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

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ABSTRACT

Cassava roots are an important source of dietary and industrial carbohydrates and suffer markedly from postharvest physiological deterioration (PPD). This paper deals with metabolomics combined with chemometric tools for screening the chemical and enzymatic composition in several genotypes of cassava roots during PPD. Metabolome analyses showed increases in carotenoids, flavonoids, anthocyanins, phenolics, reactive scavenging species, and enzymes (superoxide dismutase family, hydrogen peroxide, and catalase) until 3–5 days postharvest. PPD correlated negatively with phenolics and carotenoids and positively with anthocyanins and flavonoids. Chemometric tools such as principal component analysis, partial least squares discriminant analysis, and support vector machines discriminated well cassava samples and enabled a good prediction of samples. Hierarchical clustering analyses grouped samples according to their levels of PPD and chemical compositions.

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 south-east Asian countries. Nevertheless, as a tropical root crop, cassava is categorised as a postharvest deterioration sensitive specie (An, Yang, & Zhang, 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 in 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 the loss of acceptability of roots and it 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 taste. Primary deterioration also involves changes in oxidative enzyme activities, generating phenols including catechins and leucoanthocyanidins, which in later stages polymerise to form condensed tannins. On the other hand, 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 (Garcia, Sánchez, Ceballos, & Alonso, 2013; Sánchez et al., 2013; Zidenga, 2011; Wheatley & Schwabe, 1985; Booth, 1975; Maini & Balagopal, 1978).

Postharvest physiological deterioration (PPD) often begins rapidly within 24 h postharvest. 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 localise those regions of the cassava genome that control PPD. They concluded that the biochemical processes involved in

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the rapid deterioration of cassava are essentially wound-healing responses, which are well known in many plant species (Cortés et al., 2002).

Phytochemical analysis of compounds of low molecular weight (<500 Da) 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 used in 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 characterisation (Schulz & Baranska, 2007).

Aiming to identify changes in the chemical and enzymatic composition of four cassava genotype samples during postharvest deterioration and possibly seeking related biochemical markers, metabolomics and chemometric tools, e.g., multivariate analysis using algorithms implemented in the R language (R Core Team, 2014), 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 post-harvest physiological deterioration (fresh samples – t_{zero}, 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: 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). These cultivars were selected as they are widely used by small farmers, with morpho-agronomical characteristics and are currently lacking research efforts that will allow better applicability. The farm trials were conducted at the Ressacada Experimental Farm (Plant Science Center, Federal University of Santa Catarina, Florianópolis, SC, Brazil-27° 35′48″ S, 48° 32′ 57″ W), starting in September 2011. The experimental design was done in randomised blocks (DBCC), with 4 blocks (6.3 × 15 m^2/block) spaced at 1 m. Each block consisted of four plots (12 × 1.2 m^2/plot), spaced at 0.5 m. Cassava cuttings with 15 cm in length were used, which were planted upright, spaced 1 × 1 m. Each plot was considered a treatment. For laboratory analysis, each cultivar in each plot was mixed as a single sample. All crop management was mechanised and cultivation was done manually. The soil fertility was analysed previously and according to the results fertilisation was performed by applying calcium before cultivation (see Supplementary Table 2 for soil analysis).

2.2. Postharvest physiological deterioration (PPD)

Samples of 12-month-old cassava roots were collected and the same cultivars in each plot were mixed for the analysis of fresh samples, as well as for the 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 (0.5–1 cm) were made over the remaining root and stored at room temperature (66–76% humidity, ±25 °C). Monitoring the development of PPD and associated metabolic disturbances was performed daily after induction of PPD (see Supplementary Fig. 1 for root images during PPD). Fresh samples and those 3, 5, 8, and 11 days postharvest were collected, dried in an oven (35–40 °C), milled with a coffee grinder and stored (−18 °C) for further analysis. For the enzymatic analysis, fresh samples were collected and stored (−80 °C) until analysis.

2.3. Postharvest physiological deterioration scoring (PPD scoring)

Five independent evaluations of PPD were carried out under 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). The mean PPD score for each root was calculated by averaging the scores for the 3 transverse 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 transform spectroscopy (ATR-FTIR)

Infrared spectroscopy allows the analysis of physiological changes during postharvest physiological deterioration. Flour samples of fresh roots and from those 3, 5, and 8 days postharvest were collected for analysis. An IFS-55 (Model Opus v. 5.0; Bruker Biospin, Rheinstetten, Germany) spectrometer with a DTGS detector equipped with a golden gate single reflection diamond attenuated total reflectance (ATR) accessory (45° incidence angle) was used. A background spectrum of the clean crystal was acquired and samples (100 mg) were spread and measured directly after they were pressed on the crystal. The spectra were recorded in transmittance mode over a spectral window from 4000 to 400 cm^{-1} at a 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 data pre-processing stage (using R software), the spectra were normalised, baseline-corrected (see Supplementary Fig. 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 (Rubens, Snauwaert, Heremans, & Stute, 1999; Wilson & Belston, 1988). 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 a water bath at 55 °C, for 30 min. The mixture was centrifuged (4000 rpm/5 min), filtered through Whatman No. 2 filter paper, ethanol was removed using rotatory evaporator at 65 °C 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 were determined by the Folin–Ciocalteau reagent (FCR) method. For a total volume of 2.0 mL, 200 μL of extract were first mixed with 100 μL of FCR reagent after adding 1.40 mL of distilled water and the contents were kept at room temperature for 10 min. Later, 300 μL of Na_2CO_3 aqueous solution (20%) were added and incubated for 1 h. The absorbance was measured at 765 nm using a UV-visible spectrophotometer (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 and Singh (2011). 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) 0.002% methanolic solution (m/v) was prepared in a volumetric flask covered with aluminium foil. One millilitre of plant extract (g/mL) in methanol was mixed with 1 mL solution of DPPH (0.002%). For the blank solution, the extract was substituted with methanol. The 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 towards the DPPH radical. The 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.

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

2.6. Determination of total flavonoid content

The total flavonoid content of plant extracts was determined using the aluminium chloride colorimetric method (Woisky & Salatino, 1998 and revised by Chang, Yang, Wen and Chern (2002)) and standard solutions (0–1000 μg/mL of quercetin in 80% methanol). For that, 1 mL of extract solution was mixed with 0.5 mL 95% ethanol (v/v), 0.1 mL 1 M potassium acetate, 0.1 mL aluminium 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 min *versus* reagent blank containing water instead of the sample. Quercetin was used as the standard ($y = 0.0006x$, $r^2 = 0.98$) for the quantification of the total amount of flavonoids. Results were expressed as milligrams of quercetin equivalent per gram of dry weight (mg QE/g). Data were reported as the arithmetic means ± standard deviation (SD) for three replicates.

2.7. Determination of total monomeric anthocyanins

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

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

where A is the absorbance determined according to the equation

$$[(A_{520nm} - A_{700nm})\text{pH } 1.0 - (A_{520nm} - A_{700nm})\text{pH } 4.5] \quad \text{(A3)}$$

PM – molecular weight of cyanidin-3-glucoside (449.2 g/mol), DF-dilution factor (0.2 mL sample diluted to 2 mL, $DF = 10$), l – cuvette width (cm), ε-extinction coefficient (26,900 L/mol cm) of cyanidin-3-glucoside, and 10^3 – conversion factor g to mg (Hosseinian, Li, & Beta, 2008; Lee et al., 2005).

2.8. Determination of total carotenoids

Carotenoid content was determined as previously described (Ceballos et al., 2012). Briefly, 1 g of flour samples was added to 2 mL of cold acetone. After 10 min, 2 mL of petroleum ether were added and mixed using an Ultraturrax for 1 min. Samples were then centrifuged (3000 rpm/10 min), supernatant collected, 2 mL of sodium chloride 0.1 M were added, the solution centrifuged again (3000 rpm/7 min), dried in rotatory evaporator (55 °C), and the dried extract dissolved in 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 Eq. (4).

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

where A represents the absorbance, V the 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, Egan and Bradbury (1999) linamarin isolated from leaves as reported by Haque and Bradbury (2004), and linamarase by Yeoh, Bradbury and Egan (1997). The picrate paper was prepared by dipping a sheet of Whatman 3 mm filter paper (3 cm × 1 cm) 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, Egan, & Bradbury, 1999).

The filter paper was prepared by loading it with 50 μL of 1 M 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) were added and allowed to air dry (Bradbury et al., 1999).

2.9.2. Linamarin isolation from cassava leaves

In order to monitor cyanide contents in cassava samples, a simple method for the preparation of an acid-stabilised solution of linamarin, suitable for the preparation of standard linamarin filter paper discs, was chosen as previously reported by Haque and 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 0.1 M HCl. Five millilitres of 0.1 M HCl were added with further grinding and the pasty solution was filtered. The pink-coloured, cloudy solution was then centrifuged and the clear supernatant liquid (about 7 mL) removed with a Pasteur pipette. This solution, which also contained linamarase (inactivated in the 0.1 M HCl) and linamarin, was stored frozen in a deep freeze cabinet at −20 °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 2.1-cm diameter filter paper disc previously loaded with phosphate buffer 0.1 M 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 °C 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, as shown in Eq. (6) (Bradbury et al., 1999).

$$[\text{ppm} = A * 396] \quad \text{(A5)}$$

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 Eq. (6) (Bradbury, 2009). *Tcy* represents total cyanide and *AcCN* is acetone cyanohydrin.

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

2.9.3. Linamarase isolation from cassava leaves

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

2.9.3.1. Linamarase assay. The linamarase assay was carried out by using 1.5 mL of the homogenate previously prepared (Section 2.9.3), 0.5 mL of 5 mM linamarin in 50 mM of Na-citrate, pH 6.0 at 37 °C (Yeoh et al., 1997). After 15 min, the reaction was stopped by boiling the reaction mixture for 2 min and the glucose released was measured by the glucose oxidase method using the glucose-oxidase kit (Glucose-PAP, LAB TEST diagonostica). Briefly, 3 mL reagent were added to 0.3 mL of the sample, followed by mixing and incubation at 37 °C for 15 min 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.

$$\left[\text{Glucose}\left(\frac{\text{mmol}}{\text{L}}\right) = \frac{A_{sample}}{A_{standard}} * \frac{100}{18}\right] \quad \text{(A7)}$$

where *A* is the absorbance.

2.9.4. Total cyanide and acetone cyanohydrin in cassava roots during PPD

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

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

2.10. Enzyme activities during PPD

2.10.1. Catalase activity (CAT)

For the measurement of enzyme activity, flour samples (1 g) from different days of PPD (0, 3, 5, 8, and 11) were homogenised in 5 mL of 10 mM 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 ($y = 2.1247x$, $r^2 = 0.97$) and expressed in units (U) per milligram (U mg^{-1}, 1U = 1 mM of H_2O_2 reduced per minutes × milligrams of protein) (Aebi, 1983). The reaction mixture contained 1 mL of 50 mM potassium phosphate buffer (pH 7.0), 1 mL of 10 mM H_2O_2, and 1 mL of the extract. Protein was measured following the procedure described by Bradford (1976).

2.10.2. Determination of hydrogen peroxide (H_2O_2) content

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

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

The analysis of the SOD family of enzymes was carried out according to Fridovich (1995). Briefly, 1 g flour sample was homogenised with 10 mL of 50 mM potassium phosphate buffer (pH 7.0), centrifuged (4000 rpm/30 min) and the supernatant containing the crude enzyme extract for assay was recovered. For the total of superoxide dismutase enzyme (Total SOD), 1 mL of 0.05 M sodium carbonate buffer (pH 10.2) was added to 1 mL of enzyme extract and 0.5 mL of 0.4 × mM epinephrine. The rate of epinephrine auto-oxidation was observed by monitoring spectrophotometrically the absorbance in samples at the starting point of the reaction and 2.5 min later. SOD was expressed in units per dry weight 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 per minute of epinephrine to adenochrome at 480 nm.

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 the difference between total SOD and MnSOD.

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

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 analyses were implemented in the R (R Core Team, 2014) language (version 3.0.1), using the packages *ChemoSpec*, *Chemometrics*, *mixOmics*, *e1071*, and *pls*. All statistical analyses were performed using the same software and data were represented as mean ± standard deviation of three repetitions ($n = 3$). One-way ANOVA and Tukey HSD tests were applied to the data set when adequate.

3. Results and discussion

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

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. It is important to say that comparing different cultivars at each point of PPD scoring we did not find statistically significant differences but visual evaluation according to vascular discoloration clearly showed rapid discoloration in ORI, when compared to BRA cultivar (see Supplementary Fig. 1).

Secondary metabolites (carotenoids, phenolics, flavonoids, and anthocyanins) are found in many species of the plant kingdom and are well recognised 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 tubers up to 11 days are summarised in 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 in carotenoid contents for fresh samples and those at stage 3 of PPD were found in any cultivar, as further increases were noticed in the root pieces following injury until 3–5 days postharvest. 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, respectively. In addition, 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 IAC, followed by SAN and ORI 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 postharvest) 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, differences 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.

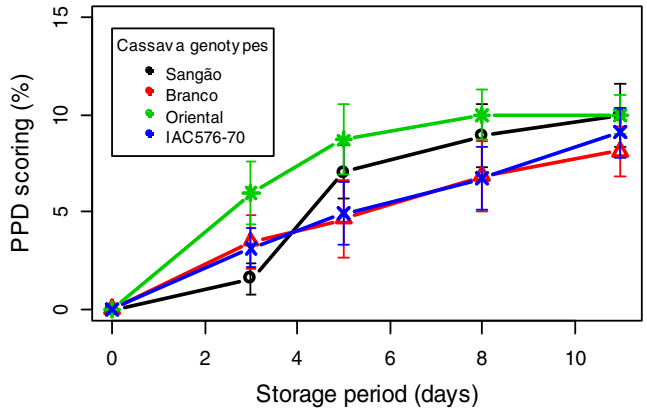


Fig. 1. Postharvest physiological deterioration (PPD) scoring of the cassava cultivars studied, from 0 (without deterioration) to 10 (100%) of deterioration evaluated in five stages of PPD. i.e., 0, 3, 5, 8, and 11 days. Scores represent means of five independent experiments of PPD scoring and 3 sliced roots for each cultivar and evaluation.

Few studies have been reported on flavonoid contents during cassava PPD. Buschmann, Reilly, Rodriguez, Tohme and Beeching (2000) reported increases in flavonoid during the first 1–2 days postharvest. After 4–6 days there was some accumulation and after 7 days there was a rapid decline. Tanaka, Data, Hirose, Taniguchi and Uritani (1983), Uritani, Data, Villegas and Flores (1984) and Uritani, Data, and Tanaka (1984) also reported increases in flavonoid contents during PPD. Our results are in accordance with those previously reported.

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). 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. (1984), Buschmann et al. (2000), Blagbrough, Bayomi, Rowan and Beeching (2010) also confirmed similar trends in phenolic compounds during cassava PPD.

IAC fresh samples showed the largest amounts of anthocyanins, followed by SAN, BRA and ORI cultivars. No significant 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 differed ($p < 0.05$) in their contents of those secondary metabolites. Taken together, the results seem to indicate that a typical genotype-dependent effect occurred for the studied feature. Rickard (1981) previously reported small increases in anthocyanins during cassava PPD.

Changes in flavonoid contents along with phenolics, anthocyanins and carotenoids indicate that the latter changes are, at least partly, due to de *novo* synthesis of those compounds and not only to qualitative changes. We found a positive correlation for flavonoids and anthocyanins with PPD ($y = 0.003x + 1.09$, $r^2 = 0.38$ for flavonoids and $y = 0.1x + 3.4$, $r^2 = 0.27$ for anthocyanins), while the total phenolic and carotenoid amounts negatively correlated to PPD ($y = -0.01x + 8.01$, $r^2 = 0.53$ for total phenolics and $y = -0.28x + 6.96$, $r^2 = 0.12$ for carotenoids, Fig. 2A–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, since upon injury a set of biochemical events takes place in a closely orchestrated cascade to repair the tissue damaged. The results reinforce previous indications that relate PPD delay to carotenoid levels and presents new insight also relating 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, Data, Villegas and Flores (1984). Increases in phenolics, flavonoids, and anthocyanins until 144 h 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.

Since abundant literature indicates that phenolics are important antioxidants in plant foods, the antioxidant activity assay was also made for cassava extracts at 3 days postharvest, since those samples showed major phenolic contents for all 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 (see Supplementary Fig. 2). 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.

Table 1
Changes in secondary metabolites and enzymes during PPD of the four cultivars studied. Values are represented as means of three repetitions in μg/g for carotenoids, flavonoids, anthocyanins, phenolics, hydrogen peroxide, and catalase activity and in units per kilogram (U/kg) of fresh weight for total superoxide dismutase, manganese and cupper/zinc dismutase. Statistical differences are represented with different letters in each column. Comparisons are related only for each group of compound reported.

Compounds	Days after harvest	Sangão	Oriental	IAC576-70	Branco
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 each column. Comparisons are related only for each group of compounds reported.					
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	579d	773b	398d	509c
	3	959c	1299a	1374b	2288a
	5	1408a	781b	1670a	1584b
	8	1318ab	1055ab	1018c	1170b
	11	1231b	1365a	1387b	1445b
Phenolics (μg/g)	Fresh samples	44.9b	64.1c	70.1c	64.0c
	3	353a	547a	514a	538a
	5	112b	170b	134b	152b
	8	155b	183b	203b	230b
	11	112b	117bc	137b	177b
Anthocyanins (μg/g)	Fresh samples	7.12c	5.01c	13.2b	6.35c
	3	8.13c	14.25a	5.84c	5.62c
	5	16.3b	5.51c	0.50d	8.96c
	8	19.04a	5.84c	6,79c	11.8b
	11	6.46c	10.7b	22.2a	42.9a
Total cyanide (mg/Kg)	Fresh samples	56.9c	33.0d	24.2d	22.8e
	3	84.7b	56.2c	60.1c	80.2a
	5	63.2c	38.2d	22.2d	55.4b
	8	60.6c	64.5b	53.4b	39.0c
	11	93.8a	79.3a	66.6a	29.2d
Acetone cyanohydrin (mg/Kg)	Fresh samples	3.64d	8.68a	10.1b	9.64a
	3	5.38b	5.88e	10.0b	6.55e
	5	6.96a	6.35d	8.91c	8.58b
	8	6.00b	8.38b	4.39d	6.90d
	11	4.84bc	7.01c	10.9a	7.98c
Linamarin (mg/Kg)	Fresh samples	53.2c	24.4d	14.2c	13.2e
	3	79.3b	50.3b	50.0b	73.7a
	5	56.2c	31.8c	13.3c	46.8b
	8	54.6c	56.1b	49.0b	32.1c
	11	89.0a	72.3a	55.7a	21.2d
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 each column. Comparisons are related only for each group of compounds reported.					
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	7.13a	7.80a	6.34c	8.74a
Hydrogen peroxide (μg/g)	Fresh samples	62.3d	97.4d	77.3e	120c
	3	41.3e	87.7e	104d	104d
	5	103c	113c	157c	117c
	8	118b	134b	195b	157b
	11	148a	189a	270a	181a
Catalase activity (μg/g)	Fresh samples	14.9e	42.5d	153a	223a
	3	112c	218a	89.1b	115b
	5	66.5d	163b	97.7b	123b
	8	296a	117c	184a	221a
	11	167b	158b	193a	253a
SOD (U/Kg)	Fresh samples	38.4b	84.8d	598a	329b
	3	29.6c	57.3e	86.2e	736a
	5	46.4a	138b	360b	270c
	8	18.1d	163a	250d	188d
	11	46.7a	131c	286c	149e
MnSOD (U/Kg)	Fresh samples	2.46c	37.8b	531a	142b
	3	20.2a	44.8a	75.9d	404a
	5	21.1a	11.6d	113c	31.2e
	8	5.11b	21.7c	74.1d	51.9d
	11	5.43b	37.9b	125b	132c
Cu/ZnSOD (U/Kg)	Fresh samples	36.0 b	47.0	67.2d	187c
	3	9.37e	12.5e	10.3e	332a
	5	25.3c	126b	247a	239b
	8	13.0d	141a	176b	136d
	11	41.2a	92.9c	162c	17.3

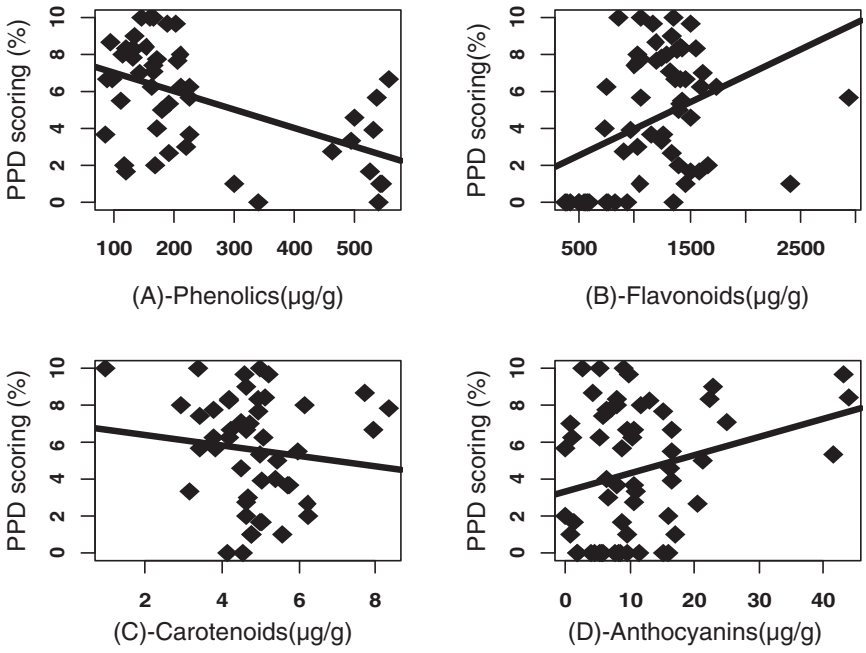


Fig. 2. Correlations between PPD and the secondary metabolites studied. A–Association with phenolic content (μg/g, $y = -0.01x + 8.01$, $r^2 = 0.53$); B–association with flavonoid content (μg/g, $y = 0.003x + 1.09$, $r^2 = 0.38$); C–association with carotenoid content (μg/g, $y = -0.28x + 6.96$, $r^2 = 0.12$); 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.

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-hydroxynitriles, 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 summarised in Table 1-I. Large variations in cyanide content and acetone cyanohydrin were found among cultivars during the 11 days of storage. When cassava roots were stored at room temperature, physiological and microbial deterioration occurred. In fresh roots, the cultivar SAN showed the highest amount of cyanide (56.9 ppm), while BRA showed the lowest (22.8 ppm). As shown in Table 1, part I, 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 revealed 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-hydroxynitrile, 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 utilised 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 postharvest and its increase in the last stage of PPD, which surely is associated with microbial deterioration. Similar results in cyanide were found by Iwatsuki, Kojima, Data and Villegas-Godoy (1984). However, Maini and 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 Tukey HSD 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 3.64 ppm (SAN) to 10.9 ppm (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, while for IAC such behaviour 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 content and linamarase activity of the root samples during PPD are also summarised in Table 1 (part I and II respectively) and for leaf samples in Supplementary Table 1. Results of leaf samples demonstrated high levels of linamarin (IAC, 1030 mg HCN/kg) and linamarase (SAN, 3.02 mmol/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 (53.2 ppm) and BRA the lowest during PPD (Table 1-I). An increase of linamarin was observed in all cultivars until stage 3 of PPD with subsequent oscillation (13.2 ppm, BRA to 89.0 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). The IAC cultivar (8.33 mmol/L) was followed by BRA (7.53 mmol/L), showing major linamarase activities in fresh samples. Mkpomg, Yan, Chism and Sayre (1990) reported values of linamarin and linamarase activity ranging from 37 to 72 ppm and from 0.19 to 2.06 mmol/L, respectively. Bradbury et al. (1999) reported for cassava flours linamarin values ranging from 4.5 to 87 ppm. 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.

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 due not to microorganisms, but having a molecular basis as an oxidative burst which commences 15 min after roots being injured (Reilly, Gomes-Vásquez, Buschmann, Tohme, & Beeching, 2003), followed by altered gene expression. The latter phenomenon is expected to play a role in cellular processes (Reilly et al., 2007) and on the accumulation of secondary metabolites (Buschmann, Rodriguez, Tohme, & Beeching, 2000). Therefore, it would be important to increase our understanding of the physiological and biochemical traits associated with 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 oxidising their thiol groups (Gill & Tuteja, 2010). In our study, during PPD, increases in hydrogen peroxide amounts were detected in all cultivars, ranging from 41.3 ppm (SAN) to 270 ppm (IAC). High levels of hydrogen peroxide were observed at the last stage of PPD (11 days postharvest), where physiological and microbiological deterioration were noticed as well (Table 1, part 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 slight decrease of CAT activity was found until stage 3 and then an accelerated increase was observed 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 postharvest, oscillating in the next stages of PPD. CAT ranged from 14.9 (SAN) to 296 (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 and Tuteja (2010) related the increases in hydrogen peroxide concentration to the augmentation 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 defence against the toxic effects of elevated ROS (Gill & Tuteja, 2010; Mittler, 2002). Results on ROS scavenging enzymes are summarised in Table 1-II. SOD values ranged from 18.1 (SAN) to 735 U/kg of dry weight (BRA). Total SOD activity increased in the tolerant cultivar BRA until stage 3 followed by a decrease. In comparison, 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 the BRA cultivar only in stage 3 followed by an increase until the last stage. The 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 (Table 1-II).

The Cu/ZnSOD activity in all cultivars ranged from 9.37 (SAN) to 332 U/kg of dry weight (Table 1, part-II). 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 behaviour 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 4 h 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. (2003, 2007). 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.

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 along the positive axis of PC1 and PC2, respectively, resulting from the FTIR spectroscopic (3000–600 cm^{-1}) data. The total variance of the data explained by the PCA model built was 83%, with 61% from PC1 and 22% from PC2 (Fig. 3A). In spite of that, some overlap of the samples of most genotypes was observed. In Supplementary Fig. 4, 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 along the positive axis of PC1 were associated with the spectral windows of 900–600 cm^{-1} and 2800–1600 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 (1200–900 cm^{-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 with carbohydrates and proteins were the best ones for cassava screening (see Supplementary Fig. 5A–D) by PCA.

In a complementary approach, PCA was conducted independently for the other metabolomic data set, i.e., the target metabolites and the enzymatic activities studied. Fig. 3B shows an evident separation from fresh samples and those with 3 and 11 days of PPD. Fresh cassava samples were found mostly in quadrant PC2+

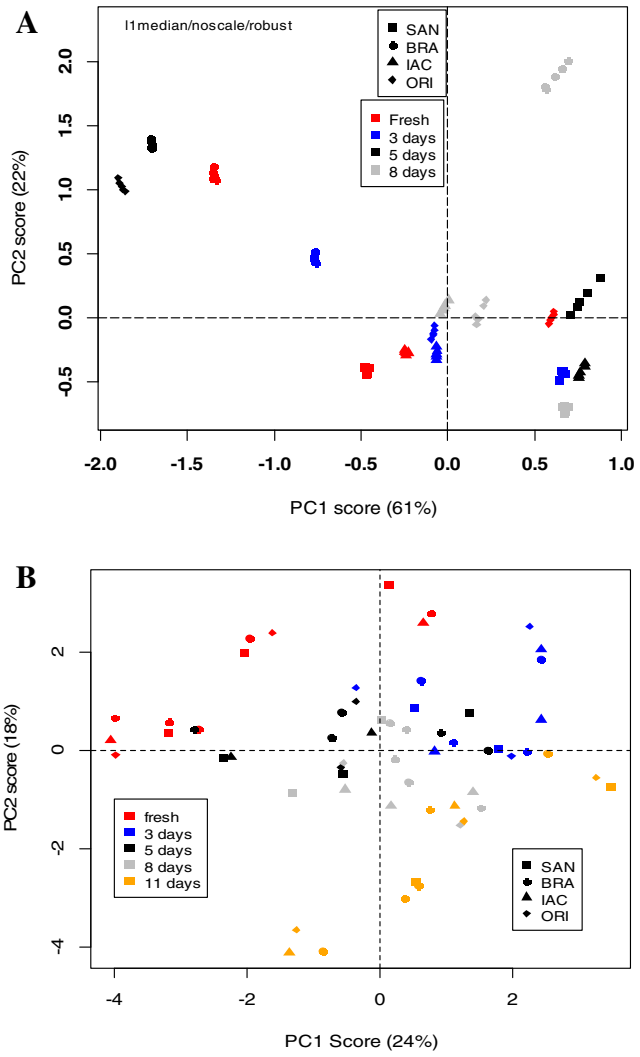


Fig. 3. First and second principal component (PCA) scores plot of cassava samples. A–ATR-FTIR spectral (3000–600 cm^{-1}) data set of cassava samples during PPD (0, 3, 5, and 8 days). B–Summary data of all metabolites, enzymes, and cyanogenic glucosides studied during PPD (0, 3, 5, 8, and 11 days).

and PC1−, while those with 11 days of PPD are mostly in quadrant PC1+ and PC2−. The total variance explained from the first 3 components was 55% (PC1: 24%, PC2: 18%, and PC3: 13%). Interestingly, no visible differences for the biochemical profiles of the samples in stages 5 and 8 of PPD were found. The loadings 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 (PPD scoring). On the other hand, the 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, while the negative axis of PC2 was explained by 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 cassava roots suffering from PPD. In addition, the results suggest that the signals at the spectral window 1600–600 cm^{-1} and the flavonoid contents are important variables determining the discrimination profile observed, indicating the need for 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, Elabadi, Platikanov, Kzaiber, & Tauler, 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

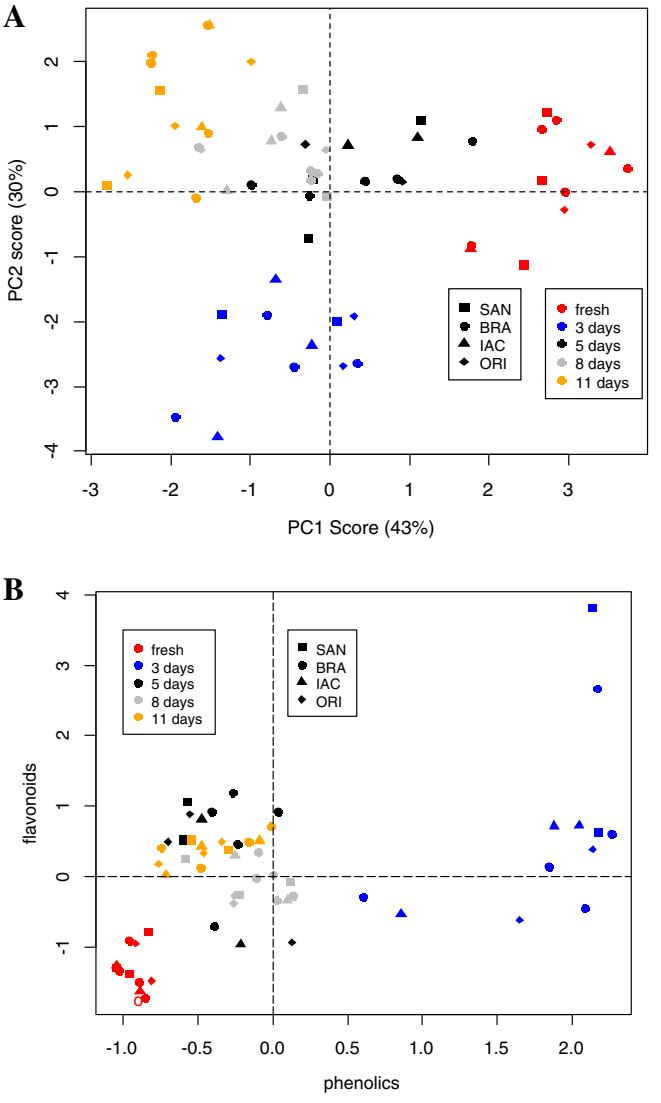
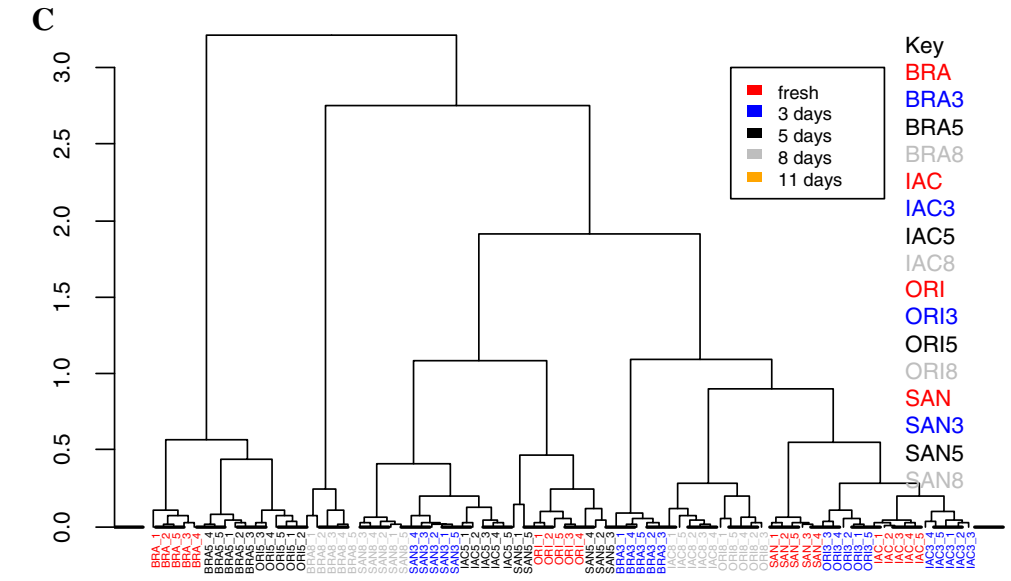


Fig. 4. (A) PLS-DA components scores plot of cassava samples during PPD in relation to all metabolites, enzymes, and cyanogenic glucosides analysed. PLS-1 (PC 1) = 43%, PLS-2 (PC 2) = 30% of variance explained. The model was built with 80% of training data and the remaining (20%) used for testing; (B) multiclass SVM classification plot of cassava samples during PPD with decision boundary $f(x) = 0$. Support vectors are represented in not filled circles. (C) Hierarchical cluster analysis (HCA) of the ATR-FTIR spectral (3000–600 cm^{-1}) data set during PPD with 75% of cophenetic correlation and (D) seriated cluster heat map of UV-visible spectrophotometric data (metabolites, enzymatic activity, and cyanogenic glucosides) studied with cophenetic correlation of 98.7%.



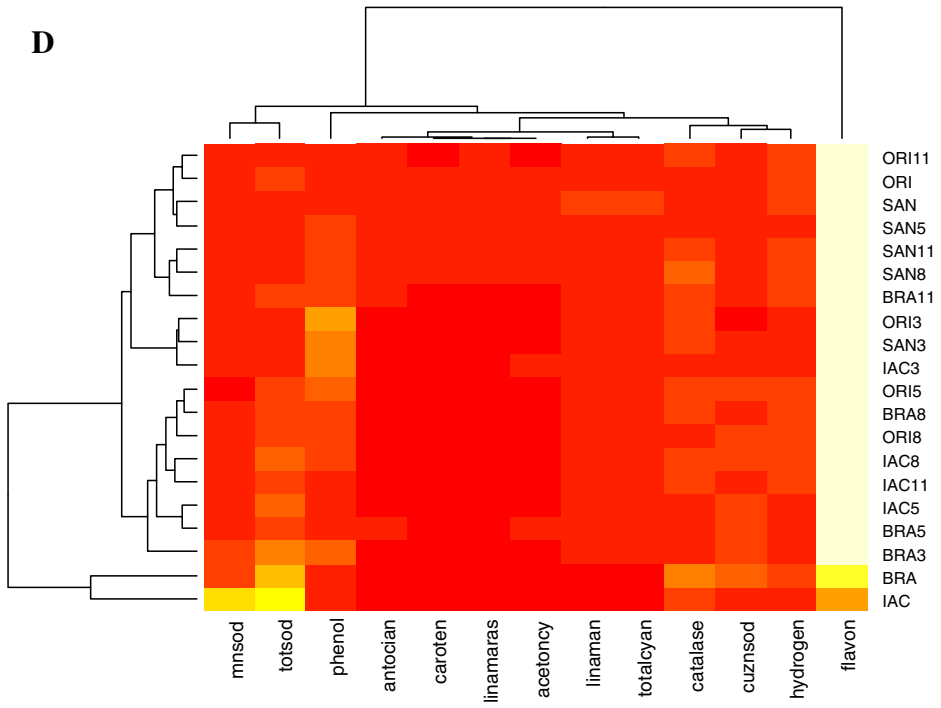


Fig. 4 (*continued*)

(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.

Fig. 4A 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 along 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. As expected, 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 machines (SVM)

SVMs are nonlinear computational learning methods based on statistical learning theory (classification supervised methods)

aiming to separate groups here defined as cultivars at different levels of PPD. The SVM method was implemented using the R package "e1071", used in a classification mode, with a radial SVM-kernel, cost of 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. 4B) using phenolics and flavonoids as target variables. Other classification methods such as artificial neural networks (ANN), K-nearest neighbours (KNN) and classification trees were also tested (data not shown), but SVM showed the lowest test classification error (see Supplementary Fig. 6A–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 (over 30 repetitions).

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 emerged (Fig. 4C). 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. 5) and previously in PCA.

When a seriated cluster heat-map was applied to the metabolites and enzymatic data set, four groups were also found, with a high similarity correlation (cophenetic correlation = 98.7%; Fig. 4D). BRA and IAC showed similarities according to their values of flavonoids, total SOD, and MnSOD activities. ORI3, SAN3, and IAC3 cultivars 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 (Supplementary Figs. 1A–H). It seems that clustering is driven by flavonoids, phenolics, total cyanide, linamarin, catalase, superoxide dismutase, and hydrogen peroxide.

4. Conclusion

The metabolomic-chemometrics approach used in this study for screening cassava roots during postharvest physiological deterioration gave rise to evidence of metabolic differentiation for the studied genotypes during PPD. A rapid screening of cassava during PPD was achieved with successful realisation 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 modelling 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 a 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 to gain more detailed insights as to the metabolic and enzymatic events related to that physiological process in root cassava genotypes.

Authors contributions

The research was conducted by the first author as PhD thesis under supervision of the last author. All co-authors contributed equally.

Conflict of Interest

None declared.

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Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2014.03.110.

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