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The role of ascorbate peroxidase, guaiacol peroxidase, and polysaccharides in cassava (*Manihot esculenta* Crantz) roots under postharvest physiological deterioration

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ABSTRACT

This study aimed to investigate the role of ascorbate peroxidase (APX), guaiacol peroxidase (GPX), polysaccharides, and protein contents associated with the early events of postharvest physiological deterioration (PPD) in cassava roots. Increases in APX and GPX activity, as well as total protein contents occurred from 3 to 5 days of storage and were correlated with the delay of PPD. Cassava samples stained with Periodic Acid-Schiff (PAS) highlighted the presence of starch and cellulose. Degradation of starch granules during PPD was also detected. Slight metachromatic reaction with toluidine blue is indicative of increasing of acidic polysaccharides and may play an important role in PPD delay. Principal component analysis (PCA) classified samples according to their levels of enzymatic activity based on the decision tree model which showed GPX and total protein amounts to be correlated with PPD. The Oriental (ORI) cultivar was more susceptible to PPD.

1. Introduction

Cassava is vital as a starchy staple throughout the developing world. The importance of cassava arises from its agronomic benefits and limited requirement for inputs. For example, cassava gives a high yield of carbohydrates, even on poor soils, has good tolerance to drought, is relatively resistant to pest infestation and disease and can be stored in the ground until required. However, once harvested, cassava is more perishable than other tubers because of it higher moisture content, greater susceptibility to physical damage and higher metabolic activity. Cassava losses are primarily affected by two types of postharvest deterioration: primary physiological deterioration, the initial cause of loss of market acceptability, and secondary deterioration from microbial spoilage (Booth & Coursey, 1974). Successful marketing of cassava is considerably constrained as the distance between production and consumption increases (Westby et al., 2004). In addition to physical loss of the crop, postharvest deterioration causes a reduction in quality resulting in price discounts that affect profitability (Naziri et al., 2014; Wenham, 1995; Westby, 2002). Furthermore, change in use can result in additional losses. For example, if harvested cassava roots cannot be marketed within two or three days of harvest, then they may be processed into dried products of low quality with corresponding low value (Westby, 2002).

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Because cassava is increasingly used as human food, special attention should be given to the development and transfer of different postharvest technologies to address the rapid deterioration of cassava roots once harvested (Sánchez & Alonso, 2012). Indeed, short storage life of harvested roots is an important constraint that limits the full realization of cassava's potential in developing countries. Cassava roots undergo rapid deterioration 24–48 h after harvest, a phenomenon known as postharvest physiological deterioration (PPD). PPD is characterized by a blue-black discoloration of the xylem vessels known as "vascular streaking", which considerably reduces the palatability and marketability of cassava roots. The rapid postharvest deterioration of cassava restricts the storage potential of fresh root to only a few days.

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

The evolution of aerobic metabolic processes, such as respiration and photosynthesis, unavoidably led to the production of reactive oxygen species (ROS) in mitochondria, chloroplasts, and peroxisomes. A common feature among the different ROS types is the capacity to cause oxidative damage to proteins, DNA, and lipids. Increasing evidence indicates that ROS also function as signaling molecules in plants to control various processes, such as defense responses against pathogens and deterioration (Apel & Hirt, 2004). The cytotoxic properties of ROS explain the evolution of complex arrays of non-enzymatic and enzymatic detoxification mechanisms in plants. For example, the peroxidases (POX) are enzymatic systems ubiquitous in fungi, plants, and vertebrates and have been associated with defense responses. Ascorbate peroxidase (APX) is the most important peroxidase in H_2O_2 detoxification, catalyzing the reduction of H_2O_2 to water by using the reducing power of ascorbate (Jebara, Jebara, Limam, & Aouani, 2005). Similarly, guaiacol peroxidases (GPX), located in cytosol, vacuole, cell wall, and apoplast, are also assumed to be involved in a range of processes related to ROS-induced stress. However, their role in the physiology and biochemistry of cassava deterioration remains to be elucidated (Ghamsari, Keyhani, & Golkhoo, 2007).

Plants store carbohydrate polymers in a number of forms. Starch is the principal form, followed by fructans and cell wall-stored polysaccharides. Primary cell walls from plants are composites of cellulose tethered by cross linking glycans (hemicelluloses) and embedded in a matrix of pectic polysaccharides (Silva et al., 2011). The high structural complexity of plant cell wall polysaccharides has led to suggestions that some components might function as latent signal molecules that are released during pathogenic infection and subsequently elicit defensive responses (Vorwerk, Somerville, & Somerville, 2004). Acidic polysaccharides are reported to be more bioactive than neutral ones, possibly because acidic groups in acidic polysaccharides can form associations with the target biomolecules, such as proteins, in hosts through electronic interactions (Zhang et al., 2015). Moreover, acidic polysaccharides have shown strong *in vitro* scavenging activities on DPPH and hydroxyl radicals (Pereira et al., 2012), as well as antioxidant capacity (Aguirre, Isaacs, Matsuhiro, Mendoza, & Zúñiga, 2009).

Conserving cassava roots in storage is economically vital; therefore, the present investigation aimed to characterize the role of APX, GPX, neutral and acidic polysaccharides, and protein contents associated with the early events of PPD in cassava storage. Changes in antioxidant enzymes and proteins were analyzed by UV–Vis spectrophotometry. Morphological and anatomical changes in acidic and neutral polysaccharides and proteins were investigated by histochemical methods.

2. Material and methods

2.1. Cassava cultivars and on-farm trials

Cassava cultivars were grown in Southern Brazil over the 2011/2012 growing season. Four cultivars were selected for this study, as follows: SCS 253 Sangão (hereinafter SAN), Branco (hereinafter BRA, a landrace), IAC576-70 (hereinafter IAC, a commercial variety), and Oriental (hereinafter ORI, a landrace). On-farm trials were carried out at the Ressacada Experimental Farm (Plant Science Center, Federal University of Santa Catarina, Florianópolis, SC, Brazil – 27°35′48″ S, 48°32′57″ W) in September 2011, using the four cassava cultivars noted above. Samples of cassava cuttings for cultivation were provided by the Santa Catarina State Agricultural Research and Rural Extension Agency (EPAGRI) at Urussanga, the official state agriculture agency. The experimental design was in randomized blocks, with 4 blocks (6.3 × 15 m/block) spaced at 1 m. Each block consisted of four plots (12 × 1.2 m/plot) spaced at 0.5 m. Cassava cuttings (15 cm long) were planted upright and spaced at 1 × 1 m. Each plot was considered a treatment, and all crop management was mechanized. Chemical analysis of soil fertility was previously done, and cultivation was performed manually, following agroecological field handlings.

2.2. Induction of PPD

Cassava root samples (12 months old) were collected for analysis of non-stored samples and for induction of physiological deterioration under controlled conditions in the laboratory. Immediately after harvest, the roots were washed, proximal and distal parts of the root were removed, and cross sections were made (0.5–1 cm) over the remaining root, followed by storage at room temperature (66–76% humidity, 25 °C). Induction of PPD was performed for 11 days. Monitoring the progress of PPD and associated metabolic disturbances was performed daily after induction of PPD. Non-stored samples and those at 3, 5, 8, and 11 days after PPD induction were collected at each time point, dried (35–40 °C) in an oven, milled with a coffee grinder (Model DGC-20N series), and kept for analysis. For enzymatic analysis, fresh samples (batch of seven roots from each cultivar) were collected, grated using a food processor (Walita-Master Plus, Brazil), and stored (−80 °C) until analysis.

2.3. PPD scoring

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

2.4. Enzymatic activities during PPD

2.4.1. Protein determination

Protein content was determined in the cassava root samples (non-stored and 3, 5, 8, and 11 days postharvest), using Coomassie brilliant blue G-250 (Bradford, 1976) with bovine serum albumin as standard ($y = 0.0159x$, $r^2 = 0.98$), and represented in mg kg^{-1}.

2.4.2. APX activity

Cassava root samples (1 g, grated samples) were collected directly into liquid nitrogen in a mortar with 2% PVPP, 1 mM PMSF, 10 mM DTT, and 0.1 mM EDTA (MW: 292.2 g mol^{-1}) in 50 mM Na–P buffer, pH 7.5. For analysis of ascorbate peroxidase (APX), the extraction buffer also contained 2 mM ascorbate (MW: 176.13 g mol^{-1}). The suspension was centrifuged (4000 rpm, 30 min, 4 °C) and the supernatant used for assay of enzymatic activity. Total APX (EC 1.11.1.11) activity was measured by monitoring the decline in absorbance at 290 nm, as ascorbate ($\varepsilon = 2.8$ mM^{-1} cm^{-1}) was oxidized for 3 min (Nakano & Asada, 1981). The assay medium consisted of 1200 µl of 50 mM potassium phosphate buffer (pH 7.0), 200 µl EDTA, 200 µl ascorbate, 200 µl of sample, and 200 µl of 0.1 mM H_2O_2 to start the reaction. APX activity was expressed in mM ascorbate min^{-1} mg^{-1} of proteins.

2.4.3. GPX activity

Guaiacol peroxidase (EC 1.11.1.7) activity was measured using a reaction medium containing 50 mM phosphate buffer (pH 7), 9 mM guaiacol, and 19 mM H_2O_2 (Lin & Kao, 1999). The kinetic evolution of absorbance at 470 nm was measured during 1 min. Peroxidase activity was calculated using the extinction coefficient (26.6 mM^{-1} cm^{-1} at 470 nm). One unit of peroxidase was defined as the amount of enzyme that caused the formation of 1 mM of tetraguaiacol per minute.

2.5. alpha-Tocopherol activity (α-TOC, or vitamin E)

Tocopherol (EC 233-466-0) activity was assayed as described by Backer (Backer, Frank, De Angells, & Feingold, 1980) with some modifications. Briefly, 1 g of cassava sample was homogenized with 5 ml of a mixture of petroleum ether and ethanol (2: 1.6, v v^{-1}), the extract was centrifuged (4000 rpm, 30 min, 4 °C), and the supernatant was used to estimate α-TOC content. To one milliliter of extract, 3 ml of 2% 2, 2-dipyridyl in ethanol were added, mixed thoroughly, and kept in dark for 5 min. The resulting red color was diluted with 4 ml of distilled water and mixed well. The resulting color in the aqueous layer was measured at 530 nm. The α-TOC content was calculated using a standard curve ($y = 0.1115x$, $r^2 = 0.96$) made with known amounts of α-TOC (0–100 mg ml^{-1}) and expressed in mg kg^{-1} of fresh weight (FW).

2.6. Histochemical analysis

2.6.1. Sample preparation

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

2.6.2. Light microscopy (LM)

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

2.6.3. Histochemical staining

LM sections were stained as follows: Periodic Acid-Schiff (PAS) used to identify neutral polysaccharides, Toluidine Blue (TB-O) 0.5%, pH 3.0 (Merck Darmstadt, Germany) used for acid polysaccharides through a metachromatic reaction (Schmidt et al., 2009), and Coomassie Brilliant Blue (CBB) 0.02% (m v^{-1}) in Clarke's solution (Serva, Heidelberg, Germany) used for protein identification (Schmidt, Maraschin, & Bouzon, 2010).

2.7. Data analysis and mining

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

3. Results and discussion

3.1. Postharvest physiological deterioration scoring (PPD scoring)

The results of PPD scoring were summarized in Fig. 1A, and images of root slices at different storage days can be found in Fig. 1B. ORI showed a high rate of deterioration when compared to the other cultivars, which agrees with findings previously reported by our research group (Uarrota et al., 2014). Statistical differences were not found (Tukey test, $p < 0.05$) among cultivars. During storage time, significant differences in PPD were only found between non-stored, samples stored during 3 days with those stored during 5, 8, and 11 days. Imaging of PPD samples (Fig. 1B) also revealed rapid deterioration of ORI samples, but also SAN samples, with samples from BRA and IAC cultivars showing the most tolerance to PPD. Since PPD scoring for these cultivars is scarce in the literature, our results will serve as a basis for future screening of these valuable genetic materials toward a better understanding of cassava root deterioration.

3.2. Enzymatic activities during PPD and multivariate analysis

Several metabolites are critical for plant growth and development and play an important role in integrating various stress signals, controlling downstream stress responses by modulating gene expression machinery and regulating various transporters or pumps and biochemical reactions (Tuteja & Sopory, 2008). On the other hand, reactive oxygen species (ROS) are continuously produced during PPD as byproducts of aerobic metabolism. Depending on the nature of the ROS species, some are highly toxic and rapidly detoxified by various cellular enzymatic and nonenzymatic mechanisms. Plants are supplied with several mechanisms to combat increased ROS levels during abiotic stress conditions. However, under other circumstances, plants appear to purposefully generate ROS as signaling molecules to control various processes, including pathogen defense, programmed cell death, and PPD (Apel & Hirt, 2004). Our recently published work has shown that some nonenzymatic mechanisms, such as secondary

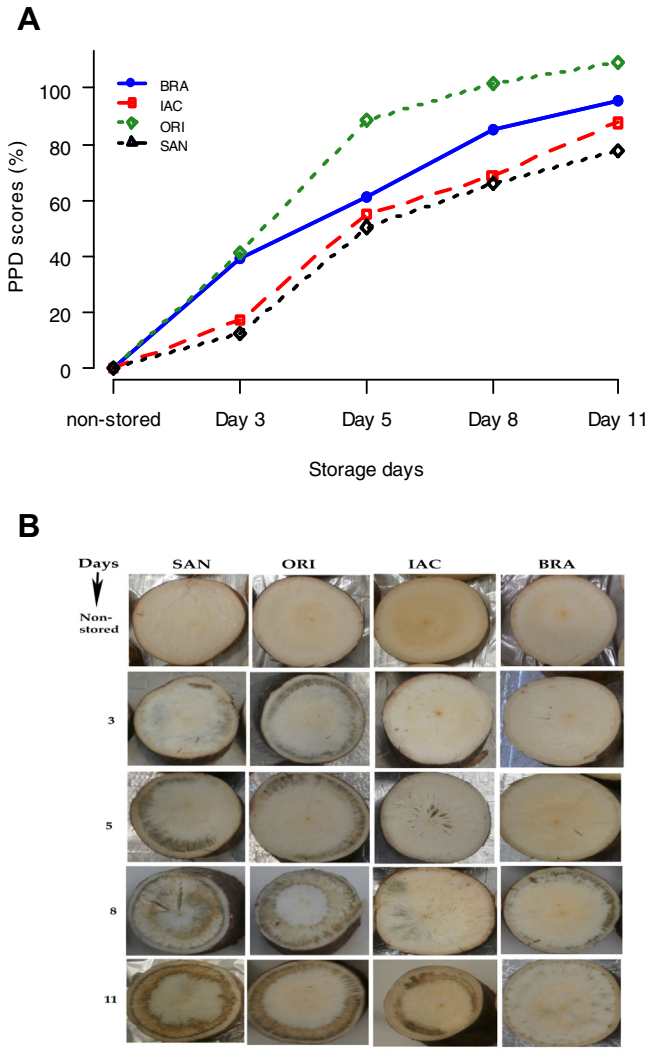

Fig. 1. (A) PPD scoring of cassava samples at different storage days (3, 5, 8, and 11 days) in four cultivars. Data are represented as means of five independent evaluations of PPD and are based on the intensity of parenchyma discoloration. (B) Root cross-sections of the four cultivars studied during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

metabolites, including phenolics, carotenoids, flavonoids, and anthocyanins, as well as certain enzymes, such as catalase, hydrogen peroxide, and superoxide dismutase, are highly involved in the process of ROS detoxification (Uarrota et al., 2014) during PPD. Ongoing experiments in our laboratory have also found hydroxycoumarins, mainly scopoletin, to be involved in PPD, as previously reported in the literature by other research groups (Sánchez et al., 2013; García, Sánchez, Ceballos, & Alonso, 2013; Zidenga, Leyva-Guerrero, Moon, Siritunga, & Sayre, 2012; Wheatley & Schwabe, 1985). Isamah (2004) observed increases of peroxidase levels in cassava roots undergoing PPD up to 24 h of storage, but decreasing thereafter. Such increase in peroxidase levels was attributed to PPD stress. Biochemical markers associated with PPD and the enzymatic activities measured were summarized in Fig. 2A–D. Specifically, APX (Fig. 2A) increased during PPD up to day 3 of storage in the SAN and IAC cultivars and up to day 5 of storage in the BRA cultivar. In the ORI cultivar, this trend was not observed. Analysis of variance of these data showed differences among cultivars along all storage days ($p < 0.05$).

Our results suggest that APX may be involved in first-line defense in order to maintain low levels of ROS formed during PPD. The second line of defense is the presence of endogenous antioxidant chemicals, some of which are the substrate of antioxidant enzymes (e.g., ascorbate for APX), while others act in a manner that is independent of these enzymes, such as phenols and anthocyanins (van Doorn & Ketsa, 2014; Apel & Hirt, 2004).

The crucial role of APX in lowering ROS has been reported in the literature (Foyer & Noctor, 2011; Gallie, 2013). Levels of APX have been found to increase in response to environmental stresses, such as water deficit, salt stress, drought, and both cold and hot temperature, in many crops (Zhang, Zhang, et al., 2013; Sato, Masuta, Saito, Murayama, & Ozawa, 2011; Wang et al., 2005). These findings support the hypothesis that APX levels in cassava roots increase as a consequence of PPD stress.

GPX activity (Fig. 2B) showed an increasing trend during PPD up to day 5 of storage, except for IAC and SAN. Significant statistical differences ($p < 0.05$) were found between ORI and BRA cultivars.

Studies reporting on GPX activity relative to PPD and stress-induced increases in ROS concentrations are scarce in the literature. According to Doorn & Ketsa, increased activity of GPX has been observed during exposure to low temperature in different crops, such as coffee, cucumber, maize and rice. Other types of abiotic stress have also resulted in an increase of GPX activity, e.g., drought (Zhang & Kirkham, 1996), hypoxia (Bai, Li, Ma, Feng, & Shu, 2010), and exposure to NaCl (de Azevedo Neto, Prisco, Enéas-Filho, do Braga de Abreu, & Gomes-Filho, 2006). In the present study, increases in GPX activity in cassava roots were observed during the first 3 days of storage. GPX can use ascorbate during oxidation reactions; therefore, these results indicate that GPX may be involved in lowering stress-induced ROS during PPD by, for example, converting hydrogen peroxide to water. Higher GPX activity was also reported in stored mango (Ding, Tian, Zheng, Zhou, & Xu, 2007), chilling injury of the peel of banana fruit stored at 5 °C (Pongprasert, Sekozawa, Sugaya, & Gemma, 2011), and stored peach fruit (Meng, Han, Wang, & Tian, 2009).

Total protein contents (Fig. 2C) in all cultivars increased up to day 5 of storage, except for BRA, which presented a small decrease at day 5, but continued to increase up to day 8. Significant differences ($p < 0.05$) in total protein contents in all cultivars and all storage days were detected. Finally, as shown in Fig. 2D, small quantities of alpha-Tocopherol were found, but no trend was identified during storage days would lead to any significant correlation with PPD in cassava roots. Increases in alpha-Tocopherol were observed only in the IAC cultivar until day 3 of storage. Tocopherols are present in all anatomical parts of plants, i.e., roots, tubers, leaves, stems and flowers (Siger et al., 2015).

The accumulation of Tocopherol varies greatly in different plant species and different plant parts as well. Tocopherols have diversified roles in plant growth and physiological processes. As an antioxidant, Tocopherol plays a vital role in conferring tolerance to several abiotic stresses, e.g., salinity, drought, metal toxicity, ozone, and UV radiation. Several reports indicate that stress-tolerant plants exhibit an enhanced level of Tocopherol, whereas sensitive ones show a decreased level under stressful conditions, leading to oxidative damage (Yao et al., 2015; Hasanuzzaman, Nahar, & Fujita, 2014). Tocopherol plays a key role in scavenging or quenching lipid peroxides, oxygen radicals, or singlet oxygen, resulting in detoxification of reactive oxygen species and thus mitigating abiotic stress-induced damage (Semida, Taha, Abdelhamid, & Rady, 2014). Tocopherol also works in coordination with other antioxidants (e.g., ascorbate) and interacts with phytohormones, such as ethylene, abscisic acid, salicylic acid, and jasmonic acid (Hasanuzzaman et al., 2014). While many studies have explored the role of Tocopherol in abiotic stress tolerance, many gaps remain with respect to its activity in the context of PPD-induced stress. Our results did not identify any relationship between Tocopherol and PPD-induced stress.

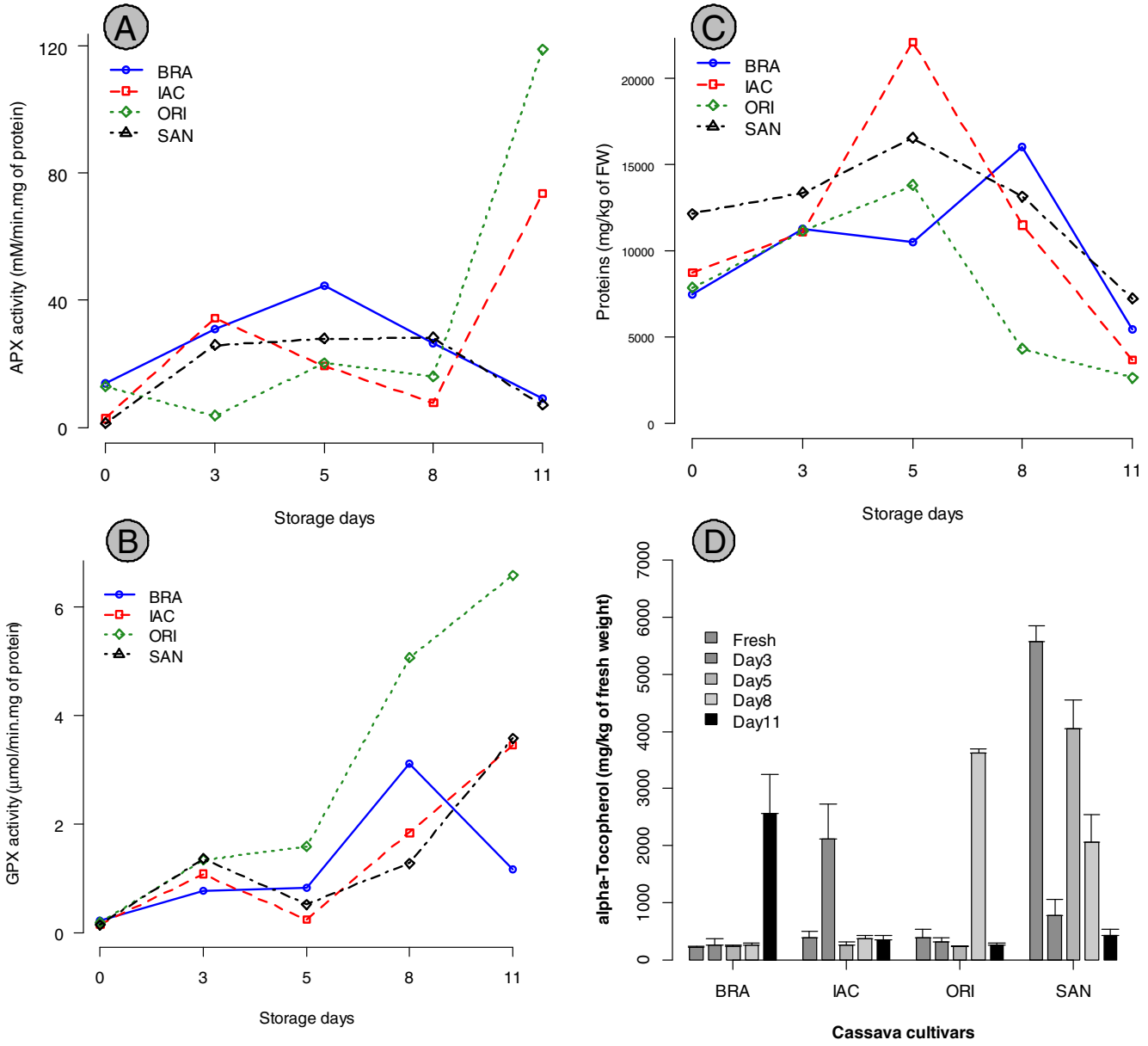

Fig. 2. Changes in enzymatic activity and protein contents during PPD of the four cultivars studied. (A) Ascorbate peroxidase (mM min^{-1} mg^{-1} of proteins); (B) guaiacol peroxidase (µmol min^{-1} mg^{-1} of proteins); (C) total protein contents (mg kg^{-1} of fresh weight), and (D) alpha-Tocopherol amounts during PPD (mg kg^{-1} of fresh weight). Data are represented as means and standard deviations of three replications ($n = 3$).

When data about enzymatic activity was summarized and correlated using Pearson's correlation coefficient (PCC), a high positive correlation was found between GPX and PPD ($r = 0.60$), followed by a moderate correlation for APX ($r = 0.35$) and a negative correlation between PPD and total protein content (Fig. 3). GPX and APX are the main antioxidant scavengers during PPD. No involvement of alpha-Tocopherol was observed in the context of PPD ($r = 0.05$). Enzymatic systems have been associated with the reduction of many stress systems in such crops as tobacco and wheat (Curvelo et al., 2013). Our results demonstrated that APX and GPX activity increased during PPD as an antioxidant mechanism against ROS formed during the PPD process and indicated a potential role of proteins in PPD delay by the negative correlation between PPD and total proteins. Future studies may extend these findings toward a better understanding of cassava deterioration.

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

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

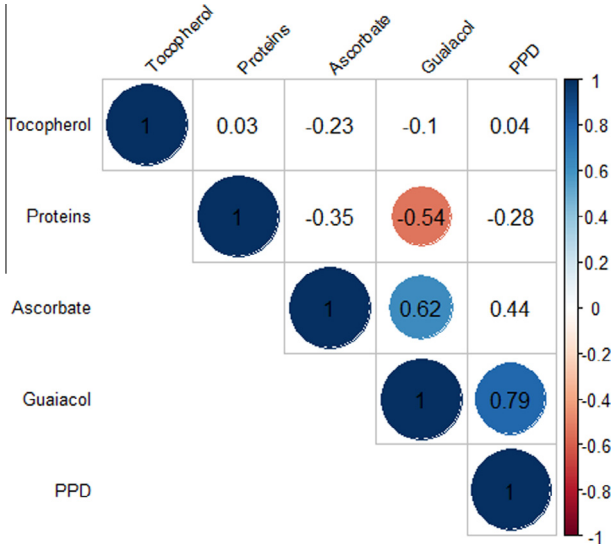

Fig. 3. Correlation matrix of enzyme data (APX, GPX, alpha-Tocopherol, and total proteins) with PPD. Crude data (non-normalized) of enzymatic activities during storage time were correlated with the level of PPD in the samples. Colors indicate the degree of correlation as represented in the matrix figure scale. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and the major group in (PC1− and PC2−). The loading values showed that samples at day 11 grouped in that component because of APX and GPX activities and PPD level. The other groups (non-stored and samples stored during 3 and 5 days) were classified according to protein content and APX activity.

A decision tree model (Fig. 4B) identified protein amounts and GPX as mainly related to PPD in cassava samples. According to our model, with protein amounts above 7238 mg kg^{-1}, 26% of samples did not deteriorate. Lower amounts of proteins and GPX activity higher than 0.23 μmol min^{-1} mg^{-1} seem to be associated with diminished deterioration in cassava roots, i.e., 19%. Such findings reinforce the strong involvement of GPX in PPD delay.

3.3. Histochemical analysis

3.3.1. Involvement of acidic and neutral polysaccharides in cassava PPD

Polysaccharides are relatively complex carbohydrates and the first biopolymers found in nature. These polymers are made up of either single or multiple monosaccharides joined together by glycosidic bonds forming large, often branched, macromolecules. They play a number of roles in biological functions like respiration, mechanical strength, source of energy, and stress tolerance (Sanandiya & Siddhanta, 2014). They also may vary qualitatively and quantitatively, depending on species, cultivar, tissue, location of cultivation, time of harvest, and duration of storage (Sills & Gossett, 2012). The high degree of structural complexity of plant cell wall polysaccharides has led to suggestions that some components might function as latent signaling molecules released during pathogenic infection as defensive responses (Vorwerk et al., 2004). They have also been implicated to possess many antibacterial and antioxidant properties (Li & Shah, 2014; Zhang, Wang, et al., 2013). Changes in plant polysaccharides, e.g., pectin and hemicelluloses, under stress conditions have been reported, and increases in lignin have also been found (Lima et al., 2014). Cell wall polysaccharides have been implicated as a promising group of antioxidant compounds (Kale et al., 2013), and their free radical scavenging activity

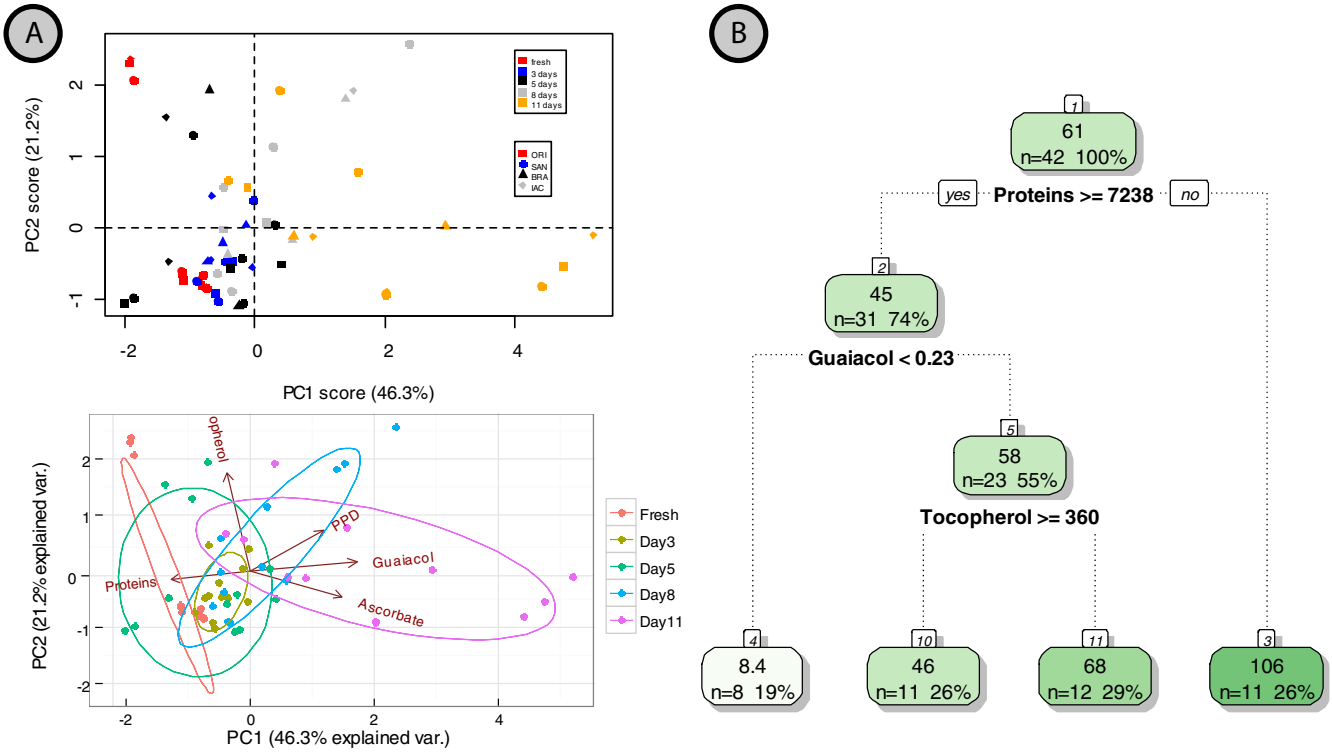

Fig. 4. Predictive models of cassava samples. (A – upper) Scores plot of the principal component analysis (PCA) model from the enzymatic activity dataset of cassava roots and the percentage of variance captured by each PC (46.3% and 21.2%, respectively) and (A – lower) PCA with the Eigen values most correlated with sample clustering. (B) Supervised method (decision tree model) of all data, taking PPD as target variable and using all enzymes to build a training model and predict expected levels of PPD in cassava roots. Decision tree shows proteins and guaiacol peroxidase as the main variables related to PPD.

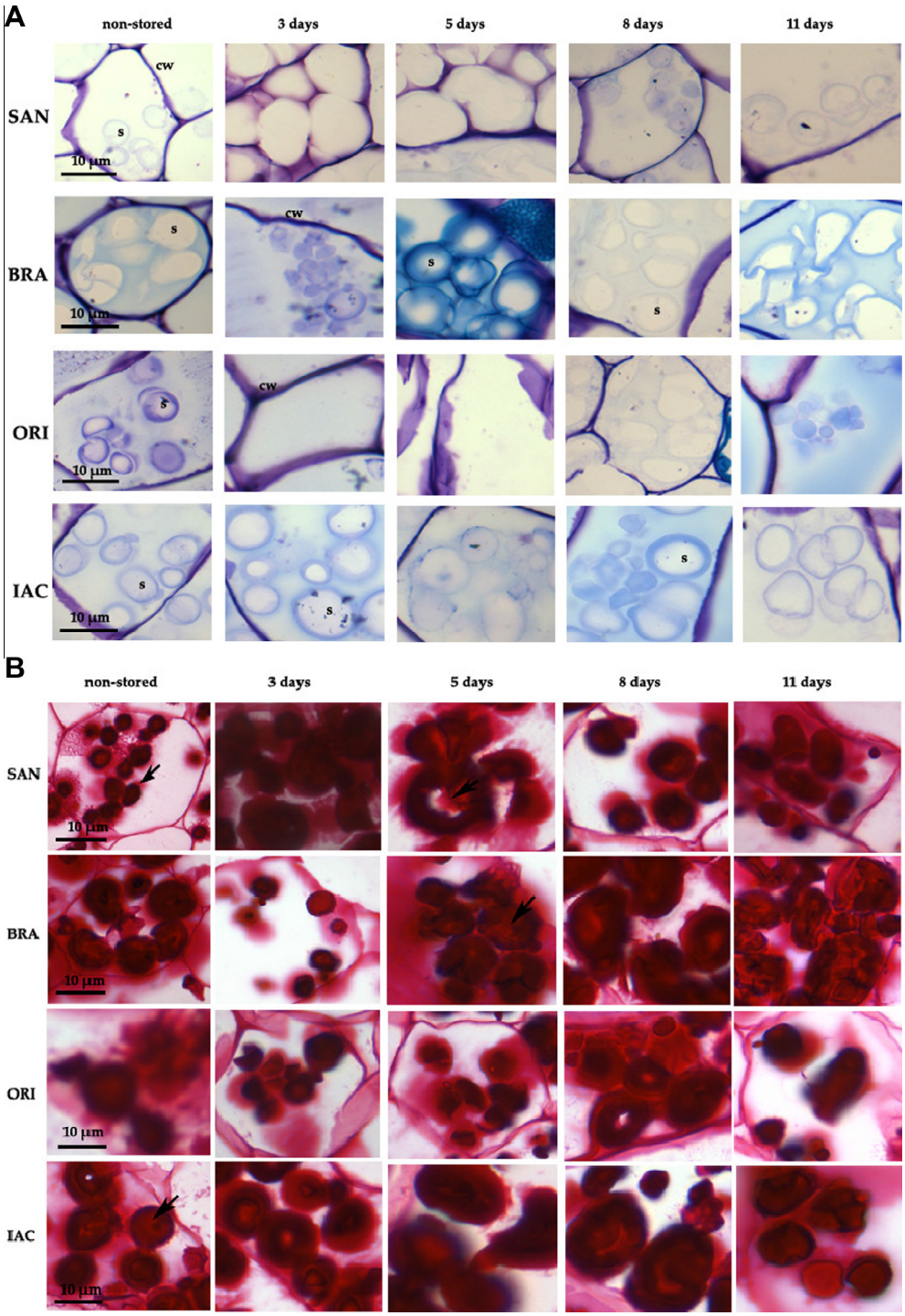

Fig. 5. Light microscopy of cassava samples during storage, as analyzed by histochemical staining. (A) Samples stained with toluidine blue (TB) to indentify changes in acidic polysaccharides. Cw indicates cell wall and S starch granules; (B) staining with Periodic Acid-Schiff (PAS) to identify neutral polysaccharides. Arrows indicate starch granules and (C) staining with Coomassie brilliant blue (CBB) to identify proteins in cassava root parenchyma. Arrows indicate cell walls and starch granules.

has been attributed to pectic polysaccharides (Mateos-Aparicio, Mateos-Peinado, Jimenez-Escrig, & Ruperez, 2010).

Cassava samples at different storage days were stained with toluidine blue (TB), and the results are summarized in Fig. 5A. All cultivars showed metachromatic reaction in the cell walls and around starch granules. This reaction was predominantly observed up to 5 days of storage in the BRA and IAC cultivars, while for other cultivars, it was observed only in the cell walls. Metachromatic reaction indicates the presence of acidic polysaccharides that are produced as oxidative stress increases in cassava samples, and their role can be attributed to a reduction in PPD stress. Degradation of starch granules can also be observed during storage. Reports attributing anatomical changes to PPD are scarce in the literature, thus making the present work the first to report anatomical alteration in relation to PPD, in particular the function of cassava primary cell walls. According to Bowen et al., 2006, a reduction in the moisture content of plant matrix generally reduces the rate of deterioration. However, oxidative stress is generally enhanced during low-water activities, such as accumulation of hydrogen peroxide and increase in lipid peroxidation (Chakraborty & Pradhan,

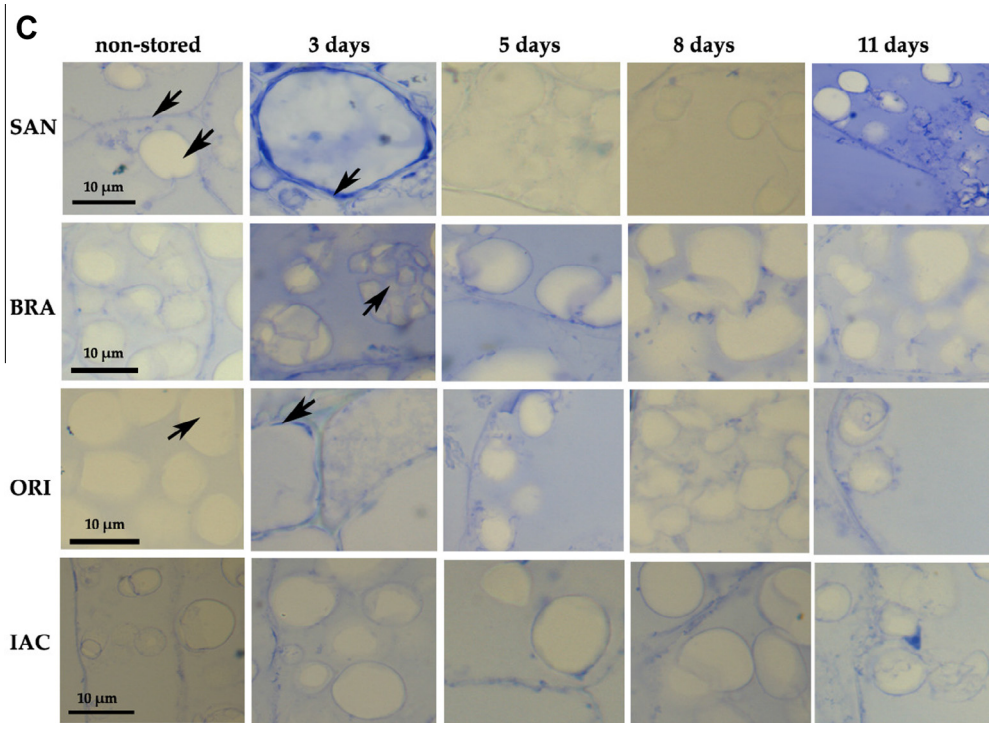

Fig. 5 (*continued*)

2012). Under these conditions, oxygen can permeate through lipid layers, such as found in cell membranes. Free radicals have been implicated in the oxidative-reductive depolymerization of carbohydrates; therefore, radicals generated by lipid oxidation may attack starch.

Samples stained with Periodic Acid-Schiff (PAS) exhibited a strong reaction for starch granules, but a lesser reaction in the cell wall of samples from all cultivars studied during 11 days in storage. The intense reaction indicates a major presence of neutral polysaccharides, namely, starch, in these samples. Starch granules can be easily observed in non-stored samples (Fig. 5B). During storage, starch is probably degraded into monosaccharides. In nearly all green plants, starch occurs as carbohydrate reserves. Starch granules consist of two very different polymers, both structural and functional: amylose and amylopectin. The functionality of starch depends on (1) the average molar mass of amylose and amylopectin and (2) its molecular structure and organization within the granule (Jankovíc, 2013). It has also been reported that the physicochemical properties of cassava starch are altered with the complexation of oxalic and succinic acids (John & Raja, 1999) during PPD (Sánchez et al., 2013).

When samples of cassava were stained by Coomassie brilliant blue (CBB), a slight reaction was found up to day 3 of storage in all samples for cell walls and around starch granules (Fig. 5C). The reaction was more intense in BRA/SAN cultivars. These findings corroborate the results of protein quantification, which showed small increases in protein rates from day 3 to 5 of storage. In general, cassava samples are poor in protein content, which explains the small reaction observed in all samples.

4. Conclusions

The results of this study revealed that the ORI cultivar is the most susceptible to deterioration. Based on BRA, IAC, SAN and ORI cultivars, GPX and APX activity generally increased to reduce PPD-induced stress during storage, and, together with total proteins, these enzymes may, therefore, play a role in PPD delay in cassava roots. Histochemical analysis demonstrated that acidic polysaccharides seem to act as barrier components of plant cell walls and may also play an important role in PPD delay in that catabolization of starch was observed during PPD. By using multivariate analysis, a descriptive model was built, and it also confirmed that GPX, protein contents, and APX all play important roles in PPD delay.

Authors' contributions

This research was conducted by the first author as part of his Ph. D. thesis under the supervision of Professor Marcelo Maraschin. Histochemical analyses were made in collaboration with the Laboratory of Plant Cell Biology, Centre for Biological Sciences, Department of Cell Biology, Embryology and Genetics (UFSC). All coauthors contributed equally.

Competing interests

The authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2015.11.025.

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