

# Study of the Early Events Leading to Cassava Root Postharvest Deterioration

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Received: 10 March 2010 / Accepted: 17 June 2010 / Published online: 27 July 2010  
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**Abstract** Cassava (*Manihot esculenta* Crantz) roots, the fourth most important food crop of the world, is the major carbohydrate source for more than 600 million people in Africa, parts of Latin America, Oceania, and Asia. Besides being a rich source of starch (~80% of root), the root is also rich in vitamin C, some carotenoids, calcium, and potassium. Upon harvest, roots begin a process of physiological decay within 24–36 h called postharvest physiological deterioration or PPD. The early events leading to PPD are not known. Research to date concerning the study of PPD has mostly focused on the signaling events several hours after harvest. Upon examination of physiological and biochemical changes occurring 3 or 4 h after cassava root detachment, changes in the nature and type of volatile compounds emitted, secondary metabolites accumulated, and changes in the expression of key genes in reactive oxygen species (ROS) turnover were observed along with a correspondent increase in tissue cytoplasmic singlet oxygen presence using radical-specific fluorescent imaging of tissue samples. It is likely that these findings have significant implications to help us understand and assist in dissection of the early events leading to the postharvest deterioration of cassava root.

**Keywords** Cassava root · Gene expression · ROS enzymes · Volatiles · Secondary metabolites · Singlet oxygen detection · Respiration and ethene

## Abbreviations

MDA	Malonyl dialdehyde
PPD	Postharvest physiological deterioration
ROS	Reactive oxygen species
( <sup>1</sup> O <sub>2</sub> )	Singlet oxygen
SOSG	Singlet oxygen sensor green

## 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. It comprises more than 80% starch, and is also rich in vitamin C, carotenoids, Ca<sup>+2</sup>, and K<sup>+</sup>. Cassava is vegetatively propagated using stem cuttings, and can grow in poor soils with minimum labor. It can remain in the soil without harvest for extended periods of time, even enduring the most adverse conditions where other food crops do not survive. This secures and guarantees a local food source for years. However, the major disadvantages of the cassava root crop are the low protein content in the root, presence of high amounts of cyanogenic glucosides, and rapid postharvest perishability of the roots. The rapid postharvest perishability of freshly harvested cassava is a problem not known in any other crop. Within 1–3 days of harvest, roots begin to develop an endogenous disorder known as postharvest physiological deterioration (PPD). It is 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. In rural areas cassava roots are usually consumed fresh. Also, the practice of a staggered harvest-

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Communicated by: Robert Paull

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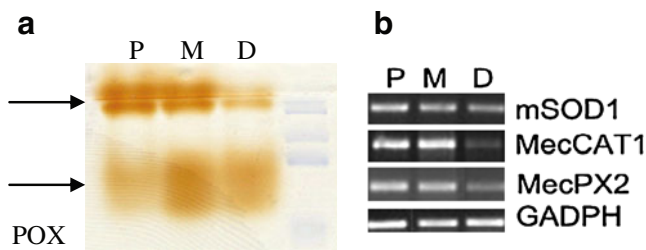
ing plan helps to minimize the problems associated with rapid PPD. A cassava root is highly perishable, thus limiting the ability to store the produce long enough for transport and consumption. Therefore, selling a 1–3 day old cassava root in urban areas represents a challenge as these roots are assumed to be already deteriorated. Cassava roots showing visible symptoms of PPD are considered to have poor eating and processing quality. Marketing remains a major factor influencing urban cassava consumption. As cassava is considered a high risk food crop in these areas, other major foods like wheat, rice, and maize are preferred (FAO 1995). Cassava production is not difficult, in sharp contrast with the high inputs and the risks involved in subsequent processing, transport, and marketing.

Increased respiration (Uritani and Reyes 1984; Hirose 1986; Uritani 1998), changes in lipid composition (Lalaguna and Agudo 1989), synthesis of ethene (Hirose et al. 1984), accumulation of secondary metabolites from the phenylpropanoid pathway, and increases in many enzyme activities such as PAL and chalcone synthase, glucanase, chitinase, proteinase inhibitors, HRGPs, invertase, catalase, dehydrogenase, peroxidase and polyphenol oxidase (Rickard 1981; Tanaka et al. 1983) have been reported during the development of PPD. In addition to detecting PAL and polyphenol oxidase (PPO) activity, cytological analyses of the wound surface in cassava roots showed the presence of phenols, lipids, carbohydrates, and lignins (Rickard 1981).

Primary deterioration due to an oxidative process was first noted by Castagnino (1943). Later, Drummond (1953) showed that the development of deterioration symptoms could be delayed by exclusion of oxygen. Averre (1967) showed that vascular streaking could be inhibited by water treatment (53°C for 45 min), or by keeping the roots under water, or by keeping them in an anaerobic condition. Several studies reported the involvement of endogenous enzyme systems, like dehydrogenases, catalases, peroxidases (Czyhrinciw and Jaffe 1951), phosphorylases (Nair and Kurup 1963), and many others. Polyphenol oxidase has been frequently implicated in the enzymatic discoloration of tissues, and peroxidase in the oxidative damage of vegetables during storage. Plumbley et al. (1981) studied the relationship between peroxidase and polyphenol oxidase activity and discoloration in harvested cassava roots and found that discolored cassava roots contained peroxidase activity in the soluble, covalently-bound, and ionically-bound fractions. Extracts from non-discolored tissue lacked activity in the latter fraction and reduced activity in the other two fractions. Polyphenol oxidase showed increased activity only in the covalently-bound fraction of discolored root tissue.

The phenolic compounds associated with the development of physiological deterioration have been identified and include scopoletin, scopolin, esculin, proanthocyani-

dins, (+)-catechin, and (+)-gallocatechin (Rickard 1985; Wheatley 1982; Wheatley and Schwabe 1985). Wheatley and Schwabe (1985) demonstrated the crucial role played by scopoletin in the development of cassava root PPD. They showed that when applied to freshly cut roots, scopoletin was able to produce intense and rapid discoloration within the tissue. Among a range of phenolic compounds applied to harvested tissue, only scopoletin caused a significant amount of discoloration. It was shown that (1) pruning of the areal portions of the plant 2–3 weeks before harvest, and (2) curing (i.e. storage under high humidity conditions to facilitate wound healing response and reduced access to oxygen), can dramatically reduce the susceptibility of the root to PPD (Booth 1976; Lozano et al. 1978; Marriott et al. 1979). Application of scopoletin (1,000 mg dm<sup>-3</sup>) to freshly cut roots from plants pruned 1, 2 or 3 weeks before harvest resulted in intense deterioration of the treated tissues regardless of the length of pruning-to-harvest interval. Exogenous application with a much lesser concentration of scopoletin (100 mg dm<sup>3</sup>) did not induce any significant discoloration or deterioration, perhaps an indication of either insufficient entry or compartmentalization within the tissues. Fluorimetric scopoletin analysis revealed the presence of greater than 170 µg g<sup>-1</sup> dry weight within proximal root sections after just 24 h of harvest (Wheatley and Schwabe 1985). Uritani (1998) compared the biochemical response of sweet potato and cassava during storage and showed that coumarins were produced only in wounded or freshly harvested cassava root, earlier than the onset of vascular discoloration. In contrast, wounded sweet potato produced no coumarins. The increase of coumarins within cassava root was greater only during primary deterioration. The coumarins identified were scopoletin, scopolin, and esculin (esculin 6-β-glucoside). The phenylpropanoid pathway enzymes, including phenylalanine ammonia lyase (PAL) and 4-coumarate: CoA ligase, are differentially regulated upon wounding of plant tissue (Lois and Hahlbrock 1992). The transcription of PAL can begin within 2–3 min of wounding in *Daucus* (29), and its activity has been shown to closely follow the progress of cassava root PPD, and peak after about 2 days of wounding (Rickard 1985). Peroxidases at the cassava root wound surface act upon these phenolic compounds. Rickard (1981, 1985) showed an increased staining for total peroxidase activity at the cassava root wound surface during the development of PPD. Apparently, accumulation of peroxidase enzyme occurs by de novo synthesis and it is induced after a lag period of about 24 h. Experiments utilizing cycloheximide to inhibit protein synthesis (Uritani and Reyes 1984) and other studies (Beeching et al. 1998) have shown that PPD is an active process involving gene expression and protein synthesis. Various secondary compounds have been listed



**Fig. 1** **a** POX activity (arrows) and **b** RT-PCR analysis using gene specific primers of cDNA prepared from cassava roots 3–4 h after harvest. (abbreviations: *SOD* superoxide dismutase, *CAT* catalase, *POD* peroxidase, *GADPH* Glyceraldehyde diphosphate dehydrogenase loading control). SOD and MDA activities in roots 3 h after harvest. The labels P=proximal, M=middle, D=distal indicate distance in root tissue from initial harvest wound here and in all figures

by Bushmann et al. (2000a,b) as thought to be involved in early cassava root PPD.

Reactive oxygen species (ROS) have also been shown to increase very early during PPD (Reilly et al. 2000, 2001, 2003) and evidence suggests that ROS and ROS turnover enzymes are involved during PPD (Chavez et al. 2000). Many experiments suggest a controlled production of ROS exists in plant defense, especially in response to wounding and pathogen attack. Reilly et al. (2003), reported a rapid oxidative burst within 15 min of harvest, signaling the start of PPD, predominantly due to a rapid production of superoxide and hydrogen peroxide. Several roles have been attributed to the accumulated ROS species, including essential functions such as cell wall repair and remodeling, induction of defense-related genes, signal transduction, and triggering host cell death. Using an RNA fingerprinting technique called cDNA-AFLP, Huang et al. (2001) showed that transcripts involved in important biochemical and physiological processes, notably oxygen stress, and carbohydrate and protein metabolism, are involved in cassava root PPD. Accordingly, the genome locations of the genes expressed in these processes have recently been mapped (Cortés et al. 2002). In an attempt to identify the entire subset of genes that are differentially regulated in cassava during PPD over a period of 0–72 h postharvest, Reilly et al. (2007) carried out a large scale cDNA microarray analyses of the cassava root transcriptome. They found 72 differentially regulated ESTs, of which 63 were up-regulated and 9 were down-regulated. Many of the up-regulated PPD specific ESTs were predicted to play roles in

cell wall repair, ROS generation and turnover, programmed cell death, ion/water/metabolite transport, signal transduction, stress response and metabolism, and protein synthesis.

Our studies were performed to further understand the nature of the very early events leading to the postharvest deterioration of cassava root. For this purpose, physiological and biochemical studies were performed on root sections obtained from an entire root. Three sections were prepared designating the section closest to harvested end of the root as proximal (P), and the sections following it as middle (M) and distal (D). We studied the volatile compound emission, production of secondary metabolites, respiration and ethene production, gene expression profile, and cytological detection of singlet oxygen ( $^1O_2$ ) species in the roots. Studies reported here increase our existing knowledge and understanding of cassava postharvest biology, particularly with regard to the early events leading to rapid postharvest deterioration of cassava root. Additional benefits may be realized owing to the global importance of this staple food crop.

## Results

### Gene Expression Profile and Enzyme Activities

Several genes in the ROS and ROS turnover pathway such as catalases, peroxidases, SOD, and MDA are expressed in cassava root within 4 h after harvest. Transcripts of SOD, catalase, and peroxidase predominate in regions closer to the wound site (Fig. 1b). We have also shown that activities of SOD, POX, and MDA are higher in the regions closer to wound site (Table 1, Fig. 1a). SOD and POX have protective roles in scavenging free radicals. MDA is a measure of lipid peroxidation.

Upon further investigation, studies to identify genes expressing within the first 4 h of harvest was performed. Examined genes included those with roles in ROS turnover, biosynthesis and metabolism, signal transduction, programmed cell death (PCD), and cell wall metabolism and remodeling (Table 2). RT-PCR clearly showed that transcripts for enzymes involved in the inter-conversion and detoxification of ROS are induced very early after harvest (Fig. 2), and are primarily in the zones closer to the cut end. Gene products for other enzymes and proteins that may be involved in cell

**Table 1** SOD and MDA activities in roots 3 h after harvest. The labels P=proximal, M=middle, D=distal indicate distance in root tissue from initial harvest wound here and in all figures

Assay	Proximal (P)	Middle (M)	Distal (D)
SOD (units of Sod/ $\mu$ g FW)	0.91	1.71	0.30
MDA ( $\mu$ moles/g FW)	6.0	4.20	4.20

**Table 2** PCR primers and related information

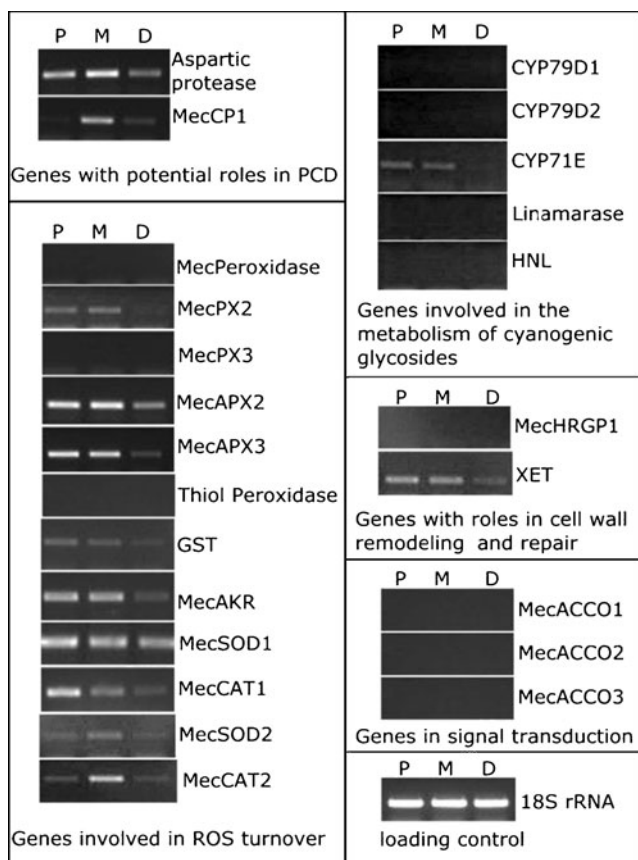
Primer name	Primer sequence (5'- 3')	length	%GC	Tm°C	Product	Accession#
CYP71E-F	GGTTCGATTTTGAGTTGGTGC	22	45.5	52.9		
CYP71E-R	GCAGGTCAGAAGAACCATT	21	47.6	50.7	511	AY217351
CYP79D1-F	GCTAAATCAACCAGAAATCCTGAAG	25	40	54.6		
CYP79D1-R	TGCAAGAGAAAACAAGATAACCCC	23	43.4	53.2	700	AF140613
CYP79D2-F	CTGATAAATCAACCAGAACTTCTGGCA	27	40.7	58.8		
CYP79D2-R	CTAACAACTCACATTCATCCCTTCCC	26	46.2	58.1	550	AY834390
APX3-F	AGGTGCTGATCACTTGAGAGAG	22	50	50.2		
APX3-R	CCGACCATCATCACATTCAA	20	45	51.2	500	AY973623
APX2-F	TTCCTTCTCCAGGTGCTCTTG	22	50	55.1		
APX2-R	CCGACCATCATCACATTCAA	20	45	51.2	344	AY973622
CP1-F	GTTGCTGCCTCTTTGAGTATGG	22	50	53.2		
CP1-R	GTTTGGGAACTGCGAGTACA	20	50	50.1	529	AY973616
PX3-F	GGCAAGTGTTCACACAAGCTG	22	50	54.8		
PX3-R	TTCCAGTCAAGACACCAACT	20	45	46.6	472	AY973612
PAL-F	GCTGCTGCTATTATGGAACA	20	45	48.2		
PAL-R	CTAGGATTCCTTCCTCCTGT	20	50	46.6	400	AF078690
PAL1-F	CTCCCACTTTCTCACCCTCC	21	57	52.5		
PAL1-R	CTGAATAACCTTGAGGAGG	20	50	49.1	550	AY036011
PAL2-F	GCAACAATCTCTCAGAATGGTC	22	45.5	50.5		
PAL2-R	TTGTGGTTGAGGAGCTTGGT	20	50	52	550	AF383152
AKR-F	GGGTTAACGAAATTGCAGCAAG	22	45.5	55.5		
AKR-R	TACAAGCGAAGCCAAAACAG	20	45	50.9	412	AY973615
Peroxidase-F	GAGCAAGTGCCTGGTGTGTGA	22	50	51.8		
Peroxidase-R	GCATATGGAATTAGAGCACTCC	22	45.5	50.1	987	AF078691
18S-F	CAGACTGTGAACTGCGAATGG	22	50	54.8		
18S-R	ATTGGAGCTGGAATTACCGC	20	50	53.3	520	AB233568
AP-F	CAAGCAGAATGCAACACTGGAG	22	50	55		
AP-R	CACGAGGTTTAGTTCAAGCC	20	50	49.2	450	AF266465
HNL-F	CATCATTGTTGGTGAGAGCTGTG	23	47.8	54.6		
HNL-R	TATGCATCAGCCACCTCTTG	20	50	51	549	AY787210
Linamarase-F	TAAGCTTGTTGGCTCTCACTAG	22	46	49		
Linamarase-R	AGGTTCTGTAGCTGAGTCTCCA	22	50	50	670	U9529
MecACCO2 F	GGGATCATACTGTTGCTCCAA	21	47.6	51.6		
MecACCO2 R	GAACCCTGTGAAGGAGCATT	21	47.6	51	500	AY973618
XET F	CTTCAATGGCTGCCTCTCAT	20	50	51.8		
XET R	CCAGAGGTAGAATTGTTGCTCTC	23	47.8	51.7	414	DT883569
QOR F	GAAGTGTACAATCCATGTCTTCG	23	43.5	50.6		
QOR R	CAAAGCATCCCTTGAGCAAG	20	50	52.5	180	DT883582
MecHRGP1 F	TTCCGGACCAAATCACCTTG	20	50	54.7		
MecHRGP1 R	ACGGTGGTGGAGGAGATTTGTA	22	50	54.9	456	AF239615

wall repair and programmed cell death are also expressed within the 4 h time frame of this experiment (Fig. 2).

#### Detection of Singlet Oxygen ( $^1\text{O}_2$ ) Species

The major sources of ROS in plants are superoxide ( $\text{O}_2^-$ ), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), and singlet oxygen ( $^1\text{O}_2$ ). The

use of a novel singlet oxygen sensor was investigated in fresh cassava roots. The roots from greenhouse grown cassava plants were sliced from the cut end (proximal, P) outward distally (distal, D) with a 0.1 mm razor blade. Simultaneous experiments were conducted without SOSG to view detectable auto-fluorescence within the root tissues. Using the listed wavelengths as well as bright field, there



**Cassava root gene expression 4 hours after harvest**

**Fig. 2** RT-PCR analysis using gene specific primers of cDNA prepared from roots of cassava 4 h after harvest. *Abbreviations:* PCD programmed cell death, CP centromere binding protein, ROS reactive oxygen species, PX peroxidase, GST glutathione transferase, AKR aldo/keto reductase, SOD superoxide dismutase, CAT catalase, CYP79 hydroxyvaline synthase, CYP71 acetone cyanohydrin synthase. HNL hydroxynitrile lyase, HRGP hydroxyproline-rich glycoprotein, XET xyloglucanendotransglycosylase, ACCO aminocyclopropane carboxyl-oxidase, rRNA ribosomal ribonucleic acid

were minimal spots of auto-fluorescence, particularly within the wavelengths necessary to visualize  $^1\text{O}_2$  (Fig. 3a–d). Fresh cassava root sections exposed to SOSG or ddH<sub>2</sub>O are shown in Fig. 4a and b. Strong fluorescence is noticed with SOSG (Fig. 4a) compared to root sections treated with ddH<sub>2</sub>O (Fig. 4b). Most of the root parenchyma cells appear to show fluorescence (Fig. 4a), especially localized in the cell wall. SOSG stains areas intensely green within the root parenchyma at the specific wavelengths known to illuminate  $^1\text{O}_2$  (Fig. 5a–c).

#### Cassava Root Secondary Metabolites

Four phenolic compounds in extracts prepared from greenhouse-grown cassava roots have been tentatively identified. By HPLC matching of all samples to standards using a diode array detector we show unequivocally that

no scopoletin is detected at day 0, 4 h postharvest, yet significant quantities of rutin were detected (Table 3). In fact, after 3 replicate analyses of each root section, we detected no scopoletin. Under our experimental conditions, authentic scopoletin standards failed to match the retention time (RT) or UV-Visible spectrum for any compound in any experimental sample. Other classes of phenolics putatively identified were: coumarin, flavanones, phenol and flavan-3-ol (pooled quantities shown in Table 3). Although there was a variable concentration difference in samples proximal and distal to the detachment point, no discernable pattern was evident. Nevertheless, of the 4 phenolic secondary metabolites, 3 tended to accumulate in the areas of tissue proximal to the wound site (Table 3).

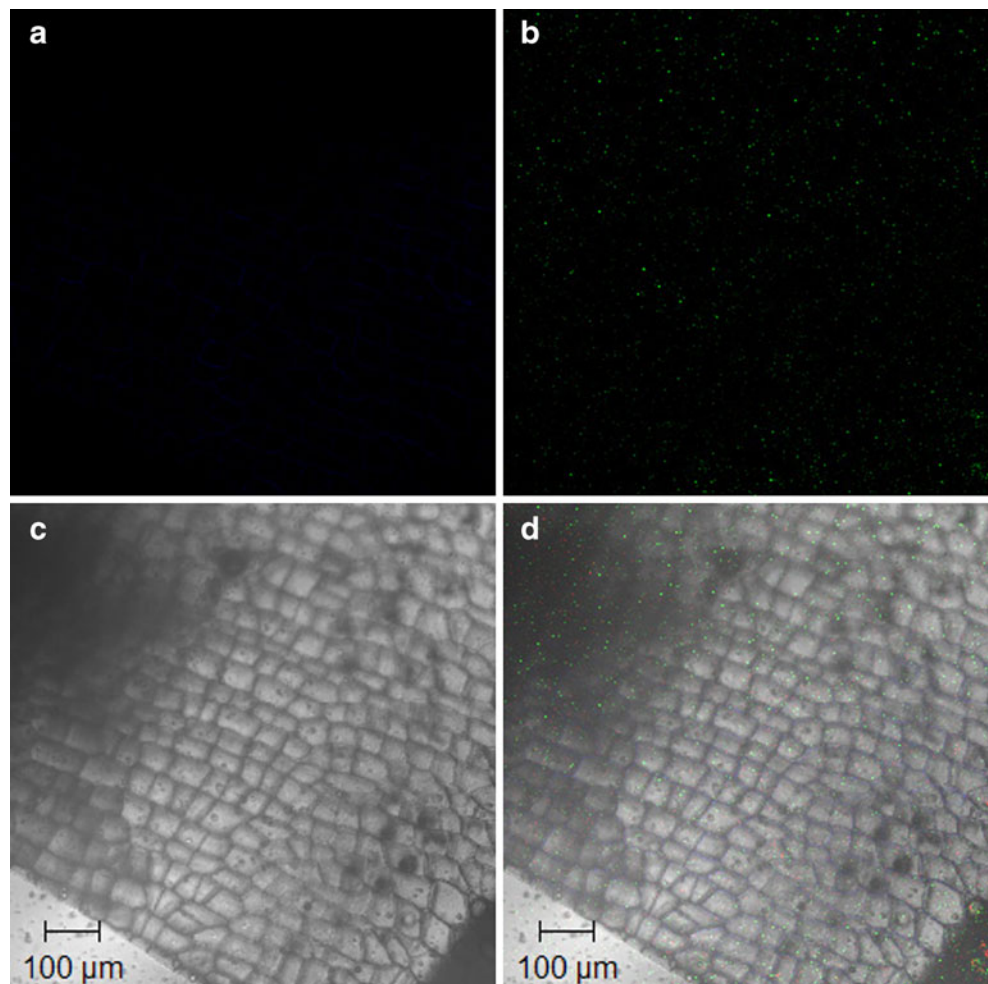
#### Respiration and Ethene Production in Cassava Root

Respiration rates of freshly harvested cassava roots are shown in Fig. 6. Cassava respiration is erratic during the first 12 h of our study, but stabilizes by day 1 at 66°F and 34°F (Fig. 6 top). At day 1 of our study, the respiration rates of the two 66°F samples increased and paralleled until day 6, peaking at about 70 ml/Kg/Hr (Fig. 6 top). The cassava root at 34°F maintained a rate at roughly 5 ml/Kg/Hr until day 6 (Fig. 6 top). The rate of ethene production increased at day 2 for the large cassava root sample (Fig. 6 bottom), and not until day 3.5 for the smaller root, both held at 66°F. However, the ethene production in the small root sample was erratic until day 6 (Fig. 6 bottom). There was no ethene detected in the root sample held at 34°F (Fig. 6 bottom). The respiration data measured by gas chromatography using thermal conductivity detection (GC-TCD) was compared directly to an infrared gas analysis (IRGA) system. The results are very similar throughout 6 days of postharvest sampling (data not shown). The initial respiration rate by day 1 was nearly identical using either GC-TCD or IRGA methods, at 20 ml/Kg/Hr, (data not shown). Thereafter, the respiration rates paralleled until day 6. The sample of cassava root analyzed by the IRGA system was much smaller than that of the GC-TCD (Table 4). The cassava sample analyzed by IRGA was only 7.2 g held at 20 ml/min compared to the GC-TCD sample which was 41.3 g and held at 100 ml/min (Table 4). The chamber sample mass to void volumes, were however, similar and therefore the respiration rates were similar as was the rate of water loss.

#### Volatile Compound Detection in Cassava Root

Volatile emissions from cassava leaves have been well studied. Hountondji et al. (2005) detected 2-butanone, 3-pentanone, (E)-2-hexenal, (Z)-3-hexen-1-ol, (E)-2-hexen-1-ol, in cassava leaves infested with the green mite (*M.*

**Fig. 3** The proximal zone (P) of cassava roots showing **a** view from  $\lambda 405$  excitation,  $\lambda 420$ – $480$  emission, **b**  $\lambda 488$  excitation,  $\lambda 505$ – $570$  emission, **c** bright field view, and **d** the images merged. Magnification  $10\times$

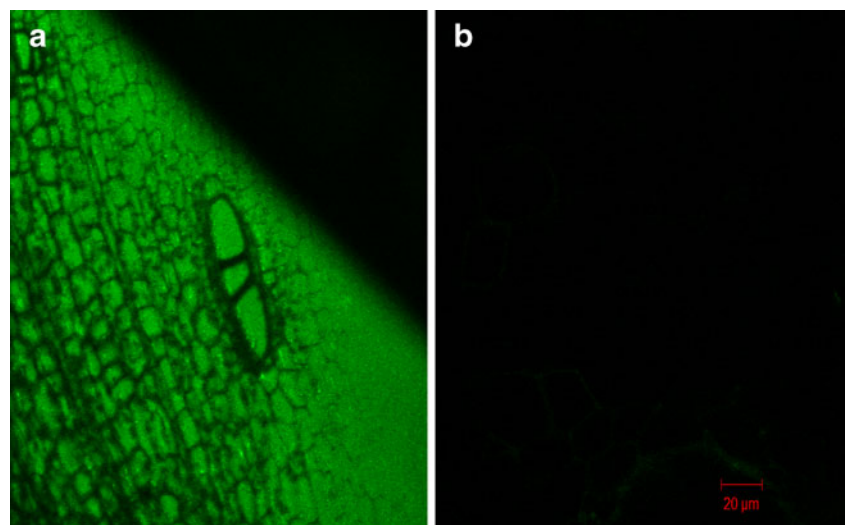


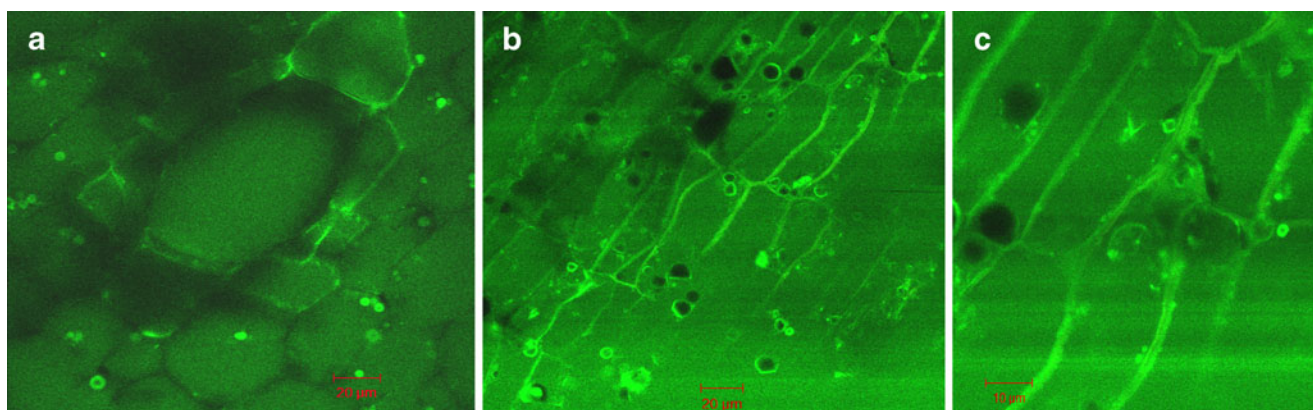
*tanajoa*). We have found these molecules in common to volatiles detected in the present root study.

Volatile molecules isolated and identified from cassava roots have not been reported with the exception of BioCassava reports from our laboratory. In our laboratory, we were able to identify and quantify 18 volatile com-

pounds from cassava roots, including 8 ketones, 1 cyanohydrin, 6 aldehydes, and 3 alcohols, by the use of Solid Phase Micro-extraction (SPME) and gas chromatography/mass spectrometry (GC/MS) (Table 5). Among these volatiles, ketones were found in the highest amount quantitatively, followed by acetone cyanohydrin, aldehydes,

**Fig. 4** Confocal images showing the cross section of cassava root tissue after **a** staining in  $50\ \mu\text{M}$  SOSG, and **b** staining in  $\text{ddH}_2\text{O}$ . Magnification  $10\times$





**Fig. 5** Confocal images of cassava root sections at 63 $\times$ , **a** 1 h after harvest (18-mo-old WT plant), **b** a proximal zone section 3 h after harvest (12-mo-old TMS 60444 cultivar), **c** magnification of image (**b**) at 105 $\times$ . Images were viewed at  $\lambda$ 488 excitation and  $\lambda$ 505–570 nm long pass emission

and alcohols. In initial experiments with cassava roots the highest volatile concentration was 2-propanone and 2-butanone at 97.2% and 1.7% of the total volatiles. Cis-3-hexenal, hexanal, trans-(E)-2-hexenal, nonanal, and decanal accounted for 0.6% of the total volatiles. The alcohols were in very low amount at 0.08% of the total volatiles. However, in more recent experiments our results show that the volatile percentages can shift from 95% ketone to about 41.1% ketone and 58.7% cyanohydrin (data not shown), owing to incomplete hydrolysis of cyanohydrins—a spontaneous process. The aldehydes remained at a low percentage at 0.19% as well as the alcohols at 0.03% (data not shown).

Volatile molecules reported in cassava leaf are shown in Table 5 as a comparison to cassava root. At this time 16 compounds have been detected by our laboratory. Among these, most are in common with cassava root, and only 5 of the volatiles shown have been previously reported by Hountondji et al. (2005).

Among the volatile compounds detected in our root experiments (Fig. 7a), 2-propanone cyanohydrin or 2-hydroxy-2-methyl propanenitrile, the metabolite released by linamarin hydrolysis during the first step of cyanogenesis (Fig. 7b) was conclusively identified (Fig. 8) by an identical mass spectrum match to the known standard (data not shown) and proposed by White et al. (1998) to exist in cassava. The final sampling included 6 replications of 3.0 g root tissue. The middle root tissue had a higher level of

acetone cyanohydrin than the proximal or distal portions, and followed a pattern similar to ketone production (Fig. 8a).

## Discussion

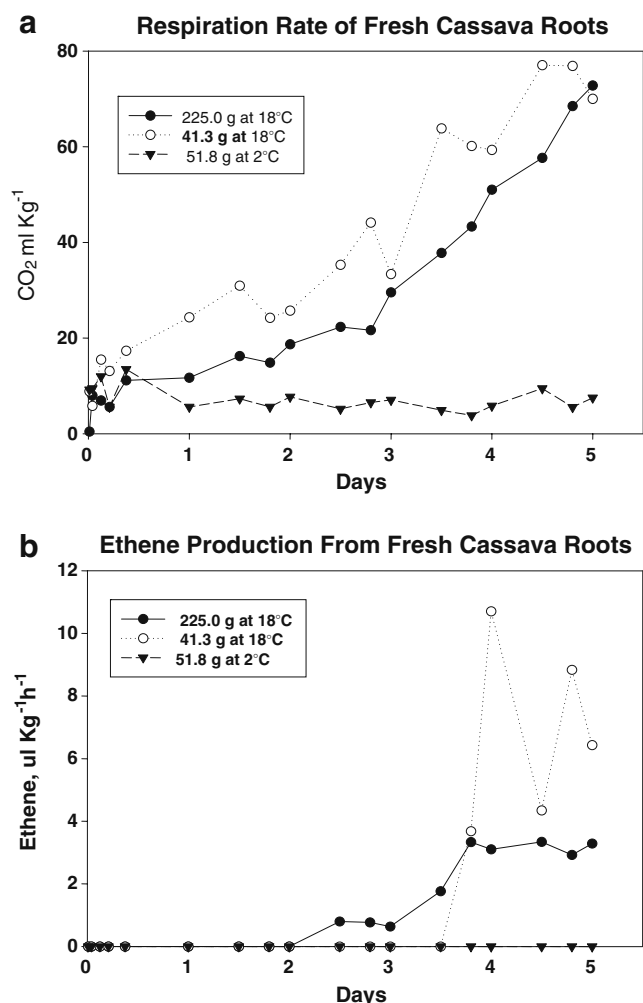
Cassava is a major food crop in the tropical and subtropical regions of the world. Since, during the process of harvesting, wounding can lead to rapid PPD, the characterization of PPD at the molecular and physiological levels have major implications for storage, utilization, and marketing of cassava. Here we have attempted to identify the early (4 h after detachment) signaling events leading to PPD of cassava roots. The development of PPD at the molecular level is quite complex (Reilly et al. 2003), and the 24 h post-detachment gene expression reported apparently is initiated very soon, within 4 h after harvest. Our analyses of the transcripts that accumulated 4 h post-detachment has shown that many of the genes that are commonly involved in cell repair, scavenging free radicals, and programmed cell death are expressed very early even before PPD is fully initiated, within the time course of our study. Also many of the ROS-scavenging enzymes seem to accumulate closer to the site of wounding, indicating that most of the gene transcription and enzyme activities leading to the onset of PPD occur very early in the root tissue proximal to the detachment wound.

The use of SOSG to detect  $^1\text{O}_2$  revealed several interesting findings. A relationship is described by Flors

**Table 3** Concentration of phenolic secondary metabolites as a function of distance from harvest wound in cassava root tissue samples

Tissue location	Scopoletin <sup>a</sup>	Rutin	Total phenolic compounds in extract
Proximal	0	1.17 $\pm$ 0.34	2.87 $\pm$ 0.20
Middle	0	0.91 $\pm$ 0.13	2.23 $\pm$ 0.10
Distal	0	0.76 $\pm$ 0.24	1.45 $\pm$ 0.18

<sup>a</sup> quantities expressed as  $\mu\text{g/g}$  (FW)  $\pm$  SD  $n=3$  measured by HPLC as described in the text



**Fig. 6** Cassava root respiration (a), and the rate of ethene production (b), at 18°C and 2°C

et al. (2006), whereby  $^1\text{O}_2$  is produced along with conjugated dienes in the presence of liposomes reacting with myoglobin, therefore showing an increase in fluorescence. The particular reaction which may generate  $^1\text{O}_2$  in cassava root thus initiating PPD is presently unknown. The first experiment revealed little auto-fluorescence within cassava tissue treated with ddH<sub>2</sub>O or SOSG (Fig. 3a–d). Root tissue stained with SOSG did in fact reveal a strong fluorescence (Fig. 4a) versus the ddH<sub>2</sub>O soaked tissue

(Fig. 4b). As we are seeing both symplastic staining and apoplastic staining, most of the root parenchyma cells appear to show fluorescence (Fig. 4a), however, upon further magnification SOSG stains parenchyma cell intensely green at the cell wall, as expected in actively-expanding plant cell tissue, where oxygen radicals are thought to play a role as site-specific oxidants involved in cell-wall loosening (Fry 1998). More pertinent to our study is the intense green fluorescence observed in unidentified structures within the cell (Fig. 5a–c). These may be organelles responsible for generating additional oxidative stress leading to cassava PPD symptoms. The results using SOSG as a  $^1\text{O}_2$  detection system provide a new tool for determining if  $^1\text{O}_2$  is involved in cassava root early PPD. The result presented suggests that a freshly harvested root does in fact show areas within root parenchyma cytoplasm where  $^1\text{O}_2$  may occur. We are presently conducting investigations with additional fluorescent probes specific to other ROS.

Our cassava secondary compound measurements (Table 4) differed from those of Uritani (1998) who found trace amounts of scopoletin upon harvest then wounding, and Bushmann et al. (2000a) who detected near 25 nmol/g fresh weights in several cassava cultivars after 1 day of harvest. Bushmann et al. (2000a) included highly PPD-susceptible cultivars including MCOL22 which is very similar to the variety used in our study; however, we detected no scopoletin. Also, unlike Uritani (1998) who detected both scopolin and esculin after 10 h of wounding, similar to Bushmann et al. (2000a), our results detected none of these coumarins 4 h after detachment (data not shown). In fact, we compared our extracts with standards for all the coumarins and flavonoids reported to occur in cassava root during early PPD, including compounds reported by Bushmann et al. (2000a, b) (data not shown). A novel study by Wheatley and Schwabe (1985), describes an 80  $\mu\text{g/g}$  scopoletin level correspondent to the highest level of PPD scored within the proximal root zone 1 day after detachment. The study also described oxidation of several phenolic and coumarin compounds externally applied at harvest followed by PPD scoring. Considering these results, along with scopoletin implications as a PPD causal agent (Bushman et al. 2000a) our results agree

**Table 4** The physical characteristics described during the greenhouse grown cassava respiration studies, including sample weight, chamber flow and chamber void volume to mass ratio, and % weight loss and temperature during 6 days of the study

Sample weight (g)	Chamber flow, ml/min	Chamber sample mass :void volume, g/ml	Weight loss %	Temp °C
7.2	20	0.360	13.8	15
41.3	100	0.413	12.5	18
225.0	100	2.25	4.9	18
51.8	100	0.518	3.8	2



**Table 5** Volatile compounds of cassava root and leaf identified using solid phase micro-extraction gas chromatography/mass spectrometry

Peak number	<sup>a</sup> KI	Root	Leaf
1	438	2-propanone	2-propanone
2	557	2-butanone	2-butanone
3	648		1-pentene-3-one
4	656		1-pentene-3-ol
5	662		3-pentanone
6	718		(E) 2-pentanal
7	728	2-hydroxy-2-methyl propanenitrile	
8	755	3-hexanone	
9	758	2-hexanone	
10	764	3-cis-hexenal	3-cis-hexenal
11	767	hexanal	hexanal
12	821	(E) 2-hexanal	(E) 2-hexanal
13	836	(Z) 3-hexen-1-ol	(Z) 3-hexen-1-ol
14	850	(E) 2-hexen-1-ol	(E) 2-hexen-1-ol
15	853	1-hexanol	
16	863	3-heptanone	
17	865	2-heptanone	
18	921		(E) 2-heptanal
19	961	3-octanone	
20	964	2-octanone	
21	972	octanal	octanal
22	1073	nonanal	nonanal
23	1173	decanal	decanal

<sup>a</sup> Kovats retention indices relative to a n-alkane series calculated on a 60 m DB-1 column

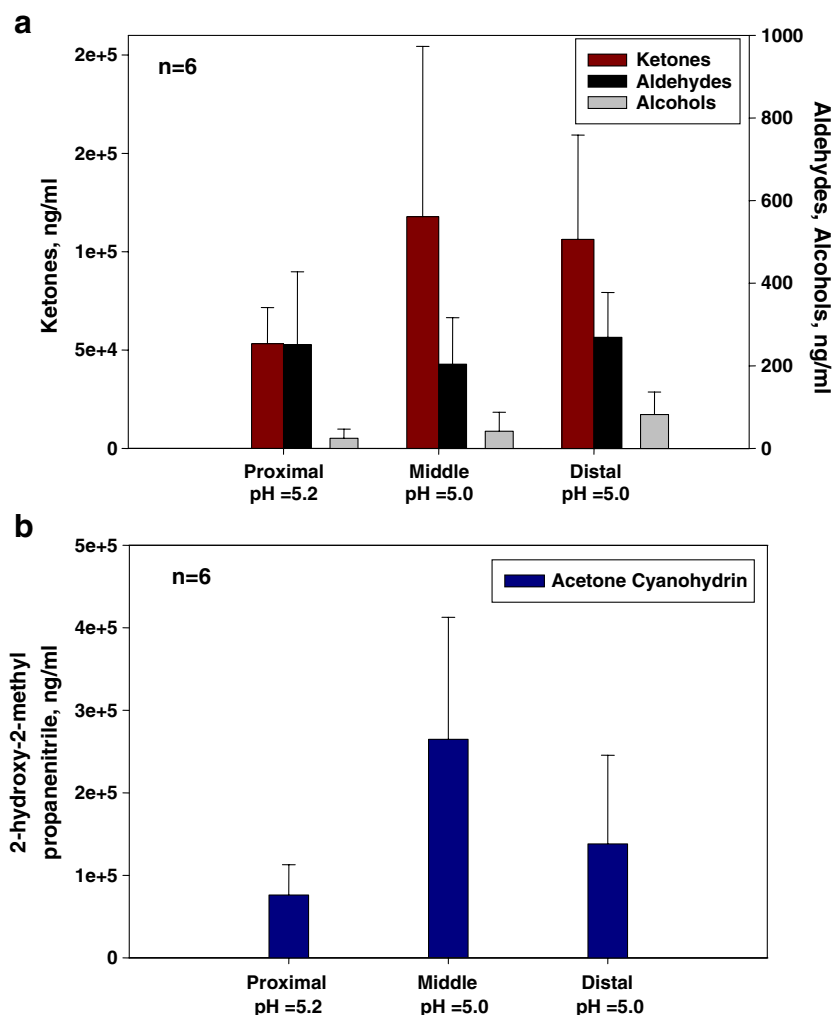
more with those of Wheatley and Schwabe (1985). Apparently scopoletin may not accumulate until well into the onset of PPD, beyond the time course of the study reported here.

The respiration rate of cassava root is not well documented. Marriott et al. (1979) showed an increase in the respiratory rate of injured cassava roots when water loss occurred from the injury site, with development of vascular discoloration. The authors showed that this was apparently independent of the extent to which vascular discoloration occurred in root sections of injured cassava, and that water stress and accompanying respiratory response are not sufficient conditions for its development. The cassava root respiration measurements were performed on samples after several days of storage, and with varying degrees of wounding (Marriott et al. 1979). In sweet potato, it was shown that wounding-induced respiration rates were activated within 24 h in the tissue as well as in the mitochondria, and this activation was maintained with an increase in the enzyme activities associated with respiration within 12 h that was sustained for almost 96 h. The respiration rate was found to be biphasic. Similarly, the respiration rate was stimulated in cassava roots as a response to wounding during 1 day of incubation, and increased within 3–4 days after incubation (Uritani 1998). The first and the second phases of respiration increases

could be due to wounding and primary physiological deterioration, respectively (Uritani and Reyes 1984). The phytohormone ethene is produced in cassava roots within 6 h of wounding (Plumbley et al. 1981; Hirose 1986), with higher ethene production in PPD-susceptible roots. Solomos and Laties (1976) suggested the activation of an alternative cyanide insensitive respiratory pathway by ethene. This pathway is known to exist in cassava tissues (Passam 1976), and it is proposed that this pathway may lead to the formation of peroxides (Chin and Frenkel 1976). However, pre-harvest pruning, a practice known to delay PPD, did not prevent ethene accumulation (Hirose 1986) thus raising questions about the role of ethene as a signal for the onset of cassava root PPD.

An analysis of respiration rate and ethene production may help in the determination of early PPD factors associated with wounded cassava root. Greenhouse-grown cassava roots show a burst in respiration followed by an increase in ethene emission by day 2 and day 3, depending on root size (Fig. 6). On the other hand, roots held at 34°F do not show an increase in respiration rate or ethene (Fig. 6). The respiration rate of cassava root is not well studied, and has only been reported on samples after several days of storage, and with varying degrees of wounding (Marriott et al. 1979), or on cut sections from 10 cm roots (Plumbley et al. 1981). Neither of these studies studied

**Fig. 7** Cassava root volatile production at day 0–3 h ( $n=6$ )  
(a). Detection of acetone cyanohydrin (b)



non-injured intact roots, nor did the authors report respiration rate or ethene production on a weight basis. This work reports cassava root respiration rates from initial harvest up to 6 days after detachment.

Several of the ketones detected in cassava root (Table 5) have been previously detected in our laboratory from barley and wheat slurries (Cramer et al. 2005), processed blueberry samples (Feng et al. 1999), fresh red raspberries (Moore et al. 2002), sweet cherries after CA storage (Mattheis et al. 1997), and apple fruits (Mattheis et al. 1991). The operating biosynthetic pathway which leads to ketone formation in fresh fruit seems to be via  $\beta$ -oxidative shortening of fatty acids followed by reduction of the carbonyl functional group on C4–C12 carboxylic acids (Paillard 1979). Alternative pathways may involve the  $\alpha$ -oxidation pathway, leading to odd chain ketone formation in fruit (Fellman et al. 2000). The formation of even chain or odd chain ketones may involve these pathways; however, the formation of 2-propanone and 2-butanone can be attributed to hydroxynitrile lyase (HNL) activity in cassava (Lauble et al. 2001). The enzyme utilizes 2-

propanone cyanohydrin and 2-butanone cyanohydrin, which are hydrolysis products of their respective cyanogenic glycosides (linamarins), and undergo further breakdown upon mechanical chewing of root tissue or fungal infestation, thus leading to HNL release of hydrogen cyanide (HCN) and a ketone depending on the initial substrate (Sharma et al. 2005) (Fig. 8). The release of a ketone, primarily acetone, and HCN from artificially-supplied substrate is well described by White et al. (1998) in cassava root, as well as their release in cassava roots over-expressed for HNL in transgenic plants (Siritunga et al. 2004). The release of a ketone and HCN is dependent on pH and temperature in cassava roots. The amount of ketones detected between each root section as well as the 2-propanone cyanohydrin first proposed by White et al. (1998) now proven to exist in cassava indicates a potential signaling event within a root 3 h after detachment, as well as an effect on cyanogenesis (Fig. 6).

Our laboratory has successfully characterized the volatile metabolites of cassava root, including the first published reports of GC/MS detection for acetone, butanone, and

acetone cyanohydrin. The ability to isolate the cassava volatile metabolites may allow an expedient test for cyanogenesis, a method to ascertain signaling within a cut root implicating early PPD, and a method to screen new cassava genotypes.

### Concluding Remarks

Our data show that many events leading to the onset of PPD occur very early after wounding, in agreement with the pioneering study of Reilly et al. (2003), where an oxidative burst was observed within 15 min of root detachment injury. The fact that these changes appear closer to the site of wounding may indicate the first cellular responses to wounding stress with a subsequent release of unique volatile metabolites, e.g. acetone, away from the detachment wound site where no corroborating molecular evidence of PPD is observed. This may indicate a potential signaling function for acetone that remains to be determined. It is interesting to note the presence of acetone cyanohydrin emissions from tissues with no demonstrable linamarase gene expression. In addition, the oxidation of coumarin, implicated in the PPD-associated vascular

darkening reaction did not occur because there was little coumarin accumulation detected in the time course of our study. These observations will help direct the future development of a cassava root with increased postharvest shelf life. It will facilitate the identification of specific metabolic pathways and proteins involved in PPD, and will advance our knowledge and understanding of the early signaling events contributing to the onset of cassava root PPD. Available data will assist in the design of effective strategies in order to delay the development of PPD, as well as determine biochemical differences between PPD susceptible and resistant cultivars. The ultimate goal will be to improve the full utilization of this staple food by people that depend heavily on its consumption.

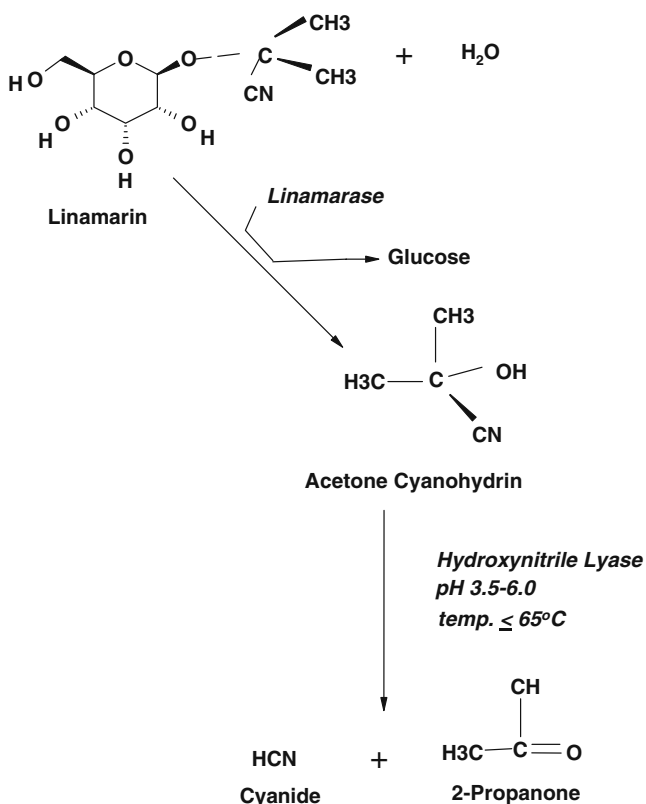
### Materials and Methods

#### Cassava Sampling

The roots were obtained from mature cassava plants grown in the Washington State University Greenhouse Facilities. Standard growth conditions were 29°C average temperature, Relative Humidity approximately 31%. Photoperiod was 14 h of light (minimum), with halide sodium vapor lighting supplement during the winter months. Supplemental lighting was not used when over 15 h of natural sunlight was available. Plants were fertilized with a 20-20-20 soluble formula at 200 ppm(mg/l) N once per week as a pot drench. Plants were allowed to dry thoroughly between water applications. Early experiments(POX and lipid peroxidation) used a wild-type *Manihot* of unknown origin. The remainder of the experiments used cultivar TMS-60444 (obtained from Dr. Nigel Taylor, ILTAB, St. Louis MO USA) Several samples of cassava roots were harvested in order to study; volatile analysis, flavonoid analysis in root sections, in situ singlet oxygen detection, respiration, and gene expression studies. The sampling technique consisted of cutting a freshly harvested root from the plant. Respiration and ethene measurements commenced immediately upon harvest. The biochemical experiments used the harvested root which was allowed to stand at room temperature then cut into proximal, middle, and distal sections. At the time of root harvest and sectioning, all of the samples were diced into sub-samples and frozen in Liquid N<sub>2</sub> and stored at -80°C until sampled further.

#### Volatile Analysis

Specific identification and quantification were carried out by using the (HS-SPME) technique. The cassava samples were sampled after either fresh harvest or from -80°C storage. A sample was prepared with cassava tissue



**Fig. 8** The biochemical pathway leading to the synthesis of cyanide and 2-propanone production known in cassava. The compound linamarin is hydrolyzed to acetone cyanohydrin, which then undergoes further oxidation to cyanide and 2-propanone(acetone) via enzyme or high pH and temperature. Adapted from White et al. (1998)

(~3.0 g), sodium chloride (1.3 g), and distilled water (4.0 mL) in a 25 mL flask using a open hole cap and Teflon-faced silicone septa. The sample was stirred at about 500 rpm for 60 min. The use of 1-pentanol (0.6 µg/mL) was included in some of the experiments as an internal standard. A SPME fiber was then exposed to the headspace of the slurry. Two fibers from a flavor assortment kit (Supelco Co, Bellefonte, PA) were tested: 65 µm polydimethylsiloxane/ divinylbenzene (PDMS/DVB), and 75 µm carboxen/ polydimethylsiloxane (CAR/PDMS). The selection of the fiber to be used for further analysis is based upon its affinity for the cassava volatiles and the symmetry of chromatographic peaks. The volatile compounds under study in this work used a PDMS/DVB fiber and were collected for 1 h based on our previous optimization studies (Cramer et al. 2005) using barley and wheat slurries.

The volatiles adsorbed onto the SPME fiber were thermally desorbed into the injection port of a HP5890/5970 GC/MS system (HP now Agilent, Avondale PA) equipped with a DB-1 column (60 m×0.32 mm i.d., 0.25 µm film thickness, J&W Scientific, Folsom, CA). Helium is used as the carrier gas. The injector and detector temperatures were 200°C and 250°C, respectively. The column temperature is initially maintained at 33°C for 5 min before increasing to 50°C at a rate of 2°C/min, and then to 225°C at a rate of 5°C/min. The sample is desorbed for 2–5 min using a SPME liner according to Yang and Peppard (1994), and set in the splitless mode. Cassava root volatile compounds were identified by comparing the MS spectra against a Wiley-NBS library. The RI was calculated based on Kovat's retention indices using a series of straight-chain alkanes (C4–C14) under the chromatographic conditions described above. The quantitative data were determined by running known standards then developing response factors based on water matrices. The final values were reported as µg/g (FW) then reported as averages with standard error.

#### Flavonoid Extraction and Analysis

The procedures of Miller et al. (1998), and Warren et al. (2003) were used to purify and quantify cassava flavonoids and related phenolics. The extraction, purification, HPLC separation, and quantitation were modified from methods described by Macheix et al. (1990). Flavonoid extracts (10 µl injections) were analyzed with an Agilent 1100 series HPLC system with autosampler, column oven, and an internal diode array detector (Agilent, Technologies, Avondale, PA) equipped with a 5 µM C-18 Zorbax-SB column and guard column (250×4.6 mm with detection at λ254 nm). The mobile phase consisted of a solvent gradient of 90:10 (0.5% phosphoric acid: 100% methanol) increased linearly to 30:70 over 40 min, maintained at 30:70 for 5 min, cleaned with

100% methanol for 13 min, and then re-equilibrated to 90:10 for 2 min, all at a flow rate of 1.0 ml min<sup>-1</sup>. We analyzed standards by retention time and repeatability, based on data obtained from standard compounds known to occur in cassava roots (data not shown). Since the qualitative data obtained consist of several as-yet unidentified compounds, only quantitative information is reported.

#### ROS Related Enzyme Assay Methods

**SOD Activity:** Enzyme extracts were added to the reaction medium, which consisted of 13 mM methionine, 75 µM NBT, 2 mM riboflavin, and 0.1 mM EDTA in a total of 3.0 mL potassium phosphate buffer pH 7.8. The reaction is light mediated, and is started by shining fluorescent light (400 µE m<sup>-1</sup> s<sup>-1</sup>) at RT. The reaction was stopped after 10 min by transferring the tubes to dark, and A<sub>560</sub> was recorded. In the absence of light the reaction failed to develop color. This served as a blank. The A<sub>560</sub> was maximum in a reaction mixture with no enzyme. A standard curve of A<sub>560</sub> was plotted as a function of concentration in SOD units using commercial SOD (Sigma) (Kumar and Knowles 1993).

**MDA Activity:** 2 g (FW) of tissue was extracted with 10 mL of 0.1% (w/v) TCA containing 2 mM Na<sub>2</sub>S<sub>2</sub>O<sub>5</sub> and centrifuged at 10,000 g for 10 min. A 1 mL aliquot of each supernatant was vortexed with 4 mL of 20% (w/v) TCA containing 0.5% (w/v) TBA and heated at 95°C for 30 min. The samples were cooled on ice for 5 min and centrifuged at 1,640 g for 20 min. The non-specific absorbance at 600 nm was subtracted from the absorbance at 532 nm. MDA equivalent, a relative measure of lipid peroxidation, was calculated on the resulting difference using the extinction coefficient of 155 nm<sup>-1</sup> cm<sup>-1</sup> (Heath and Packer 1968; Dhindsa et al. 1981).

**POX Activity:** Qualitative determination of peroxidase isozymes was performed using a method adapted from that of Miller et al. (1998). 2 g (FW) of tissue sampled as described below was extracted with 4 mL 50 mM Tris buffer (pH 8.0) containing 2 mM sodium bisulfate and 2 mM dithiothreitol (DTT). The resulting homogenate was filtered, and centrifuged at 30,000 × g. Gels were loaded with 200 µL extract and after electrophoresis (Bio-Rad Mini Protean) at 30 mA for 40 min, POX was visualized after washing with 0.2 M sodium acetate pH 5.0 containing 2 mM calcium chloride using 10 mM o-dianisidine (Sigma) mixed with 30% H<sub>2</sub>O<sub>2</sub> in a 100:1 ratio.

Singlet oxygen detection using singlet oxygen sensor green (SOSG), (Molecular Probes, Inc., Eugene, OR.)

The use of a novel singlet oxygen sensor was investigated in fresh cassava roots. A long thin root (20 cm) was

harvested from an 18 month old plant. The root was transported in a plastic bag with a wet towel to the laboratory. Within 1 h the root was sliced with a 0.1 mm razor blade from the cut end (proximal) outward distally. The sections were randomly placed into petri dishes with ddH<sub>2</sub>O or SOSG, (Molecular Probes, Eugene, OR.). The concentration of SOSG was 50  $\mu$ M from a stock consisting of 5 mM in methanol. The SOSG was held at  $-80^{\circ}\text{C}$  until the time of its use, upon use and dilution SOSG was covered in foil. The root sections were allowed to soak in the dark (foil covered) for up to 15 min. The sections were examined with a laser scanning confocal microscope, Zeiss LSM 510 (Washington State University EM Center), using  $\lambda$ 488 nm excitation and  $\lambda$ 505–570 nm emission. Individual root sections were viewed at 10 $\times$ , 63 $\times$ , and 105 $\times$  power until visible images were observed. The particular settings are suggested when analyzing biological tissues capable of generating singlet oxygen  $^1\text{O}_2$  (Flors et al. 2006). The aim of our work was to mimic a singlet oxygen detection system where rapid lipid peroxidation would occur, as well as potential protein oxidation, as outlined by Halliwell and Gutteridge (1999).

#### Root Respiration and Ethene Production

Evolved ethene and CO<sub>2</sub> production were measured by placing samples of pre-weighed groups of cassava roots into air tight flow chambers using an automated sampling system (Patterson and Apel 1984). The plexiglass chambers (18 L) at 66 $^{\circ}\text{F}$  and (6 L) at 34 $^{\circ}\text{F}$  were supplied with ethene-free air at approximately 100 ml min<sup>-1</sup>. The carbon dioxide (CO<sub>2</sub>) and ethene concentrations from each chamber were automatically measured every 8 h for 24 h using a HP 5890A gas chromatograph (Hewlett-Packard Co., Palo Alto, CA, USA). The system was equipped with a thermal conductivity detector, and a 0.53 mm $\times$ 30 m GS-Q-PLOT column (Agilent Technologies, Avondale, PA, USA) for measuring CO<sub>2</sub>, and a FID connected to a 0.53 mm $\times$ 15 m GS-Q-PLOT column (Agilent) for measuring ethene. The system uses two electronic switching valves to measure from one flow path. The oven, injector, and TCD were held at 30 $^{\circ}\text{C}$ , 200 $^{\circ}\text{C}$ , and 90 $^{\circ}\text{C}$ , respectively. The FID was held at 200 $^{\circ}\text{C}$ . The CO<sub>2</sub> column had a flow rate of 8 ml/min. The ethene column had a flow rate of 10 ml/min. The CO<sub>2</sub> was reported as CO<sub>2</sub> ml Kg<sup>-1</sup> hr<sup>-1</sup> and ethene as C<sub>2</sub>H<sub>4</sub>  $\mu$ l Kg<sup>-1</sup> hr<sup>-1</sup>. The IRGA measurements were performed using a standard LI-6262 infrared gas analyzer (LICOR Inc., Lincoln, NB, USA). The flow stream was controlled by a three-way solenoid valve switching every 4 h, whereby the CO<sub>2</sub> concentration was recorded. The respiration was monitored continuously and the rate reported as CO<sub>2</sub> ml Kg<sup>-1</sup> hr<sup>-1</sup>. The system was similar to that described by Kumar and Knowles (1993).

#### Gene Expression Studies Concerning Early PPD in Cassava Root

##### *Tissue Sampling*

Storage roots were harvested from 14-month-old greenhouse grown cassava trees and sectioned into proximal (P), mid (M), and distal (S) portions (as previously described), proximal being closest to the harvested end. Each section was immediately chopped into  $\sim$ 1 cm<sup>2</sup> chunks and quickly frozen in liquid nitrogen. Quick frozen samples were bagged, labeled, and stored in the  $-80^{\circ}\text{C}$  freezer until use.

##### *RNA Isolation and cDNA Synthesis*

For total RNA isolation, pieces were taken at random from each section from the freezer and ground to a fine powder in liquid nitrogen. Three samples of each section were ground separately. About 0.5  $\mu$ g of powder was used for RNA isolation using the RNeasy Plant Mini Kit (QIAGEN Inc., Valencia, CA) following the manufacturer's recommendations. Five  $\mu$ g of total RNA was DNase treated with the TURBO DNA-free Kit (Ambion Inc., Austin, TX) following the standard protocol, and 1  $\mu$ g of DNA-free RNA was used for cDNA synthesis using the First Stand cDNA Synthesis Kit (Fermentas Inc., Hanover, MD).

##### *Polymerase Chain Reaction*

PCR was carried out with GoTaq Green DNA polymerase (Promega Corporation, Madison, WI) with gene specific primers. The forward and reverse primer sequences are given in Table 2. For PCR, an initial denaturation at 94 $^{\circ}\text{C}$  for 2 min was followed by 30 cycles of 94 $^{\circ}\text{C}$  for 30 s, 46 $^{\circ}\text{C}$  /50 $^{\circ}\text{C}$  for 30 s, and 72 $^{\circ}\text{C}$  for 45 s. Final extension was accomplished at 72 $^{\circ}\text{C}$  for 5 min. As a loading control 18S rRNA was used.

**Acknowledgements** We gratefully acknowledge the help and services provided by Dr. Michael Knoblauch, Chris Davitt, and the Franceschi Microscopy and Imaging Center (FMIC), Washington State University. Also we thank Dr. Mohan Kumar and N. Richard Knowles for enzyme and respiration assistance. The studies are supported by grants from the Bill and Melinda Gates Foundation BioCassava Plus Project.

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