD (Table 1-I). An increase of linamarin was observed in all cultivars until stage 3 of PPD with subsequent oscillation (13.16ppm, BRA to 88.95 ppm, SAN) after this stage. Linamarase activity was observed to decrease during PPD in almost

all cultivars, except ORI that showed the opposite effect until stage 3, followed by a reduction (Table 1-II). IAC cultivar (8.33 mmol/L) was followed by BRA (7.53mmol/L) showing major linamarase activities in fresh samples. Mkpomg et al. (1990) reported values of linamarin and linamarase activity ranging from 37 to 72ppm and from 0.19 to 2.06mmol/L, respectively. Bradbury et al., 1999 reported for cassava flours linamarin values ranging from 4.5 to 87ppm. Compared to the roots, linamarin contents were higher in the leaves, confirming previous tissue-specific studies (BRADBURY, 2009; BRADBURY et al., 1999) on cyanogenic glucosides of *M. esculenta*. Taken together, these results suggest that tissue and varietal differences play a role in the catabolism of linamarin and linamarase activity.

Table 1. Part II. Changes in enzymes during PPD. Values are represented as means of three repetitions in (µg/g) for hydrogen peroxide and catalase activity, in units per kilogram (U/Kg) of fresh weight for total superoxide dismutase, manganese and cupper/zinc dismutase and in mmol/L for linamarase. Statistical differences are represented with different letters in the column. Comparisons are related only for each group of compound reported.

Group of compound	Days after harvest	Sangão	Oriental	IAC576-70	Branco
Linamarase (mmol/L)	Fresh samples	6.14b	5.48c	8.33a	7.53b
	3	6.04c	6.34b	5.88e	6.97c
	5	5.95c	4.82d	6.15d	6.87c
	8	5.68d	4.87d	6.94b	5.05d
	11	7.13[a]	7.80a	6.34c	8.74a
Hydrogen peroxide (µg/g)	Fresh samples	62.27d	97.41d	77.33e	120.47c
	3	41.25e	87.68e	103.84d	103.53d
	5	103.21c	112.78c	157.18c	117.49c
	8	117.96b	134.28b	195.46b	156.71b
	11	147.61[a]	189.34a	269.83a	180.56a
Catalase activity (µg/g)	Fresh samples	14.89e	42.50d	153.42a	222.76a
	3	112.47c	217.90a	89.09b	115.14b
	5	66.50d	162.83b	97.72b	122.83b
	8	295.56[a]	117.02c	183.70a	220.72a
	11	167.22b	158.28b	193.11a	252.88a
SOD (U/Kg)	Fresh samples	38.44b	84.77d	597.77a	328.53b
	3	29.57c	57.27e	86.18e	735.79a
	5	46.36[a]	137.97b	359.91b	270.34c
	8	18.14d	162.71a	249.86d	188.25d

	11	46.66[a]	130.82c	286.44c	149.34e
MnSOD (U/Kg)	Fresh samples	2.46c	37.81b	530.60a	142.01b
	3	20.20[a]	44.81a	75.86d	404.23a
	5	21.08[a]	11.59d	113.08c	31.23e
	8	5.11b	21.68c	74.07d	51.92d
	11	5.43b	37.90b	124.51b	132.08c
Cu/ZnSOD (U/Kg)	Fresh samples	35.98b	46.95d	67.18d	186.53c
	3	9.37e	12.47e	10.32e	331.56a
	5	25.27c	126.39b	246.83a	239.11b
	8	13.03d	141.03a	175.79b	136.33d
	11	41.23[a]	92.92c	161.93c	17.26e

(Continuation of table 1 part II)

3.3 Enzyme and reactive oxygen specie activities during PPD

As previously reported, stress conditions may lead to the accumulation of many reactive oxygen species (ROS), such as hydrogen peroxide, and to the activation or downregulation of many kinds of enzymes (AN et al., 2012). PPD has been explained as a physiological process not due to microorganisms, rather having a molecular basis as an oxidative burst which initiates 15 minutes after roots being injured (REILLY et al. 2003), followed by altered gene expression. The later phenomenon is expected to play a role in cellular processes (REILLY et al. 2007) and on the accumulation of secondary metabolites (BUSCHMANN et al. 2000a,b). Therefore, it would be important to increase our understanding of the physiological and biochemical traits associated to PPD, especially during the early stages.

Hydrogen peroxide (H_2O_2) is moderately reactive, has a relatively long half-life and high permeability across membranes. It has been well established that excess of H_2O_2 in plant cells leads to oxidative stress. H_2O_2 inactivates enzymes by oxidizing their thiol groups (GILL & TUTEJA, 2010). In our study, during PPD, increases in hydrogen peroxide amounts were detected in all cultivars, ranging from 41.25 ppm (SAN) to 269.83 ppm (IAC). High levels of hydrogen peroxide were observed at the last stage of PPD (11 days post-harvest), where physiological and microbiological deterioration were noticed as well (Table 1 part II (Table 1-II)). Statistical analyses showed significant differences among cultivars at different stages of PPD (p<0.05). A high positive correlation (r^2=0.87) was found between PPD and hydrogen

peroxide content in all studied cultivars. These results are consistent with the literature, indicating that oxidative stress may exert a toxic effect on cassava roots to adapt or tolerate under PPD conditions.

Plants, as well as other organisms, have evolved antioxidant systems to protect themselves against toxic species of oxygen. ROS scavenging enzymes, including catalase (CAT), superoxide dismutase (SOD) - including Manganese SOD (MnSOD) and copper/zinc SOD (Cu/ZnSOD) - have been demonstrated to play key roles in the removal of ROS. In the present study, a light decrease of CAT activity was found until stage 3 and then an accelerated increase until the last stage of PPD in IAC and BRA cultivars (Table 1-II). ORI, the most susceptible cultivar to PPD showed an increase of CAT in the early stages and then a decrease. The increase of CAT in SAN cultivar was observed until 3 days post-harvest, oscillating in the next stages of PPD. CAT ranged from 14.89 (SAN) to 295.56 (SAN) mM of H_2O_2/min.g (Table 1-II). Interestingly, the most tolerant cultivar (BRA) to PPD showed a high level of CAT in fresh samples. Similar results on peroxide hydrogen and CAT were also found by An et al., 2012 in cassava subjected to cold stress. Barceló (1998) showed increases in hydrogen peroxide under chemical stress. Gill & Tuteja (2010) related the increases in hydrogen peroxide concentration to the augment of CAT activity and other scavenging enzymes, corroborating the findings herein described.

The metalloenzyme superoxide dismutase (SOD) is the most effective intracellular enzymatic antioxidant, being ubiquitous in all aerobic organisms and in all subcellular compartments prone to ROS mediated oxidative stress. SOD has been proposed to be important in plant stress tolerance and provides the first line of defense against the toxic effects of elevated ROS (GILL & TUTEJA, 2010; MITTLER, 2002). Results on ROS scavenging enzymes are summarized in the Table 1-II. SOD values ranged from 18.14 (SAN) to 735.19 U/Kg of dry weight (BRA). Total SOD activity increased in tolerant cultivar BRA until stage 3 followed by a decrease. Differently, SAN, ORI, and IAC showed a decrease of SOD until stage 3 followed by an increase of SOD in the next stages of PPD. ORI and SAN (i.e., cultivars with major PPD) showed lower SOD activities in all stages of PPD.

MnSOD activity was reduced in BRA cultivar only in stage 3 followed by an increase until the last stage. ORI cultivar had a similar variation, but with lower MnSOD activity. In its turn, a higher level of MnSOD was detected for the SAN genotype at stage 3, followed by a reduction until

the last stage of PPD. In general, MnSOD activity decreased during PPD (Tab. 1-II).

The Cu/ZnSOD activity in all cultivars ranged from 9.37 (SAN) to 331.56 U/Kg of dry weight (Tab. 1). Differently, for the total SOD and MnSOD, the BRA cultivar showed an increase in the Cu/ZnSOD activity until stage 3 and, after that, a reduction was noticed. ORI, IAC, and SAN showed lower activity of Cu/ZnSOD at stage 3 and an increase until stage 8 of PPD. In general, increases of activity were observed during PPD, but such a behavior was genotype dependent. The findings herein reported corroborate those of An et al. (2012) in cassava cold stress. The authors observed increases in SOD only 4h after stress induction and then a decrease of enzymatic activity. Similar results in CAT, hydrogen peroxide, and SOD family of enzymes were found by Reilly et al. (2007; 2003). The variation in data observed may reflect that PPD is a complex phenomenon and other variables (environmental and genetic) must be viewed together for a better understanding of the physiological events occurring over the deterioration process.

Table 2. Linamarin and linamarase contents (mg/Kg) in fresh leaves of the four cassava cultivars studied. Data are represented as mean ± standard deviation of three repetitions. Different letters in the column represent significant differences (TukeyHSD test, p<0.05).

Cultivars	**Linamarin** (mg HCN /Kg fresh wt)	**Linamarase** (mmol/L)
BRA	806.65 ±1.19c	1.39c
IAC576-70	1030.26 ±5.09[a]	1.96b
ORI	822.62 ±0.82b	1.84b
SAN	558.09 ±4.50d	3.02a

[*]Different letters in the column represent statistical differences (Tukey test, p<0.05)

3.4 Chemometric analysis

3.4.1 Principal Component Analysis (PCA)

In this study, PCA was used to objectively interpret and compare the ATR-FTIR spectral data set, as well as the data resulting from metabolic profiles and enzyme fingerprints of the cassava samples under analysis. Such an experimental approach aimed to evaluate the most important biochemical events related to the deterioration changes and to discriminate cassava cultivars during PPD. Using the "ChemoSpec" and "mixOmics" R packages, PCA was applied to the raw FTIR spectral data set and to the other metabolic data (UV-visible spectrophotometric data) studied in this research. A clear separation between ORI and BRA (susceptible and tolerant-PPD genotypes, respectively) cultivars was noticed in positive axis of PC1 and PC2, respectively, resulting from the FT-IR spectroscopic (3000-600 cm^{-1}) data. The total variance of data explained by the PCA model built was 83%, with, 61% from PC1 and 22% from PC2 (Fig. 5A). In spite of that, some overlap of most samples was also observed. In supplementary figure 1, the plotted PC1 and PC2 loadings values of the spectral data are shown, representing the regions of the spectra where the differences among cultivars are more evident. The factorial contributions for discriminating the cultivars in the positive axis of PC1 were associated to the spectral windows of 600-900 cm^{-1} and 1600-2800 cm^{-1}. Such findings prompted us to perform a more detailed analysis taking into account the data set related to typical fingerprint regions of carbohydrates (1200-900cm^{-1}), proteins (1680-1540 cm^{-1}) and lipids (3000-1700 cm^{-1}) to better identify and discriminate the cultivars according to their biochemical discrepancies over the PPD. The results indicated that the spectral regions associated to carbohydrates and proteins were the best ones for cassava screening (see supplementary figures 2A-D) by PCA.
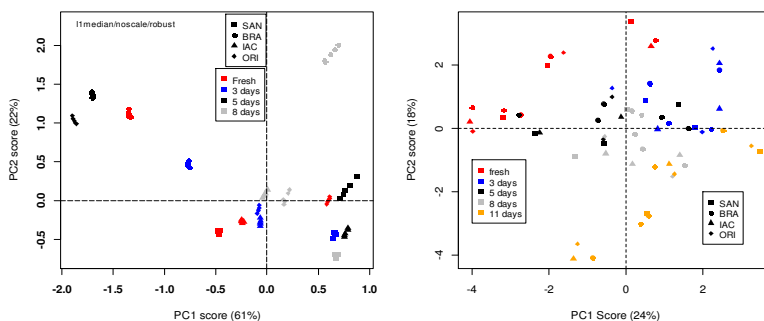
Figure 5. First and second principal component (PCA) scores plot of cassava samples. A (left) - ATR-FTIR spectral (3000-600 cm⁻) data set of cassava samples during PPD (0, 3, 5, and 8 days). B (right) - Summary data of all metabolites, enzymes, and cyanogenic glucosides studied during PPD (0, 3, 5, 8, and 11 days).

In a complementary approach, PCA was conducted independently for the other metabolomic data set, i.e., the target metabolites and the enzymatic activities studied. Figure 5B, shows an evident separation from fresh samples and those with 3 and 11 days of PPD. Fresh cassava samples were found in the positive and negative axis of PC2 and PC1 respectively, while those with 11 days of PPD in the positive and negative axis of PC1 and PC2 respectively. The total variance explained from the 3 first components was 55% (PC1 - 24%, PC2 – 18%, and PC3 - 13%). Interestingly, no differences for the biochemical profiles of the samples in the stages 5 and 8 of PPD were found. The loading values demonstrated that samples grouped in PC1+ according to their phenolic, anthocyanin, flavonoid, carotenoid, linamarin, and total cyanide contents, as well as the root deterioration degree (root scoring degree). On the other hand, negative axis of PC1 was influenced according to the values of acetone cyanohydrin, linamarin, linamarase, hydrogen peroxide, CAT, and SOD family activity. The positive axis of PC2 was explained according to the values of total phenolics and cyanide contents, and Cu/ZnSOD, as the negative axis of PC2 according to the remaining variables studied. Taking into consideration the percentage of variance explained in this second model, it is evident that the FTIR-based model showed a better performance for discrimination of the cassava roots suffering from PPD. In addition, the results suggest that the signals at the spectral window at 1600-600cm⁻¹ and the flavonoid contents are important variables determining the discrimination profile observed, indicating the need of

further studies to better investigate such findings on PPD of cassava roots. These findings are important as ATR-FTIR spectroscopy seems to be a better source of data to couple to chemometrics, i.e. PCA, because it is more cost and time effective for further discrimination of cassava roots suffering from PPD, comparatively to the biochemical assays performed.

3.4.2. PLS-DA

This classification technique finds the components or latent variables which discriminate as much as possible between two or more different groups of samples (X block), according to their maximum covariance with a target class (varieties at different levels of PPD) defined in the Y data block (OUSSAMA et al. 2012), in this case the deterioration stage. The selection of an optimal number of latent variables in PLS-DA was done using the criterion of lowest prediction error (highest accuracy) in cross validation (random subsets), i.e. of optimal prediction of y values for the external validation samples not used in the calibration step. PLS-DA was done using the package "mixOmics" for all experimental data except those from ATR-FTIR spectra. Figure 6A depicts the discriminant scores plot of PLS-DA. A separation was observed from fresh samples (positive axis of PC2 and negative axis of PC1) and those at stage 11 of PPD (positive axis of PC2 and PC1). In spite of the occurrence of 3 sample mismatches, ORI and SAN grouped in the negative axis of PC1. The total variance explained was 73%, 43% from component 1 and 30% from component 2. The loading values showed that samples grouped in x-variate 1+ (positive axis of PC1) according to flavonoid, phenolic, total cyanide, and linamarin contents, as well as root deterioration grade. In their turn, samples in x-variate 1- (negative axis of PC1) grouped according to their values of acetone cyanohydrin and total SOD. Samples were also grouped in x-variate 2+ (positive axis of PC2) as they revealed similarity in CAT activity, hydrogen peroxide and acetone cyanohydrin contents and in x-variate 2- (negative axis of PC2) by their values of phenolics, total cyanide, and linamarin contents. By applying PLS-DA, a better discrimination model was achieved in comparison to PCA, since PLS-DA was most effective in separating sample groups according to their deterioration stages.

3.4.3 Support Vector Machine (SVM)

SVM is a nonlinear computational learning method based on statistical learning theory (classification supervised method) aiming at to separate groups here defined as cultivars at different levels of PPD. The SVM method was implemented from package "e1071", used in a classification mode, with a radial SVM-kernel, cost = 4, and gamma of 0.5 as the best values found. Similarly to the PLS-DA model, our training model clearly separated fresh samples from those at stage 11 of PPD (Fig. 6B) using phenolics and flavonoids as target variables. Samples with PPD at stages 3, 5, and 8 were classified in the same group in the hyperplane (Fig. 6B). Other classification methods such as artificial neural networks (ANN), K-nearest neighbors (KNN), and classification trees were also tested (data not shown), but SVM showed the lowest test classification error (see supplementary fig. 3A-E). The predicted values (data not shown) were consistent in our classification model and the mean and standard deviation of the accuracy in a 5-fold cross validation scheme were 88.4% and 2.6% respectively.
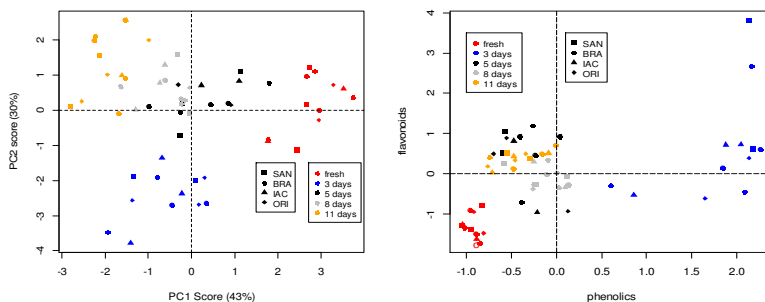
Figure 6. (A) - PLS-DA components scores plot of cassava samples during PPD in relation to all metabolites, enzymes and cyanogenic glucosides analyzed. PLS-1 (PC 1) = 43%, PLS-2 (PC 2) = 30% of variance explained. The model was built with 80% of training data and the rest (20%) used for test (B) - Multiclass SVM classification plot of cassava samples during PPD with decision boundary f(x) = 0. Support vectors are represented in circles.

3.4.4. Hierarchical Clustering Analysis (HCA)

Cluster analysis is often based on the concept of similarity. The easiest and most intuitive way to mathematically define the similarity between

two objects is based on the Euclidean distance, which was adopted in this study, without loss of generality. When HCA was applied to the FTIR spectral data, four groups emerge (Fig. 6C). The cophenetic correlation found was 75% and the similarities found were related to the spectral regions of carbohydrates and proteins as observed in feature selection (see supplementary Fig. 1) and previously in PCA.

When a seriated cluster heatmap was applied to the metabolites and enzymatic data set, four groups were also found, with a high similarity correlation (cophenetic correlation =98.7% - Fig. 6D). BRA and IAC showed similarities according to their values of flavonoids, total SOD, and MnSOD activities. ORI3, SAN3, and IAC3 were found to be grouped as a function of flavonoids. Most samples at stage 11 of PPD grouped with stages 5 and 8 samples of PPD and a clear separation between BRA and ORI at stage 8 of PPD was found. Looking for what drives the clusters, a feature selection was also applied for the 4 small molecules, CN-related compounds and enzymes (Appendix B, supplementary Figures 4A-H). It seems that clustering is driven by flavonoids, phenolics, total cyanide, linamarin, catalase, superoxide dismutase, and hydrogen peroxide.
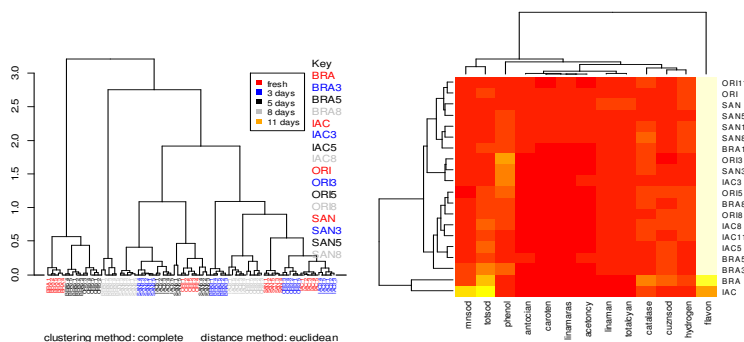
Figure 6 (continuation), (C) Hierarchical cluster analysis (HCA) of the ATR-FTIR spectral (3000-600 cm⁻) data set during PPD with 75% of cophenetic correlation and (D) A seriated cluster heat Map of UV-visible spectrophotometric data (metabolites, enzymatic activity, and cyanogenic glucosides) studied with cophenetic correlation of 98.7%.

4. CONCLUSIONS

The metabolomic-chemometrics approach used in this study for screening cassava roots during postharvest physiological deterioration gave rise to an evidence of metabolic differentiation for the studied genotypes during PPD. A rapid screening of cassava during PPD was achieved with

successful realization of the research objectives, by combining the visual inspection of cassava roots (e.g., tolerant BRA and susceptible ORI) to PPD, their biochemical profiles, the ATR-FTIR spectral data set, and the proposed chemometric tools. A clear trend was evident with fresh samples and those with 3, 5, 8 and 11 days of PPD, being well discriminated by PCA, PLS-DA, and SVM. PLS-DA and SVM modeling of the biochemical data enabled satisfactory prediction of cassava samples. PPD seems to be related negatively with phenolic compounds, carotenoids and positively with flavonoids and anthocyanins. Reactive scavenging species and enzymes such as SOD, MnSOD, and Cu/ZnSOD are activated as protective form of the oxidative stress by cells over the PPD. Finally, the results clearly indicated that further biochemical studies focusing on the t_{zero}–stage 3 period of PPD seem to be important in order to gain more detailed insights as to metabolic and enzymatic events related to that physiologiccal process in root cassava genotypes.

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6. SUPPLEMENTARY DATA

Figure 1. Loading values of Principal component 1 and 2 of the whole spectra analyzed (600-3000nm) showing the region of the spectra that more influence the separation in PCA.
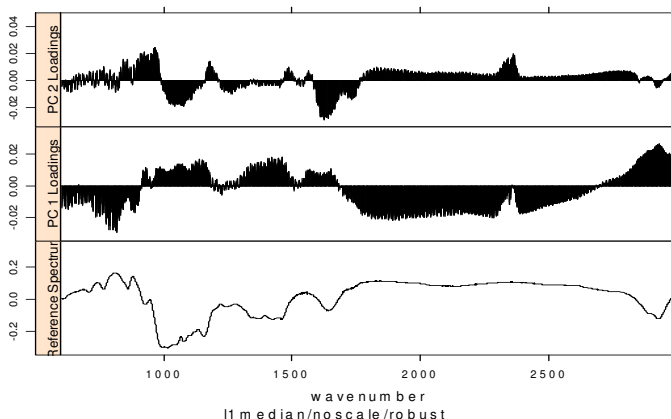
Figure 2. Feature selecting of the spectra at different frequency ranges seeking for that regions of the spectra which most contribute to separation of samples. (A) From 600 to 1700nm, (B)- From 1700 to 3000nm, (C)- From 900 to 1200nm (carbohydrate region) and (D)- From 1000 to 1680nm (protein region).
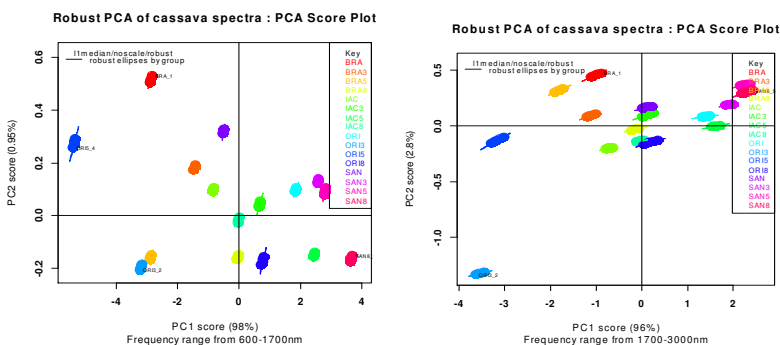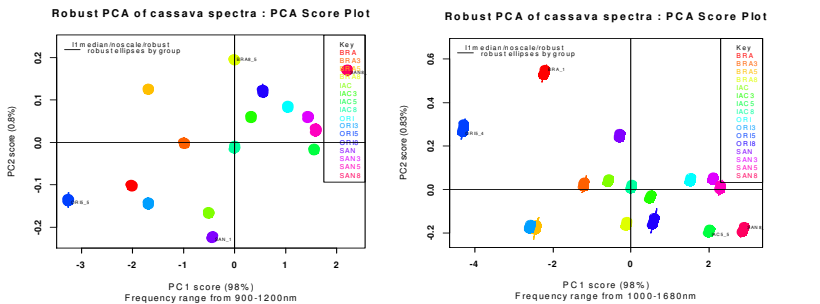
Figure 3. Missclassification errors of other classification methods tested in the study. (A: left)- Missclassification error for support vector machines (SVM), (B: right)- Missclassification error for k- nearest neighbors (KNN), (C)- Missclassification error of classification tree, (D)- Missclassification error for artificial neural networks (ANN) and (E)- Comparison of all errors tested.

Figure 3C

Figure 3D

Artificial Neural Networks
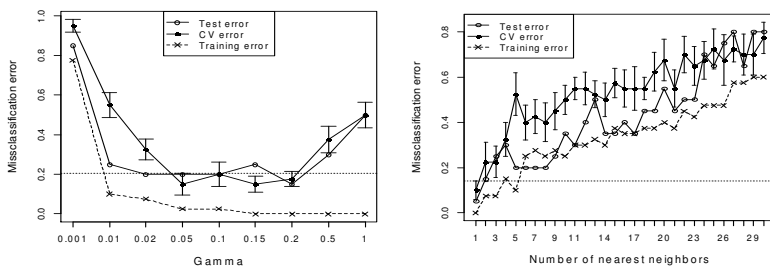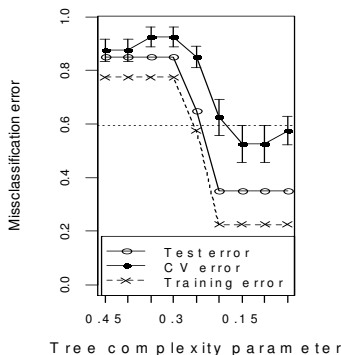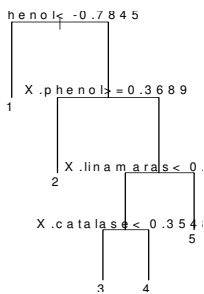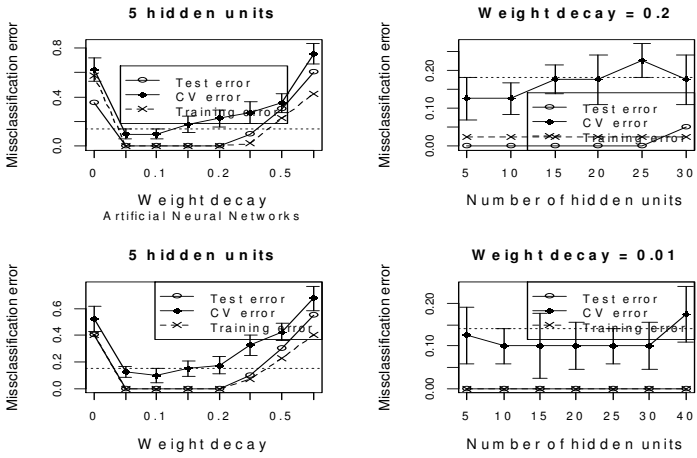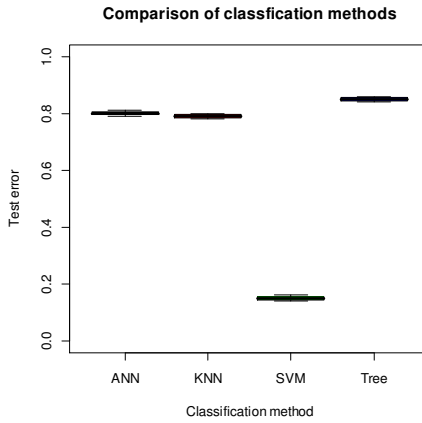
Figure 3E

Figure 4A. Seriated cluster heatmap of all variables studied (The 4 small molecules, CN- related compounds and enzymes).
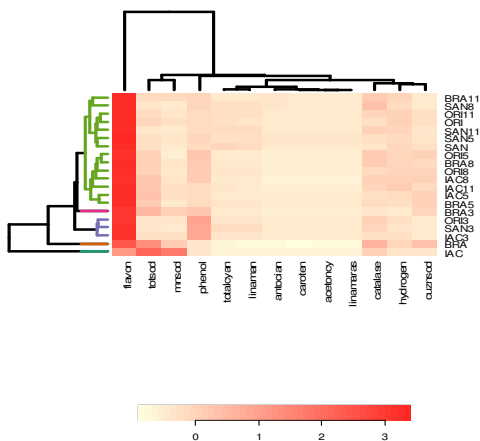
Figure 4B. Cluster analysis of all variables studied (The 4 small molecules, CN-related compounds and enzymes)
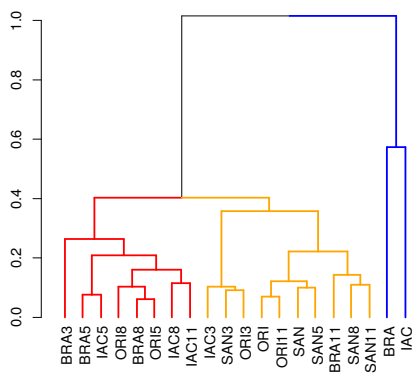
Figure 4C. Seriated cluster heatmap of the 4 small molecules.
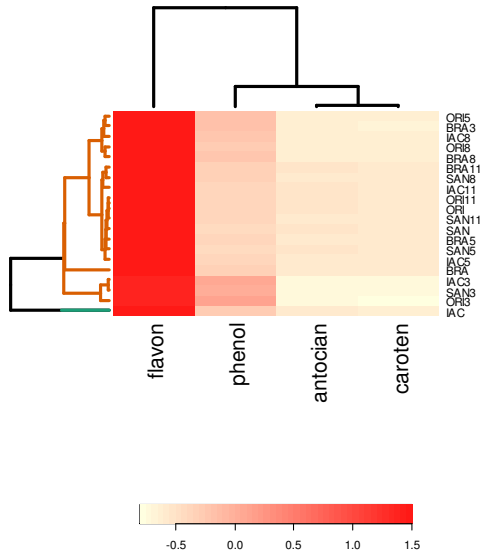
Figure 4D. Cluster heatmap of the 4 small molecules.
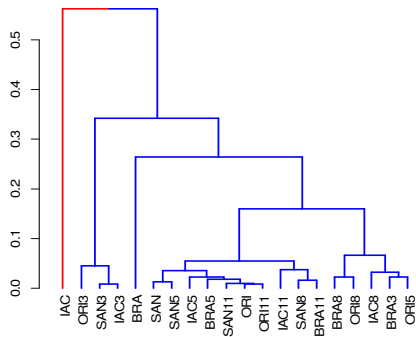
Figure 4E. Seriated cluster heatmap of CN- related compounds.
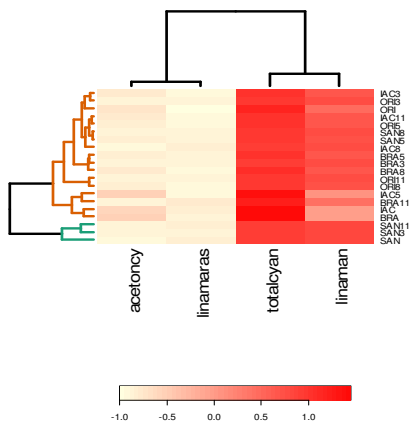
Figure 4F. Cluster heatmap of CN- related compounds.
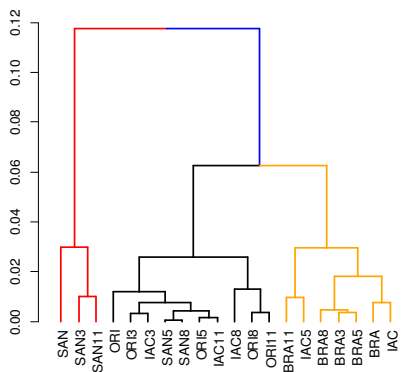
Figure 4G. Seriated cluster heatmap of enzymes.
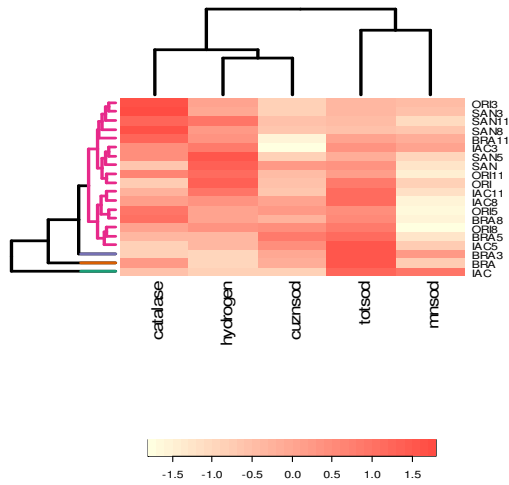
Figure 4H. Cluster heatmap of enzymes.
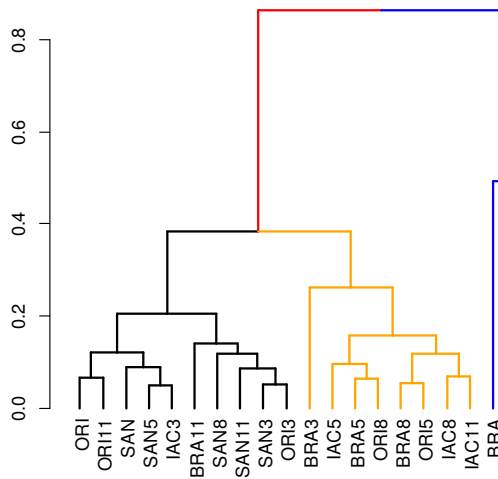
Parte III
Toward better understanding of postharvest deterioration:
Biochemical changes in stored cassava (*Manihot esculenta* Crantz)
roots

1. INTRODUCTION

Cassava (*Manihot esculenta* Crantz.) is a major tropical root crop grown in Africa, Latin America, Oceania, and Asia, feeding more than 800 million people each day. The root, which is the major edible portion of the plant, is an important source of dietary energy and comprises more than 80% starch (ISAMAH et al. 2003, ISAMAH, 2004, LYER et al. 2010). Historically, cassava has played an important role in food security as a famine reserve crop. For instance, in Eastern and Southern Africa where maize is preferred but drought is recurrent, cassava which is to, some extent drought tolerant is harvested when other crops fail. Similarly, cassava provides additional food security when armed conflicts lead to the destruction of above ground crops as it remains viable below ground for up to 36 months (ROSENTHAL & ORT, 2012). While cassava continues to be a vital subsistence crop for small scale farmers, it is also an increasingly important crop at regional and global scales (ROSENTHAL & ORT, 2012). In addition to its role in food security, cassava is being used as biofuel crop in many countries, including China, Thailand, and Brazil (ZHANG et al. 2003; DAI et al. 2006; NGUYEN et al. 2007; ZIDENGA, 2012; ZIDENGA et al. 2012). Globally, cassava is the second most important root crop after potatoes and the fifth most important crop overall in terms of human caloric intake (ROSENTHAL et al. 2012). However, subsistence and commercial utilization of cassava are affected by its short shelf-life due to a rapid postharvest physiological deterioration (PPD) process which renders the root unpalatable within 72 hours of harvest (AIDOO, 1993).
PPD is triggered by mechanical damage (which is inevitable during harvesting operations), PPD progresses from the site of damage, eventually causing general discoloration of the vascular parenchyma throughout the root. According to previous studies (BOOTH, 1975; MAINI & BALAGOPAL, 1978; WHEATLEY & SCHWABE, 1985; ZIDENGA, 2011; GARCÍA et al. 2013; SÁNCHEZ et al. 2013; UARROTA et al. 2014), cassava roots deterioration is related to two separated processes: one being physiological (also termed primary deterioration) and the other microbiological or secondary deterioration.

Physiological deterioration is usually the initial cause of loss of acceptability of roots and is shown by blue-black streaks in the root vascular tissue, which later spread, causing a more general brown discoloration, unsatisfactory cooking qualities and adverse tastes. Primary deterioration also involves changes in oxidative enzyme activities which generate phenols including catechins and leucoanthocyanidins, which in later stages polymerize to form condensed tannins (BOOTH, 1975; NOON & BOOTH, 1977; MAINI & BALAGOPAL, 1978; WHEATLEY & SCHWABE, 1985; ZIDENGA, 2011; GARCÍA et al. 2013; SÁNCHEZ et al. 2013). Microbiological deterioration is due to pathogenic rots, fermentation and/or softening of the roots and generally occurs when the roots have already become unacceptable because of physiological deterioration.

There have been several estimates of the economic impact of PPD. Losses due to PPD in cassava has been estimated to be in the range of 5–25% of the total expected value of the crop (AIDOO, 1993; WENHAM, 1995). According to a recent estimate, extending the shelf life of cassava to several weeks would reduce financial losses by $2.9 billion in Nigeria alone, over a 20-year period (ZIDENGA, 2012). The rapid postharvest perishability of freshly harvested cassava roots is a problem not known in any other root and tuber crop. Within 1–3 days of harvest, roots begin to develop an endogenous disorder, typically characterized by blue black streaking of the vascular tissues of the xylem, accompanied by an unpleasant odor and flavor. PPD profoundly impacts processing, as well as marketing of the roots (LYER et al. 2010). Several approaches have been developed to preserve cassava roots, such as underground storage, storage in boxes with moist sawdust, storage in bags combined with the use of fungicides, pruning plants before harvest, cold storage (2–4 ^{0}C) for up to two weeks, freezing or waxing the roots to prevent access to oxygen, and even chemical treatments (RAVI et al. 1996). However, these methods are too expensive or complicated for handling large volumes of roots and have been restricted mostly to high value product chains, such as the consumption of fresh cassava roots (SÁNCHEZ et al. 2013). Thus, a major goal of cassava breeding and biotechnology is to increase its shelf life by delaying the onset of PPD. Such efforts would enlarge the industrial applications of cassava worldwide (ZIDENGA, 2012).

Molecular and biochemical studies of PPD have pointed to reactive oxygen species (ROS) production as one of the earliest events in the process and many other compounds have been reported. Specific genes involved in PPD have been identified and characterized, and their expression evaluated (REILLY et al. 2007). Several secondary

metabolites and particularly hydroxycoumarins accumulate in the process (BAYOUMI et al. 2008; BAYOUMI et al. 2010). However, more research is necessary to better understand the biochemical changes involved in PPD of cassava roots. The main goal of this study was to evaluate biochemical changes involved in PPD of four cassava cultivars (at zero days of storage-hereafter designed as non-stored samples), as well as in root samples stored for up to 11 days under open air conditions. Supervised and unsupervised methods of data analysis were also used to discriminate cassava samples during the postharvest physiological deterioration.

2. MATERIAL AND METHODS

2.1 Selection of Cassava Cultivars

Cassava cultivars were provided by Santa Catarina State Agricultural Research and Rural Extension Agency (EPAGRI), experimental station of Urussanga and produced over the growing season in 2011/2012. Four cultivars were selected for this study as follows: SCS 253 Sangão (hereafter designed SAN), Branco (hereafter designed BRA, a landrace), IAC576-70 – (hereafter designed IAC, a commercial variety), and Oriental (hereafter designed ORI, a landrace). The cultivars were selected as they are widely used by small farmers and lacking research efforts.

2.2 On Farm Trials

On farm trials were carried out at the Ressacada Experimental Farm (Plant Science Center, Federal University of Santa Catarina, Florianópolis, SC, Brazil - $27^035'48"$ S, $48^032'57"$ W) in September 2011, using the four cassava cultivars above mentioned, provided by EPAGRI. The experimental design was in randomized blocks, with 4 blocks (6.3 x $15m^2$/block) spaced at 1m. Each block consisted of four plots (12 x 1.2 m^2/plot), spaced at 0.5 m. Cassava stakes with 15cm length were planted upright, spaced 1 x 1m. Each plot was considered a treatment and all land operations were mechanized. The chemical analysis of the soil's fertility was previously done, and cultivation was performed manually. For laboratory analysis, each cultivar in each plot was mixed as only one sample.

2.3 Postharvest Physiological Deterioration (PPD)

Cassava roots samples were collected from 12-months old plants for analysis Immediately after harvest, the roots were washed, proximal and distal parts of the root were removed and cross sections were made (0.5-1 cm) over the remaining root and stored enclosed as to reduce the chances of microbial contamination, at room temperature (66-76% humidity, $\pm 25^0$C). Induction of PPD was performed for up to 11 days. Monitoring the development of PPD and associated metabolic disturbances, were performed daily after induction of PPD. Non-stored samples and those with 3, 5, 8, and 11 days postharvest were collected at each point, dried (35-40^{0}C/48h) in an oven, milled with a coffee grinder (Model DGC-20N series) and kept for analysis. For enzymatic analysis, non-stored samples were collected and stored (-80^{0}C) until analysis. PPD was also induced using 2 other different methodologies (storage of entire roots and that described by Wheatley in 1982).

2.4 Postharvest Physiological Deterioration Scoring (PPD Scoring)

Five independent evaluations of PPD were carried out at laboratory conditions. A random sample of 3 sliced roots from each plant variety was scored (from 1 -10% to 10 -100%) in each point of the PPD and imaged with a digital camera (OLYMPUS FE-4020, 14 megapixel, China). The mean PPD score for each root was calculated by averaging the scores for the 3 transversal sections and five evaluations. Roots showing symptoms of microbial rotting (very different from those related to PPD) or affected by insects were discarded.

2.5 Dry Matter Content (%)

For dry matter content of cassava samples, 10 to 30g of chopped and grated fresh roots was weighed and oven-dried at 60°C, for 24h. Dry matter was expressed as the percentage of dry weight relative to fresh weight (MORANTE et al. 2010).

2.5 Polyphenol Oxidase (PPO) Activity during PPD

For PPO analysis, 2g of fresh tissue were homogenized with 0.6 g of PVPP and 8 ml of 50mM (pH 7) phosphate buffer, recovering the supernatant by filtration and centrifugation (4000 rpm, 4^{0}C, 15 min, 18 cm of rotor radius). PPO activity was measured using 2.85 ml of 0.2mM

(pH 7) phosphate buffer, 50 µl of catechol (60 mM) as substrate, and 100 µl of enzymatic extract, at 25ºC. Changes in absorbance (420 nm) were recorded over a 5 min period in a UV-visible spectrophotometer (Spectrumlab D180 – MONTGOMERY & SGARBIERI, 1975). Activity was expressed as units of activity (UA) and one unit of PPO were defined as the change in one unit of $absorbance.s^{-1}$.

2.6 Ascorbic Acid (AsA) Determination during PPD

Ascorbic acid (AsA) content was assayed as described previously with slight modifications (OMAYE et al. 1979). The extract was prepared by grinding 1 g of sample with 5 ml of 10% TCA, centrifuged (3500 rpm, 20 min), re-extracted twice, and the supernatant made up to 10 ml to 1.0 ml of extract, 1 ml of DTC reagent (2,4-dinitro phenyl hydrazine–thiourea–$CuSO_4$) was added, incubated (37^0C, 3h) and 0.75 ml of ice-cold 65% H_2SO_4 (v v^{-1}) was added, allowed to stand for 30 min, at 30^0C. The resulting color was read at 520 nm in the spectrophotometer (Spectrumlab D180). The AsA content was determined using a standard curve build with AsA (y=0.0361x, r^2=0.99, 0 to 1000 mg ml^{-1}) and the results were expressed in µg g^{-1} (ppm) of fresh weight.

2.7 Protein Extraction and Quantification from Cassava roots during PPD

At each sampling time root tissues were grated using a food processor (Walita-Master Plus, Brazil) and stored at -80 ^0C before use. The frozen tissue was ground under liquid nitrogen to a fine powder using a pre-chilled pestle and mortar. 5 g of tissue were added to a pre-chilled 50 ml tube containing 20 ml of extraction buffer (phosphate buffer 0.1 M, pH 6.4, 0.25 g of PVP), 200 µl of 1 mM DTT and 200 µl of 1 mM EDTA. Tubes were vortexed vigorously and transferred to a horizontal shaker (300rpm min^{-1}), for 1 h. Tubes were centrifuged (5000 rpm, 20 min), the supernatant recovered by filtration, transferred to a fresh tube and stored at -20 ^0C (REILLY, 2001).
The protein content of each sample was determined using the Bradford protocol (BRADFORD, 1976) with small modifications. A calibration curve was constructed using BSA (Bovine Serum Albumin, Sigma-Aldrich, MO-USA) as standard. Protein solutions were prepared in 0.15 M NaCl and a series of dilutions were prepared (0 to 100 mg ml^{-1}, y=0.0082x, r^2=0.96) to build the standard curve.

2.8 Extraction and Quantification of Soluble Sugars, Organic Acids and Scopoletin by HPLC

2.8.1 Cassava root flour Extraction for Soluble sugars, Organic acids and Scopoletin analysis

Non-stored samples and those with, 3, 5, 8 and 11 days postharvest were collected, removed the outer and inner bark, crushed with food processor previously described and dried in an oven at 35 ^{0}C (48 h). After oven drying, samples were again crushed with coffee grinder to obtain a fine powder, sieved and stored at room temperature for analysis.

Sugars and organic acids were extracted from 0.5g of cassava root flour samples in 10 ml of mobile phase (H$_2$SO$_4$ 96%, 5 mM) and determined accordingly (CHINNICI et al. 2005). Briefly, the suspension was homogenized using an ultra-turrax apparatus and mixed slowly using a horizontal shaker (Microplate shaker, 330 rpm min^{-1}), during 30 min. The suspension was centrifuged (8000 rpm, 10 min), filtered through a 0.22 µm disposable syringe membrane filter and the supernatant collected. Sugars and organic acids were analyzed by HPLC using a column, Biorad Aminex HPX 87H, equipped with a UV detector (MWDG 1365D, for organic acids), connected in series with a refractive index detector (RID G 1362A, for sugars) and an injection valve fitted with a 15 µl loop. The samples were separated isocratically at 0.6 ml min^{-1} at 30^{0}C.

Retention times and standard curves were prepared for the following sugars – glucose (Sigma–Aldrich G7528, y=26748656x-1523663, r^2=0.99), fructose (Sigma–Aldrich F2543, y=26028204x-8253663, r^2=0.99), raffinose (Sigma–Aldrich (≥99.5%), y=22680182x+45255.3, r^2=0.99), and sucrose (Sigma–Aldrich (≥99.5%) S7903, y=22582989x+727997.7, r^2=0.99) – and organic acids – citric (Sigma–Aldrich CO759, y=3281.1x+46046, r^2=0.99), malic (Sigma–Aldrich (99%) 240179, y=2498.2x+3816.4), succinic (Sigma–Aldrich (≥99%) S3674, y=1737.8x-4255.3, r^2=0.99), fumaric (SupelCo Analytical R412205, y=4047.85x-5748.3, r^2=0.99). Three consecutive injections (10 µl) were performed. Sugars and organic acids were expressed (mg g^{-1}) as mean ± standard deviation.

2.8.2 Scopoletin Extraction and Quantification during PPD

Cassava root flour samples (1 g) were placed in 50 ml falcon tubes containing 2 ml 98% ethanol (JT Baker, USA) and homogenized with an ultraturrax (IKA T18 basic) for 30 s. The suspension was vortexed (1 min), incubated (microplate shaker, 600 rpm, 30 min), and centrifuged (7000 rpm 5min^{-1}). The extract was filtered on a Whatman # 1 paper and through a 0.22 μm nylon membrane. Samples were transferred to 1.5 ml vials for HPLC (Agilent Technologies 1200 series, Waldbronn, Germany) analysis (BUSCHMANN et al. 2010). For that, samples (50 µl) were injected into an HPLC (Agilent Technologies 1200 series, Waldbronn, Germany), equipped with a reverse-phase column (Techsphere BDS C18, 250 mm × 4.6 mm, 5 μm) and a diode array detector. The column was kept at 25^{0}C and acetonitrile and 0.5% phosphoric acid (v v^{-1}) in aqueous solution were used as mobile phase. The gradient profile was 60 – 1% for 30 minutes with a 0.5 ml min^{-1} flow and 50 μl injection volume. Scopoletin was detected at 215, 280, and 350 nm and according to its retention time, using a standard compound sample (Sigma–Aldrich: scopoletin $\geq$ 99% – No. S2500). Scopoletin quantification was determined through a calibration standard curve (y=158159.59x, r^2 = 0.9993, 1–75 mg l^{-1}). Three consecutive injections (10 μl) were performed and quantifications were made on a dry weight basis, and data represented in nmol g^{-1}, as mean ± standard deviation.

2.9 Statistical Analysis

All statistical analyzes were implemented in R software (R core team-2014, version 3.1.1), using their respective packages and scripts. All statistical analyses and graphics were performed using the same software and data represented as mean ± standard deviation of three repetitions (n=3). Two-way ANOVA and multivariate analysis were applied when necessary.

3. RESULTS AND DISCUSSIONS

3.1 PPD Scoring

Results of PPD scoring of the four genotypes studied revealed the cultivar ORI as the most susceptible, followed by SAN, while BRA and IAC were found to be more tolerant (Table 1). Differences in deterioration rates were observed from tolerant cultivars (BRA, IAC) when compared with

susceptible (ORI and SAN). Similar regression coefficients were found among tolerant clones. Figure 1 shows regression models for the four cultivars during storage time. In all PPD methods, a tendency of increasing rate of deterioration during storage time were observed. A clear relationship was found when using the average value of the three methods as can be found in the figure 1.

Table 1: Comparison of methods of PPD induction in four cultivars studied. Values are represented as mean scores of five independent evaluations (n=5) in percentage (%), from zero to 100% of deterioration during different storage time

Cultivar	Method of PPD	3days	5days	8days	11days
	Root slicing	15.70	70.70	89.30	100.00
SANGÃO	Wheatley	9.30	5.70	8.60	17.10
	Entire root	0.00	35.70	15.00	26.00
Average		8.3	37.4	37.6	47.7
	Root slicing	34.90	46.40	68.60	81.50
BRANCO	Wheatley	0.00	0.70	0.00	15.00
	Entire root	0.00	0.00	0.00	1.40
Average		11.6	15.7	22.9	32.6
	Root slicing	31.50	49.30	67.20	91.00
IAC576-70	Wheatley	0.00	2.90	1.40	5.00
	Entire root	0.00	0.00	11.40	3.60
Average		10.5	17.4	26.7	21.0
	Root slicing	59.90	87.10	100.00	100.00
ORIENTAL	Wheatley	7.90	17.20	24.30	33.60
	Entire root	0.00	35.00	35.70	64.30
Average		22.6	46.4	53.3	66.0

Cassava roots deteriorate fastly when sliced (method described by Reilly, 2001-Table 1) when compared with the method of leaving the entire root under storage. For all analysis reported herein in this study, the method of root slicing was applied. This method was chosen because it is a method where cassava roots are subjected to the greatest damage or injury.
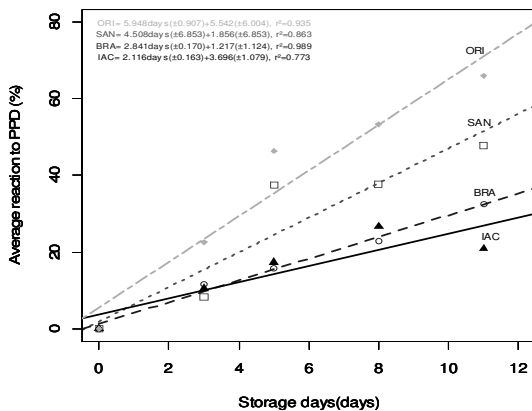
Figure 1. Average reaction to PPD (grouping together the three methods for assessing PPD) through time for the four clones involved in this study. The result of the linear regression analysis is also provided. Within parenthesis the standard errors of the parameters in the regression analyses.

3.2 Polyphenol Oxidase (PPO)

PPO has been implicated as a major causative agent of darkening in raw Asian noodles and other wheat products (ANDERSON et al. 2006), browning induced by mechanical damage in potatoes (BATISTUTI & LOURENÇO, 1985). PPO catalyses the oxidation of phenols into quinones that subsequently polymerize into brown pigments and has been also reported in avocado (GOMEZ-LOPEZ, 2002) and browning in Marula fruits (MDLULI, 2005). PPO is compartmentalized in plastids and their phenolic compounds are located in the vacuoles for most plant tissues. Action of PPO only occurs when this compartmentalization is disrupted after tissues are wounded, as observed in diseased tissues or cell disruption caused by processing and storage. The production of the browning reaction by PPO may modify plant proteins and can be more toxic to potential phytopathogens. The reactions also produce undesirable blackening in the products during food processing, or in postharvest of plant products resulting in a reduction of their sensory properties and nutritional value. Different kinds of PPO have been characterized in *Phaseolus vulgaris* (GUO et al. 2009; KHANDELWAL et al. 2010), and in banana (WUYTS et al. 2006; BAYOUMI et al. 2010).

Our results on PPO during storage of the roots from the four cultivars studied are summarized in Figure 2. By comparing the mean PPO values from each cultivar, we found that BRA and ORI did not differed statistically (p<0.05). During the storage time, a small decrease of PPO activity at day 8 was observed in IAC and SAN, but in general PPO activity varied similarly in the tolerant (BRA/IAC) and susceptible to PPD (SAN/ORI) cultivars. PPO activity from non-stored samples and those at stage 3 of PPD were significantly different from samples at stages 5, 8 and 11 (p<0.05). SAN showed a higher PPO activity than the rest of the cultivars particularly at days 3 and 5 and which may be related to the higher PPD scores for this clone.
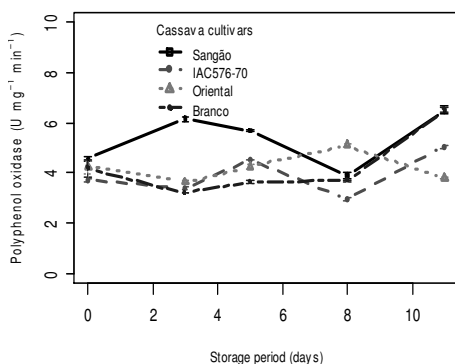
Figure 2. Changes in the activity of polyphenol oxidase in cassava cultivars during PPD. Each data point is presented as mean ± standard deviation (n = 3) in units per milligram*minutes (U mg^{-1} min^{-1} of proteins).

Taking into account non-stored samples, significant differences (p<0.05) between the cultivars SAN and IAC were observed. At day 3 of storage, SAN differed from all cultivars. At Day 5, all cultivars differed in PPO activity. At day 8 and 11 of storage, significant differences were found between BRA, SAN with ORI/IAC cultivars. When PPO were correlated with PPD (see supplementary Fig. 4A), we found a high negative correlation and a clear discrimination of tolerant clones (BRA/IAC) with susceptible (ORI/SAN). A similar trend in correlation values can be observed (0.79, 0.785 and 0.97, 0.93) respectively, meaning that the higher activity of PPO in cassava roots collaborate to reduce deterioration in that biomass. Fluctuations in PPO as reported in Figure 2 can be also

explained by differences in genotype, preharvest and postharvest conditions.

3.3 Ascorbic Acid (AsA)

L-ascorbic acid (AsA, Vitamin C) is a molecule of dietary importance to humans. It plays an important role in photosynthesis, provides significant biochemical functions as an antioxidant, enzyme co-factor, electron donor and acceptor in electron transport, a precursor for oxalate and tartrate synthesis, acts as a defense against oxidative stress and plays a possible role in cell wall metabolism and expansion (TOLEDO et al. 2003), and immune responses (QUIRÓS et al. 2009). AsA occurs abundantly in many horticultural crops and has also been reported in cassava roots (CHÁVEZ et al. 2000). A wide range of factors such as genotype, preharvest and postharvest conditions may influence AsA content. Losses of AsA during storage have been reported previously in many fruits depending on storage conditions (KABASAKALIS et al. 2000). In some fruit juices, it is purposely added for attracting consumers and has been reported to act as an antioxidant to prolong the shelf-life of the commercial product (FUNG & LUK, 1985). A progressive loss of AsA in apple fruit during storage and a subsequent increase in the number of physiological storage disorders have been reported. Lower storage temperatures reduce the loss of AsA and the incidence of storage disorders in peas, broccoli and spinach (FELICETTI & MATTHEIS, 2010). Despite the fact that several studies have been made on the effects of AsA in horticultural crops, little is known regarding to the changes in AsA contents during PPD in cassava and its correlation with a possible role in biochemical processes of deterioration.

Our results revealed that, during storage, there is a clear tendency of accumulation of AsA over time in all cultivars studied (Fig. 3). Interestingly, the clones with tolerance to PPD (IAC and BRA) tended to have the extreme contrasts for AsA. The two susceptible clones (SAN and ORI) showed at several days of evaluation AsA values intermediate in comparison with the tolerant clones. ORI showed a sharp increase in AsA after the third day of storage. SAN, on the other hand, showed a strong oscillation in AsA activity through time and its behavior is difficult to define.
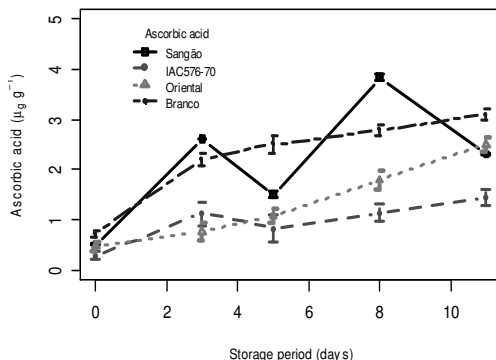
Figure 3. Changes in the concentrations of ascorbic acid in root samples of cassava cultivars during PPD. Each data point is presented as mean ± standard deviation (n = 3) in (μg g^{-1}).

Two-way ANOVA showed differences in AsA among the cultivars BRA/SAN with ORI/IAC During the storage time, non-stored samples were statistically different with samples at stages 3, 5 and 8 , 11 respectively ($p < 0.05$). Although Figure 3 shows increases in AsA during storage period until day 3 followed by oscillation in other stages, a clear correlation were found when relating levels of AsA in tolerant and susceptible clones with PPD. The tolerant clones behaved similarly and presented low negative correlations (0.785, 0.793) when compared with susceptible clones (0.969, 0.932- see supplementary Fig 4B) respectively. Again, it's possible to see that in susceptible clones levels of AsA have impact in deterioration degree. High levels of AsA presented by the tolerant cultivar (BRA) may have a role in delaying PPD.

3.4 Changes in Total Proteins and Dry Matter content

With the increasing availability of diverse biological information for proteins, integration of heterogeneous data becomes more useful for many problems particularly to understand the role of protein in postharvest deterioration of cassava, such as annotating protein functions and to predict novel protein interactions with PPD. In this study, an integrative approach of changes in total protein content in cassava roots during postharvest deterioration and the correlation of that variable with the tolerance or susceptibility to PPD have been investigated. The results of

total protein contents in the roots of the sampled cultivars are summarized in Figure 4.
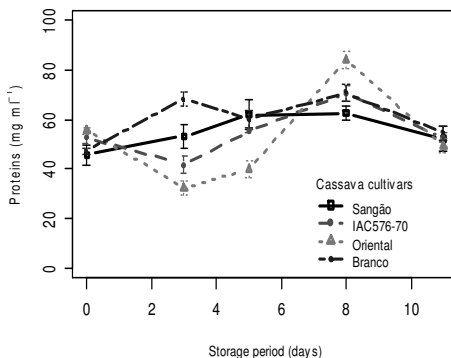
Figure 4. Changes in the concentrations of total proteins in roots of cassava cultivars during storage. Each data point is presented as mean ± standard deviation (n = 3) in (mg ml^{-1}).

An overview of the data revealed that the cassava cultivars meaningfully changed their protein amounts over the experimental period, except for days 5 and 11 of storage, revealing a genotype-specific behavior for that variable. Interestingly, a considerable increase of protein amount was observed at day 3 in BRA cultivar what apparently could be related with its tolerance to PPD. However, IAC showed a contrasting behavior. BRA differed from ORI cultivar and correlations between PPD with levels of proteins are summarized in supplementary Figure 5C. Lower negative correlations and similar trends in deterioration can be observed in all cultivars with protein levels but such results make difficulty to get us useful information related to the rule of proteins in PPD.

Dry matter content was also determined in non-stored samples and correlated with PPD at stage 3, 5, 8, and 11. The results of this research showed a positive correlation between dry matter with PPD (supplementary Fig. 4D). Cassava cultivars with the high level of dry matter are more prone to suffer from PPD. It was also observed that these correlations decrease along the PPD process. At stages 3, 5, 8, and 11 we found correlation values of 37%, 25%, 17%, and 11%, respectively and these findings confirm previous studies (CEBALLOS et al. 2012; SÁNCHEZ et al. 2013).

3.5 Scopoletin Content during PPD

PPD has been explained as a physiological process not due to microorganisms, rather, on a molecular basis, marked by altered gene expression (REILLY et al. 2003) and accumulation of secondary metabolites. Amongst these secondary metabolites are hydroxycoumarins (e.g., scopoletin) which show antioxidant properties and by oxidation and polymerization confer the typical blue/black phenotype to root cassavas suffering from PPD. Hydroxycoumarins are important in plant defense as phytoalexins due to the induction of their biosynthesis following various stress events (wounding, bacterial, and fungal infections, e.g.). Additionally, they display a wide range of pharmacological activities, including anti-coagulant (MUELLER, 2004), anti-inflammatory (SILVAN et al. 1996), antimicrobial (SMYTH et al. 2009) and anticancer (GRAZUL & BUDZISZA, 2009). However, their biosynthesis pathway in cassava is not known so far (WHEATLEY, 1982; BAYOUMI et al. 2010), but their accumulation in biomasses of that species during root deterioration has been previously reported (WHEATLEY, 1982; WHEATLEY & SCHWABE, 1985; BUSCHMANN et al. 2000; REILLY, 2001; SÁNCHEZ et al. 2013). An uptake of scopoletin regulated by the interaction between plant hormones such as salicylic acid was also reported (TAGUCHI et al. 2001).

The HPLC results of scopoletin contents during PPD are summarized in table 1 and a representative chromatographic profile for the studied cultivars during PPD is provided in supplementary Figure 1. Looking for all results of scopoletin, tolerant clones presented high levels of scopoletin at the starting point (non-stored samples) and at day 11 of PPD. Different trend were observed for susceptible clones, which presented low levels of scopoletin in non-stored samples and at day 11 of PPD. Fluctuations during PPD can be attributed to factors such genotype and PPD conditions. Correlating scopoletin with PPD (supplementary Figure 5E), we found that those cultivars with low levels of scopoletin presented high degree of deterioration (ORI/SAN) when compared with tolerants (BRA/IAC). A positive correlation were found between clones and clear separation of cultivars (tolerant with susceptible) were found. Two-way anova showed significant differences (p<0.05) in scopoletin levels in all cultivars and during storage time. BRA showed high level when compared to ORI.

Table 2. HPLC analysis of scopoletin (mmol g^{-1}) during PPD in cassava root tubers of the four cassava cultivars studied. Data are represented as mean ± standard deviation of two repetitions (n=3). Letters in the column represent significant differences (TukeyHSD test, p<0.05).

PPD days	BRA	ORI	SAN	IAC
0	91.46c	18.59b	25.99d	64.26e
3	92.11c	124.89a	45.40c	81.81d
5	95.00bc	48.17b	120.80a	123.90b
8	193.96ab	81.79ab	125.81a	214.00a
11	223.08a	54.94b	66.65b	98.10c

[*]Values are represented as mean of three repetitions (n=3) in mmol g^{-1} of dry weight. Different letters in the column represent significant statistical differences (TukeyHSD, p<0.05).

3.6 Soluble Sugar Content during PPD

Cassava starch can be converted to maltotriose, maltose, and glucose, as well as to other modified sugars and organic acids (TONUKARI, 2004). Many studies have been devoted to soluble sugars metabolism in crop species, e.g., hexoses, sucrose, and maltose of stored yam tubers increased greatly during the rest period, but much less during the sprouting period (KOUASSI et al. 1990). The study concluded that a starch-sucrose inter-conversion occurs during tuber storage. However, there has been no comprehensive evaluation of changes in the sugars contents of cassava roots during PPD.

Soluble sugars contents detected in the cassava roots during PPD are summarized in Figure 5 and supplementary table 2. Supplementary Figure 2 shows a typical chromatogram of soluble sugars detected in the BRA cultivar during PPD and Figures 5(A-E) shows changes in soluble sugars (raffinose, sucrose, glucose, fructose, and total sugars) during storage.
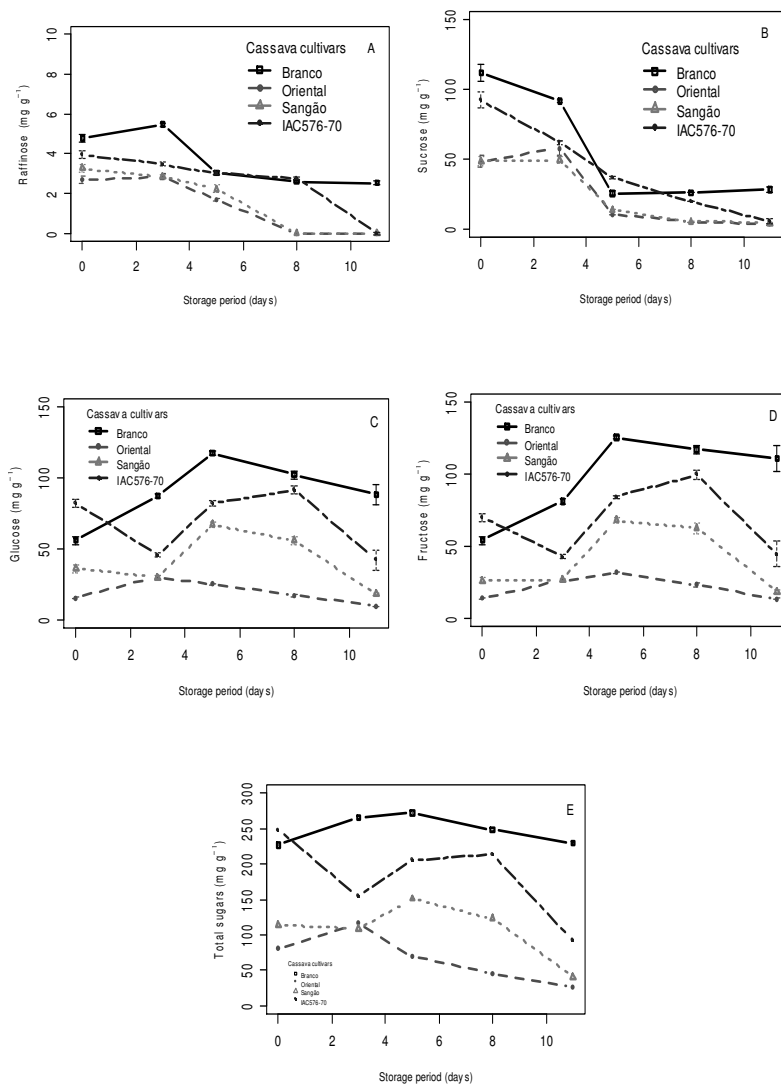
Figure 5. Changes in the concentrations of soluble sugars in cassava cultivars during storage. Each data point is presented as mean ± standard deviation (n = 3) in (mg g^{-1} in dry basis). (A) Raffinose, (B) Sucrose, (C) Glucose, (D) Fructose, and (E) Total sugars.

Significant differences (p<0.05) were found in soluble sugars amounts during storage for each cultivar (supplementary Table 2). Raffinose content was observed to decrease in all cultivars except BRA, which showed the small increase until the third day of storage (Fig 5A). Decrease in sucrose was also observed except for ORI where increases were observed until day 3 of storage (Fig 5B). Glucose, fructose and total sugar content showed different trends. A small decrease (SAN, IAC) and then increases were observed in all cultivars studied (Fig 5C-E). Results of our research clearly demonstrate that glucose and fructose were the main sugars found in all samples studied. Researchers working with susceptible and tolerant clones of cassava stored for 14 days at ambient conditions also reported similar results on soluble sugars (SÁNCHEZ et al. 2013). Our results are distinct with those reported by Sánchez et al. (2013), but why a small decrease of sugars were observed is not clear from our data.

3.7 Organic Acids during PPD

As primary metabolic products, organic acids play a regulatory role in plant growth and development. Organic acids are metabolically active solutes in cellular osmoregulation and surplus cationic balance, acting as key components in response to nutritional deficiencies, metal ion accumulation, and plant–microorganism interaction. Organic acids can also enhance resistance to diseases and inhibit oxidation during storage at low temperature, which significantly extends the storage life of plant biomasses (SUN et al. 2012). They have been also related to maintenance of membrane integrity in stress conditions (GUNES et al. 2007). The postharvest physiological deterioration properties of stored cassava remain largely unknown, and the background information on the metabolic profile of organic acids in postharvest stored cassava roots can help a better understanding of PPD.
Figure 6 (A-D) summarizes the results of the organic acid analysis performed and changes in the contents of that metabolites for the studied cultivars can be also noticed in supplementary material (Table 3 and Figure 3). The cassava roots sampled, significantly differed in their organic acids profiles (Figure 6). The main organic acids predominantly found during PPD were succinic and fumaric acids. In BRA cultivars (tolerant to PPD), succinic acid followed by malic acid were the major compounds detected (Fig 6A). Small decreases followed by increase in succinic were observed in those samples. In ORI cultivar (Fig 6B), increases in succinic and fumaric acids during PPD were also observed as

malic acid showed to be quite constant during PPD. In SAN (Fig 6C), increases in succinic until stage 8 of PPD were found as fumaric and malic acids decreased. A typical trend in IAC cultivar was not detected for the succinic and fumaric acids (Fig 6D). The chromatographic profile (supplementary Figure 3) of BRA cultivar samples (non-stored samples), 3, and 5 of PPD shows other organic acids detected in small amounts using high performance liquid chromatography (HPLC) as for instance, phytic acid (data not shown). In tolerant clones (BRA/IAC) we found succinic acid as the main acid related to PPD in those cultivars and in susceptible, fumaric was the main related.
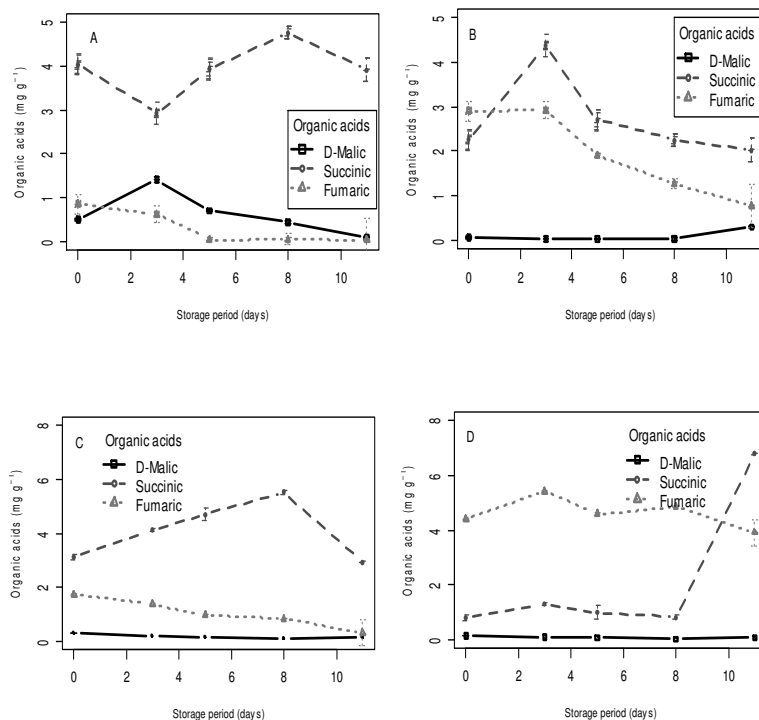
Figure 6. Changes in the concentrations of organic acids in roots of cassava cultivars during PPD. Each data point is presented as mean ± standard deviation (n = 3) in (mg g^{-1}). (A) Branco, (B) Oriental, (C) Sangão, (D) IAC576-70.

3.8 Multivariate Statistical Analysis

Multivariate analyses (nonsupervised and supervised methods) such as principal component analysis (PCA), partial least square discriminant analysis (PLS-DA) and hierarchical cluster analysis (HCA) were performed. When PCA was applied to the scaled data a clear separation was observed from non-stored samples and those with 3 days of PPD as located in PC1 (+/-)/PC2- and most samples at stages 5, 8, and 11 in PC2+. A clear separation from the tolerant and susceptible cultivar was found, as well as for the samples over the experimental period (Fig. 7A). The total variance explained by the two principal components was 63.7%, i.e., PC1 (37%) and PC2 (26.7%). The loadings plot showed that samples grouped in PC1+/PC2+ according to their values of polyphenol oxidase; in PC2+/PC1- according to fructose, scopoletin, proteins and ascorbic acid contents; in PC1-/PC2- according to glucose, raffinose, total sugars and organic acids (malic and succinic acid). Samples grouped in PC1+/PC2- showed to be similar in their fumaric acid concentrations.
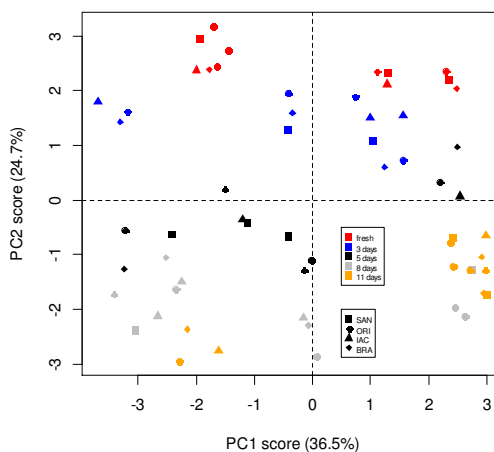
Figure 7. (A)- Scores plot of a two-component PCA model from the metabolic data set of cassava roots showing sample clustering according to their metabolic fingerprinting and the percentage of variance captured by each PC.

When a seriated cluster heatmap was applied to the data (Fig. 7B), similar results found in PCA were detected, and four major clusters were found to occur: group 1 (SAN, ORI, SAN3, ORI5); group 2 (IAC11, ORI8,

ORI11, SAN8); group 3 (BRA, IAC, ORI3, BRA3), and group 4 (SAN8, BRA11, BRA5, SAN5, IAC5, BRA8, and IAC8). A cophenetic correlation of 78.1% was found. The cluster heatmap reveals the major metabolic components that influenced the clustering noticed. Proteins and ascorbic acid were the major compounds related to the group 1, polyphenol oxidase activity for the group 2, glucose, succinic acid and total sugars contents for the group 3, and sucrose, raffinose, and malic acid for the last group.
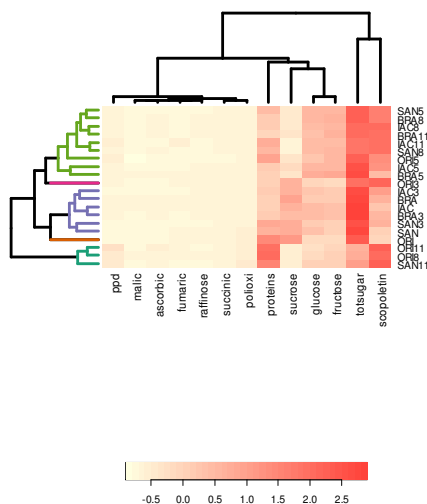
Figure 7(B)- A seriated cluster heatmap (HCA), with 78.1% of cophenetic correlation.

Using supervised methods, e.g., PLS-DA (Fig. 7C); a better separation was found (accuracy of 88.4%), comparatively to PCA. BRA grouped with IAC and ORI with SAN. Most non-stored samples and those with PPD at stage 3 were found in the same component (X-variate) and those at stage 5, 8 and 11 grouped in the same component as their presented similarities. Again, the built model was capable of predicting and separating tolerant with susceptible cultivar. The total variance explained from the axis were 80.72%, i.e., 61.23% from the latent variable 1 (x-variate 1) and 19.49% from the latent variable 2 (x-variate 2). The loading values showed that samples grouped in x-variate 1+ according to malic and fumaric acids, raffinose, sucrose and total sugars, as for the x-variate 1- according to the values of glucose, fructose, scopoletin, ascorbic acid, proteins, polyphenol oxidase and degree of PPD. Samples were also

grouped in x-variate 2+ as their revealed similarity in malic and fumaric acids, ascorbic acid and polyphenol oxidase, and in x-variate 2- according to their values of sugars described, succinic acid, scopoletin and protein amounts.
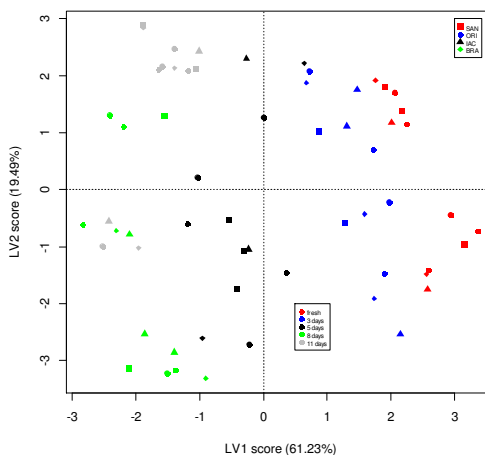
Figure 7(C)- PLS-DA components score plot of cassava samples during PPD taking into consideration all the metabolites analyzed. PLS-1 (x-variate 1) = 61.23%, PLS-2 (x-variate2) = 19.43% of variance explained.

The biochemical investigation presented in this work, combined with pattern recognition procedures, gave rise an evidence for the existence of clear metabolic differences of cassava root samples according to their deterioration state and cultivar. Many compounds are up- and down-regulated during root storage and may or may not be related to PPD. Ours findings indicate that polyphenol oxidase, ascorbic acid, proteins up-regulated in the initial stages of PPD (until 72 h) may be related to PPD. On the other hand, it is speculated that scopoletin biosynthesis is activated at the beginning of the PPD process particularly in susceptible clones, but the underlying mechanism may be better understood in the future studies. Fumaric and succinic acids were the main sugars found in tolerants (BRA/IAC) and susceptible (ORI/SAN) clones. Finally, the pattern recognition models (unsupervised and supervised ones) proposed was capable of classifying samples according to their metabolic profiles and degree of deterioration.

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5. SUPPLEMENTARY DATA

Captions for supplementary data
Figure 1. Chromatographic profiles (HPLC, detection wavelength at 350 nm) of cassava root extracts (cultivar Branco) showing the peaks of the identified hydroxycoumarins, the major peak being scopoletin.

Figure 2. Representative chromatograms (HPLC, 350 nm) of cassava root extracts (cultivar Branco) showing the major peaks of the soluble sugars identified (glucose, sucrose, and fructose).

Figure 3. Chromatographic profile (HPLC, 350 nm) of cassava root extracts (cultivar Branco) showing the peaks of the organic acids detected.

Figure 4. Correlations between PPD with Polyphenol oxidase (A), with Ascorbic acid (B),proteins (C), dry matter (D), and scopoletin (E). Linear regression correlations are also provided in the figures.

Table 1. HPLC analysis of soluble sugars (mg g^{-1}) during PPD in cassava root tubers of the four cassava cultivars studied. Data are represented as mean ± standard deviation of two repetitions (n=3). Letters in the column represent significant differences (TukeyHSD test, $p<0.05$). Numbers after cultivar name means days of PPD (e.g., BRA3 means sample of BRA cultivar with 3 days of PPD). Statistical analyses were made separately for each cultivar and each compound found.

Table 2. HPLC analysis of organic acid contents (mg g^{-1}) during PPD in cassava root tubers of the four cassava cultivars studied. Data are represented as mean ± standard deviation of two repetitions (n=3). Letters in the column represent significant differences (TukeyHSD test, $p<0.05$). Numbers after cultivar name mean days of PPD (e.g., BRA3, means sample of BRA cultivar with 3 days of PPD). Statistical analyses were made separately for each cultivar and each compound found.

1. Changes in Scopoletin (BRA cultivar)
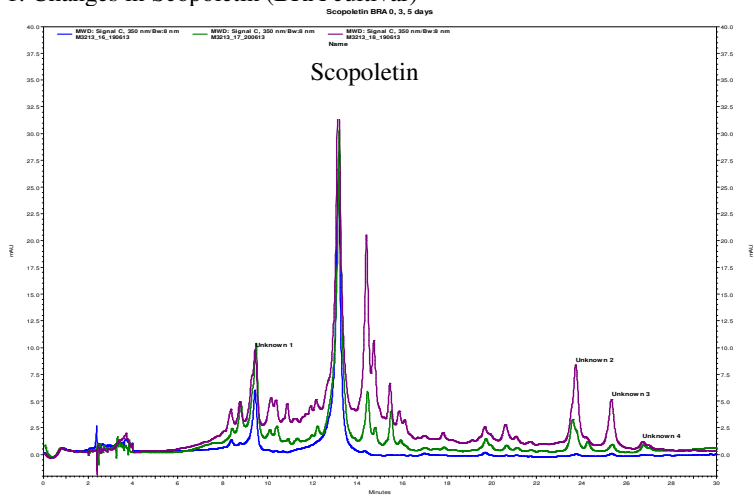
2. Changes in Soluble Sugars (BRA cultivar)
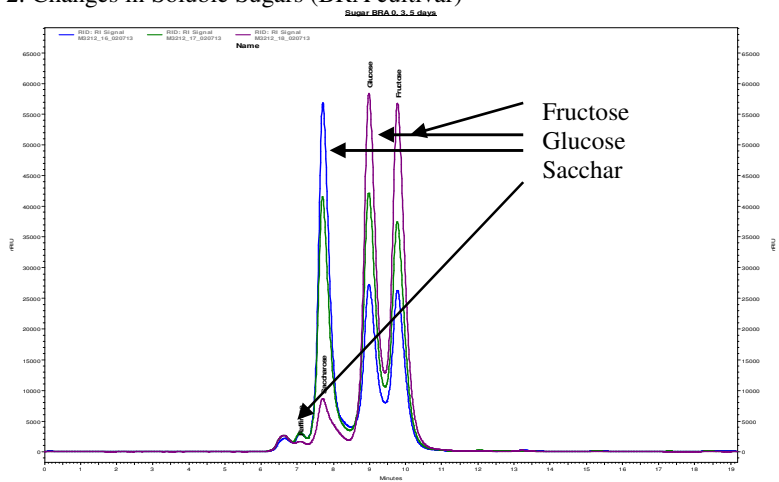
Figure 3: Changes in Organic acids (BRA cultivar)
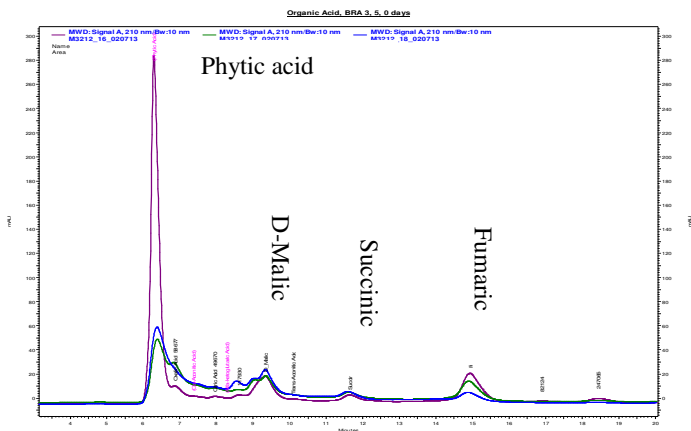
Figure 4 (A-E)
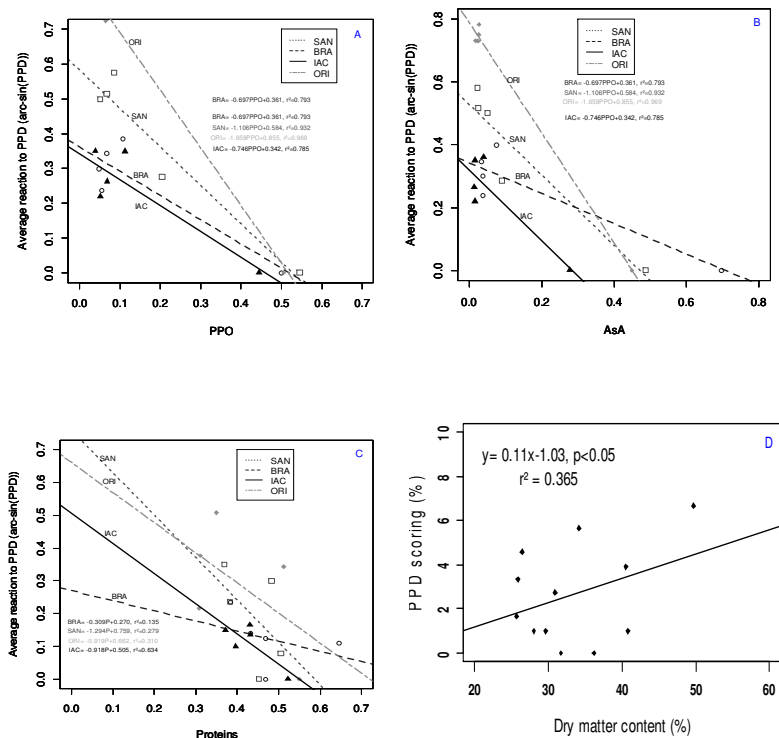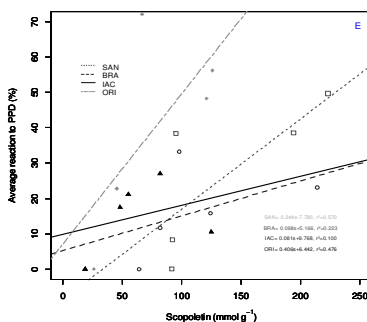
Table 1:

Sample[*]	Raffinose	Saccharose	Glucose	Fructose	Total
BRA	4.79b	112.10a	55.91d	54.19c	226.99e
BRA3	5.47a	91.48b	87.30c	81.25b	265.50b
BRA5	3.04c	25.52d	117.85a	125.74a	272.16a
BRA8	2.61cd	26.20d	102.35b	117.27a	248.43c
BRA11	2.54d	28.20cd	88.16c	111.09a	229.99d
ORI	2.69a	48.51b	15.48c	13.69c	80.37b
ORI3	2.86a	58.02a	30.23a	26.07b	117.18a
ORI5	1.70b	10.40c	25.22b	31.62a	68.94c
ORI8	0.00c	4.63d	17.09c	23.28b	45.01d
ORI11	0.00c	3.23d	9.40d	13.41c	26.05e
SAN	3.27a	48.85a	35.94c	26.06b	114.12c
SAN3	2.86a	49.20a	29.86c	26.78b	108.69c
SAN5	2.25b	13.48b	67.11a	67.94a	150.79a
SAN8	0.00c	5.24c	55.60b	62.41a	123.24b
SAN11	0.00c	4.08c	18.14d	18.40c	40.62d
IAC	3.96a	92.27a	82.26b	69.87c	248.37a
IAC3	3.47b	62.05b	45.74c	42.72d	153.98d
IAC5	3.05c	36.77c	82.08b	84.25b	206.15c
IAC8	2.76c	19.49d	91.77a	99.80a	213.81b
IAC11	0.00d	5.71e	42.23c	44.65d	92.59e

[*]HPLC sugar content (mg g^{-1}) in cassava roots during PPD. Numbers after cultivar name means days of PPD (e.g., BRA3 means sample of BRA cultivar with 3 days of PPD). Statistical analyses were made separately for each cultivar and each compound found. Different letters between column for each cultivar

means significant differences in sugar content during PPD (n=3) for the referred compound (TukeyHSD test, p<0.05).

Table 2

Sample[*]	D-Malic acid	Succinic acid	Fumaric acid
BRA	0.50c	4.04b	0.86a
BRA3	1.41a	2.93c	0.62b
BRA5	0.71b	3.94b	0.04c
BRA8	0.44c	4.77a	0.05c
BRA11	0.09d	3.92b	0.04c
ORI	0.06b	2.26b	2.90a
ORI3	0.03b	4.38a	2.92a
ORI5	0.03b	2.70b	1.90b
ORI8	0.03b	2.25b	1.27c
ORI11	0.29a	2.03b	0.77c
SAN	0.29a	3.14c	1.72a
SAN3	0.19b	4.12b	1.36b
SAN5	0.13b	4.71b	0.96c
SAN8	0.08c	5.52a	0.83c
SAN11	0.17b	2.93c	0.31d
IAC	0.14a	4.41bc	0.82c
IAC3	0.10a	5.43a	1.29b
IAC5	0.08b	4.58b	0.99b
IAC8	0.03c	4.86b	0.82c
IAC11	0.08b	3.92c	6.80a

[*]HPLC organic acid content (mg g^{-1}) in cassava roots during PPD. Numbers after cultivar name means days of PPD (e.g., BRA3 means sample of BRA cultivar with 3 days of PPD). Statistical analyses were made separately for each cultivar and each compound found. Different letters between column for each cultivar means significant differences in sugar content during PPD (n=3) for the referred compound (TukeyHSD test, p<0.05).

Parte IV
The role of Ascorbate peroxidase, Guaiacol peroxidase and Polysaccharides in Cassava (*Manihot esculenta* Crantz) roots under postharvest physiological deterioration

1. Introduction

Cassava (*Manihot esculenta* Crantz) is a perennial vegetatively propagated shrub that is grown throughout the lowland tropics. Cassava appears to be one of the earliest crops to have been domesticated and was widespread throughout the New World tropics by the late fifteenth century. Introduction to Africa occurred in the late sixteenth century (JONES, 1959) and later in Asia in the eighteenth century. The crop is typically grown between 30° north and 30° south of the Equator, in areas where the annual mean temperature is greater than 18°C. Cassava, which is believed to have originated in Latin America, has a number of attributes that have made it an attractive crop for small farmers with limited resources in marginal agricultural areas. For example, it is one of the most efficient carbohydrate-producing crops and is tolerant to low soil fertility and drought. It also has the ability to recover from damage caused by most pests and diseases, and the roots can be left in the ground for long periods as a food reserve, thus providing insurance against famine. Finally, this staple crop is well adapted to traditional mixed cropping agricultural systems and subsistence cultivation in which farmers seek to minimize the risk of total crop failure (WENHAM, 1995).

Because cassava is increasingly used as human food and in other fields, special attention should be given to the development and transfer of different postharvest technologies to solve the problem of the fast deterioration of cassava roots once harvested (SÁNCHEZ & ALONSO, 2012).

An important constraint that limits the full realization of cassava's potential in developing countries is the short storage life of harvested roots. Cassava roots undergo rapid deterioration 24-48 hours after harvest, a phenomenon referred to as post- harvest physiological deterioration (PPD). PPD is characterized by a blue-black discoloration of the xylem vessels known as "vascular streaking", which considerably reduces the palatability and marketability of cassava roots. The rapid postharvest deterioration of cassava restricts the storage potential of the fresh root to only a few days. In addition to direct physical loss of the

crop, postharvest deterioration causes a reduction in root quality, which leads to lower market prices and thus contributes to economic losses. PPD has been strongly associated with mechanical damage which occurs during harvesting and handling operations (BOOTH, 1976). Tips are frequently broken off as the roots are pulled from the ground, and separation from the plant creates a further wound. In addition, transport from field to market can result in further abrasion. In most cases, physiological deterioration develops from sites of tissue damage initially observed as blue-black discoloration of the vascular tissue. Initial symptoms are rapidly followed by a more general discoloration of the storage parenchyma. Earlier publications on the subject of cassava deterioration simply state that cassava roots will not store well, have a short storage life, will not keep for more than a few days, and are highly perishable (RICKARD & COURSEY, 1981), without giving any indication of the nature, or even the symptoms, of the deterioration processes involved. Other publications refer loosely to "rots" or "decay", giving the impression that the deterioration essentially results from microbiological infection.

The evolution of aerobic metabolic processes, such as respiration and photosynthesis, unavoidably led to the production of reactive oxygen species (ROS) in mitochondria, chloroplasts, and peroxisomes. A common feature among the different ROS types is their capacity to cause oxidative damage to proteins, DNA, and lipids. These cytotoxic properties of ROS explain the evolution of complex arrays of non-enzymatic and enzymatic detoxification mechanisms in plants. Increasing evidence indicates that ROS also function as signaling molecules in plants to control various processes, such as development and defense responses against pathogens and deterioration (APEL &HIRT, 2004). The peroxidases (POX) are associated with such biochemical and physiological processes as growth, cell formation, fruit development, ethylene biosynthesis, as well as response to various stresses. Ascorbate peroxidase (APX) is the most important peroxidase in H_2O_2 detoxification, catalyzing the reduction of H_2O_2 to water using the reducing power of ascorbate (JEBARA et al. 2005). Guaiacol peroxidases (GPX) are located in cytosol, vacuole, cell wall, apoplast, and extracellular medium, but not in organelles, and they are assumed to be involved in a range of processes related to plant growth and development. However, their role in the physiology and biochemistry of cassava deterioration remains to be elucidated (GHAMSARI et al. 2007).

Plants store also carbohydrate polymers in a number of forms. Starch is the principal form, followed by fructans and cell wall storage polysaccharides. They are important for plant metabolism and adaptation (BUCKERIDGE et al. 2000). Primary cell walls from plants are composites of cellulose tethered by cross-linking glycans (hemicelluloses) and embedded in a matrix of pectic polysaccharides. Pectins are uronic acid-rich polysaccharides and all together are important for cell adhesion, elasticity and contribute to wall tensile strength (SILVA et al. 2011). The high degree of structural complexity of plant cell wall polysaccharides has led to suggestions that some components might function as latent signal molecules that are released during pathogen infections and elicit defensive responses by the plant (VORWERCK et al. 2004). It has been reported that acidic polysaccharides are likely to be more bioactive than neutral polysaccharides, which may be due to the fact that the acidic groups in acidic polysaccharides can form associations with the target biomolecules such as proteins in the hosts through electronic interactions (ZHANG et al. 2015). Polysaccharides (neutral and acidic) are high molecular weight macromolecules that can be easily dissolved and dispersed in water under appropriate conditions. They can modulate rheological properties of foods, and are generally used as food thickeners, texture modifier, stabilizers and emulsifiers for various applications. Their have been related to have strong scavenging activities *in vitro* on DPPH and hydroxyl radicals (PEREIRA et al. 2012), anti-inflammatory activity (PEREIRA et al. 2000), antidepressant-like effects of acidic polysaccharides (WANG et al. 2010), *in vitro* inhibitory activities on both human and murine complement activation of neutral polysaccharides (BENENCIA et al. 1999), disease resistance in plants (VORWERCK et al. 2004) and antioxidant capacity (AGUIRRE et al. 2009). In view of the importance of conserving cassava roots in storage, the present investigation was performed in order to gain insights as to the role of APX, GPX, proteins and polysaccharides in cassava roots during PPD.

PPD is an active physiological process involving changes in gene expression, protein synthesis, and the accumulation of secondary metabolites (UARROTA et al. 2014). Thus, antioxidant enzymes, neutral and acidic polysaccharides and protein contents associated with the early events of PPD in cassava storage roots were investigated in this study. Changes in antioxidant enzymes and proteins were analyzed by UV-*vis* spectrophotometry. Morphological and anatomical changes in acidic and neutral polysaccharides and proteins analyzed by histochemical methods and their expression dynamics were correlated with PPD. PPD research

opens up new perspectives for engineering cassava cultivars with prolonged shelf life.

2. Material and Methods

2.1 Cassava cultivars and on farm trials

Cassava cultivars were grown in southern Brazil over the 2011/2012 growing season Four cultivars were selected for this study as follows: SCS 253 Sangão (hereinafter SAN), Branco (hereinafter BRA, a landrace), IAC576-70 (hereinafter IAC, a commercial variety), and Oriental (hereinafter ORI, a landrace). On-farm trials were carried out at the Ressacada Experimental Farm (Plant Science Center, Federal University of Santa Catarina, Florianópolis, SC, Brazil - $27^035'48"$ S, $48^032'57"$ W) in September 2011, using the four cassava cultivars as noted above. Samples of cassava cuttings for cultivation were provided by the Santa Catarina State Agricultural Research and Rural Extension Agency (EPAGRI) at Urussanga, the official state agriculture agency. The experimental design was in randomized blocks, with 4 blocks (6.3 x $15m^2$/block) spaced at 1m. Each block consisted of four plots (12 x 1.2 m^2/plot) spaced at 0.5 m. Cassava cuttings 15cm long were planted upright and spaced at 1 x 1m. Each plot was considered a treatment and all crop management was mechanized. The chemical analysis of the soil's fertility was previously done, and cultivation was performed manually, following agroecological field handlings.

2.2 Postharvest physiological deterioration (PPD)

Cassava root samples (12 months old) were collected for analysis of non-stored samples and for induction of physiological deterioration under controlled conditions in the laboratory. Immediately after harvest, proximal and distal parts of the root were removed, and cross sections were made (0.5-1.0 cm) over the remaining root, followed by storage at room temperature (66-76% humidity, 25^0C). Induction of PPD was performed for 11 days. Monitoring the evolution of PPD and associated metabolic disturbances was performed daily after induction of PPD. Non-stored samples and those at 3, 5, 8, and 11 days after PPD induction were collected at each time point, dried ($35-40^0C$) in an oven, milled with a coffee grinder (Model DGC-20N series), and kept for analysis. For enzymatic analysis, fresh samples were collected and stored (-80^0C) until analysis.

2.3 Postharvest physiological deterioration scoring (PPD scoring)

Five independent experiments of PPD were carried out in which a randomized sampling of 3 sliced roots from each plant variety was scored (from 1-10% of PPD to 10-100% of PPD) over the 11-day experimental period. The information was imaged through a digital camera (OLYMPUS FE-4020, 14 megapixel) and the results were analyzed by visual inspection of the images.

2.4 Enzymatic activities during PPD

2.4.1 Protein determination and enzyme extraction

Protein content was determined in the cassava root samples (non-stored and 3, 5, 8, and 11 days postharvest) using Coomassie brilliant blue G-250 (Bradford, 1976) with bovine serum albumin as a standard (y=0.0159x, r^2=0.975). For enzymatic activities, cassava root samples (1 g, grated samples) were collected directly into liquid nitrogen in a mortar, with 2% PVPP, 1mM PMSF, 10mM DTT, and 0.1mM EDTA (MW:292.2 g.mol^{-1}) in 50mM Na-P buffer, pH 7.5. For analysis of ascorbate peroxidase (APX), the extraction buffer also contained 2mM ascorbate (MW: 176.13 g.mol^{-1}). The suspension was centrifuged (4000 rpm/30 min, 4^0C) and the supernatant used for enzyme assay.

2.4.2 Ascorbate peroxidase activity (APX)

Total APX activity was measured by monitoring the decline in absorbance at 290 nm, as ascorbate (ε= 2.8 mM^{-1}cm^{-1}) was oxidized, for 3 min using the method of Nakano & Asada (1981). The assay medium consisted of 1200 µL of 50 mM potassium phosphate buffer (pH 7.0), 200 µL EDTA, 200 µL ascorbate, 200 µL of sample, and 200 µL of 0.1 mM H_2O_2 to start the reaction. APX activity was expressed in mM ascorbate.min^{-1}mg^{-1} of proteins in fresh basis.

2.4.3 Guaiacol peroxidase (GPX)

Peroxidase activity was measured using a reaction medium containing 50mM phosphate buffer (pH 7), 9mM guaiacol, and 19mM H_2O_2, according to the method of Lin & Kao (1999). The kinetic evolution of

absorbance at 470 nm was measured during 1 min. Peroxidase activity was calculated using the extinction coefficient ($26.6mM^{-1}cm^{-1}$, at 470nm). One unit of peroxidase was defined as the amount of enzyme that caused the formation of 1 mM of tetraguaiacol per minute. Values are expressed in fresh basis.

2.4.4 α-Tocopherol (α-TOC, or vitamin E)

Tocopherol (*α-TOC*) activity was assayed as described by Backer et al. (1980) with small modifications. Briefly, 1 g of cassava sample was homogenized with 5 mL of a mixture of petroleum ether and ethanol (2: 1.6, v.v^{-1}), the extract was centrifuged (4000 rpm/30min, 4^{0}C), and the supernatant was used to estimate α-TOC content. To one milliliter of extract, 3 mL of 2% 2, 2-dipyridyl in ethanol were added, mixed thoroughly, and kept in dark for 5 min. The resulting red color was diluted with 4 mL of distilled water and mixed well. The resulting color in the aqueous layer was measured at 530 nm. The α-TOC content was calculated using a standard curve (y=0.1115x, r^2=0.96) made with known amounts of α-TOC (0 to 100 mg.mL^{-1}) and expressed in mg.g^{-1} of fresh weight (FW).

2.5 Histochemical analysis

2.5.1 Sample preparation

For histochemical analysis, cassava root samples (non-stored and 3, 5, 8, and 11 days of PPD) were collected and small pieces were made (0.5x0.5 cm^2) for subsequent fixation in paraformaldehyde.

2.5.2 Light microscopy (LM)

Samples of cassava roots were fixed in 2.5% paraformaldehyde in 0.1 M (pH 7.2) phosphate buffer (72 h). Subsequently, the samples were dehydrated in increasing series of ethanol aqueous solutions (SCHMIDT et al. 2009; UARROTA et al. 2011). After dehydration, the samples were infiltrated with Historesin (Leica Historesin, Heidelberg, Germany). Sections 5µm in length were stained with different histochemical techniques and investigated with an Epifluorescent (Olympus BX 41) microscope equipped with Image Q Capture Pro 5.1 software (Qimaging Corporation, Austin, TX, USA).

2.5.3 Histochemical staining

LM sections were stained as follows: Periodic Acid-Schiff (PAS) used to identify neutral polysaccharides (SCHMIDT et al., 2009), Toluidine Blue (TB-O) 0.5%, pH 3.0 (Merck Darmstadt, Germany) used for acid polysaccharides through a metachromatic reaction (SCHMIDT et al., 2009), and Coomassie Brilliant Blue (CBB) 0.02% (m/v) in Clarke's solution (Serva, Heidelberg, Germany) used for proteins (SCHMIDT et al., 2010).

2.6 Data analysis and mining

All statistical analyzes and graphics were implemented in R language (R core team-2014, version 3.1.1), using the respective packages and scripts. Enzymatic activity data were represented as mean ± standard deviation of three repetitions (n=3). PPD was correlated with all enzymes studied, and Two-way ANOVA using randomized complete design was applied using the "easyanova" package. Multivariate analysis by both non-supervised and supervised techniques was applied for descriptive and predictive models. Histochemical micrographs were performed in Photoshop, version 7.

3. Results and Discussion

3.1 Postharvest physiological deterioration scoring (PPD scoring)

PPD is a major problem in cassava production, and research efforts aimed at understanding the causes underlying rapid deterioration of cassava roots after harvest. Results of PPD scoring in our research are summarized in Figure 1A, and root slices at different storage days can be found in Figure 1B. ORI cultivar showed high rate of deterioration when compared by others, results that agree with those previously reported by our research group (UARROTA et al. 2014). Statistical differences were not found (Tukey test, $p<0.05$) among cultivars. During storage time, significant differences in PPD were only found between non-stored and stage 3 samples with those at stages 5, 8, and 11 of PPD. Image analysis of PPD samples (Fig. 1B) also revealed fast deterioration of ORI/SAN samples (susceptibles) and BRA/IAC cultivars as the most tolerant to PPD. Results of PPD scoring for these cultivars are scarce in the literature. Therefore, our results will serve as basis for future screening of these

valuable genetic materials toward a better understanding of cassava root deterioration.
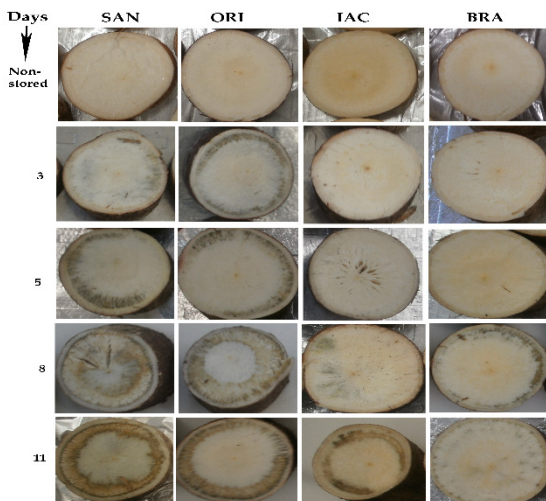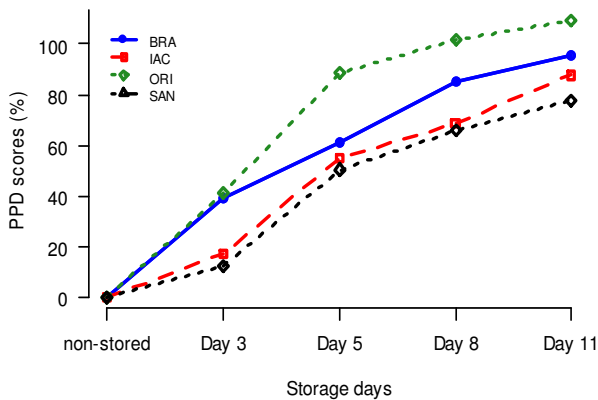

Figure 1. A (top) - PPD scoring of cassava samples at different storage days (3, 5, 8, and 11 days) in four cultivars. Data represent means of five independent evaluations of PPD and are based on the intensity of parenchyma discoloration. **B (down) -** Root cross-sections of the four cultivars studied during 11 days of storage showing parenchyma discoloration during PPD.

3.2 Enzyme activities during PPD and multivariate analysis

Several metabolites are critical for plant growth and development and play an important role in integrating various stress signals, controlling downstream stress responses by modulating gene expression machinery and regulating various transporters/pumps and biochemical reactions (TUTEJA & SOPORY, 2008). On the other hand, reactive oxygen species (ROS) are continuously produced during PPD as byproducts of aerobic metabolism. Depending on the nature of the ROS species, some are highly toxic and rapidly detoxified by various cellular enzymatic and non-enzymatic mechanisms. Plants are supplied with an excess of mechanisms to combat increased ROS levels during abiotic stress conditions. However, under other circumstances, plants appear to purposefully generate ROS as signaling molecules to control various processes, including pathogen defense, programmed cell death, and PPD (APEL & HIRT, 2004). Our recently published work has shown that some non-enzymatic mechanisms, such as secondary metabolites (phenolics, carotenoids, flavonoids, and anthocyanins), and enzymes, such as catalase, hydrogen peroxide, and superoxide dismutase, are highly involved in the process of ROS detoxification (UARROTA et al. 2014) during PPD. Ongoing experiments in our laboratory have also found hydroxicoumarins (mainly scopoletin) to be involved in PPD, as previously reported in the literature by other research groups (SÁNCHEZ et al. 2013; GARCÍA et al. 2013; ZIDENGA et al. 2012; RELLY, 2001; WHEATLEY & SCHWABE, 1985; MAINI & BALAGOPAL, 1978; BOOTH, 1975). Isamah (2004) observed increases of peroxidases up to 24 h, but decreasing thereafter, and attributed such activities to PPD. We aimed to understand the biochemical markers associated with PPD toward a better screening of these factors in the future, and our results (Figure 2 A-D), summarize the enzymatic activities measured during PPD. Specifically, APX (Fig. 2A) increased during PPD up to day 3 in the SAN/IAC cultivars and up to day 5 for the BRA cultivar. In the ORI cultivar, this trend was not observed. Analysis of variance of these data showed differences among cultivars along all storage days ($p < 0.05$).

GPX activity (Fig. 2B) showed an increasing trend during PPD up to day 5, except for IAC/SAN cultivars. In its turn, ORI genotype showed greater values of GPX during storage days. Statistical differences ($p < 0.05$) were found between ORI and BRA cultivars as GPX activity showed to be distinct over the storage period.
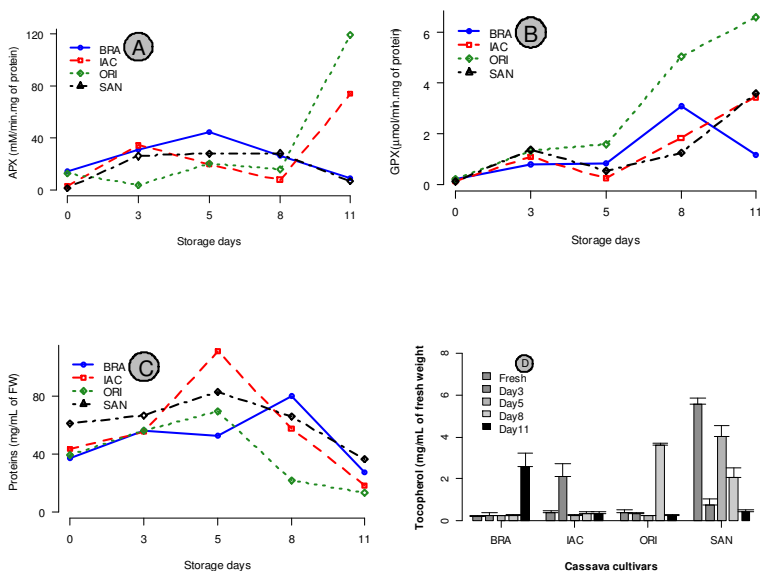
Figure 2. Changes in enzyme activity and protein contents during PPD of the four cultivars studied. A- Ascorbate peroxidase (mM min^{-1}mg^{-1} of proteins); B- Guaiacol peroxidase (µmol min^{-1}mg^{-1} of proteins); C- Total protein contents (mg mL^{-1} of fresh weight) and D-Tocopherol amounts during PPD (mg mL^{-1} of fresh weight). Data represent means and standard deviations of three replications (n=3).

Total protein contents (Fig. 2C) in all cultivars increased up to day 5, except for BRA, which presented a small decrease at day 5, but continued to increase up to day 8. Every clone had a decrease from day 8 to 11. Significant differences (p<0.05) in total protein contents in all cultivars and all storage days were detected. Finally, as shown in Figure 2D, small quantities of α-TOC were found, but no trend was identified during storage days that led to significant correlations with PPD in cassava roots. When enzyme data were summarized and correlated using Pearson's correlation coefficient (PCC), it was found a high positive correlation between GPX and PPD (r =0.60), followed by a moderate one for APX (r =0.35) and a negative correlation between PPD and total protein content (Figure 3). GPX and APX are the main antioxidant enzymes activated against ROS scavenging during PPD. No involvement of α-TOC was observed in the context of PPD (r=0.05). Enzymatic systems have been associated with the reduction of many stress systems in different crops, such as tobacco and wheat (CURVÊLO et al. 2013; GARG et al. 2012;

KWON et al. 2002). Our results clearly demonstrate that APX, GPX, and total proteins are related in PPD delay in cassava roots. A future study may be conducted to better understand the kinds of proteins involved and how they act in cassava PPD.
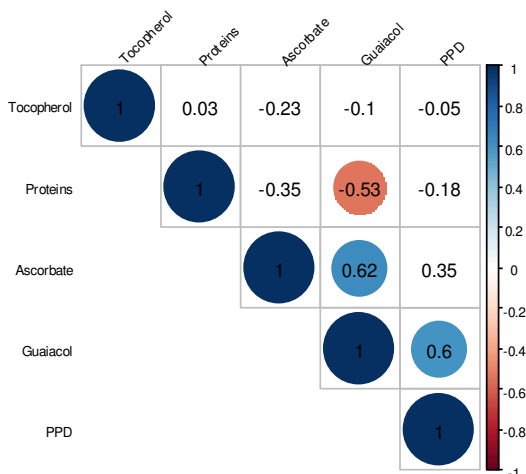

Figure 3. Correlation matrix of enzyme data (APX, GPX, α-TOC, and total proteins) with PPD. Crude data (non-normalized) of enzymes during storage time were correlated with the level of PPD in that samples. Colors represent the degree of correlation as represented in the matrix figure scale.

According to Sills and Gossett (2012), chemometric techniques that include multivariate models (e.g., principal component analysis, hierarchical cluster analysis, partial least squares discriminant analysis (PLS-DA), linear discriminant analysis (LDA), and support vector machines (SVM)) can be applied to complex and collinear data to extract relevant information. Both non-supervised (PCA) and supervised (PLS-DA, LDA, and SVM) methods reduce large datasets by combining collinear variables into a small number of latent variables (LVs), which are then used in place of the full dataset to build predictive models.

Enzyme data in this study were subjected to both non-supervised and supervised methods to better classify samples according to their biochemical behavior (see Figure 4A-B). As a result, mathematical and predictive models were constructed to screen cassava samples, and a similar profile was detected in all samples, except those at day 11 (Fig. 4A), using PCA as the best non-supervised method found. The total variance explained by PCA was 67.50%, with 46.30% and 21.20% for PC1 and PC2, respectively. Samples at day 11 were found in (PC1+/PC2-

) and the major group in (PC1-/PC2-). The loading values showed that samples at day 11 grouped in that component because of their levels of APX, GPX, and PPD. The other group (non-stored, samples at stages 3 and 5) was classified according to its protein level and APX activity.
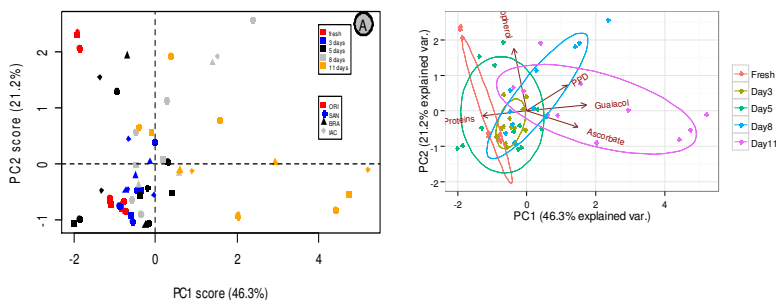

Figure 4. Predictive models of cassava samples**.** (A)- Scores plot of the principal component analysis (PCA) model from the enzyme data set of cassava roots and the percentage of variance captured by each PC (46.3% and 21.2%, respectively) and right figure showing the eigen values and sample clustering.

A decision tree model (Fig. 4B) when used found proteins and GPX as mainly related to PPD in cassava samples. According to our model, with proteins contents above 36 mg/ml, 26% of samples did not deteriorate. Lower amounts of protein and GPX activity above 0.23 µmol/min.mg seem to be associated to a diminished deterioration in cassava roots, i.e. 19%. Such findings reinforce the strong involvement of GPX in PPD delay.
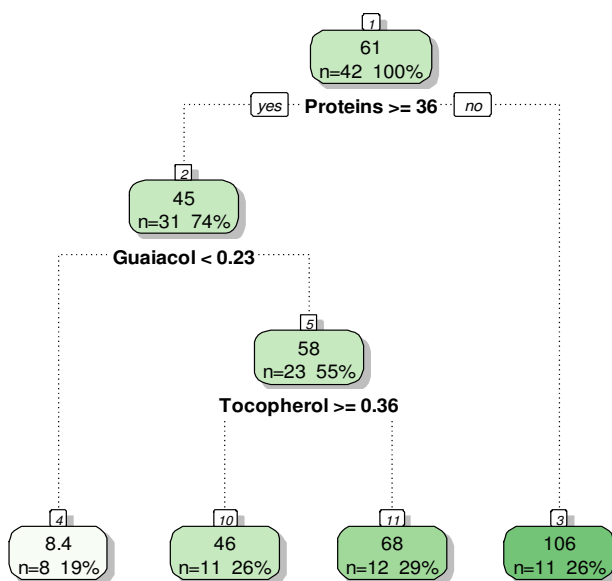
Figure 4. Predictive models of cassava samples (B)- Supervised method (Decision tree model) of all data taking PPD as target variable and using all enzymes to build a train model and predicts expected levels of PPD in cassava

3.3 Histochemical analysis

3.3.1 Involvement of acidic and neutral polysaccharides in cassava PPD

Polysaccharides are relatively complex carbohydrates and the first biopolymers found in nature. These polymers are made up of either single or multiple monosaccharides joined together by glycosidic bonds forming large, often branched, macromolecules. They play a number of roles in biological functions like respiration, mechanical strength, source of energy, and stress tolerance (SANANDIYA & SIDDHANTA, 2014), and they may vary quali/quantitatively depending on the species, cultivar, tissue, location of cultivation, time of harvest, and duration of storage (SILLS & GOSSETT, 2012). The high degree of structural complexity of plant cell wall polysaccharides has led to suggestions that some components might function as latent signaling molecules that are released during pathogen infections as defensive responses by the plant (VORWERK et al. 2004). They have also been implicated to possess

many antibacterial and antioxidant properties (LI & SHAH, 2014; ZHANG et al. 2013; ZHU et al. 2010; RAUSCH & WACHTER, 2005). Changes in plant polysaccharides (pectin and hemicelluloses) under stress conditions have been reported, and increases in lignin have been found (DE LIMA et al. 2014). Cell wall polysaccharides have been implicated as a promising group of antioxidant compounds (DOHERTY et al. 2011; KALE et al. 2013), and the free radical scavenging activity was attributed to pectic polysaccharides (MATEUS-APARICIO et al. 2010).

Cassava samples at different storage days were stained with toluidine blue (TB) and the results are summarized in Figure 5A. All cultivars showed metachromatic reaction in the cell walls and around starch granules. This reaction was predominantly observed up to 5 days of storage in BRA/IAC cultivars, while for other cultivars it was observed only in the cell walls. Metachromatic reaction indicates the presence of acidic polysaccharides that are produced as oxidative stress increases in cassava samples, and their role can be attributed to reducing PPD stress. A degradation of starch granules can also be observed during storage. Reports associating anatomical changes to PPD are scarces in the literature, thus making the present work the first to report anatomical alteration in relation to PPD, in particular the function of cassava primary cell walls. According to Bowen et al. (2006), reduction of the moisture content of the plant matrix is generally considered to reduce deterioration and is often used for food preservation. However, oxidative stress is generally enhanced at low water activities under conditions where oxygen can permeate through lipid layers as found in cell membranes. Free radicals have been implicated in the oxidative–reductive depolymerization of carbohydrates; therefore, radicals generated by lipid oxidation may attack starch.

Samples stained with Periodic Acid Schiff (PAS) exhibited a strong reaction for starch granules, but a lesser one in the cell wall of all cultivars studied during 11 days in storage. The high intensity of reaction detected is indicative of a major presence of neutral polysaccharides (i.e., starch) in these samples. Starch granules can be easily observed in non-stored samples (Fig. 5B), while their degradation is clearly visible during storage. Starch is probably degraded during storage into monosaccharides, a phenomenon previously encountered by our research group (data not shown).
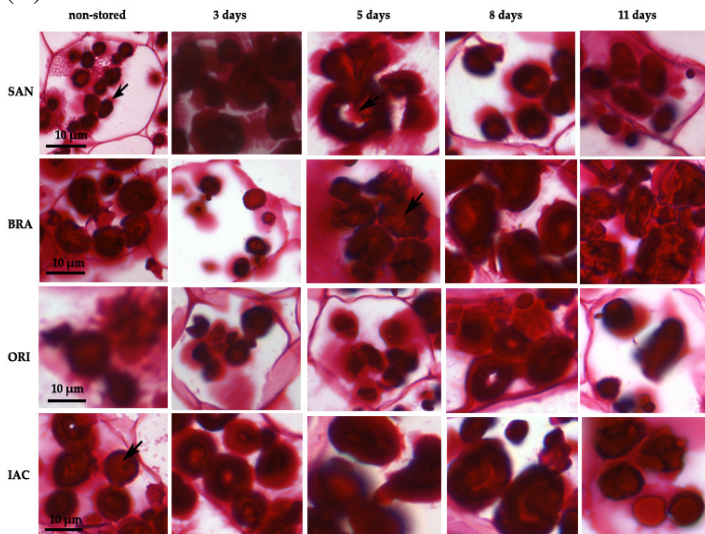
Figure 5 (A)
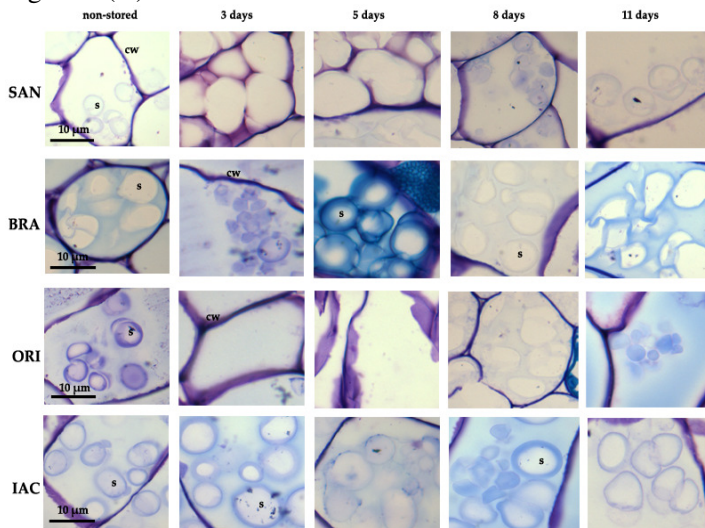
(B)

Figure 5. Light microscopy of cassava samples during storage time analyzed by histochemical staining. A- Samples stained with tolluidine brilliant blue (TB) to indentify changes in acidic polysaccharides. Cw indicates cell wall and S starch granules; B- Staining with Periodic Acid Schiff (PAS) to indentify neutral polysaccharides. Arrows indicate starch granules

Starch is the form of carbohydrate reserve in nearly all green plants, being the major carbohydrate component in food. Starch granules consist of two structurally and functionally very different polymers: amylose and amylopectin. Starch functionality depends on the average molar mass of amylose and amylopectin, as well as on their molecular structure and organization within the granule (JANKOVÍC, 2013). Physicochemical properties of cassava starch have also been reported to alter with the complexation of oxalic and succinic acids (JOHN & RAJA, 1999) during PPD (SÁNCHEZ et al. 2013). Among the starchy staples, cassava gives a carbohydrate production which is about 40% higher than that of rice and is 25% greater than that of maize, making it the cheapest source of calories for both human nutrition and animal feeding (TONUKARI, 2004). Typical composition of cassava root is moisture (60-70%), starch (24%), fiber (2%), protein (1%), and other substances including minerals (3% - TONUKARI, 2004). Delaying PPD can be a promising strategy to preserve cassava roots and starch. The assembly and network of structural polysaccharides within cell walls greatly restrict enzyme access, explaining why these glycan composites are resistant to biological degradation. Nevertheless, the enzymatic deconstruction of cell walls is of considerable biological significance as it is central to the carbon cycle and plays a key role in the pathogen invasion of plants (GILBERT et al. 2013).

When samples of cassava were stained by Coomassie blue brilliant (CBB), a slight reaction was found up to day 3 of storage in all samples for cell walls and around starch granules (Fig. 5C). The reaction was more intense in BRA/SAN cultivars. These results corroborate the results of protein quantification, which showed small increases in protein rates from day 3 to 5 of storage. In general, cassava samples are poor in protein content, which explains the small reaction observed in all samples.
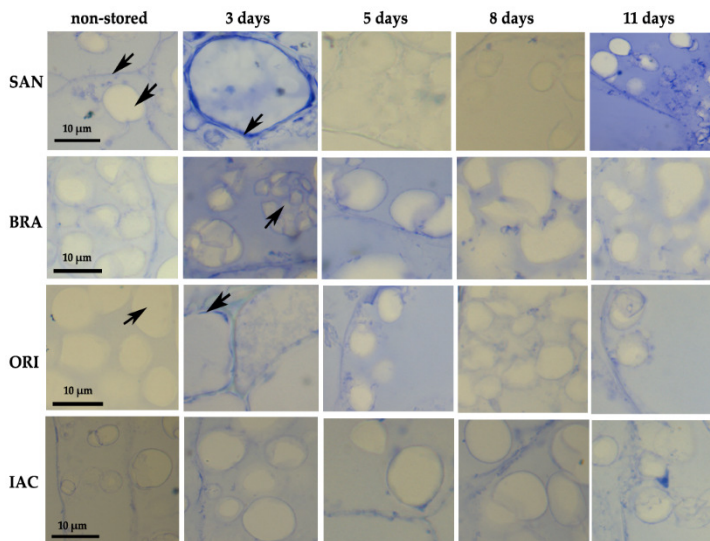
Figure 5. Light microscopy of cassava samples during storage time analyzed by histochemical staining (C) - Staining with Coomassie Brilliant Blue (CBB) to indentify proteins in cassava root parenchyma. Arrows indicate cell wall and starch granules.

4. Conclusions

PPD in cassava roots depends on the cultivar and induction method. The results of this study revealed to be the ORI cultivar more susceptible to deterioration. GPX and APX activities and total proteins are increased during PPD. Similarly, antioxidant mechanisms also take place to ameliorate ROS production during this particular stress condition, helping to delay PPD. Histochemical analysis demonstrated that acidic polysaccharides seem to act as barrier components of plant cell walls and may play an important role in PPD delay as starch catabolisation is observed during PPD. By using multivariate analysis, a descriptive model was built, showing that GPX, proteins, and APX play important roles in PPD delay.

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CONCLUSÕES GERAIS

O presente trabalho objetivou avaliar as alterações no metabolismo secundário, nos processos enzimáticos e histológicos em quatro cultivares de mandioca durante a deterioração fisiológica pós-colheita. O estudo permitiu observar que determinados metabólitos secundários são anabolisados nos estágios iniciais da deterioração (3 até 5 dias após a colheita), sendo tal fenótipo dependente da cultivar e do grau de deterioração. Carotenoides, flavonoides, compostos fenólicos, antocianinas, superóxido dismutase, catalase e peróxido de hidrogênio apresentaram concentrações superiores nos estágios iniciais da deterioração. Carotenoides, compostos fenólicos, matéria seca, polifenol oxidase, ácido ascórbico, glucose, frutose, ácido succinico, ascorbato e guaiacol peroxidases e proteínas evidenciaram um papel importante na redução ou postergação do processo de deterioração fisiológica.

A principal hidroxicumarina observada na PPD da mandioca foi a escopoletina. As cultivares mais tolerantes à deterioração mostraram níveis mais elevados deste metabólito, comparativamente às susceptíveis. Os conteúdos de escopoletina mostraram-se crescentes durante a deterioração, fato que nos permite concluir que a escopoletina poder estar envolvida na redução da deterioração no estágio inicial deste processo. A ruptura dos compartimentos celulares derivada das lesões tissulares por ocasião da colheita permitiu o contacto da linamarase com o seu substrato limanarina, fato constatado pelo incremento dos teores de ácido cianídrico até 3-5 dias de deterioração, seguido de degradação. A atividade da linamarase foi elevada nestes estágios, mostrando-se, contudo inativa nas fases avançadas de PPD. Acúmulos de polissacarídeos ácidos e neutros (e.g. celulose) foram constatados nas paredes celulares durante a evolução do processo de deterioração, um fato eventualmente associado a respostas de defesa vegetal à infecção por microrganismos, ou ainda a espécies reativas de oxigênio formadas na PPD. A hidrólise dos grânulos de amido foi detectada, subsidiando a hipótese de catabolização daquele polissacarídeo e o acúmulo do produto metabólico derivado, conforme observado durante o processo de deterioração.

Técnicas estatísticas multivariadas (métodos supervisionados e não supervisionados) e modelos de predição permitiram discriminar as amostras de acordo com as variáveis metabólicas estudadas, revelando quais variáveis bioquímicas influenciaram mais intensamente a PPD.

Modelos preditivos mostraram ser as atividades da guaiacol peroxidase, ascorbato peroxidase, superóxido dismutase e catalase e os conteúdos totais de proteínas, carotenoides e peróxido de hidrogênio as variáveis mais relevantes á determinar o atraso da PPD. O presente estudo também permitiu identicar maior tolerância à PPD da cultivar Branco, enquanto a suscetibilidade mostrou-se maior na cultivar Oriental. Por fim, considerando-se que a PPD pode ser influenciada por uma série de fatores (a)bióticos, um maior detalhamento dos estudos e uma intergração de dados a nível genômico, proteômico, transcriptômico e metabolômico corroborará à geração de um quadro de melhor entendimento dos processos bioquímicos relacionados à PPD da mandioca, com consequências positivas nos contextos agronômico e sócio-econômico afins.