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

SHAFEEQA BINTI SHAHRUDDIN

THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

FACULTY OF SUSTAINABLE AGRICULTURE
UNIVERSITI MALAYSIA SABAH
2022

UNIVERSITI MALAYSIA SABAH

BORANG PENGESAHAN STATUS TESIS

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

IJAZAH : **DOKTOR FALSAFAH SAINS PERTANIAN**

BIDANG : **HORTIKULTUR**

Saya **SHAFEEQA BINTI SHAHRUDDIN,** Sesi **2017-2022,** mengaku membenarkan tesis Doktoral ini disimpan di Perpustakaan Universiti Malaysia Sabah dengan syarat-syarat kegunaan seperti berikut:

1. Tesis ini adalah hak milik Universiti Malaysia Sabah
2. Perpustakaan Universiti Malaysia Sabah dibenarkan membuat salinan untuk tujuan pengajian sahaja.
3. Perpustakaan dibenarkan membuat salinan tesis ini sebagai bahan pertukaran antara institusi pengajian tinggi.
4. Sila tandakan (/):

[ ] SULIT (Mengandungi maklumat yang berdarjah keselamatan atau kepentingan Malaysia seperti yang termaktub di AKTA RAHSIA 1972)

[ ] TERHAD (Mengandungi maklumat TERHAD yang ditentukan oleh organisasi/badan di mana penyelidikan dijalankan

[/] TIDAK TERHAD

Disahkan Oleh,

SHAFEEQA BINTI SHAHRUDDIN
DR1611001T

(Tandatangan Pustakawan)

Tarikh : 13 Mei 2022

(Prof. Dr Mohammad bin Mohd Lassim)
Penyelia Utama

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.

10 March 2022

Shafeeqa binti Shahruddin
DR1611001T

05-4506832 pustaka.upsi.edu.my Perpustakaan Tuanku Bainun Kampus Sultan Abdul Jalil Shah PustakaTBainun ptbupsi

05-4506832 pustaka.upsi.edu.my Perpustakaan Tuanku Bainun Kampus Sultan Abdul Jalil Shah PustakaTBainun ptbupsi

CERTIFICATION

NAME	:	**SHAFEEQA BINTI SHAHRUDDIN**
MATRIC NO.	:	**DR1611001T**
TITTLE	:	**PHYSIO-CHEMICAL CHARACTERIZATION OF CHILLING AND SLOW-DRYING RESPONSES OF CACAO (*Theobroma cacao* cv. PBC 123) SEED**
DEGREE	:	**DOCTOR OF PHILOSOPHY IN AGRICULTURAL SCIENCE**
FIELD	:	**HORTICULTURE**
VIVA DATE	:	**10 MARCH 2022**

CERTIFIED BY;

Signature

1. MAIN SUPERVISOR
Prof Dr Mohammad bin Mohd Lassim

2. CO-SUPERVISOR
Assoc. Prof Dr Azwan bin Awang

ACKNOWLEDGEMENT

"In the Name of Allah, the Most Gracious and Most Merciful". Peace and blessings ofAllah be upon Prophet Muhammad. Praised be to Allah SWT, with His grace I am able to complete this thesis. Foremost, I would like to thank all people who have helped and inspired me during my study. I would like to express my deep and sincere gratitude to my main supervisor, Prof Dr Mohammad Mohd Lassim and co-supervisor, Assoc. Prof Dr Azwan Awang for all their guidance, patronage, motivation and support.

My warm thanks to all the laboratory staffs at Faculty of Sustainable Agriculture, UMS Sandakan for their kindness in providing invaluable support towards my research.

I am thankful to my family especially my parents for their unconditional support. Sincere thanks to all my friends for their kindness and moral support during my studies.

Finally, I would like to gratefully acknowledge Universiti Malaysia Sabah for their funding under UMS Great (Project Code: GUG0176-2/2017) grant, the Malaysian Cocoa Board for providing the plant materials, the support of Ministry of Higher Education (MOHE), and Universiti Pendidikan Sultan Idris (UPSI) for scholarship and financial support during my studies.

Shafeeqa binti Shahruddin
10 March 2022

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

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

LIST OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

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

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 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 (Pammenteret 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 seedsto 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-associatedfungi. On the other hand, even at high relative humidity, the onset of germinative processes requiring additional water provision does not allow water loss, causing thedecline 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 15ºC, 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 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

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.

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 (-196ºC) (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 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 needs to 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 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.

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 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.

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.

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.