Antioxidant defenses determine the maturation and physiological quality of in vitro Euterpe edulis Martius seeds

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Abstract

The palm *Euterpe edulis* Martius is native to the Atlantic Forest. The nutritional composition and antioxidant potential of its fruits are superior to those of *Euterpe oleracea* and *Euterpe precatoria*. The present study aimed to analyze the antioxidant defenses associated with *in vitro* germination of *E. edulis* and the quality of its seedlings. Seeds were harvested at 29 maturation stages (94–290 days after anthesis, DAA), and characterized in terms of budding, vigor, seedling formation, antioxidant defenses (superoxide dismutase, catalase, ascorbate peroxidase, and anthocyanins), hydrogen peroxide, lipid peroxidation, and hormonal composition. Fruit dry mass and volume did not differ from 241 DAA; whereas seed dry mass peaked 255 DAA, decreasing after this period. Seed moisture decreased throughout maturation. Seedling-related variables achieved the highest average values 227 DAA. The seeds showed maximum germination, vigor, percentage of normal seedlings, concentration of ZEA and ABA at 164 DAA. From 269 DAA, a decrease in dry mass suggested increased reserve consumption (respiration), inhibition of nutrient translocation to seeds, and reduction in water content (desiccation). The resulting greater sensitivity to environmental variations coincided with increased oxidative stress, but was counteracted by antioxidant defenses.

1 INTRODUCTION

The palm tree *Euterpe edulis* Martius (Arecaceae) is native to the Atlantic Forest. Its purple spherical fruit is similar to those of *Euterpe oleracea* Martius and *Euterpe precatoria* Martius, but has better nutritional composition and antioxidant potential (Schulz et al., 2016). The excessive extraction of palm hearts from this species has brought it to the brink of extinction and, despite its vulnerable status, its overexploitation outside natural reserves is altering the regeneration dynamics within the Atlantic Forest (Brancalion et al., 2012; Muler et al., 2014; Souza and Prevedello, 2020).

The fruit of *E. edulis* has been gaining popularity among consumers, which could divert the exploitation of this species from the harvesting of palm hearts to the sustainable growth for fruits. The fruit pulp contains many essential micronutrients, including K, Fe, Zn, P, Cu, Ca, and Mg, as well as abundant polyunsaturated fatty acids and oleic acid, but few saturated fatty acids (Pupin et al., 2018; Pereira et al., 2023). In addition, the fruits are rich in phenolic compounds, mainly anthocyanins and phenolic acids, which suggest an elevated antioxidant potential (Schulz et al., 2016).

Several biochemical and metabolic alterations in primary and secondary compounds occur during fruit maturation (Batista-Silva et al., 2018). In the early stages, intense seed metabolism is characterized by elevated respiratory activity and production of reactive oxygen species (ROS), including superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($\bullet OH^-$) (El-Maarouf-Bouteau and Bailly, 2008).

At the end of maturation, seeds experience a reduction in water content and metabolism, which may stimulate ROS production. This point coincides with reduced enzymatic antioxidant activity and

increased impportance of non-enzymatic antioxidants such as anthocyanins (El-Maarouf-Bouteau and Bailly, 2008).

ROS promote the germination of both dormant and quiescent seeds. If ROS levels are above the suitable range for germination, seeds experience oxidative damage and rapidly deteriorate; if they are below the range, germination does not occur (Mittler, 2017; Cembrowska-Lech, 2020). This balance is regulated by ROS-quenching enzymes, especially superoxide dismutase (SOD), catalase (CAT), and peroxidases. Furthermore, as homonal balancing occurs during seed development, it may influence germination, vigor and seedling formation.

We hypothesized that the production of these enzymes increased during seed maturation to ensure the physiological quality of seeds, their vigor, and the formation of normal seedlings. Therefore, the purpose of this study was to analyze the antioxidant defenses of maturing *E. edulis* seeds during *in vitro* germination, as well as the hormonal composition, and the quality of the resulting seedlings.

2 MATERIALS AND METHODS

2.1 Biological material

The fruits of *E. edulis* were harvested from 10 mother plants located in the district of Pedra Menina, in the municipalities of Dores do Rio Preto (ES) and Espera Feliz (MG) (Fig. 1a). The maximum temperature during the studied period was 28.0°C in September, while the minimum was 14.9°C in June. The maximum precipitation was 514.4 mm in January, and the maximum relative humidity was 92% in May and June (Fig. 1b, c).

The fruit harvest started 94 days after anthesis (DAA) and lasted until 290 DAA, amounting to approximately 9.7 months. The fruits were harvested manually at 7-day intervals, totaling 29 stages, and were sent to the Laboratory of Forest Seeds and Plant Tissue Culture of the Center for Agricultural Sciences and Engineering, Federal University of Espírito Santo to evaluate moisture, germination, vigor, oxidative stress, ROS, lipid peroxidation in seeds, and early seedling growth.

2.2 Seed water content

To determine the water content of seeds, four replicates with five seeds each were incubated in an oven (Adamo) at 105 ± 3°C for 24 h, according to the Rules for Seed Analysis (Brasil, 2009). The results are expressed as percentages.

2.3 *In vitro* seed germination and vigor

After cleaning the fruits, the tegument was removed, and the fruits were immersed in 2% ascorbic acid (Dinâmica) to prevent phenolic oxidation of tissues.

Using a laminar flow chamber, the seeds were immersed in 70% ethyl alcohol for 1 min, 2.5% sodium hypochlorite (Candura) for 10 min, and 3 g L^{-1} amoxicillin (Germed) for 10 min, with a triple wash in distilled and autoclaved water after each disinfecting agent. Then, the seeds were placed in test tubes (25 × 150 mm) containing 10 mL MS culture medium (Murashige and Skoog, 1962) (Sigma), supplemented with 1 g L^{-1} polyvinylpyrrolidone (Synth), 30 g L^{-1} sucrose (Dinâmica), 0.1 g L^{-1} myo-inositol (Sigma), and 6 g L^{-1} agar (Kasvi). The pH of the medium was adjusted to 5.7 ± 0.1 with 1 N KOH (Alphatec) and/or HCl (Vetec) before autoclaving.

The explants were maintained in a growth room at a temperature of 27 ± 2°C, photoperiod of 8/16 h (light/dark), and light intensity of 30 µmol m^{-2} s^{-1} until seedlings were obtained. During this period, data on germination percentage (Brasil, 2009), germination frequency, germination speed index (Maguire, 1962), and average germination time (days) were obtained (Labouriau, 1983). After 150 days, normal and abnormal seedlings (%), seedling height (cm), collar diameter (mm), root length (cm), number of roots, dry mass of shoots, roots, and total seedlings (g), and Dickson's quality index (Dickson et al., 1960) were determined.

2.4 Biochemical analyses

The seeds were stored in an ultrafreezer (Thermo Scientific) at -80°C until use.

Hydrogen peroxide and lipid peroxidation in seeds

Brielfy, 200 mg of seeds were crushed in a mill (IKA, model A11BS32) with liquid nitrogen, homogenized in 1500 µL 0.1% (w/v) trichloroacetic acid (Sigma), and centrifuged at 10,000 rpm for 15 min at 4°C.

H_2O_2 was quantified by mixing 45 µL of the supernatant with 40 µL 10 mM potassium phosphate buffer (pH 7.0) and 90 µL 1 M KI (Vetec). The concentration of H_2O_2 was determined by measuring sample absorbance at 390 nm and comparing it to a standard curve (Velikova et al., 2000).

Lipid peroxidation was quantified by determining thiobarbituric acid reactive species (Buege and Aust, 1978). Briefly, 200 µL extract and 550 µL reaction medium containing 0.5% (w/v) thiobarbituric acid and 10% (w/v) trichloroacetic acid were added to a test tube (150 × 10 mm), followed by incubation at 95°C for 30 min. The reaction was stopped by rapid cooling on ice, and readings at 535 and 600 nm were taken using an enzyme-linked immunosorbent assay (ELISA) reader (BIOTEK-Eon).

Antioxidant enzymes in seeds

For enzyme extraction, 200 mg of seeds were ground in a mill (model A11BS32) with liquid nitrogen and homogenized in 1500 µL extraction buffer containing 375 µL 100 mM potassium phosphate (pH 7.8), 15 µL 0.1 mM ethylenediaminetetraacetic acid (Calbiochem), 75 µL 10 mM ascorbic acid (Sigma), and 1035 µL distilled water. They were then centrifuged at 13,000 rpm for 10 min at 4°C (Vision, model VS-15000CFNII), and the supernatants were collected for SOD, CAT, and ascorbate peroxidase (APX) activity assays (Biemelt et al., 1998).

SOD activity was analyzed as described by Giannopolitis and Ries (1977) with some modifications. Distilled water (5 µL, blank) was added to the first two wells of the microplate, whereas 5 µL enzymatic extract was added in duplicate to the remaining wells. Next, 190 µL buffer solution consisting of 100 µL 100 mM potassium phosphate (pH 7.8), 43 µL 14 mM methionine with 0.1 µM EDTA, 15 µL 75 µM nitrotetrazolium blue (Sigma), 2 µL 2 µM riboflavin (Sigma), and 30 µL distilled water was added. The reaction medium and the sample were illuminated with a 15-W fluorescent lamp for 7 min. Absorbance was measured at 560 nm using an ELISA reader.

CAT activity was measured as described by Havir and McHale (1987). Briefly, 8-µL alliquots of enzyme extract from stages 1–25 and 5 µL from stages 26–29 were added to 91 µL (stages 1–25) or 94 µL (stages 26–29) 200 mM potassium phosphate buffer (pH 7.0), 9 µL 12.5 mM H_2O_2 (Exôdo Científica), and 72 µL distilled water. CAT activity was determined with an ELISA reader by measuring absorbance at 240 nm every 15 s for 3 min.

APX activity was measured as described by Nakano and Asada (1981). Briefly, 5 µL extract was mixed with 94 µL 200 mM potassium phosphate buffer (pH 7.0), 9 µL 10 mM ascorbic acid, 9 µL 2 mM H_2O_2, and 63 µL distilled water. APX activity was determined by monitoring ascorbate oxidation every 15 s for 3 min at 290 nm using an ELISA reader.

Anthocyanins

The extraction of anthocyanins from the seeds was carried out as described by Francis (1982). Briefly, 1.0 g of crushed seeds was weighed and 30 mL extracting solution (95% ethanol with 1.5 N HCl) was added at an 85:15 ratio and homogenized for 2 min. Then, 50 mL extraction solution was added, and the mixture was stored in the dark at 4°C for 16 h. After filtering, readings were taken at 535 nm, values were calculated using the equation (absorbance × dilution factor/98.2), and the results were expressed in mg 100 g^{-1}.

2.5 Hormones extraction and quantification

Five maturation stages were selected: 94 DAA (initial stage), 115 DAA (the stage in which germination begins to occur), 164 DAA (stage with higher germination, germination speed index, normal seedlings, and lower average germination time), 234 DAA (stage at which the fruit begins to change color, green to purple) and 290 DAA (final ripening stage). The seeds were macerated for the extraction and quantification of hormones according to Müller and Munné-Bosch (2011), with modifications (Napoleão et al., 2017; Vital et al., 2018). Fresh tissue (110 mg) was ground in liquid nitrogen, and then extracted with 300 µL of methanol:isopropanol:acetic acid, 20:79:1 (v:v:v). The samples were vortexed for 20 s four times, ultrasounded for 5 min and kept on ice for 30 min. After centrifugation at 13,000 *g* for 10 min at 4°C, 250 µL of the supernatant was collected and transferred to a tube. The extraction process was repeated three times with the pellet. The supernatants were combined and centrifuged at 20,000 *g* for 5 min at 4°C. We injected 5 µL of the obtained extract into the liquid chromatography with tandem mass spectrometry (LC-MS/MS) system (Agilent 1,200 Infinity Series, coupled with the triple quadrupole mass

spectrometer – QqQ, model 6430 Agilent Technologies). Chromatographic separation was performed using the Zorbax Eclipse Plus C18 column (1.8 µm, 2.1 × 50 mm, Agilent) in series with a 1.8 µm Zorbax SB-C18 guard column. The mobile phase consisted of: 0.02% acetic acid in water (solvent A) and 0.02% acetic acid in acetonitrile (solvent B), in a time gradient/% B of 0/5; 11/60; 13/95; 17/95; 19/5; 20/5. The flow used was 0.3 mL min^{-1} at 23 ºC. The electrospray ionization source was used at gas temperature of 300°C, nitrogen flow of 10 L min^{-1}, nebulizer pressure of 35 psi, and capillary voltage of 4,000 V.

The equipment was operated in the multiple reaction monitoring mode (MRM), in which the precursor/fragment ion masses were monitored by fragmentation tests of each molecule: auxins (indolacetic acid - IAA) (176/130), cytokinin (zeatin - ZEA) (220/136), abscisic acid (A BA) (263/153), ethylene via 1-carboxylic acid-1-aminocyclopropane (ACC) (102.1/56.2). IAA, ZEA, and ACC were scanned in the positive mode, while BA was in the negative mode. A calibration curve (0.1 to 200 ng mL^{-1}) was made to obtain the absolute quantification, using the standards for each molecule (Sigma-Aldrich). The analysis of the data obtained was performed using the Skyline software (Adams et al., 2020) and the results were presented in ng g^{-1} of fresh tissue.

2.6 Experimental design

The design was completely randomized and consisted of 29 treatments (maturation stages), four replications with 10 seeds each for biometric analyses and *in vitro* germination, and three replications in duplicate for biochemical analyses. Data were subjected to analysis of variance, Scott-Knott mean cluster test ($p < 0.05$), Pearson's correlation, and principal component analysis. All statistical analyses were performed using computational routines in R software, version 4.1.2 (R Core Team, 2023). Graphs and figures were generated using the following packages: readxl, factoextra, factoMineR, tidyverse, ggcorrplot, psych, and ExpDes.pt. For the hormonal analysis, five maturation stages were considered, in triplicate, in which analysis of variance and Tukey's test ($p < 0.05$) were performed (R Core Team, 2023).

3 RESULTS

The endosperm of *E. edulis* seeds remained watery until 94 DAA, when it changed to a gelatinous consistency, which turned solid 143 DAA. The fruits took 290 DAA to detach from the parent plant, with onset of pulp ripening 241 DAA (Fig. 2).

The morphological variables denoting fruit development varied during the maturation process (Fig. 3). Fresh fruit mass increased steadily throughout this period (Fig. 3a). Fruit dry mass and volume did not differ significantly beyond 241 DAA (mature fruit stages) (Fig. 3b, d). Seed dry mass reached a maximum 255 DAA (0.83 g) and decreased thereafter (Fig. 3c).

Seed moisture decreased during maturation, from 77–45% (94–290 DAA) (Fig. 4a). Seeds did not germinate until 108 DAA, but achieved 100% germination from 150 DAA. No statistical difference regarding the germination rate was observed from 150 to 206 DAA, and later on at 220, 234, and 283 DAA; however, rates began to oscillate 206 DAA (Fig. 4b). The seeds attained maximum vigor 164 DAA,

which coincided with a germination speed index of 1.42, mean germination time of 7 days, relative frequency of germination of 0.25, 100% germination, and 100% normal seedlings (Fig. 4b–f). Generally, seeds in the initial or final stages of maturation presented more abnormal seedlings and non-germinated seeds (Fig. 4g, h).

In the initial stages, up to 108 DAA, no seedlings emerged as there was no germination. Seedlings obtained from seeds germinated 227 DAA presented significantly greater collar diameter, root length, shoot dry mass, root dry mass, total dry mass, and Dickson quality index than other seedlings; whereas seedling height and number of roots did not differ compared to seedlings germinating 220 DAA (Fig. 5).

Antioxidant enzymatic activity, H_2O_2 levels, and lipid peroxidation increased during seed maturation; only SOD showed no change in activity (Fig. 6). CAT and APX activity, as well as H_2O_2 and lipid peroxidation showed a similar behavior, with the highest activities recorded from 269 DAA.

The production of anthocyanins by *E. edulis* seeds remained low until 241 DDA and, but increased gradually thereafter.

Most tested variables exhibited a positive correlation with each other, except seed water content and non-germinated seeds (Fig. 7). A particularly strong correlation was observed between the morphological variables of the fruit, morphological variables of the seedlings, and physiological changes related to oxidative stress in the seeds.

Principal component analysis revealed that the first two components explained 74% of the variance, with CP1 accounting for 55.6% and CP2 for 18.4% (Fig. 8a).

The variables that contributed most to CP1 and CP2 were stem diameter, fruit fresh mass, seed dry mass, number of roots, fruit dry mass, seed water content, germination, CAT, non-germinated seeds, normal seedlings, H_2O_2, seedling height, APX, and seed volume, with a contribution above the minimum value of 4% (Fig. 8b). A graphical view of the data for the first two principal components is presented in Fig. 8c.

The concentration of IAA decreased throughout the maturation stages, with its highest average 223.94 ng g^{-1} at 94 DAA, drastically reducing in the final stages 234 and 290 DAA (7.20 and 5.28 ng g^{-1}, respectively), not statistically differentiating (Fig. 9a).

The ZEA trans and cis differed from the other stages presenting their highest averages at 164 DAA (1156.67 and 1155.30 ng g^{-1}, respectively) (Fig. 9b).

The ABA had its highest average of 43.41 ng g^{-1} at 164 DAA (Fig. 9c).

The ACC obtained its highest averages of 2.74, 2.72 and 2.13 ng g^{-1} at stages 164, 290 and 234 DAA, respectively, not differing statistically from each other (Fig. 9d).

4 DISCUSSION

Seed maturation is accompanied by morphological, physiological, and biochemical changes. Here, we show that water content in the seeds decreased by 30% 269 DAA (Fig. 4a). This point, which corresponded to the lowest water content, highlights the physiological quality of recalcitrant seeds such as those of *E. edulis*.

The maximum dry mass content, germination, and vigor of the seeds indicated physiological maturity. Germination and vigor under *in vitro* conditions did not coincide with *in vivo* conditions, and caused immature seeds to present maximum germination 150 DAA (Fig. 4b), while vigor and normal seedlings were maximal 164 DAA (Fig. 4c, d, f). During this time, metabolic activity became more intense due to elevated respiratory activity, which favored the release of ROS, such as $O_2^{\cdot-}$, H_2O_2, and •OH. in addition to damage-induced loss of semi-permeability, the membrane may suffer also from lipid peroxidation (Araújo et al., 2018).

The increased activity of metabolic and respiratory processes intensifies the production of ROS, which triggers the cell's antioxidant defenses to maintain homeostasis. The accumulation of ROS and the damage they inflict are determined by the efficiency of the antioxidant system, which preserves the conformation of proteins, lipids, and DNA (Melo et al., 2021). Mitochondria and peroxisomes are the main organelles responsible for intracellular ROS production. The mitochondrial electron transport chain generates $O_2^{\cdot-}$; whereas glyoxysomes, special peroxisomes found in plants, mediate the transfer of electrons from $FADH_2$ directly to O_2 to produce H_2O_2 by β-oxidation, thereby leading to protein degradation (Kijowska-Oberc et al., 2021).

At the end of the maturation phase, whereby a reduction in water content and seed metabolism is observed, the activity of SOD, CAT, peroxidase, and APX tends to stabilize, and other protective mechanisms such as non-enzymatic antioxidants assume greater importance (Araújo et al., 2018). The same was observed here for *E. edulis* seeds, whose antioxidant enzymatic activity remained generally constant from 269 DAA, but was accompanied by an increase in anthocyanin content (Fig. 5).

The low antioxidant enzymatic activity of *E. edulis* seeds up to 262 DAA is associated with lower oxidative stress, as well as reduced metabolism (Araújo et al., 2018). Dehydrin-like proteins (DHN) immunolocalize to the cytoplasm and chromatin of the endosperm and embryo of recalcitrant mature *E. edulis* seeds (Panza et al., 2007). However, according to the authors, DHN are not sufficient to confer tolerance to desiccation. In *Araucaria angustifolia* (Bertol.) Kuntze, the presence of DHN in the nucleus is thought to protect the transcriptional machinery (Farias-Soares et al., 2012).

The observed low SOD activty suggests low $O_2^{\cdot-}$ production and, consequently, limited membrane denaturation by oxidation, which prevents the remobilization and breakdown of reserve molecules. The conservation of reserve molecules favors germination and vigor because recalcitrant seeds are naturally more sensitive to desiccation. This inability to preserve the structure of the membrane system arises from the lower activity of LEA proteins and raffinose-type sugars (raffinose and stachyose). According to Obroucheva et al. (2016), sucrose imported early from the cotyledons to the seed embryo represents a

potential osmoregulatory source and is a much more efficient reserve for the onset of growth than oligosaccharides or starch. Berjak and Pammmenter (2013) reported that metabolic “shutdown” and intracellular dedifferentiation facilitated tolerance to dessication. Because these mechanisms are not observed in recalcitrant seeds, they contribute significantly to these seeds’ sensitivity to desiccation.

CAT is effective at neutralizing high concentrations of H_2O_2 and maintaining them within controlled levels (Melo et al., 2021). In seeds of *A. angustifolia*, the genes APX and SMP (encoding SEED MATURATION PROTEIN) showed decreased expression in the cotyledons, the CAT gene was more expressed in the embryonic axes, while GPX (GLUTATHIONE PEROXIDASE) and SOD genes exhibited no significant difference after dehydration (Gasparin et al., 2021). According to these authors, the gene expression profile of recalcitrant seeds of *A. angustifolia* suggests that loss of viability after dehydration is related to decreased transcription of genes associated with tolerance to desiccation.

APX plays a key role in the ascorbate-glutathione cycle and the elimination of H_2O_2 from plant cells. This enzyme is important for cell wall biosynthesis, cell growth, differentiation, and development, in addition to its involvement in stress responses (Melo et al., 2021). APX reduces H_2O_2 to H_2O by oxidizing ascorbic acid and dehydroascorbate (Obroucheva et al., 2016).

The first change in anthocyanin content in *E. edulis* seeds occurred 164 DAA (Fig. 6e), coinciding with greater twinning and vigor. Anthocyanin content can be considered a biochemical marker for physiological processes other than the antioxidant defense system. Elevated anthocyanin levels coincided with increased ROS production, lipid peroxidation, and consequent activation of APX and CAT (Fig. 6). Anthocyanins are regulated in a coordinated manner at multiple levels through environmental signaling and seed development. A high anthocyanin content is associated with the end of the seed maturation phase due to the expression of genes linked to this developmental stage. Moreover, recent studies suggest that synthesis of this pigment is regulated through the signaling of metabolic routes that use melatonin as a precursor (Li et al., 2020).

IAA concentrations were considerably higher in the initial stage (94 DAA) of E. edulis seed maturation, which is the beginning stage of embryo and endosperm development. During this period, the concentration and localization of IAA play a key role in regulating the pattern of cell-type formation and determining the polarity of the embryo (Bewley and Nonogaki, 2017).

Cytokinins (ZEA) present in the early stages had a greater increase at the end of maturation. ZEA strongly influences cell division, subsequently causes vesicular differentiation (Miransari and Smit, 2014) and, promotes cotyledon/haustorium expansion and endosperm depletion (Bewley and Nonogaki, 2017). In addition, the accumulation of cytokinin in the final stages of maturation is essential for the nullity of ABA action.

The high concentration of ABA at 164 DAA indicates a period of high reserve accumulation, as many genes that are involved in the seed storage process are mediated by ABA (Xue et al., 2015; Ali et al., 2022).

The decrease in ABA concentrations in the final stages of *E. edulis* seed maturation reinforces its intolerance to desiccation. During the last stages of seed development, desiccation tolerance is acquired and associated with the accumulation of antioxidants, sugar, and abundant proteins in late embryogenesis (LEA), with ABA being the precursor of LEA (Bewley et al. 2013). And in seeds that do not have dormancy, as the seeds mature, their ABA content decreases (Bewley and Nonogaki, 2017).

The higher concentrations of ACC in the final stages of maturation are due to the ripening of the fruit of *E. edulis*. 1-Aminocyclopropane-1 carboxylic acid (ACC) is a prerequisite for the production of ethylene, catalyzed by ACC oxidase (Bewley and Nonogaki, 2017), with ethylene being the main hormone responsible for fruit ripening. Furthermore, ethylene has an antagonistic effect to ABA on seed dormancy, and counteracts the inhibitory effect of ABA on germination (Shu et al. 2016), which explains the increase in ACC at the expense of the decrease in ABA in *E. edulis*.

Ethylene is also involved in the regulation of endo-β-mannanase activity and in the induction of mannan hydrolysis in the endosperm (Santos and Garcia, 2023). Being mannan, the main reserve of the *Euterpe* endosperm, accumulated during maturation, causing the hardening of the endosperm, obtaining maximum deposition in the final stage (Moura et al., 2019; Nascimento et al., 2020; Lima et al., 2021).

5 CONCLUSIONS

In the present study, we show that *in vitro* germinated seeds of *E. edulis* attained maximum germination, vigor, and percentage of normal seedlings 164 DAA (immature fruit), which represents a gain of 126 days compared to seeds obtained at the last stage of maturation (290 DAA). CAT and APX activity, as well as H_2O_2 and lipid peroxidation levels, increased from 269 DDA onwards to counteract the release of ROS. Anthocyanin production by *E. edulis* seeds peaked at the last stage of maturation, with the first change in anthocyanin concentration detected 164 DAA. The initial stage (94 DAA) has the highest levels of IAA, while at 164 DAA it obtained the highest concentrations of ZEA and ABA. The ACC increases in the late stages.

Declarations

CRediT authorship contribution statement

Tamyris de Mello: Investigation, Methodology, Writing – review, and editing. **Antonio Rodrigues da Cunha Neto**, Yanara dos
Santos Taliuli, Caroline Palacio de Araujo and **Clovis Eduardo Nunes Hegedus**: Investigation, Writing – review and editing. **Breno Benvindo dos Anjos**, **Edilson Romais Schmildt** and **Adésio Ferreira**: Formal analysis. **Heloisa Oliveira dos Santos**, **José Carlos Lopes**, and **Wagner Campos Otoni**: Supervision, Writing – original draft, Writing – review and editing. **Rodrigo Sobreira Alexandre**: Conceptualization, Supervision, Project administration, Funding acquisition, Writing – original draft, Writing – review and editing.

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Data Availability Statements

Data are available in a repository and can be accessed via a link:
https://github.com/TamyrisMello/Maturation-.git

Declaration of Competing Interest

The authors declare no conflict of interest.

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References

1. Adams KJ, Pratt B, Bose N, Dubois LG, St John-Williams L, Perrott KM, Ky K, Kapahi P, Sharma V, MacCoss MJ, Moseley MA, Colton CA, MacLean BX, Schilling B, Thompson JW (2020) Skyline for small molecules: a unifying software package for quantitative metabolomics. J Proteome Res 19:1447-1458. https://doi.org/10.1021/acs.jproteome.9b00640
2. Ali F, Qanmber G, Li F, Wang Z (2022) Updated role of ABA in seed maturation, dormancy, and germination. J Adv Res 35:199-214. https://doi.org/10.1016/j.jare.2021.03.011
3. Araújo RF, Abud HF, Silva LJD, Araujo EF, Pinto CMF, Silva FWS (2018) Alterações fisiológicas e atividade de enzimas antioxidantes em sementes de pimentas *Capsicum chinense* Jacq e *Capsicum frutescens* L. durante o processo de maturação. Rev Ceres 65(6):534-545. https://doi.org/10.1590/0034-737X201865060009
4. Batista-Silva W, Nascimento VL, Medeiros DB, Nunes-Nesi A, Ribeiro DM, Zsögön A, Araújo WL (2018) Modifications in organic acid profiles during fruit development and ripening: correlation or causation? Front Plant Sci 9:1-20. https://doi.org/10.3389/fpls.2018.01689
5. Berjak P, Pammenter NW (2013) Implications of the lack of dessication tolerance in recalcitrant seeds. Front Plant Sci 4:478-511. https://doi.org/10.3389/fpls.2013.00478

6. Bewley JD, Bradford KJ, Hilhorst HWM, Nonogaki H (2013) Development and maturation. In: Seeds. Springer, New York, NY. https://doi.org/10.1007/978-1-4614-4693-4_2
7. Bewley JD, Nonogaki H (2017) Seed maturation and germination. Reference Module in Life Sciences. https://doi.org/10.1016/B978-0-12-809633-8.05092-5
8. Biemelt S, Keetman U, Albrecht G (1998) Re-aeration following hypoxia or anoxia leads to activation of the antioxidative defense system in roots of wheat seedlings. Plant Physiol 116(2):651-658. https://doi.org/10.1104/pp.116.2.651
9. Brancalion PHS, Vidal E, Lavorenti NA, Batista JLF, Rodrigue RR (2012) Soil-mediated effects on potential *Euterpe edulis* (Arecaceae) fruit and palm heart sustainable management in the Brazilian Atlantic Forest. For Ecol Manag 284:78-85. https://doi.org/10.1016/j.foreco.2012.07.028
10. Brasil. 2009. Ministério da Agricultura, Pecuária e Abastecimento. Regras para análise de sementes. MAPA/DAS/ACS. 395p.
11. Buege JA, Aust SD (1978) Microsomal lipids peroxidation. Meth Enzymol 52:302-310. https://doi.org/10.1016/s0076-6879(78)52032-6
12. Cembrowska-Lech D (2020) Tissue printing and dual excitation flow cytometry for oxidative stress - new tools for reactive oxygen species research in seed biology. Int J Mol Sci 21(22):8656. https://doi.org/10.3390/ijms21228656
13. Dickson A, Leaf AL, Hosner JF (1960) Quality appraisal of white spruce and white pine seedling stock in nurseries. For Chron 36:10-13. https://doi.org/10.5558/tfc36010-1
14. El-Maarouf-Bouteau H, Bailly C (2008) Oxidative signaling in seed germination and dormancy. Plant Signal Behav 3(3):175-82. https://doi.org/10.4161/psb.3.3.5539
15. Farias-Soares FL, Burrieza HP, Steiner N, Maldonado S, Guerra MP (2012) Immunoanalysis of dehydrins in *Araucaria angustifolia* embryos. Protoplasma 250:911-918. https://doi.org/10.1007/s00709-012-0474-7
16. Francis FJ (1982) Analysis of anthocyanins. In: Markakis, P. Anthocyanins as food colors. London: Academic Press 181-207. https://doi.org/10.1016/B978-0-12-472550-8.X5001-X
17. Gasparin E, Faria JMR, Ribeiro PR, Ligterink W, Hilhorst HWM (2021) Loss of viability during dehydration of *Araucaria angustifolia* (Bertol.) Kuntze seeds is associated with specifc changes in gene expression. Trees 35:2087-2104. https://doi.org/10.1007/s00468-021-02176-x
18. Giannopolitis CN, Ries SK (1977) Superoxide dismutases I. Occurrence in higher plants. Plant Physiol 59(2):309-314. https://doi.org/10.1104/pp.59.2.309
19. Havir EA, Mchale NA (1987) Biochemical and developmental characterization of multiple forms of catalase in tobacco leaves. Plant Physiol 67(3):450-455. https://doi.org/10.1104/pp.84.2.450
20. Kijowska-Oberc J, Staszak AM, Ratajczak E (2021) Climate change afects seed aging? Initiation mechanism and consequences of loss of forest tree seed viability. Trees 35:1099-1108. https://doi.org/10.1007/s00468-020-02072-w

21. Labouriau LG (1983) A germinação das sementes. Washington: Secretaria Geral da Organização dos Estados Americanos, 174.
22. Li D, Guo Y, Zhang D, He S, Gong J, Ma H, Gao X, Wang Z, Jiang L, Dun X, Hu S, Chen M (2020) Melatonin represses oil and anthocyanin accumulation in seeds. Plant Physiol 183(3):898-914. https://doi.org/10.1104/pp.20.00117
23. Lima ACP, Bastos DLR, Camarena MA, Bon EPS, Cammarota MC, Teixeira RSS, Gutarra MLE (2021) Physicochemical characterization of residual biomass (seed and fiber) from açaí (*Euterpe oleracea*) processing and assessment of the potential for energy production and bioproducts. Biomass Convers Biorefin 11:925-935. https://doi.org/10.1007/s13399-019-00551-w
24. Maguire JD (1962) Speeds of germination-aid selection and evaluation for seedling emergence and vigor. Crop Sci 2:176-177. https://doi.org/10.2135/cropsci1962.0011183X000200020033x
25. Mello T, Rosa TLM, Simões IM, Lima PAM, Dos Anjos BB, De Araujo CP, Hegedus CEN, Dos Santos HO, Otoni WC, Alexandre RS, Lopes JC (2021) Reserve mobilization and *in vitro* germination of *Euterpe edulis* (Martius) seeds at different maturation stages. Trees 36:415-426. https://doi.org/10.1007/s00468-021-02216-6
26. Melo GMD, Santos HOD, Oliveira TF, Cunha ARD, Pereira AAS, Guaraldo MMDS (2021) Effect of priming and different types of drying on the physiological quality of *Urochloa ruziziensis* seeds. J Seed Sci 43:e202143021. https://doi.org/10.1590/2317-1545v43250046
27. Miransari M, Smit, DL (2014) Plant hormones and seed germination. Environ Ex Bot 99:110-121. https://doi.org/10.1016/j.envexpbot.2013.11.005
28. Mittler R (2017) ROS are good. Trends Plant Sci 22(1):11-19. DOI: https://doi.org/10.1016/j.tplants.2016.08.002.
29. Moura ACF, Ribeiro LM, Mazzottini-Dos-Santos HC, Mercadante-Simões MO, Nunes YRF (2019) Cytological and histochemical evaluations reveal roles of the cotyledonary petiole in the germination and seedling development of *Mauritia flexuosa* (Arecaceae). Protoplasma 256:1299-1316. https://doi.org/10.1007/s00709-019-01375-1
30. Muler AE, Rother DC, Rancalion PS, Naves RP, Rodrigues RR, Pizo MA (2014) Can overharvesting of a non-timber-forest-product change the regeneration dynamics of a tropical rainforest? The case study of *Euterpe edulis*. For Ecol Manag 324:117-125. https://doi.org/10.1016/j.foreco.2013.09.001
31. Müller M, Munné-Bosch S (2011) Rapid and sensitive hormonal profiling of complex plant samples by liquid chromatography coupled to electrospray ionization tandem mass spectrometry. Plant Methods 7:37. https://doi.org/10.1186/1746-4811-7-37
32. Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473-497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x
33. Nakano Y, Asada K (1981) Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell Physiol 22(5):867-880. https://doi.org/10.1093/oxfordjournals.pcp.a076232

34. Napoleão TA, Soares G, Vital CE, Bastos C, Castro R, Loureiro ME, Giordano A (2017) Methyljasmonate and salicylic acid are able to modify cell wall but only salicylic acid alters biomass digestibility in the model grass *Brachypodium distachyon*. Plant Sci 263:46-54. https://doi.org/10.1016/j.plantsci.2017.06.014
35. Nascimento JRS, Neto DF, Coutinho IC, Domont GB, Nogueira FCS, Campos FAP (2020) Proteome dynamics of the cotyledonary haustorium and endosperm in the course of germination of *Euterpe oleracea* seeds. Plant Sci 298:110569. https://doi.org/10.1016/j.plantsci.2020.110569
36. Obroucheva N, Sinkevich I, Lityagina S (2016) Physiological aspects of seed recalcitrance: a case study on the tree *Aesculus hippocastanum*. Tree Physiol 36:1127-1150. https://doi.org/10.1093/treephys/tpw037
37. Panza V, Distéfano AJ, Carjuzaa P, Láinez V, Del Vas V, Maldonado S (2007) Detection of dehydrin-like proteins in embryos and endosperm of mature *Euterpe edulis* seeds. Protoplasma 231:1-5. https://doi.org/10.1007/s00709-007-0248-9
38. Pereira DCS, Gomes FS, Tonon RV, Beres C, Cabral LMC (2023) Towards chemical characterization and possible applications of juçara fruit: an approach to remove *Euterpe edulis* Martius from the extinction list. J Food Sci Technol 60:429-440. https://doi.org/10.1007/s13197-021-05342-8
39. Pupin L, Santos VS, Santos Neto JP, Fusco DO, Teixeira GHA (2018) Is the bioaccessibility of minerals affected by the processing steps of juçara fruit (*Euterpe edulis* Mart.)? Food Sci Technol 91:14-25. https://doi.org/10.1016/j.lwt.2018.01.024
40. R Core Team. R: a language and environment for statistical computing. Vienna: R Foundation for Statistical Computing. Available in: <https://www.rproject.org/>. Access at: 10 feb. 2023.
41. Santos JAS, Garcia QS (2023) Ethylene in the regulation of seed dormancy and germination: Biodiversity matters. In: Khan NA, Ferrante A, Munné-Bosch S The plant hormone ethylene. London: Academic Press, 61-71. https://doi.org/10.1016/B978-0-323-85846-5.00013-8
42. Schulz M, Borges GDSC, Gonzaga LV, Costa ACO, Fett R (2016) Juçara fruit (*Euterpe edulis* Mart.): Sustainable exploitation of a source of bioactive compounds. Food Res Int 89:14-26. https://doi.org/10.1016/j.foodres.2016.07.027
43. Shu K, Liu X, Xie Q, He Z (2016) Two faces of one seed: hormonal regulation of dormancy and germination. Mol Plant 6(1):34-45. https://doi.org/10.1016/j.molp.2015.08.010
44. Souza AC, Prevedello JA (2020) The importance of protected areas for overexploited plants: Evidence from a biodiversity hotspot. Biol Conserv 243:108482. https://doi.org/10.1016/j.biocon.2020.108482
45. Velikova V, Yordanov I, Edreva A (2000) Oxidative stress and some antioxidant systems in acid rain-treated bean plants: protective role of exogenous polyamines. Plant Sci 151(1):59-66. https://doi.org/10.1016/S0168-9452(99)00197-1
46. Vital CE, Gómez JD, Vidigal PM, Barros E, Pontes CSL, Vieira NM, Ramos HJO (2018) Phytohormone profling by liquid chromatography coupled to mass spectrometry (LC/MS). Protocols.io. https://doi.org/10.17504/protocols.io.zgf3tn

47. Xue J, Wang S, Zhang P, Zhu F, Ren X, Liu C, Zhang X (2015) On the role of physiological substances, abscisic acid and its biosynthetic genes in seed maturation and dormancy of tree peony (*Paeonia ostii* 'Feng Dan'). Sci Hort 182:92-101. https://doi.org/10.1016/j.scienta.2014.11.021

Figures

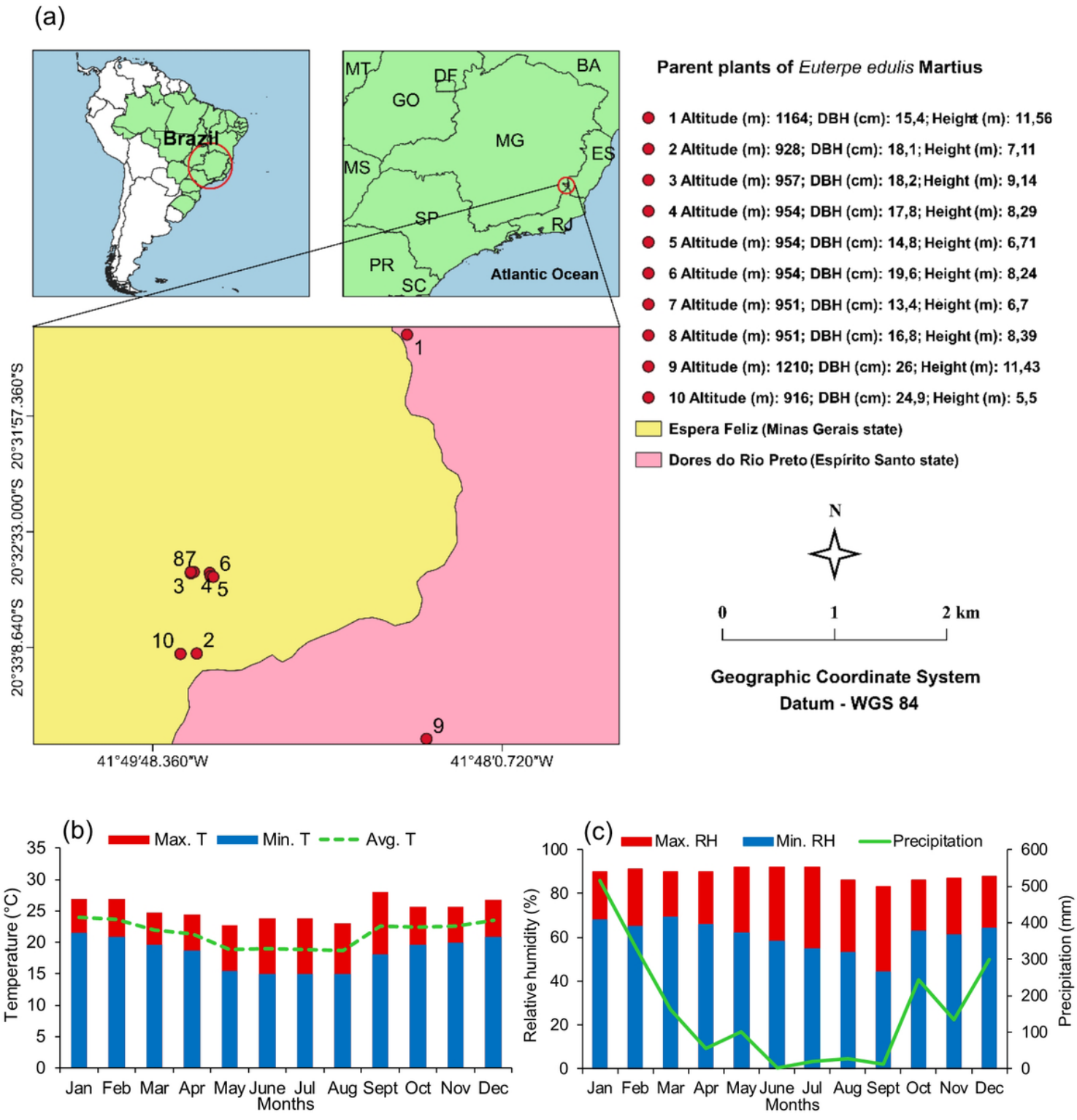

Figure 1

Physical characteristics of parent plants and their habitats. (a) Coordinates and characteristics of parent plants. (b) Maximum (Max.T), average (Avg.T), and minimum (Min.T) temperature, as well as (c) maximum relative humidity (Max.RH), minimum relative humidity (Min.RH), and precipitation from the beginning of flowering to the end of fruit harvest in the region where the *E. edulis* matrices were located. Data are relative to 2020 and were provided by the National Institute of Meteorology of Brazil. DBH, diameter at breast height (1.30 m).

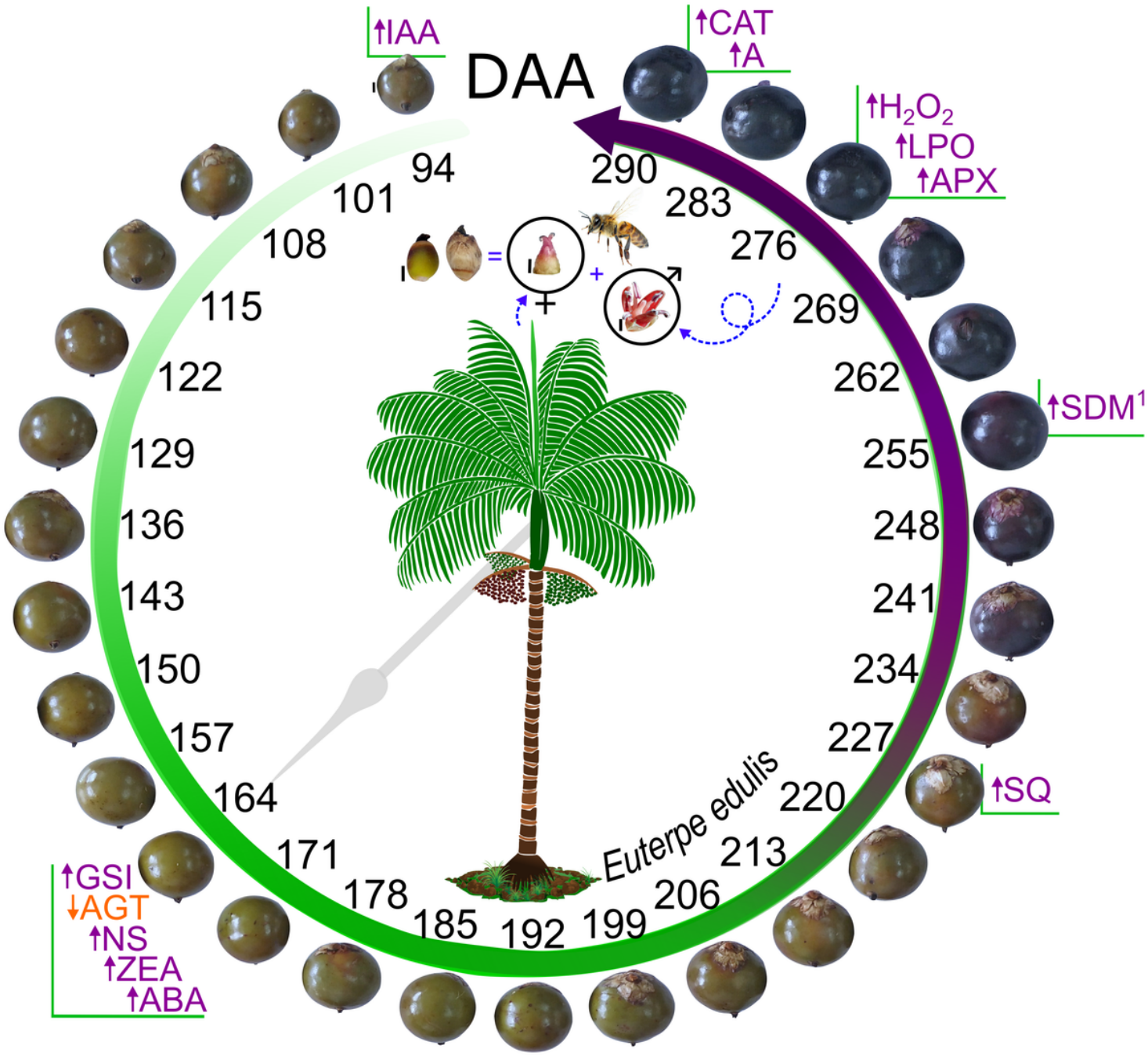

Figure 2

External morphological changes in *E. edulis* fruits during maturation (DAA, days after anthesis), highlighting some fruit/seed stages that mark important physiological changes: ↑ maximum; ↓ minimum;

IAA, indolacetic acid; GSI, germination seed index; AGT, average germination time; NS, normal seedlings; ZEA, zeatin; ABA, abscisic acid; SQ, seedling quality; SDM[1], seed dry mass; LPO, lipid peroxidation; APX, ascorbate peroxidase; CAT, catalase; and A, anthocyanin. Bar: 2 mm.

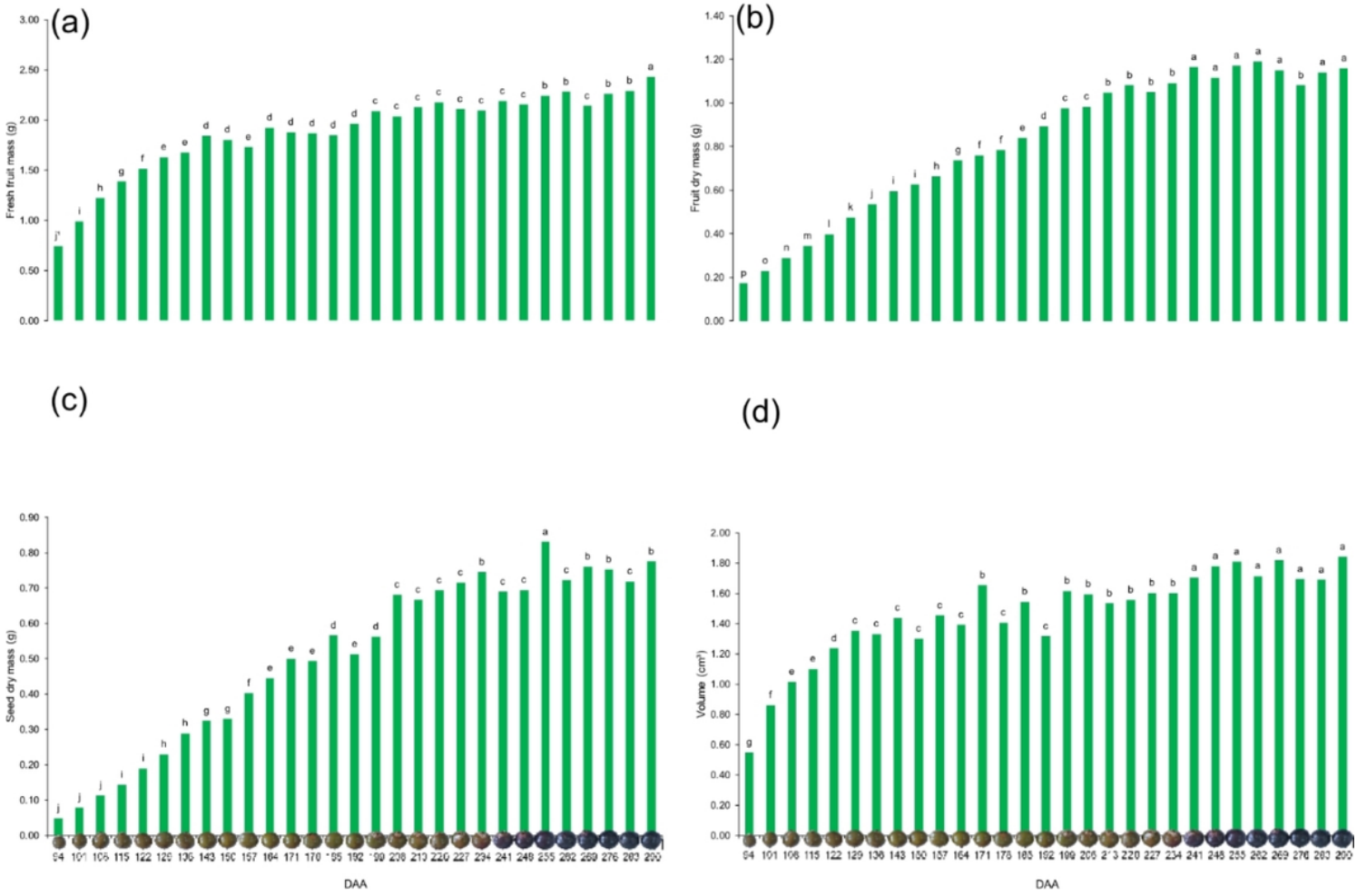

Figure 3

E. edulis fruit and seed parameters during maturation. (a) Fresh fruit mass. (b) Fruit dry mass. (c) Seed dry mass. (d) Fruit volume. DAA, days after anthesis. [1]Means not followed by the same letter differ from each other based on the Scott-Knott mean grouping test ($p < 0.05$). Fruit scale bar: 1 cm.

280

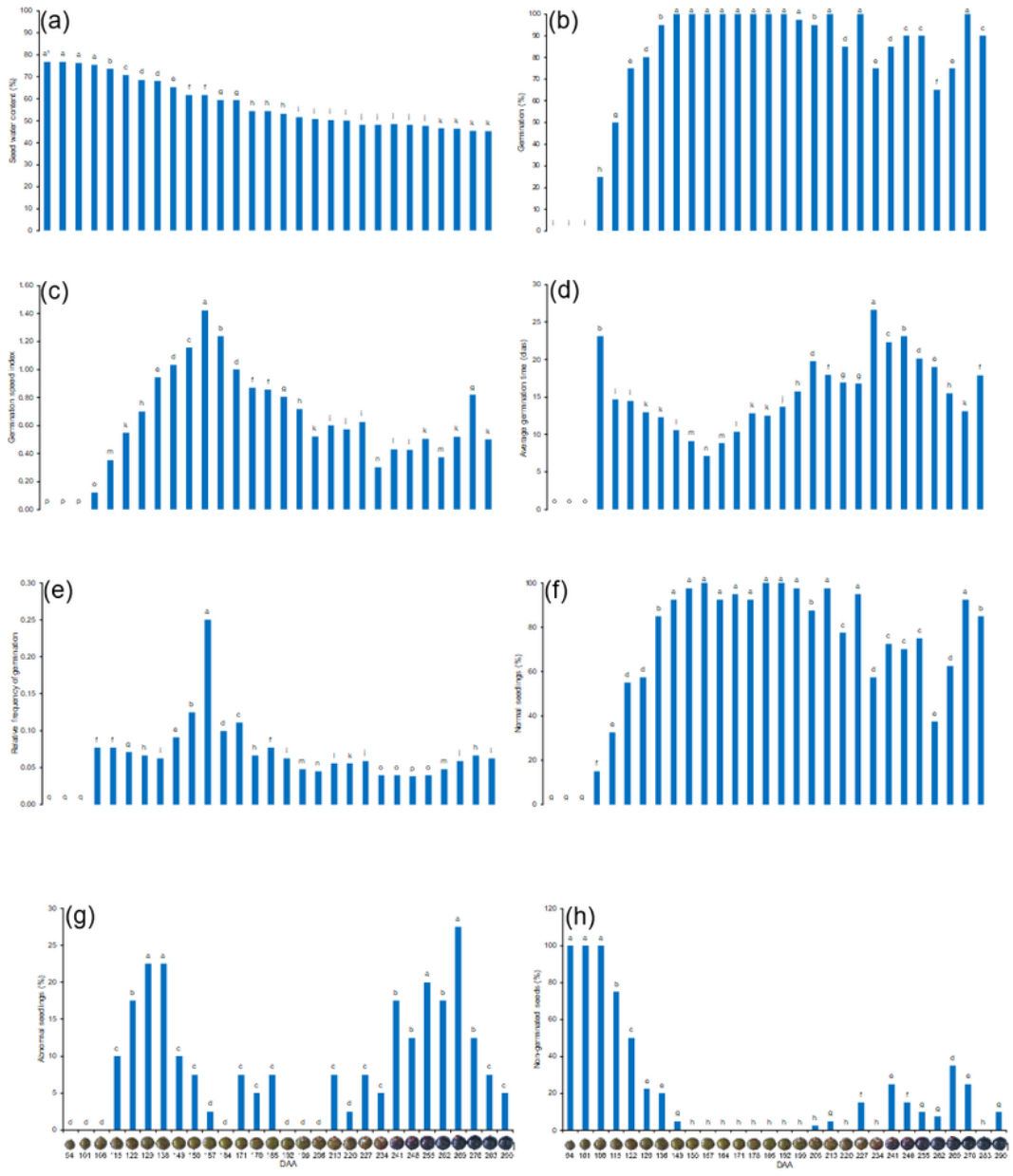

Figure 4

E. edulis seed properties during maturation. (a) Seed water content. (b) Seed germination. (c) Germination speed index. (d) Average germination time. (e) Relative frequency of germination. (f) Percentage of normal seedlings. (g) Percentage of abnormal seedlings. (h) Percentage of non-germinated seeds. DAA, days after anthesis. [1]Means not followed by the same letter differ from each other based on the Scott-Knott mean grouping test ($p < 0.05$). Fruit scale bar: 1 cm.

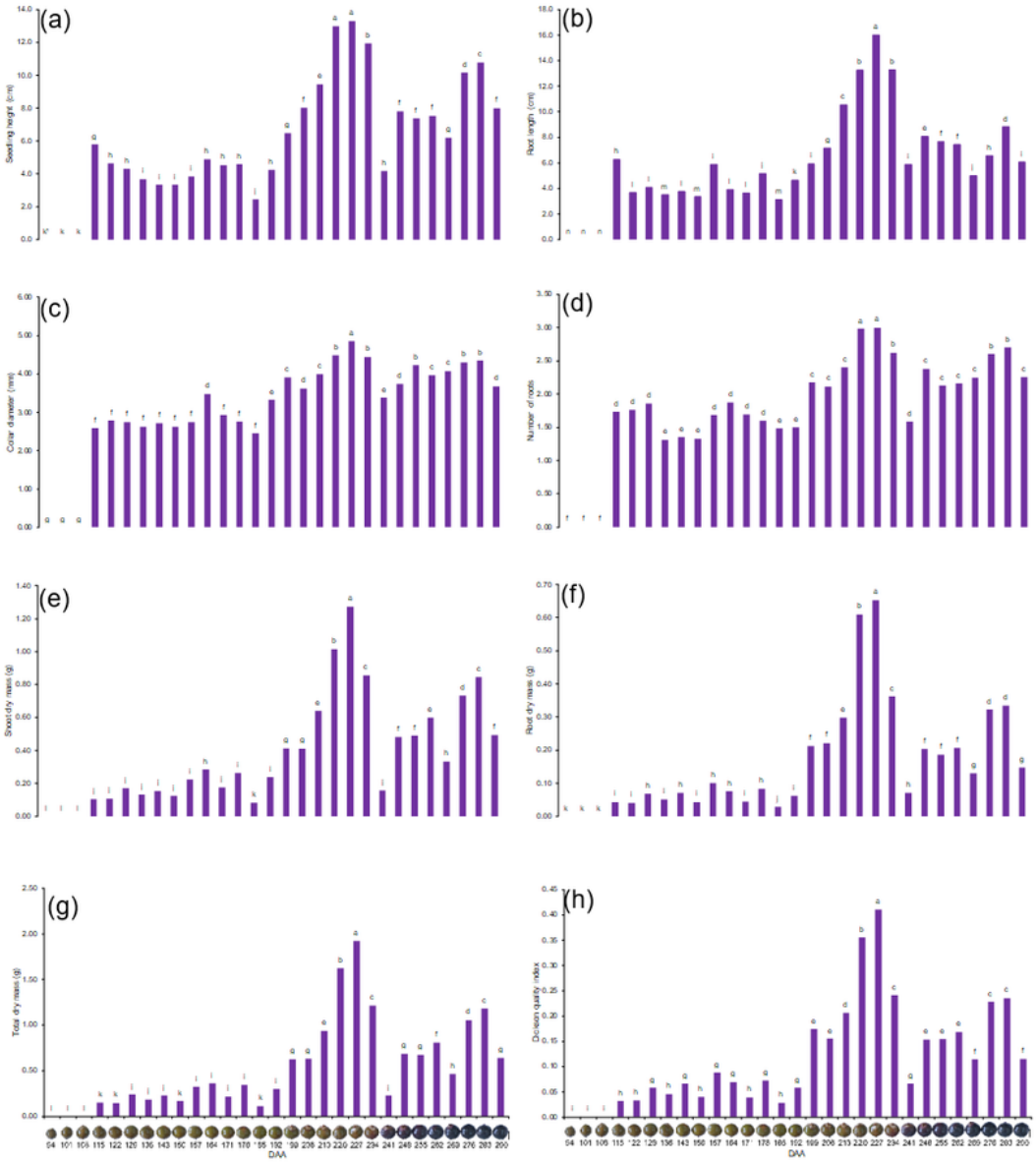

Figure 5

E. edulis seedling properties based on seed germination. (a) Seedling height. (b) Root length. (c) Collar diameter. (d) Number of roots. (e) Shoot dry mass. (f) Root dry mass. (g) Total dry mass. (h) Dickson quality index. DAA, days after anthesis. [1]Means not followed by the same letter differ from each other based on the Scott-Knott mean grouping test ($p < 0.05$). Fruit scale bar: 1 cm.

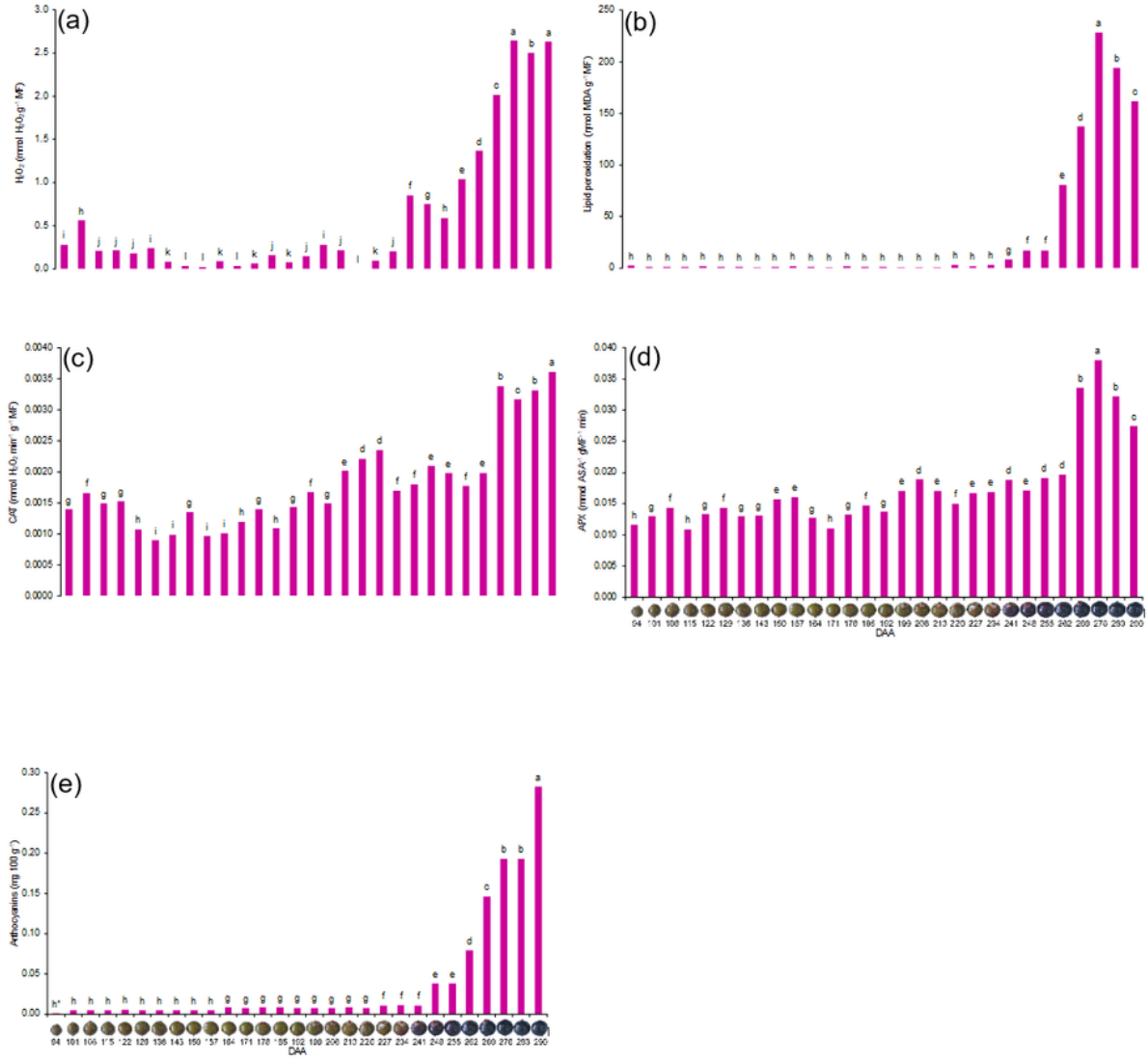

Figure 6

Levels of antioxidant defenses in *E. edulis* seeds. (a) H_2O_2. (b) Lipid peroxidation. (c) CAT. (d) APX. (e) Anthocyanins. DAA, days after anthesis. [1]Means not followed by the same letter differ from each other based on the Scott-Knott mean grouping test ($p < 0.05$). Fruit scale bar: 1 cm.

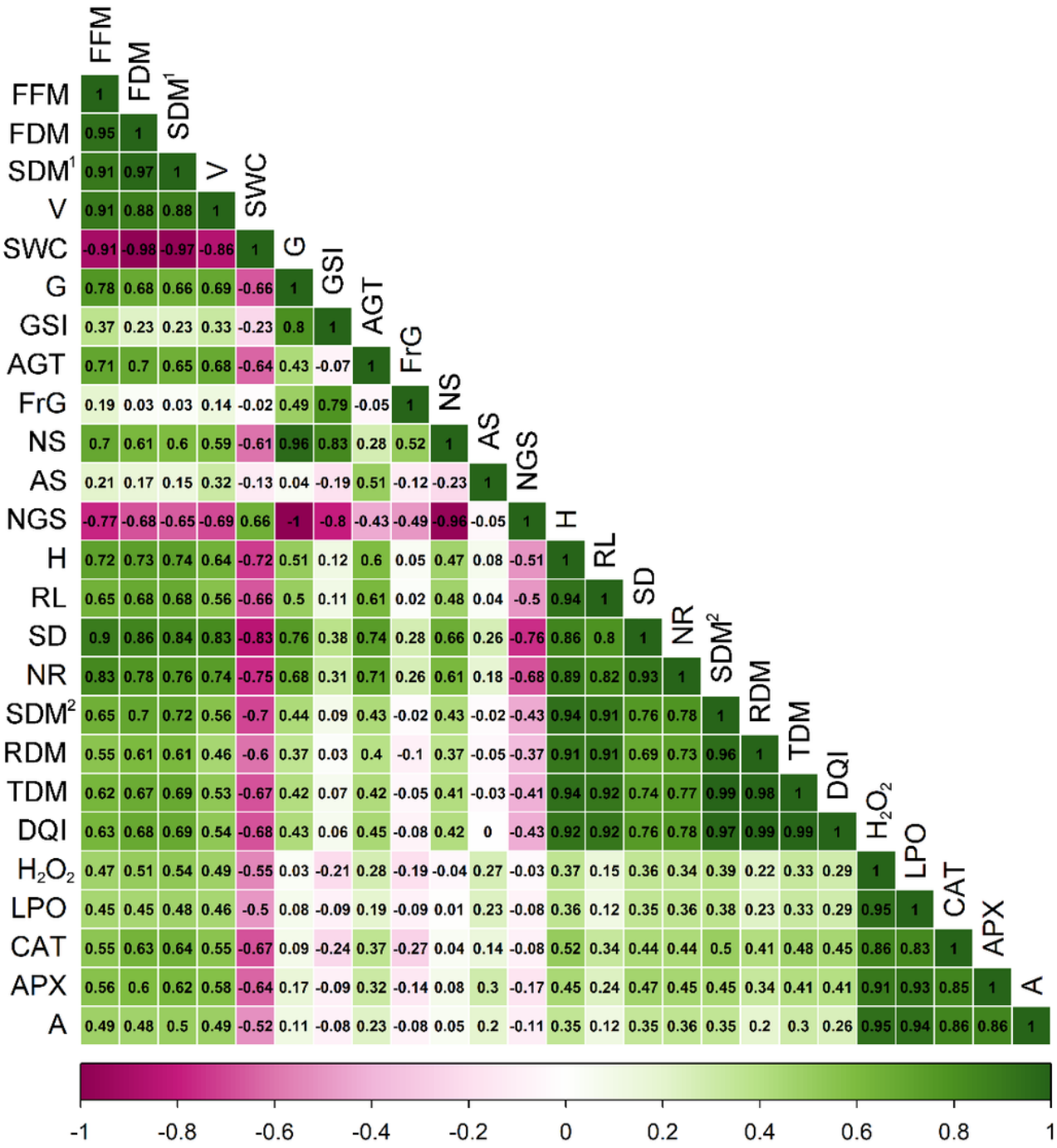

Figure 7

Pearson's correlation between the following variables: fruit fresh mass (FFM), fruit dry mass (FDM), seed dry mass (SDM^1), seed volume (V), seed water content (SWC), germination (G), germination speed index (GSI), average germination time (AGT), relative frequency of germination (FrG), normal seedlings (NS), abnormal seedlings (AS), non-germinated seeds (NGS), seedling height (H), root length (RL), stem diameter (SD), number of roots (NR), shoot dry mass (SDM^2), root dry mass (RDM), total dry mass (TDM), Dickson quality index (DQI), anthocyanin (A), ascorbate peroxidase (APX), catalase (CAT), lipid

peroxidation (LPO), and hydrogen peroxide (H_2O_2) of *E. edulis* seeds during maturation. Significance: $p < 0.05$, for values -0.21 ≤ r ≥ 0.21.

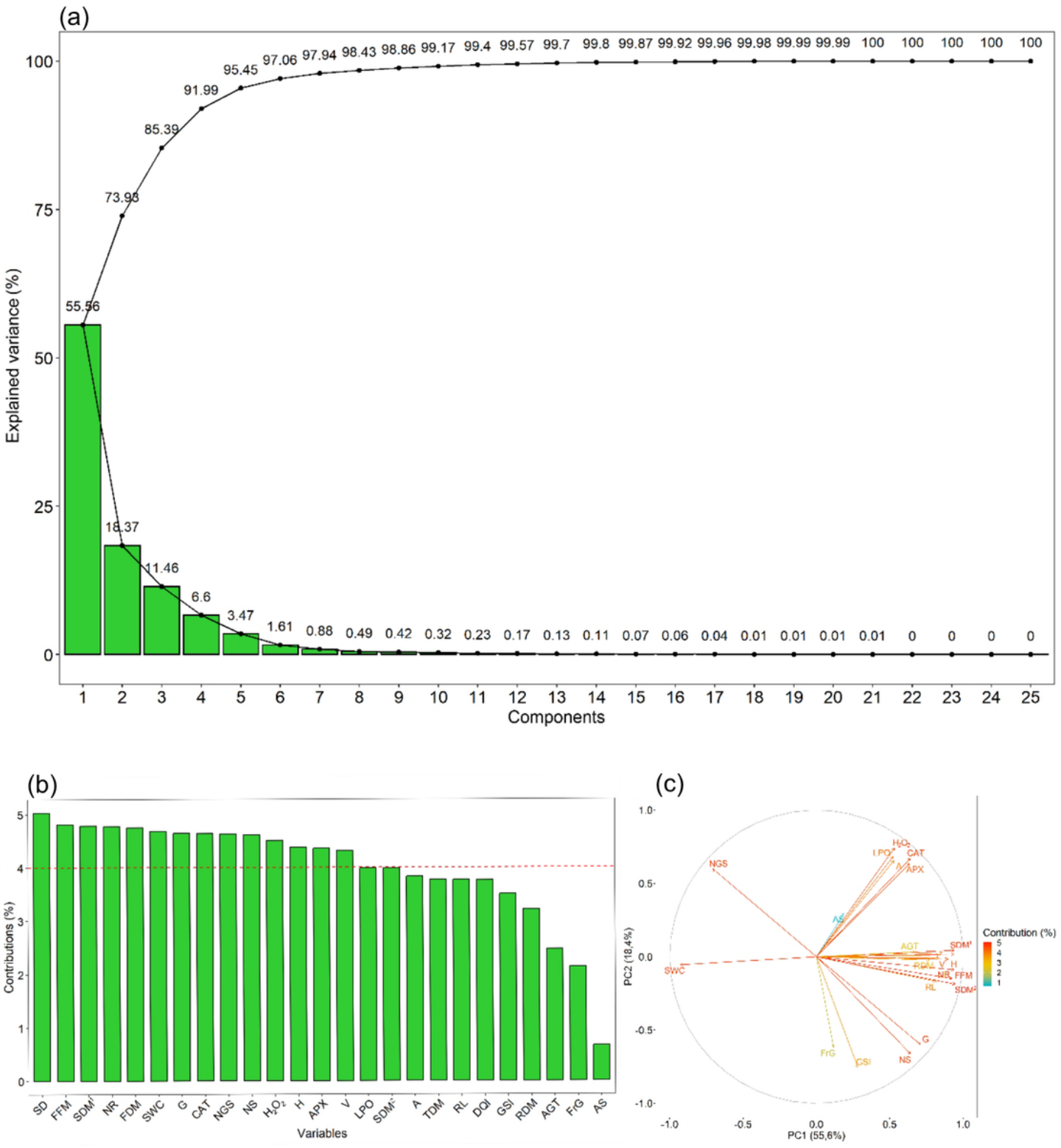

Figure 8

Principal component analysis. (a) Variance (%) explained by each of the principal components. (b) Contribution of the 25 analyzed variables to the first and second principal components (PC1 and PC2);

the dashed line represents the minimum contribution considered relevant (4%) and was obtained by dividing 100% by 25 variables. (c) Biplot graph of the variables for PC1 and PC2. Variables: fruit fresh mass (FFM), fruit dry mass (FDM), seed dry mass (SDM^1), seed volume (V), seed water content (SWC), germination (G), germination speed index (GSI), average germination time (AGT), relative frequency of germination (FrG), normal seedlings (NS), abnormal seedlings (AS), non-germinated seeds (NGS), seedling height (H), root length (RL) , stem diameter (SD), number of roots (NR), shoot dry mass (SDM^2), root dry mass (RDM), total dry mass (TDM), Dickson quality index (DQI), hydrogen peroxide (H_2O_2), lipid peroxidation (LPO), catalase (CAT), ascorbate peroxidase (APX), and anthocyanin (A).

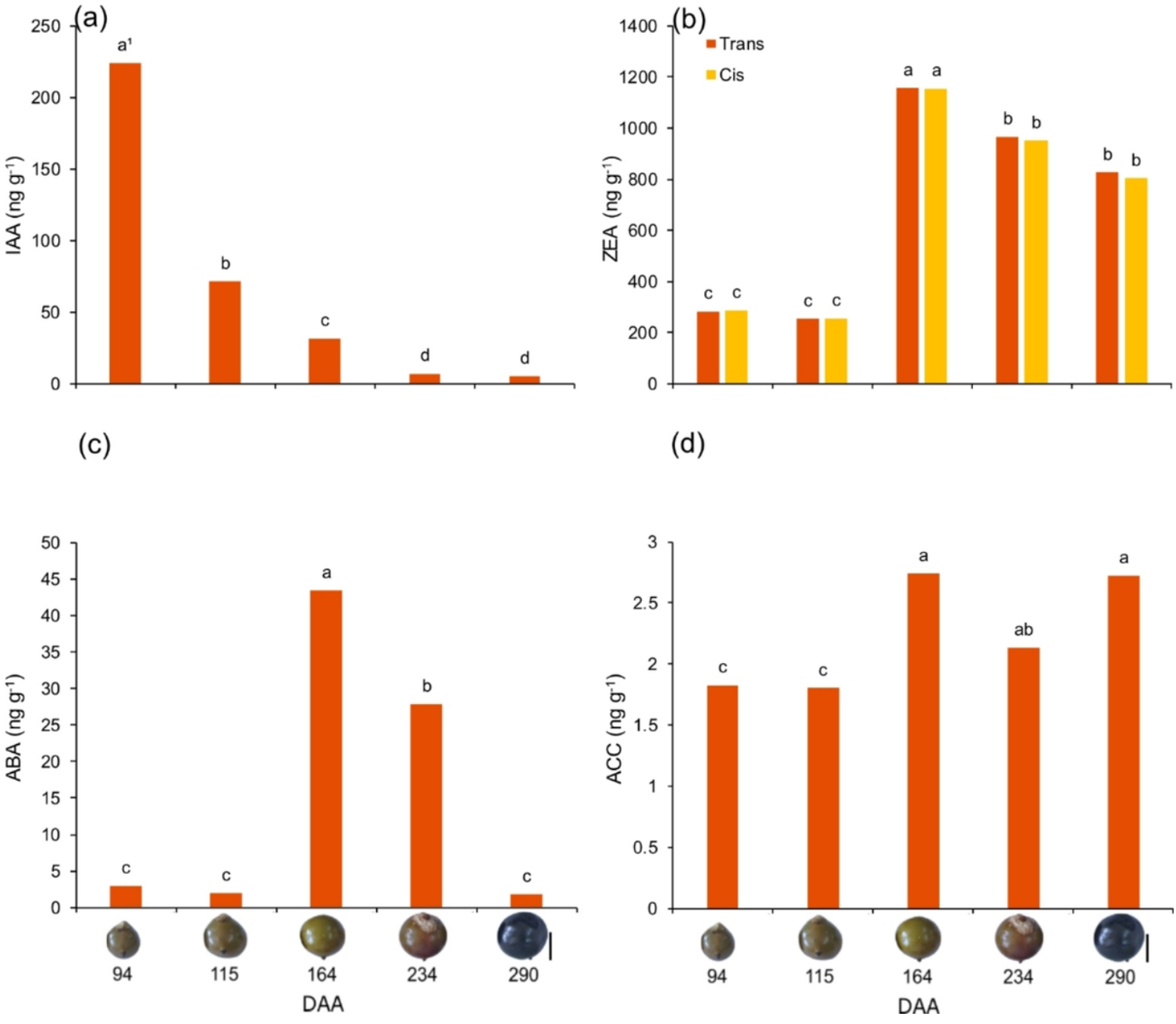

Figure 9

Homomones present at different maturation stages of *E. edulis* seeds. Indoleacetic acid - IAA (a), zeatin - ZEA (b), abscisic acid - ABA (c) and 1-aminocyclopropane-1-carboxylic acid - ACC (d). DAA, days after anthesis. [1]Means not followed by the same letter differ from each other based on the Tukey mean test ($p < 0.05$). Fruit scale bar: 1 cm.