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Metabolic Dysfunctions and Dynamic of Antioxidant Enzymes Activity in Developing Recalcitrant Cacao (*Theobroma cacao*) Seeds at Different Storage Conditions

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ABSTRACT

A potential breakthrough in improving the short-term preservation of recalcitrant cacao (*Theobroma cacao*) seeds is the specific conditions with slow-drying procedures. Thus, this study aimed to elucidate the effect of storage conditions on the physio-chemical and reactive oxygen species (ROS) antioxidant enzymes with slow-drying processes in cacao seeds. Seeds from ripened cacao pods (clone PBC 123) were demucilaged, placed in a zip-lock polyethene bag, and stored at 14°C and 16°C (40% and 80% relative humidity [RH]), room temperature (RT; 25°C), and control (0 days of storage). Seeds at 14°C tend to retain the highest respiration rate during 12 days of storage. Their impaired respiratory activity is reflected through the highest accumulation of soluble sugar during the first 6 days after storage, lower protein content with the highest antioxidant enzyme activities, indicating increased ROS production. Antioxidant enzymes involved in the glutathione-ascorbate cycle, ascorbate peroxidase, and glutathione reductase activity were crucially responsive to the oxidative status within seed cells at 14°C. In contrast, seeds demonstrated decreasing moisture content during storage (RT and 16°C, 40% RH). They displayed higher ROS (hydrogen peroxide) signalling but within the oxidative concentration threshold, giving the advantage of holding seeds with lesser exposure to oxidative stress. As the condition of 16°C, 40% RH produced lesser germinated seeds (8% to 12%) than seeds at RT during storage, it is then suggested to be the alternative to minimise seed's physio-chemical changes, contributing to the maximum germination characteristics for 12 days of storage, and thus, increase the potential for further exploration.

Keywords: Carbohydrate reserves, hydrated storage, metabolic changes, osmotic cell homeostasis, oxidative stress

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INTRODUCTION

The interrelation of seed moisture content and storage temperature, listed as one of the major factors, strongly influences recalcitrant seed damage, and it is difficult to separate them (Shibata & Coelho, 2016). Moreover, tropical weather, predominance of high temperatures and relative humidity (RH) influence storage problems and consequently contribute to low-quality products (Lisboa et al., 2017). Several adaptive mechanisms that shield cells from harm during water loss are necessary for the optimal metabolic rate in seed storage and the capacity to withstand dehydration (Corbineau et al., 2024). Therefore, saturated RH hydrated storage will be required to sustain viability in the short to medium term. The loss in viability of cacao (*Theobroma cacao*) seed is abrupt; for instance, a temperature dropped from 17°C to 15°C may kill the seeds. The research on tropical recalcitrant-seeded species is still small, with most of their longevity only being measured in days or weeks.

With all detrimental reactions (chemical, reactive oxygen species [ROS], enzymatic, respiratory, or metabolic) likely to occur, keeping seeds at least 20% to 30% hydrated may cause them to degrade quickly, especially at higher temperatures. However, water is still present, allowing defence mechanisms to function in a fully hydrated molecular state (Juan et al., 2021). More so than water content per se, the consequences for seed quality at the wider range of moist and completely imbibed seeds are dependent on functional repairing mechanisms. Soon after imbibition starts, deoxyribonucleic acid (DNA) repair gets started, and depending on how well the functional repair mechanisms are functioning, damaged genes' transcription and/or function may be affected (Pagano et al., 2017). There may also be negative responses to lowering the temperature (chilling) which, usually physically considered, such as membrane changes, protein/enzyme dysfunction, and more physiological changes that lead to loss of structural integrity and overall quality if allowed to continue over a period (Liang et al., 2020).

In the plant life cycle, seed germination and early seedling development depend mainly on the storage of carbohydrates moving in the form of soluble sugars from seed tissue to various organs such as stem and radical, which are necessary for osmotic cell homeostasis growth and maintenance (Wolny et al., 2018). Stresses related to metabolism at a specific water content may result in a breakdown of metabolic coordination in cells, which might trigger uncontrollable attacks by ROS and reduce the protection that enzymes and non-enzymes provide against oxidative damage (Hasanuzzaman et al., 2020). The detoxifying enzymes and antioxidants that make up the mechanism of cell antioxidants underpin ROS's dual role in plants. These systems might eliminate possibly harmful ROS created under stressful conditions or strictly manage ROS concentrations to govern different signalling pathways (Bailly, 2004). Elevated oxidative stress has been associated with hydrated storage, and varying species' reactions to seed storage have been attributed to the severity of the stress (Chandra et al., 2019). The metabolic pathway to the formation

of ROS and enzyme machinery involved in detoxifying is restarted during seed imbibition and subsequent metabolism reactivation (hydrated storage). Following seed imbibition, the metabolic pathway for ROS generation and enzyme machinery involved in detoxifying is reactivated. In general, superoxide dismutase (SOD), catalases (CAT), ascorbate peroxidases (APX), glutathione peroxidase (GPX), glutathione reductases (GR), dehydroascorbate reductases (DHAR), monodehydroascorbate reductases (MDAR), thioredoxins (TRX), and peroxidases (POXs) are some of the various enzymes and signalling molecules involved in ROS detoxification. All enzymes and signalling molecules are produced and active at different levels during seed germination and maturation (Zandi & Schnug, 2022).

No comprehensive data is available on the dynamics of water content-dependent changes in cacao seed viability and vigour loss under slow-drying regimes, including changing storage temperatures and relative humidity over time. Furthermore, considering ROS's multiple roles in seeds, identifying the antioxidant machinery inside the cell, particularly in terms of detoxifying enzymes, may help clarify the consequences of excessive endogenous ROS generation. Therefore, this study aims to elucidate the changes in physio-chemical and accumulation of ROS antioxidant enzymes in cacao seeds during storage.

MATERIALS AND METHODS

Treatments and Experimental Design

The Malaysian Cocoa Board in Tawau, Sabah, Malaysia, gave the PBC 123 clone's ripened cacao (*Theobroma cacao*) pods. Pusat Penyelidikan dan Pembangunan Koko, Madai, Kunak, Sabah (4.7965000487779506, 117.9670069587197) is where the cacao plants were planted. As quickly as feasible, the collected pods were sent to the Universiti Malaysia Sabah laboratory in Sandakan, Sabah. Once there, seeds were taken out of their pods, sawdust was used to demucilage them, and a soft sponge was used to clean them. One hundred and sixty (160) cacao seeds were placed in each 23 × 15 cm zip-lock polyethylene (PE) bag and kept at the following temperatures: (1) air-conditioned room temperature, RT (25±2°C, 55±5% RH); (2) 16°C, 80% RH; (3) 16°C, 40% RH; (4) 14°C, 80% RH; (5) 14°C, 40% RH. Moreover, cacao seeds with 0 days of storage are the control. A microprocessor-controlled console-style germinator (Seedburo MPG-3000, USA) was used for seed hydration.

A split-plot arrangement was used in a completely randomised design (CRD), with 3 replications (160 seeds/ PE bag per replication) for the experiment assigned during storage (seed hydration process) in the germinator. Daily, all the PE bags were opened for one to two minutes, and the seeds were gently swirled to allow for the aerated state within the bag. For a duration of 12 days, the germination characteristics, physio-chemical and ROS enzyme activity changes of the seed cell were assessed every 48 hours, and all the parameters were also measured for the control seeds at 0 days of storage.

Seed Moisture Content (MC)

Ten seeds per replication were extracted and dried for sixteen hours at 103±2°C (Bonner, 1996). The seed MC was calculated using the following formula:

$$MC\ (\%) = (\text{Fresh weight} - \text{Dry weight}) / (\text{Fresh weight}) \times 100\% \quad [1]$$

Leachate Conductivity (LC)

LC was calculated using Bonner's (1996) procedure with modification. For every replication, five seeds were extracted, weighed, and immersed in 25 mL of ultrapure water. After 24 hours, the leachate was poured off, and its conductivity was measured with an LC meter (Eutech PC2700).

Respiration Rate (carbon dioxide; CO_2 evolved)

The respiratory rate was measured using a modified method of Raudiene et al. (2017). Twenty seeds were weighed and positioned in a sealed bottle (250 mL), with the CO_2 gas sensor and infrared detector attached to the probe attachment on top (CO_2-BTA Vernier, USA). Respiration rates were calculated as mL of CO_2 generated per gram of cacao seeds per hour.

Germination Percentage and Germination Rate Index (GRI)

Germination took place in trays at a laboratory temperature (25±2°C). Fifty seeds were placed between moist cloth and monitored for 14 days. Seeds were deemed germinated when a radicle protrusion of 2 mm was observed. GRI represents the speed of germination and is computed as follows:

$$GRI\ (\%\ \text{per day}) = G1/1 + G2/2 + \cdots + Gx/x \quad [2]$$

Where G1 is germination percentage × 100 on the first day after sowing.

Sucrose and Raffinose-family Oligosaccharides (RFOs)

Sucrose and raffinose-family oligosaccharides (RFOs) were measured using a diagnostic kit of Product K-RAFGL (Megazyme Int. Ireland Ltd., Bray, Ireland).

Antioxidant Capacity (DPPH Scavenging)

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) inhibition in cacao seeds was determined by Gangwar et al. (2014). In a centrifuge tube, 200 mg of sample was used. In addition to the samples, 200 µL of distilled water was used as a control. Following that, 1 mL of DPPH (8 mg/100 mL ethanol, 80%) solution was applied to the sample and blank. This configuration

was kept at room temperature for half an hour. The tubes were then centrifuged for 10 minutes at 4000 rpm. After that, 0.5 mL of the supernatant was added to new tubes holding 1 mL of ethanol (80% v/v), and the absorbance at 517 nm was measured using a spectrophotometer (DeNovix DS-11, USA) and compared to the ethanol.

Hydrogen Peroxide (H_2O_2)

The quantity of H_2O_2 was measured in three repetitions using the approach of Velikova et al. (2000). Seed tissues (200 mg) were homogenised in 2 mL of trichloroacetic acid 0.1% (w/v) (TCA) and centrifuged (12,000 × g) for 15 minutes at 25°C. To measure absorbance at 390 nm, one millilitre of supernatant was combined with 1 mL of sodium phosphate buffer (10 mM, pH 7) and 2 mL of potassium iodide (1M) in a spectrophotometer (DeNovix DS-11, USA). The H_2O_2 concentration was computed using an extinction value of 0.28 M^{-1} cm^{-1}.

Enzyme Extraction and Determination of Protein Content

Cacao seed tissues (250 mg) were pulverised using a pestle and mortar, then homogenised in 1.0 ml of phosphate buffer (100 mM, pH 7.8) containing polyvinylpolypyrrolidone (2%). The homogenate was centrifuged (16,000 × g) (4°C) for 18 minutes, and the supernatant served as an enzyme extract. The protein content was measured using the Bradford (1976) approach. A standard curve was created using bovine serum albumin (BSA), using the equation $y = 0.573x - 0.0131$ ($R^2 = 0.99$).

Assay of Antioxidant Ascorbate Peroxidase (APX) Enzyme Activity

The test combination included 0.1 mL of enzyme solution, potassium phosphate buffer (50 mM, pH 7.0), ascorbate (0.5 mM), hydrogen peroxide (1 mM), and ethylenediaminetetraacetic acid (EDTA) (0.1 mM) in a total volume of 1 mL. After introducing an enzyme to initiate the reaction, the optical density change was measured at 290 nm (Li & Sun, 1999). An attenuation coefficient of 2.8 mM^{-1} cm^{-1} was used to calculate the APX activity.

Assay of Antioxidant Superoxide Dismutase (SOD) Enzyme Activity

SOD activity was determined utilising a method developed by Dhindhsa et al. (1981). Three millilitres of the reaction mixture contained the following: 1.5 mL of potassium phosphate buffer (100 mM, pH 7.5), 0.1 mL of Na_2CO_3 (1.5 M), 0.2 millilitres of methionine (200 mM), 0.1 millilitres of EDTA (3 mM), 0.1 mL of *p*-nitroblue tetrazolium chloride (NBT) (2.25 mM) and 0.05 millilitres of enzyme samples. An enzyme-free tube served as the control. 0.1 mL of riboflavin (60 µM) was added to the tubes, and they were left under the light of two 15 W fluorescent lamps for 15 minutes to initiate the reaction. The reaction was

prevented by shutting off the light and covering the tubes with black cloth. The absorbance was measured at 560 nm. The unit of measurement for SOD activity was U mg^{-1} protein.

Assay of Antioxidant Glutathione Reductase (GR) Enzyme Activity

GR activity was determined according to Esterbauer & Grill (1978), which involved measuring the rate of nicotinamide adenine dinucleotide phosphate (NADPH) oxidation at 340 nm (extinction coefficient = 6.22 mM^{-1} cm^{-1}). The assay mixture included NADPH (0.5 mM), oxidised glutathione (GSSG) (10 mM), EDTA (10 mM) in phosphate buffer (0.1 M, pH 7.8), and 50–100 µL of enzyme extract.

Statistical Analysis

The data were analysed using analysis of variance (ANOVA) and Pearson's correlation, with the significant means separated by the least significant difference (LSD) test at $p< 0.05$.

RESULTS AND DISCUSSION

Recalcitrant seeds often preserve an active metabolism and organelle activity. After morphogenesis, developing seeds move into a phase known as "maturation," which appears to be more metabolically and genetically active than seed drying. Known also as "reserves accumulation" periods, they entail metabolic reorganisation and the creation of store compounds, such as starch and storage proteins (Angelovici et al., 2010).

Sugar Depletion and Metabolic Dysfunction

The prolonged chilling storage on recalcitrant seeds indicates the continuous internal physiological acclimation and alterations that might cause disturbances in the distribution and function of soluble sugars in seed cells. It could be a factor in the embryo axis having less available carbohydrates (sugar deprivation) and seeds germinating at a slower rate (Morkunas et al., 2012). Similarly, all storage treatments, other than room temperature (RT), started with the lower germination rate index (GRI) (Figure 1 B) at 2 days after storage (DAS), as compared to the control (control = 0 day [storage duration]). Seeds at 14°C were consistent with the lower GRI values until 12 DAS. Moreover, soluble sugars (sucrose content; Figure 1G) for seeds at 14°C also showed a similar pattern: higher during early storage and reduced mostly after 6 DAS. Seed reserve content was stated to be linked to germination percentages and/or rates (speed) (Zhao et al., 2018). In the present study, GRI is negatively correlated with soluble sugar (sucrose; r = -0.43**).

In contrast, the protein content showed a positive correlation with both germination characteristics measured: GRI (r = 0.55**) and final germination percentage (FGP) (r = 0.63**) (Table 1). It might have to do with the depletion of reserves in the seeds meant

to sustain the developing embryo. At the same time, they are being stored (Maldonado et al., 2015), which further causes significant physio-chemical changes to occur to maintain respiration and other metabolic processes. Sugar-starved cells first adjust to the absence of carbohydrates by progressively substituting glucose with protein metabolism (Morkunas et al., 2012). During the present study, the quantification of protein (cytosolic) (Figure 1 F) concentration displayed a dramatic decrease as early as at 2 DAS for all storage treatments.

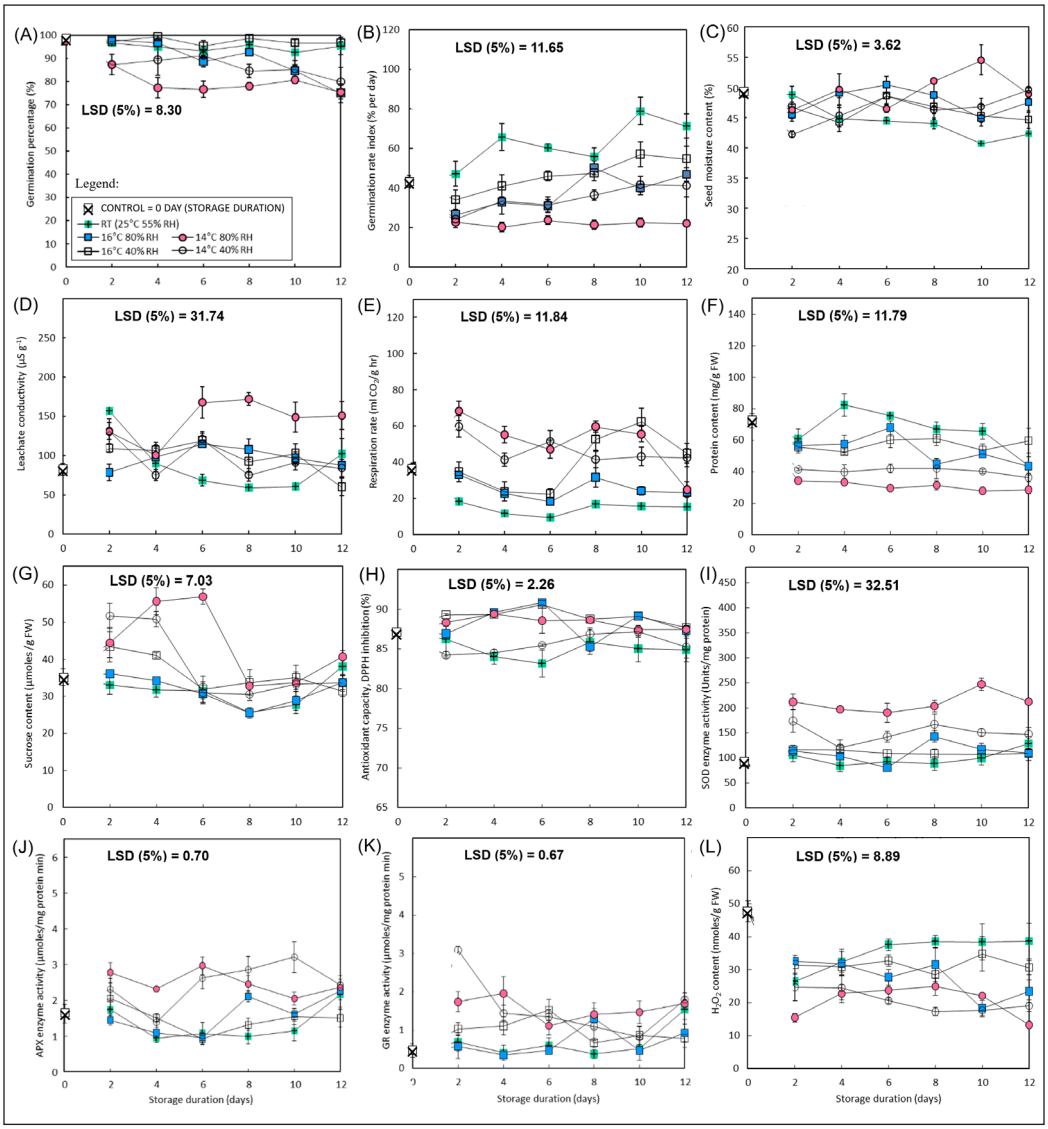

Figure 1. Effect of storage conditions of cacao seeds for 12 days of storage on the (A) final germination percentage (FGP); (B) germination rate index (GRI); (C) moisture content (MC); (D) leachate conductivity (LC); (E) respiration rate; (F) protein content; (G) sucrose content; (H) antioxidant capacity (DPPH scavenging); (I) SOD enzyme activity; (J) APX enzyme activity; (K) GR enzyme activity; (L) H_2O_2 content

Table 1
Pearson's correlation table

Correlations	LC	Respiration Rate	Sucrose Content	Protein content
FGP	-0.38**	-0.31*	-0.25*	0.63**
GRI	-0.50**	-0.54**	-0.43**	0.55**
LC	-			
Respiration rate	0.31*	-		
Sucrose content	0.31*	0.39**	-	
Protein content	-0.52**	-0.50**	-0.42**	-

Note. **, significant at $p \leq 0.001$; *, significant at $p \leq 0.05$; ns, not significant. FGP: final germination percentage, GRI: germination rate index, LC: leachate conductivity

Since those seeds are in the germination mode, de novo protein synthesis is needed. Thus, the insufficient free amino acids in cells might induce signals to start the degradation of a protein storage in the initial hours of imbibition (Rosental et al., 2014). In parallel, the decrease in cytosolic proteins is correlated with the increase of soluble sugars (sucrose content; r = -0.42**) (Table 1), which further supports their substitutional function of carbohydrates.

The other indicator of sugar starvation would be the decline in respiration rate, as shown in previous studies on yellow lupine by Borek et al. (2011). The absence of respiratory substrates is the cause of the lowered respiration rate (Morkunas et al., 2012). Most of the storage conditions showed a decreasing pattern of respiration rate (Figure 1E) at 2 until 6 DAS. It is in parallel with the positive correlation of respiration rate with sucrose content (r = 0.39**) (Table 1). However, many genes are activated in carbohydrate-depleted tissues, resulting in increased activity of relevant enzymes (Borek et al., 2013). It further explains the negative correlation shown by protein content with both respiration rate (r = -0.50**) and sucrose content (r = -0.42**) (Table 1). Seeds at chilling conditions (14°C), which maintained the higher respiration rate (Figure 1E), also displayed the lowest protein content (Figure 1F) and maintained the low values until 12 DAS. According to Da Silva et al. (2018), the increased respiratory activity throughout storage in *Jatropha curcas* seed further led to protein degradation due to the decreased pattern of soluble sugars quantified. Moreover, the increasing pattern and maintaining the highest LC (Figure 1D) (membrane damages) with the consistently lower FGP and GRI (Figure 1 A–B) showed for seeds at 14°C. It supported the significant negative correlations of LC with the germination characteristics: FGP (r = -0.38**) and GRI (r = -0.50**) (Table 1). This parallel adverse situation happened to signify the maintained low protein content (Figure 1 F) from 2 until 12 DAS at 14°C, which might be due to a sudden drop in many of the enzyme's activity, protein deterioration, and modifications to the phospholipids in the membrane that result in the loss of membrane integrity (Maldonado et al., 2015). It explains the negative correlation showed by protein content with the leachate conductivity (LC) (r = -0.52**) (Table 1).

Plants need energy and resources to deal with both biotic and abiotic stress situations; when their metabolic processes slow down due to sugar starvation, they become more susceptible to external factors (Morkunas et al., 2012). The possible reduction of adenosine triphosphate (ATP) due to impaired respiration during storage might disrupt the cells and induce the imbalance of metabolic events, further developing into more progressive deterioration within seed cells. Abiotic stress results in a rise in the radical production, or ROS, which suggests that the stressor is more influential and can cause oxidative damage (Rosental et al., 2014). In anhydrobiosis, oxygen serves as the 'fuel' to produce ROS (Bailly, 2019), but there might be lesser conditions of anhydrobiosis within seed cells during the present study.

As soon as the re-hydrated seed begins to absorb oxygen, ROS are produced and the redox state changes. However, dissolved oxygen concentration lowers when temperature increases, thus bringing about oxygen shortage at high temperatures (Brennan et al., 2016). It explains the consistency of the lower respiration rate (Figure 1E) displayed by seeds at RT. Similarly, for seeds at 16°C, both treatments displayed a lower respiration rate than seeds at 14°C. Plants always produce ROS, which can be detrimental to cells due to various metabolic pathways within individual cellular compartments or as an unavoidable consequence of electron leakage onto oxygen from the electron transport functions of mitochondria, chloroplasts, and plasma membranes (Sharma et al., 2012).

ROS attacks enzymes, damages molecules, and reduces sugar concentrations in cells, which plays a vital part in lowering antioxidant activity (Juan et al., 2021). Similar events might have happened during the present study when seeds at all storage treatments displayed decreasing soluble sugar (sucrose) (Figure 1G) at 8 DAS and kept the low values until 12 DAS. It is widely established that reduced tissue sucrose or glucose levels accelerate lipid, protein, and starch degradation (Borek et al., 2013).

Oxidative Stresses: Dynamic of Antioxidant Enzymes Activity in Developing Cacao Seeds at Different Storage Conditions

The seed moisture content (MC) and metabolic activity change significantly from the beginning of development until the final step of germination. Oxidative stress is an imbalance in forming reactive oxygen species (ROS) and an organism's ability to tolerate antioxidants (Apak et al., 2016). In this study, the increment of DPPH radical scavenging (Figure 1H) within seed cells responded more to the stress conditions during storage. Seeds in most of the storage treatments showed a higher percentage of DPPH scavenging than the control, and they further stagnated at those values during the 12 days of storage. Seeds at 14°C, 40% RH showed the gradually increasing pattern of DPPH radical scavenging activity along 12 days of storage, as well as seeds at RT, which showed a quite stagnant and lower pattern of those scavenging activities. These comparatively low initial DPPH radical

scavenging levels in seeds exposed to high temperatures point to an intentional strategy to enable ROS required for cell membrane loosening and other development processes during the early stages of germination.

The primary mechanisms of delaying ageing are cell membrane repair and enzymatic detoxification. During the present study, in such hypoxic surrounding seed cells, the overly accumulated glycolysis by-products may induce stress conditions and increased ROS production, which is reflected through the increment of antioxidant enzymes. Seeds at both 14°C, 40% RH, and 80% RH displayed quite a similar pattern: to record the higher antioxidant enzyme activity (Figure 1 I-K) than the other storage treatments, along 12 days of storage.

Results displayed the significant positive correlations of all antioxidant enzyme activity quantified with leachate conductivity (LC); APX ($r = 0.35$**), SOD ($r = 0.57$**), and GR ($r = 0.38$**) (Table 2). These indicate the significant cause of increased ROS, probably due to the fast-uncoupling oxidative phosphorylation (impaired respiration). Respiration is the primary source of electron leakage to oxygen, which produces free radicals (Francini et al., 2006). It supported the significant positive correlations of all antioxidant enzyme activity with respiration rate; APX ($r = 0.52$**), SOD ($r = 0.58$**), and GR ($r = 0.44$**) (Table 2). The seed's life span is determined by its capacity to evoke antioxidative enzyme activity to detoxify excess ROS levels (Sahu et al., 2017). Moreover, the consistently significant negative correlations of all antioxidant enzymes (APX, SOD and GR) with the germination characteristics (final germination percentage [FGP] and germination rate index [GRI] (Table 2) indicated that there might be insufficient functional antioxidant enzymes reactions on recovering oxidative stresses within seed cells. Those patterns were significantly displayed for seeds stored at 14°C. Moreover, according to Da Silva et al. (2018), one of the causes and indicators of deterioration is decreased enzyme activity during seed ageing. The stagnant or reducing only slow pattern of most antioxidant enzyme activity quantified along 12 days of storage reflected deterioration development within seed cells.

Typically, hydroxyl radical (OH^-), superoxide (O_2^-), and hydrogen peroxide (H_2O_2) are the ROS found in imbibed seeds (Pehlivan, 2017). The plasma membrane NADPH

Table 2
Pearson's correlation table

Correlations	FGP	GRI	LC	Respiration rate	H_2O_2 content
APX enzyme activity	-0.50**	-0.43**	0.35**	0.52**	-0.50**
SOD enzyme activity	-0.61**	-0.56**	0.57**	0.58**	-0.64**
GR enzyme activity	-0.38**	-0.39**	0.38**	0.44**	-0.44**

Note. **, significant at $p \leq 0.001$; *, significant at $p \leq 0.05$; ns, not significant. FGP: final germination percentage, GRI: germination rate index, LC: leachate conductivity, APX: ascorbate peroxidase, SOD: superoxide dismutase, GR: glutathione reductase, H_2O_2: hydrogen peroxide

oxidases transmit electrons from cytoplasmic NADPH to oxygen, producing O_2^-, which is then dismutated into H_2O_2 (Ishibashi et al., 2010). Hypoxia creates the O_2^- radical, an uncoupled electron molecule that can stabilise its energy through reactions with other molecules (Pehlivan, 2017). Approximately 1-2% of the oxygen absorbed by mitochondria generates O_2^- anion at the electron transport chain (complexes I and II), producing oxygen and H_2O_2 (Bailly, 2019). Those conditions might occur within seed cells in all storage treatments in this study, as they reflected the hypoxic conditions due to the higher seed MC recorded (>40%). SOD enzyme is known to be highly responsive to the changes and increase of O_2^- to normal living cells in catalysing the conversion of O_2^- to the harmless component's oxygen and H_2O_2 (Daniel & Mani, 2016). Previous studies found that higher SOD activity is necessary both at the start and end of the seed maturation process (Sharma et al., 2012).

However, as those recalcitrant seeds did not go through the maturation drying process, thus the SOD activity (Figure 1 I) within seeds displayed consistently higher activities than the control, led by seeds at 14°C, 80% RH, and followed by 14°C, 40% RH. It suggests that its role in ROS defence is particularly crucial when the seeds are immature and exhibit high levels of metabolic and respiratory activity, as well as when they are almost mature and offer protection against the production of ROS during desiccation (Sharma et al., 2012). In the present study, seeds at 14°C, 80% RH showed the highest and stagnant SOD activity along 6 DAS before increasing onwards. On the contrary, seeds at 14°C, 40% RH followed to display the lower but with a stagnant pattern of SOD activity, even though with the gradual increase of MC along 12 days of storage. The mitochondrial respiratory chain produces ROS naturally and continuously. When the re-hydrated seed begins to absorb oxygen, it produces ROS and shifts its redox state (Bouranis et al., 2007).

Higher SOD activity increases the concentration of H_2O_2 in cells, which increases the activity of antioxidant enzymes to eliminate this ROS (De Souza et al., 2018). APX involved in scavenging H_2O_2 are found in plant cells at the source of H_2O_2 production. H_2O_2 is mentioned as a possible inducer of the expression of many genes, including enzymes involved in ROS synthesis or degradation (Pehlivan, 2017). In parallel with the 15% increment of SOD activity (Figure 1I) was the higher increasing pattern of APX activity (Figure 1J) (47% increment), starting at 6 DAS, especially for seeds at 14°C, 40% RH. The synergy of functional SOD and APX activity in seed cells is further evidenced through the highly negative correlations with H_2O_2 content (SOD; r = -0.64**, APX; r = -0.50**) (Table 2). By oxidising lipids, proteins, and nucleic acids, H_2O_2 may readily pass through membranes and cause cell damage (Suresh et al., 2019), which explains more than doubled accumulation of propagated H_2O_2 (Figure 1L) within seed cells in this study. Because of its high affinity for H_2O_2, APX helps cells detoxify by controlling the intracellular H_2O_2 in each cell compartment (Sahu et al., 2017).

The later higher APX activity might also be due to their slower activity, especially during the first 6 DAS. The sugar starvation events might further have induced the stress conditions to activate more antioxidant enzymes to react. Many times, it is said that the antioxidant system becomes active towards the end of the germination or growth phase. Antioxidant enzymes are only activated when ROS levels are above a certain threshold to preserve ROS homeostasis inside the oxidative window for germination (Bailly, 2019). The rehydration process or increasing MC (Figure 1C) might cause the activation and manifestation of those ROS and further added to the newly produced ROS, which contributed to the respiratory burst; that explains the sharp increment of APX activity of seeds at 14°C, 40% RH during 6 DAS, and the levels maintained higher than the other treatments until 12 DAS. Moreover, the low pattern of LC (Figure 1D) further explains that the lower attack of ROS might be due to their lesser damage on membrane cells and lesser leaked molecules, which reduced the potential of converting H_2O_2 to more harmful molecules. Furthermore, each transition increasing or decreasing pattern of MC might contribute to the unregulated generation of ROS and is likely to occur during intermediate hydration levels where metabolic down-regulation becomes uncoordinated. Seeds at 14°C, 40% RH further recorded the consistency to accumulate among the lowest level of H_2O_2 (Figure 1 L), indicating the function of those antioxidant enzymes to reduce ROS.

The above reasons might further explain the decreasing GR activity (Figure 1 K), in parallel with the increased pattern of APX activity (Figure 1 J), for seeds at 14°C, 40% RH. GR activity, which is actively needed to complete the glutathione-ascorbate cycle, might be the pivotal indicator of the significant effects of lower protein content or depletion on sets of enzyme activity that take part in the glutathione-ascorbate cycle, or specifically in reducing oxidised glutathione to glutathione (GSH). As both processes might be needed to counter-back the increasing production of H_2O_2, the gradual reducing GR activity indicated the limited bases or sources (reduced glutathione or NADPH) for those reactions to have occurred. Moreover, the needs of NADPH usually arise from the pentose phosphate pathway; the metabolic pathway parallels glycolysis. Sugar starvation for seeds at 14°C, 40% RH, however, might cause a significant decrease in the activities of all the pentose phosphate pathway enzymes except for glucose 6-phosphate dehydrogenase. As for seeds at 14°C, 40% RH, both conditions might have occurred together to cause the gradual decreasing GR activity in this study.

During the present study, the imbibitional chilling injury seems to dominate for seeds at 14°C, 80% RH, and more than 40% RH, especially at 8 DAS onwards. Compared to seeds at 14°C, 40% RH, seeds at 80% RH showed many significant consequences on ROS attacks during storage. It displayed a gradual decreasing pattern of germination (Figure 1 A-B) and eventually worsened at 8 DAS onwards. Higher MC retention from

the earlier days of storage, with the combination of the chilling temperature, may cause stressful storage conditions or accelerated ageing, leading to ultracellular changes leading to membrane damages, solution leakage, transcription damage, and damage that cause defective or incomplete protein (enzyme) synthesis essential to germination of seed (Suresh et al., 2019). On the other hand, with the increasing pattern of SOD activity (Figure 1I), seeds at 14°C, 80% RH, however, parallelly show the uneven pattern of APX activity (Figure 1J), or increasing only slowly pattern, which indicates that these effects are the result of RNA synthesis damage, which finally leads to reduced protein synthesis and enzyme deactivation. The absence of a repair mechanism might further result in high cellular H_2O_2.

Seeds at 16°C, 80% RH showed quite a similar pattern of damages as at 14°C, 80% RH, except the ones stored in the later conditions displayed faster deterioration due to chilling injury development within seed cells. Both conditions also showed an increasing pattern and maintained to be among the highest MC (Figure 1C), especially during the first 6 DAS, which caused any damaging symptom manifested indirectly through their declining trend of germination performance (Figure 1 A–B) along 12 days of storage. As for seeds at 16°C, 80% RH, dehydration might occur only slowly (at 8 DAS onwards); thus, metabolism is thought to become imbalanced. Surprisingly, high water concentrations can cause significant intracellular damage and seed/embryo mortality (Berjak & Pammenter, 2008). In the present study, all the antioxidant enzyme activity (SOD, APX and GR) (Figure 1 I-K) measured displayed the increasing pattern at 8 DAS. Seeds at 16°C, 80% RH practically displayed the slower development of oxidative stress, with the significant ROS attack observed after the visible reducing MC at 8 DAS and the significant reducing protein content at 10 DAS.

In the present study, seeds at RT and 16°C, 40% RH, are seen to be at their best ranges, preserving the ROS concentration below the detrimental threshold of deterioration. As suggested by Bailly (2019), ROS homeostasis is directly related to a seed's capacity to germinate. When a seed's ROS concentration is within ranges that allow ROS signalling but do not injure the seed, germination is most likely to occur. Germination, on the other hand, is blocked when the level of ROS is either too low or too high. Together with the reducing MC (Figure 1C) for seeds at RT (at 4 DAS onwards) and 16°C, 40% RH (at 8 DAS onwards), however, seeds at both storage conditions displayed the lower and almost maintained a stagnant pattern of all the antioxidant enzymes activity (APX, SOD and GR) (Figure 1I–K) measured in this study. Their differences were only displayed in the higher germinability for seeds at RT than the ones at 16°C, 40% RH. However, it came together with the constraints of about 8% to 30% (RT) and 8% to 12% (16°C, 40% RH) of seeds germinated during storage. During germination, ROS signalling is thought to be a part of a complex signalling network that involves a variety of actions, such as interaction

with cytoplasmic signalling pathways, oxidative modification of gene expression inside the nucleus, and further weakening of the cell wall (Bailly, 2019). Production sources primarily determine ROS homeostasis, and if ROS production does not exceed their concentration threshold, it will not produce oxidative damage, which may further influence their germinability.

During this study, the ranges differences between 19% to 47% of H_2O_2 (Figure 1L) for seeds at RT and 16°C, 40% RH with the control seeds was recorded, with those seeds consistently exhibiting their good germination performances, along 12 days of storage. Moreover, Juan et al. (2021) added that the time needed for the repair of membrane damage and damage to other regions of the cell, as well as the initiation of antioxidant system activity to avoid the build-up of oxidative stress conditions, are likely the main causes of the delay in the germination of aged seeds. On the other hand, seeds at both storage treatments showed the least changes in their antioxidant enzyme activity, except for the elevated GR activity (Figure 1K) since 2 DAS was recorded for seeds at 16°C, 40% RH. The accumulation of GSH build-up might increase GR activity in cells, resulting in stress tolerance. Besides, at 16°C, 40% RH also displayed a considerable increase in respiration rate (Figure 1E) at 8 DAS, which coincidently occurred with the reducing MC pattern (Figure 1C). Imbibed seeds are extremely sensitive to little changes in environmental variables. Thus, environmental information must be correctly conveyed to seeds and translated into endogenous germination signals (Bailly, 2019). Considering the sugar starvation mode to have occurred at a similar timeline, the increased respiration rate but with maintained antioxidant enzyme activity and the evidence of good performance in germination indicated the efficiency of respiratory activity of seed cells at 16°C, 40% RH in response to their surrounding changes.

CONCLUSION

The present study discovered the alternatives to the easily handled short-term storage of locally produced recalcitrant cacao seeds that can be beneficial for improving the seed producer's awareness and understanding of maintaining good seed quality. Seeds at 14°C, 80% RH, and 16°C, 80% RH were found to be potentially threatened by the oxidative damages, which caused their faster deterioration development during 12 days of storage. While seeds at 14°C, 40% RH were found to respond more to starvations and depletions of their reserved food, which reduced their ability to produce sufficient energy to counter-back the strikes of oxidative stress, especially at 8 DAS onwards. In contrast, the consistency of cacao seeds quality with the minimal physio-chemical changes exhibited along 12 days of storage at 16°C, 40% RH, also demonstrated the highest germinability, lesser seeds germinated during storage (8% to 12%) with lesser fungi infestation (as compared to seeds at RT), and thus, contribute to the higher potential for further exploration. However, as this

research was limited to a specific cacao clone, specific target of fruit maturity, specific target of physio-chemical character and within a specific storage time frame, more alternatives and investigations need to be explored to amend their storability potential, and thereby, will be more practical to be used in the future.

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