

Oxidative metabolism altered by plant growth regulators in atemoia seeds (*Annona x atemoya* Mabb.) ‘Thompson’

Ana Paula Yuki Hata¹, Gisela Ferreira², Marília Caixeta Sousa³,
Breno Kennedy Lima Bezerra⁴, Camila Kissmann⁵

Abstract—The objective of this work was to validate the enzymatic activity in *Annona x atemoya* seeds submitted to treatments with plant growth regulators belonging to the group of gibberellins and cytokinins for overcoming dormancy during the germination process. Initially, the water acquisition curve was determined, where two points of phase II (72 and 144 hours) were determined to evaluate the activity of superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) enzymes in atemoya seeds after the application of two plant growth regulators. Treatments with GA resulted in higher activity of SOD enzyme, which may be favored the higher germination percentage and speed in comparison to control treatment. In cases in which SOD enzyme presented high activity, other enzymes showed the opposite, which indicates the predominantly production of superoxides during this period. The POD enzyme also has activity in seeds treated with gibberellin, which suggests interaction of ROS with GA in the process of dormancy overcoming. Treatments with cytokinin and cytokinin + gibberellin resulted in germination percentage similar to control and no activity pattern of SOD, POD and CAT enzymes that could be correlated with germination metabolism was observed.

Index terms: germination, Annonaceae, enzymatic activity, gibberellin, cytokinin.

Metabolismo oxidativo alterado por reguladores vegetais em sementes de atemoia (*Annona x atemoya* Mabb.) ‘Thompson’

Resumo—O presente trabalho teve por objetivo avaliar a atividade enzimática em sementes de *Annona x atemoya* submetidas a tratamentos com reguladores vegetais do grupo das giberelinas e citocininas para superação de dormência, durante o processo de germinação. Inicialmente, foi determinada a curva de aquisição de água das sementes, sendo determinados dois pontos da fase II (72 e 144 horas) para a determinação da atividade das enzimas: superóxido dismutase (SOD), peroxidase (POD) e catalase (CAT) em sementes de atemoia, após a aplicação dos reguladores vegetais. Os tratamentos com GA resultaram em maior atividade da enzima SOD, o que pode ter favorecido a maior porcentagem e a velocidade de germinação em comparação ao tratamento-controle. Nos casos em que a enzima SOD se apresentou elevada, as demais enzimas apresentaram atividade reduzida, o que indica a produção predominantemente de superóxidos nesse período. A enzima POD também teve atividade em sementes tratadas com giberelina, o que sugere a interação das EROS com GA no processo de superação da dormência. Os tratamentos com citocinina e citocinina + giberelina resultaram em porcentagem de germinação semelhante ao controle e não foi observado um padrão na atividade das enzimas SOD, POD e CAT que pudesse ser correlacionado ao metabolismo germinativo.

Termos para indexação: germinação, Annonaceae, atividade enzimática, giberelina, citocinina.

Corresponding author:
gisela.ferreira@unesp.br

Received: September 08, 2020
Accepted: December 03, 2020

Copyright: All the contents of this journal, except where otherwise noted, is licensed under a Creative Commons Attribution License.



¹Bachelor's degree in Bioprocess Engineering and Biotechnology, São Paulo State University (UNESP), Botucatu, SP, Brazil. Email: yuki_hata@hotmail.com (ORCID: 0000-0003-4430-9168)

²PhD, Associated Professor at Plant Biology: BBVPZ Department, Institute of Biosciences, São Paulo State University (Unesp), Botucatu-SP, Brazil. E-mail: gisela.ferreira@unesp.br (ORCID: 0000-0002-3065-2152)

³PhD in Biological Sciences - Botany, BBVPZ Department, Institute of Biosciences, São Paulo State University (Unesp), Botucatu-SP, Brazil. E-mail: marilia.kxeta@gmail.com (ORCID: 0000-0003-1386-1670)

⁴PhD in Agronomy - Agriculture, Department of Plant Production and Genetic Improvement. State University (UNESP), Botucatu, SP, Brazil. Email: breno.kennedylb@gmail.com (ORCID: 0000-0003-0745-071X)

⁵PhD, Associated Professor at Plant Biology: BBVPZ Department, Institute of Biosciences, São Paulo State University (Unesp), Botucatu-SP, Brazil. E-mail: camilakissmann@gmail.com (ORCID: 0000-0001-8636-4929)

Introduction

The seed germination process, which begins with seed hydration and metabolism reactivation and results in the protrusion of the primary root (BEWLEY et al., 2013), is influenced by several intrinsic and extrinsic factors and included in the first group reactive are the oxygen species (ROS) (GOMES; GARCIA, 2013). ROS are by-products of redox reactions generated as a result of electronic excitation, forming singlet oxygen (1O_2), or successive additions of electrons to molecular oxygen, forming superoxide ($O_2^{\cdot-}$), hydroperoxyl radical (HO_2^{\cdot}) or hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^{\cdot}) (FOYER, 2018).

Although initially recognized as toxic by-products (GILL; TUTEJA, 2010), recent studies have shown the positive participation of ROS in various processes related to seed physiology (ORACZ et al., 2007; EL-MAAROUF-BOUTEAU; BAILLY, 2008; FOYER, 2018), plant growth and development, responses to biotic and abiotic environmental stimuli (CHOUDHURY et al., 2017) and programmed cell death in plants (NATH; LU, 2015). In the case of seeds, ROS influence the expression of genes related to both seed development and germination and dormancy processes (ORACZ et al., 2007).

Increased H_2O_2 , $O_2^{\cdot-}$ and OH^{\cdot} levels were observed during the germination process of seeds of various species (MULLER et al., 2009). During water acquisition, there is increase in H_2O_2 due to the increased respiratory activity of mitochondria, β -oxidation and activity of enzymes such as NADPH oxidases (EL-MAAROUF-BOUTEAU; BAILLY, 2008; WOJTYLA et al., 2016). However, in general, the participation of these molecules in germination and dormancy processes is conditioned to their interaction with plant hormones. Several studies have reported the interaction of these molecules with plant hormones such as gibberellin (GA), cytokinins, abscisic acid (ABA), salicylic acid (AS) and jasmonates (JA) (BARBA-ESPÍN et al., 2011; GOMES; GARCIA, 2013; MHAMDI; VAN BREUSEGEM, 2018).

Thus, H_2O_2 level seems to be a central point in the regulation of seed germination and dormancy processes. Therefore, the action of the cellular enzymatic machinery is necessary to regulate the level of this compound, influencing regulation between oxidative signaling, which promotes germination and oxidative damage, which prevents or delays this process (WOJTYLA et al., 2016).

Thus, it appears that the production and accumulation of ROS may be related to overcoming dormancy, being able to stimulate seed germination through interaction with different plant regulators. Some Annonaceae species have seeds with morphological and / or morphophysiological dormancy, characterized, respectively, by the small size of the embryo and the combination of underdeveloped embryo with metabolic blocks caused, above all, by the

balance between abscisic acid (ABA) and gibberellins (GA) (BASKIN; BASKIN, 2005; YAMAGUCHI, 2008; SALAZAR-CEREZO-2018).

The use of gibberellins has been indicated in literature to overcome dormancy in seeds of several Annonaceae species such as *Annona cherimolia* Mill. (SMET et al., 1999), *A. emarginata* (Schltdl), *H. Rainer* (CORSATO; FERREIRA; BARBEDO, 2012) and *A. squamosa* L. (CHAGAS et al., 2013), among others. For *Annona x atemoya* Mabb, the use of GA_3 ($520mgL^{-1}$) and the combination of gibberellins with cytokinin has been reported (approximately $330mgL^{-1}$) (BRAGA et al., 2010).

A. atemoya (*Annona x atemoya* Mabb.), an interspecific hybrid between cherimolia (*Annona cherimolia* Mill.) and *Annona squamosa* L., produce fruits with high nutritional value, which are used in human diet (OLIVEIRA et al., 2010). As with most fruit species, atemoya propagation and cultivation are performed by grafting in order to maintain the desirable agronomic characteristics. However, in the case of atemoya, among species used for rootstock, seedlings formed from their own seeds are also used in order to avoid incompatibility with other species (BARON et al., 2011, 2019; FERREIRA et al., 2015; PINTO; FERREIRA, 2015). Obtaining atemoya rootstock seedlings is difficult due to dormancy inherent to *Annona* seeds (BRAGA et al., 2010).

Although several studies have shown the use of plant regulators to overcome dormancy of Annonaceae seeds, and considering the relationship of reactive oxygen species in the germination process, the activity of these compounds in overcoming dormancy through the application of plant regulators has not yet been studied. Such knowledge will elucidate an important chapter in the understanding of the germination process of this family using *Annona x atemoya* Mabb. as a target species, which stands out for its economic and food importance, being one of the main commercial Annonaceae species.

In this context, the aim of this work was to evaluate the effect of plant regulators (gibberellins and cytokinins) on the oxidative metabolism through the activity of superoxide dismutase, peroxidase and catalase enzymes on *Annona x atemoya* Mabb seeds.

Material and methods

Annona x atemoya 'Thompson' seeds were obtained from fruits harvested in two locations, São Miguel Arcanjo - SP (lot 1) and Pardinho - SP (lot 2). Seeds were manually removed from pulp, treated with 2% sodium hypochlorite (v / v) for 30 minutes for fungal disinfection and then washed under running water. Subsequently, seeds were stored in refrigerator at 4 ° C until use (BRASIL, 2009).

The water content of seeds was determined by the drying method in oven at 105 ° C, according to recommendations of the rules for seed analysis (BRASIL, 2009). The seed water acquisition curve was initially determined for lot 1 in order to determine the appropriate time to carry out enzymatic activity analyses. Subsequently, the water acquisition curve was performed for each treatment.

Seeds were weighed and then placed on Germitest paper roll moistened with distilled water at proportion of 2.5 times the paper weight (FERREIRA et al., 2006). Samples were incubated in germination chamber regulated at 20°C / 30°C with photoperiod of 18 hours of darkness / 6 hours of light. Four replicates of 25 seeds were used. Water mass gain per seed was evaluated by weighing seeds every hour until 12 hours and then 24, 27, 30, 33, 36, 48 hours and then every 24 hours until 192 hours (8 days). Germinated seeds (with protrusion of 1 mm of the primary root) were not weighted. Curves were plotted using the moisture percentage with subsequent regression analysis.

Two experiments were installed. Experiment 1 was carried out with the use of gibberellin and seeds collected in São Miguel Arcanjo (SP) (lot 1). Experiment 2 was performed with cytokinin alone and combined with gibberellins, using the seed lot from Pardino (SP) (lot 2). Commercial product Pro-Gibb 400® (GA₃ 40%) was used as source of gibberins and Maxcell® (N6-benzyladenine 2%) was used as source of cytokinin, both produced by Sumitomo Chemical do Brasil LTDA.

To prevent that the use of different lots compromised the results, each lot was used in an experiment and each experiment was designed with its own control treatment. Analyses were performed separately for each lot.

In experiment 1, the following treatments were applied: water (control), GA₃ 250 mg L⁻¹ and GA₃ 500 mg L⁻¹. In experiment 2, the following treatments were applied: water (control); cytokinin 250 mg L⁻¹; cytokinin 500 mg L⁻¹; cytokinin 250 mg L⁻¹ + GA₃ 250 mg L⁻¹; cytokinin 500 mg L⁻¹ + GA₃ 500 mg L⁻¹. Seeds remained immersed in the solutions of each treatment for 24 hours in aerated conditions according to recommendations proposed by Braga et al. (2010).

In both experiments, after the application of treatments, seeds were placed to germinate on germination paper roll (Germitest®) moistened with distilled water at proportion of 2.5 times the paper weight (BRASIL, 2009). Paper rolls were incubated in BOD germination chambers, regulated with alternating temperature and light (Light: 3); dark: 20°C/18h) (BRAGA et al., 2010).

The count of germinated seeds (with protrusion of 1mm of the primary root) was performed daily for 30 days. Data were used to calculate the germination percentage (%G) and the germination speed index (GSI), calculated according to formula described by Maguire (1962).

From the determination of the standard water acquisition curve, two times were chosen to analyze the enzymatic activity (72 and 144 hours, phase II of the water acquisition curve), in order to compare the activity in periods of intense metabolic activity. Thus, tests to determine the enzymatic activity were carried out concurrently with germination tests. For each of the chosen times, 4 replicates of 20 seeds were used.

For the extraction of antioxidant enzymes, seeds were macerated in liquid nitrogen (0.3 g) and homogenized in cold buffer solution (0.1 M phosphate buffer, pH 7.0) supplemented with polyvinylpyrrolidone (Pvpp). Using this extract, the superoxide dismutase activity (SOD, EC 1.15.1.1) was quantified according to Beauchamp and Fridovich (1971), whose method is based on the principle of inhibition of the nitroblue tetrazolium (NBT) photochemical reduction at 560 nm. The catalase activity (CAT, EC 1.11.1.6) was determined by monitoring H₂O₂ decomposition and the decrease in absorbance at 240 nm, and the peroxidase activity (POD, EC 1.11.1.7) was measured at wavelength of 420nm (PEIXOTO et al., 1999).

The water content of each lot was determined using three replicates of 25 seeds. For the tetrazolium test, 4 replicates of 25 seeds were used; however, this test was only carried out for seeds of lot 1 due to the limitation in the amount of seeds in lot 2. Germination tests with plant regulators were performed with 4 replicates of 25 seeds per treatment in a completely randomized design. Enzymatic activity data were obtained in triplicate for each enzyme in each treatment.

Water acquisition curves and the germination, protein and enzyme quantification data were analyzed separately for each experiment. Germination test data (% G and GSI) were submitted to one-way analysis of variance ANOVA and, in case of difference between means, these were compared by the Tukey test at 5% probability. Enzymatic activity data were submitted to two-way analysis of variance ANOVA where factors were treatments (water, GA₃ 250 mg L⁻¹ and GA₃ 500 mg L⁻¹ in experiment 1; water, cytokinin 250 mg L⁻¹, cytokinin 500 mg L⁻¹, cytokinin 250 mg L⁻¹ + GA₃ 250 mg L⁻¹, cytokinin 500 mg L⁻¹ + GA₃ 500 mg L⁻¹ in experiment 2) and water acquisition time (72 and 144h). In case of difference between means, these were compared by the Tukey test at 5% probability.

Principal component analysis (PCA) was carried out to identify similarity in treatments applied and the effects on germination percentage, germination rate index and antioxidant enzymes (superoxide dismutase, peroxidase and catalase, using the XLSTAT software (version 2019.1.1.56604, Addinsoft, USA).

Results and discussion

Atemoia seeds used to determine the initial water acquisition curve and for experiment 1 (gibberellins, lot 1) had initial water content of $31.5\% \pm 2.3\%$ and seeds of lot 2, which were stored for five months in paper bags in refrigerator that were used for experiment 2 (cytokinins and gibberellins) had water content of $7\% \pm 0.9\%$ at the time of conduction of experiments.

In the water acquisition curve of seeds of lot 1 in distilled water, it was possible to observe the change from phase I to phase II in 24 hours, reaching the beginning of phase III in approximately 8 days (equivalent to 192 hours) (Figure 1).

Water acquisition curves were also determined for seeds treated with plant growth regulators (experiments 1 and 2). In comparison to control treatment carried out with water, GA₃ treatments showed a more pronounced curve, being possible to clearly differ the three curve phases; especially in the curve of treatment with GA₃ 500 mg L⁻¹, it is possible to separate phases accurately (Figure 2). For cytokinin and cytokinin + GA₃ treatments, water acquisition curves were similar for all treatments (Figure 3).

Results regarding the water acquisition curve found in this experiment (Figures 1, 2 and 3) were similar to those reported by Ferreira et al. (2006) for the transition of phases I, II and III in atemoia cv 'Gefner' seeds from a plant population located in the municipality of Assai (PR). The fact that seeds have reached stage I (imbibition), characterized by increase in the amount of water, confirms the absence of physical dormancy (characterized by seed coat impermeability) in atemoya seeds, which was also observed in seeds of other species of the genus *Annona*, such as *Annona diversifolia* and *A. purpurea* (FERREIRA et al., 2014).

The duration of each phase is influenced by intrinsic factors - such as characteristics of the seed coat and reserves - and extrinsic to seeds - such as temperature and substrate, in addition to variations among species (KISSMANN et al., 2012). This experiment shows that the treatment of atemoya seeds with plant growth regulators based on gibberellins and cytokinins can also modify the water acquisition curve.

Seeds treated with GA₃ 250 and 500mg L⁻¹ showed higher germination percentages compared to control treatment, which did not differ from each other (Figure 4A). Seeds treated with cytokinin and cytokinin + GA₃ showed germination percentage similar to control (Figure 4B). Only treatments using cytokinin 250mg L⁻¹ and cytokinin + GA₃ 500mg L⁻¹ differed from each other ($p < 0.05$) with respect to germination percentage.

Analyzing the germination speed index (GSI), it was observed that the use of GA₃ contributed to accelerate the germination process. The GSI values of treatments with gibberellin differed from control ($p > 0.05$; Table 1). For treatments in experiment 2, seeds submitted to cytokinin + GA₃ 500mg L⁻¹ treatment showed faster germination compared to control and cytokinin treatments used alone (250 and 500mg L⁻¹) ($p < 0.05$; Table 1).

Among regulators used, GA₃ increased germination. These data reinforce information in literature that this regulator increases the germination percentage of *Annona* (OLIVEIRA et al., 2010; FERREIRA et al., 2019) including atemoia. Likewise, Braga et al. (2010) observed increase in the germination percentage of atemoia cv 'Gefner' seeds from a population in Paraná according to the increase in GA₃ up to concentration of 500 mg L⁻¹.

Since they are directly involved in the cell division process (MIRANSARI; SMITH, 2014), cytokinins have been commonly used in treatments combined with gibberellins to overcome dormancy in Annonaceae species (SOCOLOWSKI; CICERO, 2011; CORSATO et al., 2012; FERREIRA et al., 2016). This beneficial effect could be demonstrated when cytokinin was used in combination with the highest GA₃ concentration (Figure 4B). In addition to higher germination percentage in relation to the use of the lowest cytokinin concentration alone, this mixture increased the process speed, both in relation to the application of cytokinin alone (250mg L⁻¹ and 500mg L⁻¹) and in relation to control (table 1).

Regarding the enzymatic analysis of experiment with gibberellin only, there was no interaction among treatments (water, GA₃ 250 and GA₃ 500mg L⁻¹) and time (72 and 144h) for SOD, POD and CAT enzymes (Figure 5). Seeds submitted to treatment with 500mg L⁻¹ GA₃ showed higher SOD activity in relation to seeds of control treatments and 250mg L⁻¹ GA₃ (Figure 5A). Water acquisition times also differed, indicating greater activity of this enzyme in samples collected in the time of 144 hours (Figures 5B).

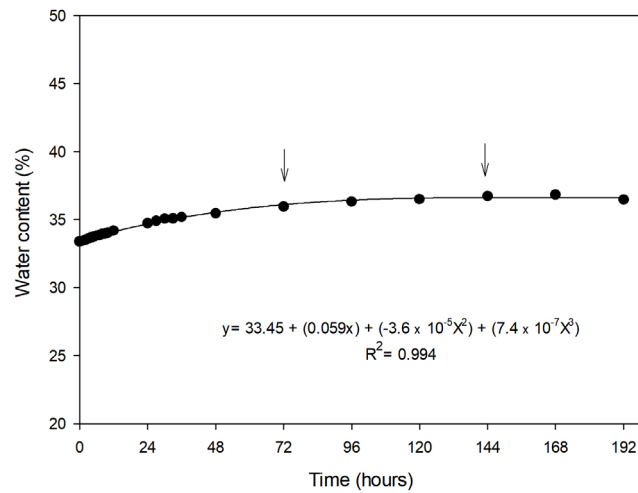


Figure 1. Water content (%) of atemoia seeds (*Annona cherimola* Mill. X *A. squamosa* L.) cv. Thompson submitted to water acquisition. Arrows indicate the period chosen for determining reactive oxygen species. Botucatu, 2017.

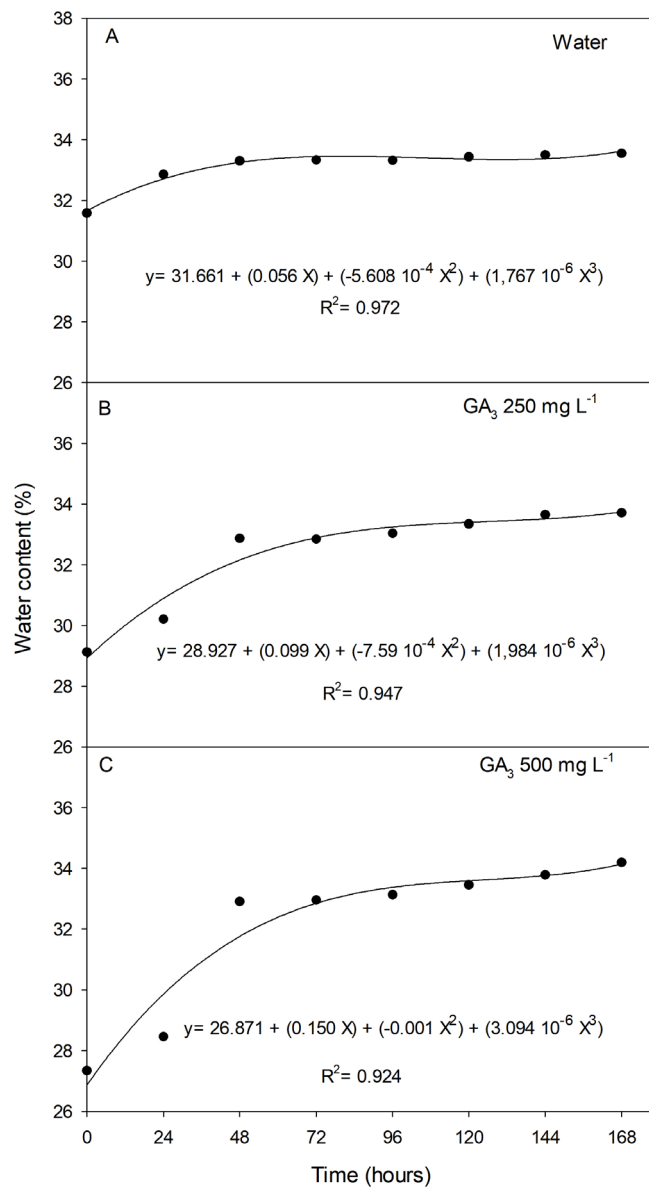


Figure 2. Water content (%) of atemoia seeds (*Annona cherimola* Mill. X *A. squamosa* L.) 'Thompson' submitted to water acquisition in water (A) and gibberellin (Pro-Gibb®) 250 mg L⁻¹ (B) and 500 mg L⁻¹. Botucatu, 2017.

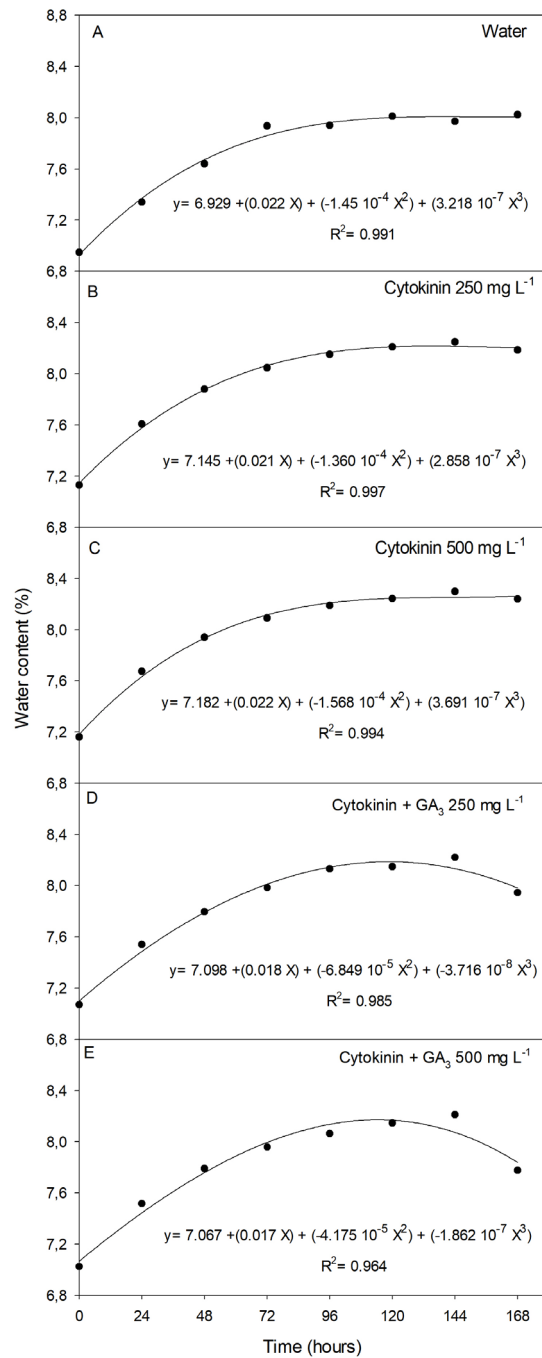


Figure 3. Water content (%) of atemoia seeds (*Annona cherimola* Mill. X *A. squamosa* L.) cv. Thompson submitted to water acquisition in water (A), cytokinin (Maxcell®) 250 mg L⁻¹ (B), cytokinin (Maxcell®) 500 mg L⁻¹ (C), cytokinin (Maxcell®) + gibberellin (Pro- Gibb®) 250 mg L⁻¹ (D) and cytokinin (Maxcell®) + gibberellin (Pro-Gibb®) 500 mg L⁻¹ (E) and 500 mg L⁻¹ stored in germination chamber at temperature of 20°C / 30°C and light (18 hours of dark / 6 hours of light) for 168 hours (7 days). Botucatu, 2017.

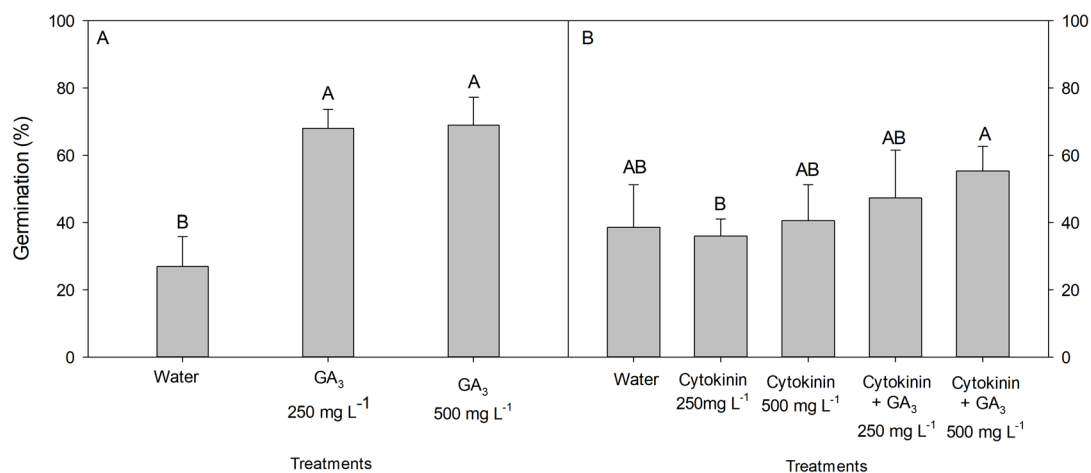


Figure 4. Germination (%) of atemoia seeds (*Annona cherimola* Mill. X *A. squamosa* L.) ‘Thompson’ submitted to treatments with gibberellin (GA₃, Pro-Gibb®) and water (control) (A, n = 4) and cytokinin (Maxcell®), cytokinin and gibberellin (GA₃, Pro-Gibb®) and water (control) (B, n = 6). Different letters indicate statistical difference by the Tukey test (p < 0.05).

Table 1. Germination speed index (GSI) of atemoia seeds (*Annona cherimola* Mill. X *A. squamosa* L.) ‘Thompson’ in water and in gibberellin (GA₃, Pro-Gibb®) (experiment 1) and cytokinin (Maxcell®) and cytokinin + gibberellin (GA₃, Pro-Gibb®) (experiment 2).

Germination speed index (GSI)				
Treatments - Experiment 1				
Water	GA ₃ 250mg L ⁻¹		GA ₃ 500mg L ⁻¹	
0.641 B	1.627 A		1.670 A	
Treatments - Experiment 2				
Water	Cytokinin 250mg L ⁻¹	Cytokinin 500mg L ⁻¹	Cytokinin + GA ₃ 250mg L ⁻¹	Cytokinin + GA ₃ 500mg L ⁻¹
0.756 B	0.742 B	0.805 B	0.970 AB	1.213 A

Different letters indicate statistical difference between treatments by the Tukey test at 5% significance.

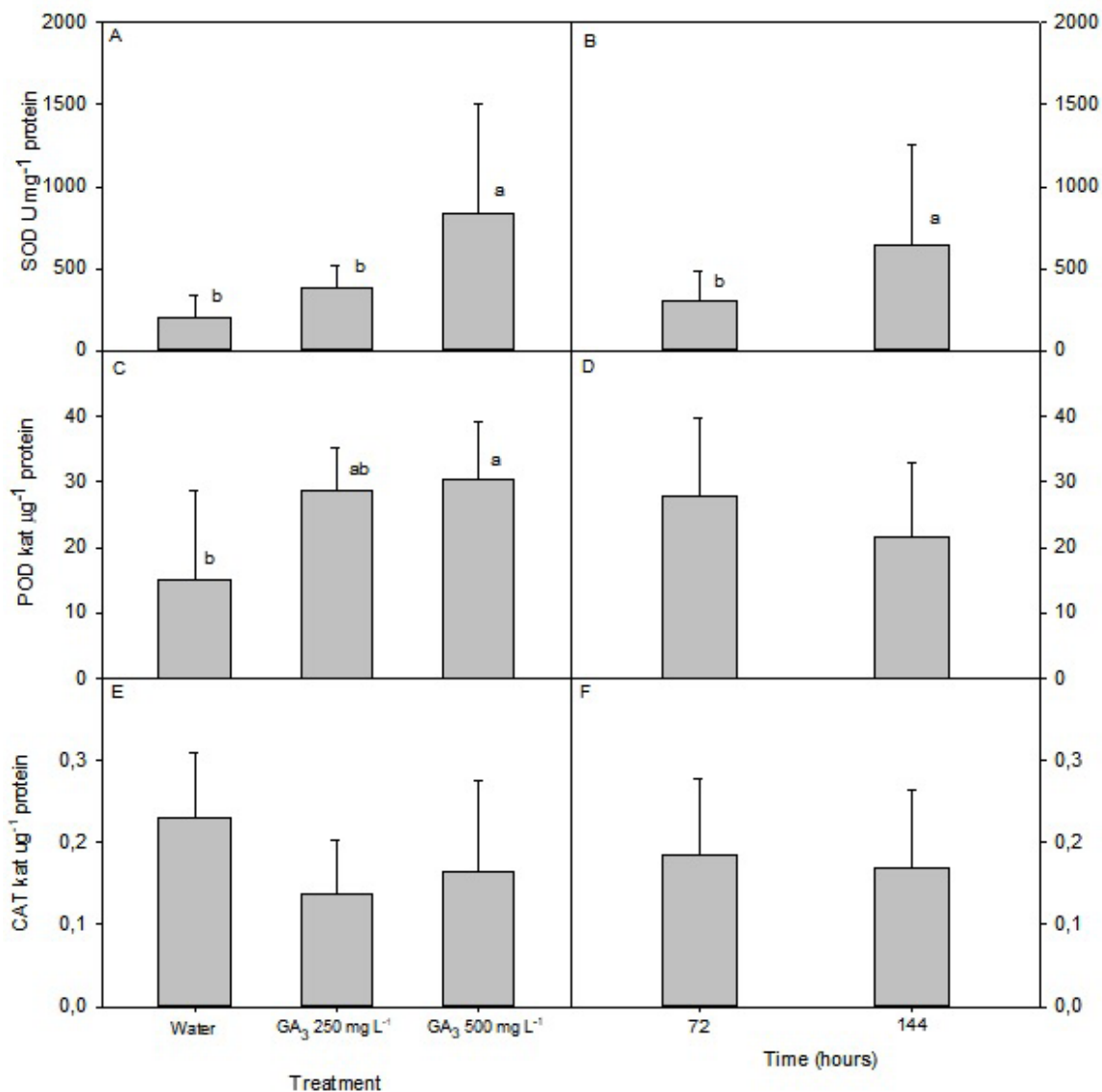


Figure 5. Superoxide dismutase (SOD, U mg protein⁻¹) (A, B), peroxidase (POD, μKat μg protein⁻¹) (C, D) and catalase (CAT, μKat μg protein⁻¹) (E, F) in atemoia seeds (*Annona cherimola* Mill. x *A. squamosa* L.) ‘Thompson’ as a function of treatments with gibberellin (GA₃) and water (control) (A, C, E) and water acquisition time (B, D, F) (n = 4). Different letters indicate differences between treatments and absence of letters indicates no difference between treatments and / or water acquisition times, both by the Tukey test (P < 0.05).

The increase in SOD activity with the use of the highest GA₃ concentration, without however observing increase in % G (in relation to the lowest GA concentration), is an indication that the highest GA₃ concentration caused greater ROS production, suggesting greater stress. In this context, several aspects must be considered. One of them is the fact that *Annona* seeds respond to GA₃ treatments with increased germination process (% and germination speed), so when seeds were soaked in solution with this regulator, physiological effects are evident, as proposed by Yamaguchi (2008) and Salazar-Cerezo et al., (2018), since gibberellin promotes the production and/or reactivation of several hydrolytic enzymes involved in the solubilization of seed reserves. These enzymes degrade reserves stored in the endosperm, forming sugars, amino acids and nucleic acids, which are absorbed and transported to

the embryo’s growth regions, accelerating germination. Thus, both 250 mg L⁻¹ and 500 mg L⁻¹ were effective in increasing the germination process. Another aspect is that this increase in metabolism, since the beginning of imbibition, can increase ROS production, since these molecules are related to places of high metabolic activity (GOMES; GARCIA, 2013). In this context, SOD, the first enzyme in the oxidative system to be activated as a form of protection against ROS, acted significantly when the highest GA₃ concentration was used, with increased activity, influencing regulation between signaling and oxidative damage, which maintained balance, avoiding damage to cells and guaranteeing germination at the same levels already achieved with the lowest GA₃ concentration, which corroborates reports by Wojtyła et al. (2016).

SOD catalyzes the dismutation reaction of the superoxide radical, transforming it into H_2O_2 , which is subsequently catalyzed by POD and CAT enzymes, whose synthesis varies according to the plant's development stage. In addition, there is also a balance in POD and CAT production, so that when one of enzymes shows high production, the other is almost nonexistent (TULLIO; ARRIGONI, 2003), as shown in Figure 5. The activity of the POD enzyme, like SOD, showed higher values in seeds treated with 500 mg L^{-1} GA_3 (Figure 5C) and there was no influence on the sample collection time (Figure 5D). CAT enzyme showed no difference in its activity due to treatments ($p = 0.122$) or to water acquisition times ($p = 0.640$) (Figure 5E, F). In this context, during the germination process, there is greater POD activity related to GA_3 application when compared to CAT.

In the second experiment, interaction between treatments (water, cytokinin 250 mg L^{-1} and 500 mg L^{-1} , cytokinin + GA_3 250 and 500 mg L^{-1}) and times (72 and 144h) for the SOD enzyme was observed ($p = 0.003$). Seeds soaked in water for 72 hours showed greater SOD activity than those that remained soaked for 144 h, different from what was observed when seeds were soaked in plant regulators. However, 72 hours after the first collection (144h), the effect of the regulators is evident, with greater SOD activity in seeds treated with the mixture of cytokinin and the highest GA_3 concentration (500 mg L^{-1}) in relation to control and use of cytokinin alone (Figure 6).

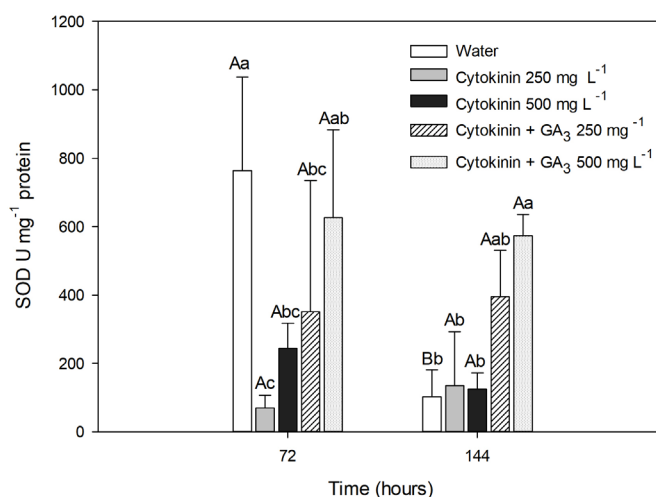


Figure 6. Superoxide dismutase (SOD, $U\text{ mg protein}^{-1}$) in atemoia seeds (*Annona cherimola* Mill. x *A. squamosa* L.) ‘Thompson’ submitted to treatments with cytokinin (Maxcell®), cytokinin and gibberellin (GA_3 , Pro-Gibb®) and water (control) ($n = 4$). Capital letters indicate difference between water acquisition times and lower letters indicate difference between treatments, both by the Tukey test ($P < 0.05$).

In the second experiment, the action of the highest GA_3 concentration associated with cytokinin is confirmed in the increase of the germination process (in relation to the use of cytokinin alone (250 mg L^{-1}) and the greater SOD activity, especially after 144h of imbibition. On the other hand, the lowest cytokinin concentration keeps stress levels low (evidenced by the low SOD activity) but with lower germination percentage, demonstrating reduced metabolic activity and inefficiency in the use of cytokinin alone. As in the first experiment, these results could be attributed to the effects of GA_3 on the increase of the germination process of seeds. In this context, as the role of gibberellin is to stimulate the activity of hydrolytic enzymes to degrade reserves and release energy for embryo development, and this increase in metabolism leads to greater ROS production (GOMES; GARCIA, 2013); therefore, with the increase in the metabolism caused by GA_3 in the germination process, greater SOD activity is observed. This relationship between increase in

reactive oxygen species and the action of gibberellin has been reported both to overcome dormancy and to stimulate the germination process; however, further studies are suggested to better understand the relationship between mechanisms involved in these processes (ORACZ et al., 2007; BARBA-ESPIN et al., 2011; MHAMDI; VAN BREUSEGEM, 2018).

For the POD and CAT activity, there was no significant difference for the interaction of factors and; therefore, the results for each factor are presented separately, with differences being observed only in relation to plant regulators (Figure 7). The highest POD activity was detected using the mixture of cytokinin and the lowest GA_3 concentration (250 mg L^{-1}) (Figure 7A). In relation to CAT, the greatest activities were observed when the lowest cytokinin and GA_3 concentrations were used, differing from control (Figure 7C).

In the analysis of principal components carried out with variables germination and enzymes in relation to the first experiment, the first component explains 70.67% of data variation and the second component explains 17.88%, thus adding 88.55% to the explanation of the total data variation. Thus, only 2 components were selected. The first component is negatively associated with catalase,

and positively associated with the other variables. Two groups were formed where seeds soaked in water (control group) were discriminated by catalase (PC1-) and GA₃-based treatments discriminated by variables germination and SOD and POD enzymes (Figure 8), making it possible to infer the participation of antioxidant enzymes in the germination process.

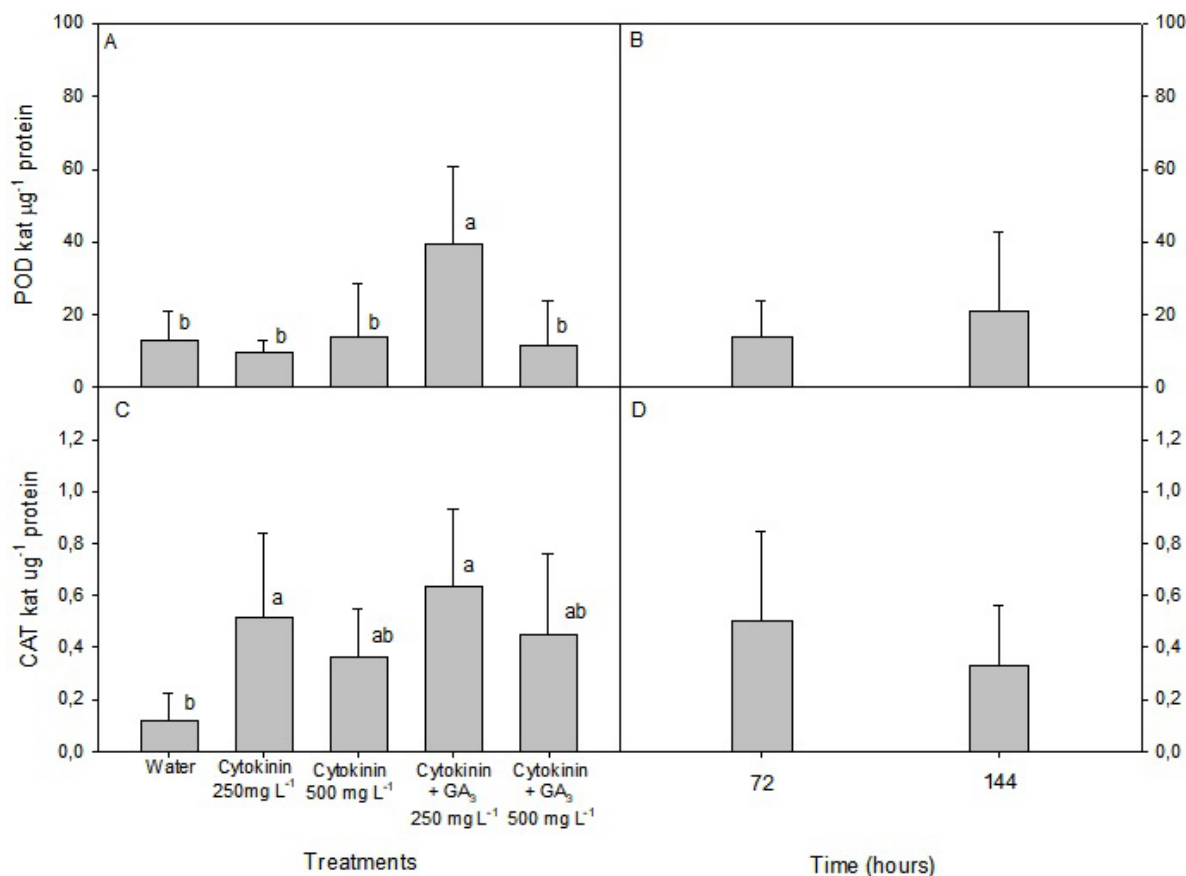


Figure 7. Peroxidase (POD, $\mu\text{Kat } \mu\text{g Protein}^{-1}$) and catalase (CAT, $\mu\text{Kat } \mu\text{g Protein}^{-1}$) in atemoya seeds (*Annona cherimola* Mill. x *A. squamosa* L.) ‘Thompson’ submitted to treatments with cytokinin (Maxcell®), cytokinin and gibberellin (GA₃, Pro-Gibb®) and water (control) (n = 4). Different letters indicate differences between treatments and absence of letters indicates no difference between water acquisition times, both by the Tukey test (P < 0.05).

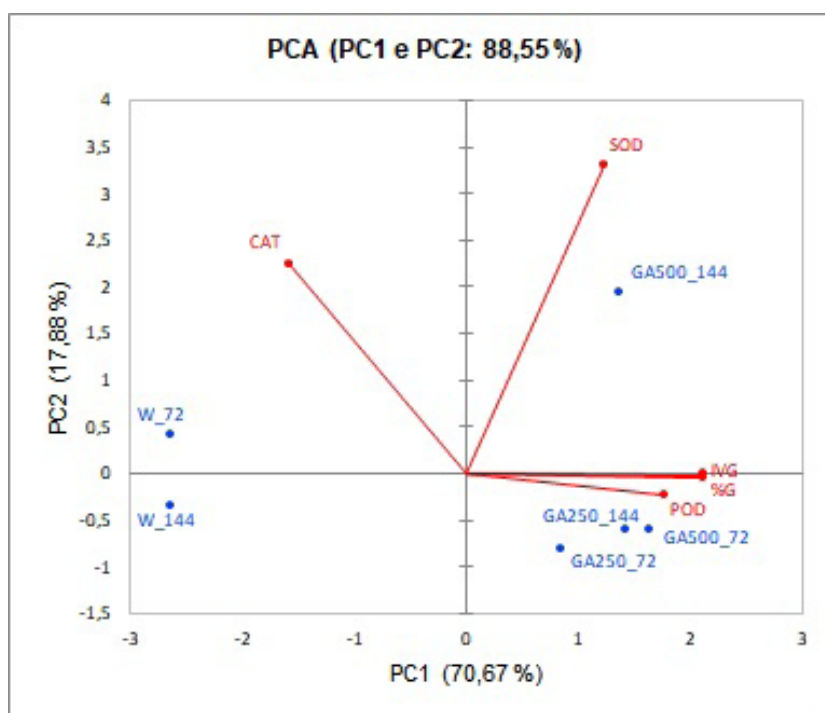


Figure 8. Two-dimensional projection of attributes related to germination (germination percentage [% G] and germination speed index [GSI]) and antioxidant enzymes (superoxide dismutase [SOD], peroxidase [POD] and catalase [CAT]) in atemoya seeds (*Annona cherimola* Mill. x *A. squamosa* L.) 'Thompson' submitted to treatments with gibberellic acid (GA_3), collected at 72 and 144 hours of water acquisition. Treatments are represented by dots, where the initial characters represent treatments (W = water, GA250 = 250 mg L⁻¹ GA_3 and GA500 = 500 mg L⁻¹ GA_3) and numbers after underline represent the collection time (72 and 144 hours of water acquisition).

In the analysis of principal components carried out with variables germination and enzymes in relation to the second experiment, the first component explains 51.08% of data variation and the second component explains 21.75%, totaling explanation of 72.83% of total data variation. Therefore, similar to the analysis of principal components of experiment 1, only 2 components were selected. The first component is positively associated with all variables, enabling the formation of two distinct groups. Cytokinin-based treatments and control treatment (PC1-) were in one of the groups and in the other, cytokinin + GA_3 -based treatments were discriminated by variables germination and antioxidant enzymes (PC1 +) (Figure 9).

In seeds treated with gibberellin, increase in the activity of SOD and POD enzymes was observed, which are involved in the antioxidant mechanism for cellular protection against ROS, produced due to the interaction of GA_3 with the cell metabolism. Seeds treated with this regulator showed higher germination rates and seed germination speeds, regardless of concentration used. The combined treatment of cytokinin and gibberellin 500mg L⁻¹ resulted in greater germination (germination speed index) and also high SOD activity, suggesting its participation in the germination process due to the presence of high amounts of superoxides and, consequently, H_2O_2 production.

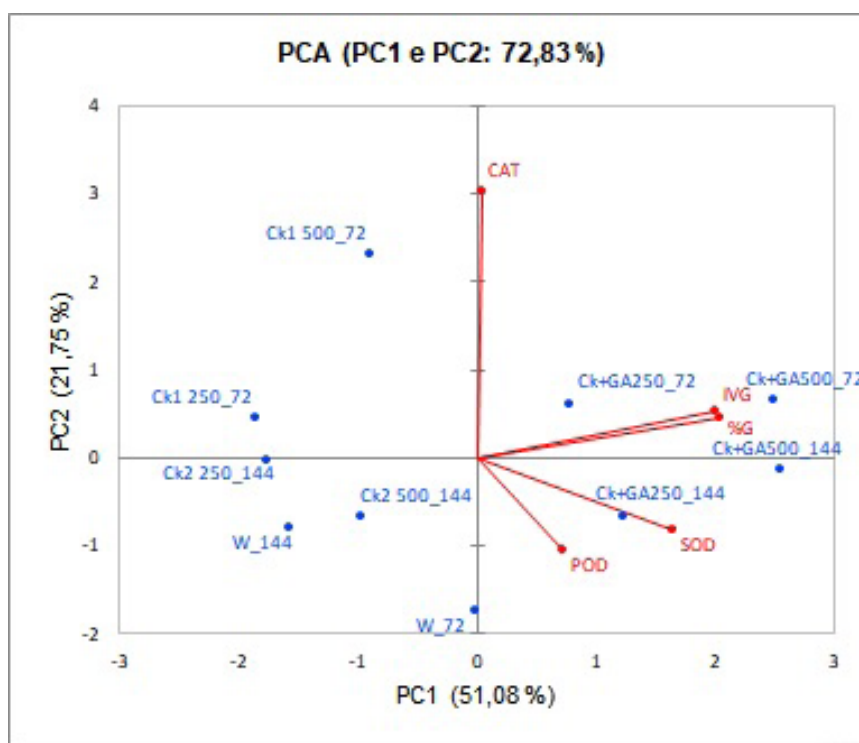


Figure 9. Two-dimensional projection of attributes related to germination (germination percentage [% G] and germination speed index [GSI]) and antioxidant enzymes (superoxide dismutase [SOD], peroxidase [POD] and catalase [CAT]) in atemoya seeds (*Annona cherimola* Mill. x *A. squamosa* L.) cv. Thompson submitted to cytokinin (Ck) and cytokinin + gibberellic acid (Ck + GA) treatments, collected at 72 and 144 hours of water acquisition. Treatments are represented by dots, where initial characters represent treatments (W = water, Ck250 = 250 mg L⁻¹ Ck, Ck500 = 500 mg L⁻¹ Ck, Ck + GA250 = 250 mg L⁻¹ Ck + GA₃ and Ck + GA500 = 500 mg L⁻¹ Ck + GA₃) and numbers after underline represent the collection time (72 and 144 hours of water acquisition).

Conclusion

The oxidative metabolism, especially of SOD, is increased as the germination speed index of atemoya seeds increases due to the use of GA₃ and the mixture of GA₃ with cytokinin.

References

- BARBA-ESPÍN, G.; DIAZ-VIVANCOS, P.; JOB, D.; BELGHAZI, M.; JOB, C.; HERNÁNDEZ, J.A. Understanding the role of H₂O₂ during pea seed germination: A combined proteomic and hormone profiling approach. **Plant, Cell and Environment**, Oxford, v.34, n.11, p.1907–1919, 2011.
- BARON, D.; ESTEVES AMARO, A.C.; PINA, A.; FERREIRA, G. An overview of grafting re-establishment in woody fruit species. **Scientia Horticulturae**, Amsterdam, v.243, n.1, p.84–91, 2019.
- BARON, D.; FERREIRA, G.; BOARO, C.S.F.; MISCHAN, M.M. Evaluation of substrates on the emergence of “araticum-de-terra-fria” (*Annona emarginata* (Schltdl.) H. Rainer) Seedlings. **Revista Brasileira de Fruticultura**, Jaboticabal, v.33, n.2, p.575–586, 2011.
- BASKIN, C.C.; BASKIN, J.M. Seed dormancy in trees of climax tropical vegetation types. **Tropical Ecology**, New Delhi, v.46, n.1, p.17–28, 2005.
- BRASIL, Ministério da Agricultura e Reforma Agrária. **Regras para análise de sementes**. Brasília: Departamento de Produção Vegetal, 2009.398p.
- BEAUCHAMP, C.; FRIDOVICH, I. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. **Analytical Biochemistry**, New York, v.44, n.1, p.276–287, nov.1971.
- BEWLEY, J.D.; BRADFORD, K.J.; HILHORST, H.W.M.; NONOGAKI, H. Germination. In: **Seeds: physiology of development, germination and dormancy**. 3rd ed. New York: Springer, 2013. p.133–181.

- BRAGA, J.F.; FERREIRA, G.; PINHO, S.Z.de; BRAGA, L.F.; SOUSA, M.P. Germination of atemoya (*Annona cherimola* Mill. x *A. squamosa* L.) cv.gefner seeds subjected to treatments with plant growth regulators. **International Journal of Science and Nature**, Daca, v.1, n.2, p.120–126, 2010.
- CHAGAS, P.C.; SOBRAL, S.T.M.; OLIVEIRA, R.R.de; CHAGAS, E.A.; PIO, R.; SANTOS, V.A. dos. Physical and chemical methods to breach seed dormancy of sugar apple. **Revista de Ciências Agrárias-Amazon Journal of Agricultural and Environmental Sciences**, Belém, v.56, p.101–106, 2013.
- CHOUDHURY, F. K.; RIVERO, R. M.; BLUMWALD, E.; MITTLER, R. Reactive oxygen species, abiotic stress and stress combination. **The Plant Journal: for cell and molecular biology**, Oxford, v.90, n.5, p.856–867, 2017.
- CORSATO, J.M.; FERREIRA, G.; BARBEDO, C.J. Desiccation tolerance in seeds of *Annona emarginata* (Schldtl.) H. Rainer and action of plant growth regulators on germination. **Brazilian Journal Plant Physiology**, Londrina, v.24, n.4, p.253–260, 2012.
- EL-MAAROUF-BOUTEAU, H.; BAILLY, C. Oxidative signaling in seed germination and dormancy. **Plant Signaling and Behavior**, Georgetown, v.3, n.3, p.175–182, 2008.
- FERREIRA, G.; DE-LA-CRUZ-CHACÓN, I.; BOARO, C.S.F.; BARON, D.; LEMOS, E.E.P.de. Propagation of Annonaceous plants. **Revista Brasileira de Fruticultura**, Jaboticabal, v.41, n.1, p.1–14, 2019.
- FERREIRA, G.; DE-LA-CRUZ-CHACÓN, I.; GONZÁLEZ-ESQUINCA, A.R. Overcoming seed dormancy in *Annona macroprophyllata* and *Annona purpurea* using plant growth regulators. **Revista Brasileira de Fruticultura**, Jaboticabal, v.38, n.3, 2016.
- FERREIRA, G.; ESQUINCA, A.R.G.; DE-LA-CRUZ-CHACÓN, I. Water uptake by *Annona diversifolia* Saff. and *A. purpurea* Moc. & sessé ex dunal seeds (Annonaceae). **Revista Brasileira de Fruticultura**, Jaboticabal, v.36, n.1, p.288–295, 2014. Número Especial.
- FERREIRA, G.; GIMENEZ, J.I.; CORSATO, J.M.; DALANHOL, S.J.; SILVA, M.A.P. da. Fisiologia da germinação e dormência de sementes de Anonaceae. In: ALMEIDA, J.R.G. da S.; OLIVEIRA-JÚNIOR, R.G. de; OLIVEIRA, A.P. de (ed.). **Anonaceae: tópicos selecionados**. Curitiba: Editora CRV, 2015.
- FERREIRA, G.; GUIMARÃES, V.F.; PINHO, S.Z. de; OLIVEIRA, M.C. de; RICHART, A.; BRAGA, J.F.; DIAS, G.B. Curva de absorção de água em sementes de atemoia (*Annona cherimola* Mill. x *Annona squamosa* L.) cv.gefner. **Revista Brasileira de Fruticultura**, Jaboticabal, v.28, n.1, p.121–124, 2006.
- FOYER, C.H. Reactive oxygen species, oxidative signaling and the regulation of photosynthesis. **Environmental and Experimental Botany**, Elmsford, v.154, n.5, p.134–142, 2018.
- GILL, S.S.; TUTEJA, N. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. **Plant Physiology and Biochemistry**, Paris, v.48, n.12, p.909–930, 2010.
- GOMES, M.P.; GARCIA, Q.S. Reactive oxygen species and seed germination. **Biologia**, Bratislava, v.68, n.3, p.351–357, 2013.
- KISSMANN, C.; TOZZI, H.H.; MARTINS, S.; HABERMANN, G. Germination performance of congeneric *Styrax* species from the Cerrado sensu lato areas and their distribution pattern in different physiognomies. **Flora: Morphology, Distribution, Functional Ecology of Plants**, Jena, v.207, n.9, p.673–681, 2012.
- MAGUIRE, J.D. Speed of germination aid in selection and evaluation for seedling emergence and vigor. **Crop Science**, Madison, v.2, n.2, p.176-177, 1962.
- MHAMDI, A.; VAN BREUSEGEM, F. Reactive oxygen species in plant development. **Development**, Cambridge, v.145, n.15, p.1-12, 2018.
- MIRANSARI, M.; SMITH, D.L. Plant hormones and seed germination. **Environmental and Experimental Botany**, Elmsford, v.99, n.3, p.110–121, 2014.
- MULLER, K.; LINKIES, A.; VREEBURG, R.A.M.; FRY, S.C.; KRIEGER-LISZKAY, A.; LEUBNER-METZGER, G. In vivo cell wall loosening by hydroxyl radicals during cress seed germination and elongation growth. **Plant Physiology**, Lancaster, v.150, n.4, p.1855–1865, 2009.
- NATH, K.; LU, Y. A paradigm of reactive oxygen species and programmed cell death in plants. **Journal of Cell Science and Therapy**, Los Angeles, v.6, n.2, p.1-2, 2015.
- OLIVEIRA, M.C. de; FERREIRA, G.; GUIMARÃES, V.F.; DIAS, G.B. Germinação de sementes de atemoia (*Annona cherimola* Mill. x *A. squamosa* L.) cv “Gefner” submetidas a tratamentos com ácido Giberélico (GA3) e ethephon. **Revista Brasileira de Fruticultura**, Jaboticabal, v.32, n.2, p.544–554, 2010.

ORACZ, K.; BOUTEAU, H.E.M.; FARRANT, J.M.; COOPER, K.; BELGHAZI, M.; JOB, C.; JOB, D.; CORBINEAU, F.; BAILLY, C. ROS production and protein oxidation as a novel mechanism for seed dormancy alleviation. **The Plant Journal: for cell and molecular biology**, Oxford, v.50, n.3, p.452–465, 2007.

PEIXOTO, P.H.P.; CAMBRAIA, J.; SANT'ANNA, R.; MOSQUIM, P.R.; MOREIRA, M.A. Aluminum effects on lipid peroxidation and on the activities of enzymes of oxidative metabolism in sorghum. **Revista Brasileira de Fisiologia Vegetal**, Campinas, v.11, n.3, p.137–143, 1999.

PINTO, E.; FERREIRA, I.M.PL.V.O. Cation transporters/channels in plants: tools for nutrient biofortification. **Journal of Plant Physiology**, Jena, v.179, p.64–82, 2015.

SALAZAR-CEREZO, S.; MARTINEZ-MONTIEL, N.; GARCIA-SANCHEZ, J.; PEREZ-Y-TERRON, R.; MARTÍNEZ-CONTRERAS, R.D. Gibberellin biosynthesis and metabolism: A convergent route for plants, fungi and bacteria. **Microbiological Research**, Jena, v.208, p.85-98, 2018.

SMET, S. DE; DAMME, P. van; SCHELDEMAN, X.; ROMERO, J.; SCHELDEMAN, X. Seed structure and germination of cherimoya (*Annona cherimola* Mill.). **Acta Horticulturae**, Brussels, v.497, p.269–278, 1999.

SOCOLOWSKI, F.; CICERO, S M. Use of growth regulators to overcome seed dormancy in -+ (Annonaceae). **Seed Science and Technology**, Zurich, v.39, n.1, p.21–28, 2011.

TULLIO, M.C.; ARRIGONI, O. The ascorbic acid system in seeds: to protect and to serve. **Seed Science Research**, Cambridge, v.13, n.4, p.249–260, 2003.

WOJTYLA, Ł.; LECHOWSKA, K.; KUBALA, S.; GARNCZARSKA, M. Different modes of hydrogen peroxide action during seed germination. **Frontiers in Plant Science**, Lausanne, v.7, n.66, p.1–16, 2016.

YAMAGUCHI, S. Gibberellin Metabolism and its Regulation. **Annual Review of Plant Biology**, Palo Alto, v.59, n.1, p.225–251, 2008.