ABRATES

Journal of Seed Science

ISSN 2317-1545
www.abrates.org.br/revista

ARTICLE

Journal of Seed Science, v.45, e202345021, 2023

http://dx.doi.org/10.1590/2317-1545v45262916

Germination and regeneration of *Eugenia involucrata* (Myrtaceae) seeds correlated with reactive oxygen species

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ABSTRACT: Seeds of the genus *Eugenia* L. (Myrtaceae) have interesting propagation strategies, and the reactive oxygen species (ROS) seem to be related to their germination and seedling production. In this study, the effects of ROS on the germination of *E. involucrata* seeds were analyzed indirectly, with the application of N-acetyl-L-cysteine (NAC), an antioxidant. The results demonstrated that the incubation in NAC suppressed and/or delayed the germination of the seeds, as well as the regeneration of roots and aerial parts. The higher the concentration of NAC, the greater the effects on seed germination and regeneration.

Index terms: inhibition of germination, recalcitrant seeds, ROS, tropical species.

RESUMO: As sementes do gênero *Eugenia* L. (Myrtaceae) possuem estratégias de propagação bastante interessantes e as espécies reativas de oxigênio (EROs) parecem estar relacionadas à sua germinação e à produção de novas plântulas. Neste trabalho foram analisados os efeitos das EROs sobre a germinação de sementes de *E. involucrata* de maneira indireta, com aplicação de N-acetil-L-cisteína (NAC), um antioxidante. Os resultados demonstraram que a incubação em NAC suprimiu e/ou atrasou a germinação das sementes, bem como a regeneração de raízes e partes aéreas. O efeito foi tanto maior quanto mais elevada a concentração do NAC.

Termos de indexação: inibição da germinação, semente recalcitrante, EROs, espécie tropical.

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Received: 04/08/2022.
Accepted: 03/14/2023.

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INTRODUCTION

Seeds with high storability have been the expected and sought after standard for agricultural production and biodiversity conservation. However, in many extremely biodiverse habitats, such as humid tropical forests, there are many species whose seeds are dispersed with high water content and are sensitive to desiccation, which makes it difficult to store them for long periods (Barbedo, 2018; Bharuth and Naidoo, 2020; Chandra et al., 2021). As an alternative to the formation of seed banks in the soil, these species have often invested evolutionarily in the development of ecological strategies such as the production of seedling banks (Barbedo et al., 2013; Subbiah et al., 2019). The seeds of *Eugenia* L. (Myrtaceae), for instance, have interesting propagation strategies, such as the ability to maintain adequate levels of hydration inside, which allows them to survive periods of drought (Inocente and Barbedo, 2019). In addition, they have a morphogenic potential to regenerate new seedlings when there is failure in the first germination or when the seeds are fractionated (Mendes and Mendonça, 2012; Calvi et al., 2016, 2017a, 2017b; Alonso et al., 2019; Alonso and Barbedo, 2020; Delgado and Barbedo, 2020), behavior similar to that observed in seeds of species of the genera *Garcinia* and *Allanblackia* (Kalia et al., 2012; Ofori et al., 2015).

Despite having the potential to produce several seedlings from a single embryo (Delgado and Barbedo, 2011), *Eugenia* seeds have a refined system of germination self-control, through which the balance between germination-stimulating and -inhibiting substances allows directing the processes for the production or not of new seedlings (Delgado and Barbedo, 2011; Amador and Barbedo, 2015). A balance between these substances, in favor of inhibition, should keep only one germination process active, until the interruption in the transport of this inhibitory substance, caused by fractionation, allows the production of a new root and, consequently, a new seedling (Amador and Barbedo, 2015). The systems involved in this control are not yet elucidated, but it is likely that oxidative processes are involved.

Reactive oxygen species (ROS) are capable of inducing biological processes, including those related to germination (Moothoo-Padayachie et al., 2016). The superoxide radical ($O_2^{\bullet -}$) is pointed out as the main ROS produced on injured surfaces of seeds that are sensitive to desiccation, with an "explosion" in the first few minutes (Roach et al., 2010; Zhou et al., 2018). One of the main sources of ROS, NADPH oxidase acts as a key enzyme in seedling germination and growth (Ishibashi et al., 2010; Yang et al., 2020). Imbibition in hydrogen peroxide (H_2O_2) can promote and accelerate the germination of some seeds, as well as stimulating the growth of seedlings (Barba-Espin et al., 2010). On the other hand, the application of N-acetyl-L-cysteine (NAC), a glutathione precursor that replenishes the stocks of this antioxidant in organisms, significantly suppresses the germination of some species (Ogawa and Iwabuchi, 2001; Ishibashi et al., 2012). Therefore, the control of germination and regeneration in *Eugenia* seeds may be closely related to the redox processes and the formation of substances capable of rapidly being distributed throughout their omnipotent tissues and inducing the differentiation of new roots and seedlings, or of inducing the formation of antioxidant compounds capable of inhibiting new germination (Amorim et al., 2020).

Studying *Eugenia* species is interesting not only due to their seeds, which are sensitive to desiccation (Delgado and Barbedo, 2012), but also because of the great potential for agricultural exploitation of many species of this genus, such as *E. involucrata* (cherry of the Rio Grande), native to Brazil, including the association of economic interests with those of the preservation of the species itself (Wagner et al., 2022). Considering that the effects of exogenous application of ROS, or ROS-generating compounds, are not yet understood for seeds sensitive to desiccation, in the present study the effects of ROS were tested indirectly, through the application of an antioxidant substance, in order to analyze the effects of this application on the inhibition of seed germination and on the regeneration of roots and seedlings of *Eugenia involucrata* DC. (Myrtaceae).

MATERIAL AND METHODS

Obtaining plant material

Ripe fruits of *Eugenia involucrata* (Figure 1A) were collected from trees of the Fontes do Ipiranga State Park (23°38' S and 46°37' W), São Paulo, SP, Brazil. Seeds were extracted manually from the fruits, washed in running water and placed on paper to absorb excess moisture. Immediately after, they were characterized for water content, dry mass content and germination percentage, as described below. Then, they were divided into two groups: seeds not subjected to drying (FS) and subjected to light drying (DS) in order to increase the intensity of absorption before incubation of the samples, since these seeds already have high water content at the beginning of the treatment, which could delay the absorption of the reagent. Drying was performed in an oven with forced air circulation at 45 °C for one hour.

Storage and fractionation of seeds

For being sensitive to desiccation and having short longevity, and aiming at assessing the influence of storage on germination rates and responses to the treatments, DS seeds were divided into three groups: without storage (less than 1 week of collection - NST) or stored inside polyethylene bags with perforations, in cold chambers at 10 °C, for 60 days (SS60) and 120 days (SS120).

In order to expose the interior of the seeds to air and to a possible stimulus to regeneration, samples of seeds from the FS and DS groups, not subjected to storage (NST), and stored for 60 (SS60) and 120 days (SS120), were subjected to longitudinal fractionation in the middle (Figure 1B). The cuts were made manually using tweezers and scalpel. The group with the fractionated seeds was referred to as CS, while the control group of whole seeds was referred to as WS.

Incubation of seeds in N-acetyl-L-cysteine (NAC) and evaluation of NAC efficiency

The seeds were incubated in NAC solutions in order to reduce the production of ROS in their tissues and evaluate their possible inhibitory role on germination, in addition to other possible physiological effects caused by this suppression. For that, seeds were placed in glass jars and submerged in solutions with three different concentrations of NAC (50 mM, 100 mM and 200 mM) and in distilled water (control group) for 3 hours. After incubation, they were washed, placed to germinate, and evaluated as described below.

In order to assess the efficiency of NAC in reducing ROS production, H_2O_2 was quantified as described below, evaluating the following groups: 1) whole (WS) and fractionated (CS) seeds exposed to air for 3 hours, which constituted the control group; 2) WS and CS seeds incubated for 3 hours in water (WS3W and CS3W); 3) WS and CS seeds incubated for 3 hours in NAC (WS3N and CS3N).

Figure 1. Fruits and seeds of *Eugenia involucrata*. A: ripe fruits and seeds used in the experiments; B: seed cut longitudinally into two similar parts. Scale = 1 cm.

Physical and physiological evaluations

Water content and dry mass content were determined gravimetrically by the oven method at 103 °C for 17 hours (ISTA, 2015), with 4 replications of 10 seeds each. The results were presented as a percentage (wet basis) for water content and in g of dry mass per seed ($g.seed^{-1}$) for dry mass.

Germination tests were carried out with 3 replications of 16 seeds each. In order to ensure that the absorption was as uniform as possible on the entire surface of the whole seeds or as precise as possible on the cutting surface of the fractionated seeds, the whole seeds were placed on Germitest paper roll and the fractionated ones were placed in Gerbox type plastic boxes lined with Germitest paper (with the cut surface facing down). In both forms (rolls and Gerbox), two sheets were used for the base and one sheet was used for the cover, moistened with water until saturation (that is, draining the excess) and kept in an air-conditioned germination room with a constant temperature of 25 °C, 100% relative humidity and continuous light (Inocente and Barbedo, 2021). Evaluations were performed on alternate days, recording the number of seeds with primary root protrusion (at least 0.5 cm of root), used to calculate the percentage and average time of germination (Santana and Ranal, 2004), and the number of seeds that produced normal seedlings (following the criteria used by Delgado and Barbedo, 2012). For fractionated seeds, germination values can exceed 100% because, for seeds of *Eugenia* species, each fraction has the potential to produce roots and seedlings, despite being monoembryonic (Delgado et al., 2022).

H_2O_2 quantifications were based on the DMAB/MBTH methodology of Bailly and Kranner (2011), with 3 replications of 5 seeds (*ac.* 1.2 - 2.0 g), which were homogenized in seven volumes of 0.1% trichloroacetic acid at 4 °C in porcelain mortar. The extracts were centrifuged at 4300 rpm for 10 minutes at 4 °C. Aliquots of 200 µL of each extract (in triplicates) were collected from the supernatant and added to 200 µL of 200 mM phosphate buffer pH 7.4 and 800 µL of potassium iodide. Each sample was homogenized and incubated at 4 °C for 1 hour. After incubation, absorbance readings of the samples were performed in a spectrophotometer at 390 nm. H_2O_2 concentrations in the tissues were determined using a standard curve, and the results were expressed in µmol $H_2O_2.g^{-1}$ of fresh mass.

Experimental design and statistical treatment

The experimental design was completely randomized in a 2 x 2 x 4 factorial scheme (drying x fractionation x reagent concentration). The data were subjected to analysis of variance considering each storage period and, when F was significant, the means were compared with each other by Tukey test at 5% probability level (Santana and Ranal, 2004). R software (R Core Team, 2018) was used for these analyses, and the Shapiro-Wilk test was used for the normality test. When necessary, the data for analysis were transformed to $(x + 0.5)^{0.5}$.

RESULTS AND DISCUSSION

The water content and dry mass content of *E. involucrata* seeds showed no significant differences between storage periods (Table 1). Regarding germination, the only difference occurred between the seeds stored for 120 days (60%) and the others (Table 1). The incubation of whole seeds without storage (NST/WS) for 3 hours in water or NAC did not alter germination or production of normal seedlings. However, both in fractionated seeds (CS) and in those stored for 60 and 120 days (SS60 and SS120), incubation in NAC reduced both germination and seedling formation, and the effect was greater with the increase in NAC concentration (Figure 2), as also observed for *Hedysarum scoparium* seeds (Su et al., 2016). This behavior only did not occur for seeds fractionated and stored for 120 days (SS120/CS), because the values were very close to zero. No important changes in this behavior were observed for seeds subjected to drying.

Table 1. Water content (in %), dry mass content (DM, in g.seed^{-1}) and germination (G, in %) of *Eugenia involucrata* seeds (without fractionation or incubation) without storage (NST) or stored for 60 (SS60) or 120 (SS120) days. Equal letters in the columns do not differ from each other by Tukey test at 5% probability level.

Storage	Water content	DM	G
NST	62 a	0.11 a	94 a
SS60	59 a	0.11 a	87 a
SS120	62 a	0.13 a	60 b

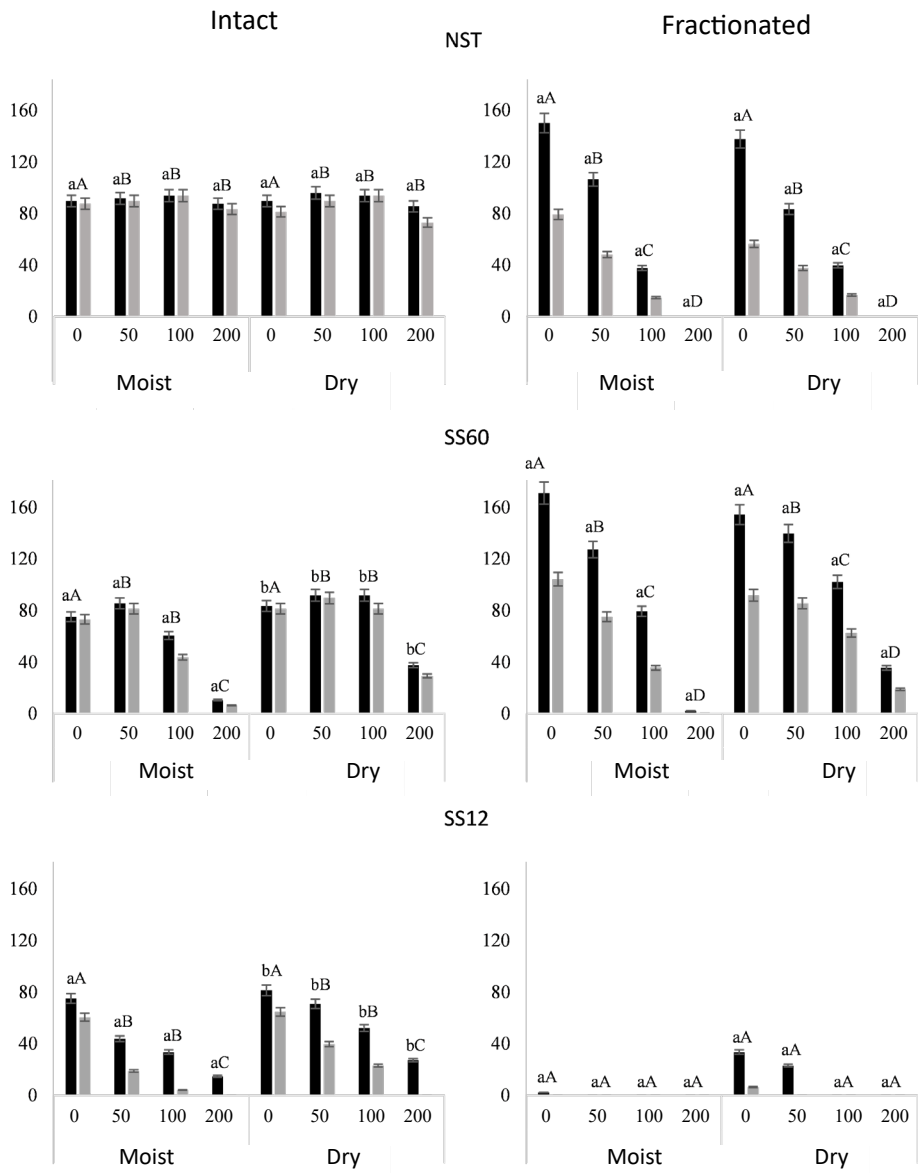

Figure 2. Percentage of seeds with root formation (black columns) and normal seedlings (gray columns) of *Eugenia involucrata* seeds without storage (NST) or stored for 60 (SS60) and 120 (SS120) days, incubated in water (0) and in NAC solutions of 50 mM, 100 mM and 200 mM. Equal letters do not differ from each other by Tukey test at 5% probability level (lowercase for drying and uppercase for reagent concentration). Coefficients of variation: for NST, 7.94%, 11.24% (intact), 29.00% and 31.62% (fractionated of NST), respectively, root formation and normal seedlings; for SS60, 19.53%, 23.88% (intact), 16.85% and 24.89% (fractionated); for SS120, 19.70%, 40.0% (intact), 32.12% and 20.31% (fractionated).

When applied exogenously, NAC replenishes glutathione stocks in seeds, inhibiting the production of H_2O_2 in the embryonic axis and reducing germination, and the effect is even greater with the increase in its concentrations (Ishibashi et al., 2013). In addition to inhibiting, NAC also delayed the germination of *E. involucrata*, as observed in the graph of mean germination time (Figure 3). The effects of inhibition and delay in germination caused by NAC have already been reported for seeds of other species (Ishibashi et al., 2013; Su et al., 2016). In addition, seeds incubated in NAC were also more susceptible to fungal contamination. The quantification of H_2O_2 showed that the incubation in NAC, in fact, reduced H_2O_2 content in the seeds, since there was no significant interaction between the factors, and seeds incubated in NAC showed values lower than the others (Figure 4).

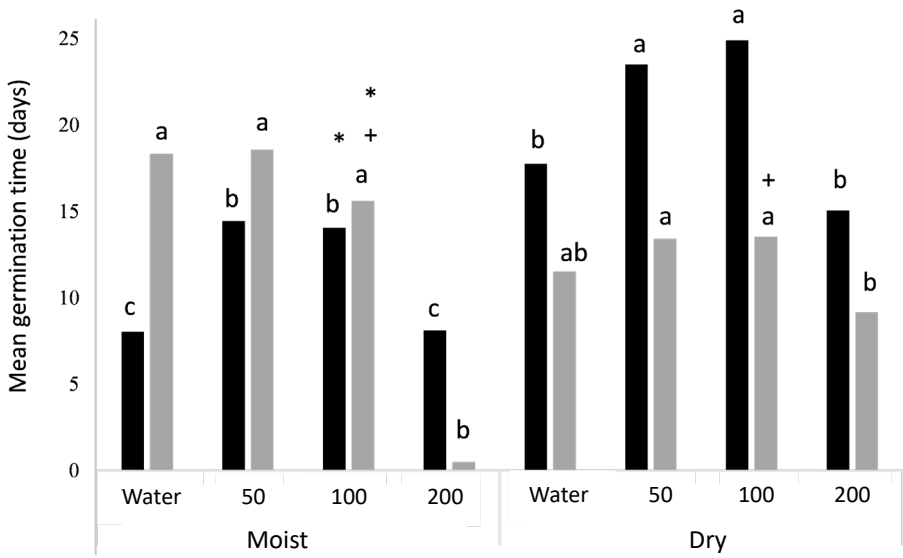

Figure 3. Mean germination time (MGT) of intact (black columns) and fractionated (gray columns) *Eugenia involucrata* seeds incubated in water and in NAC solutions of 50 mM, 100 mM and 200 mM. Columns with the same letter do not differ from each other (Tukey, 5%) in the comparison between NAC concentrations for moist or dry and for intact or fractionated. Columns with (*) do not differ from each other (Tukey, 5%) in comparisons between intact and fractionated. Columns with (+) do not differ from each other in the comparison between moist and dry. Coefficients of variation: 33.66% for intact and 28.14% for fractionated.

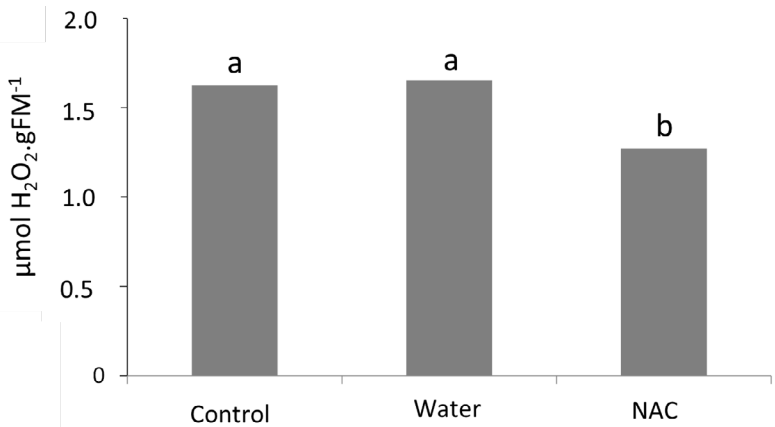

Figure 4. Quantification of H_2O_2 (µmol H_2O_2.gFM^{-1}) in *Eugenia involucrata* seeds, incubated in water and in NAC solution of 200 mM. Columns with the same letter do not differ from each other (Tukey, 5%). Coefficient of variation: 38.67%.

$O_2^{\bullet-}$ and H_2O_2 play a crucial role in the germination of *T. dregeana* and *Avicennia marina*, and there is a strong positive correlation between water uptake, ROS production rate, and maximum germination of these seeds (Moothoo-Padayachie et al., 2016). Further evidence of the positive relationship between the germination of desiccation-sensitive seeds of *T. dregeana* and *A. marina* and ROS production is that, when exposed to diphenylene iodonium, a potent inhibitor of NADPH oxidase, cellular respiration is not blocked, but germination is inhibited. In addition, the germination of *A. marina* is also compromised by exposure to dimethylthiourea, an eliminator of H_2O_2 (Moothoo-Padayachie et al., 2016).

NAC also seems to act in the suppression of ethylene, while H_2O_2 leads to its accumulation, which is related to the growth and development of plants, including the breaking of seed dormancy, germination and increase in root emergence under unfavorable conditions (Kucera et al., 2005; Ishibashi et al., 2013). Treatments that induce the generation of ROS in the embryonic axis appear to reduce the levels of abscisic acid (ABA) and suppress its inhibitory effect on germination, while treatment with NAC may reverse the inhibition of this hormone (El-Maarouf-Bouteau et al., 2014; Su et al., 2016). Gibberellic acid (GA) also plays a significant role in breaking dormancy and stimulating germination, and the production of H_2O_2 can be induced by GA in aleurone cells and suppressed by ABA. Exogenous H_2O_2 appears to promote the induction of α-amylases by GA, while antioxidants suppress the induction of α-amylases. Therefore, the H_2O_2 generated by GA in aleurone cells would promote the production of α-amylase, suggesting that H_2O_2 is a molecule that antagonizes ABA signaling (Ishibashi et al., 2012).

Other indirect pieces of evidence suggest the influence of ROS on the germination of *Eugenia* sp. seeds, such as the existence of self-regulatory mechanisms that control their germination, producing substances that inhibit new germination events as soon as the first one begins; exposure to air (when seeds are fractionated, for example) seems to break this inhibition, leading to the production of new roots (Delgado and Barbedo, 2011). *E. uniflora* seeds that were partially fractionated showed higher germination rates than intact seeds (i.e., control group that did not undergo any type of cutting), suggesting that the crack induced the differentiation of tissues that produce roots and seedlings, since intact seeds would only produce the primary root (Amador and Barbedo, 2015). A proposal for possible routes in the control of germination and regeneration in whole, fragmented, cracked and germinating seeds is presented in Figure 5.

The use of ROS as signaling molecules in plants suggests that, over time, there has been an adaptation of antioxidant mechanisms, allowing cells to overcome their toxicity and use these molecules as signal transducers (Bailey-Serres and Mittler, 2006). Enzymes that generate ROS, which make plant cells capable of producing and expanding their production for signaling purposes, have already been identified (Bailey-Serres and Mittler, 2006). And, as molecules related to cell signaling, ROS interact with other molecules and are involved with seed development, participating in the growth processes that occur early in embryogenesis and in the mechanisms linked to root protrusion during germination. ROS also play a regulatory role in gene expression during the development, dormancy, and germination of seeds (Bailly, 2004).

There is a need for studies that seek to adequately document the likely sources of ROS, as well as identifying their cellular targets and determining whether they are a connection between environmental signals and hormone signaling (Bailly et al., 2008). Evidently, the complex metabolic pathways of plants, especially seeds, hinder their complete understanding, and although indications that ROS play various roles in the germination of *Eugenia* seeds have been discovered, there is a long way to go and unravel the details involved in this relationship.

The results presented here provide insight into the action of H_2O_2, and possibly of the other ROS, on the germination of *Eugenia involucrata* seeds, as well as on the regeneration of germination in fractionated seeds. In recent years, many studies on the role of ROS have been published, but little attention has been paid to their role in the development of seeds that are dispersed while still immature or with high water content. However, as with dried seeds, these molecules also seem to play a crucial role in the stages of development and germination. Although they are unviable for storage for long periods, these seeds have a physiological universe with a multitude of adaptations that make them extremely vigorous and competitive in their habitats, and ROS certainly permeate many of them.

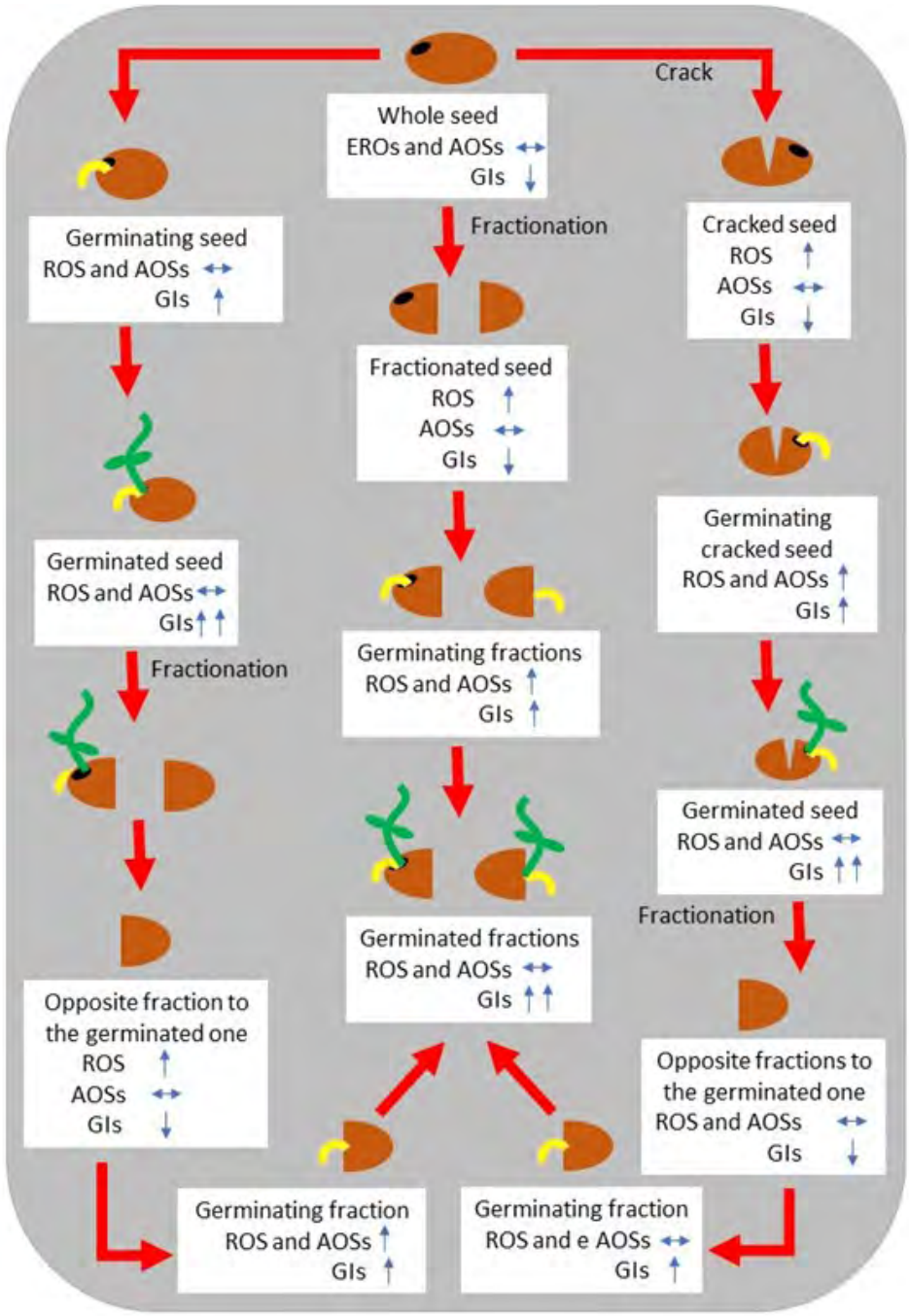

Figure 5. Proposal of the action of reactive oxygen species (ROS), antioxidant systems (AOSs) and germination inhibitors (GIs) in seeds and fractions of seeds of *Eugenia involucrata* according to the applied treatments and the germination stage. ↔: normal levels; ↑: high levels; ↑↑: very high levels; ↓: low levels.

CONCLUSIONS

Reduction in H_2O_2 concentration inhibits the germination and regeneration of roots and seedlings in *Eugenia involucrata* seeds.

ACKNOWLEDGMENTS

To the Coordination for the Improvement of Higher Education Personnel for the master's scholarship granted to I.P. Amorim and to the São Paulo State Research Support Foundation, for the financial support to the 2017/50341-0 project. To Maiara Iadwizak Ribeiro, for the help with the statistical analyses.

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