

Biochemical changes in hybrid pumpkin seeds at different stages of maturation¹

Alterações bioquímicas em sementes híbridas de abóbora em diferentes estádios de maturação

Patrícia Pereira da Silva², Antonio Carlos Souza Albuquerque Barros², Denise Cunha Fernandes dos Santos Dias³, Marcelo Coelho Sekita³ and Warley Marcos Nascimento^{4*}

ABSTRACT - This study aimed to evaluate biochemical changes in seeds of the pumpkin hybrid, 'Jabras', from fruit harvested at different stages of maturation (15, 30, 45, 60 and 75 days after anthesis). Thirty fruit were harvested at each stage, with the seeds from 15 of the fruit being extracted immediately. The remaining 15 were stored for twenty days in plastic boxes and the seeds extracted after this period. After processing and drying the seeds, the following were determined: moisture content, germination, first count and antioxidant enzyme activity (peroxidase, ascorbate peroxidase, catalase and superoxide dismutase). Seeds from the fruit harvested at 30 DAA displayed low values for germination and vigour and high antioxidant enzyme activity, indicating that they were immature and that drying possibly caused damage to the system of cell membranes. The results obtained in this study demonstrated that analysis related to changes in the activity of enzymes during development and maturation of the seeds was effective in evaluating the physiological and biochemical changes in pumpkin seeds of the 'Jabras' cultivar.

Key words: *Cucurbita maxima*. *Cucurbita moschata*. Antioxidant enzymes.

RESUMO - O presente estudo teve como objetivo avaliar alterações bioquímicas em sementes do híbrido de abóbora 'Jabras', oriundas de frutos colhidos em diferentes estádios de maturação (15; 30; 45; 60 e 75 dias após a antese), sendo que, em cada época, foram colhidos trinta frutos: quinze frutos tiveram suas sementes extraídas imediatamente e os outros quinze foram armazenados por vinte dias em caixas plásticas e, somente após esse período, tiveram suas sementes extraídas. Após o beneficiamento e secagem das sementes, foram realizadas as seguintes determinações: grau de umidade, germinação, primeira contagem e atividade de enzimas antioxidantes (peroxidase, ascorbato peroxidase, catalase e superóxido dismutase). Sementes provenientes de frutos colhidos até 30 DAA apresentaram baixa germinação e vigor e elevada atividade de enzimas antioxidantes, demonstrando que as mesmas encontravam-se imaturas e que a secagem possivelmente, provocou danos ao sistema de membranas celulares. O resultado obtido neste estudo demonstrou que a análise relacionada às alterações na atividade de enzimas durante o desenvolvimento e maturação das sementes foi eficiente para avaliar as alterações fisiológicas e bioquímicas em sementes de abóbora, cultivar 'Jabras'.

Palavras-chaves: *Cucurbita maxima*. *Cucurbita moschata*. Enzimas antioxidantes.

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*Autor para correspondência

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²Departamento de Ciência e Tecnologia de Sementes, Universidade Federal de Pelotas, Av. Eliseu Maciel s/n, Campus Universitário de Capão do Leão, Pelotas-RS, Brasil, 96.900-900, patybio55@yahoo.com.br, acbarros@ufpel.edu.br

³Departamento de Fitotecnia, Universidade Federal de Viçosa, Viçosa-MG, Brasil, dcdias@ufv.br, marcelosekita@yahoo.com.br

⁴Embrapa Hortaliças, Caixa Postal 280, Brasília-DF, Brasil, 70.275-970, warley.nascimento@embrapa.br

INTRODUCTION

The interspecific F1 pumpkin hybrid ('Jabras') was developed in 1992 by Embrapa, a result of the cross between a strain of *Cucurbita maxima* Duch (female progenitor) and *Cucurbita moschata* Duch (male progenitor), having the characteristics of greater precocity and more uniform fruit when compared to regional open-pollinated cultivars.

One factor of great importance in the process of seed production for this hybrid is to determine the point of physiological maturity of the seed in order to avoid incorrect handling that could affect quality, since there is not always a need for complete maturation of the fruit in the field; it is known that after harvesting the fruit and a further period of storage, immature seeds can complete their development, reaching maximum indices of germination and vigour.

The deterioration process begins with the physiological maturity of the seed, causing a reduction in vigour and viability. The main changes caused by deterioration are the degradation and inactivation of enzymes, a reduction in respiratory rate and a loss of cell membrane integrity. The membranes, composed of a lipid bi-layer, are the main site of the lipid peroxidation process, leading to the production of free radicals with disorganisation of the membrane system and a decline in seed vigour (MCDONALD, 1999).

Degradation is caused by oxidative stress, which is defined as a set of physiological changes resulting from the direct or indirect action of Reactive Oxygen Species (ROS), and affecting metabolic processes such as respiration, CO₂ fixation and gas exchange, among others. Excess production of ROS, such as hydrogen peroxide (H₂O₂), the superoxide radical (O₂⁻) and hydroxyl radical (HO), can damage the seeds, as they cause oxidative changes in the cells and increase the percentage of mutation (MOLLER; JENNSSEN; HANSSON, 2007).

The cells however, present a complex defence system to protect against damage caused by free radicals, involving a set of antioxidant enzymes, which catalyse reactions of molecule formation and regeneration for the capture of ROS (MARTINS; MOURATO, 2008). The system that forms these enzymatic mechanisms consists of the enzymes superoxide dismutase (SOD); catalase (CAT); peroxidases, such as guaiacol peroxidase (POD); and enzymes and metabolites of the ascorbate-glutathione cycle, such as ascorbate peroxidase (APX) and glutathione reductase (GR) (SOFO *et al.*, 2005).

The enzyme superoxide dismutase, present in cellular cytoplasm and in the mitochondrial matrix, has the initial function of catalysing the dismutation reaction

of free superoxide radicals (O₂⁻) and hydrogen peroxide (H₂O₂) (FURIAN *et al.*, 2007). Decomposition is also carried out by catalase, whose subunits are formed in the cytoplasm, and synthesis is completed in the peroxisomes (MCDONALD, 1999). Whereas peroxidase enzymes, in general, catalyse the reduction of H₂O₂ and organic peroxides in alcohols.

Alterations in the profiles of these enzymes are a very important tool in monitoring the biochemical changes resulting from the deterioration that can occur during seed maturation, especially in the final stage of seed development, as oxidation processes tend to increase during water loss (OLIVER; BEWLEY, 1997).

The principal studies into the maturation of pumpkin seeds are related to monitoring the physiological changes that occur during the maturation process, especially studies involving seed dry matter content, germination and vigour. There is still little information related to changes in enzyme activity during seed development and maturation. Given the above, the aim of the present study was to evaluate the physiological and biochemical changes in pumpkin seeds of the 'Jabras' cultivar, obtained from fruits harvested at different stages of maturation and subjected to post-harvest storage.

MATERIAL AND METHODS

The production of hybrid pumpkin seeds, resulting from the cross between *Cucurbita maxima* (female line) and *Cucurbita moschata* (male line), was carried out in the experimental area of Embrapa Hortaliças, in Brasília, DF, from May to October of 2012. Pollination was by hand during the early hours of the day and the pollinated flowers were then labelled.

The fruit was harvested 15, 30, 45, 60 and 75 days after anthesis (DAA). In each period, thirty fruit were harvested, with fifteen fruit being stored for twenty days in open plastic boxes in a ventilated area. The remaining fruit had their seeds extracted immediately after harvest, using slaked lime to remove the mucilage; following extraction, the seeds were dried and processed. After processing, the degree of moisture, germination and first germination count were determined in the Seed Laboratory of Embrapa Hortaliças. Analysis of the enzymes superoxide dismutase, catalase, peroxidase and ascorbate peroxidase was carried out in the Seed Laboratory of the Federal University of Viçosa (UFV), from June to August of 2013. The methodology of each analysis is described below:

Determining the degree of moisture: The oven method was adopted at 105 ± 3 °C for 24 hours, following the Rules for Seed Analysis (BRASIL, 2009).

Approximately 2 g of seeds from each treatment were placed in each container, with two replications. The degree of moisture was determined before and after drying.

Germination: Conducted with four replications of 50 seeds for each treatment, on a roll of germitest germination paper moistened with distilled water to twice the weight of the dry paper, in a germinator at an alternating temperature of 20 °C (16 h, dark) and 30 °C (8 h, light). Counts were taken eight days after the start of the test, and the evaluations made as per criteria established by the Rules for Seed Analysis (BRASIL, 2009).

First count: This analysis was carried out together with the germination test, counting the number of normal seedlings present on the fourth day after the start of the test. The results were expressed as a percentage (BRASIL, 2009).

Enzymatic analysis: First, the crude enzyme extract of the seeds was obtained to determine enzyme activity by homogenising in a mortar 0.3 g of seeds from each treatment in 2.0 mL of 0.1 M potassium phosphate buffer pH 6.8, supplemented with 50 mg of PVPP (Polyvinylpolypyrrolidone). The homogenate was then centrifuged at 12,000 xg for 15 minutes at 4 °C and the supernatant collected and reserved in an ice bath.

The total soluble protein content was carried out in triplicate, using a test tube with 5 mL of Bradford reagent, and adding 100 µL of the crude extract. It was then agitated, and after 15 minutes, the absorbance was read at 595 nm. Bovine serum albumin solution (BSA-Sigma) was used as the standard, with which a standard curve was obtained for the concentration range of between 0 and 1 mg mL⁻¹; the protein concentration in the samples was determined by interpolation of the standard curve.

Determination of SOD activity considered the capacity of the enzyme to inhibit the photoreduction of NBT (nitrotetrazolium blue chloride). The activity was determined by adding 50 µL of the crude seed extract to a solution containing 13 mM methionine, 75 µM NBT, 100 nM EDTA and 2 µM riboflavin in 3.0 mL 50 mM potassium phosphate buffer pH 7.8, as described by Del Longo *et al.* (1993). The Thermo Scientific™ UV-Vis GENESYS 10S spectrophotometer was programmed to read a wavelength of 560 nm. The reaction began with illumination of the test tubes in a chamber containing tubular fluorescent lamps (15 W) at 25 °C. After five minutes incubation, the lamps were switched off, thereby stopping the reaction. The blue compound (formazan) formed by photoreduction of the NBT was determined by spectrophotometer reading at 560 nm. For analysis, the test tubes considered as white received the same reagents, but were kept covered with aluminium foil and sheltered from light. One unit of SOD is defined as the amount of

enzyme necessary to inhibit 50% of the photoreduction of the NBT. To calculate the specific activity of the enzyme, the percentage of inhibition, the volume of the sample, and the protein concentration in the sample (µg µL⁻¹) were considered.

CAT enzyme activity was determined based on the amount of enzyme required to catalyse the decomposition of H₂O₂. For the test, 950 µL of 50 mM potassium phosphate buffer pH 7.0, supplemented with hydrogen peroxide at a final concentration of 12.5 mM, was placed in quartz cuvettes and the reaction initiated by the addition of 50 µL of crude extract. The variation in absorption (ΔE) was calculated by the difference in readings at 240 nm over an interval of 80 seconds. The activity of the enzyme was calculated using the molar extinction coefficient = 39.4 mM⁻¹ cm⁻¹; determination of the specific activity (µKat µg Prot⁻¹) considered the soluble protein concentration of the sample.

The enzyme peroxidase (POX) was determined by spectrophotometry, where the oxidation of guaiacol is measured in the presence of hydrogen peroxide at a wavelength of 420 nm. The results were expressed in units of peroxidase. Each unit of peroxidase corresponded to a variation of 0.001 in the absorbance value at 470 nm, per minute of reaction, per milligram of total protein.

The enzyme APX was determined based on the oxidation of ascorbate in the reaction for the peroxidase activity of the enzyme. Initially, a working solution containing a 100 µL aliquot of crude extract was prepared with 2.9 mL of 50 mM potassium phosphate buffer pH 6.0 (final volume of 3.0 mL). To this solution, ascorbate and hydrogen peroxide were added at a final concentration of 0.8 and 1 mM respectively. The variation in absorption was measured with a reading at 290 nm, and the specific activity of the enzyme (µKat µg Prot⁻¹) was calculated with a molar extinction coefficient of 2.8 mM⁻¹ cm⁻¹.

Statistical analysis: The trials were conducted in a completely randomised experimental design with four replications, in a 5 x 2 bifactorial scheme (five harvest times and two periods of fruit storage). Data were submitted to analysis of variance, and the means compared by Tukey's test (p ≤ 0.05). After the analysis of variance, regression analysis was performed using the PROC REG procedure of the SAS software (SAS 9.1.3, 2000-2004).

RESULTS AND DISCUSSION

Significant differences were seen between harvest times in relation to seed germination in the fruit stored for 20 days. Seed germination increased, with a certain stability seen between 60 and 75 DAA,

this period being considered a possible indication of the physiological maturity of the seeds, which reached maximum germination (97%) at 75 DAA (Figure 1A). It was noted that seeds obtained from fruit stored for 20 days showed greater germination compared to seeds extracted immediately after harvesting. It is important to note that 20 days' storage for fruit harvested at 75 DAA was enough to raise germination from 84% to 97%.

The negative values seen in the graph were due to the trend line that was generated from the cubic regression equation (which fit with 86.81% according to the coefficient of determination), and therefore did not present values close to 100%. However, this equation was the one that best fit the data.

Seed vigour, as determined by the test for first count, increased with age and fruit storage (Figure 1B). An increase was seen in seeds from fruit harvested at 60 DAA and stored for 20 days, stabilising as of that period. Similar behaviour was observed in seeds of the Italian pumpkin cv. Jacarezinho, where 30 days' storage of fruit harvested between 50 and 60 DAA was also beneficial to vigour (FIGUEIREDO NETO *et al.*, 2013).

Total soluble protein concentration in the seeds increased during the maturation period, with seeds from fruit stored for 20 days having greater protein concentrations up to 60 DAA, stabilising during this time (Figure 2A). It can be seen that during development, the decrease in seed water content (Figure 2B) follows the increase in the levels of reserve substances, with this possibly contributing to a greater concentration of proteins in the seeds, and consequently to germination (Figure 1A). It should also be noted that the main function of reserve proteins is the supply of amino acids for the formation of new proteins during germination.

The enzyme SOD is the first enzyme of the enzymatic barrier, and is thus considered the first line of defence against oxidative stress due to its action in the dismutation of $O_2^{\bullet-}$, transforming it into $H_2O_2 + O_2$ (GILL; TUTEJA, 2010; GRATÃO *et al.*, 2005). When analysing its activity in pumpkin seeds, a decrease was seen during the stages of maturation (Figure 3A), where seeds from stored fruit displayed in general less activity than seeds from unstored fruit.

Contrasting results were seen in cucumber seeds by Nakada *et al.* (2011), where the SOD concentration increased only up to 40 DAA. On the other hand, Vidigal *et al.* (2009), working with pepper seeds, observed an increase in the activity of this enzyme in seeds from fruit harvested at 50 DAA and stored for 6 days; in this case, the formation of free radicals in the immature seeds probably occurred during drying, activating the SOD as a cellular repair mechanism to promote the removal of molecular oxygen (O_2) in the seeds.

Drying of the seeds results in the accumulation of ROS and free radicals in the cells (NTULI *et al.*, 2011), caused by the rupture of the plastids and of the electron transport chain in the mitochondria (FERREIRA, ABREU, 2007). The increase in the concentration of these radicals is potentially toxic to cellular integrity, being able to induce the oxidation and depolymerisation of nucleic acids, the denaturation of proteins and the peroxidation of plasma membrane lipids (KRANNER *et al.*, 2010).

A decrease was seen in CAT enzyme activity throughout seed maturation, where in seeds from fruit harvested at 15 DAA and stored for 20 days, the activity was 0.66 mg, whereas in seeds from fruit harvested at 75 DAA and stored for 20 days, the concentration was 0.21 mg (Figure 3B). It should again be noted that seeds

Figure 1 - Germination (A) and first count (B) in hybrid pumpkin seeds from fruit harvested at 15, 30, 45, 60 and 75 days after anthesis (DAA) and stored for 0 and 20 days

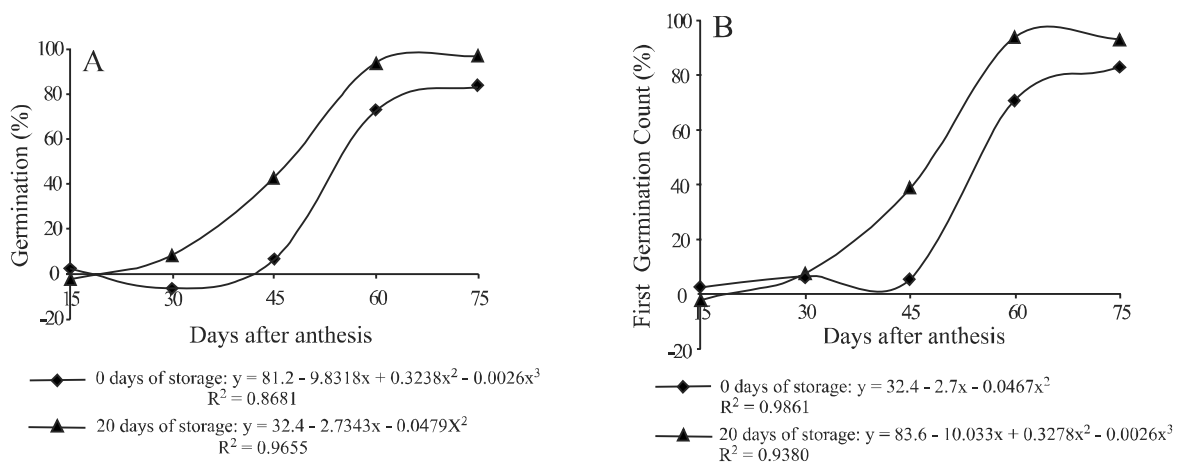


Figure 2 - Total soluble protein concentration (A) and degree of moisture (B) in pumpkin seeds from fruit harvested at 15, 30, 45, 60 and 75 days after anthesis (DAA) and stored for 0 and 20 days

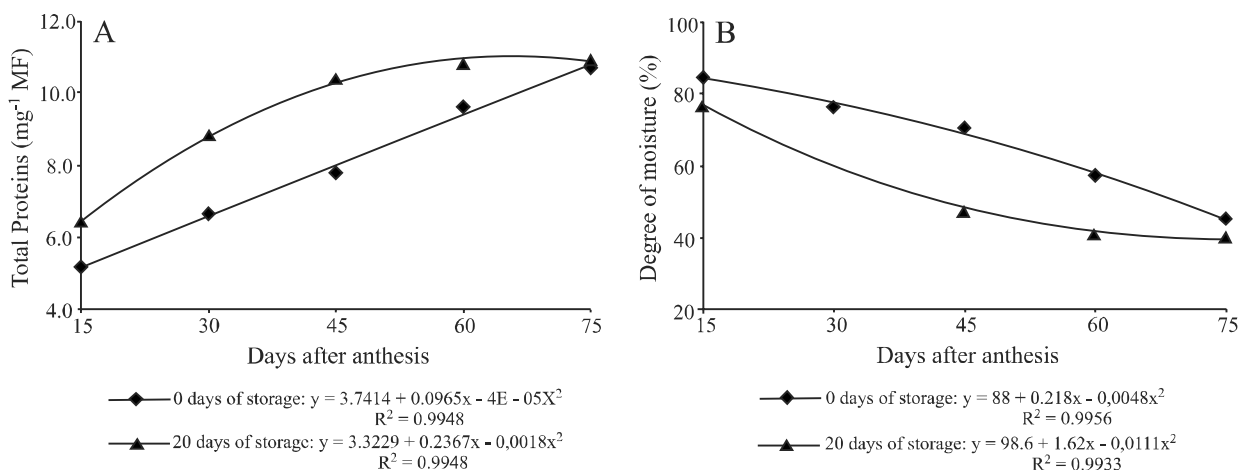
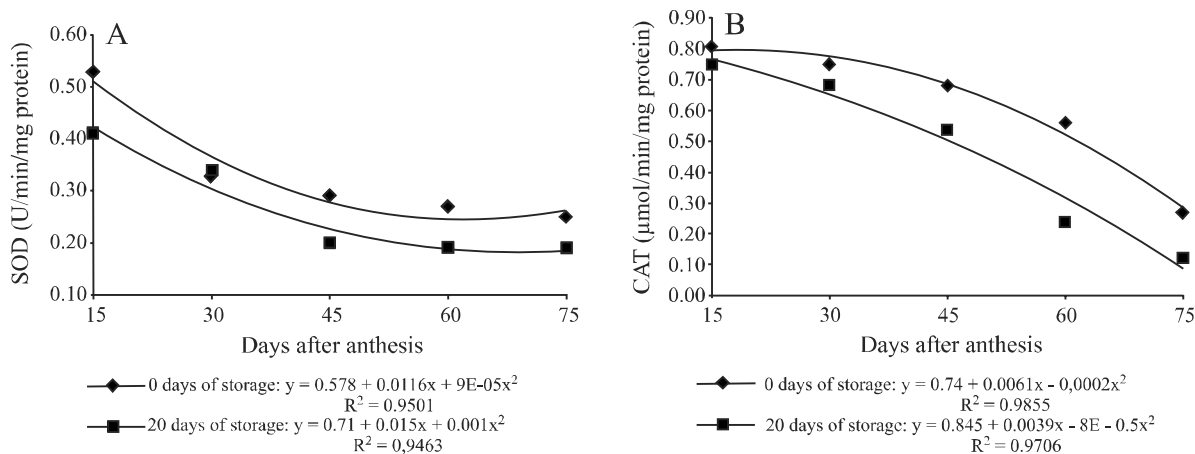


Figure 3 - Activity of the enzymes superoxide dismutase (SOD) (A) and catalase (CAT) (B) in pumpkin seeds from fruit harvested at 15, 30, 45, 60 and 75 days after anthesis (DAA) and stored for 0 and 20 days



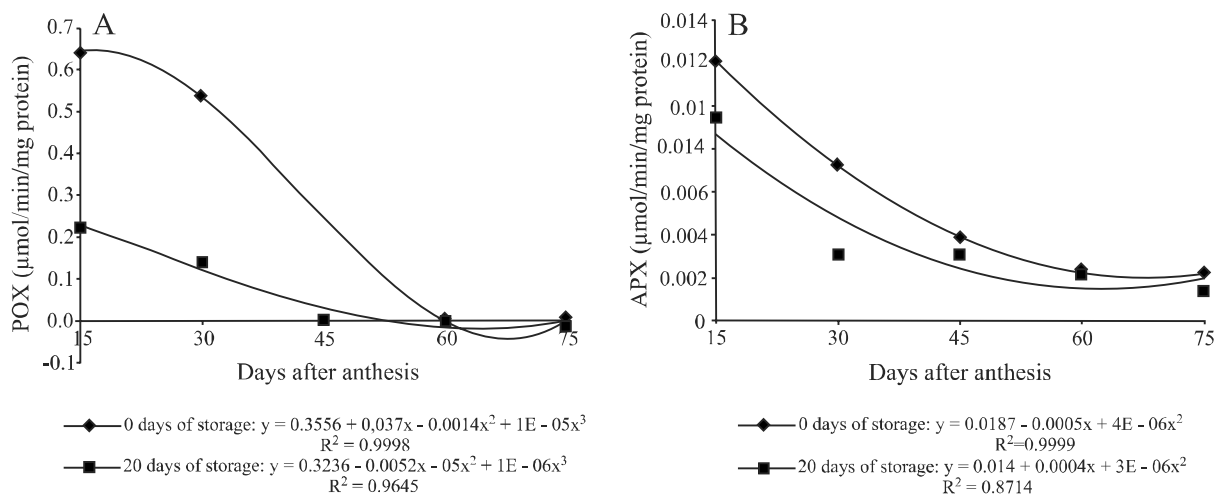
from fruit that had been stored showed less enzyme activity, thereby demonstrating a greater resistance to drying. In seeds from fruit harvested at 15 and 30 DAA, going through the drying process, considered a stress factor, probably activates the formation of free radicals due to an intolerance to desiccation, i.e. seed immaturity, which would explain the greater SOD and CAT activity. Similar results were obtained in cucumber seeds harvested at 40 DAA (NAKADA *et al.*, 2011).

The pattern of POX activity (Figure 4A) after seed drying was similar to that obtained for CAT. It was found that the activity of this enzyme was less in seeds that were beginning the maturation process, i.e. from 45 DAA, with seeds from stored fruit showing a lower concentration. A possible explanation for this is that the seeds from fruit that went through a period

of rest took more time to complete maturation, with the physiological quality of the mature seeds being preserved by remaining in osmotic equilibrium inside the fruit, showing greater viability and vigour.

The enzyme POX is widely distributed in the cellular compartments, being associated with the cell walls and membranes, organelles, vacuoles and cytosol (GILL; TUTEJA, 2010). In addition to allowing wider cell distribution than CAT, POX has a molecular mass of 35 kDa, allowing greater mobility where its action is required. It plays an important role in seed metabolism, contributing to an increase in defence mechanisms and the prevention of quality loss, mainly due to the oxidation of a great variety of hydrogen donor substances, such as for example, phenols, aromatic ring groups, diamines, ascorbic acid and amino acids, as well as some inorganic

Figure 4 - Activity of the enzymes peroxidase (POX) (A) and peroxidase ascobate (APX) (B) in pumpkin seeds from fruit harvested at 15, 30, 45, 60 and 75 days after anthesis (DAA) and stored for 0 and 20 days



ions by means of hydrogen peroxide (H_2O_2) (HORVÁTH *et al.*, 2007).

The APX enzyme, which is found mainly in the cytosol, and may be found associated with the mitochondria, peroxisomes and apoplasts (SALAMA; GAAFAR; FOULY, 2015), showed a reduction in activity throughout seed maturation (Figure 4B). More intense activity by this enzyme in the early stages of the seed maturation process indicates that its defensive action for the reduction of superoxide, with the consequent reduction in the formation of free radicals, undergoes a higher demand during this period, whether due to the process of deterioration or the immaturity of the seeds. APX has a higher affinity for H_2O_2 (μM) than do CAT and POX (mM) (SALAMA; GAAFAR; FOULY, 2015). In the present work however, it was found that CAT activity was more active in the removal of H_2O_2 from the cellular metabolism.

CONCLUSIONS

1. Seeds from fruit harvested up to 30 DAA presented low germination and vigour, and high antioxidant enzyme activity. This shows that the seeds were immature, and that drying may have caused damage to the cell membrane system, leading to the accumulation of ROS and consequently, an increase in antioxidant enzyme activity;
2. The results show that analysis of the changes in enzyme activity during seed development and maturation was effective in evaluating physiological and biochemical changes in pumpkin seeds of the 'Jabras' cultivar, from

fruits harvested at different stages of maturation and subjected to post-harvest storage.

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