Short communication

Abscisic acid and the antioxidant system are involved in germination of *Butia capitata* seeds

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ABSTRACT

Seed germination is an important step for plants without vegetative propagation and is a physiological process that begins with specific environmental cues resulting in biochemical responses. Breaking-dormancy is necessary to study germination in dormant seeds with asynchronous germination. We investigated the processes of breaking dormancy and germination of *Butia capitata* (Arecaceae) seeds, in which germination is slow and asynchronous, by operculum removal. This treatment increased germination of *B. capitata* to 90 %. Embryos of dry, imbibed, 24-hours post-operculum removal and early-germinated seeds were collected for biochemical analysis of the following: quantification of abscisic acid (ABA) and hydrogen peroxide (H_2O_2), activities of antioxidant enzymes (catalase – CAT, superoxide dismutase – SOD, glutathione reductase – GR) and histolocalization of superoxide anion (O_2^-). Decreases in H_2O_2 and ABA were recorded 24 hours post-operculum removal. Increased GR and SOD activities during imbibition, and CAT upon germination, indicate a role in controlling reactive oxygen species. Interestingly, the accumulation of O_2^- on the haustorium upon imbibition seems to be involved in germination, instead of H_2O_2. For *B. capitata* seeds, signaling from the removal of the operculum probably resulted in ABA catabolism mediated by O_2^-, which thus promoted seed germination.

Keywords: breaking-dormancy, haustorium, hormones, palm seeds, operculum, physiological dormancy, reactive oxygen species

Seed germination is one of the most important processes in the life cycle of a plant, and its success depends on environmental conditions and appropriate physiological and biochemical responses (Bewley *et al.* 2013). Reactive oxygen species (ROS) are continuously produced in metabolically active cells of seeds (Gomes & Garcia 2013). During metabolic recovery following imbibition, ROS production increases as germination progresses (Bailly 2004). The production of ROS stimulates the antioxidant system to scavenge toxic levels of those same chemicals as they interact with phytohormones and germination signaling pathways (Diaz-Vivancos *et al.* 2013). Reactive oxygen species, especially hydrogen peroxide (H_2O_2), are known to be involved in gibberellin (GA) biosynthesis and abscisic acid (ABA) catabolism during germination (Liu *et al.* 2010; Gomes & Garcia 2013). The balance between these two phytohormones induces germination or the maintenance of dormancy (Bicalho *et al.* 2015; Vieira *et al.* 2017).

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Palms (Arecaceae) are restricted to the tropics (Tomlinson 2006), but can be found in varying environments (Svenning 2001) with many species producing seeds with primary dormancy and very slow seedling recruitment over time (Pérez *et al.* 2008; Baskin & Baskin 2013; Bicalho *et al.* 2015). In the cerrado (Brazilian savanna), there are interesting and useful palm species that contribute to the survival and development of local communities. One such palm is *Butia capitata* (Mart.) Becc., popularly known as coquinho-azedo, which naturally occurs in environments with seasonal water deficit and whose fruits are used as food. The germination of *B. capitata* seeds is considered slow and asynchronous due to the physiological, or morphophysiological (see Baskin & Baskin 2014), dormancy of this species, which is related to the difficulty the embryo has in overcoming restrictions imposed by adjacent tissues (Oliveira *et al.* 2013). Overexploitation and slow natural regeneration by seeds call for conservation efforts regarding the species. However, the physiological changes, as well as the underlying signaling, involved in the process of seed germination of *B. capitata* are not well understood.

We thus performed an experiment to better understand the relationship between abscisic acid (ABA), ROS and the antioxidant system of *B. capitata* embryos during germination. To facilitate the experiment and to optimize the synchrony of germination, we employed operculum removal for overcoming dormancy since this method is known to significantly increase germination of palm species (Spera *et al.* 2001; Fior *et al.* 2011; Oliveira *et al.* 2013; Bicalho *et al.* 2016). The operculum comprises the region with micropilar endosperm and the tegument that is pushed by the embryo during natural germination. However, the natural removal of the operculum by the embryo (visible germination) in *B. capitata* (and other palm species, see Segovia *et al.* 2003, and Bicalho *et al.* 2015) is very slow and asynchronous (Oliveira *et al.* 2013), which would make the present experiment impracticable. Artificial removal of the operculum exposes the embryo to the environment, which may trigger some kind of signaling, but information regarding this is scarce. Thus, our main objective was to better understand the dynamics of ABA and ROS following operculum removal that leads to germination.

Fruits of *B. capitata* were collected in the municipality of Mirabela, state of Minas Gerais. The region is tropical with hot summers and very dry winters; the mean annual precipitation is approximately 900 mm with a seasonal water deficit, and the mean annual temperature is 22 °C with a thermal range of approximately 15 °C (*Cwa* by the Köppen system) (Köppen 1900; Alvares *et al.* 2013).

Approximately 4000 *B. capitata* fruits were collected from at least 100 trees. Four replicates of 15 seeds were sowed in distilled water and used to determine imbibition time by weighing the seeds daily until they reached a constant weight. Four replicates of 25 seeds were used for preliminary germination experiments. These seeds were surface sterilized with 5 % NaClO for 15 minutes and then rinsed three times with distilled water. The seeds were then held in distilled water for three days and subsequently transferred to transparent germination boxes lined with a double layer of filter paper moistened with distilled water, which were kept in a germination chamber at 30 °C for 30 days under a 12 h - photoperiod (Phillips, 40 µmol photons m^{-2} s^{-1}).

The opercula of the seeds were removed three days after immersion in distilled water (when imbibition stabilized). After this dormancy-breaking treatment, the germination experiment was carried out in the germination chambers as previously described with germination percentages being evaluated daily. The criterion used for visible germination was the protrusion of the cotyledonary petiole.

Embryos were extracted from seeds at different phases of the germination process: pre-imbibition (D, dry), post-imbibition (I), 24 hours after operculum removal (24 h), and early germination (G, visible germination). The sampled embryos were used for the following biochemical analyses: quantification of ABA and H_2O_2; enzymatic activity of catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR); and localization of superoxide anion O_2^-. Germination phases were independently sampled with the samples consisting of four replicates of 200 embryos.

Abscisic acid extraction was performed following Weiler (1980), with some modifications. Four replicates of 100 mg of embryos were powdered using a mortar and pestle with liquid nitrogen and then homogenized in 80 % methanol with 0.5 mg mL^{-1} ascorbic acid and 10 mg L^{-1} BHT. The samples were then added to pH 7.0 TBS buffer with the resulting solution reaching less than 10 % organic solvents. Quantification of ABA (controls, standard curve, samples and calculations) was performed strictly following the recommendations of Phytodetek® ABA Test Kits (Agdia Incorporated, Indiana, USA). Since water content increased throughout imbibition, the values of ABA quantification obtained for I, 24 h and G phases were corrected based on D phase moisture content.

Quantification of H_2O_2 was performed following Velikova *et al.* (2000). Four replicates of 100 mg of embryos were powdered using a mortar and pestle with liquid nitrogen, homogenized with 0.1 % trichloroacetic acid (TCA), and centrifuged at 12000 g for 15 minutes. The extract was reacted with 10 mM of pH 7.0 phosphate buffer and 1 M KI. The absorbance was recorded at 390 nm and the H_2O_2 content subsequently calculated from the standard curve. The values of H_2O_2 were also corrected as for ABA quantification.

Antioxidant enzyme extract was prepared following Gomes *et al.* (2013). Four replicates of 100 mg of embryos were pulverized in liquid nitrogen and homogenized in 100 mM of pH 7.8 phosphate buffer with 100 mM EDTA and 5 % PVP. The extract was then centrifuged at 12000 g (4 °C)

and subsequently used to evaluate enzymatic activity and protein content, the latter using Bradford's method (Bradford 1976).

Catalase activity was determined following Aebi (1984) in 67 mM phosphate buffer (pH 7.0) with 10 mM H_2O_2 and adequate amounts of the enzyme extract. Consumption of H_2O_2 was recorded at 240 nm for 2 minutes ($\mathcal{E}$ = 0.0394 mM^{-1} cm^{-1}). Superoxide dismutase activity was determined in a reaction buffer containing 50 mM phosphate buffer (pH 7.8), 13 mM L-methionine, 0.1 mM EDTA, 0.002 mM riboflavin, and 0.075 mM nitroblue tetrazolium (NBT) (Giannopolitis & Ries 1977). Blue formazan concentrations were read at 575 nm, and one unit of SOD was considered as the amount of enzyme necessary to reduce 50 % of the O_2^-. Glutathione reductase activity was determined in media containing 100 mM phosphate buffer (pH 7.8), 50 mM oxidized glutathione, 5 mM NADPH, and adequate amounts of the enzymatic extract, with NADPH oxidation being monitored at 340 nm ($\mathcal{E}$ = 6.2 mM^{-1} cm^{-1}) (Foyer & Halliwell 1976).

Histolocalization of superoxide anion (O_2^-) was performed strictly following Oracz *et al.* (2012). Twenty embryos of each phase were immersed in 10 mM of TRIS buffer (pH 7.0) with 1 mM of a nitroblue tetrazolium (NBT) solution for 10 minutes. The embryos were then washed three times in 10 mM TRIS buffer (pH 7.0) and photographed.

The data for ABA, H_2O_2 and antioxidant enzyme activities were tested for normality and homoscedasticity using Cochran and Levene´s test, respectively. Normal and homoscedastic or transformed data (arcsine root square) were analyzed by ANOVA followed by the Tukey test for comparisons of all means at 5 % level of probability, using Statistica 7 StatSoft® software.

The germination of *B. capitata* seeds was 88 % at 72h post-operculum removal (Fig. 1A) with great concentrations of ABA prior to imbibition (Fig. 1B). Biosynthesis of ABA has been shown to be a response to water deficit (Bray 1997), a common condition in plants growing in localities with well-defined dry seasons (Liu *et al.* 2005), as is the case for *B. capitata*. The initially high levels of ABA observed for *B. capitata* embryos reduced during imbibition (Fig. 1B). Reductions in ABA levels during imbibition were also observed by Bicalho *et al.* (2015) for macaw palm embryos, and by Dias *et al.* (2017) in the cotyledonary petiole of *B. capitata* seeds, both of which were related to leakage. In this work the reduction in H_2O_2 levels was found only 24 h after operculum removal (Fig. 1C) and the presence of H_2O_2 has been shown to induce ABA catabolism in pea seeds, thereby allowing germination (Barba-Espin *et al.* 2010). It is interesting to point out that in the present work ABA levels decreased continuously from I to early-germination phase (Fig. 1B). Concentrations of H_2O_2 were also observed to be maintained in the early-germination phase relative to 24 h post-operculum removal (Fig. 1C).

As shown by purple coloration indicating a positive reaction with NBT, O_2^- was intensely produced in the haustorium region of *B. capitata* embryos since imbibition (Fig. 2). Interestingly, the presence of O_2^- was indicated only in haustorium region, which is rich in reserves for early seedling growth. These results suggest that the haustorium has actions that go beyond reserve mobilization beginning with the first events of imbibition (see the increasing color intensity from D to G embryos of *B. capitata*; Fig. 2). The haustorium can also be assumed to play a role as a source of signaling mediated by O_2^- formation. An interesting relationship was observed here between ABA and ROS: reductions of both ABA and H_2O_2 levels following operculum removal (Fig. 1B), with an increase in O_2^- formation (Fig. 2). These results indicate that the dynamics of ROS and ABA are related to operculum removal. This leads to the suggestion that the exposure of the embryo to O_2 due to operculum removal (dormancy-breaking process) induced metabolic changes in hormonal and oxidant components. Reductions in H_2O_2 levels and increasing O_2^- formation in embryos of after operculum removal have also been observed in other palm species (TRS Santos unpubl. res.). The formation of ROS during germination is a natural process due to the reactivation of metabolism and increased respiration (Diaz-Vivancos *et al.* 2013), and can serve as a window of signaling, or the cause of damage if overproduced (El-Maarouf-Bouteau & Bailly 2008). Therefore, the activity of antioxidant enzymes during germination of *B. capitata* seeds was investigated.

Among the enzymes investigated here, SOD and GR were observed to have increased activity relative to D seeds as soon as imbibition occurred (Fig. 1E, F), while CAT activity was higher in the early-germination phase than in phases I and 24 h post-operculum removal ($P < 0.05$; Fig. 1D). The SOD and GR results indicate that imbibition in *B. capitata* seeds seems to induce or stimulate the activity of antioxidant enzymes. Indeed, that imbibition leads to increased respiration metabolism, which generates ROS and stimulates antioxidant pathways to keep redox homeostasis under control, has been widely discussed (Foyer & Noctor 2005; El-Maarouf-Bouteau & Bailly 2008; Diaz-Vivancos *et al.* 2013; Gomes & Garcia 2013). It is important to point out that CAT activity was at least 10-fold higher than that of SOD (Fig. 1D, E), suggesting that H_2O_2 was maintained at non-toxic levels in germinating seeds (higher CAT activity). Another interesting observation is that the increased SOD activity upon imbibition did not eliminate O_2^-, which leads to the conclusion that the presence of this molecule is essential for the process of seed germination. Moreover, since O_2^- is present only in the haustorium, it participates in germination itself and not only in reserve mobilization (a post-germinative event). Altogether, as germination of *B. capitata* seeds progresses successfully, the activities of CAT, SOD and GR can be assured and are essential for keeping
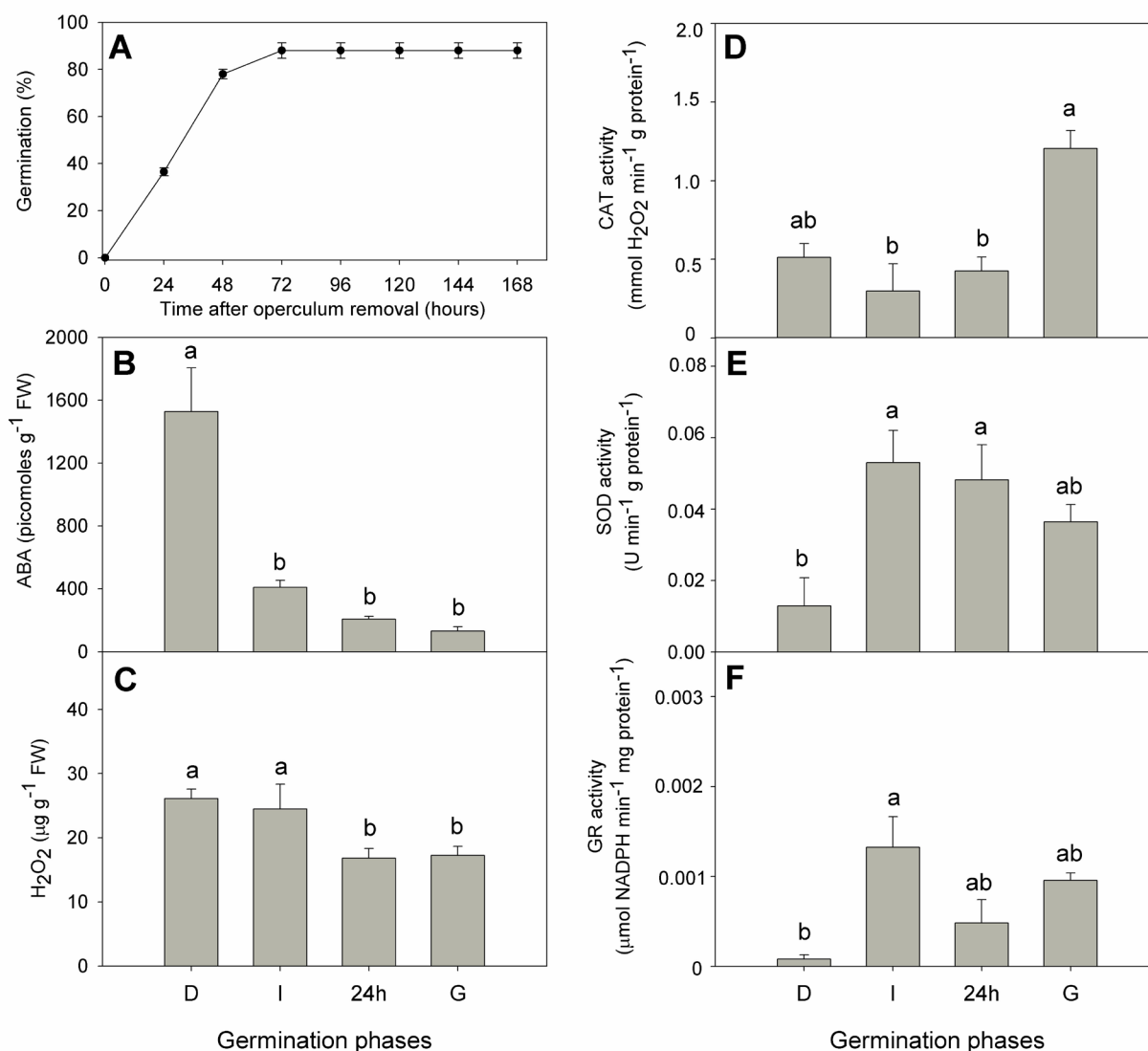
Figure 1. Germination percentage and biochemical parameters for embryos of *Butia capitata* during phases of germination. Graphics show germination percentage from the dormancy-breaking process (**A**); levels of ABA (**B**) and H_2O_2 (**C**); and CAT (**D**), SOD (**E**) and GR (**F**) activities during germination phases. Dots or bars are means ± standard error of four replicates. Means followed by the same letters do not differ according to Tukey test at 5 % of probability. ABA, abscisic acid; H_2O_2, hydrogen peroxide; CAT, catalase; SOD, superoxide dismutase; GR, glutathione reductase (y-axis); D, dry; I, imbibed; 24h, 24 hours after operculum removal; G, early-germinated (x-axis).

Figure 2. Histolocalization of superoxide anions (O_2^-) in embryos of *Butia capitata* seeds during germination. The dark blue/purple indicates the presence of the anion. D, dry; I, imbibed; 24h, 24 hours after operculum removal; G, early-germinated.

ROS under control during the recovery of metabolism, and thus allowing germination to occur.

The present study showed the involvement of ROS and antioxidant enzymes other than ABA during the germination process of the palm species *Butia capitata*. The involvement of H_2O_2 and O_2^- during the entire process was evident. Interestingly, it seems that superoxide anion is more related to germination signaling in *B. capitata* seeds than the expected H_2O_2, by interacting with ABA after operculum removal and allowing germination itself to proceed. The activity of GR and SOD had important roles in ROS scavenging during the entire process, while CAT activity must be related to the initial development of seedlings due to its increased activity in early-germination

seeds, as shown here. Taking all the enzymes together, their role in ROS scavenging seems to be secondary to their role in seed germination signalling. The marked presence of O_2^- was essential for demonstrating that the haustorium is important for palm seed germination from the beginning of the germination process. Actually, the role of the haustorium during palm seed germination itself has been neglected in previous works and needs to be investigated more deeply in *B. capitata* and in other palm species.

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