EFFECT OF STORAGE TEMPERATURE AND DURATION ON SEED VIABILITY AND SEEDLING DEVELOPMENT OF *Theobroma cacao*

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DECLARATION

I hereby declare that this dissertation is based on my original work except for citation and quotations which have been duly acknowledged. I also declare that no part of this dissertation has been previously or concurrently submitted for a degree at this or any other university.

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ABSTRACT

This study aimed to determine the effect of different storage temperature (5±1, 9±1, 13±1, 21±1, and 24±1 °C) and different storage duration (24 and 72 hours) to the seed viability and seedling development of cacao (*Theobroma cacao*), clone BR25. The seeds were tested for their viability by measuring electrolyte efflux and tetrazolium test after storage treatments. Treated cacao seeds were germinated and the developed seedlings were grown for 21 days. Germination capacity, time, and rate of the cacao seeds as well as their morphological changes in subsequent seedling development were observed and measured. Electrolyte efflux of cacao seeds was found increased as storage temperature decreased. The results showed that cacao seeds were very sensitive to low temperature, particularly below 14 °C. Cacao seeds from all storage treatments showed positive result in tetrazolium staining test, however all the seeds that stored at temperature below 14 °C were failed to develop into healthy seedlings. This suggests that cacao seeds could be stored at 20-25 °C without affecting the viability. Moreover, cacao seeds stored for 24 hours were better in retaining seed viability and seedling development than that of 72 hours.

***KESAN SUHU DAN MASA PENYIMPANAN TERHADAP KEBOLEHHIDUPAN BIJI BENIH DAN PERTUMBUHAN ANAK BENIH* Theobroma cacao**

ABSTRAK

Tujuan kajian ini adalah untuk mengkaji kesan suhu penyimpanan yang berbeza (5±1, 9±1, 13±1, 21±1, dan 24±1 °C) dan jangka masa penyimpanan yang berbeza (24 dan 72 jam) terhadap kebolehhidupan dan pertumbuhan anak-anak benih daripada biji benih koko (Theobroma cacao) dengan menggunakan klon BR25. Kebolehhidupan anak-anak benih koko telah diuji dengan mengukur efluks elektrolit dan ujian tetrazolium selepas rawatan. Biji benih koko yang telah dirawat dicambah dan dibiarkan tumbuh selama 21 hari. Kapasiti, masa, dan kadar percambahan biji benih koko dan perubahan morfologi dalam pertumbuhan anak benih seterusnya telah diperhatikan dan diukur. Efluks elektrolit biji benih koko didapati meningkat bila suhu penyimpanan menurun. Keputusan ini menunjukkan bahawa biji benih koko sangat sensitif kepada suhu yang rendah, terutamanya 14 °C ke bawah. Biji benih koko daripada semua rawatan menunjukkan keputusan positif dalam ujian tetrazolium, namun semua biji benih koko daripada penyimpanan pada suhu 14 °C ke bawah gagal dalam pertumbuhan menjadi anak benih. Hasil ini menunjukkan biji benih koko boleh disimpan pada suhu 20-25 °C tanpa menjejaskan kebolehhidupannya. Tambahan pula, biji benih koko yang disimpan selama 24 jam adalah lebih baik dalam mengekalkan kebolehhidupan biji benih dan pertumbuhan anak benih berbanding dengan penyimpanan selama 72 jam.

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LIST OF SYMBOLS, UNITS, AND ABBREVIATIONS

ANOVA	Analysis Of Variance
AS	Percentage of Abnormal Seedling
cm	centimetre
CVG	Coefficient of Velocity of Germination
DAS	Day After Sowing
DOG	Day Of Germinating
DW	Dry Weight
EC	Electrical Conductivity
CRD	Completely Randomized Design
°C	degree Celsius
g	gram
G	Germination Percentage
GI	Germination Index
HSD	Honest Significant Difference
LSD	Least Significant Difference
mL	millilitre
mS	millisiemens
M	Molar
MCB	Malaysian Cocoa Board
MGT	Mean Germination Time
%	percentage
p	probability
RO	Reverse Osmosis
SAS	Statistical Analysis System
SVI	Seed Vigour Index

LIST OF FORMULAE

CHAPTER 1

INTRODUCTION

1.1 Background

Cacao tree was first introduced into Malaysia since 17th century, and it only went commercial during the late 1970s (Lee, 2012). According to Malaysian Cocoa Board (MCB, 2017a), cocoa beans productions grew rapidly from 36,500 tonnes in 1980 to 247,000 tonnes in 1990, then followed by a continual downturn over the decades to 1,757 tonnes in 2016. The declination was mainly due to poor world cocoa prices, labour constraints, severe spread and infestation of cocoa pod borer, and competition for land use from oil palm plantation (Lee, 2012). Furthermore, cocoa industry in Malaysia is exacerbated by scarcity of knowledge and skills of the ordinary farmers, in which most of them lack of cash to purchase of modern inputs (Othman, 1990).

Almost 95 % of cacao cultivated area in Malaysia is undertaken by smallholders (MCB, 2017b). Although the government has implemented cacao cultivation program to provide planting materials (Kasin, 2012), but many of these smallholders, particularly the farmers in remote areas, tend to produce their own seedlings or sow directly from their own seed stock rather than purchase of certified planting materials (Othman, 1990; Taher, 1996). Freshly harvested cacao fruits are usually not open directly for seeds sowing, instead the pods are stored for a period of time, where the seeds could lose viability and vigour under a long period of handling or storage.

Cacao seed is highly recalcitrant (Pence, 1991; Chandel *et al.*, 1995), in which it is greatly intolerant of desiccation and sensitive to freezing. Recalcitrant seeds are very difficult to store and tend to loss their viability even in a short period of storage. They deteriorate rapidly when exposed to humid tropical conditions because they do not withstand dehydration and low temperature. In Malaysia, much planting materials have been lost as a result of poor handling and storage (Hor, 1984).

Moreover, practice of inappropriate postharvest techniques could lead to unexpected variation in growing of cacao seedlings raised from the same pod (Adu *et al.*, 2017). This may lead to the production of non-uniform and abnormal seedlings, in which it is a situation that can be avoided if cacao seeds could be stored in the right practice (Amoah, 2005). Postharvest seed handling is therefore critical for seed viability and fortunately it is the most controllable factors among the others that affecting cacao seed germination and consecutive development (Amoah, 2005).

Establishment of well growth cacao trees is controlled by mechanisms that ensure good seeds germination and seedlings development. A wide range of genotypic and environmental factors, including varietal sources of pod and bean characteristics, postharvest handling of seed storage treatments, could greatly influence germination of cacao seeds and further development into seedlings (Amoah, 2005).

Most of the postharvest researches regarding cacao nowadays are more in the area of cocoa beans storage as preserving raw materials for industrial processing purposes (Saajah and Maalekuu, 2014). Not much work has been studied in terms of postharvest management for cacao seeds storage for subsequent propagation (Saajah and Maalekuu, 2014).

In Malaysia, cacao is largely propagated by seed although vegetative propagation such as grafting and layering are possible (Hor, 1984). The demand for cacao planting materials exists throughout the year, but the seed production is limited to certain months only. Seasonal supply for continual demand has become inevitable for the farmers to store their own cacao seeds for future use. This emphasizes the need for a suitable method of seed conservation during the peak seasons.

Moreover, the current method used for conserving germplasm of cacao is by planting *ex-situ* on field (Zhang *et al.*, 2011). Nevertheless, this method is prone to natural disasters and very laborious that needs frequent replacement as the cacao trees become old and unproductive. Therefore, a more reliable method to conserve cacao germpalsm is needed.

1.2 Justification

This study was conducted to determine the effect of different storage temperature and duration to the viability of cacao seeds and their consecutive development into seedlings. Similar studies have been reported (Hor, 1984; Pence, 1991; Chandel *et al.*, 1995; Saajah and Maalekuu, 2014), however, this study was aimed to manipulate the storage of cacao seeds under different temperature conditions to check the sensitivity of recalcitrant cacao seeds over a short period of storage. The information is prime important in devising an improved method of storage to prolong cacao seed viability.

The source of seed samples in this study was selected from the clone BR25. It is one of the most widely planted commercial clone of *Theobroma cacao* in Sabah, East Malaysia (Teh *et al.*, 2006).

In this study, cacao seeds samples were stored at five (5) moderate to low temperature for 24 and 72 hours. Electrolyte leakage and tetrazolium test were then carried out to check the viability of cacao seeds for the five (5) treatments in each day. After that, the seeds were germinated and grew into seedlings in laboratory for three (3) weeks. The cacao seeds viability and germination performance into seedlings development were measured, analyzed, and evaluated respectively. Finally, the relationship of manipulating storage temperature and duration to the cacao seed viability and their subsequent seedling development were correlated.

1.3 Objectives

I. To determine the viability of cacao seeds stored under five (5) different temperatures for two (2) different storage durations.

II. To compare the performance of seeds development into seedlings between cacao seeds stored under five (5) different temperatures for two (2) different different storage durations.

1.4 Hypotheses

H_{o1} : There is no significant difference in the viability of cacao seeds stored under five (5) different temperatures for two (2) different storage durations.

H_{a1} : There is significant difference in the viability of cacao seeds stored under five (5) different temperatures for two (2) different storage durations.

H_{o2} : There is no significant difference in the seedlings development performance between cacao seeds stored under five (5) different temperatures for two (2) different storage durations.

H_{a2} : There is significant difference in the seedlings development performance between cacao seeds stored under five (5) different temperatures for two (2) different storage durations.

CHAPTER 2

LITERATURE REVIEW

2.1 Cacao Tree

Cocoa (*Theobroma cacao*), is a perennial tree from the subfamily of Sterculiaceae in the mallow family of Malvaceae. It is a worldwide cultivated plant that native to the wild tropical forests of Central America (Wright, 1999). It is an understory, shade tolerant, and moisture loving tree in well-drained soil. The economic lifespan of a cacao tree is around 25 years (Bergmann and Butler, 1985; Wright, 1999).

According to Wright (1999), cacao tree can grow up to as high as 1.8 m tall in 3-4 years, and 3.6-7 m in 10-15 years; bear fruits on its trunk and branches; a profuse branch system with wide canopy of about 1.5 m in diameter when three (3) years old, and increase to 6-7.6 m in diameter at 12 years old; leaves are alternate, smooth, bright to dark green-coloured and varying from 25-34 cm in length and 9-12 cm in width; root system consists of a stout and often very long taproot with a fibrous system of superficial rootlets; flowers are small, yellowish white to pale pink in appearance, hermaphrodite, exist either solitary or in group of clusters, arising directly from the flower cushions on tree trunk and older brunches, and are produced all year round after the plants are three (3) years old. The flowers are pollinated by midges, and only about 5 % of flowers will receive enough pollen to start fruit development (Kew Science, 2016).

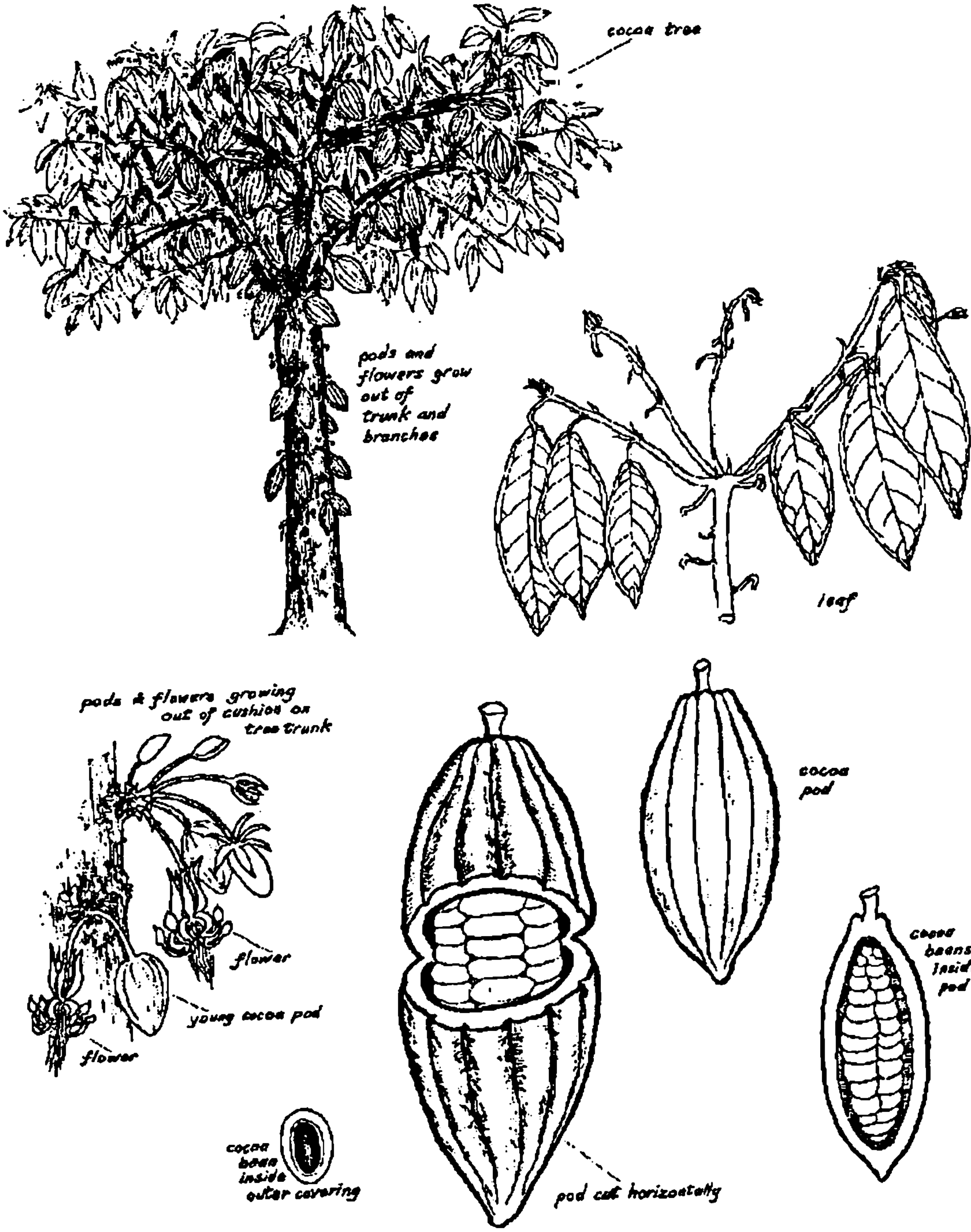

Figure 2.1 Morphology of cacao tree and fruit
Source: Bergmann and Butler, 1985

2.2 Cacao Fruit

Cacao fruit is commonly called a cocoa pod. It is usually almond-shaped, 16-21 cm in length and 22-29 cm in circumference (Wright, 1999); more or less knobble surface and lines from top to bottom; initially green or deep red while maturing and turns to yellow or reddish yellow when ripe (Bergmann and Butler, 1985) (some varieties remain green throughout ripening); many seeded that each pod contains average 30-40 pink or purple seeds, and each of them is encircled in a watery white pulp (Kew Science, 2016). The pods are usually ripe within 5-7 months after flowering (Bergmann and Butler, 1985; Niemenak *et al.*, 2010).

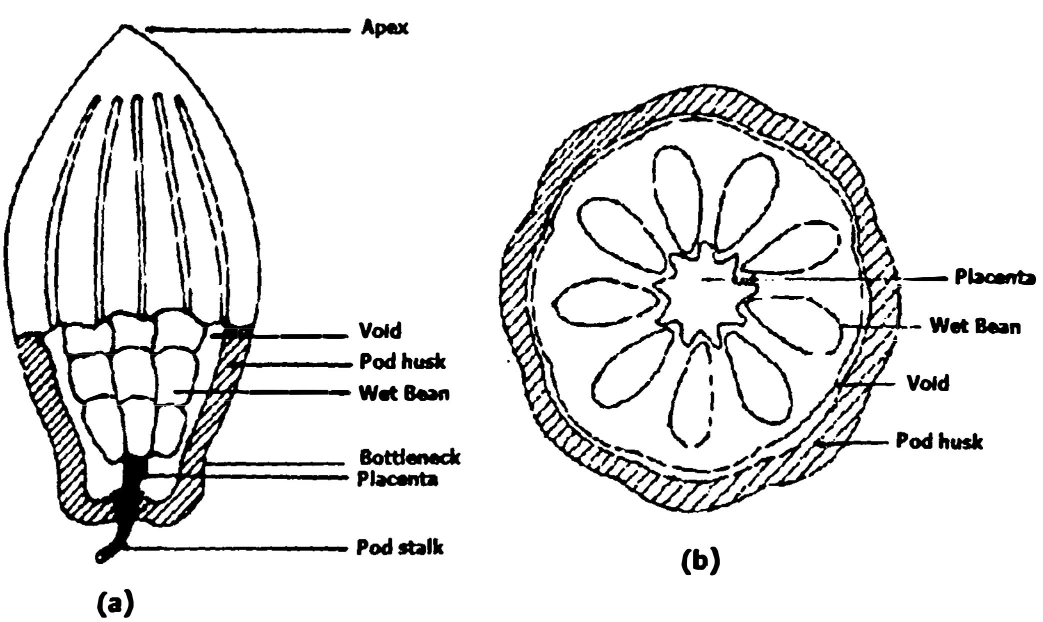

Figure 2.2 Physical features of cacao fruit in (a) longitudinal section, and (b) transverse section
Source: Fabunmi, 2004

2.3 Cacao Seed

Fresh cacao seed is embedded by testa and bitter-sweet white pulp known as mucilage on the outer surface. The testa is a tough integument that enclosing two short cotyledons, which as the food storage that support germination of the embryo, and are usually known as the kernel. The kernel is roughly responsible to only half of the total weight of a fresh seed (Wright, 1999). Weight of a seed varies with the variety and other factors such as growing condition of the mother tree.

Cacao seeds are varying considerably in shape, size, and colour. As stated by Wright (1999), the shape is sometimes flat, round and plump, or long and more or less rounded; the size varies depending on the part of the fruit occupied by the seeds, in which those at the ends are usually being smaller and also more irregular or flatter than those in the middle; the colour varies from white to deep purple depending either source from the same fruit or fruits from different varieties.

In order to obtain raw cacao to making chocolate, seeds are removed from the fruit after harvest and are processed to fermentation and drying. Otherwise, they will germinate under permissive conditions for propagating of new seedlings.

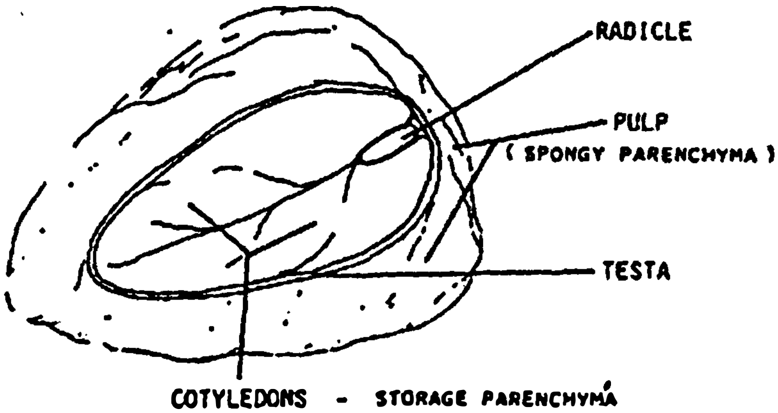

Figure 2.3 Internal features of cacao seed
Source: Christian, 2011

2.4 Cacao Seed Germination and Seedling Development

According to Niemenak *et al.* (2009), the whole cacao seedling development from onset of seed imbibition to seedling emergence takes approximately 25 days, that imbibition will complete within three (3) days (Figure 2.4A); radicle protrusion will appear on around day five (5) (Figure 2.4B); radicle elongation proceeds whereby hypocotyl will emerge and follow by the formation of root hairs (Figure 2.4C); cacao seeds will have emerged from the soil to form seedlings and the hypocotyl will form a 3-5 cm long of hook lifting the cotyledons from the ground in between day seven (7) to 18. At this stage, the cotyledons are growing upright but still closed until flushes appear.

Unfolding of the cotyledons will trigger the development and growth of the first leaves from cacao seed (Figure 2.4D). Succession of flushes will occur as more leaves continue to develop. Those unfolded leaves of the first flush are initially pale green or red in colour depending on the cultivar. They are soft and delicate, but gradually harden as getting mature (Figure 2.4E). The new unfolded leaves will take about four (4) weeks to expand for attaining their full size (Figure 2.4F). Determination on the degree of browning of the stem which the leaves are attached on, can easily distinguish the successive flushes, in which young flushes tend to grow on green stems whereas those older flushes used to appear on dark brown stem (Niemenak *et al.*, 2009).

Leaf blades are usually simple and lanceolate to ovate, while showing dimorphic features as the venation on leaves is pinnate. Leaves have long petioles and are symmetrical as they growing along; the petioles have a marked pulvinus or swelling at the end that attached to the shoot which allows the leaf to be oriented to the light. Generally, leaves that emerged on the apical meristem tend to have shorter petioles and are slightly asymmetrical (Niemenak *et al.*, 2009).

Shoot derived from seedling will have a typical orthotropic growth (Figure 2.4F), in which this shoot will later constitute to become the main trunk by achieving the physiological maturity and the apical meristem will stop its growth after one (1) to two (2) years (Niemenak *et al.*, 2009).

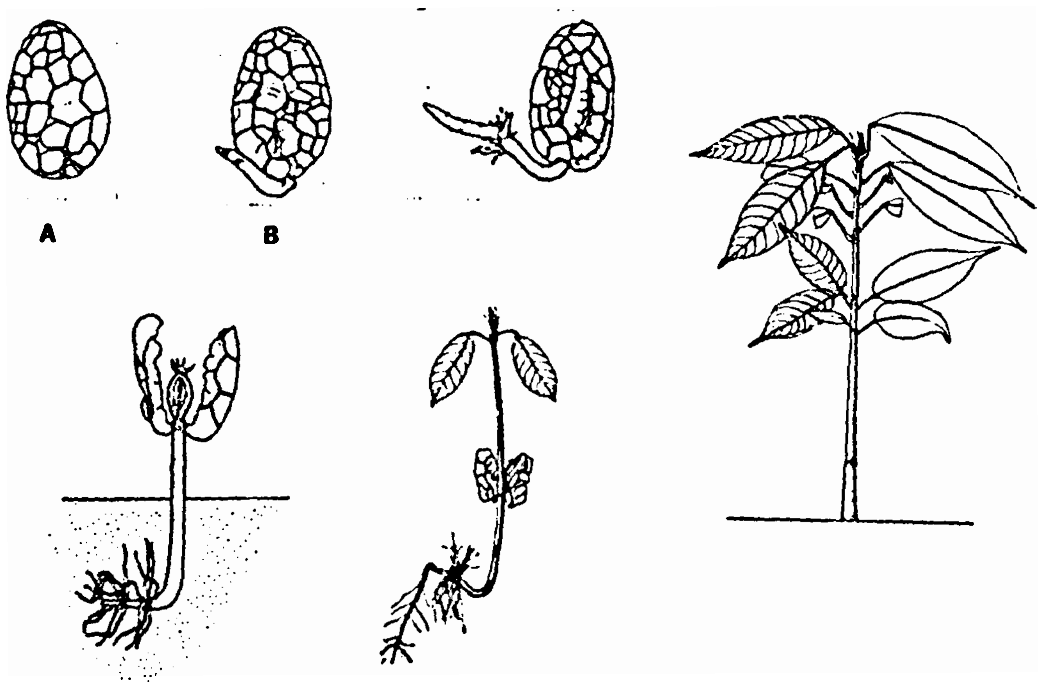

Figure 2.4 Cacao seed germination and seedling development
Source: Niemenak *et al.*, 2009

2.5 Recalcitrant Seed

Seeds that are tolerant of very little post shedding desiccation and often chilling injury are known as recalcitrant seeds (Beejax *et al.*, 1989; Pammenter and Berjak, 1999; Khandelwal, no date). Due to their rapid deterioration behaviour under dehydrated and low temperature condition, such seeds are unstorable by any of the general seed storage methods (Beejax *et al.*, 1989; Ellis *et al.*, 1990; Pammenter and Berjak, 1999).

Recalcitrant seeds are known for their short lifespan that being damaged under desiccated condition. Nevertheless, damage could occur as well even under highly moisture condition when the seed has disappointing tolerance to low temperature (Pammenter and Berjak, 1999). Previous research had done a lot of review on poor desiccation tolerance of recalcitrant seed, that recalcitrant seeds lose their viability when being dried to moisture content below a relatively high critical value, and yet there are very little scientific studies on chilling sensitivity.

Review of Farnsworth (2000) noted that recalcitrant seeds remain moist and their embryo maintain active metabolism throughout development to the time of shedding and bursts the seed tissues shortly after dispersal. Also, these seeds germinate readily within the fruit or soon after dehiscence in the natural mean for most of the time. They do not persist in the soil seed bank as well, whereby these types of embryos rapidly lose viability when they are dried or chilled (Farnsworth, 2000). Despite the reason of embryo injury, the seeds lose their stored water supply provided water available in the seed is either dried up or freezed, and eventually die owing to the fact that moisture is the continual key requirement for germination. The inability to store seeds of these species creates great challenges for the effort of germplasm conservation.

2.5.1 Storage of Recalcitrant Seed

There are varying degrees of recalcitrance based on the seed storage behaviour. A published study has proposed that types of recalcitrant seed may be grouped as low, moderate or high degree of recalcitrance (Farrant *et al.*, 1988). According to that grouping, low degree of recalcitrant seeds origin from temperate and sub-tropical regions can tolerate a fair amount of water loss and relatively low temperatures. In contrast, highly recalcitrant seeds origin from tropical forest and wetland species can tolerate very little dehydration and changes in temperature.

Generally, high moisture content of recalcitrant seeds encourages microbial contamination and therefore lead to more rapid seed deterioration (Pammenter and Berjak, 1999; Khandelwal, no date). Storage of recalcitrant seeds at sub-zero temperatures could cause the formation of ice-crystals which will disrupt cell membranes and cause freezing injury (Ellis *et al.*, 1990; Khandelwal, no date). In addition, some recalcitrant seeds are also susceptible to chilling injury that the moist seeds tend to damage and die at temperatures well above zero, typically in the range of 10-15 °C (Ellis *et al.*, 1990). Pammenter and Berjak (1999) also noted that recalcitrant seed cannot be stored at temperatures go below 15 °C. Therefore, recalcitrant seeds are used to store in growing phase of seedlings rather than as seeds for long term preservation where vegetative propagation is applicable to most common practice.

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