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Behavior of Enzymatic Antioxidant System during Development and Ripening Of Cashew Apples

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Abstract

Ripening of fruits is knowledge as an aerobic metabolic process generating reactive oxygen species (ROS) capable to initiate and enhance degenerative processes associated with fruit ripening and senescence. However, the aim of this work was to evaluate the influence of development and ripening on components of the enzymatic antioxidant system during development and ripening of early dwarf cashew clones *(Anacardium occidentale L.)*. The clones analyzed included: CCP 76, CCP 09, BRS 189 and BRS 265 in seven development and ripening stages. Superoxide dismutase (SOD) activity showed an increase during fruit ripening in CCP 09 clone at end of storage, but it remained lower in CCP 76 than in the other cultivars. CCP 09 clone showed the CAT activity 3-fold less than BRS 265 at end of storage. We conclude that changes in the activities of antioxidant enzymes indicated which nonenzymatic system plays a fundamental role during development and ripening of non-climateric cashew apples.

Keywords: BRS265 clone, Catalase, Non-climateric, Superoxide Dismutase.

1. Introduction

Independent of the species, fruit growth may be divided into distinct developmental phases, including a period of intense cell division followed by a period of cell expansion and ending with the ripening period.

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Fruit ripening has been described is an important factor influencing the compositional quality of the fruit, during ripening because they are biochemical, physiological and structural attributes that determine its quality. Thus, ripening and senescence of fruits can be seen as oxidative phenomena and selection of varieties with higher antioxidant enzyme capacity is an alternative to obtaining fruits that can be stored for longer periods without losing their quality attributes (Menichini et al., 2009). The development of oxidative stress during fruit ripening has been reported in a range of climacteric and non-climacteric fruit (Zheng et al., 2007; Larrigaudiére et al., 2009). Previous studies have also shown that cultivars differing in antioxidative metabolism also differed in their potential storage or shelf-life (Hodges et al., 2000).

Ripening of fruits is knowledge as an aerobic metabolic process generating reactive oxygen species (ROS) capable to initiate and enhance degenerative processes associated with fruit ripening and senescence (Brennan and Frenkel 1977; Rogiers et al. 1998). Under normal conditions, ROS are rapidly scavenged by various cellular enzymatic and non-enzymatic mechanisms. The enzymatic antioxidant defense in plants includes enzymes such as superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX). SOD catalyses the dismutation of superoxide (O_2^-) to hydrogen peroxide (H_2O_2), which is subsequently detoxified by CAT and APX. The non-enzymatic antioxidant system has a cellular pool of compounds such as phenolic which are capable of quenching the ROS (Noctor and Foyer, 1998; Apel and Hirt 2004). The vitamin C have redox properties and may also play a cooperative role in the protection against ROS in the plant tissue (Buchanan et al., 2012). A gradual decrease in the activities of antioxidant enzymes, lower levels of antioxidants in the reduced state and/or both can lead to accumulation of the ROS to toxic levels, thus disturbing the equilibrium of ROS production and removal (Apel and Hirt, 2004).

Given the importance of nonenzymatic metabolism in postharvest, it is imperative to understand the dynamics in the tropical fruits. In this sense, enzymatic systems of cashew apples (*Anacardium occidentale* L.) is a topic interesting, because studies evaluating the changes in components of the nonenzymatic system during ripening of early dwarf cashew clones are very limited.

2. Material and Methods

2.1 Plant material and sampling

The cashew apples (*Anacardium occidentale* L.) evaluated were manually harvested at the Experimental Station of Embrapa Tropical Agroindustry in Pacajus-CE, Brazil (4º11'26,62''S and 38º29'50,78''W) in the early hours of the day, immediately placed in plastic boxes only one layer of cashew apples protected from mechanical injury. The early dwarf cashew clones analyzed included: CCP (Clone Cashew Tree Pacajus) 76, CCP (Clone Cashew Tree Pacajus) 09, BRS (Clone Brazil) 189 and BRS (Clone Brazil) 265 in seven ripening stages scored according to the skin color of the cashew apples and nuts (Lopes et al., 2012). After harvesting, the cashew apples were transported to Embrapa at Posthartvest Physiology and Technology Laboratory in Fortaleza-CE and processed on blender (Walita®, Brazil) to obtain a pulp, which was then refrigerated at -18 °C until analysis was performed.

2.2 Determination of antioxidant enzymes

2.2.1 Protein content

Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin (Sigma-Aldrich®, Co.) as a standard. Measurements were performed in a microplate reader UV/VIS (Synergyx Mx, Biotek®, United States). Results are expressed as $mg.g^{-1}$ FW.

2.2.2 Extraction of antioxidant enzymes

The antioxidant enzyme extract was prepared following a modified version of the protocol of Yang et al. (2009). Fresh pulp (1 g) were macerated for 5 min with ice-cold in a (pH 8.0) buffer solution containing 0.05 M Tris-HCl and 0.1 mM EDTA, followed by filtration through nylon fine mesh. The filtrate rested for 1 h at 4 °C. Then the homogenates were centrifuged at 12,000 g for 15 min at 4 °C. The supernatants obtained constituted the crude enzyme extracts for determination of antioxidant enzymes activity.

2.2.3 Superoxide dismutase activity

Superoxide dismutase (SOD, EC 1.15.1.1) activity was determined by spectrophotometry, based on the inhibition of the photochemical reduction of nitroblue tetrazolium chloride (NBT, Sigma®) (Giannopolitis and Ries 1977). The absorbance was measured at 560 nm and one unit enzyme activity (UAE) was defined as the amount of enzyme required to cause a 50% reduction in the NBT photoreduction rate (Beauchamp and Fridovich 1971). The results are expressed as $U.mg^{-1}$ protein FW.

2.2.4 Catalase activity

Catalase (CAT, EC 1.11.1.6) activity was measured according to Beers and Sizer (1952). The decrease in H_2O_2 (Merck®) content was monitored by measuring the absorbance values at 240 nm and quantified using the molar extinction coefficient (ε 240 = 36 $M^{-1}cm^{-1}$). The results are expressed in $\mu mol\ H_2O_2.mg^{-1}.protein.min^{-1}$ FW.

2.2.5 Ascorbate peroxidase activity

Ascorbate peroxidase (APX, EC 1.11.1.1) activity was assayed according to Nakano and Asada (1981). The reaction was started by adding ascorbic acid, and ascorbate oxidation was measured by recording the absorbance readings at 290 nm. The APX activity was measured using the molar extinction coefficient for ascorbate (ε 290 = 2.8 $mM^{-1}.cm^{-1}$) and the results expressed in $\mu mol\ H_2O_2.mg^{-1}.protein.min^{-1}$ FW, taking into account that 1 mol of ascorbate is required for the reduction of 1 mol H_2O_2.

2.4 Experimental design and statistical analysis

A random design was used for the experiment with 4 clones and 7 ripening stages (4 x 7). Fifteen cashew apples were divided into three replicates of five cashew apples for each stage analysed. Analysis of variance (ANOVA) followed by multiple comparisons of means and significant differences between clones were determined using a Scott-Knott's test at a 5% significance level.

3. Results and Discussion

3.1. Effect of antioxidant enzymes in development and ripening of peduncles

We hypothesized that clones differing in their oxidative behavior during fruit ripening because electron transport during respiration is one of the major sources contributing to the ROS production in the non-photosynthetic plant tissue (Apel and Hirt, 2004). The ROS generation to be an intrinsic feature of senescence and fruit ripening since they could promote the process of oxidative deterioration that contributes to a general deterioration of cellular metabolism (Del Río et al, 1998; Wang and Jiao 2001). It is likely, therefore, that the antioxidant systems, which are distributed in cell organelles (Foyer and Mullineaux 1998; Jiménez et al, 1998), play an important role in both the senescence and the ripening process of fruits. The primary line of defense involving antioxidant enzymes such as SOD, CAT, and APX operate to scavenge these ROS to avoid their accumulation to toxic levels. The increase SOD activity could enhance the ability of the tissue to dismutate O^{2-}, while the enhanced CAT would contribute to the stronger elimination of H_2O_2. The results for the antioxidant enzymes of dwarf cashew peduncles at different stages of maturation are shown in Figure **1.** With regard to SOD activity, in one and two stages the clones differ at 5% significance level. Superoxide dismutase activity increased gradually for the CCP 09 clone, and the highest values for ripe cashew apples were found at stage 7 has reached activity of 5323.08 $U.mg^{-1}$protein FW, activity 22-times higher than CCP 76 clone (Figure 1A).

The BRS 189 clone reached the highest activity for superoxide dismutase at stage 5 (1056.22 $U.mg^{-1}$ protein FW) and six (1063.71 $U.mg^{-1}$ protein FW), these values are 3-times higher than those found for the CCP 76 clone in the same stages (Figure 1A). For SOD activity, the BRS 265 clone showed no difference between the first three stages of development, with peaks in the stages one, three and five, reaching the end of the ripening activity of the 932.89 $U.mg^{-1}$ protein FW (Figure 1A). Changes in the SOD activity during fruit ripening and senescence are inconclusive according some reports.

An increase in SOD activity has been reported during senescence of apples (Du and Bramlage 1994), but it decreased during fruit ripening of blackberries (*Rubus sp.*) (Wang and Jiao 2001), oranges (Huang et al, 2007), plums (Larrigaudiére et al, 2009), peaches (Zheng et al, 2007), and saskatoons (Rogiers et al, 1998).

The increase in SOD activity in CCP 09 clone indicates an increase in the capacity of fruit to neutralize the O^{2-} radicals produced during stages of fruit ripening (Figure 2A). There were differences for CAT activity among CCP 09, CCP 76 and BRS 189 clones between stages three, four and five of development (Figure 1B). At stage 7 the CCP 09 clone showed the CAT activity 3-fold less than BRS 265 clone.

The BRS 265 clone show at end of ripening (stages 6 and 7) higher CAT activity demonstrating the functioning of a highly efficient system of H_2O_2 removal (Figure 2B). APX catalyses the conversion of H_2O_2 into water using two molecules of ascorbate as a reducing power with a concomitant production of two molecules of monodeidroascorbato (MDHA). MDHA radicals rapidly disproportionate into deidroascorbate and ascorbate; the latter reaction is catalyzed by reductase monodeidroascorbato (MDHAR) using NADPH as the electron donor. The DHA is reduced back to ascorbate by the action of reductase deidroascorbate (DHAR), using gluthathione (GSH) as the reducing agent. The activity profile of APX showed no difference throughout development and ripening for clones evaluated ($p>0.05$) (Figure 1C). The red clones (BRS 189 and BRS 265) showed higher APX activity at stage 7 (Figure 1 C). The CCP 76 clone show difference significant among the three first stages of development, while CCP 76 and BRS 189 clones showed an increase slowly of APX activity at end of ripening (stages 6 and 7) (Figure 1C).

The stability of APX activity may be suggested to be responsible for the accumulation of endogenous H_2O_2 membrane deterioration and loss of tissue during ripening of clones (Kumar et al, 2011). Our data on the dynamics of primary antioxidant enzymes, SOD, CAT, and APX, implicate the development of oxidative stress to a greater extent in CCP 09 compared with the other clones.

Vitamin C is redox buffer crucial to the maintenance of a strong antioxidant system in the cell (Noctor and Foyer 1998). Lopes et al. (2012) evaluating the same cashew clones (CCP 09, CCP 76, BRS 265 and BRS 189) in the same seven stages of development and ripening stages of cashew apples showed an increase gradually in ascorbic acid and was observed the highest values are reached at the end of ripening to all the clones evaluated and authors affirmed that BRS 265 clone is richer in vitamin C reaching 279.37 mg.100 g^{-1} at stage 7. Davey and Keulemans (2004) proposed a hypothesis that early maturing apple cultivars are low in AA than the late maturing ones. Vitamin C are very important components of the ascorbate-glutathione cycle, which operates for the removal of H_2O_2 from the cell, involving oxidation of ascorbate their regeneration through enzymatic or non-enzymatic pathways (Noctor and Foyer 1998). According Chempakam (1983), the rise in ascorbic acid level during the last phase of cashew apple development may be attributed to a fall in activity of ascorbic oxidase, the enzyme involved in its degradation. Alternatively, according to the same author, the enlargement of the apple during the final phase may have lowered the concentration of the enzyme itself or the amount of Cu^{++} which is a cofactor for ascorbic acid oxidase. Studies have also shown that cultivars differing in antioxidative metabolism also differed in their potential storage or shelf-life and susceptibility to various physiological disorders (Lacan and Baccou 1998; Hodges et al, 2000). The variation in the antioxidant concentrations in different genotypes of fruits exists as it is influenced by multiple extrinsic and intrinsic factors (Davey and Keulemans 2004; Hodges and Lester 2006). Lopes et al. (2012) evaluating the same cashew clones (CCP 09, CCP 76, BRS 265 and BRS 189) in the same seven stages of development and ripening stages showed that four clones of differing in their non-climacteric ripening exhibit differences in their oxidative behavior and showed which the antioxidant capacity (ABTS method) decreased gradually for CCP 76 and CCP 09 clones during ripening, but at stage 7 the CCP 76 clone showed higher than other clones.

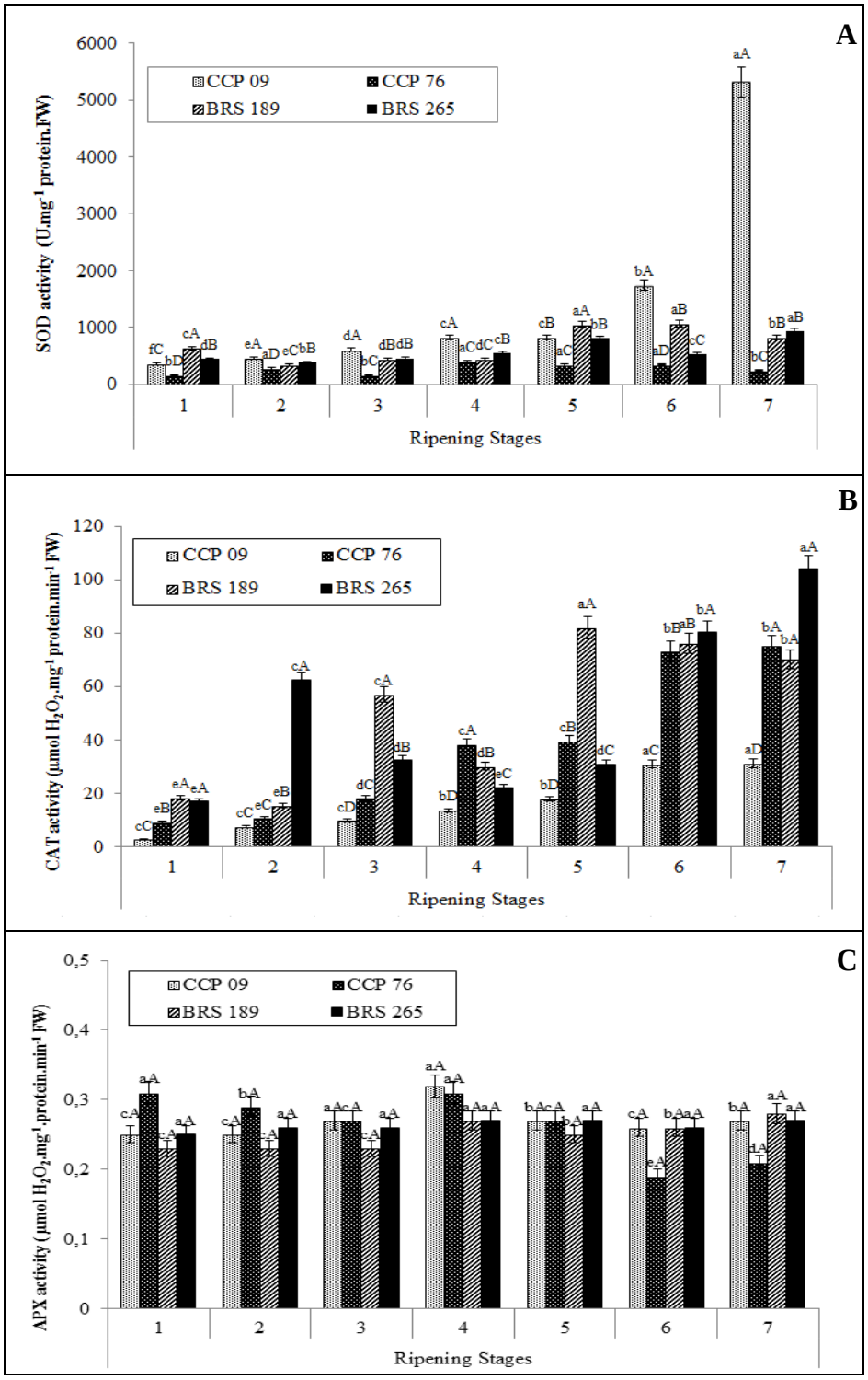

Figure 1: Antioxidant enzymes of cashew apples of early dwarf cashew clones at different development and ripening stages. (**A**) Superoxide dismutase (**B**) Catalase (**C**) Ascorbate peroxidase. Within clones, values with the same capital letters at the same stage are not significantly different at $p>0.05$ according the Scott-Knott test. Between the stages of each clone values with same small letters are not significantly different at $p>0.05$ according the Scott-Knott test. *n=3 (triplicate).

4. Conclusion

The present study has shown that four clones of differing in their non-climacteric ripening exhibit differences in their oxidative behaviour. Further work is required to explore whether the differences in antioxidant metabolism of these clones influence their long-term cold storage and susceptibility to physiological disorders. Changes in the activities of antioxidant enzymes during ripening indicated that the nonenzymatic system plays a fundamental role in the ripening of cashew apples clones.

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