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PHYSIO-CHEMICAL CHARACTERIZATION OF CHILLING AND SLOW-DRYING RESPONSES OF CACAO (*Theobroma cacao* cv. PBC 123) SEED

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DECLARATION

I hereby declare that the material in this thesis is my own except for quotations, equations, summaries and references, which have been duly acknowledged.

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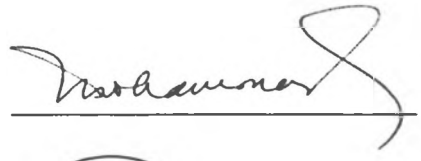
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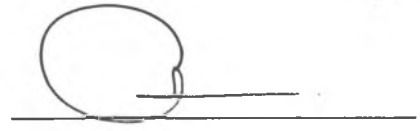
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ABSTRACT

This research explores the alternative of the easy-handled short-term storage for locally produced cacao seeds, as well as in identifying the storage windows which are considered at frequent intervals in the maintenance of high-quality seeds. The first study was conducted to evaluate the physiological quality of seeds stored at different temperatures and relative humidity. The extracted cacao seeds (PBC 123 clone) were packed in zip-lock polyethylene bags, and stored at (i) air-conditioned room temperature, RT (25±2°C, 55±5% RH), (ii) 16°C, 14°C, 10°C, (40%, 60%, 80% RH), and (iii) control (seeds freshly extracted from pods). Seeds were sampled every 24h (hours) over 144h storage period. Seeds at RT and 16°C produced the highest germination characteristics (GC) and seedling growth performances (SGP). Seeds at RT showed no variations of moisture content (MC) (46-50%) than the control. Membrane re-establishment might have occurred due to the decreased 42% of leachate conductivity (LC) at 96h. Even with the increasing (5-36%) LC over time, seeds at 16°C, 40% RH (45-52% MC) displayed a significantly higher germination rate index than seeds at 60% and 80% RH. Seeds at 10°C (44-54% MC) and 14°C (44-52% MC) displayed the poor GC and SGP over time. The exposure to the field environment for seeds at 14°C, suppressed their SGP to be similar as seeds at 10°C. In studying the physio-chemical changes affected seed quality during storage, the second study was conducted (i) to characterize the physio-chemical and microstructural changes within seeds affected by slow-drying and chilling during storage, and (ii) to elucidate the accumulation of reactive oxygen species antioxidant enzymes of seeds during storage. Seeds were packed and stored at (i) RT, (ii) 16°C, 14°C, (40% and 80% RH), and (iii) control. Seeds were sampled every 48h over 12-days storage period. Seeds at RT and 16°C, 40% RH displayed reducing (4-9%) MC at 4 and 10 DAS, respectively. Seeds at 16°C, 40% RH also showed a sharp rise of respiration rate at 8 DAS. In contrast, seeds at RT displayed the lower respiration rate along storage, with their protein content were at the same level as the control. Meanwhile, the lowest protein content displayed by seeds at 14°C, 80% RH (decreased by 53% at 2 DAS). The rapid rise of respiration rate at 2 and 8 DAS (3-4 times higher than the lowest values), and the highest soluble sugars (1-3 times higher than the lowest values) were the early chilling responses displayed by seeds at 14°C, 80% RH. Moreover, their lipid peroxidation product was thehighest (increased by 9-10% at 8 DAS). DPPH scavenging activity was the lowest for seeds at RT, decreased (3-4%) at 4-6 DAS. Seeds of the same treatment demonstrated the highest antioxidant enzymes activity; SOD, APX, POX, GR, and CAT (1-23 times higher than the lowest activity) along 12 days of storage. This is in parallel with their lowest H_2O_2 (13.24-24.92 nmol/g), and vice versa with seeds at RT (26.42-38.63 nmol/g). The SEM images of seed cells stored at the higher RH (14°C and 16°C, 80% RH) visualized the extensive structure of starch granules to fracture the plastid membranes. More damages on the membranes and plasmolysis events were observed in the seed cells of 14°C. Even though with the better quality observed for seeds at RT, incidence of 8-30% germinated seeds in-store, however, reduced their storability. Therefore, the condition of 16°C, 40% RH is suggested as the alternative to minimize physio-chemical and microstructural changes in cacao seeds, contributing among the highest GC and minimal reductions in SGP for 12 days of storage.

Keywords: seed technology, chilling and dehydration sensitivity, physically structure changes, antioxidant machinery system, metabolism-linked damage

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ABSTRAK

PENCIRIAN FISIO-KIMIA TERHADAP RESPON PENDINGINAN DAN PENDEHIDRATAN PERLAHAN BENIH KOKO (Theobroma cacao cv. PBC 123)

Penyelidikan ini meneroka alternatif penyimpanan jangka pendek yang mudah dikendalikan bagi benih koko tempatan, serta mengenal pasti julat bagi penyimpanan dengan kekerapan selang masa dalam penyenggaraan benih berkualiti tinggi. Kajian pertama dijalankan bagi menilai kualiti fisiologi benih yang disimpan pada suhu dan kelembapan relatif berbeza. Benih (klon PBC 123) diekstrak, dibungkus dalam beg polietilena berzip, dan disimpan pada (i) suhu bilik berhawa dingin, RT (25±2°C, 55±5% RH), (ii) 16°C, 14°C, 10°C, (40%, 60%, 80% RH), dan (iii) kawalan (benih segar diekstrak daripada buah). Benih disampel setiap 24 jam, sepanjang 144 jam. Benih pada RT dan 16°C menghasilkan ciri percambahan (GC) dan pertumbuhan anak pokok (SGP) tertinggi. Tiada variasi kandungan lembapan (MC) (46-50%) bagi benih pada RT berbanding kawalan. Pembentukan semula membran sel mungkin berlaku, dengan penurunan 42% kekonduksian larut lesap (LC) selepas 96 jam. Walaupun LC meningkat (5-36%) dengan masa, benih pada 16°C, 40% RH (45-52% MC) menunjukkan indeks kadar percambahan lebih tinggi daripada 60% dan 80% RH. Benih pada 10°C (44-54% MC) dan 14°C (44-52% MC) memaparkan GC dan SGP yang menurun dengan masa. Pendedahan kepada persekitaran ladang, menyebabkan SGP benih pada 14°C menyerupai prestasi buruk benih pada 10°C. Dalam mengkaji pengaruh perubahan fisio-kimia terhadap kualiti benih semasa penyimpanan, kajian kedua (i) mencirikan perubahan fisio-kimia dan mikrostruktur dalam benih dipengaruhi oleh pendehidratan perlahan dan penyejukan, dan (ii) menjelaskan pengumpulan enzim antioksidan ROS dalam benih. Benih dibungkus dan disimpan pada (i) RT, (ii) 16°C, 14°C, (40% dan 80% RH), dan (iii) kawalan. Benih disampel setiap 48 jam, sepanjang 12 hari. Benih pada RT dan 16°C, 40% RH masing-masing menunjukkan pengurangan 4-9% MC pada 4 dan 10 DAS. Kadar respirasi benih pada 16°C, 40% RH meningkat pada 8 DAS. Sebaliknya, kadar respirasi benih pada RT lebih rendah sepanjang penyimpanan, dengan kandungan protein pada tahap sama dengan kawalan. Kandungan protein terendah ditunjukkan benih pada 14°C, 80% RH (menurun 53% pada 2 DAS). Kenaikan kadar respirasi pada 2 dan 8 DAS (3-4 kali lebih tinggi daripada nilai terendah), dan gula terlarut tertinggi (1-3 kali lebih tinggi daripada nilai terendah) adalah respon awal penyejukan pada 14°C, 80% RH. Selain itu, produk peroksidasi lipid mereka yang tertinggi (meningkat 9-10% pada 8 DAS). Aktiviti penghapusan DPPH adalah terendah bagi benih pada RT, menurun 3-4% pada 4-6 DAS. Benih rawatan yang sama menunjukkan aktiviti enzim antioksidan tertinggi; SOD, APX, POX, GR dan CAT (1-23 kali lebih tinggi daripada aktiviti terendah) sepanjang penyimpanan. Ini selari dengan H_2O_2 terendahnya (13.24-24.92 nmol/g), tetapi sebaliknya bagi benih pada RT (26.42-38.63 nmol/g). Imej SEM sel benih pada RH yang lebih tinggi (14°C dan 16°C, 80% RH) menggambarkan pengembangan granul kanji menyebabkan pemecahan membran plastid. Lebih banyak kerosakan pada membran dan plasmolisis kelihatan dalam sel benih pada 14°C. Walaupun kualiti lebih baik dipaparkan benih pada RT; 8-30% percambahan semasa penyimpanan mengurangkan kebolehsimpanan. Oleh itu, keadaan 16°C, 40% RH dicadangkan sebagai alternatif bagi meminimumkan perubahan fisio-kimia dan mikrostruktur benih koko; menyumbang antara GC tertinggi dan pengurangan minimum SGP selepas 12 hari penyimpanan.

Kata kunci: teknologi biji benih, sensitiviti penyejukan dan dehidrasi, perubahan struktur fizikal, sistem antioksidan, kerosakan berkaitan metabolisme

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LIST OF ABBREVIATIONS

UA (clone)	–	Upper Amazon
PBC (clone)	–	Prang Besar Cacao
MT	–	Metric Tonnes
RH	–	Relative Humidity
RT	–	Room Temperature
SAS	–	Statistical Analysis Software
HOS	–	Hours of Storage
DAS	–	Days After Storage
MC	–	Moisture Content
FGP	–	Final Germination Percentage
GRI	–	Germination Rate Index
MGT	–	Mean Germination Time
MASS	–	Seedling Dry Biomass
SVI	–	Seedling Vigour Index
LC	–	Leachate Conductivity
SEM	–	Scanning Electron Microscope
TSS	–	Total Soluble Sugar
GC	–	Glucose Content
SC	–	Sucrose Content
RFO	–	Raffinose-Oligosaccharide Family
ROS	–	Reactive Oxygen Species
MDA	–	Malondialdehyde
DPPH	–	2,2-diphenyl-1-picrylhydrazyl
H_2O_2	–	Hydrogen Peroxide
APX	–	Ascorbate Peroxidase
CAT	–	Catalase
SOD	–	Superoxide Dismutase
GR	–	Glutathione Reductase
POX	–	Guaiacol Peroxidase

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CHAPTER 1

INTRODUCTION

1.1 Background of the Research

Seeds remained to be the most convenient and successful way of storing the genetic diversity of plant species and for producing new plants routinely for agriculture and horticulture. In general, seeds are classified into three classes depending on their drying sensitivity and storability: orthodox, recalcitrant, and intermediates. Recalcitrant seeds are difficult to keep for long periods of time and lose viability quickly. Orthodox seeds are dry seeds with extensive storage durations. Meanwhile, intermediate seeds have a character in between the two, in that their moisture content can be reduce and they can be stored for longer periods of time (generally less than 1 year) (Berjak & Pammenter, 2013).

In Malaysia, cacao (*Theobroma cacao*) is one of the most important raw commodities traded globally. Although cacao bean production has declined in recent years (2011-2018), Malaysia is still recognized as the second-largest processing center in Asia and the sixth-largest grinder in the world (ICCO, 2021). Categorized as recalcitrant, the relevance of producing cacao beans in Malaysia has not attracted much attention from local farmers because of the uncertain quality of their main planting material (seeds). Reliable sources of seed are hard to predict, together with the quantities and qualities are both too variable. Dependence on cacao beans for processing imported from other countries can reduce the potential of cacao bean production in Malaysia. Propagation of cacao trees using seedlings are still done generatively. Consequently, the handling of good quality seeds through the suitable storage methods, is vital to ensure a continuous and post-effective supply of tree

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seeds. However, since cacao regularly produces abundant fruits, little attention is paid to the storing of seeds.

Due to recalcitrant characteristic, cacao seeds are prone to drying. Recalcitrant seeds also show considerable variability among individual species, particularly with 50 to 85% of water loss that they can tolerate (Farrant et al., 1993). Metabolic "switch-off" and intracellular dedifferentiation are two factors that help with drying or desiccation tolerance. Recalcitrant seeds, unlike orthodox seeds, lack the processes that contribute considerably to their drying sensitivity (Berjak & Pammenter, 2013). Consequently, recalcitrant seeds shed at high water content can only be retained for a short time under conditions that prevent dehydration. Water loss has caused irreversible damage, resulting in a loss of seed viability (Pammenter et al., 1998). This way it is best stored at its shedding water content (FAO, 2013). The maintenance of water holding capacity in cells became vital in recalcitrant seeds to ensure the availability of sufficient hydration status for active structure and the functioning of various subcellular organelles, such as the proper conformation of protein molecules (enzymes, particularly), DNA replication, etc. (Obroucheva et al., 2016). The storability in hydrated storage, however, reduced by germination that occurs without the need for additional water, and the proliferation of seed-associated fungi. On the other hand, even at high relative humidity, the onset of germinative processes requiring additional water provision does not allow water loss, causing the decline of viability (Pammenter et al., 1994). Those seeds, especially the tropical species, are often chilling-sensitive and cannot be stored at sub-zero temperatures below 15°C, making their long-term conservation difficult (Tommasi et al., 2006). Therefore, the conservation of many tropical species in a short-term measure, ranging from a few weeks to several months.

Listed as part of the major factors which influence the recalcitrant seed damages, the interrelation of seed moisture content and storage temperature are very strong and that it is difficult to separate them (Shibata & Coelho, 2016). Moreover, the tropical weather predominance by high temperatures and relative humidity (RH) has so much influence on the storage problems which consequently contribute to the low-quality product (Lisboa et al., 2017). The ideal metabolic rate

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in seed storage and the ability to survive dehydration depends on diverse adaptation mechanisms that prevent cells from damage during water loss (Dresch et al., 2014). Thus, in a way to maintain viability in the short to medium term, the hydrated storage under saturated RH conditions will be a necessity. Evaporation, as well as the partial water vapour pressure in the air and the energy status of water in plant tissue, are all influenced by temperature, both in dry and hydrated plant tissue. When the temperature rises, the equilibrium water content for a particular RH (water activity) decreases or the equilibrium water activity for a given tissue water content increases (Dragicevic & Sredojevic, 2011). In a confined container of fixed capacity, percentage of RH drops as temperature increases under controlled desiccation or dehydration circumstances (i.e. constant RH and temperature), although not as drastically as in open space. In a sealed compartment, compression takes place. The partial pressure of water vapour increases 1.5 times when the overall pressure within a closed chamber is increased from one to one and a half atmospheres while the temperature remains constant. The saturation pressure is the same as the temperature. If it is supposed that the situation before the compression was 50% RH and 25°C, the condition after the compression is 75% RH and 25°C (Process Sensing Technology, 2021). At the higher moisture content, there are many metabolic reactions and microorganism development within the seed, which enhances deterioration and thus, reduces the quality of seeds (Salam *et al.*, 2017). Those reactions could be prevented by reducing the storage temperature (Umarani et al., 2015). High seed moisture content with temperatures reduced to subzero may disrupt cells due to the ice crystal formation which is termed as freezing injury. The loss in viability of cacao seed is found to be abrupt, for instance a temperature dropped from 17 to 15°C may kill the seeds (Chin et al., 1989). The amount of research on tropical recalcitrant-seeded species are still small, with most of their longevity can only be measured in days or weeks (FAO, 2013).

Some studies on the impact of viability potential of recalcitrant seeds have been conducted under ambient room temperature to compare with those stored at a lower temperature. For example, Sukesh & Chandrasekar (2013) conducted the storage performance experiment on *Vatica chinensis* at 28°C (ambient), 20°C, and

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12°C in sealed polyethylene bags. They found that temperature of 28°C to be detrimental to seed storage life, at which the seeds decreased 50% of their germination at the only fifth day of storage and lost their viability on the 19th day. Results further demonstrated that seeds stored at 12°C showed a more rapid loss of viability until the 15th day of storage (Umarani et al., 2015). Moreover, temperatures below 10°C have been reported to increase the loss of viability in several species of recalcitrant, such as *Hopea sp.* (4°C), *Trichilia emeria* (6°C), and *Mangifera sp.* (5 to10°C). Most of the studies suggested that both higher ambient temperatures (e.g. ±28°C) and very low temperatures (e.g. 0 to ±10°C) were detrimental to lifespan and very much species-specific. Besides, cacao (*T. cacao*) seeds will be killed if stored in temperatures below 10°C (King & Roberts, 1979).

Recalcitrant-seeded species generally progress from development to germination because of the lack of a drying stage during seed ripening (Pukacka et al., 2011). Hence, those seeds contain high moisture and active metabolism as soon as they are shed from the parent plant. These seeds usually keep the organelles fully functioning and maintain an active metabolism (Wen, 2011). While the seed moisture content exerts the greatest influence on their storage lifespan; temperature and relative humidity are the determinants of the type of deteriorative reactions (Mbofung, 2012). Recalcitrant seeds placed in the relatively high-water content environment would keep the metabolism in the seed active, leading to germination. Early germination involved three phases of an increase in seed fresh weight; Phase I, imbibition of water by the seed; Phase II, active metabolic activity but little water uptake; Phase III, radicle protrusion and additional water uptake (Bewley et al., 2013). However, holding seeds at the higher hydration level may lead to rapid degradation, particularly at higher temperatures, since all harmful reactions are likely to occur (e.g., chemical, metabolic). This process decreased the ability of the seeds to survive, which usually occurred through their physiological changes over time (Sun, 2002). Hence, seed quality implications at the greater wet range and fully imbibed seeds are based more on functional repair processes than water content per se.

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In general, water is the environment for life activity, which is also directly involved in many biochemical reactions, such as hydrolytic reactions. Water also functioned to deliver the substrates for enzymes at their active centers, and thereby, it serves as a link between compartments and cell communication (Obroucheva et al., 2016). The type and intensity of metabolism differ in different species depending on the stage of development and the concentration of water at shedding (Berjak & Pammenter, 2008), as well as there might be a wide range of differences in the post-harvest handling of recalcitrant seeds. Differences in seed germination time after storage under certain conditions have a significant impact on the degree of dehydration they tolerate, which in turn adds to the unpredictable variability (FAO, 2013). Moreover, it should be noted that initial water reduction (e.g. through early rapid-drying) does not have the same negative repercussions as severe dehydration. This is in reference to the long-term storage development of recalcitrant seeds which feasibly involved the initial rapid drying prior to storage at the ultra-low temperatures of liquid nitrogen (-196°C) (Engelmann, 2011). In contrast, the slow-drying mechanism occurred due to the embryonic axis is slowly dehydrated naturally inside the seeds. Researchers made a comparison between these two types of available recalcitrant seed storage after shedding; slow-drying is more harmful than rapid- drying because seeds retain moderate water level for long periods of time and are susceptible to damage (Berjak & Pammenter, 2013). However, the mechanism of slow-drying of persevering seed quality during storage is still poorly understood.

In recalcitrant species, variation of physiological, biochemical, and molecular disorders emerged when temperature differences are away from ideal for cellular homeostasis. The natural dehydration and rehydration (slow-drying process) might be occurred within the living recalcitrant cacao seed cells, in their process approaching equilibrium during storage. Thus, damage to membrane phospholipids can occur through chemical processes under dry conditions. However, the consequences of membrane leakage or loss of organelle compartmentation are only significant in hydrated cells when liquid water is present (Bewley et al., 2013). Many biochemical and cellular processes associated with germination are triggered by seed imbibition, including the reactivation of metabolism, the resumption of cellular

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respiration and mitochondrial biogenesis, the translation and/or degradation of stored mRNAs, DNA repair, the transcription and translation of new mRNAs, and the onset of reserve mobilization (Sershen et al., 2014). The drying rate effect on dehydration tolerance is related to the dehydration process (mechanical, and microstructural aspects) and metabolism changes (consumption of storage reserves and production of reactive oxygen species, ROS) in cells (Chandra et al., 2019). It is believed that the variable tolerance of dehydration between different plant species is due to the physical structure of the inner seed matrix, which appears to involve interactions between sugar and protein complexes with salts, organic acids, and amino acids (Angelovici et al., 2010). Consequently, to ensure proper seedling development, seeds maintain a food reserve composed primarily of proteins, lipids, and carbohydrates. Enzymatic hydrolysis of protein, lipid, and carbohydrate as well as metabolite transport is mainly based on the availability of water (Ali & Elozeiri, 2017). Moreover, soluble sugars, as the main direct substance that is essential during germination (Faria, 2006), is equally important in the development of seed germination during storage. Loss of drying tolerance in recalcitrant seeds prior to seed shedding and simultaneous reduction of soluble sugars can be a unique phenomenon to tropical seeds and is associated with the initiation and mobilization of reserves at a later stage of development. Storage molecules such as soluble sugars (mostly raffinose and sucrose) are degraded and used as an energy source for radicle projection and seedling development (Sett, 2016). Thus, germinating seeds and seedlings appear to be the phase most susceptible to fluctuations in soluble sugars because osmotic cell homeostasis needsto grow and be maintained (Maldonado et al., 2015).

One of the most important metabolic elements required for proper seed germination is respiration, or the intake of oxygen (Wang et al., 2012). When respiratory activity is hindered, seed tissues take up less oxygen, which is known to induce seed degradation in species such as soybean and cotton (Sershen et al., 2014). These processes are followed by the accumulation of ROS (mostly hydrogen peroxide, H_2O_2), as a result of a significant rise in intracellular generation during the early phases. On seed hydration, ROS acts as cellular messengers or harmful chemicals (Ali & Elozairi, 2017). Moreover, ROS are typically regarded as toxic

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chemicals in seed physiology, the accumulation of which causes cell injury and disruptions in seed growth or germination processes (seed aging). The cellular antioxidant machinery, which includes detoxifying enzymes and antioxidant chemicals, is responsible for the dual role of ROS in plants. Such processes can eliminate potentially toxic ROS, which is typically created under stressful situations, or precisely control ROS concentrations to regulate multiple signaling pathways (Bailly, 2004). According to Chandra et al. (2019), the mild dehydration stress associated with hydrated storage has been linked to an increase in oxidative stress, and the degree of this stress has recently been related to inter-species differences in seed storability. Similarly, Sershen et al. (2014) mentioned a study on resistant recalcitrant *Trichilia dregeana* seeds, which revealed ROS played a role in the biochemical trigger for germination when the seeds are somewhat dehydrated. Under normal dynamic equilibrium water content, it maintains the biological production and elimination of ROS or free radicals (Feng et al., 2017), but an imbalance between the production of ROS and the antioxidant capacity of the organism, further induces oxidative stress.

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Seed degradation begins before harvest and continues through harvest, processing, and storage, leading to ultimate death through the complete loss of germination (Shaban, 2013). Physiological and biochemical changes during germination, followed by morphological changes, are closely associated with seedling survival rate and vegetative development, which affect yield and quality (Ali & Elozeiri, 2017). The mechanisms that ensure germination and seedling development established the success of cacao plants production (Finch-Savage & Bassel, 2016). The process of seed degeneration is poorly understood, yet it is an important field of research. Therefore, knowledge about the storability of its seeds is critical and can assist create conservation measures, which is especially crucial for the establishment of new production fields by low-technology farmers.

The decline in quality of recalcitrant seed occurs at a rapid rate often due to their sensitivity to environment, relative humidity and temperature. In addition, the lack of accurate information on the storage potential of cacao seeds and their inconsistent pattern of quality after storage become challenges to the growers in

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ensuring availability of high-quality cacao seeds when required. Until now we do not have any convincing proper short-term storage conditions for cacao seeds, especially for the ones cultivated in Malaysia. Thus, experiments on combination of storage temperature and relative humidity may yield useful information regarding the loss of recalcitrant cacao seed quality during storage.

To date, reports on the locally produced cacao seed reactions in the short term of low-temperature storage are still scarce. With various storage atmospheres causing the changes in seed physiology and lifespan of recalcitrant seeds, however, the extent to which any factor(s) acts as the fundamental or critical cause for seed deterioration and poor seedling vigour is debatable. This study is significant to discover an easy-handled short-term storage, as the alternative for locally produced recalcitrant cacao seeds that can be useful in increasing awareness and understanding of seed producers and farmers in maintaining the quality of cacao seeds through specific storage methods and duration. This study could also help researchers identify critical areas where storage windows are considered at frequent intervals in the production and maintenance of high-quality cacao seeds that many researchers could not explore.

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The overall aim of this dissertation was to characterize effects of different storage conditions and duration, in association with physio-chemical changes, on seed viability, germination characteristics and their growth performance of seedlings in the nursery. This is to explore the range of storage conditions tolerance windows of locally produced recalcitrant cacao seeds. Given the above considerations, the study on recalcitrant cacao seeds was undertaken with the following objectives:

1. To evaluate the physiological quality of cacao seeds stored at different temperatures and relative humidity.
2. To characterize the physio-chemical and microstructural changes within the cacao seeds affected by slow-drying and chilling treatments during storage.
3. To elucidate the accumulation of reactive oxygen species (ROS) antioxidant enzymes of cacao seeds during storage.

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1.2 Boundaries of the Research

This research was limited to a specific cacao clone, specific target of fruit maturity (based on pod colour changes), specific target of physio-chemical character and within a specific storage time frame.

Cacao clone of PBC 123 provided by Malaysian Cocoa Board, planted in Kunak, Tawau, Sabah. Colour changes observed was from red to yellow-brownish (after 6 months of fruiting time). The target physio-chemical characterization chosen in this research primarily due to requirement (to accomplish the research objectives) in comparing the early and later symptoms exhibiting variations of seed quality after certain specific storage time.

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CHAPTER 2

LITERATURE REVIEW

2.1 Economic Importance of Cacao

Cacao (*Theobroma cacao*) is one of the most commercially important perennial tropical crops that is farmed for the beans that are used in chocolate making. Rain-fed system farming is the most common method of cacao production. A limited number of farms utilize mechanization to apply fertilizer, and the amount of fertilizer applied varies (Lahive et al., 2019). Cacao, usually cultivated in plantations or conditioning areas, has climatic variables that are completely different from the weather conditions outside that environment. The latter study also indicates that the structure and dynamics of the cacao agro-ecosystem exert some influence in the microclimate. The seasonality of microclimatic conditions inside a cacao plantation is affected mainly by the air temperature, relative humidity, and rainfall. By using a simulation model, the annual radiation and rainfall during the dry season explained 70% of the variations in annual seed yields obtained for 30 sites along the tropical cacao producing areas of the world (Zuidema et al., 2005). In addition, the changes of carbon distribution and export depended on the severity and duration of the environmental stress, as well as its occurrence in relation to flowering, pod set, and pod maturation. An estimated annual global cacao production is reported to be more than 4 million tons per season (4.7 million tons in the 2018/2019 crop season) (ICCO, 2021). It is well-known as a cash crop of huge economic significance globally, especially for the critical raw material for chocolate manufacturing. The other cacao products are reported to contribute positively to health; for instance, the polyphenols in cacao beans have been reported to exhibit anti-carcinogenic

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(Oleaga et al., 2012), anti-atherogenic and vasodilatory (Gómez-Juaristi et al., 2011) effects and they exert them mainly as antioxidants (Martorell et al., 2011).

T. cacao belongs to the family Sterculiaceae, with three main varieties; Criollo, Forastero, and Trinitario dominating the global cacao trade. Three of the world's largest cacao bean producers include Cote d'Ivoire, Ghana, and Cameroon (ICCO, 2021). Every year, the production of cacao-based products grows by roughly 12%, indicating that there is enormous potential in this crop's growth. Cacao beans are mostly exported to Europe and North America for processing into cocoa and chocolate. Cacao was brought into Malaysia for commercial production in 1950s, and it quickly became the third most important commodity product in Malaysia after oil palm and rubber, and it was identified as a crop for agricultural diversification in the Second Malaysia Plan, 1971–1975 (Arshad et al., 2015). In 2019, Malaysia ranked the second largest cacao processor (grinding industry) in the Asian and Oceania regions; and the sixth in the world (ICCO, 2021).

Plantations in tropical regions around the world cultivate *T. cacao*, which produces beans from the seeds of mature pods (Gu et al., 2013). Cacao beans begin as seeds in *T. cacao* fruit pods, which contain 30 to 40 beans embedded in a mucilaginous pulp. Astringent and unpleasant taste and flavour are the main characteristics of raw cacao. Fermented, dried, and roasted cacao is required to produce a distinctive cocoa flavour and taste. Cacao is a tropical rainforest species grown for its oil-rich seed, which is used to make cocoa and cocoa butter (Djikeng et al., 2018). It is also mildly stimulating due to the presence of theobromine, an alkaloid related to caffeine. The high-fat content of the bean kernels (cacao butter) is used in pharmaceuticals, cosmetics, and soaps. Cacao is widely consumed in the form of chocolates, and consumption is increasing due to the growing popularity of chocolate confectioneries around the world (Gu et al., 2013), as one of the world's most valuable food products. Bitter chocolate in liquid form was discovered over 3000 years ago in South America. This valuable product is made from cacao liquor and fat (cacao butter), with sugar, milk, and other additives. As a result of its pro-health properties, cacao has recently become the focus of increased scientific research (Urbanska & Kowalska, 2019).

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Raw cacao beans are one of the most nutritious foods globally, and it has been shown to protect the body from free radicals, relieve stress and depression, and protect against heart disease and many types of cancer (Djikeng et al., 2018). Fresh cacao beans contain approximately 32 - 39% water, 30 - 32% fat, 10 - 15% protein, 5 - 6% polyphenols, 4 - 6% pentosans, 2 - 3% cellulose, 2 - 3% sucrose, 1 - 2% theobromine, 1% acids, and less than 1% caffeine. It is also a good source of minerals (Urbanska & Kowalska, 2019). The presence of polyphenols is thought to be responsible for cacao beans' health-promoting properties. Polyphenols in cacao beans account for around 12 - 18% of the dry weight of the whole bean. Simple phenols, benzoquinones, phenolic acids, flavonoids, and other polyphenolic compounds present in cacao beans are the most common. Furthermore, cacao beans are a good source of nutrients (lipids, proteins, carbohydrates, minerals, and vitamins) (Djikeng et al., 2018). Although it takes 20 or more pods to extract 1 kg of processed commercial cocoa powder, the beans and pulp from a single pod are enough to meet half of a small person's daily needs (450 - 700 calories). In terms of cocoa powder, 100 g can give 10 - 20% of a young adult's energy needs and contribute significantly to a person's vitamin and mineral needs. Although cacao is not an ideal vitamin source as a single food, it is acceptable on a per unit energy basis (e.g. expressed as a quantity per calories). However, as a single dietary source, cacao is a good supplier of important minerals. For example, 50 - 100 g of processed cocoa powder can readily meet most of the necessary so-called trace minerals or elements required by humans daily. Cacao contains enough protein on a caloric or calorie basis, but the quality of the protein is questionable because the concentrations of two key amino acids, methionine, and cysteine, are too low to meet human requirements independently (Rucker, 2009).

2.2 Malaysia's Cacao Industry

Cacao has been a leading sub-sector in the economic growth and development of the producing countries, increasing world production and consumption. Malaysia has about 60 year-long experience with cacao. Since the early 1980s, the locations of cacao bean production have been dominated by (1) Sabah and, to a lesser extent, Sarawak, and (2) Peninsular Malaysia. This was a reversal of the pre-1980s status

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when Peninsular Malaysia dominated plantings. Following these structural changes over several decades, Malaysia has transitioned from a large cacao bean producer to a big cacao butter producer. The unprecedented high cacao bean prices in the 1970s and 1980s are due to the high plantation rate. However, after the 1980s, cacao planting area decreased along with cacao production due to low global cacao prices, labor shortages, competition for land use from oil palm farming, and the severe expansion and infestation of the Cocoa Pod Borer (Lee, 2013). The local supply provided cheap raw material to the grinders in the early stages of their development. The decline in Malaysia's cacao production contributing to their decreasing grinding capacity, which has caused the country to import cacao beans from Ghana, the Ivory Coast, and many other countries. Hence, beginning in the 1990s, the grinders had to rely on international markets for additional supplies of raw materials. Thereby, the trend of declining in cacao bean production, further is accompanied by a rise in grinding production. A good foresight allowed the country to develop the grindings sector into the fifth largest global player in 2013 (Lee, 2013; Arshad et al., 2015). From 1980 to 2008, the total amount of cacao grindings has grown by more than 50 times, from 6,000 tonnes to 323,653 tonnes. Its production peaked in 2008, but after that, it began to decline due to unstable supply. As of 2014, the total amount of grindings had dropped to 244,423 tonnes, a 24% decrease from the 2008 level. On the international market, instabilities in supply and high prices for cacao beans, and to some extent, insufficient local supplies, are to blame. Local bean production is declining, but grinding companies continue to thrive by sourcing supplies from overseas (Arshad & Ibragimov, 2015). The patterns continued to fluctuate until the quite major increment (about 27% of reductions from a year before) in year 2019 with 343,727 tonnes of grindings (MCB, 2021).

Cacao area planted in Malaysia increased from 123,855 ha in 1980, to 414,236 ha in 1989. Further, the decline of the cacao production area is very drastic from the 1990s till 2005 due to severe pest infestation. Continued from 2007 on-wards, with the labor constraint and the prevailing high palm oil prices, which encourage oil palm cultivation, the decline in plantation cacao planting area has been arrested by 2010. The increase in area cultivation was attained to 20,847 ha in 2011. However, the cacao cultivated area had been reduced to 13,826 ha by 2013. From 2005 (33,994 ha) to 2013 (13,826 ha), cacao areas fluctuated with a small

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decline, and during this time, strong government support was provided for cacao planting, particularly in rural and outlying areas, to improve livelihood and income while reducing poverty (MPIC, 2011). Furthermore, the rise in area cultivation targeted by the National Commodity Policy (2011-2020) to attain 40,000 ha and more than 60,000 tons by 2020 is expected to be more than welcome by the downstream industry players (Lee, 2013).These targets, however, are unlikely to be met because the cacao cultivated area declined from almost 20,000 hectares in 2010 to 13,826 hectares in 2013, following the policy's implementation, before rebounding to 17,368 hectares in 2016.

Until the year 2015, Sabah is the major producer, accounting for around 59.7%, followed by Peninsular Malaysia (33.4%) and Sarawak (6.9%) (MCB, 2015). Cacao is farmed on an estimated 15 thousand hectares by smallholding plantations, which account for 95% of total cacao production. However, as the price of cacao fell, many plantations switched to oil palm cultivation. Cacao bean output follows a similar trend to the planted area. Smallholder cacao growers dropped as well, albeit at a slower rate than estate cacao producers (MCB, 2014). In just a decade, cacao plantations shrunk by nearly half, from roughly 30 thousand hectares to 15 thousand hectares. Unfortunately, at the same period, the state's plantation decreased substantially from approximately 11,000 hectares to fewer than 900 hectares (2004-2014) (MCB, 2015). Furthermore, a drop in the global price of cocoa from RM14,323 per MT in 2015 to RM10,770 per MT in 2016 had a substantial negative impact on cacao plantation expansion, forcing many smallholders to destroy or leave their cacao plantations in favour of other commodities such as pepper and oil palm (Chizari et al., 2017). Thus, the unstable ups and downs of cacao industry in Malaysia continued after 2016, and further reduced to only 5,868 ha by 2019 (about 62% of reductions from a year before), with the total production of 1,017 MT (MCB, 2021). Cacao bean production in 2019 totaled 1,017 MT, far short of the 60,000 MT projection for 2020. On a positive note, the 2020 objective of RM6 billion for cacao based product export revenue is exceeded, as shipments in 2020 hit RM6.2 billion (MCB, 2021). Other than diversion interest to the oil palm plantation, based on the references of Brazilian experiences, the loss in the cacao area was ascribed to untreatable insect and disease problems, as well as the draw of higher returns from non-cacao projects. In Malaysia, a similar occurrence has been reported.

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The cacao plant is an agro-forestry crop, which makes it distinctive. A shade tree is required for healthy growth. This means it could be planted under a shade tree or in the forest, particularly in thinned-out woods. A significant amount of forest was destroyed in the early stages of plantation to make space for cacao plantations. Many farmers are said to have tried to reduce shade to increase productivity. In the short run, this technique has proven to be correct. However, in the long run, it harms ecosystems by lowering biodiversity and destroying the natural home of the area's insects. The disruption of the ecology, as well as inadequate farming practices, has increased insect attacks, as well as a variety of pests and diseases (Karuppuchamy & Venugopal, 2016). According to Lee (2013), the Cocoa Pod Borer (CPD) infestation was widespread and extremely detrimental to cacao bean output. This occurrence occurred as a result of a failure to recognize the disease early and poor disease control. A good agricultural monitoring and extension system would have detected early indications and taken preventative steps. Low productivity (approximately 0.5 tonnes/ha) exacerbated the infestation, despite claims that the maximum yield feasible at the MCB's experimental lab is 6 tonnes per ha. By providing good extension services, this gap can be narrowed (Arshad & Ibragimov, 2015).

With 22% of the market share in the Asia-Oceania area, Malaysia is the second-largest cacao bean processor (grinding sectors) in Asia and sixth in the global. In 2019, the value of chocolate exports was RM 1.3 billion, up 7.5% from 2018 (MPIC, 2021). The nation's food security is likely to be threatened by rising urbanization and demand increase. When the market demand exceeds industrial supplies, food prices go up. This is mostly due to the increase in input prices (e.g. fertilizers, chemicals, land, labor, and machinery). It puts a strain on the country's food production practices, which are currently inefficient. To keep up with the rising standard of life and income, household demand for all types of food is also expanding. As a result of inefficient local production methods, which are caused by outdated pre- and post- harvest technology, local production has become unstable. Due to this, imports haveincreased to meet domestic demand, allowing importers or foreign exporters to havea greater say in how much food is sold. The government is attempting to address this issue by providing local producers with a variety of awards to help them upgrade their inefficient production processes (Food &

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Beverage Industry Report Malaysia, 2020). Due to non-eco-friendly cacao bean cultivation, current cacao production processes are not sustainable, although cacao is needed in the chocolate industry. In addition to boom and bust, sustainable development attempts to achieve social, economic, and ecological success in cacao production. On the other hand, climate change in tropical regions is predicted to have a negative influence on agriculture inthe long term. According to some climate models, the rain will increase in the tropics in the future, while others forecast a decline in rainfall (Biasutti, 2013). As cacao cultivation is a low-input farming system, productivity may be subject to changing climate conditions if adaptation strategies are not used. Its fundamental flaw is that it does not take into account how cacao reacts to various environmental conditions and the possibility that it may be adapted to sub-optimal environments (Lahive et al., 2019). Other factors contributed to the rapid growth of cacao plantations in Malaysia, including superior planting materials, planting technology, government policy to encourage cacao as an intercrop with coconuts, and high favourable prices (Arshad et al., 2015).

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2.3 Cacao Production: Cultivation, Botanical, and Eco-physiology Description

Attractive cacao pricing, input subsidies, and Malaysia's fertile land are all factors that contribute to high cacao production. Unfortunately, this is not the case, as the cacao business is plagued by issues that result in severely low domestic cacao production, despite rising demand. In Malaysia, cacao production may be declining due to land size, price, and uncertain poor weather. However, the increasingagricultural land will be costly; they believe that every farmer should maximize production by making the best use of available resources. Farmers who use crop inputs efficiently can produce the highest amount of yield, maximizing their income (Fadzim et al., 2017). This eventually necessitates the acquisition and correct management of planting supplies. The efficiency of smallholder cacao farmers in Malaysia can be improved by understanding the sources of efficiency for these farmers.

The cacao tree requires hot and humid conditions to grow and can't tolerate prolonged drought which might seriously depress the tree's

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vegetative and reproductive functions. Several factors influence cacao output and quality, including genetic features of planting materials, cultivation practices, postharvest processing, and environmental or site suitability considerations. Efforts to increase cacao yields can be made through rejuvenation using high-quality seed, which comprises physical, genetic, and physiological quality. The planting material is the most important source for achieving a good yield (Sakiroh et al., 2019). Cacao trees are both seed-derived and grafted (clonal) cacao are cultivated. The trees proved to be fruitful following 2 - 3 years (contingent upon the sort and technique of cultivation) when little flowers appear on the trunk and branches. Typically, less than 5% of flowers produce fruits and are termed 'pods'. These hard-walled pods mature about 5 - 6 months after pollination and typically contain 30 - 40 seeds, which are sold into the cacao market after fermenting and drying (Lahive et al., 2019).

Before hybrid seed development initiatives, practically all cacao-producing countries employed vegetative seed propagation, which is also important in ensures uniformity. Because the majority of plants in a population are self-incompatible, using vegetatively propagated material from a single plant will provide no yield. Thus, when using budded plants, grafts, or rooted cuttings, multiclonal blends are ensured to be planted. The various procedures, which include budding, cutting roots, and grafting, are all successful. Because cacao trees are still propagated generatively, the handling required to yield high-quality seed is critical. Seed propagation is the simpler of the two methods. However, if this procedure is used, the seedlings produced will be genetically diverse. Cacao seed planting is seasonal, and because cacao pods are not available to all farmers at the optimum time for producing seed nurseries. Hence, due to seeds of cacao are non-dormant and lose viability quickly within 7 days of extraction, the farmer must store pods for at least not more than one week (Saajah & Maalekuu, 2014).

Cacao beans are the seeds extracted from cacao pods and are composed of three major components: the testa (seed coat), the embryo, and the cotyledon (Figure 2.1). The sugary, white mucilaginous pulp attached to the testa is generated during pod development from an endocarp meristem (Afoakwa et al., 2013). The fruits are oval, measure between 12 and 30 cm long, and contain about 30 to 40

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beans (seeds) embedded in a mucilaginous pulp (Lima et al., 2011), with each seed has two convoluted cotyledons, a tiny embryo, and a thin membrane, endosperm remnants, and a leathery testa. The beans can be harvested, the pulp removed from the testa, and the beans kept in polyethylene bags. This saves space and material weight while transportation. The potting media of equal parts farmyard manure, sand, and soil is enough for raising cacao seedlings. Germination will be approximately 90%. Because of their high polyphenolic content, the seeds have a reddish-brown coloured outside and a dark brown inside. The pulp is fragrant and mucilaginous, comprised of spongy parenchymatous cells that contain a carbohydrate-rich sap. The beans and pulp account for roughly one-third to one-half of the entire weight of the pod (125 - 200 g). Cacao seeds have a fat content of 40 - 50% and a protein content of 14 - 18%, depending on the harvesting time (Rucker, 2009).

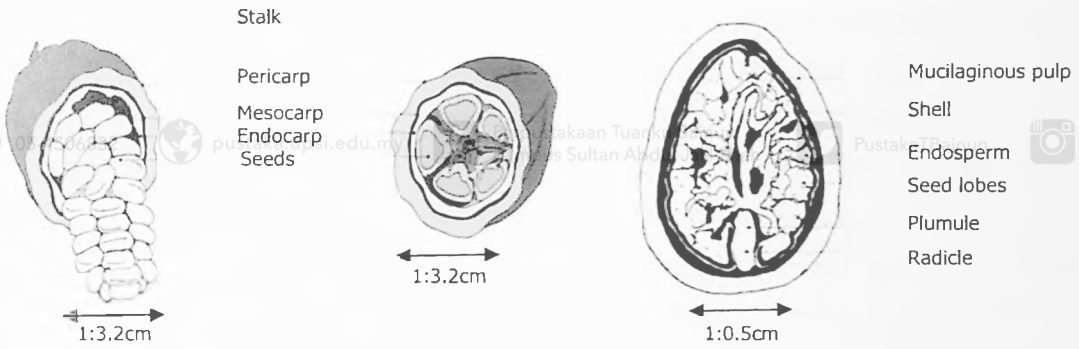


Figure 2.1 : (a) Longitudinal and transverse sections of a cacao pod [Scale: 1: 3.2cm]; and (b) Longitudinal section of a cacao seed [Scale: 1: 0.5cm]

Source : CAC/RCP 72-2013 (2013)

When growing crops on marginal land, it is critical to employ high vigour source plant materials (seeds or seedlings). The use of low-quality seeds results in seedlings with low vigour, that are not uniform, and that is less resilient to biotic and abiotic challenges, lowering productivity (Kade et al., 2013). Seed or bean quality was better when the cacao pod reached structural and functional maturity on the mother plant itself. Thus, the preservation of seed viability, particularly during storage, as well as nursery and/or field performance, has been critical to

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the survival of the cacao industry (Saajah & Maalekuu, 2014). The natural phenomenon of cacao seed deterioration during storage, which is difficult to entirely manage, is a function of storage environment conditions and the length of handling or storage, resulting in reduced germination of seed from such pods. The moisture content of seeds, humidity, storage area temperature, container, and storage length are all elements that affect the viability of recalcitrant seeds (Bahri et al., 2018).

2.3.1 Prang Besar Cacao (PBC 123) Clone

Clonal planting materials have been widely used in Malaysian cacao agriculture. Many high-yielding clones had been released and recommended for planting in prior years. The Malaysian Cocoa Board has chosen 54 cacao clones as the preferred commercial clones for farmers to plant. These 54 cacao clones are classified into four groups based on their tolerance to a wide variety of Malaysian agroclimatic conditions, good agronomic features, resistance to key pests and diseases, and high butter fat content and flavour. The Malaysian Cocoa Board Class I Commercial cacao clones are outstanding clones that are generally acceptable for planting across Malaysia, have a high output potential (>2.5 tonnes per year), and are resistant to key pests and diseases (MCB, 2012). These Class I cacao clones could be employed in the hybridization program to create new planting materials (Johnsiul & Awang, 2019). Categorized as Class I cacao clone; PBC 123 clone known as the superior Malaysian clones, a popular commercial clone in Sabah since the mid-nineties, additionally described by medium vigour and tolerant to 'Vascular Streak Dieback' disease, triggered by infection with *Oncobasidium theobromae* (Yin, 2004). During the times, the bright red pod colour, with type UA x Trinitario hybrid; PBC 123 clone is also characterized by an average-hard pod, average-smooth pod surface provided one of the highest yielding materials in the breeding program at Golden Hope Research Centre, Sabah (Teh et al., 2006). PBC 123 clone produced about 58.7 pod yield per tree, 2550 kg/ha/year of dry bean, 42 beans per pod, 1.04 g dry bean (MCB, 2012).

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2.4 Biology of Seeds

2.4.1 The Significance of Seed Storage

Plant genetic resources are basic for people and the sustainability of the planet. Accordingly, biodiversity preservation is rehearsed in an approach to ensure and preserves the abundance and variety of all species. In preventing that species and plant varieties may become extinct, the preservation of plant diversity must be done. The preservation in situ (in field collections) is risky, at which the valuable germplasm can be lost (genetic erosion) as a result of pests, diseases, and adverse weather conditions. Hence, the ex-situ (seed collection or storage) is the best and productive strategy for the preservation of plant genetic resources (Westengen et al., 2013). Seeds are on a very basic level a method for generation, which creates a remixing of genetic material and phenotype variability mostly through sexual reproduction in the plant. Other significant elements of the seeds include embryo nourishment, dispersal to another area, and dormancy during unfavorable conditions. Seed storage lifespan relies upon both intrinsic properties of the species and the external factors during storage (e.g., temperature, relative humidity, and composition of the gaseous atmosphere). Identification of a target species' seed storage behaviour is critical for seed storage purposes, i.e., conservation of plant genetic/germplasm resources (Pu et al., 2015).

2.4.2 Orthodox, Intermediate and Recalcitrant Seed Types

There are three categories into which seeds are separated by their desiccation tolerance: orthodox, intermediate, and recalcitrant. Introduced by Roberts (1973), the terms orthodox and recalcitrant already refer to the seed's ability to tolerate water loss or desiccation. The main difference of those seeds is that, for instance, the matured orthodox seeds have lower moisture contents which is a consequence of maturation drying, where there is significant water loss in the later stages of development, so that these seeds have a moisture content when shed which is usually in equilibrium with the ambient relative humidity (Kermode & Bewley, 1988). Orthodox seeds drying is not a problem to the point where all the water in the tissues cannot be frozen, either present as "glass" or bound to the surfaces of intracellular and macromolecular structures (Vertucci, 1993).

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On the contrary, water loss causing the irreversibly damaged of recalcitrant seeds, resulting in a loss in viability, although the tolerable drying rate is a variable function of the drying type and conditions (Berjak & Pammenter, 2013). Recalcitrant seeds are hydrated and metabolically active once they are shed and can germinate without additional water. Even at higher relative humidity, the onset of germinative processes that require the provision of additional water, do not allow water loss, causing the decline of viability (Obroucheva et al., 2016). When seeds show strong rehydration resistance and loss of cellular integrity (cellular and molecular) results in loss of viability, mainly as a result of theinvolvement of the oxidative process and free radical reactions. During shedding, themore actively metabolic a seed, the less water loss it will tolerate. Many recalcitrantseeds (especially of tropical origin) are additionally chilling-sensitive (causing ice-crystal damage), which cannot be stored at low, but above zero temperatures (Roberts, 1973). Moreover, the relatively high storage temperatures (15°C and above) and humidity, mostly favor the activity of fungi, thus, leading to deterioration and reducing seed quality (Santos et al., 2016).

Recalcitrant seeds are metabolically active and desiccation sensitive, which cannot be dried. Since they are hydrated, they will consequently suffer ice crystal damage at subzero temperatures. Therefore, recalcitrant seeds cannot be stored under conventional seed-banking conditions of low water content and freezing temperatures (Roberts, 1973). Despite the hydrated storage that may be suitable for some recalcitrant species to maintain an active collection, alternative means have had to be developed for long-term storage. Recalcitrant seeds, which lose their viability at relatively high-water content during dehydration might impose a serious problem for the long-term conservation of many tropical plant genetic resources. As if germination in storage occurs, it implies that the seeds must be metabolically active which indicates one of the underlying reasons as to why recalcitrant seeds are desiccation sensitive (Berjak & Pammenter, 2013). The detection of germination activation will be through the ultrastructural changes which indicate the enhancement of subcellular organization and metabolism, particularly the development and increasing complexity of membranous structures (e.g. mitochondria, endoplasmic reticulum, and plastids) (Fotouo et al., 2015). Without the additional water provided, this ultrastructural and biochemical development may further lead to an increase in desiccation sensitivity, which itself can lead to the death of the seeds (Obroucheva

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et al., 2016). Hence, the rate at which germinative metabolism proceeds may influence the significance of germination in storage and the associated increase in sensitivity.

It has since been suggested that many seeds cannot be exclusively categorized, and which fall somewhere close to the most desiccation sensitive and the most desiccation-tolerant of seeds, which are then called the 'intermediate' seeds (Berjak & Pammenter, 2013). They can be dehydrated to relatively low levels which makes conventional storage possible, but not to levels as low as those typical of orthodox seeds.

2.4.3 Long and Short-term Storage of Recalcitrant Seeds

Sensitivity to drying and low temperatures restricts the storage potential of recalcitrant seeds and their genetic conservation. In spite the hydrated storage that might be appropriate for some recalcitrant species to maintain an active collection, alternative means have must be developed for long-term storage. The cryopreservation of recalcitrant germplasm; the storage of material at the ultra-low temperatures of liquid nitrogen (-196°C) is the feasible option for the long-term storage means (Engelmann, 2011). Recalcitrant seed, however, is almost invariably too large to permit the rates of drying and cooling required for vitrification (Pammenter & Berjak, 2014). Thus, the cryopreservation process requires partial drying in a way to prevent ice crystal damage, as the fundamental process in cryopreservation is the avoidance of intracellular ice crystals that can be deadly to the seed tissue; by inducing the freezable water present to vitrify and form a glass. The formation of concentrated solution, an indistinct non-crystalline solid of very high viscosity, may effectively prevent molecular motion and so prevent individual water molecules from coming together to form embryo ice crystals. Those conditions thus preventing deleterious processes and associated aging by reducing molecular mobility which inhibits chemical and metabolic reactions (Pammenter & Berjak, 2014). If cooling rates are rapid enough, the material dried rapidly can survive to water contents low enough to permit vitrification of the remaining intracellular water rather than the development of ice crystals.

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Rapid drying was discovered to allow resistant seeds or excised axes survive at reduced water contents and to improve survival after cryopreservation. The fact that recalcitrant seeds or excised axes spend less time in a partially dried state has been related to the improvement in desiccation tolerance under rapid drying conditions (Pukacki & Juszczyk, 2015). Furthermore, unequal water distribution in seed tissues may be linked to reduce desiccation sensitivity during rapid drying of axes (Umarani et al., 2015). Meristematic tissues contain more water than other axes parts (Pammenter et al., 1998). However, unlike mature recalcitrant seed tissues, somatic embryos and immature seeds benefited from the slow-drying approach. In the latter situation, seed tissues were thought to lose water slowly and could develop desiccation tolerance during a period of prolonged dehydration. In those experiments, rapid drying was defined as dehydrating seed tissues in a laminar flow cabinet or with silica gel, while slow-drying was defined as drying seed tissues with testa or in closed containers (Liang & Sun, 2000). Moreover, previous studies revealed that some seed species which had been dried rapidly may pass through intermediate water content range before damage can accumulate to lethal levels and survived to lower water contents than those dried slowly (Umarani et al., 2015). Rapid drying, however, does not in any way induce desiccation tolerance; the condition rather of the stress being applied faster than the damage accumulates, which will save plenty of hours for the tissue to survive in the partially dehydrated state (Pammenter & Berjak, 2014). Moreover, irrespective of the drying rate, there were findings showed that there is a lower limit to which seeds or seed parts can be dried without total loss of viability, which appears to coincide with the level at which the remaining intracellular water is non-freezable (Jaganathan et al., 2017) and is suggested to be structure-associated.

Recalcitrant seeds generally show considerable variability between species and within individual species, particularly concerning the amount of water loss that can be tolerated and the rate at which drying can occur. Moreover, most of the studies on the response of recalcitrant seeds to dehydration were within a narrow range of drying rates. Recalcitrant seeds or seed axes' responses on drying depends on the rate at which water is lost (Berjak & Pammenter, 2013). Generally, seeds or seed axes that are dried very rapidly can survive to the lowest water contents (Kioko et al., 2006). During drying, the rapid dehydration method allowed insufficient time for the

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accumulation of damage to lethal levels, compared to slow-drying. On the other hand, aqueous-based degradative processes result from the disturbance of on-going metabolism (Walters et al., 2001), at which those damage accumulates during slow-drying, and leads to viability loss at relatively high-water contents. However, there is an absolute lower limit below which recalcitrant seeds will not survive, irrespective of the drying rate. It appears, therefore, that there are at least two types of damage (e.g., 'metabolism-induced damage' and 'desiccation damage') that can occur when desiccation-sensitive seeds are dried. On the other hand, rapid drying of whole seeds may decrease the water contents below which viability was lost, in which the removal of stabilizing water from intracellular surfaces below the lower limit may cause direct rapidly lethal damage. More studies claimed that the optimal drying rate is related to a minimal level of combined damages from mechanical and metabolic stresses during dehydration (Liang & Sun, 2000).

Slow drying seeds were exposed to high water contents for extended periods during which they may experience continued germinative development, further eventually increased the desiccation sensitivity and thus the seeds or seed parts die at relatively high-water contents (Liang & Sun, 2000). The short lifespan of recalcitrant seeds will be from days to weeks, and a few weeks to a few months for species adapted to tropical environments, and up to about 3 years for several species adapted to temperate environments (Pu et al., 2015). Some of the consequences of recalcitrant tissues dehydration are membrane deterioration (plasmalemma and mitochondria), protein denaturation, reduction of respiratory rate, and reduction in ATP level. Therefore, under very slow drying conditions, seed axes may be damaged by various deleterious processes that take place during the period of prolonged dehydration, ranging from the disruption of metabolic regulation to the failure of antioxidant systems (Liang & Sun, 2000).

The theoretical basis for the equilibrium dehydration method is that the optimal drying rate is related to a minimal level of combined damages from mechanical and metabolic stresses during dehydration (Liang & Sun, 2000). The storage in an environment where the atmospheric relative humidity is in equilibrium with the seed's water content (often 100% RH), or commonly referred to as wet storage, is the method used to maintain high water content (De Vitis et al., 2020).

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Although this enhancing the storage lifespan, it can lead to other problems such as the initiation of germinative metabolism. Early germination involved three phases of increase in seed fresh weight. It began with imbibition of water by the seed (Phase I), followed by the lag phase where there was an active metabolic activity but little water uptake (Phase II), and later to radicle protrusion with the fresh weight gain driven by additional water uptake (Phase III) (Bewley et al., 2013) (Figure 2.2). The length of time it takes for these events to occur varies by species and is affected by germination conditions. In a way to know the appropriate water in avoiding germination metabolism, it can be referring to the five (I to V) levels of hydration in seed tissues identified by Vertucci & Roos (1990) (Figure 2.3). At level V (high water content; >0.75 $gH_2O\ g^{-1}$wb, wet basis) and the water properties in the tissue are similar to those of a dilute solution; the metabolism is normal, and seeds germinate. As the water content decreases to level IV, properties of water are similar to a concentrated solution where the interaction between water and solutes becomes stronger; water content is inadequate for cell growth and germination, but respiration occurs with the synthesis of protein and nucleic acid. On removal of water (level III, 0.45–0.25 $gH_2O\ g^{-1}$ wb), synthesis of protein and nucleic acid is not significant together with some respiration. In level II (0.25–0.08 $gH_2O\ g^{-1}$wb) only low-level catabolic events are slowly produced. The solution becomes concentrated and viscous, having the properties of a glass in level II and III. At very low water content (level I <0.08 $gH_2O\ g^{-1}$wb), water is tightly associated with macromolecular surfaces and there is no metabolic activity (Pammenter & Berjak, 2000).

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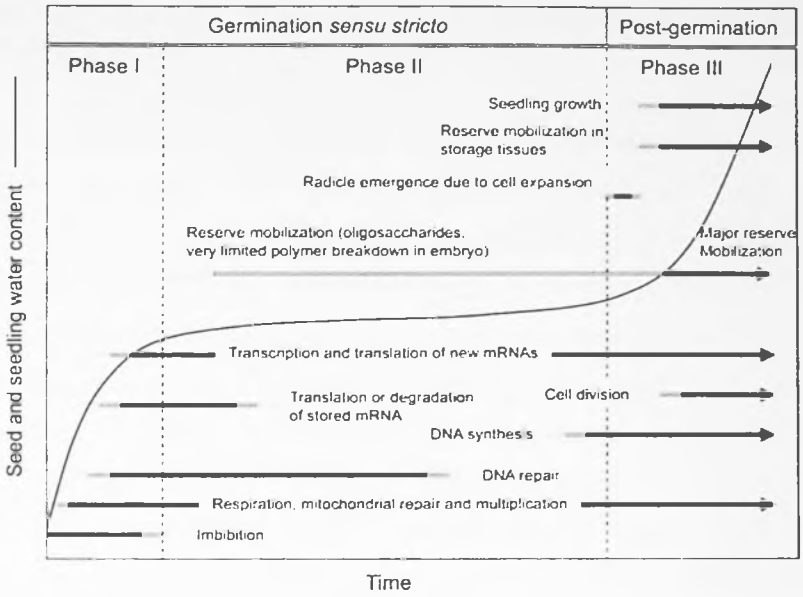


Figure 2.2 : Time course of physical and metabolic events occurring during germination and post-germination (Phases I, II and III). The curve shows a stylized time course of water uptake

Source : Nonogaki et al. (2010)

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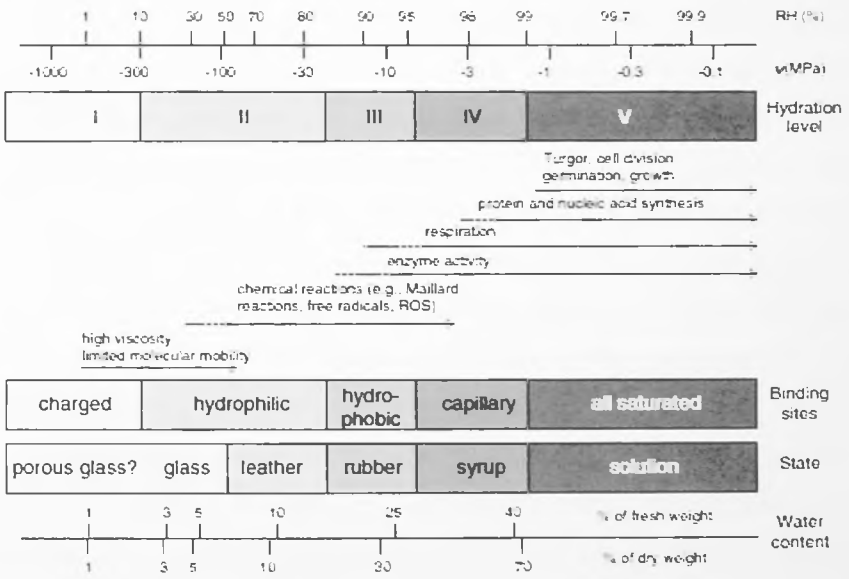


Figure 2.3 : Hydration levels and associated metabolic/chemical activities in seeds

Source : Bewley et al. (2013)

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2.5 Role of Storage Conditions on Reducing Water Loss in Recalcitrant Seed

The three major factors which influence the damages caused to stored recalcitrant seeds are (i) seed moisture content, (ii) rate of drying, and (iii) storage temperature. Recalcitrant seeds characterized by the high seed moisture content, since they do not undergo maturation drying before shedding from the mother plant. As the germination of desiccation-sensitive seeds declines rapidly as seed moisture content decrease, the condition is closely linked to seed decay and death of recalcitrant seeds (Gasparin et al., 2017). However, there was study proposed that seeds can be dried to a certain level termed as 'critical water content' (Finch-Savage et al., 2007). In a way to avoid mechanical damage to the cell membrane as well as macromolecules, maintenance of seed volume by retaining high seed moisture content in the recalcitrant seeds is quintessential. The high level of moisture content in recalcitrant seeds, however, are exposed to another type of damage, i.e. metabolism linked damage which caused by ROS-mediated lipid peroxidation, protein and DNA denaturation.

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As the primary control of all activities, the seed moisture content is the most important factor in maintaining viability during storage. The seed moisture will come to an equilibrium with the moisture in the storage atmosphere based on the differences in the vapour pressures and the chemical nature of the seeds. Thus, the metabolic rates can be minimized by keeping seeds in a dry state. Moreover, the lower temperature would also be one of the most convenient ways of prolonging the storage lifespan of seeds, which it decreases metabolism and helps prevent water loss. In differentiating patterns of seed storage behaviour, there are two practical aspects to be considered: (i) the effect of desiccation (dehydration) on seed viability; (ii) the response of seed longevity (period of seed survival) to the storage environment. The 'maturation drying' process known as a late phase of development which will be going through by most seeds, during which they lose most of the tissue water and come into equilibrium with the relative humidity of the surrounding atmosphere (Gasparin et al., 2017). In recalcitrant-seeded species, however, hydration resulted in not clearly discernable switch from developmental to germinative metabolism (Salam et al., 2017). They are sensitive to drying and low

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temperatures, which complicates their prolonged conservation (Suma et al., 2013). Moreover, recalcitrant seeds are characterized by large size and mass. The advantage of large seeds is their slower desiccation rate due to lower ratio of seed surface to seed volume and better survival, because large seeds maintain higher hydration for a longer time (Obroucheva et al., 2016). The appropriate water content conditions should be identified to minimize the deterioration of seed during storage (Lisboa et al., 2017; Umarani et al., 2015) as well as to maintain their vigour performance after storage.

Decreasing metabolism and helps preventing water loss through the reduction of temperature is the most convenient way of prolonging the storage lifespan of seeds. Storage of recalcitrant seeds in the hydrated state is generally accepted to be the best way in which to retain viability in the short-term (Berjak et al., 1993). At lower temperatures, problems encountered with hydrated storage of recalcitrant seeds are reduced, at which both germinative metabolism and fungal metabolism are significantly slowed. As a complex phenomenon characteristic of many plants originating in tropical or subtropical climates, each species of recalcitrant seed seems to have a specific temperature at which it incurs chilling injury (King & Roberts, 1979). A plant with chilling sensitive character may suffer injury and eventually dies on exposure to chilling temperatures. Defined as a change in metabolism as a consequence in altered physiological function, chilling injury occurs as a result of exposure to a chilling stress, which occurs below a critical temperature (Sanchez et al., 2019) and is dependent on the plant species concerned and its origin or provenance. The nature and severity of the injury is a function of the species, plant part, maturity, and metabolic state, as well as being dependent on the duration and severity of the chilling stress. The degenerative tissue injury which is not the initial events that lead to alteration of metabolic processes, is the responses of plant tissues to chilling stress (Albornoz et al., 2019). In fact, chilling stress causes a primary event and followed by a series of secondary physiological and physical events that are damaging (lead to visible symptoms and cell death) (Van Hasselt, 1990). The primary event that leads to metabolism alteration and impaired functioning of the tissue is very instantaneous, and even to claim as reversible if the chilling stress is removed after a brief exposure (Albornoz et al., 2019).

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On the other hand, knowledge of processes and mechanisms involved in the acquisition and maintenance of desiccation tolerance in orthodox seeds are important in understanding of the basis of seed desiccation sensitivity, especially to compare with seeds with recalcitrant behaviour. The inability or perhaps only the very limited ability to express the processes and mechanisms conferring desiccation tolerance, which may preclude the dramatic decline in water content during the final phase of pre-shedding development, is occurred in developing seeds of desiccation-sensitive exceptional species (recalcitrant seeds). With the acquisition of desiccation tolerance, the intra-cellular physical characteristics which become modified, which include deposition of insoluble proteins within vacuoles together with the accumulation of starch and lipid reserves, thus providing mechanical resilience against cell collapse.

Desiccation tolerance in seeds is a developmental process rather than a response to maturation drying or environmental conditions. Dehydration may be the signal to switch on the desiccation program in developing seeds (Finch-Savage & Bassel, 2016). As the membranes are considered a primary target of desiccation injury in most proposed mechanisms of desiccation tolerance, thus, the membrane stabilization is thought to be a key mechanism of desiccation tolerance. Desiccation tolerance is understood to include the ability of cells to become air-dry without loss of viability, and to successfully rehydrate (Castro et al., 2017). The period between dehydration and rehydration, is primarily referred to as life span or longevity in the dry state. Generally, water content and temperature are the crucial factors controlling seed longevity. The cytoplasm turns into a glassy state at low water contents and temperatures, in which molecular motion is slow and, the ageing as well (Ballesteros et al., 2020). During the loss of seed viability many biochemical and biophysical changes occur, which lead to deterioration of seeds or seed axes. Moreover, there is a great variability in seed longevity among accessions within a species, as the life span results from genetic controls of the seed maturation program as well as from environmental conditions during growth and after physiological maturity (Walters et al., 2005).

Recalcitrant seeds or seed axes responses on drying depends on the rate at which water is lost (Liang & Sun, 2000). Generally, seeds or seed axes that are

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dried very rapidly can survive to the lowest water contents (Kioko et al., 1998). During drying, rapid dehydration method allowed insufficient time for the accumulation of damage to lethal levels, compared to slow drying. An aqueous based degradative processes result from the disturbance of ongoing metabolism (Walters et al., 2001), at which those damages accumulate during slow dehydration, and leads to viability loss at relatively high-water contents. However, there is an absolute lower limit below which recalcitrant seeds will not survive, irrespective of the drying rate. It appears therefore, that there are at least two types of damage (i.e. 'metabolism-induced damage' and 'desiccation damage') that can occur when desiccation-sensitive seeds are dried. Rapid drying of whole seeds may decrease the water contents below which viability was lost, in which the removal of stabilizing water from intracellular surfaces below the lower limit may cause the direct, rapidly lethal damage. In contrast with slow drying, the rapid drying has brought about uneven across the seed tissues as seen by the time course of decline of axis water content. This uneven dehydration during rapid drying may contribute to a better survival of rapidly dried seeds (more hydrated in meristematic tissues area), and therefore less prone to desiccation damage (Xia et al., 2012). More studies claimed that the optimal drying rate is related to a minimal level of combined damages from mechanical and metabolic stresses during dehydration (Liang & Sun, 2000).

Due to the desiccation sensitivity, the viability is first slightly reduced as the fresh recalcitrant seeds begin to dry. The moisture content then begins to be reduced considerably to a certain point termed the "critical moisture content", and with a further continues drying, the viability is eventually reduced to zero. Recalcitrant seeds must then be stored at their shedding water content which termed as "hydrated storage" (seeds maintained under saturated relative humidity conditions), since even mild dehydration adversely affects viability (FAO, 2013). However, the seeds will germinate in storage (Berjak et al., 1993), as they will become more desiccation sensitive, which at this point indicates the end of the useful storage life span. The hydrated storage of recalcitrant seeds, therefore, is strictly a short-term measure, from a few days for seeds of many tropical species to perhaps a year or a little longer for seeds of chilling-tolerant temperate species that can be maintained at temperatures close to zero. In the past three decades, there

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were development of several methods for long-term storage of a base collection as the alternative means, such as 'moist' storage, partially dry storage and cryo-storage, however, have their own limitations (Chin et al., 1989).

Under any controlled desiccation conditions could be used (i.e. constant RH and temperature) (Liang & Sun, 2000), the amount of water vapour in the air expressed as a percentage of the amount required to reach saturation at the same temperature (percent RH). RH is inversely related to temperature and extremely sensitive to changes in temperature. With a more consistent temperature in the system, their RH will likewise be consistent (Dragicevic & Sredojevic, 2011). In an open space, at constant moisture level and temperature, % RH is directly proportional to the total pressure. However, the value of % RH is limited to 100% as vapour pressure (p) cannot be greater than saturation pressure (ps). In a closed container of fixed volume, % RH decreases as temperature increases, but not quite as strongly as in open space. Following the formula, % RH = p/ps x 100; the RH of a system decreases as the temperature rises because pressure rises but vapour pressure remains constant. Similarly, as a system's temperature drops, the RH rises because vapour pressure falls while pressure remains constant. On the other hand, the RH of a system falls when the total pressure of the system decreases because the pressure decreases, but the vapour pressure does not change because the temperature does not change. Similarly, as a system's total pressure rises, the RH rises as well, until saturation is attained (Process Sensing Technologies, 2021).

2.6 Deterioration Development of Recalcitrant Seed in Chilling and Slow-drying Storage Conditions

Decreasing metabolism helps in preventing water loss through the reduction of temperature (chilling) is the most convenient way of prolonging the storage lifespan of seeds. Storage of recalcitrant seeds in the hydrated state is generally accepted to be the best way in which to retain viability in the short term (Berjak & Pammenter, 2013). At lower temperatures, problems encountered with wet storage of recalcitrant seeds are reduced, at which both germinative and fungal metabolism are significantly slowed (Dalziell & Tomlinson, 2017). As a complex phenomenon

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characteristic of many plants originating in tropical or subtropical climates, each species of recalcitrant seed seems to have a specific temperature at which it incurs chilling injury (Obroucheva et al., 2016). A plant with a chilling sensitive character may suffer injury and eventually dies on exposure to chilling temperatures. Defined as a change in metabolism as a consequence of altered physiological function, chilling injury occurs as a result of exposure to chilling stress, which occurs below a critical temperature and is dependent on the plant species concerned and its origin or provenance. The nature and severity of the injury are a function of the species, plant part, maturity, and metabolic state, as well as being dependent on the duration and severity of the chilling stress (Lukatkin et al., 2012). Chilling stress causes a primary event and is followed by a series of secondary physiological (alteration of metabolism) and physical events that are damaging (lead to visible symptoms and cell death) (Liang et al., 2020). The primary event that leads to metabolism alteration and impaired functioning of the tissue is very instantaneous and even to claim as reversible if the chilling stress is removed after a brief exposure (Rurek et al., 2015). Changes in membrane permeability, respiration, protoplasmic streaming, and metabolic changes involving enzyme systems, sugars, and other chemical substances, are considered as the main physiological symptoms. Considered as the other secondary event, physical symptoms, include necrotic lesions, increased susceptibility to decay organisms, cessation of growth, and ultimately death (Lukatkin et al., 2012).

2.7 Mechanical Damages in Response to Slow-drying Mechanisms of Recalcitrant Seeds During Storage

The germination of desiccation-sensitive seeds declines rapidly as seed moisture content decrease (Lee et al., 2016), the condition is closely linked to seed decay and death of recalcitrant seeds. In most studies, researchers found that generally, the critical moisture content is slightly higher than the level of water which is non-freezable (0.35 g g^{-1}) (Finch-Savage et al., 2007). Water properties in dried tissues appear to be of primary importance in desiccation tolerance and in determining survival during the long-term storage of seeds. Mechanical damage is associated with volume reduction on the loss of water. Recalcitrant seeds which are shed from the

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mother plant with high seed moisture content subsequently, resulted in seed dehydration over time results in structural damage to the vacuoles, cytoskeleton, and cellular membranes, culminating in the loss of seed viability (Gonzalez-Morales et al., 2016). Cytoskeleton (constituted by microtubules and microfilaments) plays a major role in imposing the organization of the cytoplasm and nucleus (Berjak & Pammenter, 2013). The failure of the cytoskeleton to reassemble following water imbibition, causing permanent damage to cell membrane systems of dehydrated recalcitrant seed. It is seen as a major damaging factor because it leads to a lack of intracellular support and structural organization.

As the membranes are considered a primary target of desiccation injury in most proposed mechanisms of desiccation tolerance, thus, membrane stabilization is thought to be a key mechanism of desiccation tolerance (Shivaraj et al., 2018). As the cellular membranes are the primary sites of injury, various cytoplasmic solutionsmay leak out when the collapse of membrane. Generally, the cells remain essentially viable during dehydration, and the membrane injury may occur upon rehydration ofdry organs. Drying changes the physical properties of the membrane phospholipids, which go from the liquid crystalline phase to a gel phase compared to at a hydrated state. As if the water is removed from the phospholipid head groups, the hydrated lipid that is in the liquid crystalline phase at physiological temperatures will be in the gel phase at the same temperature. These could be changes in permeability, compartmentation, and membrane-bound enzyme activity, as the membrane and membrane-protein organizations are lost (Oliver et al., 2020). Membranes which consist of a lipid bilayer, with each type of cellular membrane has unique lipid-lipid and lipid-protein interactions, play important roles in the growth and survival of plants at chilling temperatures. Those conditions made the chloroplast, mitochondrial, endoplasmic reticulum, plasma, and tonoplast membranes each would respond differently towards chilling stress (Murata & Nishida, 1990). A well-known theory was proposed by Lyons (1973), who explained that the exposure of the cellular membranes to chilling temperatures has caused the physical phase transition from a flexible liquid-crystalline to a more solid gel structure. Later studies reported that, as lipids control the physical state of cell membranes, thus the transition causing membranes with a high percentage of saturated fatty acid chains to solidify (Taiz & Zeiger, 1988). Moreover, as the fluidity of the membrane decreases, it affected the

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activities of membrane proteins and enzymes, thus causing the proteins and enzymes to no longer function normally. Permeability refers to the freedom of water and solutes that can pass through a membrane; thus, the increase of solute leakage would be the first symptom of membrane permeability enhancement. Influenced by the concentration gradient, solute leakage is a passive event that occurs after exposure to chilling stress, as the consequence of increased permeability (Lukatkin et al., 2012).

2.7.1 SEM Observation on Seed Deterioration Development

SEM micrographs of materials are used to evaluate moisture transfer mechanisms throughout the drying process and to interpret the rehydration qualities or textural properties of the dried materials (Thuwapanichayanan et al., 2011). There is limited information on the mechanism of moisture movement during drying, and the process may involve molecular diffusion, capillary flow, surface diffusion, and other factors that affect drying characteristics, so the moisture transfer mechanism hasn't been described in full detail. During the drying process, moisture could be transferred from parenchyma cells to the surrounding drying media in two ways: either directly through pores or open structures on the surface layer, or indirectly through parenchyma cells' internal matrix (Xiao & Gao, 2012). The microstructure of fresh and dried samples can be a potent tool and compelling evidence for examining the changes in sample properties during the drying process. The structure of the seeds changes dramatically as they are dried. Water loss causes a decrease in cell volume, an increase in solute concentration, a change in intracellular pH, protein denaturation, and changes in membrane and DNA structure (Martins et al., 2019).

The method and rate of drying influence density characteristics, with slow-drying yielding a fully consolidated product and rapid drying yielding a solid with cracks and holes with a lower density (Al-muhtaseb, 2004). The damaged cells have a dense, amorphous structure and heavily folded cell walls, which are most likely the result of pressure caused by swelling cells located deeper in the cotyledon (Neya et al., 2004). According to Martins et al. (2019), imbibed recalcitrant *Eremanthus erythropappus* seeds demonstrated complete cell organization. The plasmalemma was still attached to the cell walls. Cell contents were damaged, and

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membranes detached as soon as the seeds were dehydrated. Such a response is frequent in recalcitrant seeds, either before or after the commencement of germination. Sample matrix microstructure qualities are directly related to the drying behaviour of plant materials, rehydration properties after a sample have been dried, and other properties (Yang et al., 2010). When the material is dried, the internal moisture gradient creates stresses in the cellular structure, resulting in a collapse of the structure in response to physical changes in shape and dimension or volume. Moisture diffusion from the inside to the outside of the cell is affected by cell wall disruption (Thuwapanichayanan et al., 2011). SEM micrographs can be used to investigate, interpret, and even anticipate the rehydration and textural aspects of dried materials. The changes in sample microstructure are strongly related to the stress created in the tissue, which might be caused by shrinkage. Shrinkage occurs when materials are heated and moisture is lost during the drying process, which is a simultaneous heat and mass transfer process. Furthermore, each substance has unique shrinking characteristics. Non-uniform shrinkage inside the product causes two forms of internal stresses: thermal stress from temperature gradients within the material and hydro stress from moisture gradients within the material. When the combination of temperature and hydro stress surpasses the binding force between material cells, a crack or burst phenomenon occurs, which can modify the macrostructure and microstructure of the product throughout the drying process (Xiao & Gao, 2012).

2.8 Physiological and Biochemical Changes Responses on Slow-drying Mechanisms of Recalcitrant Seeds during Storage

Seed storability, which includes both vigour and viability, is a key feature for sustaining natural populations and commercial operations in areas where members of the genus are farmed grown (Chandra et al., 2019). The poor storability of recalcitrant seeds has been frequently reported, however, the processes underlying seed death during storage remain uncertain. Knowledge of processes and mechanisms involved in the acquisition and maintenance of desiccation tolerance in orthodox seeds is important in understanding the basis of seed desiccation sensitivity, especially to compare with seeds of recalcitrant behavior. Following

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morphogenesis, developing seeds enter a 'maturation' stage, which seems to be a more active stage in gene expression and metabolism than the seed drying itself. Also known as 'reserves accumulation' periods, it involves re-organization the metabolism and storage compound synthesis,including starch, storage proteins, and lipid (Angelovici et al., 2010). With the acquisition of desiccation tolerance, the intra-cellular physical characteristics become modified, which include deposition of insoluble proteins within vacuoles together withthe accumulation of starch and lipid reserves, thus providing mechanical resilience against cell collapse. Seeds from these species appear to have completed their maturation program during storage, building food reserves that were used followingthe switch to the germination mode (Faria, 2006). For seedlings to grow and develop(during germination), the storage components like soluble sugars (mainly raffinose, stachyose, verbascose, and sucrose) are degraded and used as an energy source (Sett, 2016).

The inability or perhaps only the very limited ability to express the processes and mechanisms conferring desiccation tolerance, which may preclude the dramatic decline in water content during the final phase of pre-shedding development is occurred in developing recalcitrant seeds. Recalcitrant seeds are characterized by the high seed moisture content since they do not undergo maturation drying before shedding from their mother plant. This reduction in moisture content continues untilhygroscopic equilibrium is reached. From then on, changes in moisture content are associated with changes in relative humidity (Bareke, 2018). Although the hydratedstorage may enhance the storage lifespan of recalcitrant seeds, it can lead to other problems such as initiation of germinative metabolism which may reduce their storability potential. During development, recalcitrant seeds stored reserves as soluble sugars, whereas more tolerant species accumulated insoluble reserves (Zaritzky, 2015). Following the early stages of seed germination, seeds accumulate significant amounts of storage compounds that serve as nutrients and energy reserves. When seeds are hydrated, embryonic reserves are mobilized, marking the beginning of the germinative process (Dias et al., 2018), protein synthesis, and consequence radicle protrusion (Ali & Elozeiri, 2017). Following seeds germination progression, storage-metabolites are converted into nutrients and energy, when the seedling is still heterotrophic (Galili et al., 2014). The enzymatic process is used to degrade food reserves in each compound. The results of food reserve degradation

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are transferred to the growing point in the form of simple carbohydrate compounds, where they can be used as an energy source for growth (Sakiroh et al., 2019).

There is a process of respiration in the hydrated seeds during storage, thus the food stores held in the cotyledons employed as energy reserves in the process of seed growth are further depleted (Ali & Elozeiri, 2017). Commonly, the effects of respiration on seed lifespan are in the form of heat and water vapour. The heat generated as a result of energy scattering in seeds during storage might directly reduce the viability and vigour of the seeds and further, the seeds will experience aging and deterioration. On the other hand, it is also suggested that food reserves in seeds diminish as storage time increases. This further causes the seeds to lose energy and eventually grow at a slower rate, as well as their moisture content as a medium of metabolic processes (Bahri et al., 2018). Moreover, seed germination and reserve mobilization are highly controlled processes that are influenced by environmental factors such as temperature as well as developmental factors. The development of a seed is highly controlled, with various transcript, protein, and metabolite transitions occurring in concert throughout its progression (Galili et al., 2014). On the other hand, reorganization of subcellular structures and activation of metabolism are among the wake-up calls of a dry seed in a dense, oxygen-poor atmosphere (Rosental et al., 2014). Seed water content greatly determines the viability of seeds to maintain lifespan, hence, the water content of recalcitrant seeds is kept high. However, the production of storage compounds, whose generation by respiration becomes limited due to oxygen penetration limitations in dense hydrated seed tissues, necessitates a significant quantity of energy. The need for oxygen, and so energy production, becomes especially important during reserve metabolite accumulation and seed desiccation, when thick seed tissues limit oxygen penetration and consequently mitochondrial energy production (Galili et al., 2014). Thus, this suggests that little progress should be expected in terms of increasing the storability of these recalcitrant seeds with any treatment applied after harvesting.

Physiological and biochemical changes during germination, followed by morphological changes, are closely connected to seedling survival rate and vegetative development, both of which affect yield and quality (Ali & Elozeiri, 2017). Seeds with

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high vigour can rapidly form and transfer raw materials to the embryo shaft, enhancingdry matter accumulation. Good plant development is characterized by high dry weightand is affected by the speed with which roots access nutrients in the soil, as well asthe increase in the number and length of plant roots (Bahri et al., 2018). Thus, the high dry weight can illustrate the sufficient and efficient utilization of food reserves in seeds. Sucrose and certain raffinose series oligosaccharides (RFOs) appear invariably to build in orthodox seeds during maturation drying, and high sucrose concentrations are also prevalent in desiccated resurrection plant tissues (Berjak & Pammenter, 2008). The non-specific solutes (such as salts and sugars) affect membranes at low or intermediate hydration, as they reduce the stress on membranes through their osmotic effect, but only if the solutes remain between the membranes (Wolfe & Bryant, 1999). Similarly, Pammenter & Berjak (1999) suggested that specific sugars replace the water normally associated with membrane surfaces, in which the lipid head-group spacing is maintained and prevents the damaging liquid- to gel phase-transition. Moreover, during dehydration, high viscosity, amorphous, super-saturated solutions formed by sucrose and certain oligosaccharides slowed molecular diffusion, and thereby slowing down chemical reactions.

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Separate intercellular bodies of seed stored carbohydrates, proteins, lipids, and phosphate act as an energy source and carbon skeleton during seed hydration. Many metabolic processes were triggered by seed imbibition, including the activation or fresh synthesis of hydrolytic enzymes, which resulted in the hydrolysis of stored starch, lipid, protein hemicellulose, polyphosphates, and other storage materials into a simple form suitable for embryo uptake. Furthermore, increased oxygen consumption may cause activation or hydration of mitochondrial enzymes involved in the Krebs cycle and electron transport chain (Ali & Elozeiri, 2017). The carbohydrates in seeds are made up of oligosaccharides as well as non-starch and starch polysaccharides. Commonly, both raffinose and sucrose are employed as energy sources during early germination and imbibition, as well as reserves in the embryo (Da Silva et al., 2017). The decreased carbohydrate content was caused by carbohydrate hydrolysis into simpler molecules, which were subsequently utilized as energy for growth during the germination phase. Carbohydrates are stored in the seeds as starch, and the compounds must be hydrolyzed into a simpler component.

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Degrading starch can be accomplished by two methods: amylolysis or hydrolysis and phosphorolysis. The amylolysis process yields glucose, whereas the phosphorolysis process yields glucose and dextrin by degrading amylose and amylopectin with the help of phosphate ions. Glucose was also transported to the developing point (root and shoot) (Sakiroh et al., 2019). Imbibed seeds may acquire sucrose as a result of sugars interconversion within the seed. The mobilization of seed storage reserves, on the other hand, can cause sucrose to accumulate. As part of early post-germination growth, these sugars are required to supply energy for germination, before further depleted and used-up.

Seed damage and seed quality decline are affected by the relative temperature and humidity of the storage space, period, and the original moisture content of the seed. Increased seed water levels boost enzyme activity, particularly hydrolysis enzymes (Bahri et al., 2018). The presence of free water and the maintenance of suitably high hydration status is principally responsible for the activation of all organelles and cytoplasm, as well as for compartmentation and metabolic traffic between compartments. At optimal cell hydration levels, the whole enzymatic machinery continues to operate based on energy production and an active protein-synthesizing system, giving the entirety of biochemical reactions typical of mature seed metabolism and preparing cells for growth initiation. This suggests the concept of metabolic readiness aimed at germination readiness, which is also evident in the attempt to protect metabolic activity from the onset of hazardous desiccation (Obroucheva et al., 2016). Proteases degraded proteins during germination and growth (Kim et al., 2011). A previous study revealed that *T. cacao* seed protein levels increased during ripening and decreased during germination (Biehl et al., 1982). The total protein is the nitrogen content of cotyledons, whereas dissolved proteins are proteins that are easily absorbed by the embryonic axis.

Changes in dissolved protein content occurred as a result of changes in protein degradation during germination. In the germination process, the active protein hydrolyses the protein into shorter chains and amino acids, which are then transformed into energy or growth components. During germination, there occurs not just protein breakdown, but also the synthesis of amino acids or new proteins, which is required for growth. The longer the germination period, the lower the

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protein concentration, because during embryonic growth, nitrogen (N protein) was utilized for the construction of new structures as the germination stage progressed (Sakiroh et al., 2019).

The activity of protein-synthesizing machinery is a key indicator of metabolic activity. Concerning embryonic axes metabolic activity, it is suggested that they remain metabolically active within the moisture range between shedding and desiccation-induced viability loss. DNA synthesis continues, ribosomal machinery is active, and protein synthesis can be performed on long-lived mRNA, and sucrose reserves can be rapidly mobilized to meet metabolic demands, high respiration, and high energy expenditures (Obroucheva et al., 2016). The ability of the embryo to generate new proteins during germination can be an important function that is sensitive to hydration because protein synthesis in embryonic tissues decreases as the water deficit increases (Machado et al., 2017). Proteolytic enzymes play a critical role in the use of stored protein in the metabolism of germinating seeds. The hydrolysis of stored proteins released free amino acids, which aid in protein synthesis in the endosperm and embryo and thus the germination process. Furthermore, drying and rapid seed dehydration processes have an impact on DNA integrity. During seed germination, seeds generate a range of repair processes, including the repair of membranes, proteins, and DNA. Phytohormones play an important function in stress response via responsive protein-mediated stress. Improved germination performance has also been linked to higher expressions of genes and proteins involved in water transport, cell wall modification, cytoskeletal organization, and cell division, as well as increases in protein synthesis potential, post-translational processing capacity, and targeted proteolysis (Ali & Elozeiri, 2017).

The mobilization of starch, protein and lipid reserves in seeds is usually thought to occur after germination. However, some studies have found that this occurs during germination (Zhao et al., 2018). Recalcitrant species usually characterized by large seeds have an advantage in low-light circumstances, where their higher protein and lipid reserves, or more advanced development, can contribute to growth (Bareke, 2018). According to Pukacka et al. (2011), *T. cacao* seeds have a high lipid content, and the conversion of sucrose to oil and lipids via glycolysis is a key metabolic activity in growing seeds. The lipid content of *T. cacao*

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seeds began to increase throughout the fruit maturation phase, while it declined during the germination phase (Sakiroh et al., 2019). Lipases, which catalyze the hydrolysis of ester carboxylate bonds, releasing fatty acids and organic alcohols, and the reverse reaction (esterification) (Ali & Elozeiri, 2017). Lipase is a hydrolysis enzyme that hydrolyses lipid to fatty acids and glycerol, increasing free fatty acid content as seed viability decreases. Whitefield (2005) states that due to enhanced lipase activity, cacao seed-free fatty acids are elevated in stored seeds with high water content. Changes in enzyme activity, respiration rate, increased fatty acid, and decreased availability of food reserves are biochemical degradation indicators in seeds. The high fatty acid concentration of the seeds indicates fatty acid build-up since it is not converted further into energy, causing the seed to lose energy while germinating (Bahri et al., 2018). Germination, including the formation of cotyledon radicles and the growth and development of the embryo, will continue to be supported by lipid hydrolysis (Sakiroh et al., 2019). The breakdown of lipids, on the other hand, is slower and requires more energy resources (De Souza et al., 2018). Because sucrose is the substrate for lipid production in developing seeds and the end product of lipid breakdown, it may be largely regarded as a regulatory factor in studies of lipid metabolism mechanisms. Storage lipid mobilization in germinating seeds starts with lipases hydrolyzing triacylglycerols in oleosomes into free fatty acids and glycerol. Furthermore, embryo progress from the quiescent to active phase is mostly determined by the use of stored ATP and storage lipid breakdown products. The glyoxylate cycle is known to play an important role in lipid breakdown in oilseeds, where stored lipid is transformed into glucose, the primary respiratory fuel during germination and hence seedling development (Ali & Elozeiri, 2017). Moreover, the accumulation of non-reducing sugars, sucrose, and certain oligosaccharides act as the glasses that protect macromolecules from denaturation while also minimizing liquid crystalline gel phase transformations of the lipid bilayer of membranes. The behavior of membrane phases during dehydration and rehydration is critical to the survival of seeds and other anhydrobiotic tissues. The deployment of endogenous amphipathic molecules into membranes in response to water loss may be a requirement for desiccation tolerance; by lowering the water content at which membrane lipid phase changes occur, these molecules help desiccation-tolerant organisms maintain their membrane integrity. They must be reversible, allowing the membranes to return to a functional state following rehydration. During rehydration,

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the presence and operation of repair mechanisms are observed (replacement of damaged rRNA, repair of DNA lesions, and protein-synthesizing systems) (Zaritzky, 2015). During the desiccation of recalcitrant seeds, cellular membranes are among the primary sites of damage. This is accomplished through the oxidative attack, which promotes phospholipid breakdown and the loss of membrane structural integrity (Pukacka et al., 2011).

The dynamics of ROS and the enzymes involved in reserve metabolism are suggested to be critical for controlling germination and the mobilization of seed reserves (Dias *et al.*, 2018). The loss of viability of recalcitrant seeds during drying and storage is exacerbated by oxidative stress (Bailly, 2004; Kranner & Birtic´, 2005). The metabolic "switch-off," dedifferentiation of organelles, and reduction of the elements of the end of the membrane system interact, had subsequently limiting unregulated metabolism and the membranous targets of consequent generation of free radicals or reactive oxygen species (ROS) in orthodox seeds. Proteins, lipids, and nucleic acids are among the macromolecules that ROS react with, causing cell damage and disruption of cell function. Membrane lipids are peroxidized, resulting in membrane disintegration and cell death. A decrease in carbon uptake during a waterdeficit may limit sugar transport and lipid deposition in developing seeds, explainingthe observed lower seed weight associated with water deficit (Handley, 2016). The production of ROS in recalcitrant seeds is still controlled by the antioxidant system, even directly after seed shedding. However, as a result of unbalanced metabolism and the impairment of respiratory electron transport during seed dehydration, ROS formation dramatically increases (Pukacka et al., 2011).

The high level of moisture content in recalcitrant seeds, further, caused the exposure to another type of damage, i.e. metabolism-linked damage which is likely caused by ROS-mediated lipid peroxidation, protein, and DNA denaturation. ROS cause damage and disruption of cell function when they react with cellular macromolecules such as proteins, lipids, and nucleic acids. This damage causes membrane lipid peroxidation, which leads to membrane breakdown and cell death. However, ROS also plays an important role as signaling molecules, particularly in response to various stresses (Pukacka et al., 2011).

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Free radicals or ROS are naturally produced in plant metabolism, and plants must be endowed with antioxidant molecules (i.e. tocopherols, ascorbic acid, and carotene, and the enzymes can be exemplified with superoxide dismutase, catalase, ascorbate, and different peroxidases) and scavenging mechanisms, i.e. enzymic systems that neutralize potentially toxic activated oxygen (Obroucheva et al., 2016). The previous study found that there was a decline of viability below the critical water content, and it was correlated with increased lipid peroxidation and cellular leakage in recalcitrant *T. cacao* seeds (Li & Sun, 1999), at which the activities of free radical-scavenging enzymes, i.e. ascorbate peroxidase, peroxidase, and superoxide dismutase, decreased rapidly during the same time. As the embryonic axis contained large amounts of sugars, raffinose, and stachyose, desiccation damage did not appear to be caused by lack of sugar-related protective mechanisms, these results subsequently support a hypothesis that enzymic protection against free radicals plays an important role in the desiccation sensitivity of recalcitrant tissues during desiccation (Li & Sun, 1999).

Several studies have shown that the damage to the antioxidant system is a major factor in the loss of viability of recalcitrant seeds. For instance, it was found that recalcitrant *Quercus robur* embryos viability declined as free radical damage and cell membrane permeability increased (Feng et al., 2017). The earlier previous study also relates the loss of the germination capability of recalcitrant *Shorea robusta* seeds to a decreased effectiveness of the antioxidant system (Tommasi et al., 2006). Metabolism-linked damage, considered as a consequence of the failure of coordination of metabolism, is most likely mediated by ROS (compounds that are powerful oxidizing agents and contain oxygen), leading to aqueous-based oxidative degradation, which particularly occurs at high to intermediate water contents. Damage accumulation, and the water content at which death occurs through this process, are strongly influenced by the drying rate.

As the aerobic organisms undergo the respiration process, and with oxygen as the terminal electron acceptor, electrons may 'leak' from the electron transport chain and directly reduce molecular oxygen to superoxide (O_2), a free radical ROS (Gilí & Tuteja, 2010). Superoxide further can be disputed by superoxide dismutase (SOD) to hydrogen peroxide (H_2O_2) and O_2. Hydrogen peroxide is a non-free-radical

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ROS that can form the highly reactive hydroxyl radical (OH^-), which can directly oxidize unsaturated bonds in the fatty acid components of lipids, and proteins, and nucleic acids. Initially, ROS are normal components of aerobic metabolism with an important role in cell signaling, and the pro-oxidant and antioxidant status is in balance, preventing excessive pro-oxidants from causing damage but permitting ROS signaling. However, there is an increase in production under conditions of stress, physical damage, desiccation, and chilling stress (Gilí & Tuteja, 2010). Under non-stressful conditions the production of these oxygen free radicals is a normal part of cell metabolism under aerobic conditions, and they are usually inactivated by antioxidants and free radical scavenging enzyme systems (Ali & Elozeiri, 2017); a) Non-enzymatic (glutathione [GSH], ascorbic acid [AsA], and a-tocopherol, which is lipid-soluble); and b) Enzymic (SOD, a range of catalases and peroxidases, and glutathione reducíase [GR]) (Gilí & Tuteja, 2010). Failing antioxidant activity in the axes might eventually cause the death of the seeds.

2.9 Summary

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The recalcitrant characteristic of cacao seeds with chilling and dehydration-sensitivity, make them unsuitable for prolonged storage. Thus, it poses some of the more significant challenges to seed technologists and growers in ensuring the availability of high-quality seeds when required. Recalcitrant seed procurement activities are frequently constrained by a lack of knowledge about precise recalcitrant seed handling techniques. The establishment of long-term storage on recalcitrant seeds using liquid nitrogen (-196°C) (cryopreservation), considered as the outcome of drying rate theory exploration. The initial rapid drying of seeds prior to storage, revealed to be the feasible method in reducing risk of damaged cells, further, to restore their viability during cryopreservation. In the contrary, under slow drying conditions, it is crucial that the water content of recalcitrant seeds remains high during short-term storage, to prevent dehydration stress that would ultimately lead to loss of viability. The major physiological element of the post-shedding life strategy is the preservation of a high hydration state, which is where all of the germination-preparation activities will take place. Decreasing moisture content under certain values or a general physiological deterioration with time, both factors

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contribute to the loss of viability. While the appropriate storage condition is important to maximize the seed's lifespan, it also contributes to the seed quality at the end of the storage. Method of lowering storage temperature could be practical as to minimize water loss, to reduce their metabolic activity, and to reduce infestation of seed associated myco-flora. However, the choice of impropriate storage conditions may also induce to the other rapid metabolism alteration and damages. Each species of recalcitrant seed seems to have a specific temperature at which it incurs desiccation and chilling injury. Hence, to develop a simple protocol with easy-handled post-harvest methodology, an equilibrium hydrated storage could be tested, in which cacao seeds will be allowed to equilibrate with the storage conditions of known temperature and relative humidity.

Mechanical and metabolism-linked damage revealed to contribute to the short lifespan of recalcitrant seeds during storage. Due to the high seed moisture content of recalcitrant seeds, maintaining seed volume by sustaining high seed moisture content is critical to avoid mechanical damage to the cell membrane and other components. The minimal mechanical changes may also eventually minimize and lowered the rate of deterioration caused by metabolism-linked reactivity. The presence of free water and the maintenance of a suitably high hydration status are principally responsible for the active state of all organelles and cytoplasm,as well as compartmentation and metabolic traffic between compartments. The whole enzymatic machinery proceeds to operate based on energy production and an active protein-synthesizing system at optimum cell hydration levels, giving the entirety of biochemical reactions typical of mature seed metabolism and preparing cells for growth commencement. This refers to the concept of metabolic readiness as it relates to germination readiness, as well as the aim to protect metabolic activity from harmful dehydration and chilling injury.

The ongoing dehydration and rehydration, as well as the continuous respiratory activity within living cells of recalcitrant seed may further induced their chilling and dehydration sensitivity. Food reserves mainly the soluble sugars represent a potential source of osmotic balancing and being a much more efficient component for growth initiation as to maintain substrates prior for respiratory activity. The increment of ROS activity generated mostly from the normal and an

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impaired respiratory activity. Hence, their detoxified mechanisms (e.g. antioxidant enzymes activities) played the important role in explaining seed deterioration development. Some previous studies revealed the oxidative stress development through the water loss which may as well reduced the efficiency of antioxidant enzymes activities. This is in reference to the requirement of a certain level of water in their cells structure for most enzymes to maintain their original conformation, allowing them to perform at their best. Since reduction in moisture content per se is deleterious to seed lifespan, thus, providing the hydrated storage with the aim of maintaining cells repairing mechanisms is necessity in explaining their comparisons. On the other hand, in acknowledging the variation of negative impacts due to metabolism-linked damage caused by ROS in high level of moisture content, the extent to which it could be minimize may as well through the maintenance of appropriate storage conditions.

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CHAPTER 3

MOISTURE EQUILIBRATION, PHYSIOLOGICAL QUALITY AND SEEDLING GROWTH OF CACAO SEEDS IN RELATION TO STORAGE CONDITIONS AND DURATIONS

3.1 Introduction

Seed deterioration results in the accumulation of harmful by-products. This decreased the ability of the seed to survive, which usually occurred through their normal physiological changes over time (Sun, 2002). While the seed moisture content exerts the greatest influence on their storage longevity, temperature and relative humidity are the determinants of the type of deteriorative reactions (Mbofung, 2012). At harvest maturity, the seed contains a hydrated glass state influenced by the amount of water within the seed. Since the stability of the glass state is very important for long-term seed survival, the initial seed quality should be preserved, and the seed must be properly stored between the time of harvest and the planting of a subsequent crop (Mbofung, 2012). The frequent fluctuations of relative humidity, however, have caused the variations of water content in the seed, which further complicates the processing and storage of seeds to minimize their loss of viability and vigour (Lisboa et al., 2017; Suma et al., 2013). The thermodynamic properties ofthe water within the seed will further decide the type of reaction that may take placewithin the seed (Vertucci & Roos, 1990). The drying or rehydration process may cause a change in water content on a fresh mass basis, reciprocal to the fresh tissuemass (Sun, 2002). The ideal metabolic rate in storage that could operate at a level that maintains the integrity of the embryos contributed to the conservation of morestored food reserves in the seed (Patil & Krishna, 2016).

Recalcitrant seeds of particularly tropical species are very often chilling-sensitive, and the temperature ranges of at least 10 to 15°C can cause injury and

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loss of viability (Tommasi et al., 2006). The exposure of the seeds at a slightly above critical chilling range may alleviate the effects and may delay the development of injury symptoms (Hatton, 1990). Categorized as recalcitrant, cacao seeds will be killed if stored in temperatures below 10°C (King & Roberts, 1979). High seed moisture content with temperatures reduced to subzero may disrupt cells due to the ice crystal formation which is termed as freezing injury. Temperatures above the freezing point, however, show variations of deleterious effects, and it became difficult to explain in terms of freezing damage as the temperature approaches 16 to 18°C for some recalcitrant species, including cacao (Hor, 1984).

While the appropriate storage condition is important to maximize the seed's lifespan, it also contributes to the seed quality at the end of the storage duration. The mechanisms that ensure germination and seedling development established cacao plants production successfully (Finch-Savage & Bassel, 2016). The ability to predict seed viability is of particular importance in the planning process of the agriculture industry to ensure that resources are allocated where and whenever they are needed. However, not all viable seeds have enough vigour to carry on producing good and healthy plants. Hamid (2015) suggested that the successful assessment ofgermination might be incomplete without observation of seedlings. The losses of seed vigour are related to a reduction in the ability of seeds to perform, which could be due to physiological deterioration (Shaban, 2013). A variety of storage atmospheres causing changes in the physiology and shelf life of recalcitrant seeds. However, the extent to which any factor(s) acts as the basic or critical cause for seed deteriorationand poor seedling vigour is debatable.

Despite all the trials to assess the ideal storage conditions for recalcitrant cacao seeds, there is still a lack of report on the correlation of viability and vigour performance of cacao seeds after storage. The common method to expose seeds to high temperature and moisture conditions will cause accelerated aging of the seed in the laboratory. The relatively slow deterioration may occur at low temperatures and moisture. Such treatments might yield useful information regarding the loss of seed viability and vigour during storage. Therefore, the objective of this study was toevaluate the physiological quality of cacao seeds stored at different temperatures and relative humidity (RH).

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3.2 Materials and Methods

3.2.1 Plant Material

Ripened cacao pods of PBC 123 clone were provided by the Malaysian Cocoa Board Tawau, Sabah. The cacao plants were planted at Pusat Penyelidikan dan Pembangunan Koko Madai, Kunak, Sabah (4.7965000487779506, 117.9670069587197). The harvested pods were transferred soonest possible to the Laboratory of Seed Technology, Faculty of Sustainable Agriculture, Universiti Malaysia Sabah (UMS), Sandakan, Sabah, Malaysia. Upon arrival, pods were temporarily stored at air-conditioned room temperature (25±2°C) and were used within one week. Seeds were extracted from pods, and mucilage was removed using sawdust and cleaned using a soft, dry sponge.

3.2.2 Storage Condition and Duration Treatments

The cacao seeds were packed into 28 x 20 cm of zip-lock polyethylene bags (250 seeds per bag) for 3 replications and stored at; (i) air-conditioned room temperature, RT (25±2°C, 55±5% RH), (ii) 16°C, 80% RH, (iii) 16°C, 60% RH, (iv) 16°C, 40% RH, (v) 14°C, 80% RH, (vi) 14°C, 60% RH, (vii) 14°C, 40% RH, (viii) 10°C, 80% RH, (ix) 10°C, 60% RH, (x) 10°C, 40% RH, and (xi) control (seeds freshly extracted from pods) (Figure 3.1). Seed hydrating procedures involved the use of a microprocessor-controlled console-style germinator (Seedburo Equipment Company, MPG-3000, United States). Seeds were sampled every 24 hours for duration up to 144 hours.

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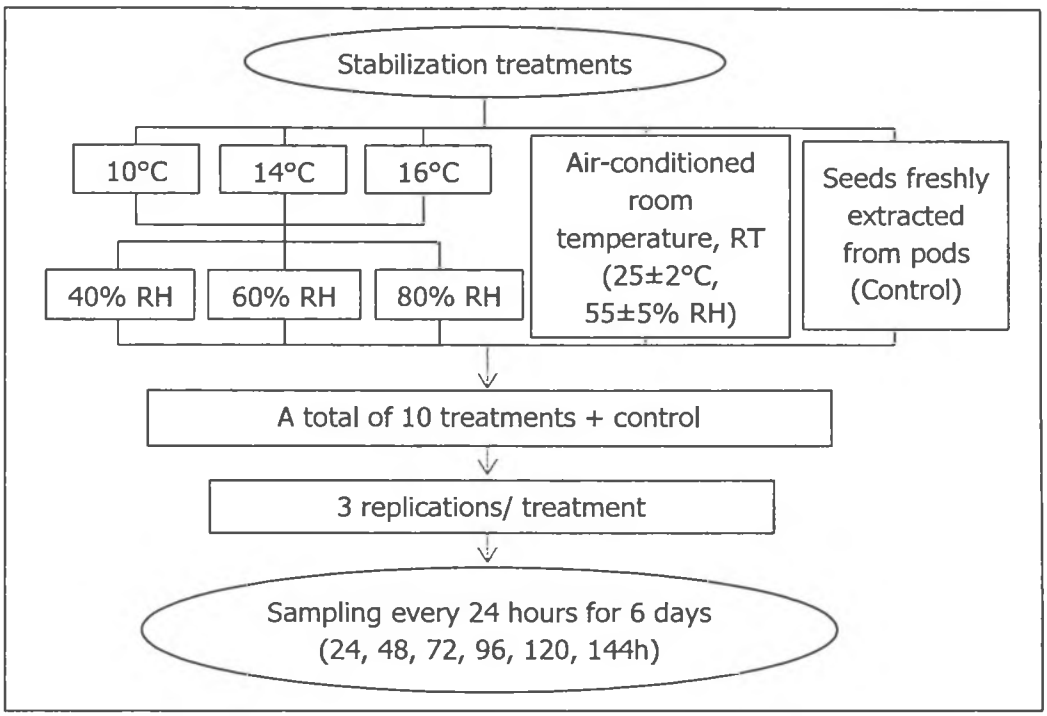


Figure 3.1 : Experimental procedures of cacao seeds stabilization at different storage temperatures and relative humidity

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3.2.3 Measurement of Seed Moisture Content

Moisture content (MC) was analyzed using 10 seeds per replication by the low constant temperature oven method. Seeds were dried at 103±2°C for 16±1 hours (ISTA, 2014) in an oven (Memmert Model-UNB 100, The Netherlands). The following formula determined moisture percentage:

$$MC\ (\%) = \frac{(\text{Fresh weight} - \text{dry weight})}{\text{Fresh weight}} \times 100$$

3.2.4 Measurement of Final Germination Percentage

Seed germination was conducted in trays in a laboratory maintained at 25±2°C. Fifty seeds per replication were kept between moist towels and observed until 21 days. Seed germination was recorded daily and accumulated as the final germination percentage until last day of observation (day 21).

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Seeds were considered germinated when a 2 mm protrusion of the radicle was visible (Michalak et al., 2013). Final germination percentage was calculated as follows:

$$\text{Final germination percentage (\%)} = \frac{(D1 + D2 + \ldots + D21)}{\text{Total no. of seeds tested}} \times 100$$

Where D1= Number of germinated seeds on the first day after germination test, D2= Number of germinated seeds on the second day after germination test

3.2.5 Measurement of Germination Rate Index

The germination rate index (GRI) reflects the germination percentage on each day of the germination period, with the higher GRI values indicating higher and faster germination (Kader, 2005). The following formula calculated GRI:

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

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Where G1= Germination percentage x 100 on the first day after sowing, G2= Germination percentage x 100 on the second day after sowing

3.2.6 Measurement of Mean Germination Time

Mean germination time (MGT) reflects the average germination speed of a population of seeds that have germinated. The lower MGT indicates, the faster a population has grown (Kader, 2005). The following formula calculated MGT:

$$\text{MGT (day)} = \frac{\sum fx}{\sum f}$$

Where f= seeds germinated on day x

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3.2.7 Measurement of Leachate Conductivity

The conductivity of seed leachate was determined by the method derived from Bonner (1996). Five seeds per replication for each treatment were drawn, weighed, and soaked in 25 mL ultrapure water. After 24 hours, the leachate was decanted, measured using the Eutech PC2700 Conductivity Meter, and expressed in $\mu S\ g^{-1}$.

3.2.8 Measurement of Seedling Growth

Thirty germinated seeds were sown in black polyethylene bags (22 x 10 cm) filled with a mixture of 20% chicken manure and 80% topsoil. Seedlings were grown under a rain shelter with 70% shade. Observations were made every week for the emergence of the shoot (shoot length, cm), and the number of leaves, starting from 14 days after sowing.

At the end of week 8, seedlings were harvested, and the remaining soil medium on roots was cleaned carefully with water to avoid damage. To measure dry matter production, shoots and roots were dried in an oven at 80°C for 24 hours, and the shoot and dry root biomass were recorded (Vanitha et al., 2005). The number of internodes and root length were also measured.

3.2.9 Measurement of Seedling Vigour Index

The early seedling growth and germination traits (seedling vigour index, SVI) (Kulkarni et al., 2007) were evaluated and calculated using the following formula:

$$SVI = (s + r) \times G$$

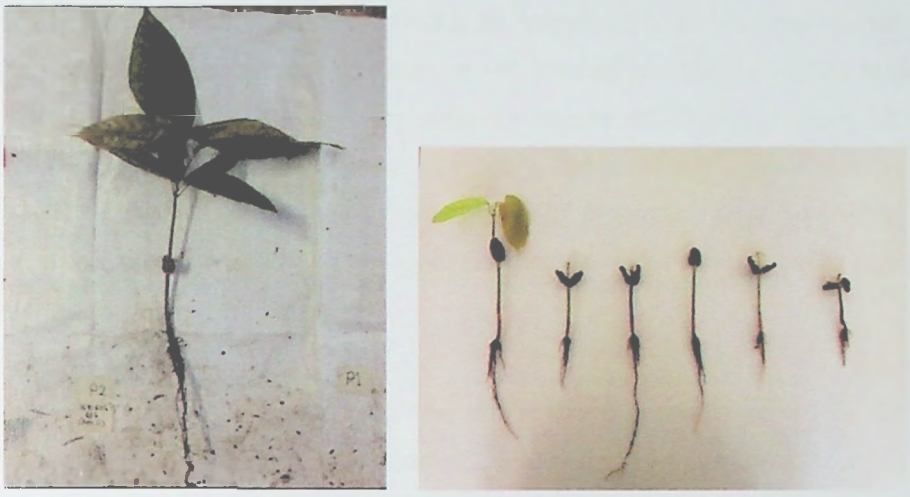
Where s = shoot length (in cm), and r = root length (in cm), at the end of data collections, G = final percentage germination

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3.2.10 Evaluation of Normal, Abnormal and Dead Seedlings

The percentage of normal (morphologically complete), abnormal and dead seedlings were recorded at the end of the test period (at the end of week 8). Dwarfed, stunted, and tip dry seedlings were common deformities observed in this study (Figure 3.2).



(a) (b)

Figure 3.2 : (a) Example of normal seedling (morphologically complete); (b) Example of abnormal (morphologically complete, with dwarf, stunted appearance) and dead seedlings (morphologically incomplete, tip dry seedling)

3.2.11 Statistical Analysis

The experiment was arranged in a completely randomized design (CRD), witha split plot arrangement (storage condition as the main plot and the storage durationsas the subplot) and with 3 replications. The data were analyzed using ANOVA, and the significant means were separated by least significant difference (LSD) ($P< 0.05$).

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3.3 Results

3.3.1 Seed Moisture Content

Freshly extracted cacao seeds (control) recorded 49.06% moisture content (MC) (Table 3.1). Cacao seeds stored at all conditions showed no significant differences of MC over the first 24 hours of storage (HOS) as compared to the control. Seeds at RT and those of control showed no variation in MC throughout the 144 HOS. However, seeds at 16°C, 80% RH showed significantly higher MC ranging from 52.29 to 56.23%, after 48 to 120 HOS. Seeds at 16°C, 60% RH exhibited a significant increase in MC after 120 hours and attained the highest MC (53.95%) after 144 HOS. While seeds at 16°C, 40% RH maintained a somewhat stable level of MC, with a significant reduction to 44.94%, after 96 HOS.

On the other hand, reducing the storage temperature to 14°C, 60% RH resulted in a significant decrease of seed MC, and the values remained low (43.91 to 46.89%) after 48 to 120 HOS. Whereas seeds at 14°C, 40% RH, and 80% RH showed a significant decrease of MC to 45.75 and 45.61% after 120 and 144 HOS, respectively. On the contrary, seeds at 10°C, 80% RH showed a significant decrease of MC to 43.54% at one point after 48 HOS. Seeds at 10°C, 60% RH exhibited a significant reduction of MC attaining the lowest MC of 44.56% after 144 HOS. Meanwhile, seeds at 10°C, 40% RH showed a significant increase of MC to 53.99% at one point after 72 HOS.

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Table 3.1 : Effect of stabilization treatments on the seed moisture content of cacao seeds for 144 HOS

Storage condition		SEED MOISTURE CONTENT (%)						
		Storage duration (hours)						
		0 (Control)	24	48	72	96	120	144
10°C	40% RH	49.06±1.30^{bc}	48.46±0.97^{bc}	50.34±0.11^{b}	53.99±3.02^{a}	46.62±0.90^{c}	46.40±0.87^{c}	47.83±0.60^{bc}
	60% RH	49.06±1.30^{ab}	46.73±1.87^{abc}	48.15±4.37^{ab}	49.09±0.99^{a}	47.73±2.50^{ab}	46.00±2.51^{bc}	44.56±4.19^{c}
	80% RH	49.06±1.30^{a}	46.20±3.59^{bc}	43.54±4.02^{c}	50.99±3.62^{a}	51.45±1.11^{a}	48.71±4.52^{ab}	47.36±1.90^{b}
14°C	40% RH	49.06±1.30^{b}	52.16±0.59^{a}	49.46±0.81^{ab}	47.71±2.78^{bc}	47.49±3.08^{bc}	45.75±1.47^{c}	45.46±3.23^{c}
	60% RH	49.06±1.30^{a}	46.40±1.65^{abc}	43.91±0.45^{c}	45.54±1.04^{bc}	44.65±0.57^{c}	46.89±0.09^{abc}	48.49±0.17^{ab}
	80% RH	49.06±1.30^{a}	47.64±0.05^{ab}	45.59±2.80^{b}	49.16±0.23^{a}	48.33±1.86^{ab}	50.44±0.63^{a}	45.61±0.83^{b}
16°C	40% RH	49.06±1.30^{a}	49.67±0.47^{a}	49.36±0.58^{a}	48.97±3.50^{a}	44.94±1.45^{b}	51.69±2.47^{a}	49.53±0.96^{a}
	60% RH	49.06±1.30^{b}	51.48±1.22^{ab}	45.18±4.52^{c}	49.11±1.41^{b}	51.61±1.74^{ab}	52.76±1.27^{a}	53.95±3.27^{a}
	80% RH	49.06±1.30^{d}	49.09±0.79^{d}	52.46±0.80^{bc}	56.23±2.57^{a}	52.29±1.00^{bc}	54.19±0.41^{ab}	51.00±1.16^{cd}
RT (25°C, 55% RH)		49.06±1.30^{ab}	48.14±0.05^{ab}	50.11±1.02^{a}	48.82±0.55^{ab}	46.95±0.46^{b}	47.88±0.82^{ab}	46.37±0.17^{b}

Note: Values are expressed as mean±SE. Mean values by different alphabets within the column are significantlydifferent at P ≤ 0.05 using LSD test. *LSD (5%) = 3.06*

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It is interesting to note that seed MC displayed significant correlations (Table 3.2) with some of the germination characteristics, and seedling vigour or growth performances variables; i) a significant positive correlations with FGP ($r=0.32^{**}$), LC ($r=0.16^{*}$), SVI ($r=0.30^{**}$), SDB ($r=0.33^{**}$), NORM ($r=0.30^{**}$), and ii) a significant negative correlations with both the MGT ($r= -0.18^{*}$) and DEAD ($r= -0.32^{**}$). Seeds are hygroscopic; they absorbed and released moisture to the atmosphere until both the seed (vapor pressure of moisture in the seed) and RH of the atmosphere (vapor pressure of atmospheric moisture) reached equilibrium. With seeds mostly recorded MC in the ranges of 40% and above, they have the full wetted conditions for any metabolic events to occur. In this study, cacao seeds especially the ones stored at 16°C and RT for at least 144 HOS, benefited the characters to retain high MC and produced higher seed viability and vigour. However, differences of moisture-retaining characters displayed for seeds at each treatment might induce different types and rates of changes or deterioration within seed during storage.

Table 3.2 : Pearson's correlation table

Corr	FGP	GRI	MGT	LC	SVI	SDB	NORM	ABN	DEAD
Seed MC	0.32^{**}	0.10	-0.18^{*}	0.16^{*}	0.30^{**}	0.33^{**}	0.30^{**}	-0.02	-0.32^{**}

Notes: **, significant at $P \leq 0.001$; *, significant at $P \leq 0.05$. MC: seed moisture content, FGP: final germination percentage, GRI: germination rate index, MGT: mean germination time, LC: leachate conductivity, SVI: seedling vigour index, SDB: seedling dry biomass, NORM: normal seedling, ABN: abnormal seedling, DEAD: dead seedling.

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3.3.2 Final Germination Percentage (FGP)

Control seeds germinated 97.50% (Table 3.3). Regardless of the RH, seeds at 16°C showed no significant differences from the control, with germination of 88.33 to 98.33% throughout 144 HOS. The same pattern was observed for seeds stored at RT, with an FGP of 95.8 to 100%. Among the chilling temperatures (10 and 14°C), seeds at 14°C, 40%, and 80% RH showed the insignificantly higher FGP (than the control) throughout 144 HOS, from 66.67 to 77.50% and from 68.00 to 78.67%, respectively (Table 3.3). This was followed by seeds at 14°C, 60% RH, which showed a consistent decrease of FGP (from 86.67 to 58.33%) over the first 96 hours after storage. The treatment further maintained the lower FGP (69.17%) after 144 HOS.

On the other hand, seeds at 10°C, 60% RH showed a decreasing pattern of FGP to produce their highest FGP (65.00%) after 144 HOS (Table 3.3). Whereas seeds at 10°C, 80%, and 40% RH recorded lower FGP compared to the control, but with inconsistent fluctuating values with increased storage duration. The treatments displayed the lowest FGP (53.33 and 59.33%, respectively) after 144 HOS.

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Table 3.3 : Effect of stabilization treatments on final germination percentage of cacao seeds for 144 HOS

Storage condition		FINAL GERMINATION PERCENTAGE (%)						
		Storage duration (hours)						
		0 (Control)	24	48	72	96	120	144
10°C	40% RH	97.50±2.50^{a}	78.33±3.82^{b}	62.67±9.50^{c}	74.17±3.82^{b}	58.33±2.89^{c}	42.67±9.50^{d}	59.33±1.15^{c}
	60% RH	97.50±2.50^{a}	90.83±8.78^{a}	77.50±7.50^{bc}	82.50±6.39^{b}	65.00±2.50^{d}	70.83±3.82^{cd}	65.00±10.50^{d}
	80% RH	97.50±2.50^{a}	71.67±9.46^{bc}	49.17±1.44^{de}	43.33±7.64^{e}	64.17±9.83^{c}	73.33±10.88^{b}	53.33±6.29^{d}
14°C	40% RH	97.50±2.50^{a}	77.50±5.00^{b}	71.67±3.82^{bc}	70.83±7.64^{bc}	72.50±7.50^{bc}	66.67±3.82^{c}	75.83±1.44^{b}
	60% RH	97.50±2.50^{a}	86.67±3.82^{b}	78.33±1.44^{bc}	75.83±3.82^{cd}	58.33±8.78^{f}	65.00±10.00^{ef}	69.17±7.64^{de}
	80% RH	97.50±2.50^{a}	76.00±2.00^{bc}	70.67±3.06^{bc}	74.00±2.00^{bc}	68.00±4.00^{c}	76.67±3.06^{bc}	78.67±1.15^{b}
16°C	40% RH	97.50±2.50^{a}	96.67±2.89^{ab}	97.50±2.50^{a}	95.83±1.44^{a}	88.33±6.29^{b}	97.50±0.00^{a}	94.17±1.44^{ab}
	60% RH	97.50±2.50^{a}	93.33±1.44^{a}	94.67±5.48^{a}	91.17±6.33^{a}	94.17±6.11^{a}	97.50±4.29^{a}	95.83±1.68^{a}
	80% RH	97.50±2.50^{a}	95.83±1.44^{a}	92.50±5.00^{a}	94.17±5.20^{a}	95.00±2.50^{a}	95.00±4.33^{a}	98.33±2.89^{a}
RT (25°C, 55% RH)		97.50±2.50^{a}	99.17±1.44^{a}	95.83±1.44^{a}	100±0.00^{a}	100±0.00^{a}	98.33±1.44^{a}	96.67±2.89^{a}

Note: Values are expressed as mean±SE. Mean values by different alphabets within the column are significantlydifferent at P ≤ 0.05 using LSD test. *LSD (5%) = 8.89*

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3.3.3 Germination Rate Index (GRI)

Despite the insignificant differences in FGP of seeds at 16°C and RT as compared to the control (Table 3.3), more variations showed in their speed of germination or GRI (Table 3.4). Control seeds had an average GRI value of 43.4% per day. Seeds at RT showed an increase of GRI with increased storage duration, to produce the highest GRI (75.38% per day) after 144 HOS.

After 24 HOS, seeds at 16°C, 80%, and 60% RH exhibited significantly lowerGRI than the control. The GRI values remained low throughout 144 HOS (Table 3.4). Those treatments recorded GRI ranging from 22.01 to 32.8% per day and 18.54 to 22.21% per day, respectively. Whereas seeds at 16°C, 40% RH showed significantlylower GRI than the control, for the first 48 HOS. The values, however, increased significantly to display GRI at the same level as the control (40.9 to 45.85% per day) after 120 HOS.

Regardless of RH, seeds at 14°C showed a significantly lower GRI after 24 HOS, as compared to the control. Those GRI remained low throughout the 144 HOS; 14°C, 40% RH (15.88 to 23.69 % per day), 14°C, 60% RH (12.21 to 19.03% per day) and 14°C, 80% RH (17.49 to 21.91% per day) (Table 3.4). Despite the fluctuating patterns of GRI throughout this study, seeds at 14°C (40%, 60%, and 80% RH) coincidently showed no significant differences of GRI with seeds at 16°C, 60% RH. As one of the indicators of seed vigour, the lower GRI of 16°C, 60% RH as compared to the other RH significantly justified the better seed quality showed withinthe other treatments of 16°C (40% and 80% RH).

The same pattern of poor GRI values was observed for seeds at 10°C (40%, 60%, and 80% RH), as those treatments showed significantly lower GRI than the control after 24 HOS. Thereafter, the results remained the lowest GRI values (12.39 to 15.75% per day) after 144 HOS.

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Table 3.4 : Effect of stabilization treatments on germination rate index of cacao seeds for 144 HOS

Storage condition		GERMINATION RATE INDEX (% PER DAY)						
		Storage duration (hours)						
		0 (Control)	24	48	72	96	120	144
10°C	40% RH	43.4±2.33^{a}	15.38±0.87^{b}	13.23±1.14^{bc}	16.38±0.58^{b}	12.23±1.08^{c}	8.41±1.74^{d}	12.7±0.31^{c}
	60% RH	43.4±2.33^{a}	18.98±1.07^{b}	15.66±1.58^{c}	12.42±1.11^{d}	13.38±0.29^{d}	16.38±0.17^{c}	15.75±1.49^{c}
	80% RH	43.4±2.33^{a}	17.21±1.46^{b}	8.93±0.87^{e}	7.43±0.70^{e}	11.2±0.78^{d}	14.44±0.93^{c}	12.39±0.91^{cd}
14°C	40% RH	43.4±2.33^{a}	19±0.68^{c}	23.69±0.38^{b}	20.9±1.59^{c}	15.88±1.74^{d}	16.43±0.18^{d}	19.54±0.51^{c}
	60% RH	43.4±2.33^{a}	17.67±0.65^{bcd}	15.93±0.57^{d}	19.03±0.35^{b}	12.21±0.87^{e}	16.58±0.89^{cd}	18.72±0.14^{bc}
	80% RH	43.4±2.33^{a}	19.16±1.27^{c}	17.49±1.07^{c}	19.09±0.37^{c}	18.04±0.56^{c}	21.91±0.55^{b}	21.96±0.43^{b}
16°C	40% RH	43.4±2.33^{b}	40.61±1.11^{c}	31.23±0.73^{d}	39.03±0.65^{c}	39.58±0.33^{c}	40.9±1.70^{c}	45.85±1.45^{a}
	60% RH	43.4±2.33^{a}	18.54±0.85^{c}	20.22±1.49^{b}	22.21±0.79^{b}	20.01±1.91^{c}	18.76±0.24^{c}	18.57±0.47^{c}
	80% RH	43.4±2.33^{a}	22.01±0.13^{d}	29.35±0.73^{c}	30.23±1.26^{c}	32.8±0.37^{b}	30.87±0.63^{bc}	31.23±0.96^{bc}
RT (25°C, 55% RH)		43.4±2.33^{c}	42.51±0.23^{c}	53.2±0.82^{b}	59.79±1.69^{b}	75.83±1.64^{a}	77.01±0.13^{a}	75.38±1.33^{a}

Note: Values are expressed as mean±SE. Mean values by different alphabets within the column are significantlydifferent at P ≤ 0.05 using LSD test. *LSD (5%) = 2.15*

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3.3.4 Mean Germination Time (MGT)

Control seeds recorded an average germination time of 2.6 days (Table 3.5). Other than GRI's variable, different values of mean germination time (MGT) might also be one of the early indicators on the changes of metabolic events (towards germination) within seeds, which were found to significantly differ between storage treatments in this study. Only seeds at RT showed a significant decrease (42%) in MGT as compared to the control, along with the increased storage duration. The values remained to be low (from 1.53 to 2.43 of MGT) throughout 144 HOS.

Seeds at 16°C, 40% RH showed the least differences of MGT as compared to the control throughout 144 HOS (Table 3.5). The treatment recorded means of 2.63to 3.83 days to germinate. Whereas seeds at 16°C with the higher RH (60% and 80% RH) recorded significantly higher, but gradually reducing values of MGT than the control, along with the increased storage duration. After 144 HOS, those treatments recorded MGT of 4.14 and 4.64 days, respectively. The same pattern was recorded for seeds at 14°C with the higher RH. After 144 HOS, those treatments displayed MGT of 3.98 and 4.63 for seeds at 14°C, 80%, and 60% RH, respectively.

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On the other hand, seeds at 14°C, 40% RH exhibited a higher MGT than the control, but with a fluctuating value throughout 144 HOS (Table 3.5). The treatment further recorded an MGT of 4.67 days after 144 HOS. The same pattern was observed for seeds at 10°C, regardless of the RH. The ones stored at 80% RH were observed to display among the longest time (highest MGT) to germinate (5.55 to 7.28 of MGT) especially after 48 to 144 HOS. The one stored at 40% and 60% RH followed the pattern to record among the longest time to germinate especially at 120 to 144 HOS (5.04 to 5.31 of MGT).

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Table 3.5 : Effect of stabilization treatments on mean germination time of cacao seeds for 144 HOS

Storage condition		MEAN GERMINATION TIME (DAYS)						
		Storage duration (hours)						
		0 (Control)	24	48	72	96	120	144
10°C	40% RH	2.63±0.32^{c}	5.70±0.54^{ab}	5.55±0.11^{ab}	5.20±0.44^{b}	5.98±0.40^{a}	5.76±0.34^{ab}	5.31±0.20^{ab}
	60% RH	2.63±0.32^{d}	5.43±0.11^{bc}	5.91±0.81^{b}	7.10±0.20^{a}	5.99±0.20^{b}	5.14±0.37^{c}	5.04±0.23^{c}
	80% RH	2.63±0.32^{f}	4.62±0.62^{e}	6.64±0.93^{bc}	7.28±0.42^{a}	6.70±0.42^{bc}	6.26±0.45^{c}	5.55±0.53^{d}
14°C	40% RH	2.63±0.32^{d}	5.07±0.04^{ab}	4.51±0.33^{bc}	4.07±0.54^{c}	5.48±0.80^{a}	5.00±0.19^{ab}	4.67±0.26^{bc}
	60% RH	2.63±0.32^{c}	6.05±0.27^{a}	6.01±0.18^{a}	5.86±0.09^{a}	5.60±0.18^{a}	4.50±0.25^{b}	4.63±0.26^{b}
	80% RH	2.63±0.32^{d}	5.00±0.24^{a}	4.88±0.35^{a}	4.38±0.04^{ab}	4.60±0.06^{ab}	3.87±0.14^{c}	3.98±0.21^{bc}
16°C	40% RH	2.63±0.32^{b}	2.85±0.09^{b}	3.83±0.19^{a}	2.84±0.15^{b}	2.81±0.03^{b}	3.16±0.32^{ab}	2.60±0.15^{b}
	60% RH	2.63±0.32^{c}	6.26±0.26^{a}	5.66±0.89^{a}	4.94±1.29^{b}	4.24±0.54^{b}	4.74±0.43^{b}	4.64±0.10^{b}
	80% RH	2.63±0.32^{d}	4.57±0.06^{a}	3.67±0.08^{b}	3.47±0.27^{bc}	3.34±0.08^{c}	3.70±0.26^{bc}	4.14±0.38^{ab}
RT (25°C, 55% RH)		2.63±0.32^{a}	2.43±0.13^{a}	2.23±0.03^{ab}	2.11±0.08^{abc}	1.69±0.08^{bc}	1.53±0.05^{c}	1.53±0.16^{c}

Note: Values are expressed as mean±SE. Mean values by different alphabets within the column are significantly different at $P \leq 0.05$ using LSD test. *LSD (5%) = 0.68*

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Table 3.6 shows the Pearson's correlations between germination variables (FGP, GRI and MGT) and seed/seedling vigour variables (LC, SVI, SDB, I/N, LEAF, NORM, ABN and DEAD). Among those variables, only GRI showed significant correlations with all the vigour variables measured. The results showed highly positive correlations with LC ($r=0.18^{**}$), SVI ($r=0.68^{**}$), SDB ($r=0.51^{**}$), I/N ($r=0.31^{**}$), LEAF ($r=0.29^{**}$) and NORM ($r=0.70^{**}$). Whereas GRI also showed the significant negative correlations with ABN ($r=-0.32^{**}$) and DEAD ($r=-0.65^{**}$).

On the other hand, the FGP variable shows a strong and positive correlation with SVI ($r=0.83^{**}$), SDB ($r=0.66^{**}$), I/N ($r=0.38^{**}$), LEAF ($r=0.49^{**}$) and NORM ($r=0.80^{**}$) (Table 3.6). It showed negative correlation with ABN ($r=-0.18^{*}$) and DEAD ($r=-0.79^{**}$) variable. Furthermore, the MGT variable showed significant negative correlation with most of the vigour variables; LC ($r=-0.24^{**}$), SVI ($r=-0.64^{**}$), SDB ($r=-0.48^{**}$), I/N ($r=-0.26^{**}$), LEAF ($r=-0.28^{**}$) and NORM ($r=-0.69^{**}$). In contrast, it only showed positive correlation with ABN ($r=0.32^{**}$) variable.

Table 3.6 : Pearson's correlation table

Corr	LC	SVI	SDB	I/N	LEAF	NORM	ABN	DEAD
FGP	0.14^{*}	0.83^{**}	0.66^{**}	0.38^{**}	0.49^{**}	0.80^{**}	-0.18^{*}	-0.80^{**}
GRI	0.18^{**}	0.68^{**}	0.51^{**}	0.31^{**}	0.29^{**}	0.70^{**}	-0.32^{**}	-0.65^{**}
MGT	-0.24^{**}	-0.64^{**}	-0.48^{**}	-0.26^{**}	-0.28^{**}	-0.69^{**}	0.32^{**}	0.64^{**}

Notes: **, significant at $P \leq 0.001$; *, significant at $P \leq 0.05$. FGP: final germination percentage, GRI: germination rate index, MGT: mean germination time, MC: seed moisture content, LC: leachate conductivity, SVI: seedling vigour index, SDB: seedling dry biomass, I/N: seedling internode, LEAF: leaf number, NORM: normal seedling, ABN: abnormal seedling, DEAD: dead seedling

3.3.5 Seed Leachate Conductivity

Control seeds recorded an average of 82.12 $\mu S\ g^{-1}$ of leachate conductivity (LC) (Table 3.7). The higher the LC value, indicating the bad conditions of the cell's membrane within the seed, and vice versa. In this study, seeds at 16°C exhibited significantly higher LC values (101.97 to 128.3 $\mu S\ g^{-1}$) after only 24 HOS. Seeds at 16°C, 80%, and 40% RH further showed the increasing pattern of LC with the increased storage duration. Among those treatments, seeds at 16°C, 40% RH, was

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observed to show a higher range of LC values (128.3 to 197.42 μS g^{-1}) throughout 144 HOS. Seeds at 16°C, 60% RH showed the increased pattern of LC at the beginningof storage, but the values slowly decreased after 96 HOS to record the LC value backto the same level as the control (98.54 μS g^{-1}) after 144 HOS (Table 3.7). Similarly, seeds at RT exhibited the increase pattern of LC over the first 72 HOS, but then, showed the reducing pattern of LC with the increased storage duration. The treatment was observed to display the lowest LC value (78.2 μS g^{-1}) after 144 HOS.

More fluctuated pattern of LC displayed for seeds at 14°C. Regardless of RH, seeds at 14°C exhibited LC values at the same level as the control, ranging from 79.04 to 89.37 μS g^{-1}, after 24 HOS (Table 3.7). Only seeds at 14°C, 60% RH showed the increasing LC values with the increased storage duration, to produce the significantly higher LC value (139.77 μS g^{-1}) after 144 HOS. In comparison, seedsat 14°C, 40%, and 80% RH showed similar fluctuating patterns of LC throughout 144 HOS. Those treatments recorded the LC values of 67.91 and 105.8 μS g^{-1}, respectively. On the other hand, seeds at 10°C, 60%, and 80% RH displayed LC values atthe same level as the control after 24 HOS (Table 3.7). Both treatments showed an increasing pattern of LC along the increased storage duration to produce 99.46 and 40.53 μS g^{-1}, respectively, after 144 HOS. Meanwhile, seeds at 10°C, 40% RH exhibited the highest LC value (128.12 μS g^{-1}) after 24 HOS. The treatment further showed a decreasing pattern of LC with the increased storage duration but reverted to display among the highest values (129.76 μS g^{-1}) after 144 HOS.

In this study, there were low (significant) correlations show for seed LC and germination characteristics (FGP, GRI, and MGT) (Table 3.6), which indicated their positive influences on the germination processes. However, the fact that the seed LC patterns fluctuating within 144 HOS justified more on the active metabolic events (membrane changes due to damaging and repairing mechanisms) which might have started to maintain the homeostasis within cells.

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Table 3.7 : Effect of stabilization treatments on leachate conductivity of cacao seeds for 144 HOS

Storage condition		LEACHATE CONDUCTIVITY ($\mu S\ g^{-1}$)						
		Storage duration (hours)						
		0 (Control)	24	48	72	96	120	144
10°C	40% RH	82.12±6.64^{c}	128.12±10.94^{a}	100.40±9.95^{bc}	108.03±4.80^{ab}	84.49±1.50^{c}	90.07±9.27^{bc}	129.76±12.14^{a}
	60% RH	82.12±6.64^{ab}	64.80±3.26^{bc}	55.59±6.35^{c}	85.34±4.95^{ab}	80.97±3.64^{ab}	80.24±10.97^{ab}	99.46±10.87^{a}
	80% RH	82.12±6.64^{c}	84.52±3.83^{c}	94.26±11.82^{c}	83.01±5.00^{c}	103.62±8.50^{bc}	120.37±5.68^{ab}	140.53±8.63^{a}
14°C	40% RH	82.12±6.64^{bc}	89.37±10.46^{bc}	113.06±1.80^{a}	93.33±1.90^{ab}	71.31±5.85^{bc}	101.77±8.17^{ab}	67.91±3.66^{c}
	60% RH	82.12±6.64^{c}	79.04±1.70^{c}	69.52±11.65^{c}	86.01±5.74^{c}	110.88±7.71^{b}	139.69±1.43^{a}	139.77±8.38^{a}
	80% RH	82.12±6.64^{c}	87.56±1.65^{bc}	73.62±9.51^{c}	118.90±2.00^{a}	75.04±11.69^{c}	93.63±4.65^{bc}	105.80±9.81^{ab}
16°C	40% RH	82.12±6.64^{d}	128.30±6.76^{c}	144.54±12.77^{bc}	137.89±10.56^{c}	178.24±11.09^{ab}	169.12±12.89^{b}	197.42±10.90^{a}
	60% RH	82.12±6.64^{c}	101.97±1.94^{abc}	113.70±5.24^{ab}	123.61±11.02^{a}	96.06±3.56^{bc}	95.16±7.60^{bc}	98.54±4.08^{bc}
	80% RH	82.12±6.64^{d}	106.75±1.78^{bc}	88.81±2.18^{cd}	111.59±9.50^{b}	123.71±9.83^{b}	157.68±4.61^{a}	147.84±9.27^{a}
RT (25°C, 55% RH)		82.12±6.64^{c}	119.87±9.38^{b}	175.14±10.72^{a}	169.75±11.35^{a}	97.54±6.01^{bc}	105.65±9.14^{b}	78.20±11.63^{c}

Note: Values are expressed as mean±SE. Mean values by different alphabets within the column are significantlydifferent at $P \leq 0.05$ using LSD test. *LSD (5%) = 22.52*

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3.3.6 Seedling Growth Performance: Normal, Abnormal, and Dead Seedling

Control seeds produced 100% normal seedlings (Table 3.8). The same pattern was established by seeds stored at RT throughout 144 HOS. This was followed by seeds at 16°C, which showed no dead seedlings with a greater percentage of normal seedlings than the abnormal (physical appearances: dwarfed, stunted, and tip dry seedlings) ones throughout 144 HOS. Regardless of RH, seeds at 16°C exhibited 100% of normal seedlings after 24 HOS. Seeds at 16°C, 40% RH were observed to exhibit the least percentage of abnormal seedlings (6.7%) after 72, 96, and 144 hours after storage. In contrast, seeds at 60% and 80% RH showed higher abnormal seedlings (ranging from 6.7 to 33.3%) after 48 until 144 HOS.

Regardless of RH, seeds at 14°C showed the consistent reducing pattern of normal seedlings along 144 HOS (Table 3.8). Among the treatments, seeds at 14°C, 80% RH were observed to display the highest reduced percentage (92%) from 80 to 6.7% of normal seedlings after 144 HOS. The same treatment was also observed to produce the higher increased percentage (82%) from 13.3 to 73.3% of dead seedlings throughout 144 HOS. Comparing seeds at 10°C, the ones stored at 40% RH show a greater percentage of normal seedlings (ranging from 46.7 to 60%) over the first 72 HOS, as well as their dead seedlings (ranging from 40 to 46.7%) (Table 3.8). The treatment later exhibited 100% of dead seedlings until 144 HOS. In contrast, seeds at the higher RH (10°C, 60%, and 80% RH) displayed lesser normal seedlings (ranging from 13.3 to 46.7%), along with the greater dead seedlings (ranging from 33.3 to 80%, respectively) over the first 96 HOS. Both treatments further exhibited 100% of dead seedlings until 144 HOS.

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Table 3.8 : Effect of stabilization treatments on seedlings performance of cacao seeds for 144 HOS

NORMAL SEEDLING (%)

Storage condition		Storage duration (hours)						
		0 (Ctrl)	24	48	72	96	120	144
10°C	40% RH	100^{a}	53.3^{b}	46.7^{b}	60^{b}	0^{c}	0^{c}	0^{c}
	60% RH	100^{a}	26.7^{b}	13.3^{bc}	26.7^{b}	13.3^{bc}	0^{c}	0^{c}
	80% RH	100^{a}	46.7^{b}	20^{c}	0^{c}	13.3^{c}	0^{c}	0^{c}
14°C	40% RH	100^{a}	26.7^{b}	33.3^{b}	13.3^{bc}	13.3^{bc}	33.3^{b}	0^{c}
	60% RH	100^{a}	46.7^{bc}	40^{bc}	53.3^{b}	13.3^{d}	13.3^{d}	26.7^{cd}
	80% RH	100^{a}	80^{ab}	60^{bc}	33.3^{d}	26.7^{de}	40^{cd}	6.7^{e}
16°C	40% RH	100^{a}	100^{a}	100^{a}	93.3^{a}	93.3^{a}	100^{a}	93.3^{a}
	60% RH	100^{a}	100^{a}	66.7^{b}	80^{ab}	100^{a}	73.3^{b}	86.7^{ab}
	80% RH	100^{a}	100^{a}	93.3^{a}	100^{a}	100^{a}	86.7^{a}	73.3^{a}
RT (25°C, 55% RH)		100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}

ABNORMAL SEEDLING (%)

Storage condition		Storage duration (hours)						
		0 (Ctrl)	24	48	72	96	120	144
10°C	40% RH	0^{a}	6.7^{a}	6.7^{a}	0^{a}	0^{a}	0^{a}	0^{a}
	60% RH	0^{b}	6.7^{b}	6.7^{b}	20^{a}	20^{a}	0^{b}	0^{b}
	80% RH	0^{b}	20^{a}	6.7^{b}	20^{a}	26.7^{a}	0^{b}	0^{b}
14°C	40% RH	0^{b}	6.7^{b}	26.7^{a}	6.7^{b}	6.7^{b}	0^{b}	20^{a}
	60% RH	0^{d}	13.3^{bc}	6.7^{cd}	13.3^{bc}	26.7^{b}	6.7^{cd}	67^{a}
	80% RH	0^{c}	6.7^{c}	20^{b}	20^{b}	33.3^{a}	20^{b}	20^{b}
16°C	40% RH	0^{a}	0^{a}	0^{a}	6.7^{a}	6.7^{a}	0^{a}	6.7^{a}
	60% RH	0^{d}	0^{d}	33.33^{a}	20^{bc}	0^{d}	26.7^{ab}	13.3^{c}
	80% RH	0^{c}	0^{c}	6.67^{bc}	0^{c}	0^{c}	13.3^{b}	26.7^{a}
RT (25°C, 55% RH)		0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}

DEAD SEEDLING (%)

Storage condition		Storage duration (hours)						
		0 (Ctrl)	24	48	72	96	120	144
10°C	40% RH	0^{c}	40^{b}	46.7^{b}	40^{b}	100^{a}	100^{a}	100^{a}
	60% RH	0^{d}	66.7^{bc}	80^{b}	53.3^{c}	66.7^{bc}	100^{a}	100^{a}
	80% RH	0^{e}	33.3^{d}	73.3^{bc}	80^{b}	60^{c}	100^{a}	100^{a}
14°C	40% RH	0^{c}	66.7^{a}	40^{b}	80^{a}	80^{a}	66.7^{a}	80^{a}
	60% RH	0^{d}	40^{c}	53.3^{b}	33.3^{c}	60^{b}	80^{a}	66.7^{a}
	80% RH	0^{d}	13.3^{cd}	20^{c}	46.7^{b}	40^{b}	40^{b}	73.3^{a}
16°C	40% RH	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
	60% RH	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
	80% RH	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
RT (25°C, 55% RH)		0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}

Note: Values are expressed as mean. Mean values by different alphabets within the column are significantly different at P≤0.05 using LSD test. *LSD (5%) = NORMAL (22.57); ABNORMAL (9.47); DEAD (17.12)*

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Seedling performances derived from seeds subjected to different storage treatments in this study indicated the pattern of deterioration was directly displayed through the exposure of seedling establishment processes to the external environment. Seeds stored at 14°C showed better germination characteristics than seeds at 10°C, however gradually postulated the negative effects on their seedling vigour. The similar displayed pattern of dead seedlings for both treatments (10 and 14°C) as early as at 24 HOS, indicated dominations of storage temperature over different RH reflected through their poor quality of germination characteristics, which later failed to support their seedling establishment.

3.3.7 Seedling Dry Biomass

In this study, control seeds recorded an average seedling dry biomass (SDB) of 1.78 g (Table 3.9). With the variety of causes to influence the pattern of SDB in this study, the positive and higher significant correlations with the germination characteristics justified the high-quality seeds to produce the higher seed or seedling vigour. Similar domination (as normal, abnormal, and dead seedlings variables) on different storage temperatures was displayed for SDB variable (better SDB showed for seeds at RT, followed by seeds at 16°C).

Seeds at RT produced similarly high SDB values as the control throughout the 144 HOS. The treatment recorded an SDB of 1.69 to 2.03 g per plant. The same pattern was recorded for seeds at 16°C, 60% RH, but with lower values of SDB (1.4 to 1.81 g per plant) throughout the 144 HOS, compared to the seeds at RT. Moreover, seeds at 16°C, 80% RH recorded the highest SDB (2.25 to 2.44 g per plant) over the first 72 HOS (Table 3.9). Then, SDB values declined significantly with the increased storage duration to produce among the lowest SDB (1 g per plant) after 144 HOS. Meanwhile, seeds at 16°C, 40% RH recorded significant reductions of SDB after 72 HOS to produce SDB of 1.34 g per plant after 144 HOS.

On the other hand, the poor seed quality is continually reflected through the significantly lower SDB derived from seeds stored earlier at 14°C and followed by seeds at 10°C. Seeds at 14°C (40%, 60%, and 80% RH) recorded the reduced pattern of SDB with the increased storage duration (Table 3.9). Those treatments lowered

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SDB to 0.4 and 0.3 g per plant, respectively, after 144 HOS. Moreover, seeds at 10°C, 40%, and 80% RH showed a gradual reduction of SDB with the increased storage duration (Table 3.9). Those treatments recorded SDB of 1.27 and 1.07 g per plant after 72 and 96 HOS, respectively. Meanwhile, seeds at 10°C, 60% RH recorded the fluctuated pattern of SDB over the first 48 HOS but it continued to decline with the increased storage duration. The treatment recorded SDB ranging from 0.46 to 1.66 g per plant for over 96 HOS.

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Table 3.9 : Effect of stabilization treatments on seedling dry biomass of cacao seeds for 144 HOS

Storage condition		SEEDLING DRY BIOMASS (g per plant)						
		Storage duration (hours)						
		0 (Control)	24	48	72	96	120	144
10°C	40% RH	1.78±0.27^{a}	2.11±0.20^{a}	1.64±0.18^{ab}	1.27±0.09^{b}	-	-	-
	60% RH	1.78±0.27^{a}	1.17±0.12^{b}	1.66±0.12^{a}	0.73±0.32^{c}	0.46±0.29^{c}	-	-
	80% RH	1.78±0.27^{a}	1.67±0.45^{ab}	1.32±0.36^{bc}	1.26±0.09^{bc}	1.07±0.42^{c}	-	-
14°C	40% RH	1.78±0.27^{a}	1.26±0.89^{b}	0.82±0.08^{c}	0.96±0.15^{bc}	1.37±0.28^{ab}	0.82±0.08^{c}	0.35±0.05^{d}
	60% RH	1.78±0.27^{a}	1.02±0.10^{b}	1.00±0.23^{b}	0.97±0.21^{b}	0.46±0.08^{cd}	0.78±0.13^{bc}	0.32±0.02^{d}
	80% RH	1.78±0.27^{a}	1.37±0.32^{ab}	1.26±0.28^{b}	1.01±0.13^{bc}	0.79±0.34^{cd}	0.96±0.10^{bc}	0.43±0.03^{d}
16°C	40% RH	1.78±0.27^{ab}	1.82±0.15^{ab}	1.95±0.07^{a}	1.48±0.10^{bc}	1.27±0.15^{c}	1.24±0.08^{c}	1.34±0.06^{c}
	60% RH	1.78±0.27^{a}	1.81±0.18^{a}	1.40±0.16^{a}	1.62±0.36^{a}	1.80±0.33^{a}	1.53±0.31^{a}	1.64±0.25^{a}
	80% RH	1.78±0.27^{b}	2.25±0.18^{a}	2.44±0.15^{a}	2.38±0.13^{a}	2.02±0.23^{ab}	1.44±0.29^{c}	1.00±0.02^{d}
RT (25°C, 55% RH)		1.78±0.27^{a}	2.01±0.35^{a}	1.99±0.12^{a}	1.69±0.10^{a}	1.70±0.10^{a}	2.03±0.21^{a}	1.73±0.17^{a}

Note: Values are expressed as mean±SE. Mean values by different alphabets within the column are significantlydifferent at P ≤ 0.05 using LSD test. *LSD (5%) = 0.42*

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3.3.8 Number of Leaves, Number of Internodes, Shoot Length, and Root Length

Growth variables of seedlings derived from seeds subjected to different storage treatments (at 48, 96, and 144 hours after storage) are presented in Table 3.10 (number of leaves and internodes) and 3.11 (shoot and root length). For the number of leaves variable measured, the control seeds recorded the increasing number of leaves (3 to 7 leaves per plant), with the increase of days after sowing (DAS) until 42 DAS. Besides, the control seeds also produced 3 internodes, with an increase (14.27 to 22.17 cm) of shoot length and 18 cm of root length.

Among seedlings derived from seeds stored at the chilling conditions, seeds at 10°C, 40% RH were observed to produce among the higher number of leaves (6 to 8 leaves) and shoot length (15.4 to 24.1 cm), which are in parallel with their higher normal seedlings (46.7 to 60%) (Table 3.8) produced until 72 HOS. The pattern, however, was discontinued the extension of storage duration (until 144 HOS), as the storage treatment recorded 100% of dead seedlings. Even though with the higher survival rate, seeds stored at the higher RH (10°C, 60%, and 80% RH), display the reducing pattern of normal seedlings after 96 HOS and recorded 100% dead seedlings after 120 HOS (Table 3.8). The poor seedling development was also reflected through the low number of leaves and shoot length, as well as their root length produced throughout 144 HOS. A similar pattern showed for seeds stored at 14°C, as seedlings derived from those treatments displayed reducing quality of seedling development through reducing pattern of normal seedlings produced (Table 3.8), number of leaves and shoot length, as well as their root length produced especially after 96 until 144 HOS. As for the number of internodes variable, a more significantly lower number of internodes displayed after 96 until 144 HOS, for seeds stored at 10 and 14°C than the control, RT and 16°C.

On the other hand, seeds stored at RT and 16°C were observed to produce a consistently higher seedling development quality reflected through the significantly higher percentage of normal seedlings (Table 3.8), seedling dry biomass (Table 3.9), number of leaves (6 to 11 leaves) (Table 3.10), as well as the consistently higher with no variations of shoot length (17.17 to 28.27 cm) (Table 3.11), root length (16.5

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to 23.23 cm) (Table 3.11), and number of internodes (2 to 4 internodes) (Table 3.12) displayed throughout 144 HOS.

Table 3.10 : Effect of stabilization treatments on the number of leaves and internodes of cacao seedlings

Duration (hours)	Storage condition		NO. OF LEAVES					NO. OF I/NODES
			14 DAS	21 DAS	28 DAS	35 DAS	42 DAS	
	Control		3^{ab}	4^{cd}	5^{a}	5^{cd}	7^{cd}	3^{c}
24h	10°C	40% RH	5^{a}	7^{a}	7^{a}	9^{a}	11^{a}	5^{a}
		60% RH	0^{c}	3^{d}	3^{c}	3^{d}	5^{d}	3^{c}
		80% RH	4^{a}	5^{ab}	6^{ab}	7^{ab}	7^{cd}	3^{c}
	14°C	40% RH	3^{a}	6^{ab}	6^{ab}	7^{c}	7^{cd}	3^{c}
		60% RH	1^{bc}	4^{cd}	5^{ab}	6^{c}	8^{bc}	3^{c}
		80% RH	3^{a}	6^{ab}	6^{ab}	6^{c}	7^{cd}	4^{ab}
	16°C	40% RH	4^{a}	5^{bc}	6^{ab}	7^{bc}	9^{ab}	4^{ab}
		60% RH	4^{a}	4^{cd}	5^{bc}	5^{cd}	9^{ab}	4^{bc}
		80% RH	5^{a}	5^{a}	6^{ab}	8^{ab}	8^{ab}	3^{c}
	RT (25°C, 55% RH)		5^{a}	5^{a}	7^{a}	8^{ab}	9^{ab}	4^{ab}
	Control		3^{c}	4^{bc}	5^{c}	5^{ab}	7^{c}	3^{ab}
48h	10°C	40% RH	4^{bc}	4^{bc}	4^{c}	4^{c}	6^{c}	3^{ab}
		60% RH	3^{c}	3^{c}	4^{c}	4^{c}	5^{c}	2^{b}
		80% RH	2^{cd}	4^{bc}	5^{c}	5^{ab}	6^{c}	3^{ab}
	14°C	40% RH	5^{ab}	5^{b}	5^{bc}	6^{ab}	8^{ab}	3^{ab}
		60% RH	1^{d}	4^{bc}	5^{c}	5^{ab}	8^{bc}	4^{a}
		80% RH	5^{a}	7^{a}	7^{a}	7^{a}	8^{ab}	3^{ab}
	16°C	40% RH	5^{ab}	5^{bc}	6^{ab}	7^{a}	9^{ab}	3^{a}
		60% RH	3^{c}	4^{bc}	4^{c}	5^{bc}	7^{c}	3^{ab}
		80% RH	4^{ab}	5^{b}	6^{ab}	7^{ab}	8^{ab}	3^{ab}
	RT (25°C, 55% RH)			5^{bc}	7^{ab}	8^{a}	11^{a}	4^{a}
	Control		3^{c}	4^{ab}	5^{ab}	5^{abd}	7^{ab}	3^{a}
72h	10°C	40% RH	4^{ab}	5^{ab}	6^{ab}	7^{ab}	8^{ab}	4^{a}
		60% RH	2^{c}	4^{c}	4^{c}	5^{cd}	6^{bc}	3^{a}
		80% RH	2^{c}	3^{c}	3^{c}	3^{d}	3^{c}	4^{a}
	14°C	40% RH	6^{a}	6^{ab}	6^{ab}	6^{c}	7^{ab}	3^{a}
		60% RH	2^{c}	4^{ab}	5^{bc}	6^{bc}	7^{ab}	3^{a}
		80% RH	4^{ab}	5^{ab}	6^{ab}	7^{ab}	7^{ab}	4^{a}
	16°C	40% RH	4^{ab}	5^{ab}	6^{ab}	6^{ab}	8^{ab}	3^{a}
		60% RH	3^{bc}	4^{c}	4^{c}	5^{cd}	7^{ab}	3^{a}
		80% RH	5^{a}	6^{a}	7^{a}	8^{a}	9^{a}	4^{a}
	RT (25°C, 55% RH)		4^{ab}	4^{bc}	6^{ab}	6^{ab}	7^{ab}	4^{a}

Note: Values are expressed as mean. Mean values by different lowercase alphabets within the row are significantly different at $P \leq 0.05$ using LSD test. *LSD (5%) = NO. OF LEAVES (1.17)*

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Duration (hours)	Storage condition		NO. OF LEAVES					NO. OF I/NODES
			14 DAS	21 DAS	28 DAS	35 DAS	42 DAS	
	Control		3^{ab}	4^{a}	5^{ab}	5^{ab}	7^{ab}	3^{ab}
96h	10°C	40% RH	0^{d}	0^{b}	0^{c}	0^{c}	0^{c}	0^{c}
		60% RH	3^{c}	4^{a}	4^{ab}	5^{ab}	8^{ab}	3^{ab}
		80% RH	3^{bc}	4^{a}	5^{ab}	5^{ab}	6^{bc}	2^{b}
	14°C	40% RH	4^{ab}	5^{a}	6^{ab}	6^{a}	8^{ab}	3^{ab}
		60% RH	0^{d}	3^{a}	4^{b}	4^{b}	5^{b}	3^{ab}
		80% RH	4^{ab}	5^{a}	5^{ab}	5^{ab}	5^{b}	3^{ab}
	16°C	40% RH	4^{ab}	4^{a}	5^{ab}	6^{ab}	7^{ab}	3^{ab}
		60% RH	5^{a}	5^{a}	5^{ab}	6^{a}	9^{a}	4^{a}
		80% RH	4^{ab}	5^{a}	6^{a}	7^{a}	8^{ab}	3^{ab}
	RT (25°C, 55% RH)		4^{ab}	4^{a}	4^{ab}	5^{ab}	7^{ab}	4^{a}
	Control		3^{ab}	4.33^{a}	5^{ab}	5^{ab}	7^{ab}	3^{a}
120h	10°C	40% RH	0^{c}	0^{c}	0^{d}	0^{d}	0^{d}	0^{d}
		60% RH	0^{c}	0^{c}	0^{d}	0^{d}	0^{d}	0^{d}
		80% RH	0^{c}	0^{c}	0^{d}	0^{d}	0^{d}	0^{d}
	14°C	40% RH	0^{c}	1^{c}	3^{bc}	3^{c}	5^{bc}	4^{a}
		60% RH	0^{c}	3^{ab}	4^{ab}	4^{bc}	4^{c}	2^{bc}
		80% RH	2^{bc}	2^{bc}	2^{cd}	2^{cd}	2^{cd}	1^{c}
	16°C	40% RH	4^{a}	4^{a}	5^{ab}	6^{ab}	6^{ab}	3^{ab}
		60% RH	3^{ab}	4^{a}	4^{ab}	5^{ab}	8^{a}	3^{a}
		80% RH	3^{ab}	4^{a}	5^{a}	7^{a}	7^{ab}	3^{ab}
	RT (25°C, 55% RH)		4^{a}	4^{a}	5^{ab}	6^{a}	7^{ab}	4^{a}
	Control		3^{ab}	4^{ab}	5^{ab}	5^{abc}	7^{ab}	3^{ab}
144h	10°C	40% RH	0^{c}	0^{d}	0^{d}	0^{e}	0^{d}	0^{d}
		60% RH	0^{c}	0^{d}	0^{d}	0^{e}	0^{d}	0^{d}
		80% RH	0^{c}	0^{d}	0^{d}	0^{e}	0^{d}	0^{d}
	14°C	40% RH	0^{c}	2^{d}	3^{c}	3^{d}	5^{bc}	3^{ab}
		60% RH	1^{bc}	3^{c}	3^{c}	3^{cd}	3^{c}	1^{c}
		80% RH	4^{a}	6^{a}	6^{a}	7^{ab}	8^{a}	3^{ab}
	16°C	40% RH	4^{a}	4^{ab}	6^{ab}	7^{a}	8^{a}	3^{ab}
		60% RH	3^{a}	4^{ab}	4^{bc}	5^{bc}	9^{a}	3^{ab}
		80% RH	3^{ab}	4^{ab}	5^{ab}	5^{abc}	6^{ab}	2^{bc}
	RT (25°C, 55% RH)		3^{a}	4^{bc}	5^{ab}	6^{ab}	7^{ab}	4^{a}

Note: Values are expressed as mean. Mean values by different lowercase alphabets within the row are significantly different at $P \leq 0.05$ using LSD test. *LSD (5%) = NO. OF LEAVES (1.17)*

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Table 3.11 : Effect of stabilization treatments on shoot and root length of cacao seedlings

Duration (hours)	Storage condition		SHOOT LENGTH (cm)					ROOT LENGTH (cm)
			14 DAS	21 DAS	28 DAS	35 DAS	42 DAS	
	Control		14.27^{ab}	17.67^{ab}	19.44^{a}	20.53^{ab}	22.17^{ab}	18^{ab}
24h	10°C	40% RH	17.11^{a}	19.24^{ab}	20.78^{a}	21.29^{ab}	24.12^{ab}	18.64^{ab}
		60% RH	4.92^{c}	8^{d}	9.83^{b}	11.67^{c}	12.67^{c}	15.07^{b}
		80% RH	14.19^{ab}	14.75^{c}	15.57^{ab}	16.49^{bc}	16.78^{c}	19.05^{ab}
	14°C	40% RH	12.75^{ab}	15.33^{ab}	17.58^{a}	17.92^{ab}	19.42^{ab}	16.17^{ab}
		60% RH	9.39^{bc}	14.83^{bc}	16.44^{a}	17.83^{ab}	19.17^{bc}	19.33^{ab}
		80% RH	14.45^{ab}	17.34^{ab}	18.55^{a}	19.14^{ab}	21.36^{ab}	20.37^{ab}
	16°C	40% RH	16.57^{a}	21.1^{a}	22^{a}	24.13^{a}	27.23^{a}	19.37^{ab}
		60% RH	14.27^{ab}	18.43^{ab}	19.53^{a}	20.87^{ab}	22.68^{ab}	18.6^{ab}
		80% RH	16^{a}	19.8^{ab}	20.43^{a}	21.4^{ab}	23.33^{ab}	20.92^{a}
	RT (25°C, 55% RH)		15.27^{a}	20.2^{ab}	21.23^{a}	23.93^{a}	27.03^{ab}	21.43^{a}
	Control		14.27^{ab}	17.67^{ab}	19.44^{a}	20.53^{ab}	22.17^{ab}	18^{ab}
48h	10°C	40% RH	11.18^{bc}	12.13^{b}	12.65^{b}	13.53^{c}	15.35^{c}	16.9^{c}
		60% RH	13.33^{ab}	14.5^{ab}	15.33^{ab}	16.67^{c}	20.17^{ab}	19^{ab}
		80% RH	12.25^{ab}	14.92^{ab}	16.33^{ab}	18.42^{ab}	18.83^{c}	18.83^{ab}
	14°C	40% RH	12.22^{ab}	14.67^{ab}	14.94^{b}	15.11^{c}	17.11^{c}	13.5^{c}
		60% RH	8.89^{c}	16.61^{ab}	18.17^{ab}	18.39^{ab}	20^{ab}	16.67^{c}
		80% RH	16.71^{ab}	18.38^{a}	19.67^{a}	20.38^{ab}	21.13^{ab}	17^{bc}
	16°C	40% RH	17.2^{a}	20.6^{a}	21.1^{a}	24.3^{ab}	27.27^{a}	18.1^{ab}
		60% RH	10.53^{c}	15.4^{ab}	16.6^{ab}	17.77^{bc}	19.03^{bc}	16.83^{c}
		80% RH	15.57^{ab}	18.87^{a}	20.17^{a}	21.2^{ab}	25.7^{ab}	20.58^{ab}
	RT (25°C, 55% RH)		16.7^{ab}	19.87^{ab}	21.6^{a}	24.97^{a}	27.4^{a}	23.23^{a}
	Control		14.27^{ab}	17.67^{ab}	19.44^{a}	20.53^{ab}	22.17^{ab}	18^{ab}
72h	10°C	40% RH	14.54^{ab}	15.55^{ab}	16.63^{ab}	16.95^{ab}	19.59^{ab}	16.04^{a}
		60% RH	8.94^{c}	13.53^{cd}	14.42^{cd}	15.22^{cd}	16.61^{cd}	17.53^{a}
		80% RH	6.83^{c}	8.67^{d}	9^{d}	9.17^{d}	9.17^{d}	19.17^{a}
	14°C	40% RH	14.33^{ab}	16.67^{ab}	17^{ab}	17.5^{ab}	19^{bc}	21.33^{a}
		60% RH	9.15^{bc}	15.31^{ab}	16.68^{ab}	17.21^{ab}	18.47^{c}	19.71^{a}
		80% RH	13.45^{ab}	15.11^{bc}	15.61^{bc}	16.39^{bc}	16.83^{cd}	19.67^{a}
	16°C	40% RH	15.73^{a}	20^{ab}	20.67^{ab}	22.53^{ab}	24.73^{ab}	18.9^{a}
		60% RH	13.07^{ab}	16.8^{ab}	17.43^{ab}	19.37^{ab}	20.9^{ab}	17.24^{a}
		80% RH	16.73^{a}	21.4^{a}	22.83^{a}	23.93^{a}	27.23^{a}	19.37^{a}
	RT (25°C, 55% RH)		15.47^{a}	20.33^{ab}	22.33^{a}	23.63^{a}	26.73^{ab}	17.63^{a}

Note: Values are expressed as mean. Mean values by different lowercase alphabets within the row are significantly different at $P \leq 0.05$ using LSD test. *LSD (5%) = SHOOT LENGTH (3.90)*

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Duration (hours)	Storage condition		SHOOT LENGTH (cm)					ROOT LENGTH (cm)
			14 DAS	21 DAS	28 DAS	35 DAS	42 DAS	
	Control		14.27^{ab}	17.67^{ab}	19.44^{a}	20.53^{ab}	22.17^{ab}	18^{ab}
96h	10°C	40% RH	0^{e}	0^{d}	0^{d}	0^{d}	0^{d}	0^{d}
		60% RH	7.83^{cd}	12.5^{c}	12.83^{c}	13.17^{c}	14.92^{c}	10.83^{c}
		80% RH	10.22^{bc}	13.22^{bc}	$14.11b^{c}$	15.06^{bc}	15.78^{c}	13.56c
	14°C	40% RH	11.33^{ab}	12^{c}	13.83^{c}	14.17^{c}	$18.33b^{c}$	17.33^{ab}
		60% RH	4.33^{de}	9.03^{c}	11.94^{c}	12.22^{c}	13.17^{c}	12.59^{c}
		80% RH	9.65^{cd}	11.64^{c}	12.97^{c}	13.89^{c}	15.28^{c}	14.03^{bc}
	16°C	40% RH	15.2^{ab}	18.43^{ab}	19.03^{ab}	20.3^{ab}	22.53^{ab}	17.57^{ab}
		60% RH	16.7^{a}	19.57^{a}	20.1^{ab}	21.57^{ab}	23.6^{ab}	20.27^{a}
		80% RH	16.63^{a}	20.2^{a}	20.57^{ab}	21.3^{ab}	24.43^{ab}	18.11^{ab}
	RT (25°C, 55% RH)		12.57^{ab}	19.6^{a}	22.07^{a}	24.17^{a}	27.77^{a}	18.13^{ab}
	Control		14.27^{ab}	17.67^{ab}	19.44^{a}	20.53^{ab}	22.17^{ab}	18^{ab}
120h	10°C	40% RH	0^{d}	0^{d}	0^{d}	0^{d}	0^{f}	0^{d}
		60% RH	0^{d}	0^{d}	0^{d}	0^{d}	0^{f}	0^{d}
		80% RH	0^{d}	0^{d}	0^{d}	0^{d}	0^{f}	0^{d}
	14°C	40% RH	7^{c}	9.5^{c}	9.83^{c}	9.83^{c}	11^{de}	13.53^{bc}
		60% RH	7^{c}	12.33^{bc}	12.67^{bc}	12.67^{bc}	13.92^{cd}	14.83^{ab}
		80% RH	7.5^{bc}	8.25^{c}	8.25^{c}	8.58^{c}	8.92^{e}	12.33^{c}
	16°C	40% RH	15.2^{a}	19.83^{a}	20.9^{a}	22.27^{a}	24.2^{ab}	18.5^{ab}
		60% RH	11.73^{ab}	16.6^{ab}	17.9^{ab}	18.8^{ab}	19.95^{bc}	17.07^{ab}
		80% RH	10.17^{ab}	15.1^{ab}	15.63^{ab}	17.03^{ab}	18.93^{cd}	17.29^{ab}
	RT (25°C, 55% RH)		11.53^{ab}	19.7^{a}	20.83^{a}	23.57^{a}	28.27^{a}	19.37^{a}
	Control		14.27^{ab}	17.67^{ab}	19.44^{a}	20.53^{ab}	22.17^{ab}	18^{ab}
144h	10°C	40% RH	0^{d}	0^{d}	0^{d}	0^{d}	0^{d}	0^{d}
		60% RH	0^{d}	0^{d}	0^{d}	0^{d}	0^{d}	0^{d}
		80% RH	0^{d}	0^{d}	0^{d}	0^{d}	0^{d}	0^{d}
	14°C	40% RH	6.33^{c}	9.42^{c}	10.92^{c}	12.33^{c}	14.33^{c}	15.33^{bc}
		60% RH	6.67^{bc}	12.67^{bc}	12.67^{bc}	14.33^{c}	15^{c}	14.5^{c}
		80% RH	14.28^{a}	16.83^{ab}	18^{ab}	18.5^{ab}	19.17^{bc}	16.39^{ab}
	16°C	40% RH	12.27^{a}	17.97^{ab}	19.03^{ab}	21.63^{ab}	23.73^{ab}	16.5^{ab}
		60% RH	11.8^{ab}	16.23^{ab}	17.87^{ab}	19.47^{ab}	21.42^{ab}	17.93^{ab}
		80% RH	8.93^{ab}	14.77^{ab}	15.3^{ab}	16^{bc}	17.17^{c}	19.47^{ab}
	RT (25°C, 55% RH)		10.6^{ab}	19.43^{a}	21.07^{a}	23.43^{a}	28.07^{a}	21.2^{a}

Note: Values are expressed as mean. Mean values by different lowercase alphabets within the row are significantly different at $P \leq 0.05$ using LSD test. *LSD (5%) = SHOOT LENGTH (3.90)*

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3.3.9 Seedling Vigour Index

In this study, the control seeds recorded an average 39.75 of seedling vigour index (SVI) (Table 3.12). Among all treatments, only seeds at RT showed significantly higher SVI than the control. The values remained to be high, ranging from 44.37 to 48.39 of SVI throughout 144 HOS. The same pattern was observed for seeds at 16°C, 40% RH, in which the SVI increased significantly and consistently among the highest (41.82 to 45.06 of SVI) over the first 72 HOS. As mentioned earlier in this study, seeds at RT and 16°C also produced among the highest FGP. The calculation of SVI, as to measure the multiplication of plant height (shoot and root) and FGP; the higher SVI recorded for seeds at RT and 16°C indicated their higher viability and vigour thanthe other treatments in this study.

On the other hand, no significant differences in SVI throughout 144 HOS for seeds at 16°C, 80% RH, and after 24 HOS for seeds at 16°C, 60% RH (Table 3.12). Seeds at 16°C, 60% RH then demonstrated a fluctuating pattern of SVI with increased storage duration. The treatment further remained the high values (36.07 to 41.09 of SVI) to be at the same level as the control, after 96 HOS.

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Regardless of RH, seeds at 14°C recorded significantly lower SVI than the control, but with the fluctuating pattern of values throughout 144 HOS (Table 3.12). Seeds at 14°C, 40% RH, showed more drastic results with about 29% decreased in SVI after 24 HOS than the control. The pattern of SVI further fluctuated before displayed the lowest values after 144 HOS (SVI = 24.73), as compared to seeds at 60% and 80% RH. The fluctuation patterns might be due to the variations of abnormal seedlings recorded, with not so many significant differences from their normal seedlings (Table 3.10).

The same pattern was observed for seeds at 10°C, 80% RH, which display lower SVI values after only 24 HOS (Table 3.12). The results further fluctuated with the increased storage duration to produce SVI values ranging from 14.78 to 22.34. Meanwhile, seeds at 10°C, 60%, and 40% RH showed a more gradual reducing SVI values for over 96 hours (SVI = 28.96 to 16.68) and 72 hours (SVI = 33.64 to 26.43) of storage, respectively.

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Table 3.12 : Effect of stabilization treatments on seedling vigour index of cacao seeds for 144 HOS

Storage condition		SEEDLING VIGOUR INDEX						
		Storage duration (hours)						
		0 (Control)	24	48	72	96	120	144
10°C	40% RH	39.75±3.31^{a}	33.64±5.98^{b}	26.21±2.63^{c}	26.43±1.80^{c}	-	-	-
	60% RH	39.75±3.31^{a}	28.96±10.04^{b}	28.86±8.09^{b}	28.54±8.70^{b}	16.68±1.80^{c}	-	-
	80% RH	39.75±3.31^{a}	30.25±6.32^{b}	18.08±7.49^{cd}	14.78±2.60^{d}	22.34±3.31^{c}	-	-
14°C	40% RH	39.75±3.31^{a}	28.17±9.93^{b}	21.87±0.77^{c}	24.77±0.95^{b}	26.37±8.63^{b}	16.32±2.22^{c}	24.73±11.70^{b}
	60% RH	39.75±3.31^{a}	33.71±1.09^{bc}	28.72±2.89^{c}	36.44±4.13^{ab}	15.11±8.61^{d}	27.44±10.33	28.64±0.35^{c}
	80% RH	39.75±3.31^{a}	31.04±3.47^{b}	26.94±1.27^{bc}	27.01±1.19^{bc}	20.87±6.27^{d}	23.54±11.20^{cd}	27.96±0.82^{bc}
16°C	40% RH	39.75±3.31^{a}	45.06±2.06^{a}	44.21±0.14^{ab}	41.82±0.60^{abc}	38.16±4.45^{c}	41.63±0.10^{abc}	37.86±2.75^{c}
	60% RH	39.75±3.31^{a}	38.57±3.55^{abc}	33.94±2.41^{c}	34.78±2.76^{bc}	41.09±3.47^{a}	36.07±4.05^{abc}	38.03±2.83^{abc}
	80% RH	39.75±3.31^{a}	42.39±1.99^{a}	43.97±0.78^{a}	44.27±0.43^{a}	40.41±1.05^{ab}	35.90±3.67^{b}	36.20±0.78^{b}
RT (25°C, 55% RH)		39.75±3.31^{a}	48.06±3.00^{a}	48.49±0.81^{a}	44.37±0.25^{ab}	45.90±2.65^{a}	46.83±0.39^{a}	47.60±0.72^{a}

Note: Values are expressed as mean±SE. Mean values by different alphabets within the column are significantlydifferent at P ≤ 0.05 using LSD test. *LSD (5%) = 5.08*

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3.4 Discussion

During the present study, the stabilization treatment (controlled temperature and RH) aimed to balance an equal distribution of moisture within the seed. Lisboa et al. (2017) reported that recalcitrant seeds placed in the relatively high-water content environment would keep the metabolism within seed active, leading to germination. During the present study, the MC of cacao seeds was recordedto be in the range of 43 - 57% throughout 144 HOS. The results suggest that thoseseeds were already in their Phase II (refer Figure 2.2 in Chapter 2) of water uptake but with different metabolic activities among seeds and storage conditions. It is also supported by Vertucci (1993), who reported that among five hydration levels classified in the recalcitrant and orthodox seed tissues, respiration activity is first observed in the hydration level III (20 - 30% of MC) and only completed germination if fully hydrated, hydration level V is present (>50% of MC; or it is generally achieved only by contact with liquid water) (refer Figure 2.3 in Chapter 2).

In sealed storage, seed MC determines the RH, as the inside atmosphere will be in equilibrium with the seed MC and further remain at that level. Furthermore, the exchange of water vapor is allowable between seeds with a high-water content inside the semipermeable packages and the external environment, thereby reducing or increasing the seed MC (Dresch et al., 2014). During the present study, the stabilization treatment did show an impact on the viability and vigour of cacao seeds inside the re-sealable polyethylene bags. Cacao seeds were found to be tolerated with a temperature of 16°C and RT. Regardless of the RH, seeds at 16°C and RT showed the highest final germination percentage (FGP) with no significant differences with the control throughout 144 HOS. A similar previous attempt was made on recalcitrant *Trichilia emetic* seeds by Kioko et al. (2006), which also indicates that there were no changes in viability for seeds either at 16°C or ambient (25°C).

However, those seeds were recorded to attain total germination in-store within 40 and 60 days for seeds at the ambient and 16°C, respectively. Further

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extension of storage duration for seeds at RT, might probably contribute to the increased of germinated seeds, as those treatment (with 46 - 50% MC) have already exhibited about 8 - 10% of germinated seeds while still inside the re-sealable polyethylene bags during the present study. On the contrary, seeds at 16°C showed a much lower (<5%) germinated seeds, within just 144 hours (6 days) of storage. On the other hand, the leachate conductivity (LC) variable was to indicate the barrier effectiveness of cell membranes. The higher LC reflected the badly structured or damaged membranes and is usually associated with the seed deterioration process, which could eventually reduce the seed vigour (Salam et al., 2017). According to Bewley *et al.* (2013), the higher storage temperature could ameliorate the membrane damagesthrough the humidification or 'melting' of the dry membrane to a more fluid gel state. Thus, less leakage or damage might occur, further explains the reduction of LC valuesafter 96 hours and towards 144 HOS for seeds at RT. Umarani et al. (2015) also reported that slow-drying (drying of seed tissues with testa or in closed containers) at the ambient (25 - 28°C) temperature could help to reduce the mechanical or physical damage to the seeds due to the delay time taken to cross the critical seed MC. The lowest metabolic rate of recalcitrant seed appears to be at the least desiccation-sensitive stage, usually coinciding with their natural shedding water content (Berjak & Pammenter, 2013). Throughout the present study, MC for seeds at RT was at the same level as the control, as well as their LC values declined to thesame level as the control with the increased storage duration, which perhaps due tothe membranes were able to re-establish their functional integrity. The amount of moisture the air can hold also depends on the storage temperature. Higher temperatures will hold more moisture in the air than cooler ones (Bui et al., 2019) explained that the constant adjustments in the seed MC during storage might furtherstabilize the MC to be the same level as the control. The consistent pattern of MC and reduced LC of cacao seeds at RT might attribute to the lesser harmful conditionsfor seed cells of which results in the higher viability, as well as the higher availabilityof food reserves for the seeds to produce vigorous seedlings (Adu et al., 2017). However, at such storage, the ongoing metabolic activity could expose those seeds to increase respiration, heating, and fungal invasion. The extension of the storage duration

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might further reduce seed viability and vigour (Suma et al., 2013). The other recalcitrant species, such as *Campomanesia adamantium* seeds under laboratory conditions (25°C) storage with a water content of 31.2%, were also found to exhibit primary root protrusion while still inside the plastic bag (storage) and allowed for the development of fungi such as *Aspergillus sp.* Similarly, in this study, storing cacao seeds at RT has resulted in more germinated seeds and fungi invasion on seeds was discernible with the extension of storage duration. Seeds at RT also showed the higher and consistent increasing pattern on the speed of germination or germination rate index (GRI) and decreasing pattern of mean germination time (MGT) with the increased storage duration, which also supported that the continuous higher metabolic activity might have occurred within seed during storage. In some ways, the RT condition was observed to maintain the seed vigour performance, and further might cause the rapid development of raw materials (food reserved) transmission directed to the embryo. Those favorable conditions could also increase the efficiencyto utilize food stocks, and thus, contribute to the higher accumulation of dry matter within the seed (Bahri et al., 2018). Similar observations were obtained during the present study, as those seeds at RT continuously showed higher seedling vigour performances through the production of 100% normal seedling, higher SVI, as well as their seedling dry biomass was at the same level as the control throughout 144 HOS.

In this study, seed MC showed positive correlations with FGP, and the FGP results were maintained higher for seeds at 16°C (40%, 60%, and 80% RH) throughout 144 HOS. Even with the higher FGP, cacao seeds at 16°C, 60%, and 80% RH exhibited significantly delayed germination through the lower GRI and higher MGT than the control. Besides, seeds at 16°C, 80% RH also showed a significantly higher MC during the middle, and towards the end of storage duration for seeds at 16°C, 60% RH. According to Bewley et al. (2013), there will be a water potential gradient between the seeds and their surroundings due to an increase of cell expansion through the conversion of stored polymeric reserves to more osmotically active building blocks seed, e.g., starch to sugars. The greater concentration of solutes further lowered the water potential in the seed. It resulted in a driving force

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for water uptake during Phase II, which explains the increased seed MC with increased storage duration. Reductions in water potential and the low storage temperature further resulted in the slow metabolism activity in seed, which might cause the extension of time required during Phase II to prepare for germination (Bewley et al., 2013). Besides, as the seeds stayed longer at the intermediate water contents, at which aqueous-based deleterious processes would occur ranging from the disruption of coordinated regulation of metabolism to the failure of antioxidant systems (Liang & Sun, 2000), which explains the maintained level of GRI and the increased of MGT especially for seeds at 16°C, 60% RH, towards the end of storage duration. Since repairing the disturbed metabolism within the seed is time-consuming process; for instance, to improve the membrane damage, to restart the antioxidant system activity, and to prevent oxidative stress, which results in further delay in germination (Suma et al., 2013). Liang & Sun (2000) also concluded that slow-drying at high RH was harmful to *T. cacao* seed tissues. Similar observations were obtained by Suma et al. (2013), who found that both the germination percentage and time taken for germination were drastically affected at higher RH than lower RH, regardless of the species. Besides, delayed germination could also be one of the earliest signs of deterioration by crop seeds. Moreover, seeds at 16°C, 60% RH, which showed higher FGP in the present study, coincidently exhibited insignificantly lower GRI and higher MGT with those seeds at 14°C (40%, 60%, and80% RH). The results suggest that the initial metabolic or respiration activity; the synthesis of new proteins and additional antioxidants within seed at 14°C, regardless of the RH, may have been as an attempt to adjust to the chilling temperatures, which further caused the extension of time required during storage to prepare for germination. Whereas storage of 16°C, 60% RH might provide more favorable conditions to permit the early initiation of germination.

Among those seeds at 16°C, the ones stored at 40% RH exhibited significantly higher GRI and lower MGT throughout 144 HOS. Seeds in hydrated condition do not leak solutes when placed in water. Report to the contrary, seeds at 16°C, 40% RH showed among the highest LC values, and it steadily increased with the increased storage duration. According to Liang & Sun (2000), the water potential of cacao seed

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tissues tends to change rapidly under low RH. Thus, the well-organized seed tissues might be disturbed by the uneven and rapid volumetric change and further caused more mechanical stresses. In this case, small dehydrations might cause the rapid shift in seed membrane from more fluid to less fluid gel state, which further restricts their motion. Once the seeds are exposed to higher water content or vapor from surroundings, it results in a phase transition back to the hydrated bilayer membrane, and severe leakage might occur during this transition phase (constant dehydration and rehydration process). It is also supported by Rahayu et al. (2014), who found that the higher the membrane leakage value is usually proportionate to the lower seed MC or at the dehydrated conditions. The metabolic activity prior to germination of seeds at 16°C, 40% RH, however, seems to counteract their membrane changes, which further exhibited the higher GRI and lower MGT. Those vice versa relationships were also supported by Liang & Sun (2000), which suggested that the mechanical and metabolic stress within seed tissues during drying could have been minimizing when their water potential increases and decreases, respectively. The trend of high and stable MC of seeds at 16°C, 40% RH throughout 144 HOS, might be due to consistent heat and water formed with the increased metabolic activity within the seed (Bahri et al., 2018; Umarani et al., 2015). On the other hand, a different pattern of results showed for their seedling vigour performance. Even though there were no dead seedlings recorded for seeds at 16°C, there were still variations in normal and abnormal seedlings produced compared to different RH throughout 144 HOS. Seeds at 16°C, 40% RH generally showed better germination performance and produced the lowest percentage of abnormal seedling compared to the others stored at the higher RH. However, seeds at the higher RH showed better performance on their seedling growth. In this study, cacao seed MC also showed positive correlations with the SVI, seedling dry biomass, normal seedling, and the negative correlation with the dead seedling. Results suggest that cacao seeds with higher MC might contain higher growth potentials and are supported by Bahri et al. (2018). Similar observations were obtained, as the seeds at 16°C, 80% RH showed the increase of MC, as well as the higher seedling dry biomass after 24 to 72 hours in storage. However, the extension of storage duration might further cause the stored food reserves to be used up through the increasing metabolic activity in

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preparation of germination, which reduced the availability for the seedlings to emerge (Saajah & Maalekuu, 2014). This explains the reducing pattern of seedling dry biomass after being storedfor 96 hours for seeds at 16°C, 80% RH.

The small but significant fluctuated patterns on seed MC at all storage conditions in this study indicating that the lower temperatures (10, 14, and 16°C) are efficient for maintaining the cacao seed MC, even in semipermeable re-sealable polyethylene bags and this was supported by Dresch et al. (2014). Categorized as the chilling sensitive species, cacao seeds were found unable to withstand 10 and 14°C for longer storage duration. The chilling-sensitive plant suffers an injury and eventually dies with the continuous exposure to chilling temperatures. Similar observations were made in this study on seeds at 14°C (40%, 60%, and 80% RH), which showed the subsequent gradual decline in FGP with the increased storage duration. Whereas seeds at 10°C showed fluctuated patterns with much lower FGP, especially at the RH of 40% and 80%. The decreased of germinability and increased seed death, both symptoms are typical chilling injury usually occurred during early imbibition of orthodox seeds. The fluctuations of seed performance during storage are in agreement with Lewis (2002), which indicated that the physiological function of chilling-sensitive plant species could be abruptly and dramatically affected by the exposure of chilling temperatures. Liang & Sun (2000) also added that chilling injury occurs due to altered physiological function and appearance with adverse effects. While the adjustments of seed MC constantly occurred during hydrated storage, the membrane phase transitions at the same time being imposed to the temperature below which chilling injury occurs will further induce chilling stress (Bewley et al., 2013). Indication through the prolonged gradual increase of leakage while chilling stress was imposed, suggesting that the low temperature might also interfere with the normal and proper reorganization of the membrane. In this study, the increasing patterns of LC were shown for seeds at 14°C (60% and 80% RH) and seeds at 10°C (40%, 60%, and 80% RH), especially after 96 hours and towards the end of storage duration. Even though the LC values recorded for seeds at 10 and 14°C are mostly lower than seeds at 16°C, seed imbibition (rehydration) at lower temperatures was reported to be more detrimental (Bewley et al., 2013). As the membranes begin

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rupturing, the cellular membranes are unable to function normally. They might be attributed to the leakage of several compounds such as amino acids, organic acids, inorganic ions, sugars, phenolics, and proteins from the imbibed seeds (Harttman et al., 2011). Those 'leakiness' may also be responsible for the delayed germination and reduced relative growth rate. Besides, recalcitrant seeds start to lose viability at the relatively high-water content, which is often in the existence of their freezable (free) water; far above that at which a glassy state and non-freezable water (hydration level II; <20% of MC) can exist (Zaritzky, 2015). Similar observations were made in this study, with both temperatures (10 and 14°C) exhibited seed MC in the range of 43 - 53% throughout 144 HOS. Despite the high range of MC, seeds at 14°C showed significantly lower MC values as compared to the control at some points after storage, especially towards the end of storage duration for seeds at 14°C, 40% RH, in the middle of storage duration for seeds at 14°C, 60% RH, as well as at the early and towards the end of storage duration for seeds at 14°C, 80% RH. Considering that the moisture equilibration was included in this hydrated storage, reduction of MC might be due to the increase of water potential within the seed, caused by the slow-down of metabolic activity at the lower storage temperature. There will be a reductionin the exotherm (process releases heat) peak as the decrease in seed tissue moisture occurred with the presence of freezable water (Dragicevic & Sredojevic, 2011). It is also supported by Zaritzky (2015), who reported that seeds maintaining the high water content showed resistance to rehydration which might be due to the involvement of the oxidative process and free radical reactions, which further leads to loss of cellular and molecular deterioration integrity.

The readily aqueous phase within seed during hydrated storage will enhance the deteriorative processes possibly due to the constraint on chemical processes (Dragicevic & Sredojevic, 2011) because of the increased viscosity. On the other hand, seeds at 10°C were observed to show the least fluctuations of MC throughout 144 HOS compared to seeds at 14 and 16°C. The poor germination performance (low FGP, GRI, and high MGT) of seeds at 10°C suggests that their MC's steady pattern might be due to more non-viable seeds imbibed or absorbed moisture than the viable seeds. According to Bewley et al. (2013), this indicates that

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non-viable seeds have a higher affinity on the continuous water uptake due to their poorly structured un- intact cellular membranes, water potential retained zero, and most solutes are released out to the apoplast. Besides, the increased seed MC could be one of the signs of the imbibitional chilling phenomenon during storage. Furthermore, their GRI and MGT values also showed parallel adverse effects for both seeds at 10 and 14°C. Regardless of the RH, seeds at 10 and 14°C exhibited lower and inconsistent GRI patterns with the higher MGT, which started after 24 HOS. Among those treatments, only seeds at 14°C, 80% RH, showed lower MGT throughout 144 HOS. The results suggested that those seeds might become more susceptible to chilling stress once the germination had begun, and it was supported by Lewis (2002). As germinative metabolism proceeds, it driven the changes of the cell nature and further affected the water thermodynamic chemical potential (Dragicevic & Sredojevic, 2011). The authors further suggested that the function proportions of the different hydration levels of water changed, even with no changes in the water content of seed tissues. The altered and unregulated metabolism could occur in the hydration level III and IV, which further adversely affected the repairing processes at those hydration levels.

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On the other hand, variations of the pattern can be seen through their seedling growth performance. Even though seeds at 14°C managed to produce a better germination performance compared to seeds at 10°C, the exposure of germinated seeds to the real controlled field environment, however, suppressed their seedling growth. The results further proved that the seed deterioration is not restricted to the onset of germination. Besides, the decreased FGP and increased seed death with the increased storage duration suggest that those seeds might lose vigour before they lose the ability to germinate (Shaban, 2013). The results indicate that the temporary injury might be repaired prior to seed germination. Still, as the storage duration extended, the conditions might become harsh and thus, accelerated seed damages. The enhanced metabolic activity due to the priority on the germination process further caused the seedling growth to become susceptible and critically influenced by the chilling stress (Ali & Elozeiri, 2017). No specific pattern of seed germination and seedling, growth performance can be seen

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through seeds at 10°C; thus, their survival and growth ability are dependent on their very own vigour responses. It is supported by the fact that the performance of seed (individual) vigour will be depended on the ability of their embryo axes, including the meristematic cells (radicle and plumule) to germinate and could still produce a normal or weak seedlingunder ideal conditions even if parts of the cells in the seed are deteriorated (Mbofung, 2012).

3.5 Conclusion

The present study confirming that recalcitrant cacao seeds were susceptible to chilling injury. The cooler the storage temperature, the more damage was infested upon the seeds. Extended storage at 10°C beyond 24 hours resulted in delayed seeds germination and consistently produced higher abnormal and dead seedlings. Despite being prone to fungal infestation, cacao seeds could be stored at 16°C (regardless of RH) and RT (25±2°C) for at least 144 hours and still produced among the highest germinability and lower abnormal, with no dead seedlings recorded. However, more germinated seeds (about 8 to 10%) and fungal invasion was evident during storage at RT, which further reduced their storability compared to seeds at 16°C. Further, regardless of the RH, seeds at 16°C exhibited minimal fungal invasion with no seeds germinating during storage. The moisture content of cacao seeds was recorded to be in the range of 45 - 56% (wetted conditions) throughout 144 hours of storage. The seeds might already be in their Phase II of water uptake (germination phases) but with different metabolism rates among storage conditions. Cacao seeds that retained higher moisture content had a relatively low germination rate index. Despite the deterioration associated with seed aging and increased pattern of leachate conductivity (membrane damage), the storage condition of 16°C, 40% RH showed more potential to improve germination characteristics, with less reduction in seedling performances. Evaluations on the other biochemical changes, including factors that contribute to oxidative stress (metabolism-linked damage), may help further understand the deterioration development of recalcitrant cacao seeds during storage.

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CHAPTER 4

CHARACTERIZATION OF PHYSIO-CHEMICAL, MICROSTRUCTURAL CHANGES, AND OXIDATIVE STRESSES ASSOCIATED WITH SLOW-DRYING AND CHILLING SENSITIVITY IN CACAO SEEDS

4.1 Introduction

Previous findings in Chapter 3, suggested that cacao seeds stored at different combination temperatures and RH; 10°C, 14°C, 16°C (40%, 60%, and 80% RH), and RT (25°C, 55% RH), managed to hold their moisture content to be at least in the range of hydration level IV (43 to 57% MC), along 144 hours of storage. However, holding seeds at least at the hydration level III (20 to 30%) may lead to rapid degradation, particularly at higher temperatures, since all harmful reactions are likely to occur (e.g., chemical, ROS, enzymic, some respiratory or metabolic), but there is still the presence of water to allow defense mechanisms in a completely hydrated molecular condition (Bewley et al., 2013). Their volumes increased (sharply) along with the increase of hydration levels. The reformed spatial relationships among organelles and constituents might cause stresses, particularly when the membrane systems are impaired, along the compartmentalization is compromised. Hence, the implications for seed quality at the greater range of wet and fully imbibed seeds are based more on functional repair processes than on water content per se. DNA repair is activated soon after imbibition begins, and the transcription and/or function of damaged genes will be compromised depending on the sufficiency of the functional repair mechanisms (Bewley et al., 2013). 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

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(e.g., cessation of protoplasmic streaming, alterations in respiration rates) that lead to loss of structural integrity and overall quality if allowed to continue over a period (Lukatkin et al., 2012).

Observations from some authors have shown that slow drying is more harmful because the seeds maintain an intermediate water level for a longer period and are susceptible to damage. Meanwhile, they may pass through this critical range of water so quickly during the fast-drying process to survive further drying. However, in few studies, for example, Pukacka et al. (2011) found that either rapid or slow dehydration of recalcitrant *Quercus robur* seeds to hydration level II is not a response for further reductions in seed germination but induces different specific reactions in the embryonic axis. It is tempting to consider seed drying events during slow drying due to the embryonic axis is slowly dehydrated naturally inside the seeds, and thus, the storage time may appear to be critical to maintaining germination than the temperature (storage conditions). Hence, another experiment was conducted to evaluate the physio-chemical and microstructural changes, and oxidative stresses aspects of cacao seeds for 12 days of storage at 14°C and 16°C (40% and 80% RH), and RT (25±2°C, 55±5% RH). A scanning electron microscopy (SEM) technique was used to observe structural changes in samples to better understand the effect of different osmo-dehydration treatments at the cell level on mass transfer and texture characteristics (Deng & Zhao, 2008). The appearance of cells in dry seeds that have been fixed and microscopically observed without contact with water demonstrates the importance of physical structure and its maintenance to preserve viability upon rehydration. The damage of membrane phospholipids could occur in dry conditions via the chemical processes, but the consequences of membrane leakage or loss of organellar compartmentation are significant in a hydrated cell only in which liquid water is present (Bewley et al., 2013).

The macroscopic properties of materials are changed by the modifications of their microstructure. For example, during drying, biological materials are shrunk simultaneously with moisture diffusion and can therefore influence the rate of moisture removal. Thus, density and porosity are important physical properties

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that characterize the texture and the quality of dry and intermediate moisture materials (Wang & Brennan, 1995).

Physiological, biochemical, and subsequent morphological changes are strongly related to seedling survival and vegetative growth that affect yield and quality (Ali & Elozeiri, 2017). In the plant life cycle, seed germination and early seedling growth 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 (Maldonado et al., 2015). Metabolism stresses at a given water content could cause metabolic coordination breakdown in cells, leading to uncontrolled free-radical (including reactive oxygen species, ROS) attacks and reducing enzyme and non-enzyme protection from oxidative damage (Li & Sun, 1999). The dual function of ROS in the plant is based on the machinery of cell antioxidants, involving the detoxifying enzymes and antioxidants. Such mechanisms may eliminate potentially toxic ROS produced under stressful circumstances, or control ROS concentrations tightly to regulate various pathways for signaling (Bailly, 2004). Hydrated storage has been linked to an increase in oxidative stress and the intensity of the stress has been reported to be responsible for differences in responses of seed storage between species (Chandra et al., 2019). This suggestion is supported by a study made on recalcitrant *Trichilia dregeana* seeds which showed that ROS could become part of a biochemical germination trigger when the seeds are slightly dehydrated (Sershen et al., 2014). The metabolic pathway to the production of ROS and enzyme machinery involved in detoxification is resumed following seed imbibition and consequent metabolism reactivation (during hydrated storage). Generally, ROS detoxification involves a series of different enzymes and signaling molecules, which are produced and active at different levels during seed germination and maturation, e.g. superoxide dismutases (SOD), catalases (CAT), ascorbate peroxidases (APX), glutathione peroxidase (GPX), glutathione reductases (GR), dehydroascorbate reductases (DHAR), monodehydroascorbate reductases (MDAR), thioredoxins (TRX), peroxiredoxins (PRX), glutaredoxins (GRX), and peroxidases (POXs) (Pagano et al., 2019).

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The amount of detailed data on the dynamics of cacao seeds viability and vigour loss as a function of water content under slow drying regimes (different storagetemperatures and RH during storage) is limited. Moreover, under the light of the involvement of ROS dual functions in seed, characterizing the cellular antioxidant machinery in specific of detoxifying enzymes, may reflect in understanding the effectsof the over-accumulation of endogenous ROS production. Therefore, the objectives of this study were, (i) to characterize the physio-chemical and microstructural changes within the cacao seeds affected by slow-drying and chilling treatments during storage, and (ii) to elucidate the accumulation of reactive oxygen species (ROS) antioxidant enzymes of cacao seeds during storage.

4.2 Materials and Methods

4.2.1 Plant Material

The plant material as described in Chapter 3, Subsection 3.2.1.

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4.2.2 Storage Condition and Duration Treatments

The cacao seeds were packed into 23 x 15 cm of zip-lock polyethylene bags (160 seeds per bag) for 3 replications and stored at; (i) air-conditioned room temperature, RT (25±2°C, 55±5% RH), (ii) 16°C, 80% RH, (iii) 16°C, 40% RH, (iv) 14°C, 80% RH, (v) 14°C, 40% RH, (vi) control (seeds freshly extracted from pods). Seed hydrating procedures involved the use of a microprocessor-controlled console-style germinator (Seedburo Equipment Company, MPG-3000, United States). Seeds were sampled every 48 hours (2 days) for durations of 12 days storage (Figure 4.1). During storage, all polyethylene bags were opened daily, and the seeds were quick gently stirred, to allow the aerated condition within polyethylene bag.

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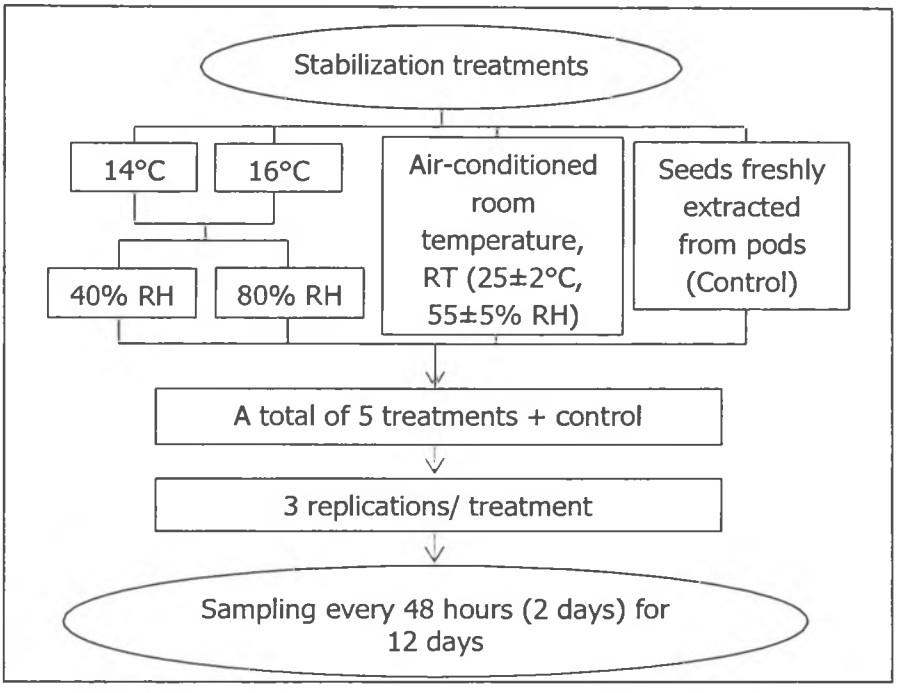


Figure 4.1 : Experimental layout of cacao seeds stabilization at different storage temperatures and relative humidity

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4.2.3 Measurement of Seed Moisture Content

The measurement of seed moisture content as described in Chapter 3, Subsection 3.2.3.

4.2.4 Measurement of Germination Percentage

The measurement of germination percentage as described in Chapter 3, Subsection 3.2.4.

4.2.5 Measurement of Germination Rate Index

The measurement of germination rate index as described in Chapter 3, Subsection 3.2.5.

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4.2.6 Measurement of Mean Germination Time

The measurement of mean germination time as described in Chapter 3, Subsection 3.2.6.

4.2.7 Measurement of Seedling Growth

The measurement of seedling growth as described in Chapter 3, Subsection 3.2.8.

4.2.8 Measurement of Seedling Vigour Index

The measurement of seedling vigour index as described in Chapter 3, Subsection 3.2.9.

4.2.9 Evaluation of Normal, Abnormal and Dead Seedlings

The evaluation of normal, abnormal and dead seedlings as described in Chapter 3, Subsection 3.2.10.

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4.2.10 Measurement of Leachate Conductivity

The measurement of leachate conductivity as described in Chapter 3, Subsection 3.2.7.

4.2.11 Estimation of Respiration Rate (Carbon Dioxide Gaseous)

Respiration rate as carbon dioxide (CO_2) production was measured by a modification of the CO_2 measurement system procedure proposed by Raudiene et al. (2017). Twenty freshly after-stored seeds were weighed and put into the hermetically closed container (250 mL), with the probe attachment (infrared detector, CO_2 gas sensor; Model No. CO_2-BTA Vernier, USA) on its top.

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The sensors measured CO_2 concentrationby a digital interface that connected straight to the data collection and was recordedafter every minute. Respiration rates were expressed as mL CO_2 produced per g of cacao seeds per hour, and calculated by the following formula:

$$\text{Respiration rate (mL } CO_2\text{/g hr)} = \frac{\text{Change of } CO_2 \text{ concentration x Volume of the container}}{\text{Sample weight x Sampling period}}$$

The measurement range of the CO_2 sensors was 1000 ppm (parts per million volumetric) and up to 10,000 ppm in the extended measurement range (±10% of reading). The response time of the sensors was 95% full-scale reading in 120 seconds. The normal operating temperature and humidity range of the sensors was 25°C (±5°C) and 5-95% RH (non-condensing environment), respectively.

4.2.12 Scanning Electron Microscopy (SEM) Observation

Scanning electronic microscopy was applied to evaluate the damages from the slow-drying and chilling conditions exposure, following the method proposed by Ignatz et al. (2019). Seeds were cut by transverse-section using a razor blade; the radical cells of the meristematic axis and the cotyledon mesophyll were visualized. Samples were then sputter-coated with a gold layer using a Quorum Q150R Thin-Film Coater. Later, they were examined under SEM (Hitachi S-3400N, Japan), with images subsequently contrast adjusted in Adobe Photoshop CS6 (Version 13.0).

4.2.13 Biochemical Analysis: Sample Preparation

The after-stored cacao seeds were sampled and frozen with liquid nitrogen and stored at -80°C for further biochemical analysis.

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4.2.14 Measurement of Total Soluble Sugar

This method was carried out according to Nath et al. (2015). A 100 mg of seeds were homogenized with 5 ml of 80% (v/v) ethanol and centrifuged at 3000 rpm for 10 minutes. Supernatants from 3 times extraction (with 80% ethanol) were collected into 25 mL volumetric flasks. The extract (0.3 mL) was pipetted in separate tubes from each treatment and placed in a boiling water bath (WiseBath, WSB-model, Germany) for 3 minutes to evaporate ethanol. Each test tube was added with 1 mL pure water and 4 mL of (0.2% w/v) anthrone reagent (200 mg in 100 mL H_2SO_4) and placed in ice-cold water. The blank reagent was prepared with 1 mL of distilled water and 4 mL of anthrone reagent. The intensity of colour was measured using a spectrophotometer (DeNovix DS-11, USA) at 600 nm. A standard curve was prepared using 10 mg glucose per 100 mL distilled water with the equation was $y = 1.45x + 0.32$ (R^2=0.84).

4.2.15 Measurement of Glucose, Sucrose, and Raffinose-family Oligosaccharides (RFOs) Content

Methods to quantify glucose, sucrose, and RFOs were based on a diagnostic kit (Product K-RAFGL, Megazyme Int. Ireland Ltd., Bray, Ireland). Aliquots at 200 µL of ethanol 80% (v/v) sugar extracts were added to three test tubes labelled A, B, and C. Tube A was directly assayed for glucose. Sucrose was hydrolyzed to glucose and fructose in Tube B through digestion with 200 µL of invertase in 200 µL of 50 mmol L^{-1} NaOAc buffer (pH 4.5). RFOs were hydrolyzed into galactose, glucose, and fructose in Tube C through digestion with 200 µL of α-galactosidase and invertase in 200 µL of 50 mmol L^{-1} NaOAc buffer. Three milliliters of GOPOD reagent were addedto each tube, and all solutions were incubated at 50°C for 20 min. Glucose concentrations were determined using glucose (standard), and spectrophotometer (DeNovix DS-11, USA) absorbance was read at 510 nm. Tube A absorbance was used to calculate glucose concentration. The absorbance difference between Tubes A and B was to calculate sucrose concentration since sucrose was hydrolyzed (by invertase) to glucose + fructose. The absorbance difference between Tubes B and C was to calculate RFOs concentration since sucrose and RFOs were converted (by invertase and α-galactosidase) to equivalent moles of glucose.

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4.2.16 Measurement of Lipid Content

The procedure was adapted from Bligh & Dyer (1959). Ten milliliters of methanol (80% v/v, MeOH) and 5 mL chloroform (CHCl) were addedto a 100 mL conical flask containing 1 g of sample, and vortexed for 2 minutes. Fivemilliliters of CHCl were added a second time, and the mixture was vigourously shaken for 2 minutes. After adding 10 mL of distilled water, the mixture was vortexed for another two minutes. The layers were separated by centrifugation at 2000 rpm for 10 minutes. By using a pipette, the lower layer was transferred to a pear-shaped flask. A second extraction was performed with 10 mL of 10% (v/v) MeOH in CHCl by vortexing for 2 minutes. The CHCl phase was added to the first extract after centrifugation. Rotavapor (Heidolph, Model LB-RE52A, USA) was used to evaporate the residue, which was then dried at 104°C for 1 hour to ensure complete dryness. Lipid content was calculated by the following formula:

$$\text{Lipid content (mg/g)} = \frac{\text{Weight of lipid in aliquot x Volume of the chloroform layer}}{\text{The volume of aliquot x 100}}$$

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4.2.17 Measurement of Antioxidant Capacity (DPPH Scavenging)

DPPH inhibition in cacao seeds was determined following Gangwar et al. (2014). In a centrifuge tube, 200 mg of sample was taken (in triplicate). Instead of the sample, 200 microliters of distilled water were used as a control. The sample and blank were then treated with 1mL of DPPH (8 mg/100 mL ethanol, 80%) solution. For 30 minutes, this setup was left at room temperature (vortexed in between). After that, the tubes were centrifuged at 4000 rpm for 10 minutes. Following that, 0.5 mL of supernatant was poured into new tubes containing 1 mL of ethanol (80% v/v), and the absorbance at 517 nm was measured against the ethanol using a spectrophotometer (DeNovix DS-11, USA).

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Each crude extract was analyzed three times. The percentage of inhibition was calculated against blank:

$$\text{DPPH inhibition (\%)} = \frac{\text{A blank} - \text{A sample}}{\text{A blank}} \times 100$$

Where, blank is the absorbance of the control reaction (containing all reagents except the test compound) and the sample is the absorbance of the test compound.

4.2.18 Measurement of Hydrogen Peroxide (H_2O_2)

The amount of H_2O_2 was measured in 3 replicates using the Velikova et al. (2000) method. Seed tissues (200 mg) were homogenized in 2 mL of 0.1 percent (w/v) trichloroacetic acid (TCA), then centrifuged at 12,000 g for 15 minutes at 25°C. Onemilliliter of supernatant was mixed with 1 ml of 10 mM sodium phosphate buffer (pH7) and 2 mL of 1 M potassium iodide to measure absorbance at 390 nm using a spectrophotometer (DeNovix DS-11, USA). The H_2O_2 content was calculated using an extinction coefficient of 0.28 M^{-1} cm^{-1} and expressed as nmol g^{-1}.

Calculations:

$$\text{Volume activity (Units/mL)} = \frac{\text{Absorbance} \times V_q}{\text{Extinction coefficient} \times V_s}$$

$$H_2O_2 \text{ content (Units/g)} = \frac{\text{Units/mL}}{\text{Sample (g)/mL}}$$

where, V_q = reaction volume into cuvette (in mL)

V_s = volume (in mL) of sample used

4.2.19 Measurement of Lipid Peroxidation (MDA Content)

Lipid peroxidation was determined by estimation of the malondialdehyde (MDA) content according to Heath & Packer (1968). A 200 mg of liquid nitrogen crushed sampleswere homogenized in triplicate with 0.5 percent (w/v) 2-thiobarbituric acid

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(TBA) dissolved in 20% (w/v) trichloroacetic acid (TCA). The homogenate was incubated for 30 minutes in a water bath (WiseBath, WSB-model, Germany) at 100°C before being shifted to 0°C for 15 minutes to stop the reaction. After that, the sample was centrifuged at 11,000 g for 10 minutes, and the absorbance of the supernatant wasmeasured at 532 and 600 nm using a spectrophotometer (DeNovix DS-11, USA). Values of absorption recorded at 600 nm were subtracted from the values recordedat 532 nm. The concentration of MDA present was calculated using the extinction coefficient of 155 mM^{-1} cm^{-1} and expressed as nmol MDA g^{-1}.

Calculations:

$$\text{Volume activity (Units/mL)} = \frac{\text{Absorbance} \times V_q}{\text{Extinction coefficient} \times V_s}$$

$$\text{Lipid peroxidation (MDA content) (Units/g)} = \frac{\text{Units/mL}}{\text{Sample (g)/mL}}$$

where, V_q = reaction volume into cuvette (in mL)

V_s = volume (in mL) of sample used

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4.2.20 Preparation of Enzyme Extraction and Determination of Protein Content

This method was carried out according to Li & Sun (1999). Cacao seed tissues (about 250 mg) were ground with a pestle and mortar and then homogenized with 1.0 ml of 100 mM phosphate buffer (pH 7.8) containing 2% polyvinylpolypyrrolidone (PVPP). The homogenate was centrifuged at 16,000 x g (4°C) for 18 min, and the supernatant was used as enzyme extract.

Protein content of the enzyme extract was determined according to the method of Bradford (1976). A standard curve was prepared using bovine serum albumin (BSA), with the equation was y = 0.573x - 0.0131 (R^2=0.99). The results were expressed as mg per g.

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4.2.21 Assay of Antioxidant Ascorbate Peroxidase (APX) Enzyme Activity

In a total volume of 1 mL, the assay mixture contained of 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbate (AsA), 1 mM hydrogen peroxide, 0.1 mM EDTA and 0.1 mL enzyme solution. The reaction was started by adding an enzyme, and the change in optical density was measured at 290 nm (Li & Sun, 1999). APX activity was calculated following an extinction coefficient of 2.8 mM^{-1} cm^{-1} and expressed as µmol AsA mg^{-1} protein min^{-1}.

Calculations:

$$\text{Volume activity (Units/mL)} = \frac{\text{Absorbance} \times V_q}{\text{Extinction coefficient} \times V_s}$$

$$\text{Enzymes activity (Units/mg)} = \frac{\text{Units/mL}}{\text{mg protein/mL}}$$

where, V_q = reaction volume into cuvette (in mL)

V_s = volume (in mL) of sample used

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4.2.22 Assay of Antioxidant Catalase (CAT) Enzyme Activity

CAT activity was measured using a procedure of Aebi (1983). In a total volumeof 1 mL, the assay mixture contained of 50 mM phosphate buffer, pH 7.0, containing 10 mM H_2O_2 and 20 µL enzyme solution. CAT activity was determined by measuringthe decomposition of H_2O_2 at 240 nm; at 0 to 3 minutes. CAT activity was calculated following an extinction coefficient of 0.0436 mM^{-1} cm^{-1} and expressed as µmol H_2O_2 mg^{-1} protein min^{-1}.

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Calculations:

$$\text{Volume activity (Units/mL)} = \frac{\text{Absorbance} \times V_q}{\text{Extinction coefficient} \times V_s}$$

$$\text{Enzymes activity (Units/mg)} = \frac{\text{Units/mL}}{\text{mg protein/mL}}$$

where, V_q = reaction volume into cuvette (in mL)

V_s = volume (in mL) of sample used

4.2.23 Assay of Antioxidant Superoxide Dismutase (SOD) Enzyme Activity

CAT activity was measured using a procedure of Dhindhsa et al. (1981). Three millilitres of reaction mixture containing 0.1 mL of 1.5 M Na_2CO_3, 0.2 mL of 200 mM methionine, 0.1 mL of 3 mM EDTA, 0.1 mL of 2.25 mM of p-nitroblue tetrazolium chloride (NBT), 1.5 mL of 100 mM potassium phosphate buffer (pH 7.5), 1 mL of distilled water, and 0.05 mL of enzyme samples. As a control, a tube without enzyme was used. The reaction was started by adding 0.1 mL of 60 µM riboflavin and placing the tubes for 15 minutes beneath a light source of two 15 W fluorescent lamps. By turning off the light and covering the tubes with black cloth, the reaction was stopped. At 560 nm, absorbance was measured. An illuminated blank without protein resulted in the greatest reduction of NBT and, as a result, the greatest absorbance at 560 nm. SOD activity is calculated per mg protein by subtracting the absorbance of the blank from the absorbance of the sample. SOD activity was measured in units of U mg^{-1} protein. One unit of activity is the amount of protein required to inhibit a 50% initial reduction of NBT in the presence of light.

4.2.24 Assay of Antioxidant Glutathione Reductase (GR) Enzyme Activity

Glutathione reductase activity was determined using activity was determined according to Esterbauer & Grill (1978), which involved measuring the rate of NADPH oxidation at 340 nm (extinction coefficient = 6.22 mM^{-1} cm^{-1}). The assay

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mixture included 0.5 mM NADPH, 10 mM oxidized glutathione (GSSG), 10 mM EDTA in 0.1 M phosphate buffer pH 7.8, and 50 - 100 µL of enzyme extract. The GR activity was measured in µmol NADPH mg^{-1} protein min^{-1}.

Calculations:

$$\text{Volume activity (Units/mL)} = \frac{\text{Absorbance} \times V_q}{\text{Extinction coefficient} \times V_s}$$

$$\text{Enzymes activity (Units/mg)} = \frac{\text{Units/mL}}{\text{mg protein/mL}}$$

where, V_q = reaction volume into cuvette (in mL)
V_s = volume (in mL) of sample used

4.2.25 Assay of Antioxidant Guaiacol Peroxidase (POX) Enzyme Activity

Guiaicol peroxidase (POX) activity was measured using a procedure of Jebara et al. (2005). About 1.5 mL of 0.1 M phosphate buffer (pH 7.0), 1 mL of 10 mM guaiacol, 0.1 mL enzyme extract, and 0.1 mL of 12.3 mM H_2O_2 were included in the 3 mL assay mixture. POX activity was measured by the oxidation of guaiacol at 470 nm (extinction coefficient = 26.6 mM^{-1} cm^{-1}). Enzyme activity was measured in µmol guaiacol oxidized mg^{-1} protein min^{-1}.

Calculations:

$$\text{Volume activity (Units/mL)} = \frac{\text{Absorbance} \times V_q}{\text{Extinction coefficient} \times V_s}$$

$$\text{Enzymes activity (Units/mg)} = \frac{\text{Units/mL}}{\text{mg protein/mL}}$$

where, V_q = reaction volume into cuvette (in mL)
V_s = volume (in mL) of sample used

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4.2.26 Statistical Analysis

The experiment was arranged in a completely randomized design (CRD), witha split plot arrangement (storage condition as the main plot and the storage durationsas the subplot) and with 3 replications. The data were analyzed using ANOVA, and the significant means were separated by least significant difference (LSD) test at $P < 0.05$.

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4.3 Results

4.3.1 Physical and Physiological Status of The Cacao Seed: Seed Moisture Content

Seeds at the non-chilling conditions [RT, 16°C, 40% RH and 16°C, 80% RH] tended to decline their moisture content (MC) with time, especially for seeds at RT and 16°C, 40% RH. Along with storage durations, seeds at RT showed a significantly lower (8 to 17%) of MC than the control, at 4 days after storage (DAS), and later consistently exhibited low MC (Figure 4.2). In the end, seeds at RT showed the lowest MC at 12 DAS, as compared to the other treatments. On the contrary, seeds at 16°C, 40% RH, and 16°C, 80% RH showed no significant changes of MC than the control, during early days of storage. Thereafter, seeds at 16°C, 40% RH showed a gradual and significant decline (8 to 9%) of MC at 4 DAS and, 8 to 12 DAS. Meanwhile, seeds at 16°C, 80% RH showed a significant decline (9%) of MC only at 10 DAS but reverted to the same level as the control at 12 DAS. On the other hand, seeds at the chilling conditions (14°C, 40% RH and 14°C, 80% RH) exhibited a more inconsistent pattern of MC. Seeds at 14°C, 40% RH showed a significantly lower (8 to 14%) of MC than the control, at 2 to 4 DAS. The pattern, however, gradually increased back by 9 to 15% at 6 to 12 DAS, which further showed among the highest seed MC at 12 DAS, as compared to the other treatments. Meanwhile, seeds at 14°C, 80% RH showed no significant changes of MC as compared to the control during early days of storage but later showed a significant increase (10%) of MC, only at 10 DAS to record the highest MC values in the seeds. The changes pattern of MC for each of the treatments could have been due to the structural changes in seed depending on their storage conditions (moisture equilibration processes), which further affected the rate of water intake or loss. Seeds stored at the higher RH (80% RH) seem to retain the higher values of MC during almost all the storage durations.

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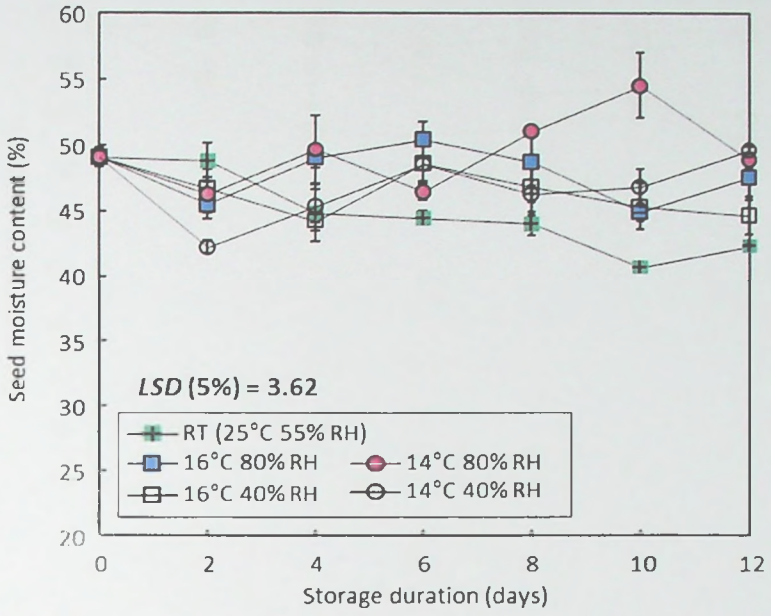


Figure 4.2 : Effect of storage conditions on the moisture content of cacao seeds for 12 days of storage

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4.3.2 Final Germination Percentage (FGP)

Seeds at the non-chilling conditions [RT, 16°C, 40% RH and 16°C, 80% RH] produced a better percentage of germination until the end of data collections. Along with storage durations, seeds at RT and 16°C, 40% RH exhibited consistently higher final germination percentage (FGP), and showed no significant differences with the control, until 12 DAS (Figure 4.3). Seeds at 16°C, 80% RH showed the same pattern of FGP during early days of storage, before significantly decline (13%) at 10 DAS. At 12 DAS, the treatment exhibited the lowest FGP as compared to the other treatments. On the contrary, seeds at the chilling conditions (14°C, 40% RH and 14°C, 80% RH) produced a significantly lower (10%) of FGP as compared to the control, at 2 DAS. Seeds at 14°C, 40% RH, continually maintained the FGP at the same level as the control at 6 to 8 DAS, before declined significantly (12 to 18%) until 12 DAS. Meanwhile, seeds at 14°C, 80% RH showed a following significant decline (17 to 23%) of FGP at 4 to 12 DAS, to further consistently produced among the lowest FGP recorded in the seeds until 12 DAS.

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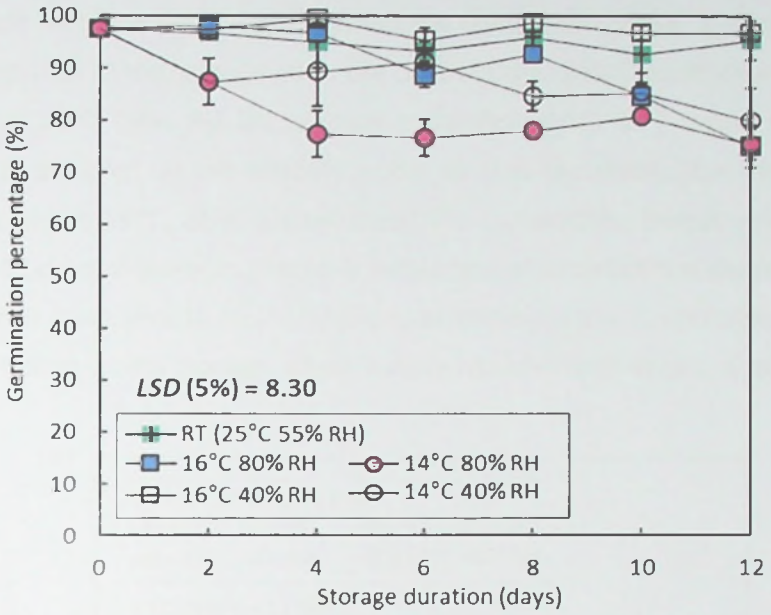


Figure 4.3 : Effect of storage conditions on the final germination percentage of cacao seeds for 12 days of storage

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4.3.3 Germination Rate Index (GRI) and Mean Germination Time (MGT)

Seeds at the non-chilling conditions [RT, 16°C, 40% RH and 16°C, 80% RH] continually showed a dominant characteristic of better quality on seed physiological parameter evaluations. In terms of germination rate index (GRI) or speed of germination, for example, seeds at RT exhibited a significantly higher (34%) GRI than the control at 4 DAS (Figure 4.4). The values were later maintained throughout the storage durations, before significantly increased by 17% at 10 DAS, to record the highest GRI of the seeds. Seeds at 16°C, 40% RH followed to show the gradual increase of GRI with no significant differences than the control, along 12 days of storage. Meanwhile, seeds at 16°C, 80% RH exhibited a significantly lower (39%) GRI than the control, at 2 DAS. The values later maintained before increased significantly by 47% at 8 DAS. On the other hand, seeds at the non-chilling conditions (14°C, 40% RH and 14°C, 80% RH) showed quite similar patterns of GRI with the previous treatment stated (16°C, 80% RH), especially during the early days of storage. Both treatments, 14°C, 40% RH, and 14°C, 80% RH, exhibited significantly

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lower (44 and 47%, respectively) GRI than the control, at 2 DAS. The values were further maintained at the same level as the control throughout the storage durations, with seeds at 14°C, 40% RH led to show a significantly (1 to 2 times) higher GRI than seeds at 80% RH, at the majority of the storage durations. It is interesting to note that seeds at 14°C, 80% RH exhibited the consistently lowest values of GRI throughout 12 days of storage. The early symptoms of deterioration shown, as those seed cells need more time to fix any changes or damages due to the exposure to the stressful condition during storage, which further reduced their speed of germination.

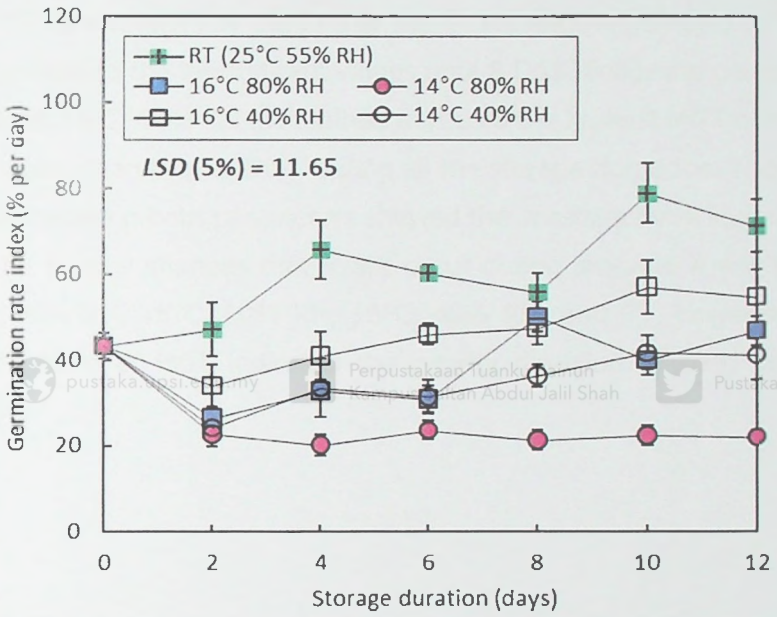


Figure 4.4 : Effect of storage conditions on germination rate index of cacao seeds for 12 days of storage

Mostly, seeds at the non-chilling conditions [RT, 16°C, 40% RH and 16°C, 80% RH] recorded the lower number of days needed to achieve the optimum germination percentage for each of the seeds lots. Seeds at RT led the pattern to record the consistently insignificant lower mean germination time (MGT) than the control during the early days of storage, and later significantly exhibited a significant decline (36 to 46%) of MGT at 10 DAS (Figure 4.5). The treatment recorded the lowest MGT values of the seeds towards the end of storage durations. Both seeds at 16°C, 40% RH and 16°C, 80% RH showed the higher MGT during the early days of storage, with about

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34% and 41% higher than the control, respectively. Seeds at 16°C, 40% RH however, showed a significant decline of 16% of MGT at 4 DAS. Thereafter, the treatment consistently showed the gradual decline (26 to 41%) of MGT to be at the same level as the control until the end of storage durations. Meanwhile, seeds at 16°C, 80% RH recorded a significant decline (32 to 56%) of MGT at 8 DAS to be at the same level as the control and further recorded among the lowest MGT of the seeds at 12 DAS. Starting with a quite similar pace as the seeds at 16°C, both seeds at 14°C, 40% RH and 14°C, 80% RH exhibited the significantly higher (47 and 48%, respectively) MGT than the control, at 2 DAS. However, seeds at 14°C, 40% RH led to show better values of MGT (lower MGT) by the further significant decline (25%) of MGT at 4 DAS and later maintained the insignificant values until 8 DAS. Following germination rate index, seeds at 14°C, 80% RH maintained to record the highest MGT of the seeds (1 to 2 times higher than the control), during all the storage durations. Those negative inter-relation between both parameters showed the consistently in line of more time needed to fix for any changes that might occur during storage. A few treatments, e.g., 14°C, 40% RH, 16°C, 80% RH, 16°C, 40% RH, and RT, however, displayed decreasing pattern of MGT indicated the priority direction of seed cells towards germination.

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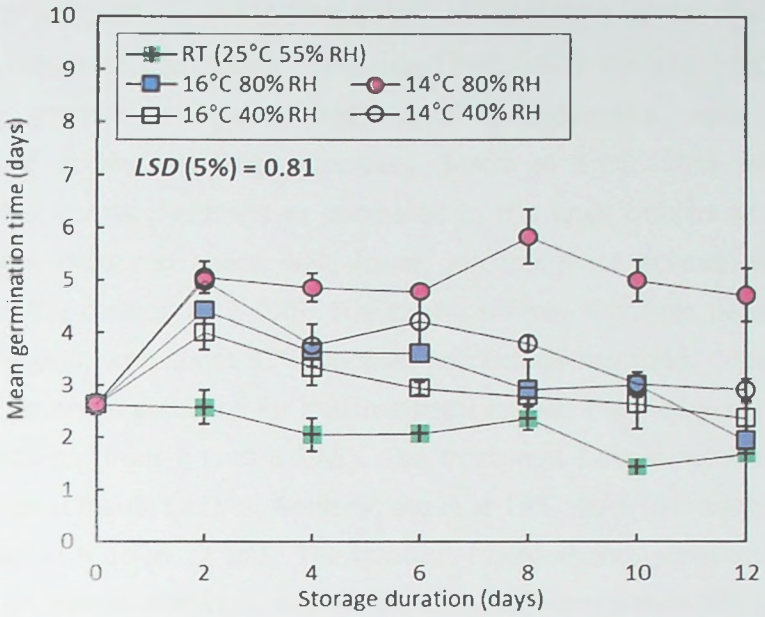


Figure 4.5 : Effect of storage conditions on mean germination time of cacao seeds for 12 days of storage

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4.3.4 Seedling Growth Status

Seeds at the non-chilling conditions [RT, 16°C, 40% RH and 16°C, 80% RH] exhibited better seedlings performance through the indicator of no dead seedlings recorded throughout the data collections. Seeds at RT led the positive patterns by recorded 100% of normal seedlings throughout 12 days of storage (Figure 4.6). This was followed by seeds at 16°C, 40% RH which produced a consistently higher ratio of normal and abnormal (normal: abnormal) percentage at every point of storage duration and only showed the significant decline (27%) of normal seedlings at 12 DAS. Meanwhile, seeds at 16°C, 80% RH showed the consistent ratio of normal and abnormal seedlings percentage at the same level as the control during early days of storage (until 6 DAS). Thereafter, the treatment showed a significant 33 and 27% declined of normal seedlings at 8 and 10 DAS, respectively. The declining pattern of normal seedlings was parallel with the increase of abnormal and dead seedlings, especially towards the end of storage durations. Seeds at 16°C, 80% RH further recorded the highest percentage of abnormal seedlings, and maintained the

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insignificantly low pattern of dead seedlings, as compared to the control. On the other hand, seeds at the chilling conditions (14°C, 40% RH and 14°C, 80% RH) continue to exhibit low quality and vigour characteristics, with the lowest percentage of normal seedlings recorded. Seeds at 14°C, 80% RH however, showed higher normal seedlings as compared to the ones treated with 40% RH. The treatment produced about 40% lower percentage of normal seedlings, as compared to the control, at 2 DAS. The lowest normal seedlings percentage was recorded at 4 DAS, with about 33% declined percentage recorded. Combinations of normal and abnormal seedlings for this treatment exceed the increasing percentage of dead seedlings (from 2 until 6 DAS). The treatment further recorded 100% of dead seedlings at 8 until 12 DAS. Similarly, seeds at 14°C, 40% RH recorded 100% of deadseedlings at 8 up to 12 DAS. The treatment beforehand, produced the lowest percentage of normal seedlings, with about 67% differences than the control, at 2 DAS. Seeds at 14°C, 40% RH were also observed to produce the abnormal seedlings only at 2 DAS. The percentage of dead seedlings was recorded to exceed the combination of normal and abnormal seedlings, at 4 DAS until the end of storage durations. Furthermore, the higher normal seedling produced for seeds at 14°C, 80% RH than 14°C, 40% RH at 2 until 6 DAS, probably due to only seeds with superior characteristics could survive in 14°C, 80% RH.

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Figure 4.6 : Effect of storage conditions on seedling growth status of cacao seeds for 12 days of storage

Seeds at the non-chilling conditions [RT, 16°C, 40% RH and 16°C, 80% RH] produced higher seedling dry biomass (MASS), which indirectly indicated the higher quality and vigour status, as compared to the other treatments. Seeds at RT led the positive pattern to exhibit the highest MASS, which was also recorded to be 28% significantly higher than the control, at 2 DAS (Figure 4.7). The results maintained to show significantly higher (about 15 to 24%) values than the control during most of the storage durations (from 6 until 12 DAS). Seeds at 16°C, 40% RH followed to exhibit about 4% to 6% significantly higher MASS than the control at 2 to 4 DAS. The values, however, showed a gradual declining pattern which later recorded the insignificantly lower values of MASS than the control, at 6 DAS until DAS. Meanwhile, seeds at 16°C, 80% RH exhibited about 11% insignificantly higher MASS than the control, at 2 DAS. Thereafter, seeds at 16°C, 80% RH declined significantly (26 to 53%) to be recorded as among the lowest values of MASS until the end of storage durations. As for seeds at the chilling conditions (14°C, 40% RH and 14°C, 80% RH), the data collections stopped until 6 DAS due to 100% of dead seedlings recorded for both conditions at 8 until 12 DAS. Seeds at 14°C, 80% RH exhibited a declining pattern but with higher values of MASS as compared to the ones treated with 40%

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RH. Seeds at 14°C, 80% RH showed a significant decline (about 29 to 56%) of MASS than the control, at 2 DAS until 6 DAS. Meanwhile, seeds at 14°C, 40% RH showed a significant decline (54%) of MASS than the control, at 4 DAS and maintained the values until 6 DAS. The self-heterogenous character of seed-lots might contribute to some fluctuated patterns of MASS recorded in this study. Align with the germination characteristics displayed in the other results earlier, only seeds at RT and 16°C, 40% RH showed the promising potential of seedling establishment, not significantly lower than the control throughout 12 days of storage.

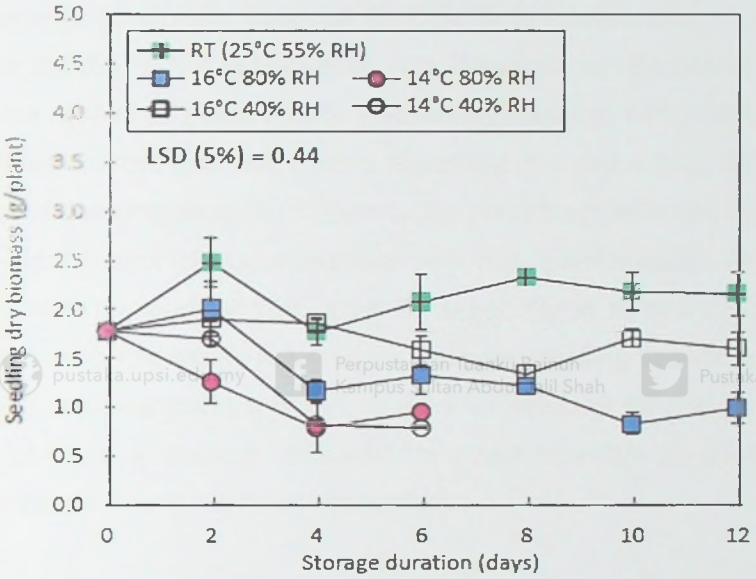


Figure 4.7 : Effect of storage conditions on seedling dry biomass of cacao seeds for 12 days of storage

Seedling vigour index (SVI), as one of the parameters used to evaluate the seedling growth status during this study, also indicated that most seeds at non-chilling conditions [RT, 16°C, 40% RH and 16°C, 80% RH] exhibited better and higher values as compared to the seeds at the chilling conditions. From the non-chilling conditions, seeds at RT and 16°C, 40% RH exhibited the higher SVI along 12 days of storage (Figure 4.8). Seeds at RT showed a significantly higher (21%) than the control at 2 DAS, which was recorded as the highest SVI (50.53) of the seeds. Thereafter, the values declined significantly (17%) at 4 DAS, to be at the same level as the control until towards the end of storage durations. The positive pattern was

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followed by seeds at 16°C, 40% RH, which showed the insignificantly higher values of SVI as compared to the control during all storage durations. Meanwhile, seeds at 16°C, 80% RH managed to maintain the insignificantly higher SVI than the control, during the early days of storage. The results declined significantly (30%) at 10 DAS than the control to further produced among the lowest SVI at 12 DAS. On the other hand, the SVI for both seeds at the chilling conditions (14°C, 40% RH and 14°C, 80% RH) were only managed to be collected until 6 DAS, due to 100% of dead seedlings recorded at 8 to 12 DAS. Seeds at both conditions (14°C, 40% RH and 14°C, 80% RH) showed a significant decline (45% and 33%, respectively) of SVI during early days of storage (2 to 4 DAS). Seeds at 14°C, 80% RH maintained the low SVI, to exhibit among the lowest SVI of the seeds, with the significant decline (30% to 48%) of SVI than the control at 2 until 6 DAS. Meanwhile, seeds at 14°C, 40% RH showed no significant differences from the control especially at 2 and 6 DAS. SVI parameter which measured the multiplication of germination percentage with the shoot and root length gave the indicator of vigour together with their germinability. The significant decreasing pattern for seeds at 16°C, 80% RH especially at 10 and 12 DAS, showed the consistency of germinability followed by the poor seedling growth exhibited. On the other hand, a similar pattern of SVI showed for seeds at RT and 16°C, 40% RH throughout 12 days of storage, indicated their fair potential in producing seed/ seedling quality at the same level as the control.

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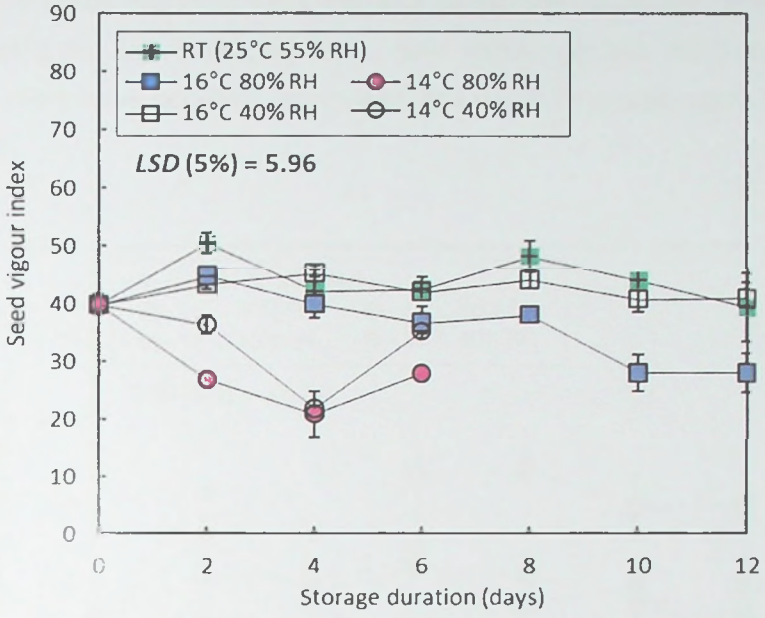


Figure 4.8 : Effect of storage conditions on seedling vigour index of cacao seeds for 12 days of storage

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4.3.5 Leachate Conductivity (LC)

Among the non-chilling conditions [RT, 16°C, 40% RH and 16°C, 80% RH], only seeds at RT showed a significantly higher (48%) of LC than the control, at 2 DAS (Figure 4.9). The treatment later showed a significant decline back to the same level as the control until the end of storage durations. Meanwhile, seeds at 16°C, 40% RH, and 16°C, 80% RH recorded the significantly higher (31 and 33%, respectively) LC than the control, only at 6 DAS, and later reverted to the same level as the control until the end of storage durations. Among these non-chilling conditions, seeds at RT showed the lowest LC values of the seeds at most of the storage durations (at 6, 8, and 10 DAS). On the other hand, both the chilling conditions (14°C, 40% RH and 14°C, 80% RH) showed a significant increase (37%) of LC, at 2 DAS. Seeds at 14°C, 40% RH further showed the significant higher (32%) of LC at 6 DAS, before reverted to the same level as the control until the end of storage durations. Meanwhile, seeds at 14°C, 80% RH showed a significant increase (45 to 52%) of LC at 6 until 12 DAS. The treatment (14°C, 80% RH) maintained to exhibit majority the highest LC

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values of the seeds recorded until the end of storage durations. The highest LC reading during the first 2 DAS, however, gave some signs the more cell membrane transitions might have occurred along with the moisture equilibration processes of seeds at RT.

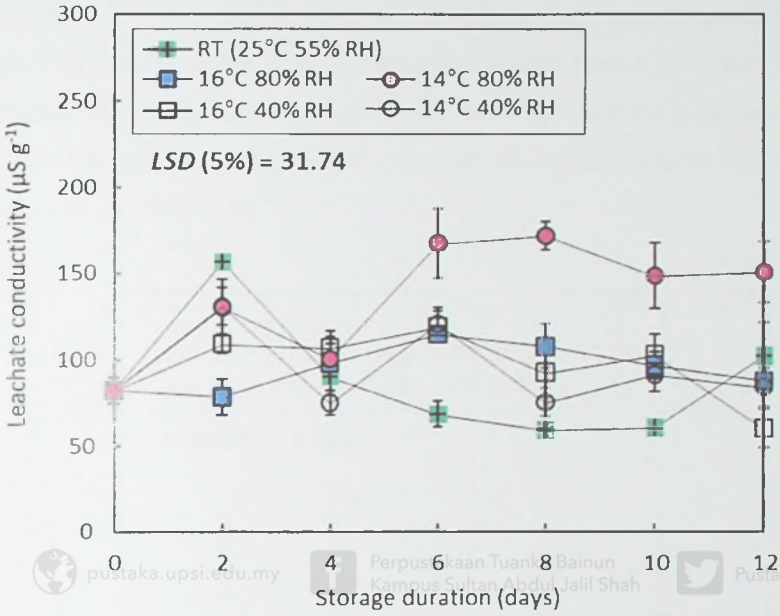


Figure 4.9 : Effect of storage conditions on leachate conductivity of cacao seeds for 12 days of storage

4.3.6 Respiration Rate

Seeds at the non-chilling conditions [RT, 16°C, 40% RH and 16°C, 80% RH] demonstrated the lower respiration rate at most of the storage durations. Seeds at RT showed the significant lowest (50 to 74% lower than the control) respiration rate at 2 until 12 DAS (Figure 4.10). Meanwhile, seeds at 16°C, 40% RH, and 16°C, 80% RH showed quite similar declining (13 to 18%) pattern of respiration rate during early days of storage (from 2 until 6 DAS). Seeds at 16°C, 40% RH further showed a sharply significant increase (16%) of respiration rate than the control at 8 DAS, and later maintained the high values until the end of storage durations. On the other hand, seeds at the chilling condition (14°C, 80% RH) showed a significant increase

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(32 to 46%) of respiration rate along the 12 days of storage durations, which further recorded the highest respiration rate at most of the storage durations as compared to the other treatments. Meanwhile, seeds at 14°C, 40% RH showed an insignificantly higher respiration rate than the control, until the end of storage durations. The respiration rate parameter indicated changes of metabolism occurred within seed cells. The higher but stagnant or reducing pattern displayed for seeds at 14°C, further indicates some sort of impaired respiration may have occurred. On theother hand, the abrupt increasing pattern of respiration rate especially for seeds at 16°C, 40% RH indicated the major changes of metabolic activity in maintaining homeostasis within seed cells.

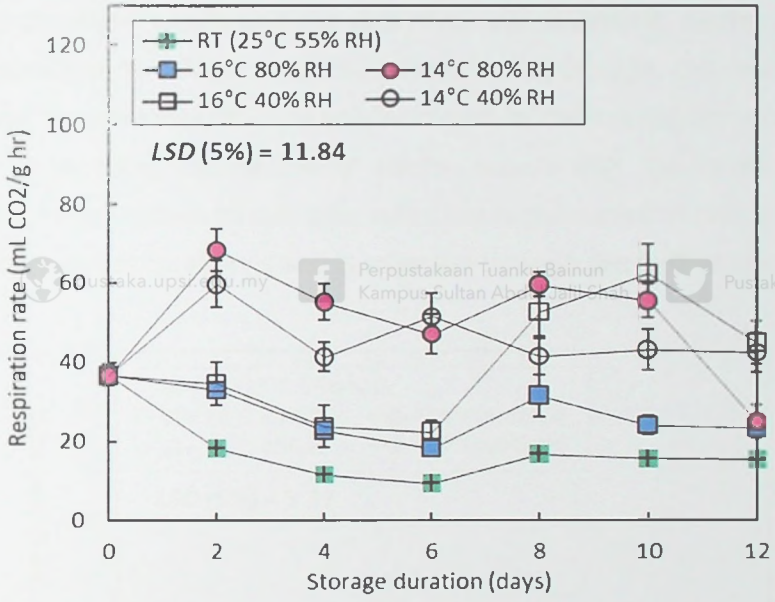


Figure 4.10 : Effect of storage conditions on the respiration rate of cacao seeds for 12 days of storage

4.3.7 Biochemical Status of the Cacao Seed: Total Soluble Sugar

Seeds at the non-chilling conditions [RT, 16°C, 40% RH and 16°C, 80% RH] demonstrated a significant decrease (24 to 73%) of total soluble sugar (TSS) than the control, at 2 DAS (Table 4.11). Among the treatments, seeds at RT showed the lowest TSS (at 2 and 4 DAS) but later gradually increase with the increased storage

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durations. Meanwhile, both seeds at 16°C, 40% RH, and 16°C, 80% RH showed a significant (gradual) decreasing pattern of TSS than the control from 2 until 10 DAS. Seeds at 16°C, 40% RH demonstrated the insignificantly lower TSS as compared to the ones at 80% RH at most of the durations. Both treatments further maintained their values at 12 DAS. On the other hand, seeds at the chilling conditions(14°C, 40% RH and 14°C, 80% RH) exhibited much higher values of TSS during most of the storage duration, as compared to the non-chilling condition treatments. Both treatments showed a significant (42 to 48%) lower of TSS, at 6 DAS. Moreover, different fates showed later, as the seeds at 14°C, 40% RH showed the insignificant increasing pattern of TSS towards the end of storage duration. Vice versa with seeds at 14°C, 80% RH which continued to show a decrease of TSS up to 8 DAS and maintained the lower values until the end of storage durations. As the recalcitrance may be dependent on soluble sugars to survive during storage, the results displayed may indicate the efficiency of using soluble sugars in maintaining homeostasis within seed cells. Decreasing the pattern of soluble sugars with the increasing storage durations for most treatments may also indicating sugar depletion or starvation which enhances their stress conditions and is reflected through the further seed longevity.

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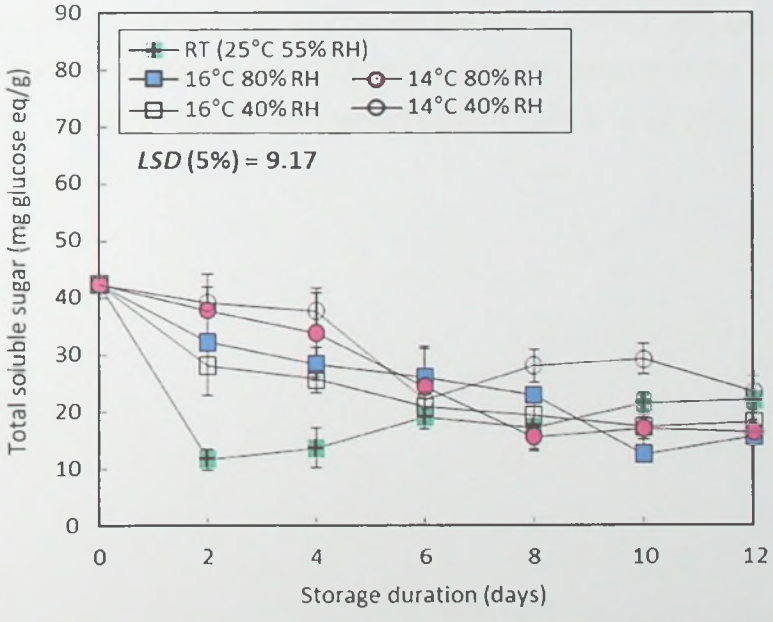


Figure 4.11 : Effect of storage conditions on total soluble sugar of cacao seeds for 12 days of storage

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4.3.8 Glucose, Sucrose, and RFOs Content

Except for seeds at RT, all the storage condition (chilling and non-chilling) treatments showed a significant increase (31 to 42%) of glucose content (GC) in seeds than the control, at 2 DAS (Figure 4.12). Seeds at the non-chilling conditions (16°C, 40% RH and 16°C, 80% RH) showed the sharp decreasing (46 and 34%, respectively) pattern from 2 until 4 DAS. The treatments later showed the significant decreasing pattern of GC at 6 DAS, before maintained the values until the end of storage durations. While seeds at RT were maintained to show the GC values at the same level as the control from 2 until 6 DAS. The GC values later showed a significant decrease (45%) of GC at 8 DAS, before maintained the level of values until the end of storage durations. Moreover, seeds at the chilling conditions (14°C, 40% RH and 14°C, 80% RH) demonstrated quite similar sharp decreasing (78 and 48%, respectively) pattern of GC from 4 until 6 DAS. Seeds at 14°C, 40% RH later maintained to show the lowest GC as compared to the other treatments, until the end of storage durations. Vice versa with seeds at 14°C, 80% RH which showed among the highest GC until the end of storage durations. As parts of the simple sugars, glucose content appeared to be important to reflect the efficiency of using sugars for the seed cells to keep survive during storage. The chilling conditions (14°C) seem to induce those seeds to accumulate glucose content especially during the first 6 days of storage. Vice versa, only RT showed the least influenced to keep the glucose content at the same ranges as the control, with the minimize decreasing pattern from 8 to 12 DAS.

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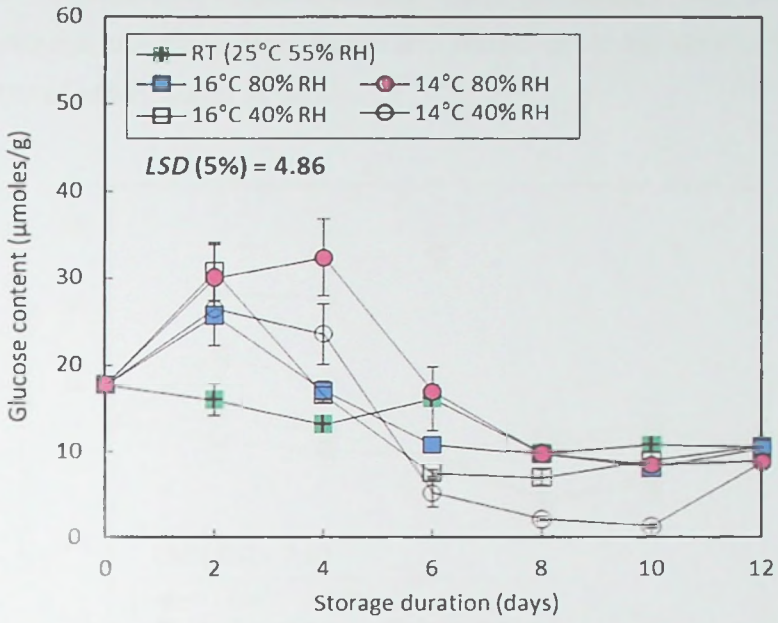


Figure 4.12 : Effect of storage conditions on glucose content of cacao seeds for 12 days of storage

Except for seeds at RT and 16°C, 80% RH, all the storage condition (chilling and non-chilling) treatments showed a significant increase (19 to 32%) of sucrose content (SC) in seeds than the control, at 2 DAS (Figure 4.13). Seeds at 16°C, 40% RH further reverted to be at the same level as the control until the end of storage durations. While seeds at RT and 16°C, 80% RH maintained the SC at the same level as the control since early storage, before showed the significant decreased (21 to 27%) of SC at some points in the middle of storage (at 8 to 10 DAS). On the other hand, only seeds at the chilling conditions (14°C, 40% RH and 14°C, 80% RH) continued to accumulate significantly higher (up to 31% and 38%) of SC than the control until 4 and 6 DAS, respectively. Both treatments later showed the sharp decreased (39 and 42%) of SC at 6 and 8 DAS, respectively, to be at the same level as the control until the end of storage durations. As compared to the simple sugar, disaccharides, such as sucrose content plays the important role in maintaining the homeostasis within seed cells, and as parts of important metabolites used in defensive mechanisms. The increase of sucrose content at first may help to activate defensive systems within seed cells. However, the seed aging

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process may continueto enhance and may reflect the negative feedback with the increased storage durations. The significant impact could be viewed through the performances of seeds stored at 14°C.

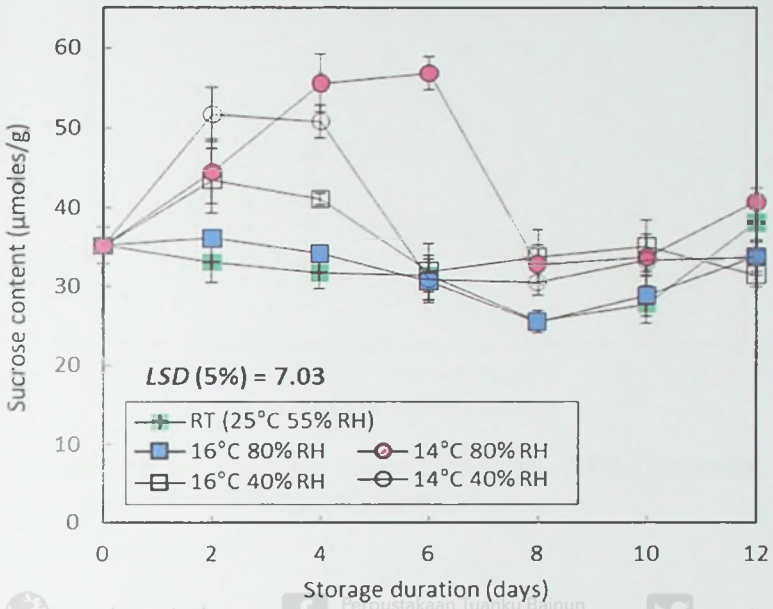


Figure 4.13 : Effect of storage conditions on sucrose content of cacao seeds for 12 days of storage

There were slight differences or changes observed on RFOs content in cacao seeds stored at both the chilling and non-chilling conditions. Only seeds at 14°C, 80%RH showed a significant increase (51 to 57%) of RFOs content at 2 to 4 DAS (Figure4.14). Meanwhile, seeds at RT and 16°C, 40% RH showed a significant increase (42 to 46%) of RFOs only during the earlier storage (at 2 DAS). All the treatments later reverted to the same level as the control until the end of storage durations. On the other hand, only seeds at 16°C, 80% RH and 14°C, 40% RH displayed no significantdifferences on RFOs content than the control, throughout 12 days of storage. More complexity mechanisms are needed in using or degrading the oligosaccharides (RFOs), which cause the slower changes pattern displayed for all storage treatmentsin the present study.

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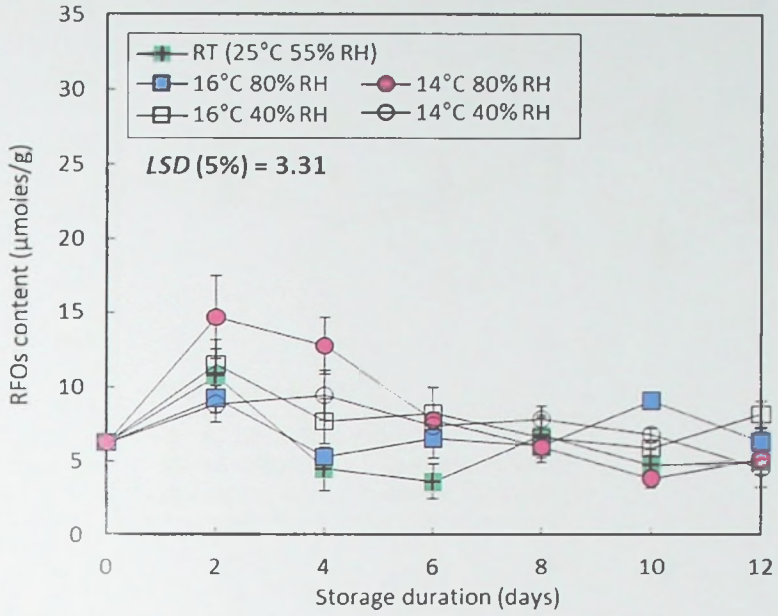


Figure 4.14 : Effect of storage conditions on RFOs content of cacao seeds for 12 days of storage

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4.3.9 Lipid Content

There were slight differences or changes observed in lipid content in cacao seeds stored at both the chilling and non-chilling conditions. Only seeds at 14°C, 40% RH and 16°C, 40% RH showed significant increases (11 to 14%) of lipid content during early storages (at 2 and 4 DAS, respectively) (Figure 4.15). On the other hand, the significant changes in lipid content were displayed at 10 DAS in cacao seeds at all storage conditions. Only seeds at 14°C, 80% RH, and 16°C, 80% RH showed a significant increase (16 to 17%) of lipid content. While seeds at RT, 16°C, 40% RH, and 14°C, 40% RH showed a significant decrease (16 to 20%) of lipid content. All the treatments were then displayed reverted values to the same level as the control at 12 DAS. The consistent pattern of lipid content displayed for most of the storage treatments indicated the unaffected feedback especially during the first 6 days of storage. Vice versa, the small changes (increasing or decreasing) pattern of lipid content further might indicate more significant effects to reflect the mechanisms that may be occurred within seed cells during storage.

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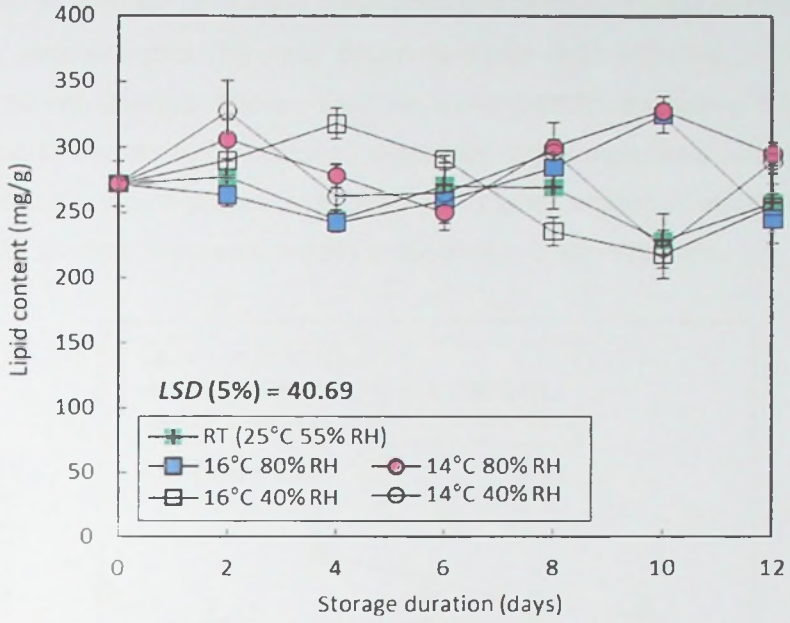


Figure 4.15 : Effect of storage conditions on the total lipid of cacao seeds for 12 days of storage

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4.3.10 Protein Content

Seeds at the non-chilling conditions [RT, 16°C, 40% RH, and 16°C, 80% RH] dominate the others to exhibit the significantly higher protein content. Seeds at RT led to exhibit the highest protein content of the seeds, during all storage durations. The values maintained to be at the same level as the control, along with the increased of storage durations, before further demonstrated a significant decrease (40%) of protein content than the control, at 12 DAS (Figure 4.16). Meanwhile, both seeds at 16°C, 40% RH, and 16°C, 80% RH demonstrated a significant decrease (22 to 23%) of protein content, at 2 DAS. Both treatments later maintained their values until the end of storage durations, with seeds at 16°C, 40% RH showed the significantly higher protein content than the ones stored at 80% RH, towards the end of storage durations (at 8 and 12 DAS). On the other hand, seeds at both the chilling conditions (14°C, 40% RH, and 14°C, 80% RH) demonstrated a significantly lower (42 to 62%) protein content than the control, along the 12 days of storage. Seeds at the 14°C, 80% RH showed the lowest protein content as compared to the other treatments

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throughout the 12 days of storage. The consistent pattern of seed protein content in this study demonstrated the sync and continuous different kind of metabolisms occurred during storage. There might be more protein degrading together with synthesizing processes to be occurred in maintaining homeostasis within seed cells. Higher protein content probably displays the higher vigour potential, which was aligned with the importance of protein in sustaining seed longevity.

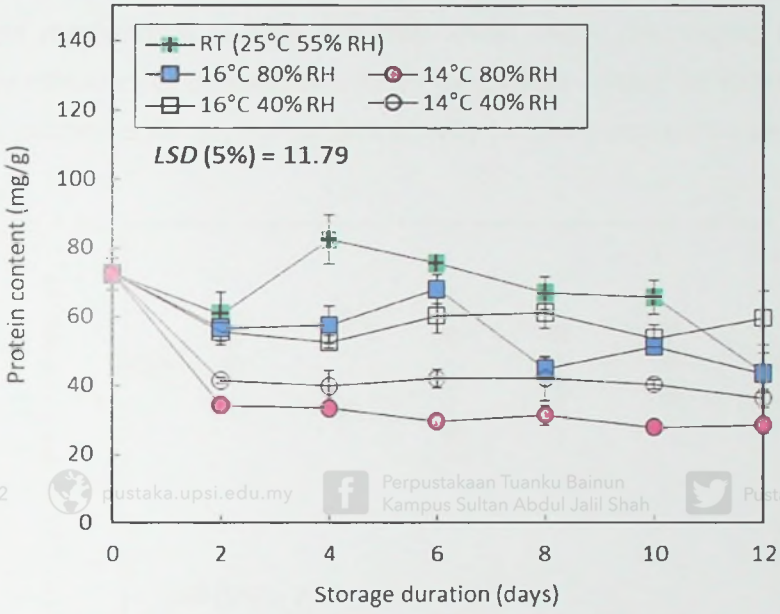


Figure 4.16 : Effect of storage conditions on a total soluble protein of cacao seeds for 12 days of storage

4.3.11 Antioxidant Capacity (DPPH Scavenging)

Seeds at the non-chilling conditions [RT, 16°C, 40% RH, and 16°C, 80% RH] displayed different changes patterns of antioxidant capacity throughout 12 days of storage. Seeds at RT showed a gradual significant decrease (3 to 4%) of antioxidant capacity than the control, at 4 to 6 DAS before reverted to the same level as the control until the end of storage durations (Figure 4.17). Earlier changes of antioxidant capacity pattern displayed for seeds at 14°C, 40% RH with the significant decrease (2 to 3%) of antioxidant capacity than the control at 2 to 4 DAS, before reverting to the same level as the control until the end of storage durations. On the other hand, the different

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fate of antioxidant capacity displayed by the other treatments; seeds at 16°C, 40% RH showed the earlier changes by the significant gradual increase (2 to 4%) of antioxidant capacity than the control, at 2 to 6 DAS. Followed by seeds at 16°C, 80% RH, and 14°C, 80% RH which showed a significant increase (2 to 4%) of antioxidant capacity than the control, at 4 to 6 DAS. All the treatments later reverted to the same level as the control until the end of storage durations. The not so many differences showed for antioxidant capacity indicates the potentially fair level of spaces for any antioxidant mechanisms to have occurred within seeds cells during storage. This hinted the efficiency of antioxidant mechanisms might further be dependent on the activation processes for any antioxidant activity to take place within seed cells.

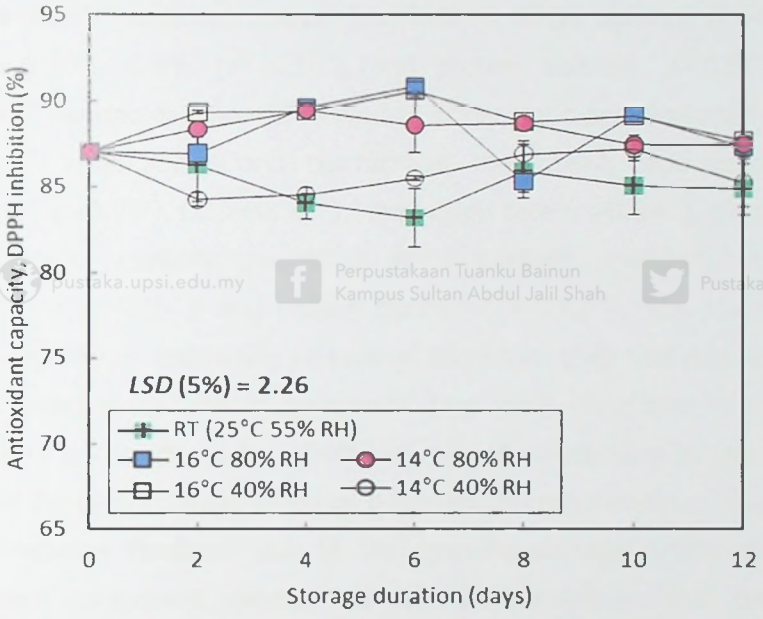


Figure 4.17 : Effect of storage conditions on antioxidant capacity (DPPH inhibition) of cacao seeds for 12 days of storage

4.3.12 Pearson's Correlation

Supporting results shown in the Pearson's correlation (Table 4.1), which demonstrated that one of the seed physiological status parameters measured, FGP, displayed the significantly positive correlations with some of the physiological and biochemical parameters measured in this study; total soluble sugar ($r=0.29^{*}$),

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protein content ($r=0.63^{**}$), LC ($r=-0.38^{**}$), respiration rate ($r=-0.31^{*}$), sucrose content ($r=-0.25^{*}$) and lipid content ($r=-0.20^{*}$). The results indicate initial symptoms reflected the high or low seed quality. The highly positive significant correlations with the protein content justified their importance in the germination mechanism within seed cells. On the contrary, the membrane damages, the increased respirationrate, and the accumulation of sucrose content may indicate the significant negative impacts in limiting the progress of the germination process.

Moreover, GRI parameter showed the significant correlations with all the physiological and biochemical components measured in this study; MC ($r=-0.38^{**}$), LC ($r=-0.50^{**}$), respiration rate ($r=-0.54^{**}$), total soluble sugar ($r=-0.19^{*}$), glucose content ($r=-0.28^{*}$), sucrose content ($r=-0.43^{**}$), RFOs content ($r=-0.30^{*}$), lipid content ($r=-0.40^{*}$), DPPH ($r=-0.30^{*}$), and protein content ($r=0.55^{**}$). On the contrary, MGT parameter followed behind to show the significant correlations with almost all the physiological and biochemical component, vice versa with GRI parameter; MC ($r=0.26^{*}$), LC ($r=0.57^{**}$), respiration rate ($r=0.59^{**}$), glucose content ($r=0.27^{*}$), sucrose content ($r=0.46^{**}$), RFOs content ($r=0.31^{*}$), lipid content ($r=0.38^{*}$), DPPH ($r=0.24^{*}$), and protein content ($r=-0.62^{**}$). The results indicated that seeds with higher respiration rates tend to reduce their GRI and have a higher tendency to need more time to germinate after some durations of storage. The respiration rate is considered to reflect the rate of metabolism in seed cells. This explains that the accumulation of some biochemical components in seed cells may reflect the negative feedback due to the stressful storage conditions. Only the protein content component showed the opposite correlations with both GRI and MGT. This showed the needs and importance of protein content to be sustained during seed growing processes.

On the other hand, MASS and SVI showed the mostly same pattern of significant correlations with some of the physiological and biochemical component measured in this study; (MASS): MC ($r=-0.44^{**}$), LC ($r=-0.26^{*}$), respiration rate ($r=-0.46^{**}$), glucose content ($r=0.30^{*}$), and protein content ($r=0.58^{**}$); (SVI): MC ($r=-0.30^{*}$), LC ($r=-0.29^{*}$), respiration rate ($r=-0.42^{**}$), glucose content ($r=0.29^{*}$), lipid

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content ($r=-0.19^{*}$), and protein content ($r=0.67^{**}$). On the other hand, the correlations between seedling growth performances with some of the physiological and biochemical component measured in this study through their NORMAL, ABNORMAL, and DEAD parameters. (NORMAL): LC ($r=-0.33^{**}$), respiration rate ($r=-0.33^{**}$), sucrose content ($r=-0.24^{*}$), glucose content ($r=0.20^{*}$), and protein content ($r=0.77^{**}$); (ABNORMAL): glucose content ($r=0.21^{*}$), sucrose content ($r=0.22^{*}$), RFOs content ($r=0.36^{**}$), and protein content ($r=-0.25^{*}$); (DEAD) MC ($r=0.25^{*}$), LC ($r=0.31^{*}$), lipid content ($r=0.20^{*}$), glucose content ($r=0.29^{*}$), and protein content ($r=-0.70^{**}$). Again, protein contentcomponent has proven to show the highly positive significant correlations with MASS, SVI, NORMAL and DEAD. The straight positive and negative relationships towards those seedling growth characteristics indicated the significant influence of maintaining higher protein content within seed cells during storage.

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Table 4.1: Pearson's correlation table

Correlations	MC	LC	Respiration rate	Total soluble sugar	Glucose content	Sucrose content	RFOs content	Lipid content	Protein content	DPPH
FGP	ns	-0.38**	-0.31*	0.29*	ns	-0.25*	ns	-0.20*	0.63**	ns
GRI	-0.38**	-0.5**	-0.54**	-0.19*	-0.28*	-0.43**	-0.30*	-0.40**	0.55**	-0.30**
MGT	0.26*	0.57**	0.59**	ns	0.27*	0.46**	0.31*	0.38**	-0.62**	0.24*
MASS	-0.44**	-0.26*	-0.46**	ns	0.30*	ns	ns	ns	0.58**	ns
SVI	-0.30*	-0.29*	-0.42**	ns	0.29*	ns	ns	-0.19*	0.67**	ns
NORMAL	ns	-0.33**	-0.50**	ns	0.20*	-0.24*	ns	-0.25*	0.77**	ns
ABNORMAL	ns	ns	ns	ns	0.21*	0.22*	0.36**	ns	-0.25*	ns
DEAD	0.25*	0.31*	0.44**	ns	-0.29*	ns	ns	0.20*	-0.70**	ns
MC	-									
LC	0.31*	-								
Respiration rate	0.23*	0.31*	-							
Total soluble sugar	ns	-0.22*	0.25*	-						
Glucose content	ns	ns	ns	0.54**	-					
Sucrose content	ns	0.31*	0.39**	0.38**	0.64**	-				
RFOs content	ns	ns	0.25*	0.25*	0.54**	0.40**	-			
Lipid content	ns	0.30*	0.20*	ns	ns	ns	0.19*	-		
Protein content	ns	-0.52**	-0.50**	ns	ns	-0.42**	ns	-0.28*	-	
DPPH	0.30*	0.25*	ns	ns	ns	ns	0.22*	ns	ns	-

Notes: **, significant at P ≤ 0.001; *, significant at P ≤ 0.05; ns, not significant. FGP: final germination percentage, GRI: germination rate index, MGT: mean germination time, MC: moisture content, LC: leachate conductivity, SVI: seedling vigour index, MASS: seedling dry biomass, NORMAL: normal seedling, ABNORMAL: abnormal seedling, DEAD: dead seedling, DPPH: DPPH scavenging.

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4.3.13 Ascorbate Peroxidase (APX) Enzyme Activity

Seeds at the non-chilling conditions were measured to have lower APX enzyme activity than seeds at the chilling conditions, along with the increased storage durations. All seeds at the non-chilling conditions [RT, 16°C, 40% RH, and 16°C, 80% RH] showed insignificant changes in APX enzyme activity than the control, at all the storage durations. On the contrary, seeds at the chilling conditions; 14°C, 80% RH displayed a significant increase (30 to 45%) of APX enzyme activity than the control, at all storage durations (except at 10 DAS) (Figure 4.18). Meanwhile, seeds at 14°C, 40% RH showed a significant gradual increase (33 to 49%) of APX enzyme activity at 6 to 10 DAS. The treatment still showed a significantly higher APX enzyme activity than the control, until 12 DAS. It is interesting to note that seeds at 14°C, 40% RH displayed the abrupt increasing pattern of APX enzyme activity, starting from 6 until 10 DAS. In general, there might be an increase of ROS or free radicals to induce this kind of pattern within seed cells. This was followed by seeds at 16°C, 80% RH which displayed the abruptly increasing APX enzyme activity at 8 until 12 DAS. The quick flashback on their germination and seedling growth performances, seeds at both conditions, however, showed the decreasing pattern of those seed and seedling quality characteristics. This might indicate that irreversible deterioration development within seed cells might be occurred.

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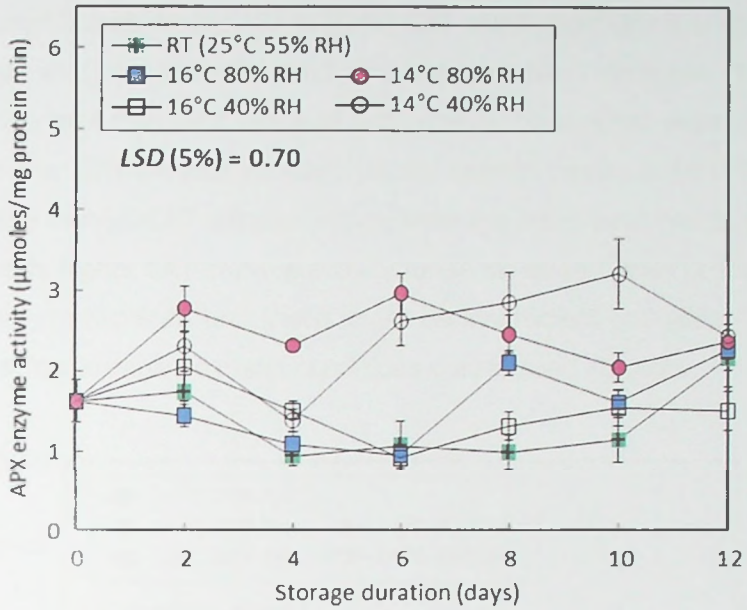


Figure 4.18 : Effect of storage conditions on APX enzyme activity of cacao seeds for 12 days of storage

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4.3.14 Catalase (CAT) Enzyme Activity

Along with the extension of storage durations, seeds at the non-chilling conditions continued to exhibit the lower (antioxidant) enzyme activity as compared to seeds at the chilling conditions. Seeds at the non-chilling conditions [RT, 16°C, 40% RH, and 16°C, 80% RH] showed insignificant values of the CAT enzyme activity than the control, throughout the 12 days of storage durations (Figure 4.19). Seeds at RT were measured to record the lowest values of the CAT enzyme activity, at all the storage durations. The treatment, however, showed significantly higher (74%) CAT enzyme activity in seeds than the control, at 12 DAS. Seeds at 16°C, 80% RH which showed much higher CAT enzyme activity than the ones at RT, later showed a significant increase (65%) values than the control, at 12 DAS. On the other hand, seeds at both the chilling conditions exhibited the significantly higher CAT enzyme activity from the early days until the end of storage durations. Seeds at 14°C, 40% RH, and 14°C, 80%

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RH showed significantly higher (82 to 91%) CAT enzyme activity than the control, at 2 DAS. Seeds at 14°C, 80% RH later showed the sharp decrease (17%) of CAT enzyme activity at 4 DAS, and seeds at both chilling treatments were maintained to exhibit the higher CAT enzyme activity than the control. Seeds at 14°C, 80% RH also led to show the highest CAT enzyme activity than the other treatments, until 12 DAS. The consistently higher CAT enzyme activity for seeds at 14°C may reflect the further breaking-point, to highlight that there might be insufficient antioxidant enzymes to cope with the increasingly stressful conditions during seed storage.

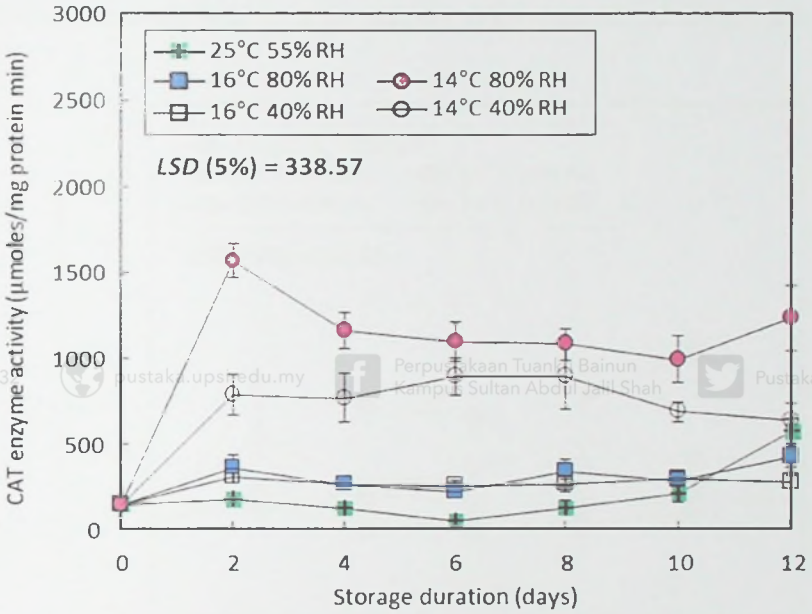


Figure 4.19 : Effect of storage conditions on CAT enzyme activity of cacao seeds for 12 days of storage

4.3.15 Superoxide Dismutase (SOD) Enzyme Activity

Seeds at the non-chilling conditions showed significantly lower SOD enzyme activity than seeds at the chilling conditions. Most of the significantly lowest SOD enzyme activity was displayed by seeds at RT, along with the extension of storage durations (Figure 4.20). The treatment showed the insignificant changes of SOD enzyme activity, to be at the same level as the control at 2 to 10 DAS. The treatment later showed a significant increase (29%) of SOD enzyme activity than the control, at 12

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DAS. Seeds at both 16°C, 40% RH, and 16°C, 80% RH showed the insignificant changes of SOD enzyme activity to be at the same level as the control along with the extension of storage durations. On the contrary, seeds at both the chilling conditions (14°C, 40% RH and 14°C, 80% RH) showed a significant increase (48 to 57%) of SOD enzyme activity, at 2 DAS. The treatments later maintained higher values than the control until 12 DAS. Seeds at 14°C, 80% RH led to display the significantly higher SOD enzyme activity, as compared to the other treatments along the 12 days of storage durations. As a part of the frontline in antioxidant enzyme mechanisms, the higher SOD enzyme activity displayed for seeds at 14°C, 80% RH reflected the consistent or increased ROS or free radicals to be neutralized within seed cells.

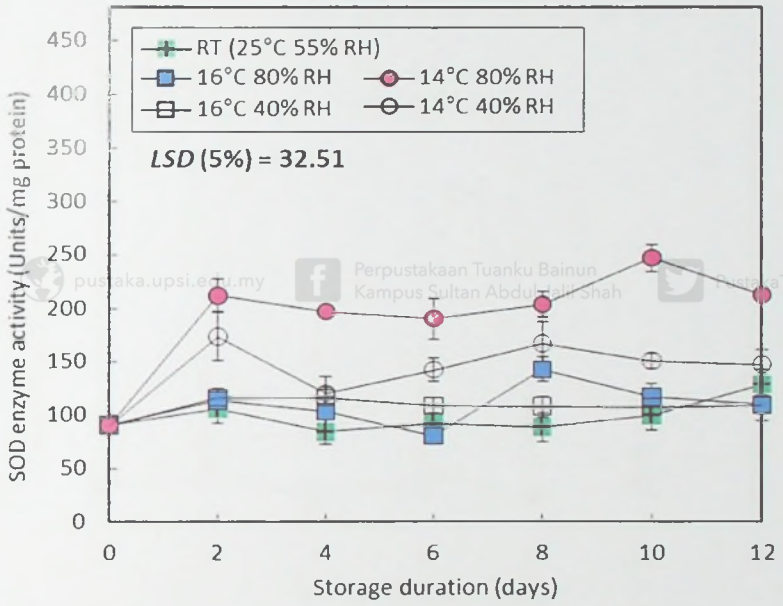


Figure 4.20 : Effect of storage conditions on SOD enzyme activity of cacao seeds for 12 days of storage

4.3.16 Glutathione Reductase (GR) Enzyme Activity

Seeds at all the non-chilling conditions showed no significant changes of GR enzyme activity to be at the same level as the control along with the extension of storage durations (except for at least at one duration for each of the treatments). On the contrary, seeds at the chilling conditions (14°C, 40% RH and 14°C, 80% RH) showed

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significantly higher (73% to 85%) GR enzyme activity than the control at 2 DAS (Figure 4.21). The sharp decreasing (17%) pattern showed for seeds at 14°C, 40% RH at 4 DAS, and later maintained the values until 6 DAS. Thereafter, the values increased back to display among the highest GR enzyme activity at 12 DAS. Meanwhile, seeds at 14°C, 80% RH displayed significantly higher GR enzyme activity than the control, at all the storage durations (except at 6 DAS). Following seeds at the 40% RH, seeds at 14°C, 80% RH displayed among the highest GR enzyme activity at 12 DAS. As one of the intermediate antioxidant enzymes, the higher pattern of GR enzyme activity indicates their inclusive character to react efficiently in reducing harmful effects of ROS or free radical existence within seed cells.

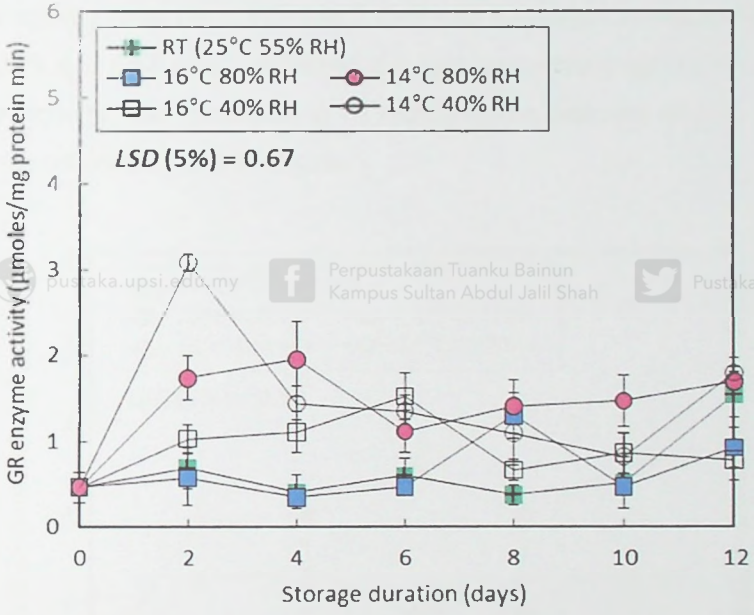


Figure 4.21 : Effect of storage conditions on GR enzyme activity of cacao seeds for 12 days of storage

4.3.17 Guaiacol Peroxidase (POX) Enzyme Activity

Seeds at the non-chilling conditions were recorded to have among the significantly lowest POX activity as compared to seeds at the chilling conditions, along with the extension of storage durations. Among the non-chilling treatments, seeds at RT showed the majority insignificantly lowest POX enzyme activity, at 2 to 10 DAS (Figure

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4.22). The treatment later showed a significant increase (44%) of POX enzyme activity, then the control, at 12 DAS. Seeds at 16°C, 80% RH showed much higher POX enzyme activity also showed significantly higher (35%) values than the control, only at 8 and 12 DAS. While seeds at 16°C, 40% RH showed no significant changes of POX enzyme activity than the control, at all storage durations. Consistent with the other antioxidant enzyme activities stated before, seeds at both the chilling conditions (14°C, 40% RH and 14°C, 80% RH) displayed significantly higher POX enzyme activity at all storage durations. Seeds at 14°C, 80% RH dominated to show the significant increase (60%) of POX enzyme activity than the control at 2 DAS and later maintained to show the highest values (as compared to the other treatments) until 12 DAS. Followed by seeds at 14°C, 40% RH which showed a significant increase (48%) of POX enzyme activity than the control at 2 DAS and retained the values until 12 DAS. In line with APX and CAT which reflected the first detoxifying actions made by SOD; POX enzyme activity also contributing to reducing the harmful effects on the ROS orfree radical existence or accumulation.

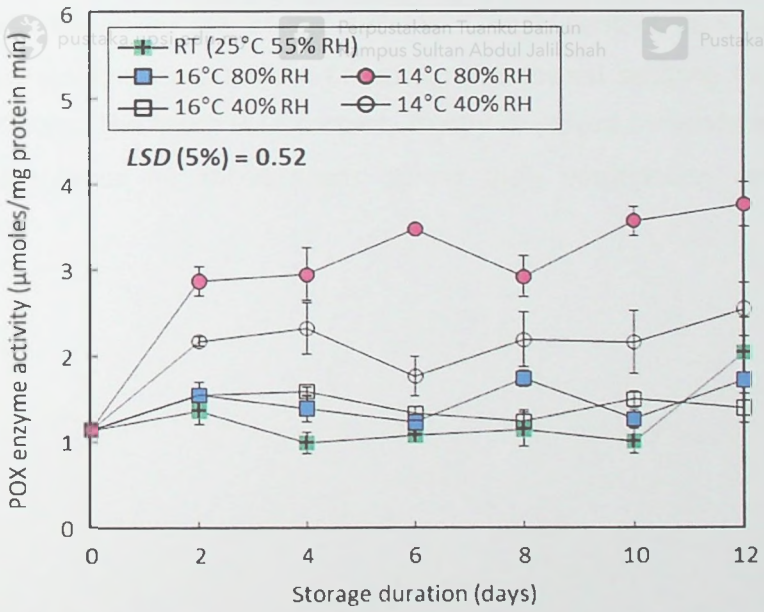


Figure 4.22 : Effect of storage conditions on POX enzyme activity of cacao seeds for 12 days of storage

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4.3.18 Hydrogen Peroxide (H_2O_2) Content

Seeds at the non-chilling conditions were found to show higher values of hydrogen peroxide (H_2O_2) content than the chilling conditions. Despite the higher H_2O_2 content showed by those treatments, all the values were not exceeding the values displayed by the control. For example, seeds at RT showed a significantly lower (45%) H_2O_2 content than the control, at 2 DAS (Figure 4.23). The treatment later showed a significant increase (12%) of H_2O_2 content at 4 DAS, and further maintained the values until 12 DAS. Seeds at both 16°C, 40% RH and 16°C, 80% RH then followed to show among the highest H_2O_2 content as compared to the other treatments at 2 until 8 DAS. Seeds at 16°C, 80% RH later decreased (28%) sharply to further displayed among the lowest H_2O_2 content than the other treatments, until 12 DAS. On the other hand, seeds at both the chilling conditions (14°C, 40% RH and 14°C, 80% RH) displayed the lowest H_2O_2 content as compared to the other treatments, at all storage durations. Seeds at 14°C, 80% RH showed the highest decreasing (68%) pattern of H_2O_2 content than the control, at 2 DAS. Followed by seeds at 14°C, 40%RH with a significant decrease (48%) of H_2O_2 content than the control, at 2 DAS. Widely known as one of the main ROS, H_2O_2 content also reflected the growth signaling within seed cells in producing the normal seedling from seed, in optimal conditions. The lower H_2O_2 content, mostly displayed by seeds at 14°C may reflect disadvantages for those seeds during their germination and seedling establishment.

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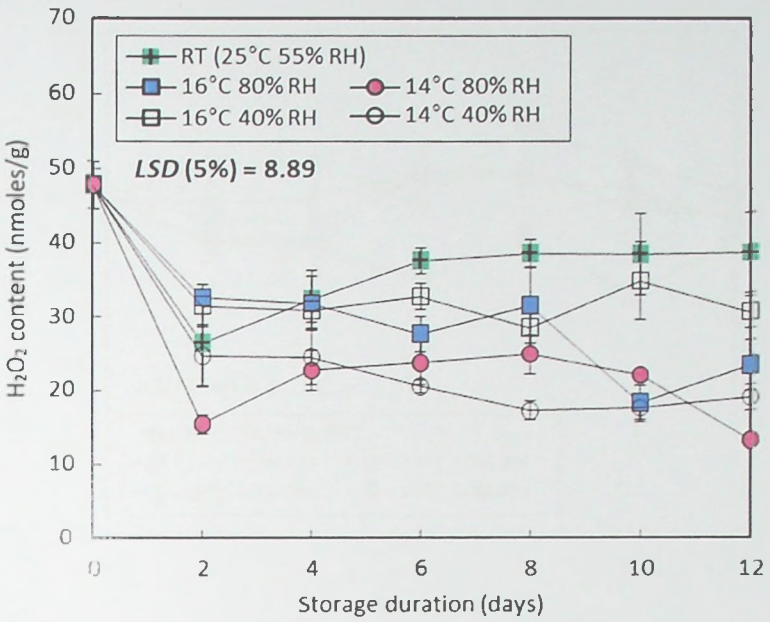


Figure 4.23 : Effect of storage conditions on H_2O_2 content of cacao seeds for 12 days of storage

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4.3.19 Lipid Peroxidation (MDA Content)

Seeds at the non-chilling conditions [RT, 16°C, 40% RH, and 16°C, 80% RH] displayed the insignificant changes of the MDA content accumulation to be at the same level as the control, along with the extension of storage durations. On the contrary, seeds at the chilling conditions (14°C, 40% RH and 14°C, 80% RH) showed changes of the MDA content accumulation at least at one point of the storage durations (within 6 to 10 DAS) (Figure 4.24). There were quite a gap values displayed in between seeds at 14°C, 40% RH, and 14°C, 80% RH. Seeds at 14°C, 80% RH showed significantly higher (17 to 31%) MDA content accumulation than the ones at 40% RH, from 2 until 10 DAS. It is interesting to note the wide differences of lipid peroxidation displayed for seeds at 14°C, 40% RH, and 14°C, 80% RH. Lipid peroxidation, which indicated the loses of membrane lipid mainly through the attack from ROS or free radical, may further reflect the destructive levels displayed between storage treatments.

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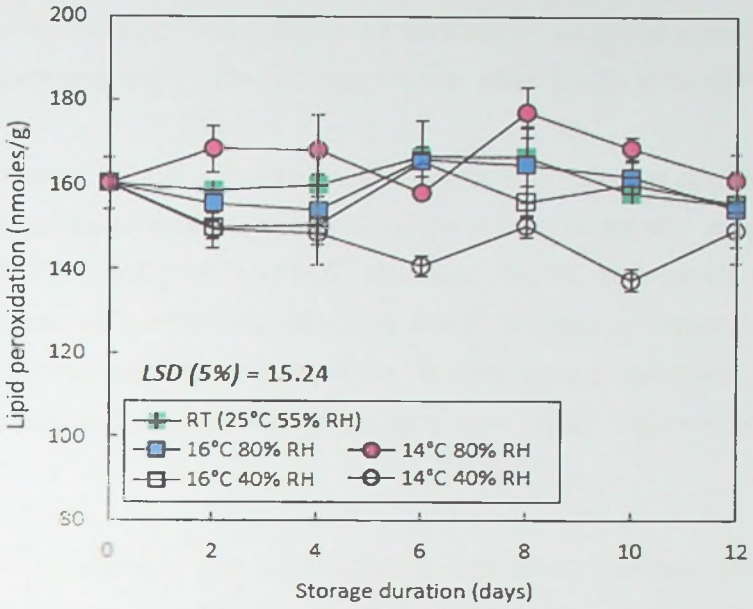


Figure 4.24 : Effect of storage conditions on lipid peroxidation (MDA content) of cacao seeds for 12 days of storage

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4.3.20 Pearson's Correlation Table

Supporting results shown in the Pearson's correlation (Table 4.2), which demonstrated that some of the seed physiological status parameters measured; FGP and GRI displayed the significantly negative correlations with all the antioxidant enzymes activity measured in this study; APX, CAT, SOD, GR, and POX. Aligned with the character of MGT, which always showed the significant opposite results with GRI; it displayed the positive correlations with all the antioxidant enzymes activity measured in this study; APX, CAT, SOD, GR, and POX.

On the other hand, the seedling growth components; MASS, SVI, and NORMAL also displayed significantly negative correlations with all the antioxidant enzymes activity measured in this study; APX, CAT, SOD, GR, and POX. On the contrary, only the DEAD component displayed significant positive correlations with all the antioxidant enzymes activity measured in this study; APX, CAT, SOD, GR, and POX. The consistent correlations of those antioxidant enzymes activity with all the

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seed physiological status and the seedling growth components suggest the insufficient or inefficient reactions of all antioxidant enzymes activity towards the harmful conditions, which affected the reducing seed quality exhibited in this study.

Moreover, LC and respiration rate components together displayed significant positive correlations with all the antioxidant enzymes activity measured in this study; APX, CAT, SOD, GR, and POX. However, the MC component only displayed significant positive correlations with SOD and POX enzymes activity. Those results also indicated that oxidative stresses may be developed progressively, aligned with the biophysical and structural changes that may occur within seed cells during storage.

Besides, some of the biochemical components; sucrose content displayed significantly positive correlations with all the antioxidant enzymes activity measured in this study; APX, CAT, SOD, GR, and POX. Similarly, the lipid content component also displayed significant positive correlations with some of the activity of the antioxidant enzymes measured in this study, CAT, SOD, GR, and POX. On the contrary, protein content consistently displayed significantly negative correlations with all the antioxidant enzymes activity measured in this study; APX, CAT, SOD, GR, and POX. In the metabolically active recalcitrant seeds, the increased or maintain values of biochemical components, such as sucrose content and lipid content indicated their vital importance for seed survival during storage. However, the higher significant values of correlations with protein content components indicatedthe probability of faster deterioration than their survival maintenance during storage.

The other component, lipid peroxidation only displayed significant positive correlations with LC. Moreover, H_2O_2 content displayed significantly negative correlations with all the antioxidant enzymes activity measured in this study; APX, CAT, SOD, GR, and POX. On the contrary, H_2O_2 content displayed significant positive correlations with some of the seed physiological status components (FGP and GRI), as well as their seedling growth performances (MASS, SVI, and NORMAL). Moreover,

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LC and respiration rate components together displayed significantly negative correlations with H_2O_2 content. It is interesting to note the dynamic character of H_2O_2 content in the present study as it may act in positive or negative ways may be depended on their relative situations within seed cells during storage.

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Table 4.2 : Pearson's correlation table

Correlations	APX enzyme activity	CAT enzyme activity	SOD enzyme activity	GR enzyme activity	POX enzyme activity	Lipid peroxidation	H_2O_2 content
FGP	-0.50**	-0.65**	-0.61**	-0.38**	-0.67**	ns	0.55**
GRI	-0.43**	-0.58**	-0.56**	-0.39**	-0.58**	ns	0.39**
MGT	0.42**	0.69**	0.69**	0.51**	0.65**	ns	-0.48**
MASS	-0.54**	-0.59**	-0.58**	-0.26*	-0.61**	ns	0.56**
SVI	-0.59**	-0.67**	-0.67**	-0.37**	-0.72**	ns	0.59**
NORM	-0.63**	-0.73**	-0.72**	-0.52**	-0.73**	ns	0.70**
ABN	ns	ns	ns	0.22*	ns	ns	-0.29*
DEAD	0.58**	0.69**	0.67**	0.45**	0.71**	ns	-0.62**
MC	ns	ns	0.24*	ns	0.24*	ns	ns
LC	0.35**	0.51**	0.57**	0.38**	0.56**	0.21*	-0.38**
Respiration rate	0.52**	0.60**	0.58**	0.44**	0.48**	ns	-0.28*
Total soluble sugar	ns	ns	ns	ns	ns	ns	0.33**
Glucose content	ns	ns	ns	ns	ns	ns	ns
Sucrose content	0.33**	0.50**	0.40**	0.48**	0.51**	ns	-0.25*
RFOs content	ns	0.27*	ns	ns	ns	ns	-0.24*
Lipid content	ns	0.25*	0.36**	0.40**	0.30*	ns	ns
Protein content	-0.73**	-0.83**	-0.86**	-0. 67**	-0.88**	ns	0.68**
DPPH	ns	ns	ns	ns	ns	ns	ns

Correlations	APX enzyme activity	CAT enzyme activity	SOD enzyme activity	GR enzyme activity	POX enzyme activity	Lipid peroxidation	H_2O_2 content
APX enzyme activity	-						
CAT enzyme activity	0.68**	-					
SOD enzyme activity	0.67**	0.88**	-				
GR enzyme activity	0.48**	0.63**	0.64**	-			
POX enzyme activity	0.65**	0.88**	0.90**	0.61**	-		
Lipid peroxidation	ns	ns	ns	ns	ns	-	
H_2O_2 content	-0.50**	-0.66**	-0.64**	-0.44**	-0.61**	ns	-

Notes: **, significant at $P \leq 0.001$; *, significant at $P \leq 0.05$; ns, not significant. FGP: final germination percentage, GRI: germination rate index, MGT: mean germination time, MC: moisture content, LC: leachate conductivity, SVI: seedling vigour index, MASS: seedling dry biomass, NORM: normal seedling, ABN: abnormal seedling, DEAD: dead seedling, DPPH: DPPH scavenging, APX: ascorbate peroxidase, CAT: catalase, SOD: superoxide dismutase, GR: glutathione reductase, POX: guaiacol peroxidase, H_2O_2: hydrogen peroxide

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4.3.21 Scanning Electron Microscope (SEM) Observations

The microstructural changes of cacao seeds to different storage conditions were determined by comparing the state of meristematic cells and cotyledons before and after storage treatment. Possible variations of the embryonic axis and cotyledon cell volume were observed during the storage process. The control, which consisted of the seeds freshly extracted from pods, presented compact cells with the irregular shape of a cell wall, starch granules, and some evidence of ruptured plastid membranes, signs of starch granule gelatinization, considerable numbers of perforated and divided cells were also observed [Figure 4.25(a-d)]. Good conditions of the mesophyll cotyledon with rare cell damages can be observed [Figure 4.25(e-f)]. The most likely explanation, however, is that the damage at the higher temperature was the result of water stress imposed as the seeds progressed towards germination.

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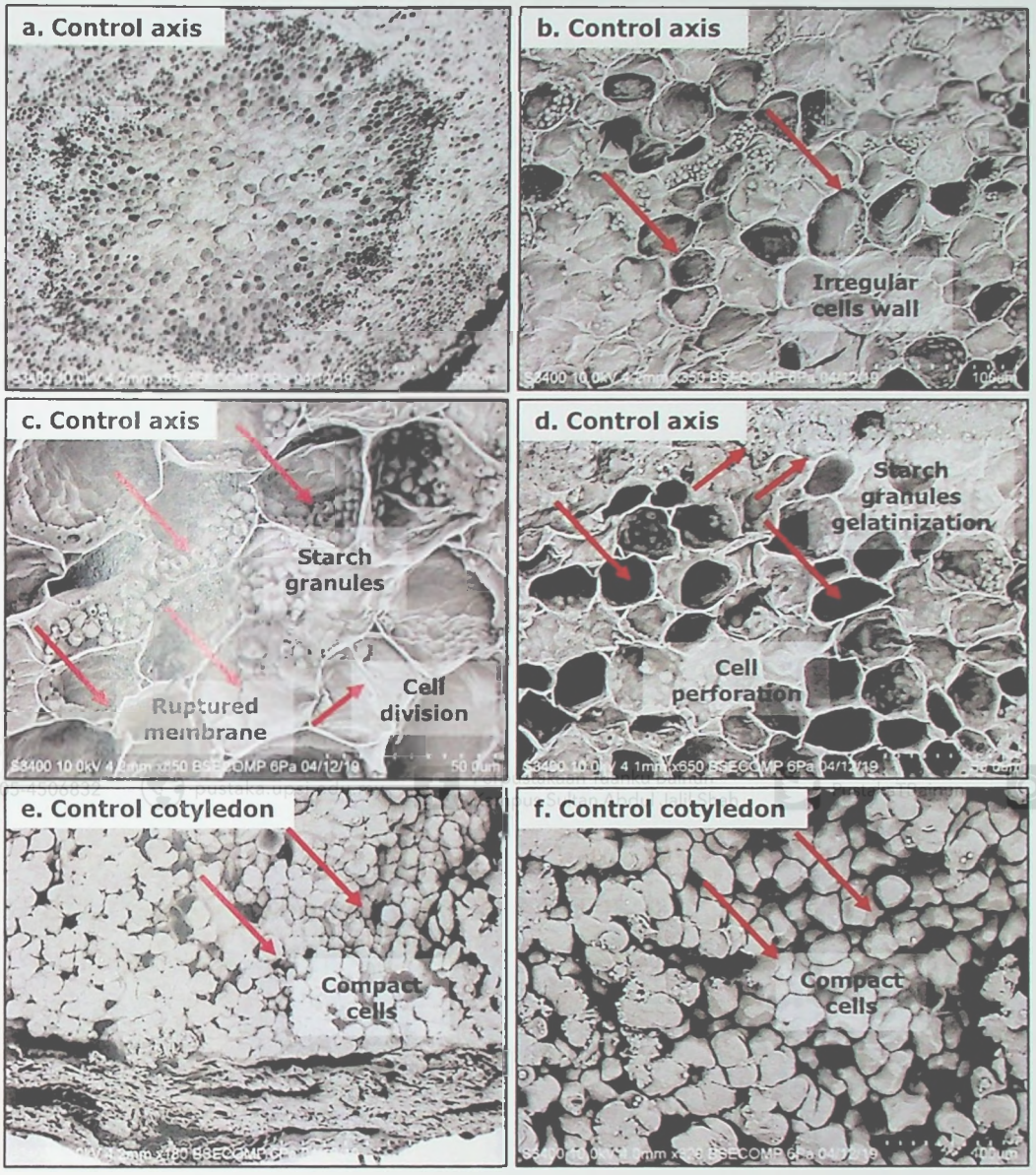


Figure 4.25 : Scanning electron micrographs of axis meristematic (a-d) and cotyledon (e-f) cells of the freshly extracted cacao seeds

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4.3.22 Seeds Stored at RT (25±2°C, 55±5% RH)

Starting from 2 DAS, the procambium cells of the radicle apex of seeds at RT showed the turgid and compact with irregular rounded shape structure of cells, also presented numerous intercellular spaces and presence of considerably scattered starch granules (Figure 4.26). Along with the extension of storage durations, cells showed the symptoms of shrinkage, which might be due to the internal stresses to pull the membrane tissues further apart (from 4 until 12 DAS). A folding and thickening of the cell walls and more pores/ intercellular spaces were observed started from 6 DAS, and more irregular cells shape were observed started from 8 DAS. Those visible structures made cell walls more resistant and stable. Moreover, cells appeared to be relatively active throughout the storage durations through the increased mass number of starch granules with the extension of storage durations. The presence of internal starch granules was also visualized by the distinct swollen rounded structure, and fracturing the plastid membranes, especially when it became more extensive than the membranes. The shrunk and dense plastid membranes might also indicate minimal damage to the surface, especially when more rigid dense cell membranes were visualized along with the extension of storage durations. Starch granules also showed the signs of surface cracking at 2 DAS and increased along with the increased storage durations. Cell's fractured consequences are also evidenced by the mass accumulation of degraded starch granules (gelatinization of granules to starch gel) and protein matrix mainly deposited near the cortex region and epidermis of developing radicle. Numerous pores, perforated cells, and intercellular spaces were found within cells as well as between adjacent cell walls (visible more at 8 until 12 DAS), which might further expand their rehydration capacity between cells. The intercellular spaces reduced, with time due to pressure from expanding cells as well as the development of a rigid dense layer on the shrunk cells. However, there was an increment in the pores and perforations, which indicate aging and progression towards germination during storage.

In general, cotyledon (mesophyll) cells were found in a solid structure and seemed to be resistant to fracturing, limiting observation to the exterior of the cytoplasm, cell walls, and a few isolated starch granules (Figure 4.27). Furthermore,

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the irregular pectinaceous material structure was detected on cotyledon cells especially at 4 DAS, which further made more sturdy and well-glued cells structure nearer seed coat. In general, cotyledon cells are maintained to display good condition with numerous ruptured cells detected and scattered along with the extension of storage durations.

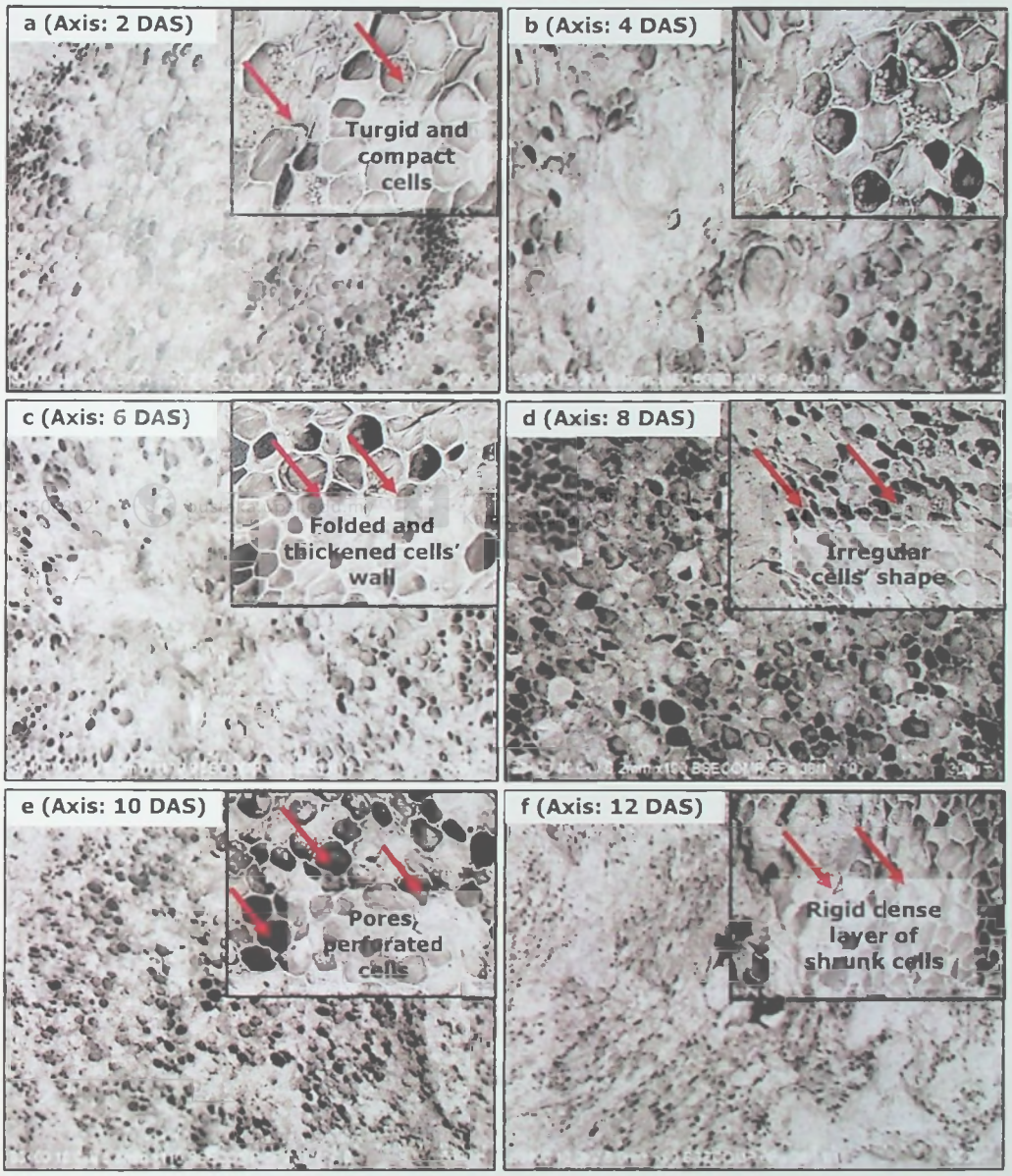


Figure 4.26 : Scanning electron micrographs of axis meristematic cells of cacao seeds stored at RT

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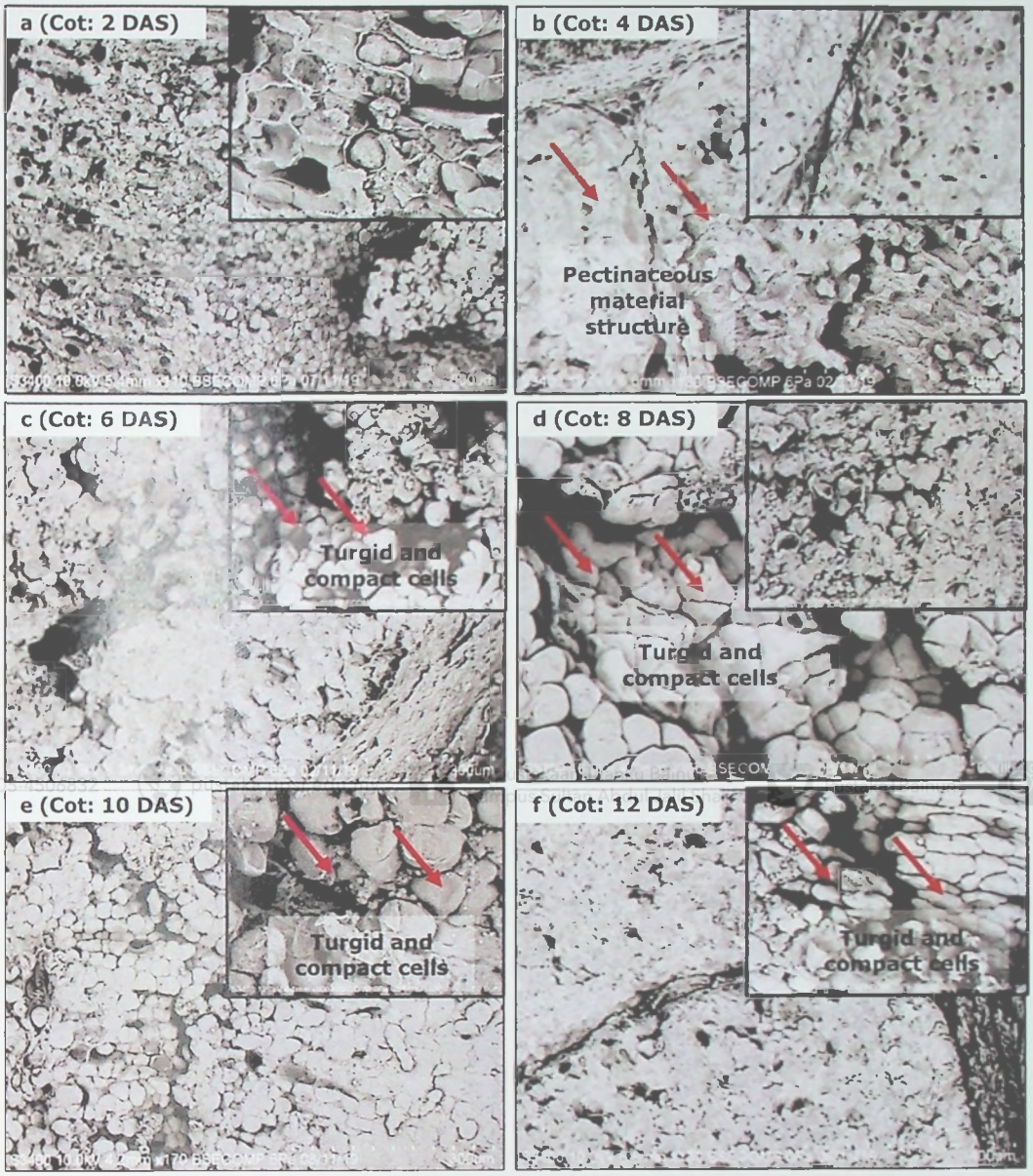


Figure 4.27 : Scanning electron micrographs of cotyledon cells of cacao seeds stored at RT

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4.3.23 Seeds Stored at 16°C, 40% RH, and 80% RH

During early storage durations, cells of the procambium region of the apex of radicle of seed at 16°C, 40% RH show the compact and turgid structure of cells, with irregular, rounded shape cells, also presented numerous intercellular spaces and the presence of multiple of scattered starch granules, spotted especially at the center (develop into pith) region (Figure 4.28). Dense structured (cell membranes) cells were observed at 2 DAS, but further exhibited more loosen cells (not so the much-swollen structure of starch granules seen from inner parts). A folding and thickening cell walls, more pores/ intercellular spaces, with more regular cells shape, were observed started at 6 DAS. Those visible structures made cell walls more resistant and stable. On the contrary with the seed at RT, there were lesser pores and starch granules were found on the surface of the folded, homogenous, and tight cells, whilethey still retained their original shape structure of seed at 16°C, 40% RH.

There were developing cotyledon cells deterioration observed for seeds at 16°C, 40% RH (Figure 4.29). At 6 DAS onwards, there was a spatially scattered pattern of damages on cotyledon cells, which were located peripherally near seed coat or at the edge of the inner cells, but rarely for cells in the interior of the tissues. Most of the cells seemed to maintain the well-cohesive structure between cells, which caused the visual of turgid and strong structures among cotyledon cells.

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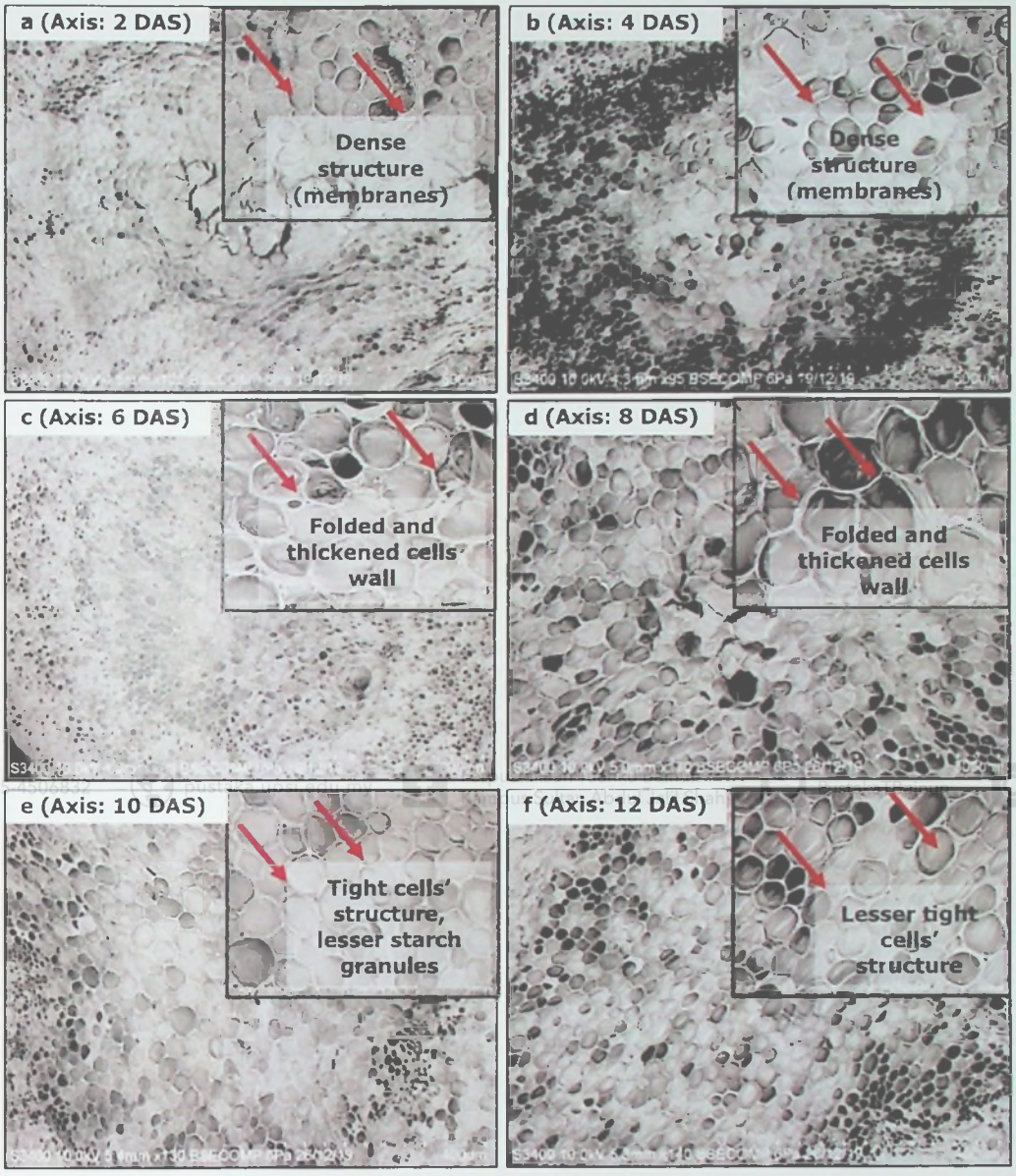


Figure 4.28 : Scanning electron micrographs of axis meristematic cells of cacao seeds stored at 16°C, 40% RH

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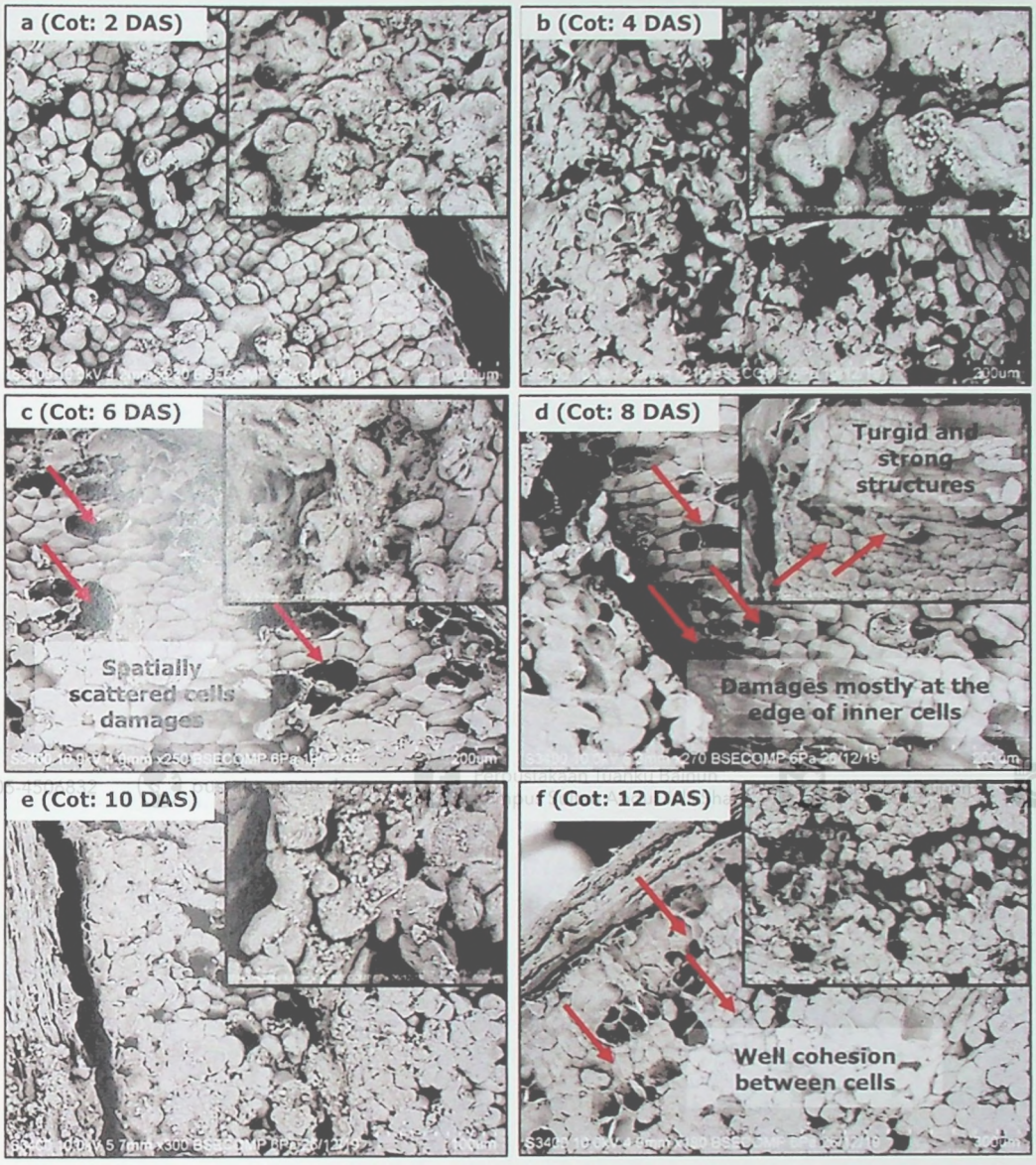


Figure 4.29 : Scanning electron micrographs of cotyledon cells of cacao seeds stored at 16°C, 40% RH

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At 2 DAS, the procambium cells of the radicle apex of seeds at 16°C, 80% RH showed the turgid and compact with regular rounded shape structure of cells, also presented more considerably scattered starch granules than seeds at 16°C, 40% RH (Figure 4.30). From 4 until 12 DAS onwards, cells showed the symptoms of shrinkage, as well as wrinkled, tight, and compact structure of cell membranes, but with the less thick cell walls. The shrunk and dense cell membranes might indicate minimal damage to the surface, especially when more rigid dense cell membranes were visualized along with the extension of storage durations. However, there were also more pores, perforated cells, and intercellular spaces during the same time, which cause more irregular cells shape, were observed from 6 until 12 DAS. This might further expand their rehydration capacity between cells. There was a numerous and scattered pattern of lesions displayed on cell membrane especially from 6 until 12 DAS. The increased pores, perforations, and signs of lesion indicated aging (programmed cell death) and might be a progression towards germination during storage. The increased mass number of starch granules significantly displayed at 6 DAS, as their membrane tore. Besides, the presence of internal starch granules was also visualized by the distinct swollen rounded structure, and fracturing the plastid membranes, especially when it became more extensive than the membranes. A folding and thickening of the cell walls were observed only at 12 DAS.

In general, cotyledon (mesophyll) cells were found in a solid structure and seemed to be resistant to fracturing during 2 DAS (Figure 4.31). Furthermore, the irregular pectinaceous material structures were also detected on cotyledon cells especially at 2 to 4 DAS, which further visualized more sturdy and glued cells structure. The materials probably derived from the content of shrunk cells spotted, which might be due to plasmolysis events that occurred. In general, cotyledon cells were maintained to display good condition with numerous ruptured cells detected and scattered along 12 days of storage. Besides, the loosened cell-to-cell structure was increasingly visible, with some plasmolysis events appeared at their cotyledon cells during 12 DAS.

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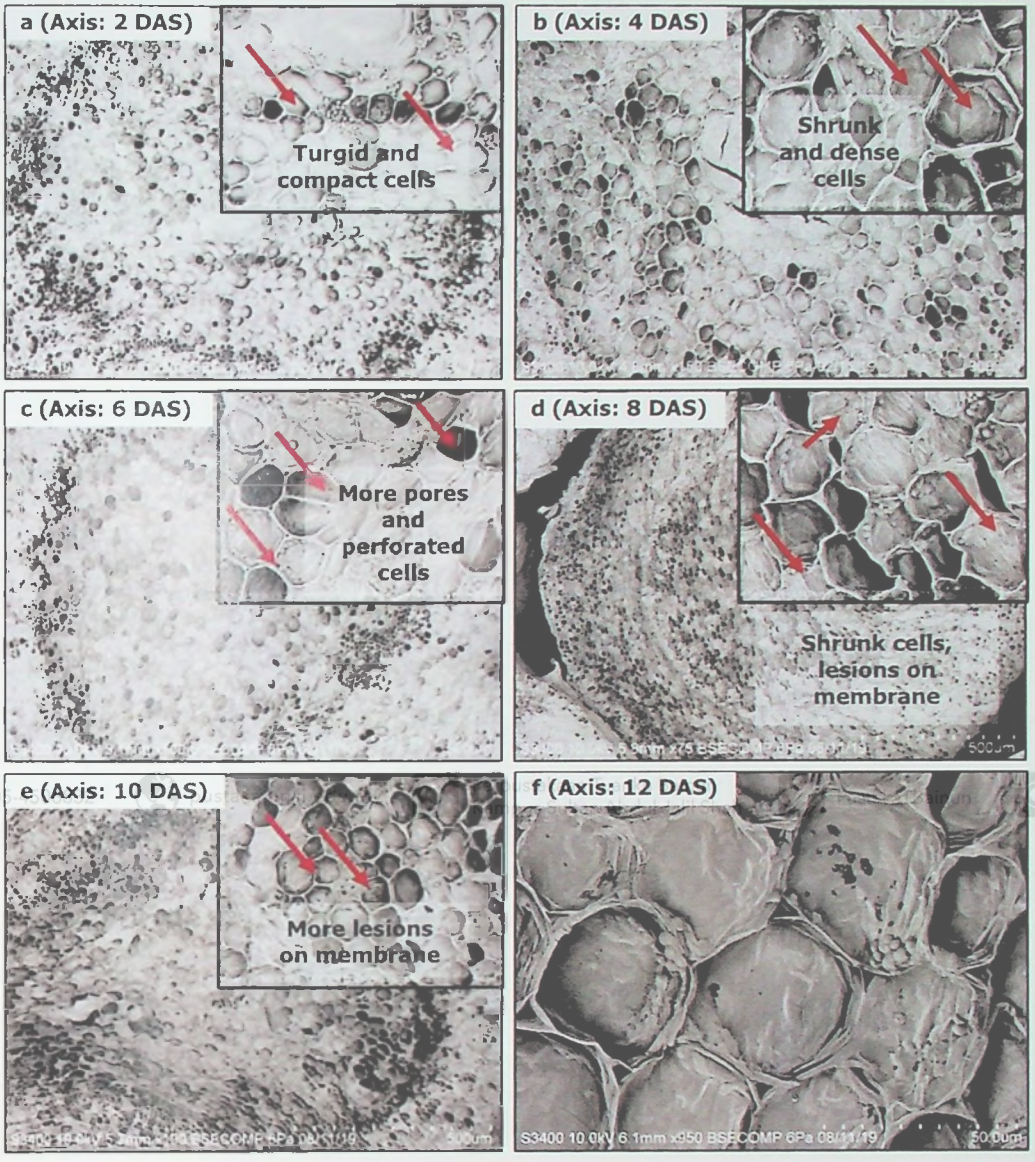


Figure 4.30 : Scanning electron micrographs of axis meristematic and cells of cacao seeds stored at 16°C, 80% RH

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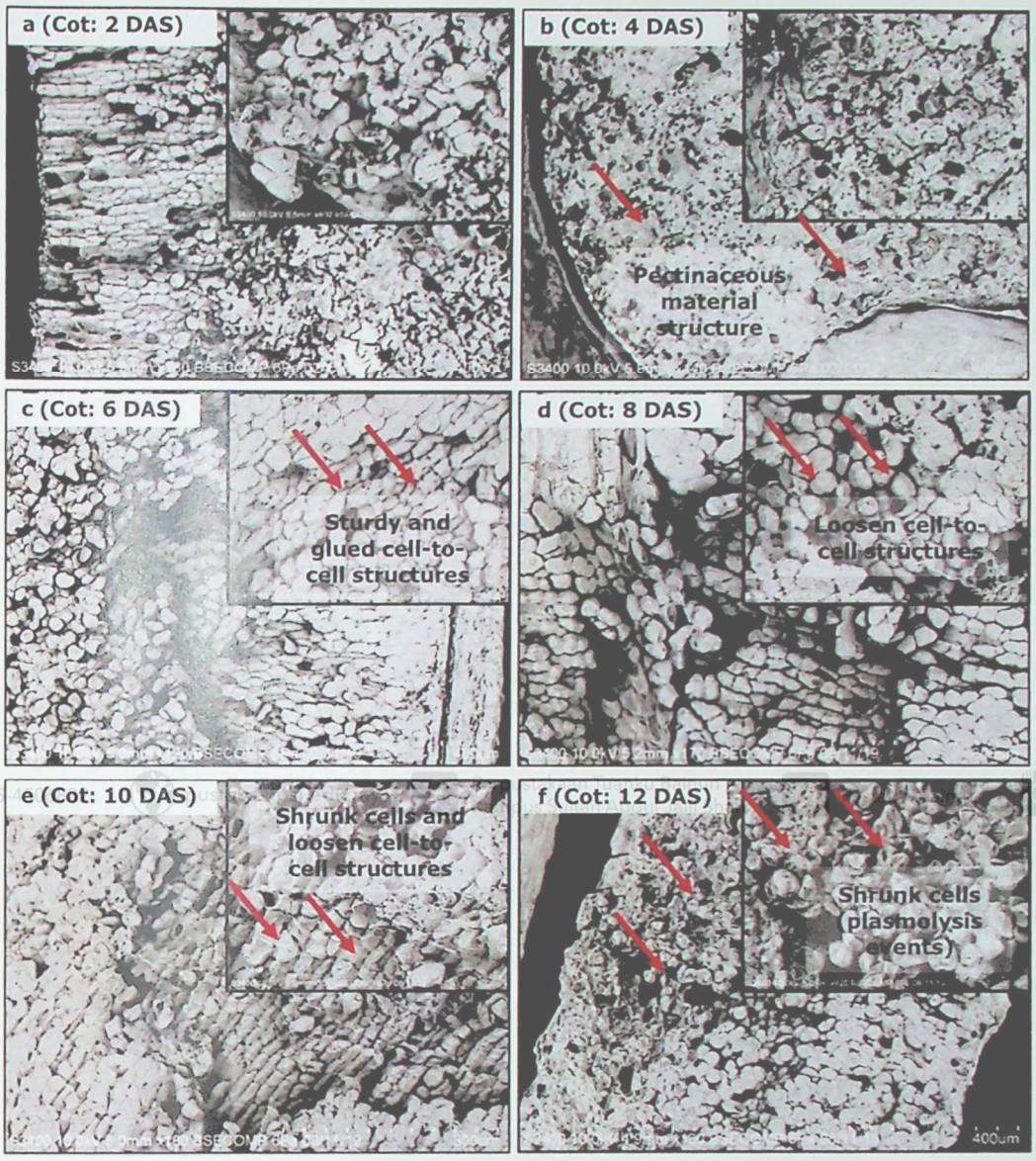


Figure 4.31 : Scanning electron micrographs of cotyledon cells of cacao seeds stored at 16°C, 80% RH

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4.3.24 Seeds Stored at 14°C (40% RH and 80% RH)

Starting from 2 DAS, the procambium cells of the radicle apex of seeds at 14°C, 40% RH showed the irregular shape structure of cells, also presented the mass cells content and starch granules scattered all around the area (Figure 4.32). More regular cells shape with shrinkage events were visualized afterward (until 6 DAS). The presence of internal starch granules was also visualized by the distinct swollen rounded structure, and fracturing the cell membranes, especially when it became more extensive than the membranes. The shrunk and dense cell membranes might also indicate minimal damage to the surface. The symptoms of loosening internal cell content, cell wall ruptured, and membrane cell lesions were observed to be present started from 8 DAS onwards. Numerous scattered starch granules were still observed until 12 DAS might be due to the continuous membrane cells ruptured.

In general, cotyledon (mesophyll) cells were found to develop numerous kinds of deteriorations from 2 until 12 DAS (Figure 4.33). Masses of starch granules and scattered cell contents probably derived from leaked or damaged cells were present since 2 DAS. More ruptured or damaged cells were observed to be located near the seed coat. The patterns of deterioration developed together with the increase of storage duration. Their cotyledon cells also displayed the signs of imbalance osmotic gradient conditions through the more and frequent plasmolysis events that occurred during storage; spotted from 4 until 12 DAS at the scattered areas. Cells also seemed to have a brittle structure with less cohesive cell-to-cell appearances until 12 DAS.

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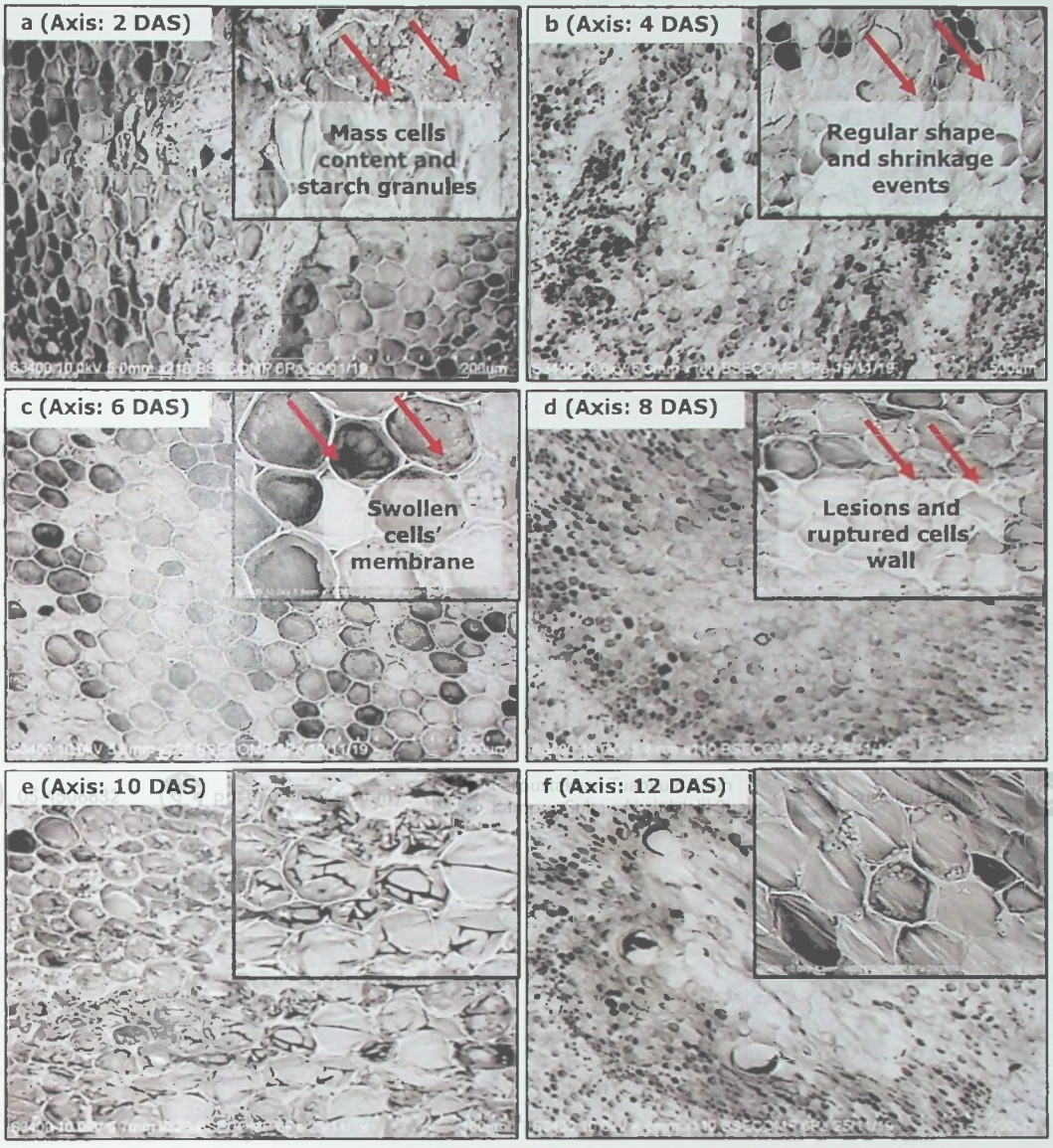


Figure 4.32 : Scanning electron micrographs of axis meristematic cells of cacao seeds stored at 14°C, 40% RH

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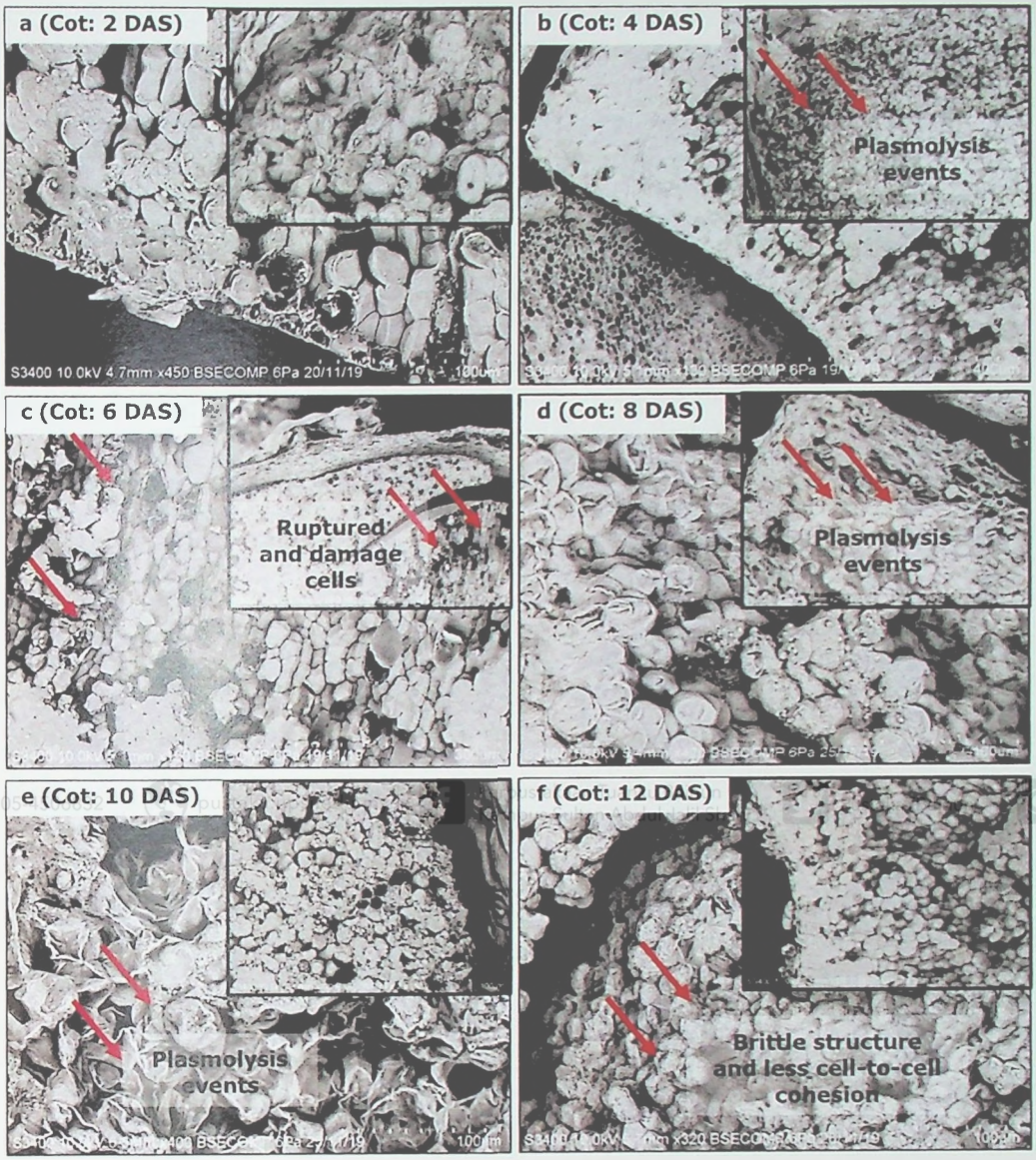


Figure 4.33 : Scanning electron micrographs of cotyledon cells of cacao seeds stored at 14°C, 40% RH

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Starting from 2 DAS, the procambium cells of the radicle apex of seeds at 14°C, 80% RH showed the dense and compact, with irregular rounded shape structure of cells and also the presence of considerably scattered starch granules (Figure 4.34). As the cells became shrunk at 4 DAS, more membrane cell lesions started to occur consistently until 12 DAS. The keep-changing cell wall or shapes were observed together with the occurrence of pores, and perforations. This might further expand their rehydration capacity between cells, and as well, indicated aging (programmed cell death) and might be the progression towards germination during storage. The characters of less thick, shrunk, wrinkled membrane cells, implying a harder texture and irregular cells shape of seeds at 14°C, 80% RH. Those characters also came together with the presence of masses of internal starch granules which were observed to fracture the membrane cells; spotted at 10 and 12 DAS.

In general, cotyledon (mesophyll) cells were found in a solid structure, which seemed to maintain the well-cohesive structure between cells, which caused the visual of turgid and strong structures among cotyledon cells (Figure 4.35). There were developing cells deterioration were observed in scattered locations, whole over the areas; included the interior locations of the tissues and frequently observed in masses pattern near the seed coat.

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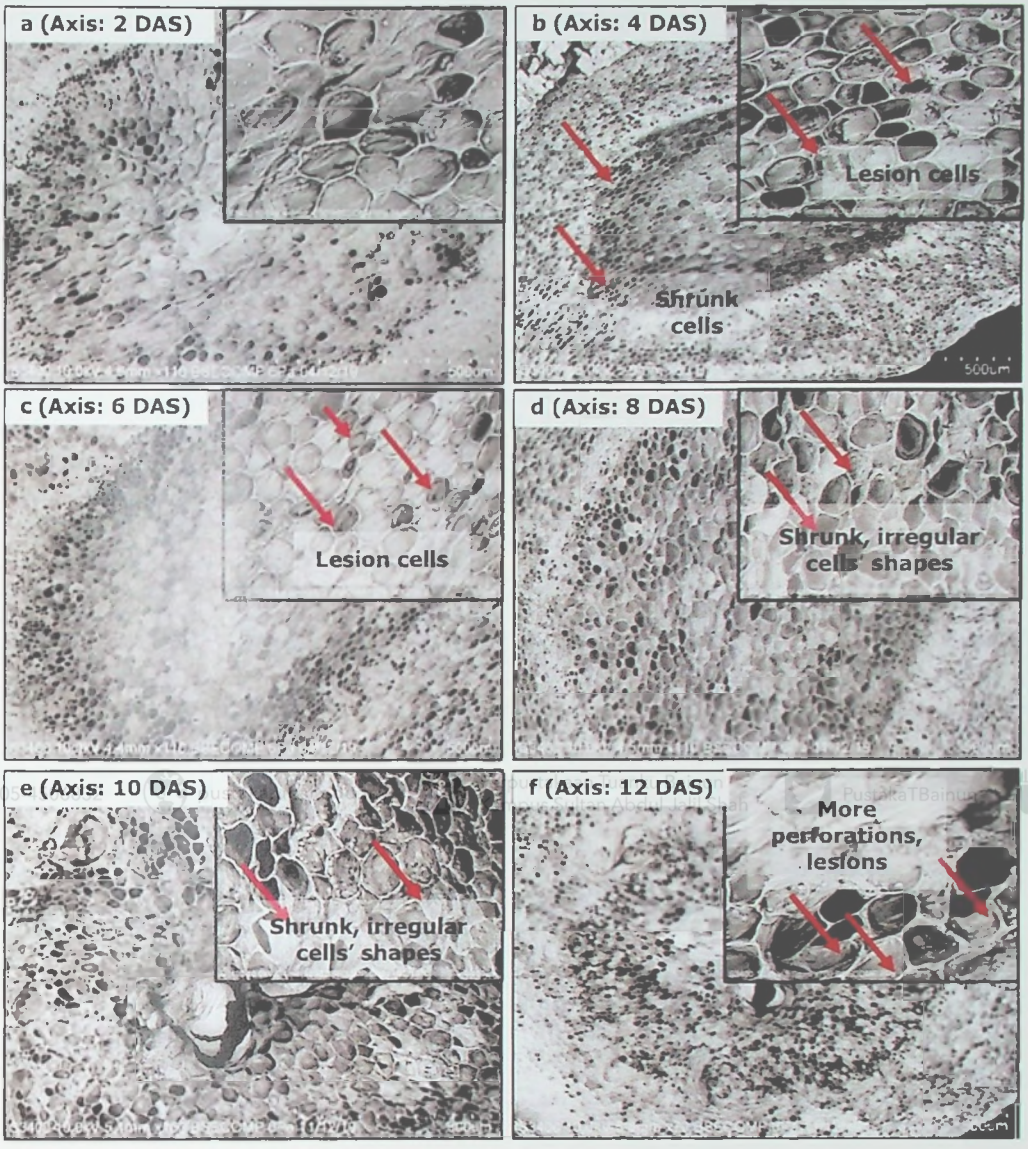


Figure 4.34 : Scanning electron micrographs of axis meristematic cells of cacao seeds stored at 14°C, 80% RH

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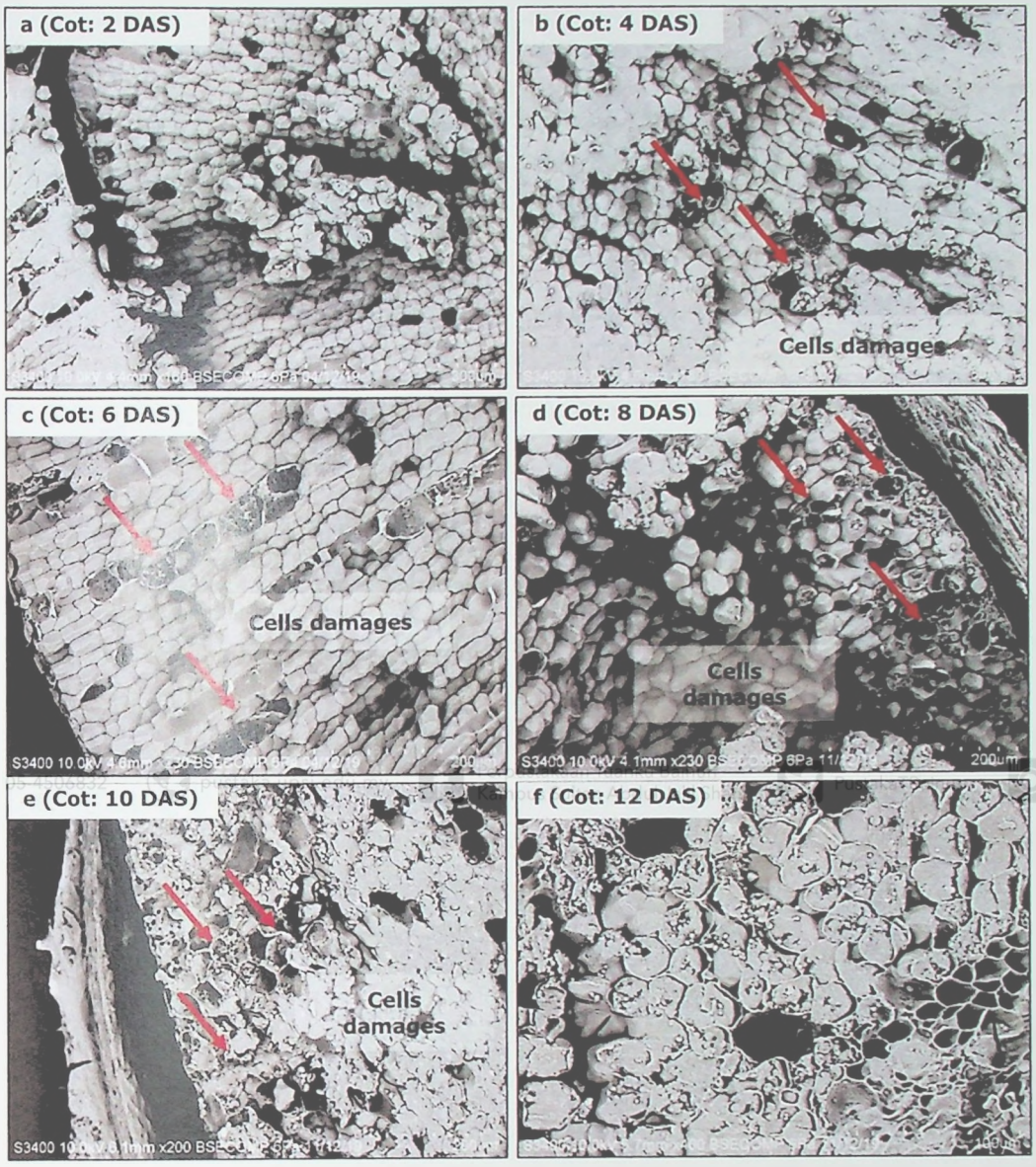


Figure 4.35 : Scanning electron micrographs of cotyledon cells of cacao seeds stored at 14°C, 80% RH

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4.4 Discussion

4.4.1 Physiological and Microstructural Changes Affected Viability and Vigour Status of Cacao Seeds Along 12 Days of Storage

Based on the previous preliminary investigation (refer to Chapter 3), it is clear from the results obtained that cacao seeds stored at temperatures higher than the chilling temperature (14°C); RT (25±2°C, 55±5% RH) and 16°C exhibited higher viability and vigour status. Along with the extension of storage durations (from 6 days in a preliminary study to 12 days during the present study), more significant differences of physiological status can be seen for seeds stored at different temperature and RH conditions. The drying rate effect on dehydration tolerance is related not only to metabolism (physico-chemical aspects) control but also to the dehydration process itself (mechanical aspects) (Liang & Sun, 2000). When the transition from Phase I to Phase II of the germination process occurs, considering that the metabolic processes have already begun in Phase I and that, in Phase II, a small amount of seed water is absorbed due to some changes in osmotic potential caused by the degradation of the reserves (Bewley et al., 2013). A comparison among recalcitrant cacao seed tissues stored at different slow-drying conditions was to test the hypothesis that responses of repairing mechanisms may vary in tissues differing in sorption characteristics due to chilling and dehydration sensitivity development.

During the present study, all the storage treatments except for seeds at RT showed the reducing pattern of MC at 2 DAS. The trend of losing and gaining moisture content (MC) represents a non-equilibrium situation partly due to the high or increasing viscosity of the tissue, which slows the structural adjustments toward equilibrium. Seeds at RT and 16°C, 40% RH displayed the significant reducing (4 to 9%) pattern of seed MC at 4 and 10 days after storage (DAS), respectively. Cacao seeds at RT demonstrated a gradual and significantly higher germination rate index (GRI) than the control, and their final germination percentage (FGP) reached the same levels as the control from 2 until 12 DAS. The slight but significant decline of seed MC at RT, however, did not affect their leachate conductivity (LC) negatively. Parallel with the positive correlations ($r=0.31^{**}$) of seed MC and LC; seeds at RT showed the sharply declining pattern of LC, especially at 4 DAS, and maintained the lowest LC until 10 DAS. The gradual decreased of seed MC might cause a decrease

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in cell metabolism (Soares et al., 2015), and together with the low LC, which might induce to more efficient deposition of insoluble storage reserves for seeds at RT. The decreasing trend of LC after their peak value at 2 DAS, suggesting the positive feedback effects of mild dehydration on cacao seeds at RT which might be due to the sufficient water content of the cytoplasm for metabolic (repairing) activities to be maintained or activated, and further to remain in close equilibrium with the associated conditions. In this study, only seeds at RT showed the consistency of reducing or maintaining the lower MC especially at 4 DAS onwards.

The effects of mild dehydration were displayed through the changes of seed microstructural conditions. During early storage (2 DAS), seeds at RT recorded MC at the approximate hydration level IV to V (48.82%) which was suggested to be in the physical state of 'syrupy' (Walters et al., 2002). A decreasing trend of MC afterward caused more visualization of the amorphous deposits (through SEM observation) which may have been the result of dehydration of salts and cytosolic constituents (Moore et al., 2006), especially at the cotyledon cells for seeds at RT, at 4 DAS onwards. The highest MC recorded for seeds at RT during 2 DAS is in parallel with the highest LC reading which might then justify the negative effects of higher seed MC during storage especially for seeds at RT. The stretched appearance of the hydrated cells was displayed at the meristematic axes (up to 6 DAS). However, as the water capillary forces reduced concomitantly with the reductions of seed MC, which further caused more folded, collapsed cell walls against the cellular content (Deng & Zhao, 2008), and reductions of cellular volume (from 4 to 12 DAS) to be estimated at the meristematic axes. Note that the cell collapse and the folding of the cell walls to accommodate volume reduction due to dehydration (Bewley et al., 2013). On the other hand, as the mature seed tissues are filled with storage materials, it further displayed evidence on the abundance of coalescing starch granules (at the meristematic cells), as well as the minimum structural disruptive events of the cotyledon cells until 12 DAS. Seeds at RT consistently showed the lowest respiration rate of all storage durations and maintain to display the highest viability and vigour throughout 12 days of storage.

Cacao seeds stored at 16°C, 40% RH found to be comparable with the ones at RT. Despite the same reducing trend of MC (at 10 to 12 DAS) as compared to

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seeds at RT (at 4 DAS onwards), more hydrated (higher MC) seeds at 16°C, 40% RH produced insignificantly higher FGP than the ones at RT. Based on the SEM observation, seed cells at 16°C, 40% RH displayed a more compact structure (at the meristematic axes) at 2 DAS, but then it became loosen with the stretched appearance and folded cell walls against the cellular content, until 12 DAS. The cells seemed to display sufficient tension to retain water through the strong capillary forces (approximate hydration level IV). A decreasing trend of MC especially at 8 DAS onwards has caused visualization on the spatially scattered pattern of damages on cotyledon cells, which are located peripherally near seed coat or at the edge of the inner cells, but rarely for cells in the interior of the tissues. Those conditions suggested the slow loosen water capillary forces and further displayed the symptom of hydrophobic water-binding state (character) which shall develop when reaching hydration level III, as suggested by Walters et al. (2002).

The enhanced permeability of cell membranes (higher LC) however, is associated with the metabolite loss, inability to maintain electrical, chemical, or pH gradients, and mixing of cellular components that are normally separated (Cherussery et al., 2015). This might cause physiological and biochemical disruption through essential processes such as hydrolysis, macromolecules biosynthesis, and respiration (Ali & Elozeiri, 2017). The direct indicator would be the loss of viability and vigour, which also discernible in the present study through the negative correlations of LC with most of the seed physiological status evaluated; FGP ($r=-0.38^{**}$), GRI ($r=-0.50^{**}$), MASS ($r=-0.26^{**}$), SVI ($r=-0.29^{**}$) and NORMAL ($r=-0.33^{**}$), as well as the positive correlations with MGT ($r=0.57^{**}$) and DEAD ($r=0.31^{*}$). The higher LC showed for seeds at 16°C, 40% RH suggests that they might then consume more on the maintenance of cell membrane re-structural processes to cope with the readiness for germination. The cells damages (lyses) might increase together with the release of solutes (increased of LC), which further induced the gradual decreasing pattern of seed MC for seeds at 16°C, 40% RH, especially at 8 to 12 DAS. The cytoplasm condenses as dehydration takes place and the intracellular components become more crowded. Such conditions give a variety of unnecessary interactions, contributing to protein aggregation and denaturation as well as the fusion of organelle cell membrane (Wheeler & Hyman, 2018). The slight increasing pattern of MC for seeds at 16°C, 40% RH (at 6 DAS) was also

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found coincidently at the point before the increased pattern of their respiration rate (at 8 to 12 DAS). Thisfurther evidencing that the increased of MC was unlikely due to the water generates from the intense respiration, but rather due to the enhancement of water driven through cells as their structures changed. The rise of respiration rate later reflected that there might be more processes of compensation to accommodate the disrupted metabolisms along with the increased of storage durations.

However, those disturbances may not lead to worse visible injuries or changes in growth and development rates, as disturbances in the physiological processes are reversible to stability (Lukatkin et al., 2012), as per revealed through the significantly higher GRI than the control, for seeds at 16°C, 40% RH. Rapid seed germination is usually accompanied by most recalcitrance, including cacao seeds, as revealed in this study. However, the prerequisites of the short storage lifespan of mature recalcitrant seeds are not only high-water content accompanied by thin water-permeable seed coat but also fully developed embryo structure at dispersal time (Obroucheva et al., 2016). These properties make up the structural readiness for germination in most seeds, supported by the mobilization of massive seed reserves. The slight differences recorded for SVI between seeds at 16°C, 40% RH, and RT, but with more significantly higher MASS showed for seeds stored at the latter. This indicates that the accumulation of massive seed reserves and lesser disruptive events within cells during seed development (in storage) at RT might justify their contribution to the normal and well-developed seedling, than seeds at 16°C, 40% RH.

On the other hand, seeds at 16°C, 80% RH which seemed to keep more water within cells, displayed the gradually decreasing pattern of FGP especially at 6 to 12 DAS. As evidenced by SEM images, the consequences of retaining aqueous phase within seed cells during storage at 16°C, 80% RH manifested by the characteristics of wrinkled, less thick cells wall structure with more lesions detected on plastid membranes (at the meristematic axes) along with the increased storage duration. Located at the approximate hydration level IV, strong water capillary forces factor seemed to cause the water retention more at the meristematic axes, as there were more swollen starch granules at the internal space of the cells. Lesser damages were observed at the cotyledon cells, but the events of cells plasmolysis were observed

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towards the end of storage durations. Although respiration rate decreased and maintained lower than the control, a quite strong leakage (recorded as among the highest LC) appeared in seeds at 16°C, 80% RH might cause the attenuation of DNA synthesis (Obroucheva et al., 2016). As there was less deposition of insoluble reserve materials in cells, the vacuolated cells might tend to change their structural volume (cells collapse) due to the osmotic solute gradients. In the hydration range where seed deterioration is rapid through metabolic activation, observable respiration and possibly membranes are still hydrated; seeds at 16°C, 80% RH seemed to increase their dehydration sensitivity. Decreasing pattern of seed MC started from 8 DAS for seeds at 16°C, 80% RH coincidently displayed the viability reductions faster than seeds at 40% RH; indicated by the decreasing pattern of FGP, as well as the lower vigour status (GRI, SVI, and MASS), and the increasing percentages of abnormal anddead seedlings. Eventually, the increasing trend of LC and MC of seeds at 16°C, 80%RH further suggested that the slower repair mechanisms might become even more inoperative along with the increasing "unregulated" metabolisms.

Classified as the chilling temperature, seeds at 14°C, 80% RH and 14°C, 40% RH revealed similar poor seed germination characteristics and seedling performances. It was suggested that the chemical rather than physical (e.g., structural) damage is inferred from the observation that membrane abnormalities were most frequent in slowly dried axes even though they were having similar water contents (Wesley-Smith et al., 2001). Recorded to attain lower MC than seeds at 14°C, 80% RH, the symptom of hypertonic conditions displayed at the cotyledon cells (under SEM observation) of seeds at 14°C, 40% RH especially at 4 until 12 DAS, indicating that there might be the osmotic solutes gradients transition. The process caused the cells to shrink and further gradually increased the seed MC. Cells at the meristematic axes, however, displayed the positive feedback to exhibit the less irregular shape of cell walls with the stretched appearances which further evidencing the strong water capillary forces on cells. The events of plastid membranes ruptured were also observed might be due to the swollen of the hydrated internal materials (e.g., starch granules). On the other hand, seeds at 14°C, 80% RH displayed more turgid with most of the cotyledon cells might be filled with the reserved materials from 2 until 12 DAS. However, the high MC displayed started from early storage durations might cause the strong capillary forces to have existed. This further

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explains the ruptured cells at whole over the areas of cotyledon cells, including cells in the interior of the tissues. More water retention to be detected at the meristematic axes as characterized by the wrinkled, less thick with the irregular shape of cells wall's structure and more lesions were observed on plastid membranes. The lesions (might be due to the water retention) which appeared as early as at 4 DAS might indicate the increase of leakage and seemed to be concomitantly displayed the sharp declining pattern of FGP as well. Respiration rate was somewhat irregularly elevated after that, and germination was mostly low, which might be due to irreversible metabolic dysfunction and incompletely oxidized intermediate accumulation, which further caused repair processes to become inoperative. It has also been noted that solute leakage is one of the most common occasions of chilled tissue phenomena. In response to drying and rehydration, the membrane lipid structure initially exhibited reversible shifts between gel and liquid crystalline phases; however, reversibility declined as viability was lost (Soares et al., 2015). This explains the consistently higher LC showed for seeds at 14°C, 80% RH at 8 DAS onwards, together with the decreasing pattern of seed FGP and seedling growth performance, which indicated the symptom of irreversible membrane damages.

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4.4.2 Dynamic of The Physio-chemical Status of Cacao Seeds in Relation to Slow-drying and Chilling Sensitivity Along 12 Days of Storage

Recalcitrant seeds normally keep organelles fully functioning and maintain active metabolism. Following morphogenesis, developing seeds enter a 'maturation' stage, which seems to be a more active stage in terms of gene expression and metabolism than the seed drying itself. Also known as 'reserves accumulation' periods, it involves reorganization of the metabolism and storage compound synthesis, including starch, storage proteins, and oil (Angelovici et al., 2010). Protein, lipid, and carbohydrate enzymatic hydrolysis and transport of metabolites are primarily based on water availability (Ali & Elozeiri, 2017). During the present study, the slow-drying storage treatments seemed to initiate changes in the physical structure of the seed internal matrix (evidenced through SEM observations) and thus attributed to the variability of physiological and biochemical changes of seeds.

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a. Soluble Sugar Content in Cacao Seeds During Storage

Soluble sugars which are also known to be the primary direct substance essential during germination (Jennifer et al., 2002), are of similar importance for development towards germination on recalcitrant cacao seeds during storage. The loss of dehydration tolerance in recalcitrant seeds prior to seed shedding along with the concurrent decline of soluble sugars and protein associated with stress could be a phenomenon that is unique to tropical seeds and is related to both initiation and mobilization of the reserves during the final stages of development. According to Wen (2011), the recalcitrant *Livistona chinensis* embryos which underwent correlative changes in the physiological effects of seed production with acquisition and loss of cryotolerance; the embryos demonstrated the redifferentiation after dedifferentiation in the subcellular organization, an increase followed by a decrease in total soluble sugar content. A similar subcellular re-organization might occur in this study, as the seeds also underwent the physiological changes of developing or enhancing their dehydration sensitivity, which further displayed the decreasing pattern of total soluble sugar of seeds at all storage treatments. Reports from previous studies on orthodox seeds emphasized the importance of the soluble sugar increment (especially sucrose and RFOs) to survive dehydration during maturation (Zhao et al., 2018). During the present study, almost all the storage treatments, showed the increment of soluble sugars (glucose, sucrose, and RFOs) quantified during early days of storage (mostly until 6 DAS), which led by seeds at 14°C, 80% RH. Since recalcitrant embryos are in the germination mode of growth, the probable main source of soluble sugar synthesis is starch, as a common food reserve in seeds to maintain their energy metabolism during moist/hydrated storage (Faria, 2006). The supporting evidence was detectedunder SEM observations in this study, at which cells (especially at the meristematic axes) did not seem to have been abundantly packed with starch grains and it is a typical feature of actively metabolizing recalcitrant seeds (Pammenter & Berjak, 1999). A marked decrease in deposits of starch may well have provided the sugars required for the growth processes following the germination (Sershen et al., 2014). Plastids-containing starch, as the characteristic of the seed cells for all storage treatments, were no longer evident mostly at 4 DAS (for seeds at 14°C), and 8 DAS (for seeds at 16°C) onwards.

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Even though recorded to be in the wetted-seed conditions, the reducing trend of MC for seeds at RT was observed likely to exhibit the starch granules visual (at the meristematic axes) as a reflection of extended maturation. According to Angelovici et al. (2010), the seed maintains a low level of metabolic activity during post-ripening may retain its viability, and the same pattern showed for seeds at RT. During the present study, seeds at RT which displayed among the lowest respiration rate, also consistently accumulated among the lowest total soluble sugar, glucose, sucrose, and RFOs as compared to the other treatments. In addition, to serve as a respiration substrate, sugars (e.g. sucrose and glucose) are also carbon sources to produce metabolites; amino acids, lipids, proteins, and complex carbohydrates (e.g. starch or cellulose) (Gimenez et al., 2014). In parallel with the highest FGP and increasing GRIof seeds at RT than the other treatments, the rapid inter-conversions of sucrose to simple sugars (hexoses), while those hexoses lead to sucrose synthase, might be happened as to maintain their cell homeostasis (Rosa et al., 2009). In this study, the negative correlations between soluble sugars (glucose, sucrose, and RFOs) and GRI further evidencing the involvement of soluble sugars for seed development towards germination. The decreasing pattern of membrane alteration (lower LC) might cause seed cells at RT to remain low but metabolically active, which further cause the extensive vacuolation and increase in the size of cells (Faria et al., 2004), as well as to display the higher viability and vigour seedling performances after storage.

The membrane alterations for seeds at 16°C (higher LC recorded than RT) might attribute to more leaked substrates or enzymes to be used for further metabolic activity. However, seeds at 16°C seemed to benefit from the adverse conditions. Even though with the higher soluble sugar accumulation for seeds at 16°C than seeds at RT, but there might be a reduction in germination inhibitors, membrane alteration, and protein degradation occur that enhance germination vigour (Angelovici et al., 2010). For instance, seeds at 16°C, 40% RH with the reductions of MC at 8 DAS onwards, coincidently displayed the higher pattern of sucrose content (than the other treatments) and lower protein concentration (than seeds at RT) recorded together with the maintained high viability and seedling vigour performances as the control. Astudy made by Li & Sun (1999) also found that the increase of sucrose levels was also seen in cacao seeds in response to drying. In the slowly dehydrated developing recalcitrant *Machilus thunbergii* seeds, an increase in the sucrose

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concentration may indicate a protective role for sucrose (Connor & Sowa, 2003). The discrepancy between the seeds seems to be universal not only due to dehydration tolerance butalso due to the ready access of respiratory substrates for increasing the metabolism associated with radicle emergence development (Steadman et al., 1996). Thus, starch hydrolysis also functioned to produce sucrose for the sake to reduce dehydration sensitivity in the metabolically active embryo during moist storage (Faria, 2006), especially in terms to which the starvation of sugars might be avoided. While DNA repair enzymes are unstable during storage and lose their function, resulting in gradually delayed repairing mechanisms and ultimately delayed germination, these enzymes could still regenerate during the dehydration and rehydration cycles (Long et al., 2014). More rapid dehydration and rehydration cycles within seed cells might contribute to the higher viability and vigour of seeds at 16°C, 40% RH than at the higher RH (16°C, 80% RH). The extension of storage duration might further cause vigour performances to lag behind viability due to more impaired biochemical changes that occurred, and this pattern displayed for seeds at 16°C, 80%RH.

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In general, low temperature increases soluble sugar levels. Soluble sugars can act directly either as negative signals or as plant sensitivity modulators and can also take important roles in cell responses to distant signals caused by stress (Rosa et al., 2009). According to Zhang et al. (1995), the cytoplasm viscosity declines at a slight chilling as result of increased colloid dispersion and structural formations decay. However, the strong and long-term chilling further caused the viscosity to increase due to the coagulation of structural proteins. The condition might cause a slower rate of metabolic events within seeds and further increased the accumulations of soluble sugars (glucose, sucrose, and RFOs) during early storage durations (until 6 DAS) in this study. In addition, mostly leveled LC values displayed for seeds at 14°C before increased at some point after storage. This indicates that there might be fewer additional leaked substrates or inducers to increase metabolic activity within seeds. In this study, the higher MC for seeds at 14°C, 80% RH suggested the faster deterioration to have occurred than seeds at 14°C, 40% RH. Their LC values were found to have fluctuated at the high range andan increasing pattern was observed to maintain at the highest values from 8 until 12 DAS. Concomitantly, the final sharp increase of MC displayed for seeds at 14°C, 80%RH

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might be due to more damaged cells and more cells volume changes which caused the higher water uptake in seeds. According to Lukatkin et al. (2012), instead of the rapid changes in membrane phase transition caused by low-temperature exposure (imbibitional chilling), the increase in LC is most likely due to chilling induced water stress, which is significantly weaker in a water-saturated atmosphere. As the changes do not occur immediately after the start of chilling, the significant breakdown or disruptions of membranes which might allow more enzymes to contact with their substrates, are likely to be secondary disorders (Ofosu-Ansah et al., 2013). The hypothesis of the changes in membrane phase prevailed in the 1970s as to which chiller-induced changes in the molecular order of membrane lipids could cause chilling injuries amongst the primary events which trigger a reaction at lower temperatures. These changes exacerbate the damage by causing a shift in intracellular pH, a decrease in ATP levels, metabolic imbalances, and an increase in membrane permeability (Lukatkin et al., 2012).

According to Connor & Sowa (2003), recalcitrant *Quercus alba* acorns (embryonic axis) showed an increase of sucrose concentration but coupled with declining viability. Further, it suggested that sucrose was no longer used for growth and development, or that the enzyme activity in deteriorating seeds or both of starch was broken down. Though there may have been protected membranes in Q. alba tissue in the combination of high sucrose concentrations and high axis MC, the mechanism did not work to maintain viability that quickly fell after few days (Connor & Sowa, 2003). In this study, the increasing trend of MC, together with the higher pattern of sucrose content especially during early days of storage (until 6 DAS) for seeds at 14°C, concomitantly showed the reducing pattern of FGP and lower GRI than the other treatments. Besides, as both seeds at 14°C, 40% RH and 80% RH were at the same level of hydration (level IV), the pattern of attaining MC within seeds, however, were differed and different damages of cells could be observed under SEM. Instead of the significantly lower LC, seeds at 14°C, 40% RH were observed to maintain their higher germination performances compared to 14°C, 80% RH. The plasmolysis events (from 4 until 12 DAS) were detected in some areas of cotyledon cells of seeds at 14°C, 40% RH. This further indicates the reductions of metabolic events with the reduced MC as to activate any metabolic events within cells, thus,

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damages could be reduced, as well. However, reducing metabolic events within recalcitrant cacao seeds might not be a suitable solution, as the seeds need to continue being activated to compensate for damages caused by exposure to the chilling storage temperature.

b. Sugar Depletion and Decreasing Trend of Protein Content in Cacao Seeds During Storage

The prolonged chilling storage on recalcitrant seeds indicates the continuously internal physiological acclimation and alterations that might cause disturbances in the distribution and function of soluble sugars in seed cells. This may contribute to a decrease in available carbohydrates in embryo axes (i.e. sugar starvation) and a lower germination rate in seeds (Morkunas et al., 2012). A similar pattern showed in the present study, as the storage treatments other than RT started with the lower GRI as compared to the control at 2 DAS, and some (seeds at 14°C) were even consistent with the lower GRI values until 12 DAS. Most of the soluble sugars showed a quite similar pattern; higher during early storage and the pattern reduced mostly after 6 DAS. This may be pertaining to the depletion of reserves in seeds intended to provide energy for the growing embryo during storage (Maldonado et al., 2015), which further cause the significant physiological and biochemical changes occur to maintain respiration and other metabolic processes (Rosa et al., 2009). Struck of sugar starvation might cause a specific sequence of events in plant cells. Cells subjected to sugar starvation initially adapt to the lack of carbohydrates by gradually replacing carbohydrate metabolism with protein and lipid metabolism. Under sugar starvation, the increased activity of proteolytic enzymes and autophagic processes observed are undoubtedly adaptive processes that allow cells to maintain a constant energy supply (Morkunas et al., 2012). Brouquisse et al. (1992) reported that during sugar starvation of maize root meristems included the reduction of total protein content to overcome their acclimation and survival phases. Borek et al. (2012) also reported that the in-vitro studies of lupine and garden pea embryo axes showed that the concentration of protein (especially the soluble fraction) in starving cells decreased significantly. During the present study, the quantification of protein concentration (in cytosolic soluble fraction) displayed a dramatic decrease as early as at 2 DAS, for allstorage treatments. Since those seeds are in the germination (growing) mode, de novo protein synthesis is needed. Thus, the insufficient free amino acids in cells might

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induce signals to start degradation on protein storage during the first hours of imbibition (Rosental et al., 2014). This explains the decreasing pattern of protein content starting in the early days in hydrated storage. Moreover, the decrease in cytosolic proteins is found to be correlated with the increase of soluble sugars (especially sucrose content) ($r=-0.42^{**}$), which further supports their substitutional function of carbohydrates.

The other indicator of sugar starvation would be the decline of respiration rate as shown in some previous studies made by Morkunas et al. (2000) on embryos of garden pea and by Borek et al. (2011) on yellow lupine. The decreased respiration rate is driven by a lack of respiratory substrates rather than mitochondrial degradation (Morkunas et al., 2012). In the present study, most of the storage conditions showed a decreasing pattern of respiration rate at 2 until 6 DAS. However, many genes are activated in carbohydrate-depleted tissues, resulting in increased activity of relevant enzymes (Borek et al., 2013). This explains the negative correlation showed by protein content and respiration rate ($r=-0.50^{**}$). Seeds stored at the chilling condition (14°C) which maintained the higher respiration rate, also displayed the lowest protein content and further consistently maintain the low values until 12 DAS. According to Da Silva et al. (2018), a decrease in soluble protein content in *Jatropha curcas* seed embryos during storage, as evidenced by increased respiratory activity throughout storage, leading to protein degradation. As the cells age, the evolutionary for the metabolic reorganization might involve more autophagy on cytosolic proteins and even degradation of whole organelles. Plastids, ribosomes, and endoplasmic reticulum are consumed relatively early, while plasma membrane, mitochondria, and peroxysomes survive for longer periods (Morkunas et al., 2012). Brouquisse et al. (1992) further categorized the phase as cell disorganization, when the level from all metabolites and enzymatic activity are decreased substantially and the changes are irreversible, leading to death. In the present study, evidence through the increasing pattern and maintain highest LC (membrane damages) with the consistently lower values of FGP, GRI, seedling growth performances and, also recorded the highest number of abnormal and dead seedlings for seeds at 14°C. This parallel adverse situation happened to signify the maintained low values of protein content from 2 until 12 DAS at 14°C; a sharp decrease in synthesis activity of many enzymes, degradation of protein, and changes in membrane phospholipids leading

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to membrane integrity loss (Connor & Sowa, 2003). This decrease in protein content could be attributed to numerous mechanisms, including an increased rate of protein loss in cotyledons and embryonic axis, disruption to the protein synthesizing machinery, and the creation or activation of significant quantities of proteolytic enzymes during seed deterioration (Maldonado et al., 2015). The author further reported that there was a massive loss of cell protein during storage in recalcitrant seeds of *Shorea robusta*.

c. Lipid Content of Cacao Seeds at The Slow-drying and Chilling Conditions During Storage

Fat or lipid components in wild species seeds may play additional roles, such as controlling the developmental transition from dormancy to germination, assisting seed defense and dispersal, and fueling seedling growth following germination (Zhao et al., 2018). Seed reserve content was stated to be linked to germination percentages and/or rates (speed) (Soriano et al., 2014; Zhao et al., 2018). In the present study, GRI is correlated with all the soluble sugars (glucose; $r=-0.28^{*}$, sucrose; $r=-0.43^{**}$, and RFO; $r=-0.30^{*}$) and lipid content ($r=-0.40^{**}$). In contrast, only the protein content showed a positive correlation ($r=0.55^{**}$) with GRI. It is worth to be noted that seeds are highly biochemically heterogeneous (Zhao et al., 2018). According to Morkunas et al. (2012), the increased use of cytosolic and storage proteins during sugar starvation is followed by more intense catabolism of lipids aimed at supplying respiratory substrates. The significant negative correlations of lipid with protein content ($r=-0.28^{*}$) further justified the useful contribution of protein content for lipid biosynthesis, prior to the used of lipid as the reserved substrates during crucial sugar depletion (Borek et al., 2013). However, De Souza et al. (2018) who did study on the recalcitrant and lipid-rich rubber seeds emphasized that the use of lipids only occurs after the loss of moreimmediate energy resource supplies (sugars), such as starch. Thus, the sugar feeding may significantly retard the breakdown of storage lipid. It is widely accepted that storage oil reserve mobilization begins after germination (Bewley et al., 2013) and no reserves are required for its completion (Rosental et al., 2014). Accordingly, the maintained values of lipid content in seeds for most storage treatments during the present study (until 6 DAS), further indicate that there might be sufficient (soluble) sugar or protein content for the cells to maintain their homeostasis.

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However, there were different patterns of significant changes showed for seeds at all treatments towards the end of storage durations (at 8 until 12 DAS). In carbohydrate-depleted conditions, lipid conversion into sugars is significantly slowed in yellow lupin seeds. When the carbohydrate content of tissues was reduced, the intermediates of storage lipid breakdown could be used as respiratory substrates but not for sugar synthesis (Borek et al., 2013). The decreasing pattern of lipid content for some of the treatments during the present study indicated that there might be the progress of lipid breakdown (development to germination); the gradual significant decrease of lipid content displayed for seeds at 16°C, 40% RH after 6 to 10 DAS. Supported by the sharp increment of respiration rate at 8 DAS onwards, lipid breakdown might be an act as the substrates for respiration. Some studies also indicated that during seed germination, storage lipid is converted into sucrose, as one of the primary carbon transport forms in plants (Graham, 2008). However, as revealed in the present study, there were no significant correlations of lipid content with soluble sugars, especially sucrose, which indicated the significantly reduced pattern of lipid content for seeds at 16°C, 40% RH, might be mainly due to reflections of compensating the depleting substrates directly for respiratory activity. This is also supported by Borek et al. (2013), who discovered that not all storage lipid is converted to sucrose during lupin seed germination. Some of it is used as a respiratory substrate and in the synthesis of amino acids. The breakdown of lipid for seeds at 16°C, 40% RH after 6 to 10 DAS might have occurred together with biosynthesis of amino acid and protein as their functional repairing mechanisms. This might also contribute to the maintained high values of protein content until 12 DAS as compared to the other storage treatments.

On the other hand, slow drying would expose seeds to relatively high moisture contents and, as a result, high metabolic rates, with the possibility of continued triacylglycerol synthesis and thus increased lipid content (Nkang et al., 2003). Other than functioning as "cotyledonary sinks" to avoid the build-up of starch hydrolysis products, the increasing pattern of lipid content displayed for seeds at 16°C, 80% RH and 14°C, 80% RH at 8 until 10 DAS, explains the negative feedback on the increasing LC values throughout the storage. This is also supported through the significant positive correlation displayed between lipid content and LC ($r=0.30^{*}$). The increased LC might also indicate destructuralization of the plasma membrane which explains

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the poorgermination characteristics for seeds at 16°C, 80% RH and 14°C, 80% RH at 8 until 12 DAS. Under hypoxic conditions, energy deficiency and disrupted membrane structure are likely to activate membrane proteins that integrate stress. The plasma membrane H^+-ATPase regulates plant growth and development by controlling plant cell chemical potential and nutrient transport. Plasma membrane ATPase activity is lipid dependent: destructuralization of the plasma membrane decreases ATPase activity, while the reintroduction of lipids restores inhibition and enhances ATPase-specific activity. While lipids are required for membrane bilayer structure, changes inmembrane lipid composition can impact enzyme conformation and aggregation statein the lipid bilayer (Chen & Boss, 1991).

4.4.3 Oxidative Stresses: Metabolic Dysfunctions as The Secondary Effects After Membrane Disruption of Cacao Seeds During Storage

Considering the different activation of various metabolic processes with seed water uptake, the rise in respiration and the initiation of sugar consumption can be observed minutes after the onset of imbibition (Gimenez et al., 2014). In this study, there was an initial (at 2 DAS) sharp increase in respiration rate for seeds at 14°C, 80% RH and 14°C, 40% RH which could be due in part to the hydration and activation of mitochondrial enzymes involved in the citric acid cycle and electron transport chain. Lags in respiration as carbon dioxide production stabilized or increases only slowly, might be due to the activation of the cytosolic glycolysis during germination is faster than the production of efficient mitochondria. Changes in the carbohydrate levels resulting from chilling are correlated with impaired respiration and activity of carbohydrate enzymes (Gimenez et al., 2014). Similarly, seeds at 14°C displayed an increase of soluble sugars measured especially during early days of storage (at 2 to 6 DAS) in the present study. Besides, pyruvate could also build up because of citric acid cycle deficiencies or the oxidative phosphorylation (electron transport chain). Those pyruvates would be temporarily redirected into the fermentation pathway, which does not involve oxygen (Bewley et al., 2013). Nonetheless, the toxicity of fermentation products can be a limiting factor for seed germination (Rosental et al., 2014), which explains the poor germination characteristics for seeds stored at 14°C. More previous studies found that the increase in respiratory activity during chilling

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and their prolonged high activity after cold exposure may suggest that dysfunction of metabolism is irreversible and that there is an incompletely oxidized intermediate accumulation. The mechanism of stimulation is unknown. However, as mentioned previously, the increased levels of LC indicated more membrane leakage events, which increased the possibility to cause more uncoupling of oxidative phosphorylation. These changes will result in a loss of membrane integrity and compartmentation, solute leakage, a decrease in mitochondrial oxidative activity, an increase in the activation energy of membrane-bound enzymes, metabolic disruption and imbalance, toxic substance accumulation, and chilling injury symptoms (Lukatkin et al., 2012).

Plants require energy and resources to cope with abiotic and biotic stress conditions, hence in sugar starvation conditions, when metabolic processes slow down, the plants were more vulnerable to stress factors (Morkunas et al., 2012). The possible reducing ATP due to impaired respiration during storage might disrupt the cells to induce the imbalance metabolic events which might develop to more progressing deterioration within seed cells. The increased generation of free radicals, including ROS, in response to abiotic stress factors, indicates a greater influence of the stress factor, which can lead to 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. However, Rosental et al. (2014) stated that free radicals and ROS damages are most prevalent in dry or lower wet seed ranges (hydration level I to III), but additional mechanisms of damage become possible with a higher seed MC (hydration level II to IV) (Morkunas et al., 2012).

The commencement of oxygen uptake in the re-hydrated seed results in the formation of ROS and a shift in the redox state. However, the concentration of dissolved oxygen lowers when temperature increases, thus bringing about oxygen shortage at high temperatures (Brennan et al., 2016). This explains the consistency of the lower respiration rate displayed by seeds at RT. The same explanation goes for seeds at 16°C, as both treatments displayed a lower respiration rate than seeds at 14°C. ROS are always formed in plants due to the inevitable leakage of electrons onto oxygen from the electron transport activities of chloroplasts, mitochondria,

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and plasma membranes, or as a by-product of various metabolic pathways located in various cellular compartments, which can pose a threat to cells (Sharma et al., 2012).

It was intriguing to see if sugar starvation, or at least sugar depletion, is accompanied by changes in the redox status of the cell, as in the case of plant response to other stress stimuli (Rosental et al., 2014). The consequences of ROS attack on all enzymes cause damage to the molecules and reduces sugar concentration in cells, which can play a significant role in the reduction of antioxidant activity (Mansouri-Far et al., 2015). Similar events might have happened during the present study when seeds at all storage treatments displayed the decreasing soluble sugar content (glucose, sucrose, and RFOs) at 8 DAS and keep the low values until 12 DAS. It is well established that decreased sucrose or glucose levels in tissues promote lipid, protein, and starch breakdown (Borek et al., 2013). In this study, the decreasing soluble sugars, however, were in parallel with the increased lipid content for seeds at 14°C, 80% RH, and 16°C, 80% RH. As mentioned earlier, the highest LC values displayed for seeds at 14°C, 80% RH, might cause de-lipidation to have occurred on the plasma membrane (due to uncoupling oxidative phosphorylation). Thus, sugars or other free metabolites will be used to synthesize lipid as the compensation for membrane damage and fixing the system supplying energy, rather than substrates synthesis for respiratory activity. However, the irreversible membrane damage shown as the LC maintains the highest until 12 DAS, concomitantly occurred together with decreasing pattern of viability and vigour of seeds stored at 14°C, 80%RH.

Leakage points can occur as result of the emergence of membrane domains with various configurations as result of cold-induced changes in lipid phases, or membrane damage, particularly lipid damage. Other than reducing sugar content, ROS or free radicals can cause lipid peroxidation, which damages cell membranes and lipoproteins (Daniel & Mani, 2016), and ultimately cause destruction. Although the main mechanisms for seed deterioration are unknown, some studies have shown that lipid peroxidation caused by oxidative damage can result in the inactivation and/or depletion of key enzymes of recipient protein transport or ion channels, as well as impairment of RNA and DNA synthesis (Mansouri-Far et al., 2015). In the

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present study, seeds at 14°C, 80% RH recorded among the increasing pattern and the highest level of malondialdehyde (MDA) (at 8 DAS onwards), as one of the final products of stress-induced lipid peroxidation of poly-unsaturated fatty acids. This was followed by much lower levels of MDA displayed by seeds stored at 16°C, 80% RH. Membrane damage due to lipid peroxidation is reported to happen frequently in dry conditions. In this study, even though most of the stored seeds may contain higher MC (>40%) during storage, the changes in the cell's water potential might create lags to produce some dehydrated pockets within cells to be exposed to the consequences of mild dehydration. For example, in some areas in cotyledon cells for seeds stored at 16°C, 80% RH displayed the plasmolysis symptoms, observed underthe SEM. Moreover, in respect to dehydration tolerance, Bewley et al. (2013) stated that the wetted-seed condition may create a dangerous hydration range which can induce inefficient respiratory activity to generate ROS, and antioxidant protection andregeneration mechanisms may be inefficient.

Together with the lower MDA values, seeds at 16°C, 80% RH also displayed much lower LC than the ones at 14°C, 80% RH. However, the increasing lipid content which was in parallel with the reducing MC indicated that the compensation from the (membrane) damages occurred due to the higher MC retained within seeds at 16°C, 80% RH, during the first 6 DAS. Both treatments indicated some similarities in their characters, as those seeds recorded the consistently higher or increasing pattern of MC, especially during the first 6 DAS, as compared to the other treatments. The continuation of an increasing pattern of MC displayed for seeds stored at 14°C, 80% RH. On the contrary, further decreasing protein content which occurred in parallel with decreasing pattern of MC for seeds at 16°C, 80% RH hinted the imbalanced metabolism-linked mechanisms within cells, at 8 DAS onwards. It further caused the sharp decreasing pattern of germination (at 8 DAS) and continuation of the gradually decreasing seedling growth performances until 12 DAS. The ability to generate ATP, which is heavily influenced by metabolic activity, correlates with the percentage of seeds that germinate and the subsequent growth of seedlings (Rosental et al., 2014).Moreover, due to the character of retaining higher MC, both treatments further displayed the other similarity, which was the development of lysigenous (lysis) aerenchyma (in the cortex of embryonic axes), through the increasing visual of intercellular spaces or pores under SEM observation. Those lysis

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mechanisms are a developmental-controlled programmed process in which certain parenchymatous cells are designed to self-destruct while the surrounding cells survive. Besides, it appears likely that the pressures that cause aerenchyma production results in ATP depletion, possibly because of rapidly uncoupled electron transport. A decrease in mitochondrial respiration causes an increase in NADH and a decrease in ATP. Membrane fusions causing more calcium influx, oxidative stress can produce uncoupling between respiration and ATP synthesis, and this depletion of ATP can besufficient to induce program cell death (PCD) (Bouranis et al., 2007). Under hypoxicconditions within seeds, desperation for oxygen content might induce the development of air channels, which traverse both the hypocotyl and cotyledons as reviewed under SEM. Similar interconnected channels have been identified in developing seeds *B. nicolai*. A high level of interconnectivity between these air spaces serves to improve tissue porosity, allowing for gas exchange within the developing/germinating embryo (Borisjuk & Rolletschek, 2009).

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

The seed MC and metabolic activity vary substantially from the start of development to the end of germination. As a result, the sources of ROS in seeds are likely to differ significantly (Bailly, 2004). Oxidative stress is described as an imbalance between the generation of ROS and the antioxidant capability of the organism. The term "total antioxidant capacity" refers to the collaborative (additive and possibly synergistic/antagonistic) action of all antioxidants present in a complex sample, most researchers regard it as a more useful parameter for assessing antioxidative defenses of the organism (Apak et al., 2016). During the present study, DPPH radical scavenging activity was evaluated, and out of all the variables measured in this study, their positive correlation with MC ($r=0.30^{**}$), indicates their responsiveness when the surroundings became more hypoxic with more water uptake or retaining water within seed cells. Together with the positive correlation with LC ($r=0.25^{**}$), further indicates the cell's defensive development with the increasing leaked molecules. The increment of DPPH radical scavenging activity within seed cells in this study, however, responded more to the stress conditions being exposed to them during storage, which further results in the negative and positive correlations with GRI ($r=-0.30^{**}$),

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and MGT ($r=0.24^{**}$), respectively. Seeds at almost all the storage treatments showeda higher percentage on DPPH scavenging than the control, and further stagnant at those values along 12 days of storage. Only 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 activity levels in seeds exposed to high temperatures point to an intentional strategy to enable (rather than quench) ROS required for cell membrane loosening and other development processes during the early stages of germination. This might somehow occur for seeds stored at RT, as about 8 to 30% of seeds germinated during the storage in this study. Since ROS are intracellular signaling molecules in hydrated tissues, they are expected to perform this role in hydrated recalcitrant seeds as well (Sershen et al., 2014).

The primary mechanisms of delaying aging 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 enzymes activity than the other storage treatments, along 12 days of storage. During the present study, results displayed the significant positive correlations of all antioxidant enzymes activity quantified with LC; APX ($r=0.35^{**}$), CAT ($r=0.51^{**}$), SOD ($r=0.57^{**}$), GR ($r=0.38^{**}$), and POX ($r=0.56^{**}$). These indicate the significant cause of increased ROS probably due to the fast-uncoupling oxidative phosphorylation (impaired respiration). Respiration is the principal cause of electron leakage to oxygen, resulting in the generation of free radicals (Francini et al., 2006). This supported also with the significant positive correlations of all antioxidant enzymes activity with respiration rate; APX ($r=0.52^{**}$), CAT ($r=0.60^{**}$), SOD ($r=0.58^{**}$), GR ($r=0.44^{**}$) and POX ($r=0.48^{**}$). The seed's life span is determined by its capacity to evoke antioxidative enzymes activity to detoxify excess ROS levels (Sahu et al., 2017). Moreover, the consistent significant negative correlations of all antioxidant enzymes with all the germination characteristics and seedling performances measured in this study, indicated that there might be insufficient functional antioxidant enzymes reactions on recovering

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oxidative stresses within seed cells. Those patterns were significantly displayed for seeds stored at 14°C. According to Da Silva et al. (2018), one of the causes and indicators of deterioration is decreased enzyme activity during seed aging. Most of the previous studies showed that superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (POX), ascorbate peroxidase (APX), and glutathione reductase (GR) are among the main antioxidant enzymes in plants that operate in different subcellular compartments and respond in concert when cells to scavenge free radicals that are detrimental to seed viability (Sharma et al., 2012; Suresh et al., 2019). In this study, there was less to be observed on the reduced enzyme activity during seed aging. However, the stagnant or reducing only slowly pattern of most antioxidant enzymes activity quantified along 12 days of storage, reflected deterioration development within seed cells.

Commonly detected ROS in imbibed seeds is superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^-). Active mitochondria are most likely one of the principal generators of ROS in developing or germinating seeds, producing superoxide (Pehlivan, 2017). The plasma membrane NADPH oxidases, which convert electrons from cytoplasmic NADPH to oxygen, are also a primary source of O_2^-, which is then dismutation to H_2O_2 (Ishibashi et al., 2010). Besides, the reduction of oxygen (hypoxic conditions) results in the creation of the O_2^- radical, which is an uncoupled electron molecule that can react with other molecules to stabilize its energy (Pehlivan, 2017). It is estimated that 1 - 2% of the oxygen consumed by mitochondria forms O_2^- anion at complexes I and II of the electron transport chain, resulting in 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%). Among the frontline of antioxidant enzymes activity, SOD is known to be highly responsive to the changes and increased of O_2^- to normal living cells, in catalyzing the conversion of superoxide 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 (Martins et al., 2012; Sharma et al., 2012). However, as those recalcitrant seeds did not go through the maturation drying process, thus the SOD activity within seeds displayed consistently higher activities than the control; led by seeds stored at 14°C, 80% RH, and followed by the ones at 40% RH. This suggests that its role in ROS defense is

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most important during the stages when the seeds are not fully formed, exhibiting significant metabolic and respiratory activity, and during the final stage of maturity, providing protection against ROS creation during desiccation (Sharma et al., 2012). In the present study, there was a positive correlation displayed between SOD activity and MC ($r=0.24^{*}$). An obvious pattern could be seen to compare seeds at 14°C, 80% RH, and 40% RH. Seeds at 14°C, 80% RH showed the highest and stagnant SOD activity along 6 DAS, before increased onwards. The increasing pattern is coincidently parallel with the increasing MC. 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. ROS production by the mitochondrial respiratory chain is a natural and ongoing process. The commencement of oxygen uptake in the re-hydrated seed results in the formation of ROS and a redox state shift (Bouranis et al., 2007). In parallel with the stagnant or increasing only slowly pattern showed for respiration rate for both treatments, the pattern of SOD activity displayed might also be due to the stagnant pattern of oxygen available within the seeds (hypoxic conditions).

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Higher SOD activity raises the H_2O_2 concentration in cells, increasing the activity of antioxidant enzymes, which removes this ROS (De Souza et al., 2018). APX and CAT, which are 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 expression of many genes, including enzymes involving in ROS synthesis or degradation (Pehlivan, 2017). In parallel with the 15% increment of SOD activity, was the higher increasing pattern of APX (47% increment) which started at 6 DAS especially for seeds at 14°C, 40% RH. By oxidizing lipids, proteins, and nucleic acids, this H_2O_2 can easily went through membranes and cause cell harm (Suresh et al., 2019); which explained more than doubled accumulation of propagated H_2O_2 within seed cells in this study. Due to its strong affinity for H_2O_2, APX modulates the intracellular H_2O_2 present in all cell compartments and, unlike CAT, participates in cell detoxification (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. It has frequently been reported that antioxidant system activation occurs late in the germination or development process: antioxidant enzymes are activated only when ROS levels are

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above a particular threshold, to maintain ROS homeostasis within the oxidative window for germination (Bailly, 2019). Recorded to be among the lowest MC values displayed for seeds at 14°C, 40% RH during the first 6 DAS, it might cause the imbalance of water distribution among cells which were in parallel with the evidence of plasmolysis (under SEM observation) events that occurred at some cotyledon areas. This further suggested that there might be some trapped ROS at the dehydrated pockets areas detected. Rehydration process or increasing MC 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 and similar events mentioned by Bailly (2004). Moreover, the low pattern of LC further explains on 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; for instance, producing OH^- through Fenton reaction. Moreover, each of the transition increasing or decreasing patterns of MC might contribute to the unregulated generation of ROS and is likely to occur during a time of intermediate hydration levels where metabolic down-regulation becomes uncoordinated. This idea is supported by the fact that oxidative stress occurred when *Mimusops elengi* seeds crossed the hydration level interval where ROS were created during drying (Tang, 2012). Seeds at 14°C, 40% RH further recorded the consistency to accumulate among the lowest level of H_2O_2, indicated the functional of those antioxidant enzymes to reduce ROS. The lesser membrane damage for seeds at 14°C, 40% RH, may also reduce their potential to be exposed to stressful conditions contributing to lipid peroxidation. This explains the very least consequences of ROS attacked could be detected for seeds at 14°C, 40% RH than the ones at 80% RH. Moreover, those seeds displayed a reducing glucose pattern to points approaching zero which reflected the very low turnover process for sugar used as the substrate, due to the reducing metabolism which is in parallel with their aging process. A study made by Barros et al. (2004) indicated that low-glucose yeast growth appears to elicit long-term adaptation changes that restore increased respiratory activity while decreasing mitochondrial ROS emission (Couée et al., 2006). The reducing pattern of pivotal glucose, together with the consistent of lower protein content and impaired respiration within seeds stored at 14°C, 40% RH, might diminish more ATP production

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and increasing ROS scavenging reactivity, which further caused exhaustion and exhibit reduction in vigour; and similar intolerant to oxidative stress conditions of agedseeds mentioned by Dussert et al. (2006). The ability to generate ATP, which is heavily influenced by metabolic activity, but also correlates with the percentage of seeds that germinate and the subsequent growth of seedlings (Rosental et al., 2014).

The above reasons might further explain the decreasing GR activity, in parallel with the increased pattern of APX activity, 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 enzymes activity that takes part in the glutathione-ascorbate cycle, or specifically in reducing oxidized 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 enzymes of the pentose phosphate pathway, except for glucose 6-phosphate dehydrogenase. As for seeds at 14°C, 40% RH, both conditions might be occurred together to cause the gradual decreasing GR activity in this study.

During the present study, the imbibitional chilling injury seems to be dominant for seeds stored at 14°C, 80% RH than the ones at 40% RH especially at 8 DAS onwards. Compared to storage conditions of 14°C, 40% RH, seeds stored at 14°C, 80% RH showed many significant consequences on ROS attacked during storage. It displayed the gradual decreasing pattern of germination characteristics and seedling performance and eventually became worsen at 8 DAS onwards. The high MC retention from the earlier days of storage, with the combinations of the chilling temperature, may cause all the stressful storage conditions or accelerated aging, leading to ultracellular changes leading to damage to the membranes, solution leakage, transcription damage, and damage that cause defective or incomplete protein (enzyme) synthesis essential to germination of seed (Suresh et al., 2019). As

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such, it was expected that the activity of CAT and POX would alleviate, which is associated with increased SOD activity. The pattern does increase but at the different levels and rate of increments for each storage treatment in this study. Catalase has one of the highest turnover rates among all enzymes (Daniel & Mani, 2016). During the present study, CAT displayed a highly significant activity during 2 DAS for seeds stored at 14°C, 80% RH and followed by seeds at 14°C, 40% RH than the other storage treatments. The pattern reduced afterward, and further stagnant at the highest values until 12 DAS, for seeds at 14°C, 80% RH. The most distinct increase in CAT shows that a possible ROS production is along the path to hydrogen peroxide production (De Souza et al., 2018). On the other hand, with the increasing pattern of SOD activity, seeds at 14°C, 80% RH however, parallelly showed the irregular pattern of APX and CAT activity, or increasing only slowly pattern, which appears that these consequences are the result of RNA synthesis damage, which eventually leads to decreased protein synthesis and enzyme deactivation (Mansouri-Far et al., 2015). Under abiotic stress the concentration of H_2O_2 or ROS in the cells can rise significantly, reaching a threshold that can remove cells that are damaged beyond repair by initiating programmed cell death (PCD) (Bouranis et al., 2007). The lysigenous aerenchyma structural development can be seen under SEM observation for seeds stored at 14°C, 80% RH, as part of the PCD process. The absence of a repair mechanism might further result in high cellular H_2O_2. The uncontrollable H_2O_2 produced might induce the formation of OH^- radicals through Fenton reaction with reduced Fe^{2+}, thus promoting the generation of deteriorative causative free radicals. Hydroxyl radicals may degrade polysaccharides through oxidative scission of backbone bonds, a mechanism that may be implicated in cell wall loosening processes, allowing cell expansion and radicle protrusion to occur (Bailly, 2004). Moreover, in parallel with the increasing H_2O_2 and might consequently the OH^- pattern, seeds at 14°C, 80% RH also showed the significantly gradual increasing of POX activity along 12 days of storage. Considering the POX activity in auxin catabolism and the formation of wall components, as well as the demand for H_2O_2 as a substrate, it can be deduced that the variation activity of this enzyme occurs at times when the seed's components exhibit a greater synthesis of the wall or differentiation of cell types associated with the end of the embryo maturation process (De Souza et al., 2018).

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Seeds at 16°C, 80% RH showed quite a similar pattern of damages as in 14°C, 80% RH, except the ones stored in the later conditions displayed the faster deterioration due to chilling injury development within seed cells. Both conditions also showed the increasing pattern and maintained among the highest MC especially during the first 6 DAS, which cause any damaging symptom manifested indirectly through their decreasing pattern of germination and seedling performances, along 12 days of storage. As for seeds at 16°C, 80% RH, there might be the dehydration occurs only slowly (at 8 DAS onwards), and thus, metabolism is thoughtto become imbalanced. At surprisingly high-water concentrations, this can cause significant intracellular damage (referred to as metabolism-linked damage) and seed/embryo mortality (Berjak & Pammenter, 2008). In the present study, almost all the antioxidant enzymes activity measured (SOD, APX, GR, and POX) 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 attacked being observed after the visible reducing MC at 8 DAS, as well as through the significant reducing pattern of protein content at 10 DAS. ROS attack on proteins can modify proteins in a variety of ways, some of which are direct and others of which are indirect. Proteins can be indirectly changed by coupling with fatty acid peroxidation breakdown products (Sharma et al., 2012). This is also supported through the increasing pattern of LC and MDA produced for seeds at 16°C, 80% RH especially at 8 DAS onwards. The symptoms of PCD also occurred for seeds stored at 16°C, 80% RH, as their development towards the mechanisms was quite clearly shown under SEM observation (more membrane lesions observed at 6 DAS onwards). The overly reducing pattern of H_2O_2 at 10 DAS, further hinted at the increased oxidative stress. The very low ROS signaling (on germination) resulted in significantly lower germination and the consistently decreasing pattern of seedling performance.

In contrast with all the arguments discussed above, seeds at RT and 16°C, 40% RH are seen to be at their best ranges, keeping the amount of ROS below the damaging level of degradation. As suggested by Bailly (2019), a seed's ability to germinate is directly dependent on ROS homeostasis: seed germination is likely to occur only when the seed's ROS concentration is contained within values that allow ROS signaling but not cause harm. Germination, on the other hand, is blocked when the level of ROS is either too low or too high. Together with the reducing MC for

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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 stagnant pattern of all the antioxidant enzymes activity (SOD, APX, GR, CAT, and POX) measured in this study. Their differences only displayed on the higher germinability and seedling performances, for seeds at RT than the ones at 16°C, 40% RH. Regarded as a part of a complex signaling network, ROS signaling on germination included their distinct roles ranging from interaction with cytoplasmic signaling pathways, oxidative regulation of gene expression within the nucleus, and further cell wall weakening (Bailly, 2019). ROS homeostasis mostly depends upon production sources, and if the ROS production did not surpass their concentration threshold, it will not cause oxidative damage, which might further affect their germinability and seedling's performance. During this study, the ranges differences in between 19 to 47% of H_2O_2 for seeds stored at RT, and 16°C, 40% RH with the control seeds was recorded; with those seeds consistently exhibited their good germination and seedling growth performances, along 12 days of storage. In this study, seedling measurements evaluations shall be made independently following germination, due to their characteristics such as SVI and MASS which were correlatedwith percentage germination (FGP) and speed of germination (GRI). This is also supported by Finch-Savage & Bassel (2016) who stated that a seedling from a late germinating seed will always be smaller than a seedling from quick germination at the time of sowing. Moreover, Mansouri-Far et al. (2015) added that it is likely that the time required for membrane damage repair and damage to other parts of the cell, as well as the initiation of antioxidant system activity to prevent the buildup of oxidative stress conditions, causes a delay in the initiation of germination in aging seeds. On the other hand, seeds at both storage treatments showed the least changes in their antioxidant enzymes activity, except for the elevated GR activity since 2 DAS was recorded for seeds at 16°C, 40% RH. The accumulation of GSH build-up might increases GR activity in plants, resulting in stress tolerance. A similar increment of GR activity has been recorded to react to the abiotic stresses in pea and *Reaumuria soongorica* (Habibi, 2014). Besides, seeds at 16°C, 40% RH also displayed a significant increase of respiration rate at 8 DAS, which was coincidently occurred with the reducing MC pattern. Imbibed seeds are extremely sensitive to little changes in environmental variables. Thus, the environmental information must be accurately communicated to seeds and transformed into endogenous germination signals (Bailly,

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2019). Considering the sugar starvation mode to have occurred at a similar timeline; the increased respiration rate but with maintained antioxidant enzymes activity, and the evidence of good performance in germination and seedling growth indicated the efficiency of respiratory activity of seed cells at 16°C, 40% RH in responding to their surrounding changes.

4.5 Conclusion

Condition of RT and 16°C, 40% RH appeared to be convenient along 12 days of cacao seeds storage. Their mild dehydration patterns contributed to the least seed microstructural changes and thus, lessened cell damages. However, seeds at RT reduced their storability due to 8 to 30% (>50% higher than seeds at 16°C, 40% RH) of germination incidence during storage. Serving as reflections of the intense metabolic events, retaining higher seed MC during storage also exhibited their internal structural matrix damages and worsened with time. Though with much lower impaired respiration, the high LC of seeds at 16°C, 80% RH increased their dehydration sensitivity at 8 DAS on-wards. The variation of soluble sugars accumulation in cacao seeds reflected the effectiveness of utilizing the free metabolites during storage, which further affecting the catabolism of other vital components, such as protein and lipid. The intense respiration but with lower metabolic events of seeds at chilling conditions (14°C) further exposed their active metabolism site to be attacked by ROS, accelerated the loss of vigour and their eventual decline in viability during the extended storage (>6 DAS), especially the ones with higher MC (14°C, 80 %RH).

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CHAPTER 5

SUMMARY, CONCLUSIONS, AND RECOMMENDATIONS

5.1 Summary

In evaluating the physiological quality of cacao seeds stored at different temperatures and relative humidity, results from the first experiment further suggested that the lower storage temperatures; 10 °C, 14 °C, and 16°C showed efficiency in maintaining the high MC (>40%) within seed throughout 144 HOS. However, as the chilling stress strikes, the reductions in vigour (seedling growth) were precedent rapidly before the viability of seeds at 14°C (40%, 60%, and 80% RH), and more unpredictable adverse performances displayed for seeds at 10°C (40%, 60%, and 80% RH). The prolonged storage duration further made the adverse effects became more visible. Seeds at 16°C (40%, 60%, and 80% RH) and RT showed better performances in maintaining the unchanged viability. The favourable storage conditions made those seeds produced higher germinability, vigour and seedling growth performance than the other treatments. However, the increase of germinated seeds (8 - 10%) and more discernible fungi on seeds at RT during storage might further reduce their storability. Among seeds at 16°C, the ones stored at 40% RH demonstrated the higher potential to exhibit more consistent quality (with no seeds germinated during storage) along 144 HOS. The slight but significant changes of their seed MC somehow could be the differences attained in between the high and low vigour seed or seedling performances.

More investigation on the physiological and biochemical changes may help in explaining the possible factors and consequences caused by the dynamic of MC changes within cacao seed. Therefore, the following experiment and analyses in Chapter 4 were performed to investigate and provide additional information in

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characterizing the specific responses of cacao seeds on chilling or desiccation sensitivity development.

The second objective of this research was to characterize the physio-chemical and microstructural changes within the cacao seeds affected by slow-drying and chilling treatments during storage. Differences in the concert of thermodynamic reactivity had first led to the changes of bio-physical and microstructural characters within the hydrated cacao seed cells being exposed to different storage conditions. With the different rates of MC increment, seeds stored at the higher RH (14°C, 80% RH and 16°C, 80% RH) tend to retain higher MC along 12 days of storage and induced the occurrence of more metabolic events. Seeds at both treatments shared similar cell structural characters, as they displayed the wrinkled, and shrunk cells with the visible internal molecules, indicating the increased of surface areas available for metabolism reaction's site, and seeds at both treatments displayed the symptoms of lysigenous aerenchyma development with the increasing storage duration. Besides, seeds at 14°C, 40% RH also tend to gradually increase their MC, and parallelly displayed more signs of cell wall disruption, cell deformation, the collapse of cell structure, and even lesions. In contrast, seeds stored at RT and 16°C, 40% RH tend to reduce their MC, and exhibited signs of folding and thickening cell wall, along 12 days of storage.

Respiratory activity within seed cells stored at different storage treatments played vital roles in leading those seeds to germination or deterioration development. Seeds stored at the chilling conditions (14°C) tend to increase and consistently retained their respiration rate among the highest levels recorded along 12 days of storage. The third objective of this research was to elucidate the accumulation of reactive oxygen species (ROS) antioxidant enzymes of cacao seeds during storage. The effects of mostly impaired respiratory activity reflected through the highest accumulation of soluble sugar content (glucose, sucrose, and RFOs) during the first 6 DAS, the consistently lower protein content with the irregular pattern of lipid content, as well as recorded among the highest and increasing pattern of antioxidant enzymes activities, which also indicated the increased of ROS production along 12 days of storage, for seeds stored at 14°C. The lower metabolism of seed cells at chilling conditions, further extent or exposed their active metabolism site to

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be attacked by ROS. Antioxidant enzymes involved in the glutathione-ascorbate cycle; APX and GR activity, found to be crucially responsive to indicate the oxidative statuschanges within seed cells during storage, especially for seeds stored at 14°C. As forseeds demonstrated the decreasing MC throughout 12 days of storage (RT and 16°C, 40% RH), those seeds displayed the higher ROS (H_2O_2) signaling than the other treatments, but still below or within oxidative concentration threshold, which gave the advantage of holding those seeds such in controlled conditions with lesser exposure to the oxidative stress. Overall, both RT and 16°C, 40% RH exhibited the better quality for cacao seeds storability for 12 days but came together with the constraints of about 8 to 30% (RT) and 8 to 12% (16°C, 40% RH) of seeds germinated incidence during storage. On the other hand, 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. Whileseeds at 14°C, 40% RH were found to be responded more to the 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.

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5.2 Conclusions

Recalcitrant cacao seeds stored at chilling or non-chilling with slow-drying conditions resulted in the retention of MC within seed cells during storage. Thus, the method of storing in the slow-drying conditions was successful in retaining the seed MC to allow the continuation of metabolic reactions within seeds cells after extraction from pods. Further evaluations on physiological quality of cacao seeds during storage highlighted through the significant impacts of the consistently lower and decreasing pattern of viability and vigour for seeds stored at 14°C (regardless the RH), indicated the poor and significant effects of storing seeds at the chilling conditions, and this is vice versa with seeds stored at 16°C and RT. The characterization on the physio-chemical and microstructural changes further reflected through the increasing LC values along the storage durations, and damages illustrated ranging from the microstructural to oxidative stresses within seed cells stored at the 14°C. Seeds stored at the higher RH (regardless the

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temperature; 14°C and 16°C, 80% RH) tend to retain higher MC, further contributed to the faster deterioration development led by the ones at the chilling condition along 12 days of storage. Meanwhile, seeds stored at the non-chilling conditions with the lower RH such as 16°C, 40% RH, and RT (25°C, 55% RH) however, tend to reduce their MC eventually, led by the latter condition. More frequent rehydration and dehydration processes within seed cells during storage in these conditions contributed to non-fatal microstructural (physical) changes of the seed inner matrix, due to more activation of repairing mechanisms came together with the impaired metabolic events, which further lessened their potential metabolism-linked damages. This results further elucidated through the lower ROS accumulation reflected through the lower antioxidant enzymes activity as the significant responses involved in antioxidant machinery system within seed cells, and this is vice versa with seeds at 14°C.

5.3 Recommendations

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The consistency of cacao seeds quality with the minimum changes of physio-chemical and microstructural exhibited along 12 days of storage at 16°C, 40% RH. Moreover, those seeds also demonstrated the highest germinability, no dead seedlings, minimum abnormal seedlings, lesser seeds germinated during storage (8 - 12%) with lesser fungi infestation (as compared to seeds at RT), and thus, contribute to the higher potential for further exploration.

The findings from this study will benefit the industry as it discovered the alternatives and guidelines of the easy-handled short-term storage of the locally produced recalcitrant cacao seeds that can be beneficial for improving the seed handlers' awareness and understanding of maintaining good seed quality using the specific storage method and duration. It provides a preliminary account of the characterization of physio-chemical changes associated with cacao seed quality in response to chilling and slow-drying conditions during storage. Thus, the method (used in this research) of cacao seeds extraction, packaging, and storage (at 16°C, 40% RH) for at least 12 days, could be used as the guidelines by the seed handlers, as per the expectations for the seed quality to be at least in the same

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ranges as the freshly harvested seeds in pods. Moreover, the outcome of this research could possibly be used as the yardstick to reduce the windows tolerance in managing seeds quality during the gap before replanting for the other locally produced cacao clones.

Furthermore, a deeper study should be attempted in the optimization of their post-shedding/ storage management to may at least reduce the gap of tolerance windows of the locally produced cacao seeds. The storage temperatures in between the ranges used in this study, for example, the temperature of 15 °C and, 17 to 24°C, with different RH, seem to gain potential in determining more suitable tolerance windows and are significant to be studied in the future.

In the future study, with an assurance of the continuous and more samples (cacao pods/ seeds) should be taken into consideration for establishing a more robust and accurate pattern of evaluations of quality during storage. This might be useful to ensure the continuous evaluations on the other seed biochemical changes, including factors that contribute to oxidative stress; the other parts involved in a complex signaling network, ROS signaling on germination/ deterioration including their distinct roles ranging from interaction with cytoplasmic, oxidative signaling pathways, and further cell wall weakening. Moreover, the use of SEM together with transmission electron microscopes (TEM) may provide more supportive evidence in elucidating physical changes of seed inner matrix, in relation to the changes of organelles' functions within seed cells during storage.

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