UNIVERSITI TEKNOLOGI MARA

GERMINATION BEHAVIOUR AND REGENERATION POTENTIAL OF *Syzygium malaccense* FROM SEED FRACTIONS

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Thesis submitted in fulfilment
of the requirements for the degree of
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ABSTRACT

Syzygium malaccense (Malay apple) is an underutilized member of the Myrtaceae family with economic potential, especially for rural communities. However, commercial expansion is hindered by the availability of planting materials. Numerous seedless fruits are observed and conventionally would present significant challenges for regeneration. In addition, seedless or seeded fruits are also very vulnerable to animal predation. Thus expanding the cultivation of this fruit species for commercial purposes can be limited by the availability of planting materials. Preliminary study have found that the monoembryonic seeds of this species had the unique ability to regenerate into full plant even after loss of half of their reserves and devoid of plumule-radicle axis This study focused on the germination behaviour of fragmented seeds to understand the regeneration potential with the objectives to evaluate the relationship between reserve reduction and sprouting potential through systematic fractionation, the influence of light condition, assessing the growth performance and the impact of natural predation on viability. Healthy seeds collected from a single tree located in Tanjung Sepat, Selangor with their morphometrics and weight recorded were subjected to five different cutting treatments to evaluate the in vivo sprouting from seed fractions. Subsequently, induced ripening of half-riped fruits collected from different localities were subjected to different light conditions to observe the regeneration from seed fractions. Full plants successfully developed from seed fractions in the dark were assessed for their growth performances. Predated seeds fractions from fruits grouped into damage categories based on visual assessment were assessed for their sprouting, and growth performances. Results demonstrated that a seed was capable of producing multiple plants when cut into even quarters with significantly reduced mass. The highest full plant development rate was 1.5 with longitudinally halved and quartered fractions, respectively, which was significantly contrasted to a full plant regeneration rate of 0.93 shown by the intact seeds. Most remaining fractions rooted without shoot development over a period of five months. In the event of full plant development success, the cotyledonary surfaces showed re-rooting after the full plants and portions of cotyledons attached to the full plants were cut off, confirming new cell development from the seed fractions. When sprouted in the dark, the longitudinally quartered fractions had full plant regeneration rate increased to 1.8. One of the seeds could yield up to four entire plants following longitudinal cutting into four fractions and dark sprouting, supporting the potential of dark sprouting in inducing the development of more full plants from seed fractions. The findings showed that early shoot emergence in less than 40 days was correlated to taller plants with larger leaves. Late sprouting into entire plant yielded smaller plants, regardless of fraction size (weight). Results of field-collected fruits and seeds with varying degrees of predation demonstrated that partially predated seeds were mostly capable of producing viable plants. However, severe internal damage, mostly caused by borers that resulted in multiple tunnels within seeds, was lethal. The adaptive strategy through multiple plant production following seed fragmentation enhances population sustainability and offers a valuable resource for planting material production in nurseries, reducing reliance on pristine seeds. Collectively, this research provides a comprehensive framework for efficient propagation of *S. malaccense,* advocating for a protocol that integrates strategic seed sourcing, utilization of predated seeds, controlled fragmentation and optimized dark sprouting.

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LIST OF SYMBOLS

Symbols

%	Percentage
°C	Degrees Celsius
cm	Centimeter
g	Gram
lux	Luminous Flux Per Unit Area
m	Meter
mg	Milligram
mm	Millimetre
N	Number of Samples or Counts
Nil	Nothing or Zero
nm	Nanometer

LIST OF ABBREVIATIONS

Abbreviations

ANOVA	Analysis of Variance
DNA	Deoxyribonucleic acid
ISSR	Inter-simple Sequence Repeats
IUCN	International Union for Conservation of Nature
LED	Light-Emitting Diode
LSD	Least Significant Difference
p-value	Probability value
RAPD	Random Amplified Polymorphic DNA
SSR	Simple Sequence Repeat

LIST OF NOMENCLATURES

Nomenclatures

A	Amplitude Ratio, (No Units)
C	Centroid of Pipe, Inches

CHAPTER 1
INTRODUCTION

1.1 Background of Study

Seeds are vital units for plant reproduction. A seed, regardless of size, is capable of undergoing metabolic activities and developing into an autotrophic plant. In nature, germination of seeds usually occurs when the seeds are dispersed away from the mother plants. Some seeds will germinate right away, meanwhile, some are delayed due to dormancy, which is affected by environmental factors such as light and temperature as well as the genetic factor (Bentsink & Koornneef, 2008).

Seed dispersal mechanisms represent the diverse biological and physical strategies, ranging from abiotic factors, like wind and water, to biotic vectors like animals. Vibrant color and fleshy texture of fruits are clear indicators of animal dispersal strategy. The fruits and seeds within the fruits are vulnerable to injuries mainly in their interactions with animals at pre- and post-dispersal, depending on the way they are handled by the animals (Ekramirad et al., 2016). Plant regeneration from injured or even damaged seeds is important, especially in the unattended natural environment. In many cases, the injured seeds remain regenerative; they tolerate some degree of injury in harnessing the services of their partnering animals.

Some members from Myrtaceae, the eighth largest family of flowering plants, are among those that have been found to show in vivo regeneration even from the fragmented seeds left in the natural environment (Thurlby et al., 2000, 2012; Delgado et al., 2010, 2022; Blando et al., 2013; Alonso et al., 2019; Tsan, 2023). Most plant species from this family are polyembryonic and could easily tolerate damages on some parts of their seeds. However, some species from this family are monoembryonic. Among these monoembryonic species, a limited number of them have been studied for plant regeneration from the cut seeds to corroborate the likelihood of polyembryony. Surprisingly, some could even produce full plants from fragments at the absence of the plumule-radicle axes (Au, 2013; Anto et al., 2018). Such regenerative capability is thought to be an important adaptation to survive predation by animals while maintaining ecosystem stability. For the species in this family that have been listed in the International Union for Conservation of Nature, plant development from seed fractions

could be of significant importance to preserve the germplasm of the species ex situ (IUCN, 2024). However, the knowledge on the embryology of this family upon fragmentation remains patchy.

Some *Eugenia* and *Syzygium* species are among the documented members in the family of Myrtaceae that regenerate with complete separation of a monoembryonic seed into fractions (Lughadha & Proenca, 1996; Thurlby et al., 2000, 2012). Within the last few decades, a few economically important species in these two genera in the neotropical regions and Africa have been studied for regeneration following seed cutting treatments (Delgado et al., 2010). These plant species have been acknowledged for providing edible fruits and medicines, while others are timbers that are useful for living. Given the similarities in seed characteristics and regeneration behavior observed in *Eugenia* species, it is hypothesized that monoembryonic *Syzygium* seeds, when fragmented, will also exhibit a similar regeneration mechanism. While the unique plant development potential from seed fragments has been suggested to support the natural population resilience of these species, the agronomists also hope to utilize these phenomena alongside stem cutting and grafting means for increasing the planting material production for horticultural and economic purposes (Amador & Barbedo, 2015).

In Malaysia, *Syzygium* spp. are important members of Myrtaceae. Some rare fruits of *Syzygium* show the potential for future commercialization, but many remain less studied in the forests. The indigenous *Syzygium malaccense* was chosen as the test species for this study in view of its current economic value among the rural people (Nair, 2017). This forest species has been found producing seedless fruits on some trees, while the factors contributing to this phenomenon are not known. In the case where seeds are formed within fruits, the seeds are monoembryonic, contrasting with some other *Syzygium* spp. that contain multiple embryos within the seed coat. Cut seed propagation technique is yet to be studied as a non-conventional multiplication means for this species. Regenerative seed fractions are also beneficial for the production of planting materials for selected landraces of this species for the commercialization of the fruits or product development from their other plant parts in the future. This species was also justified as the study candidate based on the fact that the fully ripe fruits that are shed from trees are frequently damaged by the hoarding animals.

1.2 Problem Statement

Numerous seedless fruits are observed and conventionally would present significant challenges for regeneration. In addition, seedless or seeded fruits are also very vulnerable to animal predation and insect borers. Thus, expanding the cultivation of this fruit species for commercial purposes can be limited by the availability of planting materials. Studies on the vegetative propagation through stem cutting and budding for the *Syzygium* genus, including *S. malaccense*, recorded low success rates (Lebrun et al., 1998; Ryadin et al., 2014; Tacloy, 2017; Swadhin et al., 2019). The vegetative propagation of *Syzygium* spp. is further affected by varietal sensitivity, as different cultivars show significant differences in their physiological responses to identical treatments. Additionally, the stem cutting propagation of *S. malaccense* needs extra attention as the cuttings are vulnerable to rotting due to fungal attacks. The difficulty of propagating this species vegetatively is also probably due to the presence of tanniferous cells, which are present in most species of the Myrtaceae family. The high concentration of tannins can inhibit cellular regeneration and interfere with the rooting process (Lebrun et al., 1998).

Fragmentation of seeds serves as a potential alternative propagation strategy. A recent study has discovered that species in this family can regenerate even after half of their reserves have been removed. Preliminary work carried out for the past few years found that *Syzygium myrtifolium* and *S. malaccense* could regenerate well following seed fractionation. The ability to develop full plants was even observed with the small *S. myrtifolium* seeds of only 4 to 6 mm, following cutting into four much smaller portions (Tsan, 2023). The studies on in vivo plant regeneration from the seed fractions were limited to growth success, while the mechanism of regeneration from cotyledonary surfaces is yet to be investigated.

Further work is needed to understand the development of functional plants from seed fragments despite the loss of a high amount of reserves in *S. malaccense* seeds. There is a significant lack of understanding regarding the minimal amount of reserves required to support the development of a full plant. Currently, it remains unknown whether the ability to regenerate is limited to larger seed fragments possessing a larger seed reserve or if the seed of this species has a physiological resilience that allows even minimal tissue portions to regenerate. Furthermore, the extent to which a seed can sustain the loss of its initial reserves without compromising the long-term vigor and

functional development of the resulting plant has not been studied.

Aside from the internal limitation, the regenerative capability from seed fragments might depend on the external environmental factors. Like other forest species, the regeneration of intact or predated *S. malaccense* from seeds typically happens under the low light conditions of a forest understory or the tree's own canopy. In a relatively large whole seed, the abundant stored nutrients allow the embryo to remain in a heterotrophic state for a period of time, making it less vulnerable to the surrounding light conditions. However, when a seed is fragmented, the stored nutrients are significantly reduced. This leads to the hypothesis that fragmented units may require an earlier or a specific light stimulus to initiate photoautotrophy to survive. Therefore, the optimal light condition for the in vivo regeneration of *S. malaccense* from seed fragments needs to be studied in order to come up with a suitable protocol applicable in horticulture.

While the meristematic and totipotent cotyledons within Myrtaceae can be associated with polyembryony (Nacata & de Andrade, 2020; Tsan, 2022), the growth performances of the regenerated plants also need investigation. In polyembryonic species within the Myrtaceae family, seeds can contain both a zygotic embryo and multiple nucellar embryos. Due to a lack of molecular evidence, it remains unknown whether fragmentation-induced regeneration yields a genetically uniform or a diverse mixture of offspring. In other documented species, plants that are zygotic exhibited superior characteristics, while nucellar plants showed the opposite. For *S. malaccense*, it is currently unknown if the multiplication from cut seeds will result in different growth rates of plants, depending on whether the plants are all nucellar type, all zygotic plants, or a heterozygote. Such information is important for the in vivo seed fragmentation technique that offers simplicity, contrasting with the in vitro plant tissue culture that depends on the nutrients and hormones incorporated into the culture medium to enable the regeneration of new cells.

While controlled seed fractionation could be effective for supplying more planting material, information on the regeneration and growth performances of the partially predated *S. malaccense* seeds collected from the fields should also be established. There is a lack of evidence to determine if partially predated seeds in nature can exhibit the same performance as systematically fragmented seeds. Understanding their performances is essential for developing a robust protocol that salvages predated or damaged seeds into a high-yielding resource to increase planting materials.

1.3 Research Objectives

Therefore, this research work was set to:

i. To evaluate the relationship between reserve reduction (size and fresh weight) and the sprouting potential of *S. malaccense* seed fractions obtained through systematic longitudinal and transversal sectioning.
ii. To determine the influence of light conditions on regeneration from quartered seeds.
iii. To assess the growth performance of plants regenerated from quartered seeds in expressing polyembryony.
iv. To investigate the plant regeneration potential from naturally insect-damaged or infested seeds.

1.4 Research Questions

The research questions in this study are:

i. Could the smaller seed fractions sprout and produce full plants following systematic longitudinal or/and transversal seed cutting into halves and quarters?
ii. Does light affect full plant development from quartered seeds?
iii. What is the growth performance of plants formed from seed fractions?
iv. Are insect pest-infested or damaged seeds capable to regenerate to ensure the population health of this plant species in the natural environment?

1.5 Hypotheses

H_0: There is no significant difference in sprouting and full plant development capability following systematic longitudinal or/and transversal seed cutting into halves and quarters.
H_1: There is a significant difference in sprouting and full plant development capability following systematic longitudinal or/and transversal seed cutting into halves and quarters.

H_0: There is no significant difference in the rate of full plant development from quartered seeds under different light conditions.
H_1: There is a significant difference in the rate of full plant development from quartered

seeds under different light conditions.

H_0: There is no significant difference in the growth performance of plants regenerated from quarters of a seed in expressing polyembryony.
H_1: There is a significant difference in the growth performance of plants regenerated from quarters of a seed in expressing polyembryony.

H_0: Seeds with insect infestations or damage from the field mostly do not develop into full plants.
H_1: Seeds with insect infestations or damage from the field mostly develop into full plants.

1.6 Scope of Work

This research work first studied the seed morphometrics and reserve effects expressed as the weight of fractions as the basic determinants for the sprouting potential of seed fractions. Then, the second experiment aimed to induce the development of more full plants from single seeds following fractionation into quarters. The experiment was designed based on a higher rate of full plant development from the quartered fractions in a preliminary trial carried out on the darker benches in the laboratory. It was hypothesized that the photosynthesis of the green colour cotyledons was hampered under dim conditions, posturing the need to develop shoots to source light as a survival strategy. In the subsequent work, the plants obtained from fractions of a single seed were measured for their growth performance. Lastly, to validate the findings obtained with seed incisions, seeds having varying severities of damage due to insect pest predation were collected from the natural sites and examined. Regeneration and growth from the seeds with defects were analysed against the polarity of the seeds, as concluded from preceding experiments.

1.7 Conceptual Framework

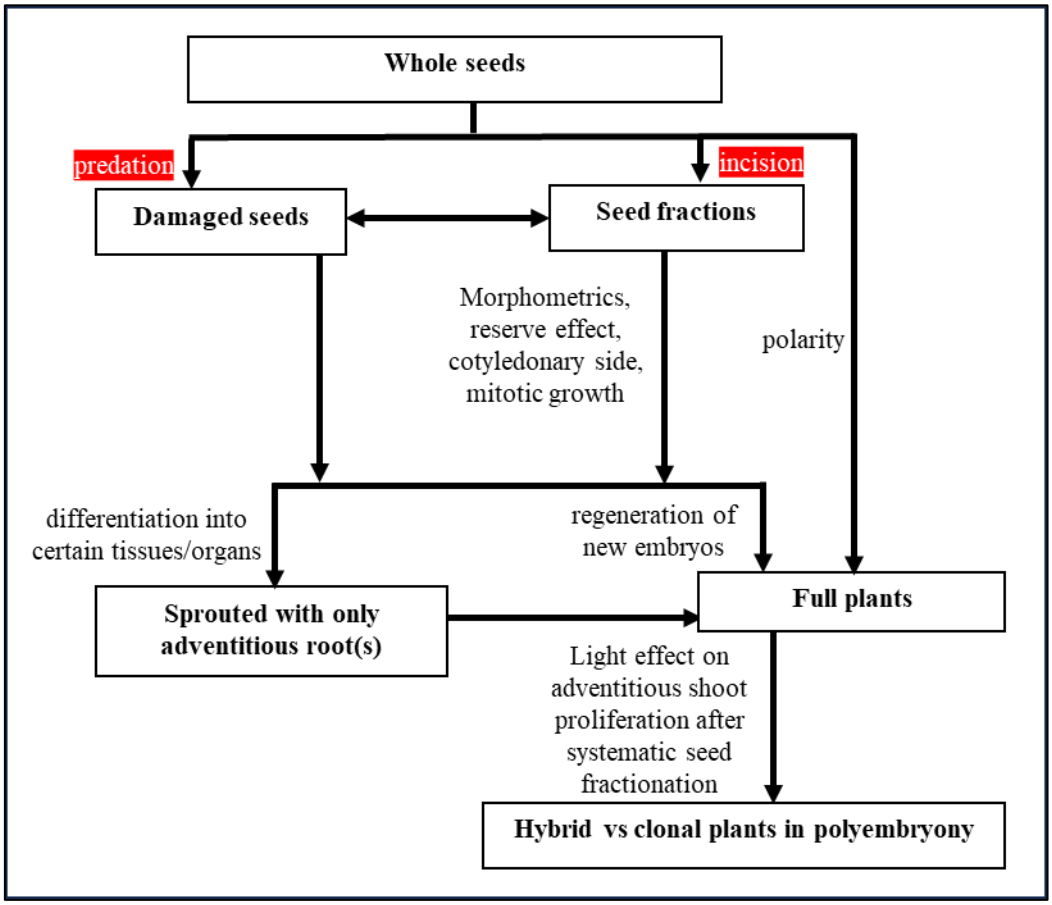


Figure 1.1 Conceptual Framework of the Research

1.8 Significance of Study

From this study, the ecologists have access to useful information about the regenerative ability of *S. malaccense* despite fragmentation or insect pest problems. The development of adventitious shoots from the seed fractions becomes a concern for both the natural stand sustenance and horticultural benefits. The growth performance differences among seed fractions illustrate the potential clonal properties in plant regeneration with the seed cutting approach. For the conservation and breeding program of *S. malaccense*, both the clonal and biparental genomes of this fruit species can be preserved with single seeds for future use. The information from this study may also yield new insights or lead to other research areas, especially those related to the signaling cascades that trigger the hormonal changes in the cotyledons, leading to the unique in vivo multiple plant development from a seed, as well as resprouting potential with further injuries. The information gathered from this work is also hoped to be useful

in the much more complicated biochemical studies later that investigate the cross-talk of hormones in the pluripotency and totipotency in the cotyledons.

1.9 Limitation of Study

Like other studies, this particular research work had its own limitations, especially in test material availability. The experiments were highly dependent on the availability of fruits with seeds of *S. malaccense* in conditions needed for fulfilling the objectives mentioned above. Both the seeds that were free from damage, as well as predated seeds in sufficient quantity, delayed the planned experiments. Aside from that, some trees produced entirely seedless fruits and were devoid of test materials for the research work. Other problems included inconsistent flowering and fruiting times of the identified trees. Actual site visits were needed to know fruit availability, while the owners of the trees generally did not follow the flowering of their trees well. With some budget constraints in this research work, site visits and fruit collections were limited within the states of Selangor, Negeri Sembilan, Malacca, and Johor, Malaysia.

CHAPTER 2
LITERATURE REVIEW

2.1 Myrtaceae

The Myrtaceae species is widely distributed across Southeast Asia, Australia, the Pacific Island and even in the Neotropics. To date, the Myrtaceae family has 126 accepted genera, consisting of 6,000 species. These plants are valued for their ornamental qualities, uses in traditional medicine for their health benefits, and for producing aromatic oils from their leaves and stems (Wilson, 2010; Nunes et al., 2016; Pazzini et al., 2021; Vadu et al., 2023)

A defining characteristic of the Myrtaceae family is its remarkable regenerative ability. For example, some species in the *Eucalyptus* group are known to resprout after fires. They have developed adaptive reproductive strategies to ensure their survival. Many species possess large cotyledons and exhibit polyploidy, which allows them to thrive in adverse environmental conditions. Apomixis, a form of asexual reproduction where embryos develop without fertilization, has also been observed in several species of this family (Wilson, 2010). This trait, along with polyembryony (the formation of multiple embryos in a single seed), is an important strategy to compensate for the loss of fitness due to reduced seed mass. These adaptations are particularly crucial given that the seeds of many members of the Myrtaceae family are dispersed by animals (Krishnamurthy et al., 1997; Wilson, 2010)

In the wild, fleshy fruits of members from the Myrtaceae family are eaten by various animals, including bats, birds, foxes, rodents, monkeys, and squirrels. The frugivores will swallow the whole fruit or partially feed on the fruits (Sinue 2012). However, this plant-animal interaction is beneficial because it helps in the seed dispersal. When dispersers like birds and monkeys swallow the fruits, they defecate or regurgitate the seeds at a new site, successfully dispersing the seeds. However, it is also noteworthy that some of the seeds may also serve as food for the insects. Seed predation by small insects affects the sprouting potential of the seed.

2.2 *Syzygium malaccense*

S. malaccense is an underutilized species in the Myrtaceae family. It is commonly known as Malay apple. The fruit of the tree is also known as French cashew, mountain apple, nakavita and pomerac. The locals in Malaysia call it *jambu bol*, *jambu Melaka*, *jambu jamaika*, and in some parts of Malaysia, it is known as *jambu keling*.

S. malaccense is a woody tree reaching up to 20 m. The flowering of this woody species is erratic, usually for one to two months when in season. The tree bears large fruits which are usually bell-shaped, rather watery, and have a sweet taste. The fruit is normally glossy red, while fruits of white colour are less common. The edible stage of *S. malaccense* fruit is when it is more than 80% in ripeness. The fruits have high nutritional value as a source of fiber, minerals, vitamins, and reducing sugars (Nunes et al., 2016; Batista et al., 2017; Tao et al., 2020). Ripe fruits are typically consumed raw or as part of a mixed fruit salad. In addition to being eaten fresh, some entrepreneurs are adding value to fruit production by turning it into functional food and low-cost wine in the Caribbean (Hosein et al., 2015). In Southeast Asia, the fruits are also pickled and preserved as snacks, candies, sauces, and even canned fruit (Whistler & Elevitch, 2006).

S. malaccense fruit usually contains one or two large seeds, though some trees produce seedless fruits (Yusnita et al., 2017; Nikhil & Upadhya, 2024). The seeds are subglobose to hemispherical in shape and are recalcitrant, which means they lack the protective mechanisms to survive dehydration. They do not undergo a dormancy phase and remain metabolically active after being shed from the tree. Therefore, the seeds must germinate rapidly, and a drop in their moisture content below a critical threshold leads to irreversible cellular damage and death (Asomaning et al., 2011; Nair, 2017).

The plant is thought to originate from either the Indo-Malayan region or Southeast Asia. The tree is generally found and can be planted in humid tropics with rainfall of approximately 1,500 mm or more per year. The plant is now commonly cultivated in many parts of the warm, humid regions. It needs to be irrigated when planted in areas with dry seasons.

S. malaccense has a promising marketing value. The fruit has medicinal values (Lim, 2012; Batista et al., 2017; Quenon et al., 2022). It has also been reported to contain a high content of phenolic compounds and antioxidant ability (Nunes et al., 2016; Savi et al., 2020; Pazzini et al., 2021). To date, plants are still used in traditional medicine in some parts of the world to treat infectious diseases. The antioxidants in the fruits were

also reported to potentially be used as natural antioxidants in processed food, increasing their economic exploitation (Lim & Rabeta, 2013). The value of *S. malaccense* is not only limited to the consumability of its fruit; other parts of the tree, like the bark, leaves, roots, and seeds, were reported to have anti-inflammatory properties (Oliveira et al., 2011; Mitra et al., 2012; Arumugam et al., 2014). The presence of anti-bacterial and anti-inflammatory compounds in the extract of *S. malaccense* plant justifies the medicinal uses of the plant species against infectious diseases (Quenon et al., 2022; Vadu et al., 2023). Besides, the trunk of the tree is also used as timber, especially for making boats, rafts, and canoes.

Despite the potential of *S. malaccense* as a significant income for the rural people, it has not been grown on a large scale due to its erratic fruiting behaviour. The fruits typically shed from the tree within a day or two once ripen after approximately two months from the fruit set. At postharvest, they are also highly perishable with a shelf life of only four to six days at a temperature of 28°C. Due to its fragility and short shelf life, the fruits do not fulfil market standards for export and, therefore, are sold only in local markets (Hosein et al., 2015).

In the matter of plant preservation, *S. malaccense* is one of the many species in the Myrtaceae family that needs to be protected (Ryadin et al., 2014). Earlier reports also indicated that the species was threatened for existence due to anthropogenic activities. Seeds serve as an integral component in the preservation of this flowering plant. The seed within the fruit is the main mechanism of reproduction in the natural environment. Thus, the germinability of seeds is important for the population stability of this species and ecosystem resilience. Efforts for ex situ conservation of this species could also be beneficial for future human and germplasm uses. However, conservation efforts of many of the world's threatened plant species are hindered by the lack of understanding of fundamental biological information and their seed biology (Van der Walt et al., 2020)

2.3 Seed Germination

The germination of seeds, as an important seed biology, is a complex physiological process. The process is important for population growth, especially in unattended natural ecosystems. According to Bewley & Black (1978), germination begins with imbibition or the uptake of water by the dry seed, and is completed when

the radicle extends from the embryo and penetrates the medium that surrounds it. The germinating seeds generally have increased respiration rates. Simultaneously, protein synthesis and other metabolic processes will trigger the growth and development of cells during germination to enable the emergence of the radicles and plumules, culminating in the development of full plants.

Numerous studies have been conducted to gain a better understanding of how germination occurs in nature. Germination is regulated by the interaction of the physiological stage of the seed and environmental conditions, in which each plant species needs a set of specific requirements for this process, such as the availability of water, light, and temperature. While these environmental conditions influence the success of seed germination, the seeds themselves have the potential to optimize their own timing for germination with respect to environmental conditions to reduce competition while gaining benefits from the surrounding resources (Zhang et al., 2014). The timing of germination can have a significant impact on the subsequent performance and successful establishment of the plants into adulthood as the tender seedlings are vulnerable to moisture deficit conditions.

2.3.1 Water Requirements

Water availability is an important environmental factor influencing seed germination, yet species within the same family can exhibit varying degrees of tolerance to water stress (Baskin & Baskin, 2025). Recalcitrant or desiccation-sensitive seeds must have access to water within short periods after they are shed from mother plants. These seeds typically lack a dormancy phase and germinate quickly to send their roots into the soil to find water to escape desiccation-induced death. These seeds die when faced with water-lacking conditions, especially in the natural environment (Schütz et al., 2002). On the other hand, orthodox seeds tolerate dryness well. They turn dormant and form a soil seed bank. They show staggered germination with periodic rain. Dormancy in orthodox seeds ensures their survival in the field.

Syzygium seeds are notably desiccation-sensitive. They fail to survive when moisture levels decline (Asomaning et al., 2011; Nair, 2017). For *S. malaccense*, germination generally occurs beneath the canopy, where the moist and shaded microenvironment provides optimal conditions for sprouting. Similar results were obtained in several *Eugenia* species, revealing that germination success declined

significantly under increasing water stress levels (Inocente & Barbedo, 2019).

Certain Myrtaceae species thrive in habitats that remain submerged throughout the year. For instance, seeds of *Eugenia stipitata* can survive under submerged conditions for up to 12 months, irrespective of the water depth. The moisture content of the seeds of *E. stipitata* remained stable and even capable of resprouting after 12 months (Calvi et al., 2017). The seeds of *Leptospermum lanigerum* and *Melaleuca squarrosa* have also demonstrated the ability to germinate under complete submersion. However, prolonged flooding significantly affects the seedling vigor and long-term survival (Zacks et al., 2018).

2.3.2 Temperature Requirements

Another requirement for seed germination is a suitable temperature. This is to allow metabolic processes to proceed before radicle protrusion from the seed structures can occur (Bewley & Black, 1978). While temperature is typically a primary driver of seed metabolism, species-specific responses can vary (Nacata & Andrade, 2020).

In forest ecosystems, temperature is intrinsically linked to light penetration, while light conditions change with the movement of the sun and the forest structure (Foster, 1986; Martínez et al., 2021). The temporary heterogeneity usually favors seed germination. Tropical recalcitrant seeds like *Syzygium* spp. require a warm and moist environment on the forest floor for rapid germination. However, the subsequent growth of the seedlings varies depending on the species. Members within Myrtaceae family have diverse germination and growth rates. Seedlings of pioneer species demand more light after germination success and may die with sprouting under heavy shade. On the other hand, climax species enjoy the cooler conditions under shade in their early life, waiting for canopy opening and gaining faster growth in the later stage.

2.3.3 Light Requirements

The light condition also plays an important role in various photomorphogenic processes in plants, including stages of germination, growth of the seedling, and the formation of plant architecture (Suzuki et al., 2010; Pocock, 2015; Lee et al., 2016; Landi et al., 2020; Repeat, 2020). In plants, light serves two purposes: supplying energy and providing signals for stress response (Goh et al., 2012). Meanwhile, in seeds, light

functions only to provide necessary signals because energy is supplied within the seed reserves (Sugahara et al., 2004; Farooq et al., 2022).

Light from the environment activates the phytochrome photoreceptor in plants, which then translates these signals into a biological response (Chen et al., 2004; Pons, 2000). These photoreceptors, referred to as phytochromes and cryptochromes, are found in the cotyledons of seeds, and they detect the light that reaches them, regulating germination and their subsequent photomorphogenesis (Chen et al., 2004; Lee et al., 2016; Jeong & Sivanesan, 2018).

Both light quality and intensity affect plant physiological processes. Light quality refers to specific wavelengths of light present in a light source. It is often described in terms of color, temperature, and spectrum, for instance, red and blue light (Jeong & Sivanesan, 2018). Light quality helps regulate hormones like gibberellic acid and abscisic acid (ABA) that are required for seed germination (Wei et al., 2023). Blue light is reported to inhibit germination, while red light induces the process. The complex interplay between light quality and hormone signaling is important for seed germination, and there is still much to be understood, as light requirements for each species differ.

Light intensity refers to the amount of light that reaches the surface. It is commonly measured in lux. Different plant species react to light availability differently. Therefore, understanding how different light affects seed germination and seedling performance is important for population establishment success in natural ecosystems and also for horticulture. In modern horticulture, the use of light-emitting diodes (LED) in nurseries offers a precise and energy-efficient method of tailoring light spectra and intensity to optimize seed germination and plant growth (Massa et al., 2010; Singh et al., 2015; Berkovich et al., 2017; Ryong & Sivanesan, 2018; Lazzarin et al., 2020; Zhang et al., 2020; Paradiso & Proietti, 2022; Rahman et al., 2024).

Meanwhile, some plants, particularly those from forest or shaded environments, rely on low light conditions for successful germination (Suzuki et al., 2010; Blando et al., 2013; Wei et al., 2023). Such low-light conditions are appropriate for the germination of many seeds from the Myrtaceae family. Generally, large-seeded species thrive better in low light conditions because larger seeds contain more energy reserves to be exhausted over a longer period (Foster, 1986; Myers & Kitajima, 2007; Nameth et al., 2012). In the case of dark germination, the environment that is insufficient for photosynthesis could have triggered a biological process in the seed to induce

germination. In tissue culture, it is generally believed that adventitious root and shoot formation favors low light conditions. The advantages of inducing seed germination in darkness in horticulture can potentially reduce the reliance on energy-intensive lighting systems, leading to more sustainable and cost-effective planting material production.

However, subsequent plant growth in darkness often exhibits shade avoidance syndrome, which results in elongated hypocotyls and internodes, etiolation, hyponastic leaves and reduced branching (Franklin & Whitelan, 2005; Repeat, 2020; Wang et al., 2020; Wei et al., 2023). The terms photomorphogenesis (light-grown) and skotomorphogenesis (dark-grown) are used to explain the plant's responses to the variations of light conditions in the environment (Cocco et al., 2022; Mawphlang & Kharshiing, 2017; Wei et al., 2023). During skotomorphogenesis, seedlings grown in the dark focus on a single goal: reaching light. This is achieved through rapid elongation of the hypocotyl. Once the seedling perceives light, de-etiolation begins, and it changes into a normal photomorphogenic form.

2.4 Seed Predation Effect

The successful establishment of plants in their early growth phase in nature is determined by more than just the adaptations to surrounding abiotic factors. In the event of seed germination and establishment of seedlings, many fruits with seeds in them depend on animals to bring them away from mother trees for better survival rates (Janzen, 1971). While the fruits and seeds could possibly be damaged by the invertebrates on the standing trees, dispersing animals that treat the fruits as sources of nutrients and water could also cause damage to the seeds. Some predators consume the entire fruits with seeds, resulting in total seed loss and reduced plant reproductive efficiency. On the other hand, partial seed consumption has been documented in many plant species, particularly those with large seeds (Loayza et al., 2015). While whole seed germination is common, damaged seeds may not necessarily be fatal in nature (Vallejo-Marín et al., 2006). Partially predated seeds could also be germinable when the damages are within tolerable levels (Perea et al., 2018; García-Hernandez et al., 2023). The degree of damage caused to the seed can vary depending on the predator. For example, some predators may only injure the outer layer of the seed, while others may bore into and feed on the seed. Some insects can introduce pathogens and kill the seeds

(Kremer & Spencer, 1989). The level and type of seed predation can have a significant impact on the regeneration process of plant species (Vallejo-Marín et al., 2006).

2.4.1 Protection by Seed Coat

Plants can evolve and develop adaptive strategies to counter predation. These adaptive strategies may include traits such as thick seed coats and hard shells. The external part of a seed that consists of the seed coat and hilum is generally liable to protect the embryo, which is made up of the cotyledon, plumule, and radicle. Different plant species have their own unique seed structures, which have also frequently been referred to in the botanical classification of plant species (Lughadha & Proenca, 1996).

Some seeds have woody and hard seed in order to protect them from predators. As the external barrier against injuries, a thick seed coat reduces possible seed damage but it can influence seed germination significantly (Noor et al., 2016). In other words, the thickness of the seed coat influences seed dormancy. Dormancy or failure in germination is caused by the inability of the seed to absorb water or seed coat hardness and impermeability (Joshi et al., 2006).

In the seed-predator relationships, the injuries caused by the predators open up the seed coat for facilitated water uptake. Exposed tissues also have increased surface area for water absorption, resulting in increased seed growth and vigour. In fact, partial seed predation is a reproductive strategy for these seeds, which are covered by a hard seed coat as the seeds gain an enhanced germination rate and speed after their physical dormancy has been overcome with scarification (Vallejo-Marín et al., 2006; Anto et al., 2018).

2.4.2 Protection of Regenerative Axis

In contrast to seed coat protection, recalcitrant seeds are vulnerable to damage by animals because they have thin seed coats to absorb moisture quickly to avoid desiccation-induced death (Asomaning et al., 2011). Sprouting of damaged seeds is probably a survival strategy in nature.

In general, seeds with a thin seed coat possess some unique physiological properties to fulfil their role in the plant's life. In the wild, partial seed predation has been found beneficial to species that do not have dispersers other than the foraging

animals (Anto et al., 2018). In other words, the seeds endure some degree of damage due to handling by the animals.

For the seeds having a thin seed coat, they could be equipped with unique structures to ensure the protection of the vital parts and sprouting success. The embryos within their seed coat play essential roles in tolerating physical damage. Studies on oak species regeneration have revealed that animals typically cause damage only to the basal portion of the seeds. Predators tend to attack non-vital parts of the seeds, which results in no damage to the embryo (Perea et al., 2011; García-Hernandez et al., 2023). Interestingly, the studies have shown that partially predated seeds exhibit faster regeneration and emergence, possibly to reduce the risk of desiccation and fungal infestation (Perea et al., 2011).

From the biochemical point of view, Branco et al. (2002) reported that some seeds protect their embryos well with some compounds to survive animal predation. For example, acorns have higher concentrations of tannins in the apical ends that contain their plumule-radicle axes, making this region unpalatable. The tannins serve as a natural defense mechanism against predation. Therefore, many acorns could still regenerate and produce viable plants even after they have been heavily predated by insects (Hou et al., 2010; Fukumoto & Kajimura, 2014).

In *Syzygium* seed germination, the fleshy outer layer of the fruit needs to be broken down either by natural weathering or through the ingestion of animals. Fruits of *S. malaccense*, in particular, are at risk of being preyed upon by fruit flies, monkeys, birds, and bats. Partial seed predation by bats and birds is generally mutualistic, while seed regeneration impairment is not usually noted (Lughadha & Proenca, 1996). However, fruit flies lay their eggs on fruits, and their larvae bore into the flesh and seeds, causing damage to the cotyledons and seed germination failure.

In the natural environment, the abundance of both the fruits or seeds and predators can be affected by climatic factors like the onset of rain as well as the availability of alternate food sources (Loayza et al., 2015; Harrington et al., 2020). The relationships between the seeds and the animals are complex, being essential to achieve ecological stabilization and ecosystem health.

2.4.3 Reserve Effect Hypothesis

Despite differences among the seeds in tolerating the loss of their biomass, the

fate of damaged seeds is dependent on the remaining reserves in general. As indicated by Bonfil (1998), the amount of reserves initially available to a seedling may influence its chances of survival and continued growth. This is because damaged seeds with a larger portion of reserves still have sufficient energy for tissue replacement and even germination (Foster, 1986). Thus, large seeds are generally more advantageous in facing greater biomass loss to animals through the reserve effect hypothesis. For example, Wyatt & Broyles (1997) reported that large seeds of *Gustavia superba* could produce normal plants despite losing more than 50% of seed mass. Seeds of *Garcinia imberti* could also retain a high probability of regeneration with more than half of the seed content removed. Similar regenerative capabilities have been observed in the oak species, where the large cotyledon reserves of acorns contribute to successful regeneration and help overcome the effects of seed predation (Hou et al., 2010). On the other hand, large seeds of *Calatola laevigata* (Icacinaceae) and *Protium copal* (Burseraceae) died with the loss of small parts of their seeds (Vallejo-Marín et al., 2006). These seeds become vulnerable to the pathogenic fungi even with small injuries, leading to the death of the seeds.

Some studies have analyzed the relationship between the size or weight of the seed fragments and their sprouting potential. The researchers used at least the simplest scoring that ranges from 0% to 100% for the severity of seed damage in the germinability studies by Fukumoto and Kajimura (2014). In validating the survival and growth of damaged seeds, seed cutting treatments followed by in vivo sprouting of the cut seeds have also been initiated under controlled conditions in laboratories and greenhouses (Delgado et al., 2010; Amador & Barbedo, 2015; Anto et al., 2018). These studies with the cut seeds provided not only findings with regard to the sprouting potential following loss of reserves, but also the polarity in entire plant regeneration from the seed fractions was another important aspect revealed for further research work.

After sprouting, damage due to predation can also occur during the tender young plant stage. Hence, it is important for the young plants to also be able to resprout to tolerate any herbivory and physical damage (Harms & Dalling, 1997). The potential of subsequent shoot emergence, through resprouting, after the first shoot has been lost to grazing animals has also frequently been associated with the reserves or size of remaining cotyledons. Young plants having sufficient energy with their larger cotyledonary fractions usually have a higher chance of survival as compared to those left with smaller amounts of reserves or with reserves totally lost to the herbivores. The

effect of seed size on the size of the seedling is called the seed-seedling hypothesis, and this hypothesis states that large seeds will give rise to large seedlings (Green & Juniper, 2004a; Souza & Fagundes, 2014)

2.5 Regenerative Cotyledons

While sprouting with plumule-radicle axes is well understood, in vivo cotyledonary regeneration potential with some species reported in the past is an exclusive adaptation. Wyatt and Broyles (1997) found the ability of seeds to regenerate from cotyledonary fragments in the forest understory, as there is a higher probability of being predated by herbivores on the forest ground. For example, cotyledonary fragments of *Gustavia superba* sprouted in the forest soil. Detailed studies confirmed that a high percentage of the cotyledonary parts of its large seeds were able to regenerate into new plants. A damaged and exposed portion of the seed helped in metabolic activities for new cell regeneration, leading to sprouting devoid of plumule-radicle axis (Anto et al., 2018).

2.5.1 Embryology Related Factor

Within Myrtaceae, there are broadly three different types of embryos, namely eugenoid, myrcioid, and myrtoid (van Wyk & Botha, 1984). Embryos with large, fleshy cotyledons and a small hypocotyl are known as eugenoid embryos. Myrcioid embryos are those with leafy cotyledons that are larger than the hypocotyl. Last but not least, the myrtoid, also known as pimentoid, is one with a well-developed hypocotyl and relatively small, narrow cotyledons. These types of embryos have been associated with the ability to develop new roots and even entire plants, although some of their biomass has been removed (Silva et al., 2005; Amador & Barbedo, 2015). With regards to the capabilities to form functional plants from sections of seeds, a limited number of *Eugenia* spp. and *Syzygium* spp. have been studied, but many other genera, as well as members from other families, are yet to be investigated (Delgado et al., 2010; Biffin et al., 2014).

2.5.2 Injury-induced Embryonic Cell Development

In the matter of more than one plant obtained from the seed fragments, Amador & Barbedo (2015) emphasized that the development of new tissues was associated with the injuries of the seeds. They suggested that the exposed surfaces in the seed fragments triggered some physiological processes that could induce the formation of new roots and full plant development, while this could not take place when the cotyledons remained intact and attached to the differentiated plumule-radicle axis. It is also likely that after germination has begun, the seedling produces chemicals that prevent the differentiation of new embryonic tissues by spreading such compounds from the germinating region to the rest of the seed. As a result, any new roots or plants could only be developed after the seed portion is entirely separated from the growing plant or at least adequately spaced from the growth area of the developing plant.

Within the Myrtaceae family, some *Eugenia* species demonstrated sprouting when seeds were cut into smaller fragments, attributed to the presence of meristematic tissues throughout the seeds (Teixeira & Barbedo, 2012; Calvi et al., 2017). For instance, the seeds of *Eugenia stipitata*, which regenerated embryos after fractionation, had full plants growing from the seed regions opposite to the parts containing the existing embryonic axes, as the cut seeds were induced to be mitotic with injuries (Anjos & Ferraz, 1999, as cited in Amador & Barbedo, 2015).

Further studies on *Eugenia* species, such as *Eugenia cerasiflora*, *Eugenia umbelliflora*, and *Eugenia pruinosa*, have confirmed the capacity for seed fraction regeneration (Delgado et al., 2010). Researchers found that regeneration was possible for cut seeds containing embryonic parts, with additional sprouting occurring in different seed segments after fragmentation. This suggests that seed separation triggers a stimulation for differentiation into new embryos. Nevertheless, the results showed that the second sprouting only happened when the seed was fragmented, while intact seeds produced only one seedling each. The seed separation triggered some kind of stimulation for the differentiation into a new embryo. These findings align with earlier descriptions of eugenoid seed behavior, where seeds of some *Eugenia* species lacked or had only tiny differentiated embryonic axes, indicating the potential for any seed part to regenerate into normal plants (van Wyk & Botha, 1984)

In the genus *Syzygium*, limited research has been conducted on cut seed regeneration. Past studies on a few species have demonstrated similar in vivo sprouting

success, with some seed fractions developing into full plants while others only rooted without shoot development (Sivasubramaniam & Selvarani, 2012; Tsan, 2023). The findings suggested the existence of meristematic tissues in the cotyledons of these *Syzygium* species.

The aforementioned ability of seeds to survive partial loss of reserves or even embryonic axes is primarily attributed to the meristematic or mitotic cells in the cotyledons. While a single plant cell is theoretically totipotent, this is not always the case in nature. As the propagation unit of many flowering plant species, a seed contains embryos, mainly the radicle or root-to-be and plumule or shoot-to-be. The embryo is the most important structure of the seed. Being linear or rudimentary, it consists of strands of regenerative tissues which will develop into an entire plant (Agyili et al., 2007). A seed also contains reserves stored in endosperm or cotyledons and the outer protective seed coat. Besides the differentiated embryos, it is, however, not impossible to find regenerative tissues in other parts of the seed, for example, the cotyledons (Lughadha & Proenca, 1996; Prataviera et al., 2015).

Seed maturity also influences the regeneration of seeds. Teixeira & Barbedo (2012) reported that mature seeds have a greater potential to regenerate than immature ones. This is because the meristematic tissues in the mature seeds are capable of further differentiation, including the formation of new embryos. The maturity of seeds also influences the water content in the seed. However, the maximum regeneration capacity of the seeds and the limitations on their maturation and germination have not been fully explained.

2.5.3 Mitotic Cell within Vascular Tissue

The unique full plant development ability was also reported with the cut seeds of *Garcinia* species. This ability is shared by many members of the *Garcinia* genus such as *Garcinia gummi-gutta*, *Garcinia kola*, *Garcinia mangostana*, *Garcinia atrovidis*, *Garcinia hombroniana,* and *Garcinia prainiana* (Joshi et al., 2006; Asomaning et al., 2011; Noor et al., 2016). The existence of the undifferentiated tissues in the cotyledons of *Garcinia* was reported by Joshi et al. (2006). The remaining seed could also produce roots and shoots, provided that the cut seed contained vascular tissues. By the internal structures, the seed cut transversely contained a thread of tissues consisting of mitotic cambium in the vascular bundles along its entire length, providing a plausible

explanation for the ability of the cut seed to form a full plant. Noor et al. (2016) found that seeds of *Garcinia mangostana, G. atrovidis, G. hombroniana* and *G. prainiana* did not have differentiated embryos. However, each shoot protruding from each seed segment has its own procambium, which can differentiate into an individual plumule and radicle. This has been proposed to be an evolutionary strategy of the *Garcinia* species to exploit mammalian frugivory in seed dispersal (Joshi et al., 2006).

The mechanisms responsible for generating any plant organ from plant cells and tissues are complex and involve complex gene hierarchies, which are coordinated spatially and temporally to regulate cell divisions, gene expression, and hormonal functions (Chandler, 2008). The induction of in vivo regeneration from somatic cells in plants is uncommon, and such new cell formation capability, followed by differentiation into adventitious shoots and roots, remains less studied. However, injuries to seeds may trigger biochemical processes similar to those involved in stem cutting propagation, leading to the development of adventitious roots and shoots from exposed cotyledonary surfaces (Amador & Barbedo, 2015). While auxins are essential for regeneration, the concentration of cytokinins also plays a significant role in determining cell proliferation and differentiation in seeds with physical defects.

2.6 Genetic of Plants Regenerated from Seed Fractions

While sexual reproduction through seed is common, asexual propagation through tissues other than plumule-radicle axes, on the other hand, is known with tissue culture methods, also known as micropropagation or in vitro propagation. In vitro propagation is carried out in a sterile and controlled environment in an artificial nutrient medium, mostly enriched with hormones (Blando et al., 2013; Rose et al., 2013; Muthukumar et al., 2020; Thormann et al., 2020). Under such a growth medium, newly dividing cells eventually become totipotent, of which some will re-differentiate and commit to a developmental pathway that results in somatic embryogenesis (Mahrous et al., 2023). In vitro regeneration is particularly beneficial for asexually propagating genotypes without inducing genetic variability (Blando et al., 2013; Rohela et al., 2013; Kamle et al., 2014). This propagation technique is suitable for plant breeders who wish to clone plants with desired traits. However, the downside of this technique is that it is a rather expensive technology and it requires skilled personnel.

While previous studies have shown that members of the *Syzygium* genus could

successfully be propagated in vitro (Cid et al., 1999; Blando et al., 2013; Kamboj et al., 2023; Mahrous et al., 2023), in vivo clonal plant regeneration from fractionated seeds is yet to be investigated as a more accessible and cost-effective technique. This intriguing technique involves inducing embryo development from cotyledon tissue, but not all plant species have the potential for propagation through seed cutting. The relationship between plant regeneration from seed fragments and the plant's unique embryological characteristics, such as apomixis, polyploidy, and polyembryony, also remains underexplored.

2.6.1 Apomixis

Apomixis is an asexual mode of reproduction through seed. It comprises gametophytic (apospory and diplospory) and sporophytic apomixis. Gametophytic apomixis refers to asexual seed production where the embryo develops from an unfertilized diploid (2n) egg cell in an embryo sac. It requires the formation of a female gametophyte that does not undergo meiosis. This ensures that the egg cell retains a full complement of maternal chromosomes, allowing for the production of clonal offspring that are genetically identical to the parent plant. On the other hand, sporophytic apomixis refers to adventitious embryos that arise somatically from maternal tissues, specifically the nucellus or integument, through mitotic division.

Some members of Myrtaceae family can be apomictic and exhibit the occurrence of adventitious embryony (van Wyk, 1978; Wilson, 2010; Souza-Pérez & Speroni, 2017). Apomixis has been reported for several *Syzygium* species like *Syzygium cumini, S. paniculatum* and *S. jambos*, and some species from the *Psidium* and *Eugenia* genera (Lughadha & Proenca, 1996; Thurlby et al., 2012; Da Luz-graña et al., 2025). The asexual embryos were reported to be derived from the ovules in adventitious embryony.

However, apomixis may reduce genetic diversity within each group while increasing genetic divergence between populations (Thurlby et al., 2000; Hojsgaard & Hörandl, 2019). This is because apomixis results in offspring that are genetically identical to the parent. There is little to no genetic variation among the individuals within a population. This means that all the plants in a population will have very similar genetic makeups. If different populations of the same species are isolated from each other, they will each develop their genetic variants through mutations and other

processes. Since apomixis limits genetic variation within each population, these populations can become more distinct from one another over time, leading to increased genetic differences between populations.

Despite these genetic implications, apomixis is highly valued in agriculture due to its potential to produce high-quality, genetically uniform crops at a lower cost (Barcaccia & Albertini, 2013). This reproductive strategy allows seeds to reproduce asexually, ensuring the reliable propagation of desirable traits from the mother plant. As a result, the study of apomixis in the regenerative tissues of *Syzygium* species remains a promising area of research, particularly since the apomictic potential of seed fractions in *Syzygium* species has yet to be fully investigated.

2.6.2 Polyploidy

In addition to apomixis, polyploidy has been linked to asexual reproduction capabilities in seeds. Polyploidy is characterized by the presence of more than two complete sets of chromosomes. Several authors found a strong association between polyploidy and apomixis (Thurlby et al., 2000; Da Luz-graña et al., 2025). Almost all apomictic species are polyploids. Research by Thurlby et al. (2000) found a strong association between polyploidy and apomixis in *Syzygium paniculatum*. By analyzing genotypic traces from embryo tissues of varying ploidy levels, they detected low genetic variation, suggesting the occurrence of apomixis in this species (Da Luz-graña et al., 2025).

Polyploid plants are said to thrive even in stressful environmental conditions, including overcoming seed predation effects (Pham, 2021; Li et al., 2024). This phenomenon is a strategy to compensate for the loss of fitness due to loss of seed mass. This adaptation is important, especially when the seed dispersal is by animals. The role of polyploidy in regeneration has also been highlighted, with studies showing that polyploid plants often possess superior regenerative capacities compared to their diploid counterparts, further helping their survival and propagation in challenging environments (Yali, 2022).

2.6.3 Polyembryony

Polyembryony is another phenomenon associated with apomixis.

Polyembryony is a process in which more than one embryo is formed per seed, and these embryos might either originate from the zygote or by adventitious embryogenesis, originating from the differentiation of the nucellus cells (Lakshmanan & Ambegaokar, 1984; Kishore, 2016). This phenomenon is common in tropical and subtropical trees and shrubs within the Myrtaceae family. Previous literature has also noted that *Syzygium* spp. may exhibit polyembryony, particularly in cases where multiple plants were found within a single seed (Malik et al., 2005; Mendes-Rodrigues et al., 2005; Sivasubramaniam & Selvarani, 2012; Thurlby et al., 2012; Woo et al., 2019; Nacata & de Andrade, 2020; Rekha et al., 2020). Both internal (e.g., plant genetics, physiological status) and external factors (e.g., environmental conditions, fruit positioning) can influence polyembryony (Rekha et al., 2020).

Adventitious embryony can also be naturally induced due to stress conditions such as drought or nutrient limitation. Additionally, the regeneration of adjoining cells, potentially triggered by biotic or abiotic stresses, may release hormones that stimulate cell division and proliferation in nucellar cells, leading to adventitious embryony (Maheshwari, 1952).

Given that apomixis is an asexual reproductive mechanism, polyembryony that is totally or partially based on apomixis results in clonal offspring with reduced genetic diversity, as the plant inherits the entire genome from the mother plant. In the genus *Citrus* and *Garcinia*, polyembryony has become a form of vegetative propagation, as the maternal (nucellar) embryos produced are genetic clones of the mother tree. However, this biological advantage poses a significant challenge for plant breeders as seedlings derived from embryos are morphologically indistinguishable from those originating from adventitious embryos. Through seed fragmentation, the induction of multiple sprouting in relation to apomixis and polyembryony remains an area requiring further study (Lakshmanan & Ambegaokar, 1984; Delgado et al., 2010).

In other cases, a single seed can sometimes contain both asexual (nucellar) embryos and a sexual (zygotic) embryo (Mendes-Rodrigues et al., 2012; Ochoa et al., 2012). The presence of these different embryos within one seed means that the resulting seedlings can have different genotypes. This coexistence of multiple embryos is considered a reproductive strategy that can compensate for low germination rates or a lack of successful fertilization (Shaanker & Ganeshaiah, 1997). By increasing the number of potential offspring from a single seed, a mother plant can enhance the overall probability that at least one of its seedlings will survive and grow successfully (Delgado

et al., 2010; Mendes-Rodrigues et al., 2005).

To understand which type of embryo is responsible for a particular seedling, researchers use various identification methods. One of these methods is morphological characterization. Morphological identification relies on observable physical traits, or phenotypes, such as a seedling's height, leaf size, and stem diameter (Kishore, 2016). While this approach can provide a quick assessment, its findings are often debated among scientists. Due to the lack of visual distinction, the genetic fidelity study has been used in past research to evaluate whether or not the offspring are genetically identical to the mother plant.

CHAPTER 3
SEED RESERVE AND SPROUTING BEHAVIOR OF SEED FRACTIONS

3.1 Introduction

Seed regeneration is important for the survival of many plant species. The ability of seeds to recover from biotic disturbances such as predation that cause physical damage to seeds is crucial for the population resilience of plants, especially in the unattended natural environment. Some plant species are susceptible to even small injuries to seeds, while others tolerate a substantial amount of mass loss due to animal consumption and sprout normally, in particular when the regenerative axes of plumules and radicles are unharmed. For the cases of seed fragment survival at post-predation by granivores, it is usually presumed that the remaining reserves and nutrients can affect their growth potential and fitness. According to the reserve effect hypothesis, the regenerative energy and young plant growth are positively related to the amount of reserves in a seed. A seed fraction having more reserves is more likely sprout faster in comparison to one having fewer reserves after animal gnawing. Past studies on regeneration from seed fragments also generally indicated that seed morphometrics, particularly seed size and reserve distribution, play an essential role in determining the success of young plant establishment (Foster, 1986; Silva et al., 2005). Larger seeds generally offer better chances for successful sprouting due to their higher nutrient content, which supports the early stages of development (Harms & Dalling, 1997).

Within the Myrtaceae family, members of several genera, especially *Eugenia* and *Syzygium* species, have been reported to regenerate well from seed fragments, demonstrating unique adaptability that could be important for both natural population maintenance and agricultural applications (Sivasubramaniam & Selvarani, 2012; Tsan, 2023). Understanding how seed size, reserves, and polarity of the seed affect regeneration potential can offer valuable insights for conservation efforts and practical propagation strategies. In general, the regenerative capacity of these seeds also depends on how much reserves are within the seed, but it also responds to the fractionation type in relation to polarity (Teixeira & Barbedo, 2012). Studies on *Eugenia*, *Syzygium,* and a few other species in the Myrtaceae family have demonstrated that even seeds with up

to 75% of their reserves removed could still regenerate, suggesting that seed fragments retained significant regenerative potential despite loss of mass (Blando et al., 2013; Delgado et al., 2022).

S. malaccense has been of particular interest in the in tropical regions due to its economic potential and ecological importance. The ability of *S. malaccense* to regenerate from seed fragments could not only offer a strategy for population resilience in natural ecosystems but also provide a non-conventional means of propagation for horticultural purposes, while its germination is significantly hindered by desiccation, as it is recalcitrant in nature (Amador & Barbedo, 2015). Previous studies have explored the sprouting potential of intact and fragmented seeds, less is known about the impact of seed cutting treatments or polarity on sprouting success and full plant development from fragmented seeds. This chapter pertained to Objective 1: To evaluate the relationship between reserve reduction (size and fresh weight) and the sprouting potential of *S. malaccense* seed fractions obtained through systematic longitudinal and transversal sectioning.

3.2 Materials and Methods

This section outlines the materials utilized in the study. It also explains the methods employed to conduct the research effectively.

3.2.1 Test Material

The fruits of *S. malaccense* were collected from a single tree in a durian orchard located in Tanjung Sepat, Selangor (2°38'56"N, 101°35'39"E) in the end of November 2021. It was the only fruiting tree at that point of time. The tree was grown in a flat terrain in the orchard, which is a coastal site. It was a more than 10 years old tree and has fruited many times, generally once or twice yearly, but not at exactly demonstrated seasonal fruiting patterns.

The fruits were overripe and soggy at the time of fruit collection. Therefore, the seeds were extracted from the fruits in the field before transportation to the laboratory. A total of 300 seeds were taken and placed loosely in plastic bags. The seeds were then brought back to Universiti Teknologi MARA Jasin Campus, Malacca, Malaysia (2°13'16"N, 102°27'8"E), in an air-conditioned vehicle.

On the following day, the seeds were cleaned of fruit flesh residues manually. Then, the seeds were washed thoroughly under slow-running tap water for 10 minutes. Afterwards, they were laid out as a single layer on paper towels to air dry for 2 hours in the air-conditioned laboratory to remove surface water before conducting the study on the in vivo sprouting from seed fractions (Figure 3.1).



Figure 3.1 Cleaned Seeds Before Being Laid Out as Single Layers for Air Drying to Remove Surface Water

3.2.2 Study Location

The research was conducted at the Plant Science Laboratory of Universiti Teknologi MARA (UiTM), Jasin Campus, Malacca, Malaysia.

3.2.3 Experimental Procedure

The experimental procedure consisted of a series of steps, including test material preparation, seed weighing, fractionation, sprouting, and resprouting. Each stage was meticulously executed to ensure accurate data collection and analysis for the study.

3.2.3.1 Seed Morphometrics and Weight

A total of 200 air-dried intact seeds were randomly selected and divided into five groups and then randomly subdivided into four sub-groups, each with 10 seeds.

Each seed was numbered for identification purposes and determined for its morphometrics and weight. The length and breadth of each seed were measured using a Vernier caliper to the nearest 0.01 mm. After measuring the size, the seeds were weighed individually and accordingly using an analytical balance with an accuracy of 0.0001 g to determine their fresh weight. These morphometric and weight data were recorded prior to seed cutting treatments and were also used to evaluate the relationship between seed size and sprouting potential.

3.2.3.2 Medium Preparation

Sand was used as a medium in assessing the sprouting of the seed fractions. Sand was taken from the UiTM Jasin compost house. The sand was sieved to obtain sand with particle size in the range of 0.2 to 2.0 mm (fine to coarse sand) for uniformity of sprouting medium across all studies in this research work (ISTA, 2019). Then, sand was washed several times under running water to remove impurities, followed by sun drying until completely dry before use.

3.2.3.3 Seed Cutting Procedure

The five groups of seeds were randomly subjected to five different cutting treatments, respectively, to determine the regenerative zones of the seeds. Each treatment was replicated four times, each being the sub-group as mentioned above in 3.2.3.1. The control treatment was intact seed with no seed cutting treatment (C0) (Figure 3.2a). For the seed cutting treatments, the seed parts (left, right, distal, and proximal) were identified based on the position of the hilum and a small pit on the seed (Figure 3.2a). These parts were consistently utilized in systematic seed cutting treatments using a sharp knife.

For halved seeds, the seed laid in a position of Figure 3.2a was either cut longitudinally from the hilum to the base (C2L) or transversely (C2T), which is perpendicular to that performed for C2L. For C2L, the right half was named C2L(R), and the other half was C2L(L) (Figure 3.2b). For C2T, the seed fraction with hilum was labelled C2T(P) and the one with the distal portion was C2T(D) (Figure 3.2c). Each cut seed was weighed using an analytical balance to an accuracy of 0.0001 g (Figure 3.3).

To cut a seed into quarters, the seed was cut into four longitudinal sections in

the C4L treatment. In the C2L2T treatment, the seed was first cut into two longitudinally and then halved transversally. The different types of seed fractions obtained from C4L were C4L(L), C4L(CL), C4L(CR), and C4L(R) (Figure 3.2d). Meanwhile, C2L2T resulted in C2L2T(LP), C2L2T(LD), C2L2T(RP), and C2L2T(RD) (Figure 3.2e). Each cut seed was also weighed using an analytical balance, as described above.

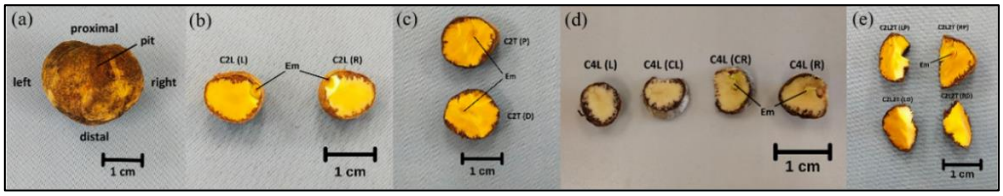


Figure 3.2 Seed Cutting Treatments: (a) Whole Seed with Indication of Left, Right, Proximal, Distal and Pit Positions, Respectively, (b) Seed Cut Half Longitudinally (C2L) to Obtain Left [C2L(L)] and Right [C2L(R)] Fractions, (c) Seed Cut Half Transversely (C2T) to Obtain Proximal [C2T(P)] and Distal [C2T(D)] Fractions, (d) Seed Cut into Quarters Longitudinally (C4L) to Obtain Left [C4L(L)], Center Left [C4L(CL)], Center Right [C4L(CR)] and Right [C4L(R)] Fractions, (e) Seed Cut Half Longitudinally Followed by Halving Transversely (C2L2T) to Obtain Left with Proximal [C2L2T(LP)], Left with Distal [C2L2T(LD)], Right with Proximal [C2L2T(RP)] and Right with Distal [C2L2T(RD)] Fractions



Figure 3.3 Weighing the Seed Fraction using Analytical Balance

3.2.3.4 Sprouting Procedure

Sprouting of seed fractions was carried out in sand medium in plastic containers as mentioned above. Each plastic container was filled with approximately 500 g of sand

(Figure 3.4), and 80 mL of tap water was added to moisten the sand. Containers were labelled accordingly.

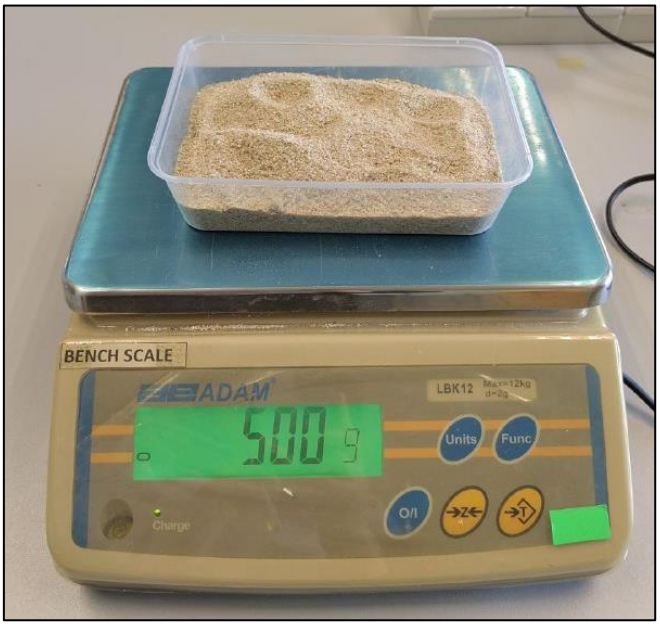


Figure 3.4 Weighing of Sand using Electronic Balance

Whole seeds and seed fractions were sown randomly in moistened sand medium in labelled plastic boxes and recorded in the MS Excel file (Figure 3.5). This experiment with five treatments, each replicated four times, was based on a completely randomized design. There were 10 seeds in each replicate. Before sowing into the moist sand, there was no surface sterilization of the cut seeds because a preliminary trial of surface sterilization of cut seeds using 10% sodium hypochlorite solution destroyed the cotyledonary surfaces and killed the cut seeds, while non-sterilized cut seeds sprouted with minimal microbial contamination.

Sprouting of the whole seeds and seed fractions was evaluated under ambient light conditions near the window in the laboratory. The window blinds were partially open to avoid direct sunlight but allow natural diffuse light to reach the seed fractions in the containers. With the aid of a HOBOWARE® datalogger, varied diffuse light intensities were recorded. The light intensity ranged from above 1,000 lux from 8 a.m. to 4 p.m., depending whether it was a sunny or cloudy day, but the light intensity decreased to below 1,000 lux after 4 p.m. and was 11.8 lux from 7 p.m. to 6 a.m. in the next morning.

The plastic covers were opened for 5-10 seconds daily to allow for gas exchange

and sufficient oxygen for sprouting. Then, the boxes were closed again to ensure sprouting was always in an enclosed condition. Tap water was sprayed onto the sand medium using a small sprayer when necessary to always ensure a moist condition for sprouting procedures.

The intact seeds or seed fractions were recorded as sprouted with the emergence of radicle or adventitious root of at least 2 mm in length. After root emergence, the whole and cut seeds were observed for the emergence of shoots. Rotting whole seeds or seed fractions were recorded and discarded immediately. Sprouting assessment was terminated after 30 consecutive days of no sprouting being observed.



Figure 3.5 Whole Seed and Seed Fractions Sown in Sand in Labelled Plastic Container

3.2.3.5 Resprouting Procedure

Once the initial sprouting and full plant formation had occurred, a portion of the cotyledon attached to the full plant was excised to monitor for re-sprouting potential. The remaining cotyledonary fragment was replanted in its original sowing position to assess the potential for resprouting. Resprouting was recorded when a new root, at least 0.2 mm in length, developed from the remaining cotyledon fragment. Resprouting observations were terminated after 30 consecutive days of no new growth being observed.

3.2.4 Data Collection

3.2.4.1 Whole Seed Morphometrics and Weight

Each numbered whole intact seed in the sub-groups (replicate) of each treatment, as described above in 3.2.3.1 and 3.2.3.3, was measured for length and diameter using a Vernier caliper to the nearest 0.01 mm, as explained in 3.2.3.1 (Seed Morphometrics and Weight). Each seed was also weighed using an analytical balance to an accuracy of 0.0001 g as mentioned above.

3.2.4.2 Seed Fraction Weight

After the seeds were subjected to the four types of cutting treatments, each seed fractionwas weighed accordingly using the analytical balance (Figure 3.3)

3.2.4.3 Sprouting Rate

Rooting and full plant development from whole and cut seeds were recorded on alternate days to obtain data regarding their sprouting time.

The rate of full plant development and the rate of root development without shoot emergence were calculated separately for each type of seed fraction using the following formula in Bányai & Barabás (2002):

$$Rate = \left(\frac{\sum_{i=1}^{k} n_i}{N}\right) \qquad (3.1)$$

where,

n_i = number of sprouted experimental units (whole seeds or seed fractions, respectively) on the i^{th} day (not the accumulated number)

N = total number of experimental units

k = last day of the sprouting procedure

Therefore, the total sprouting rate for each treatment of C0, C2L, C2T, C4L, and C2L2T, respectively, was the sum of its full plant development rate and rate of rooted seed fractions without shoot development. For instance, the total sprouting rate of C2L is the rate of full plant development for [C2L(L) + C2L(R)] + rate of rooted fractions with no shoot emergence for [C2L(L) + C2L(R)]. Total sprouting rate of each seed fractionation treatment was another indicator of propagation efficiency, as the rooted fractions could have the potential to be induced to develop shoots, culminating their contributions as functional planting materials.

For each seed fractionation treatment, its full plant development rate and total sprouting rate, and their respective periods taken for rooting and full plant development, were analysed against whole and cut seed weight to test the reserve effect hypothesis for sprouting potential from seed fractions.

3.2.4.4 Resprouting Rate

Re-rooting and re-occurrence of full plant development from remaining whole or cut seed after excision of the initially developed full plant were also recorded on alternate days. The resprouting rate was calculated similarly to the above formula, based on the proportion of remaining whole or cut seeds that successfully resprouted following the removal of the earlier full plant, in comparison to total resprouted whole or cut seeds. The time taken to resprouting was also recorded.

3.2.5 Statistical Analysis

The data were tested for homogeneity of variances at P=0.05. The whole seed length, breadth and fresh weight of seeds applied in each seed cutting treatment were subjected to analysis of variance (ANOVA). Similarly, the weight of C2L(L), C2L(R), C2T(P), and C2T(D) was also tested for homogeneity of variances, followed by ANOVA. A similar statistical procedure was also applied to weight of C4L(L), C4L(CL), L(CR), L(R), C2L2T(LP), C2L2T(LD), C2L2T(RP), and C2L2T(RD).

In the sprouting assessment, independent-samples Kruskal-Wallis tests were carried out for full plant regeneration rate and total sprouting rate, respectively. Mean separations were performed with Mann-Whitney U-tests at P = 0.05. Full plant regeneration from seed fractions was also plotted against seed fraction weight. A plot

was also presented with regard to the time taken for rooting and full plant development from the seed fractions.

Lastly, the rate of re-sprouting from the remaining whole and cut seeds following the excision of their initial full plant from the cotyledonary surfaces was described. The time taken for resprouting from each type of remaining seed fractions was also described.

3.3 Results

3.3.1 Whole Seed Morphometrics and Weight

Table 3.1 shows the sizes (length and diameter) and weight of *S. malaccense* seeds used in the five different seed cutting treatment groups. The seeds were not significantly different in length, diameter, and fresh weight (Table 3.1). By size, the seeds have a mean length and breadth of 24.73±0.51 mm and 28.25±0.55 mm, respectively, with coefficients of variation of 8.89 to 10.19% (Appendix A: A1). The seeds had a mean fresh weight of 12.6408±2.5086 with a coefficient of variation (CV) of 19.85%, indicating greater variations in seed weight (Appendix A: A1).

Table 3.1
Morphometrics and Weight of Seeds Subjected to Seed Fractionation Treatments

Treatment	Length (mm)*	Diameter (mm)*	Weight (g)*
C0	24.73±0.16	27.90±0.33	12.0591±0.4525
C2L	24.56±0.26	28.20±0.35	12.6244±0.6959
C2T	24.98±0.62	28.33±0.23	12.7594±0.5471
C4L	24.88±0.64	28.23±0.45	12.6925±0.8682
C2L2T	24.50±0.71	28.59±1.04	12.6832±0.8556
Grand mean	24.73±0.51	28.25±0.55	12.6408±2.5086
P-value	>0.05	>0.05	>0.05

* mean±SD

3.3.2 Seed Fraction Weight

Levene's test for equality of variance revealed homogeneity for seed fraction weight following C2L and C2T treatments ($p>0.05$). Halved seeds following C2L and C2T had a mean weight of 6.1612±0.6122 g with a coefficient of variation (CV) of 10% (Figure 3.6; Appendix A: A1). Meanwhile, a similar test for homogeneity of variances for the quartered seeds resulted from C4L and C2L2, also indicated that the seed

quartering procedures were carried out fairly. Following further reduced mass in the seed quarters, these fractions had mean weight of 3.0741±0.3702 g with CV of 12% (Figure 3.6; Appendix A: A1).

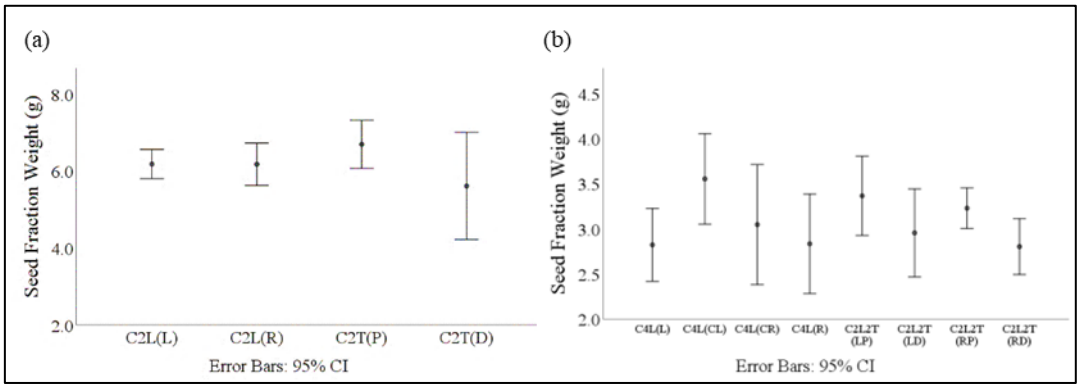


Figure 3.6 Weight of Seed Fractions Following C2L, C2T, C4L and C2L2T; Means Within Chart are not Significantly Different at 5% Level of Significance

3.3.3 Effects of Seed Reserve and Cutting Treatment on Regeneration of Seed Fractions

The evaluation of the regeneration of seed fractions of *S. malaccense* was analysed based on their sprouting and full plant formation rate. Seed cuttings resulted in two types of post-fractionation sprouting: seed fractions either developed into full plants or only rooted. All intact and cut seeds exhibited hypogeal sprouting, and the cotyledon remained underground (Figure 3.7).

In general, germination of the intact *S. malaccense* seed began with the formation of the primary root from one of the cotyledons in the region closer to the distal end of the seed, followed by the formation of a shoot from the almost central area of the cotyledons (Figure 3.7). The proximal end of the seed refers to where the hilum is located, as explained in 3.2.3.3, while the opposite end is called the distal end. Whole intact seeds demonstrated more than 90% sprouting, or a sprouting rate of 0.93 (Table 3.2; Appendix A: A1). All the sprouted seeds were capable of producing full plants.

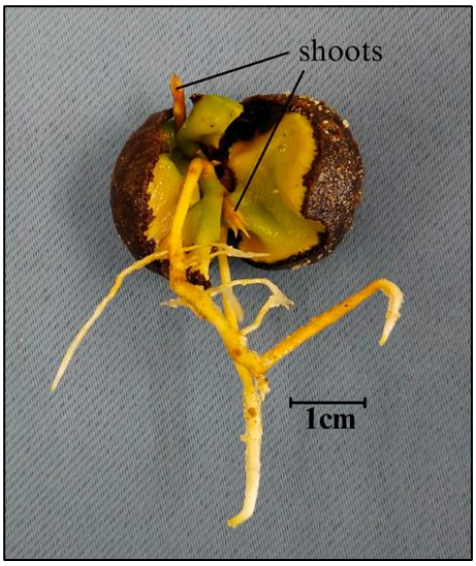


Figure 3.7 Hypogeal Germination from Intact Seed (Treatment C0); Two Shoots Development from a Single Root System in an Intact Seed was Possible

Table 3.2
Regenerative Capability as Affected by Seed Sectioning

Seed sectioning	Full plant regeneration rate	Total sprouting rate*
C0	0.93 c	0.93 d
C2L	1.5 a	1.98 c
C2T	1.2 b	1.95 c
C4L	1.5 a	3.4 b
C2L2T	1.4 ab	3.95 a

* full plant development + rooting without shoot development
Means having the same letter within column are not significantly different at P=0.05 using Mann-Whitney U-tests

Cutting the seeds into fractions was not lethal for *S. malaccense*. In fact, the results exhibited that at least one of the fractions could regenerate into a full functional plant in most cases. For the treatments in which seeds were cut into two parts, either longitudinally or transversally, it was possible for both seed fractions to develop into full plants, regardless of the fraction size (weight) (Figures 3.8 - 3.10). Figures 3.8 and 3.9 demonstrate that the distribution of full plant regeneration (△) was not related to fraction weight. These figures also reveal the pairs of fractions, i.e., those having similar whole seed weight (Y-axis), that both formed full plants or only one fraction capable of forming a full plant.

The fractions obtained following treatment C2L showed the highest full plant formation rate, i.e., 1.5, in comparison to seeds treated with C2T that achieved a 1.2 full plant regeneration rate (Table 3.2). C2L(R) provided the highest number of full plants, i.e., 32 full plants were formed with 40 C2L(R) cut seeds, while the total of 40 C2L(L)

fractions provided 28 full plants (Figures 3.8; Appendix A: A1). Figure 3.8a and 3.8b demonstrated that 21 out of 40 seeds with similar whole seed weight according to the y-axis formed full plants with both their separated fractions following C2L (Figure 3.8).

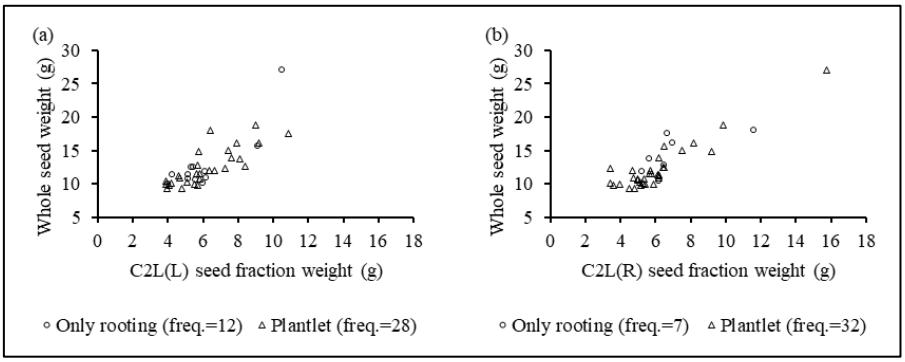


Figure 3.8 Reserve Effect on Sprouting of C2L Seed Fractions; N = 40; (a) C2L(L), (b) C2L(R); 1 Seed Did Not Develop Any Full Plant with Both Fractions; 18 Seeds Had Only One (Any One) Fraction Developed a Full Plant Each; 21 Seeds Had Both Fractions Developed Full Plants Each

For seeds that were subjected to treatment C2T, the proximal fractions produced more full plants (29 full plants) than the distal portions (19 full plants) (Figure 3.10; Appendix A: A1). However, C2T was less appropriate for propagation of this woody fruit tree species through the seed cutting technique. Only 8 seeds out of 40 seeds experimented with were found capable of forming two full plants with both their separated proximal and distal cut seeds (Figure 3.10). The remaining 32 out of 40 seeds developed a full plant each with either of the separated fractions.

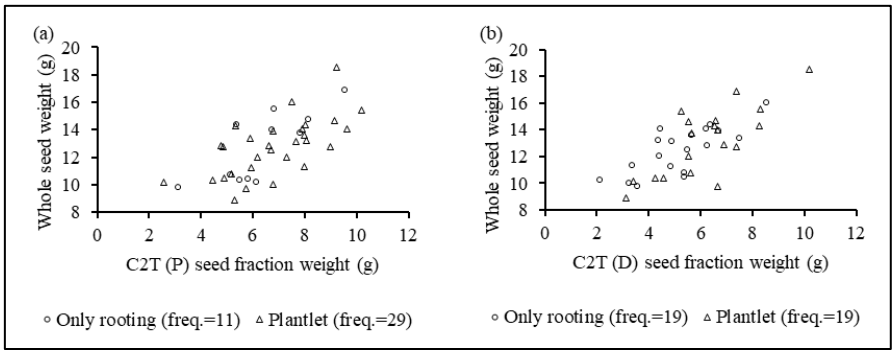


Figure 3.9 Reserve Effect on Sprouting of C2T Seed Fractions; N = 40; (a) C2T(P), (b) C2T(D); 32 Seeds Had Only One (Any One) Fraction Developed Full Plant Each; 8 Seeds Had Both Fractions Developed Full Plants Each

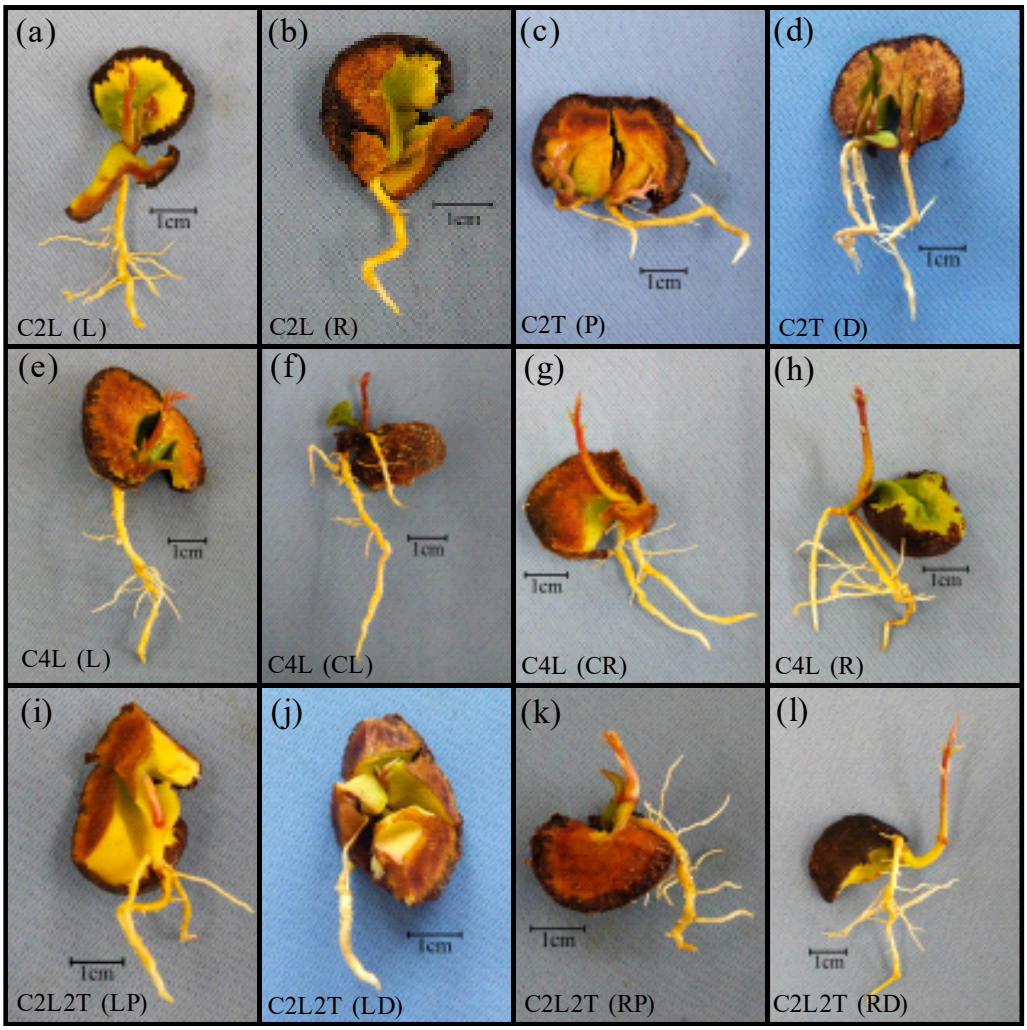


Figure 3.10 Full Plant Development from Seed Fractions: (a) C2L(L), (b) C2L(R), (c) C2T(P), (d) C2T(D), (e) C4L(L), (f) C4L(CL), (g) C4L(CR), (h) C4L(R), (i) C2L2T(LP), (j) C2L2T(LD), (k) C2L2T(RP), (l) C2L2T(RD)

Meanwhile, for the treatments in which seeds were cut into four parts, the cut seeds demonstrated normal full plant development in up to three out of four fractions of a single seed (Figures 3.11 and 3.12). This again proved that full plant development from *S. malaccense* cut seeds was not in accordance with the reserve effect hypothesis, where the amount of reserves in seeds determines the growth of the full plant in the event of loss of seed mass.

For seeds subjected to treatment C4L, the treatment revealed a higher full plant formation rate as compared to seeds subjected to treatment C2L2T (Table 3.2). For the seeds subjected to this treatment of C4L, the central quarters of C4L(CL) and C4L(CR) developed a higher number of full plants as compared to the leftmost or rightmost quarters (Figure 3.11; Appendix A: A1). Both types of seed fractions developed a total of 47 full plants. In contrast, the left and right parts [C4L(L) and C4L(R)] of the seeds,

upon fractionation, showed a higher probability of root protrusion, which was not followed by shoot emergence (Figure 3.11). With C4L, four seeds proved their ability to form three full plants with their quartered fractions (Figure 3.11, Appendix A: A1).

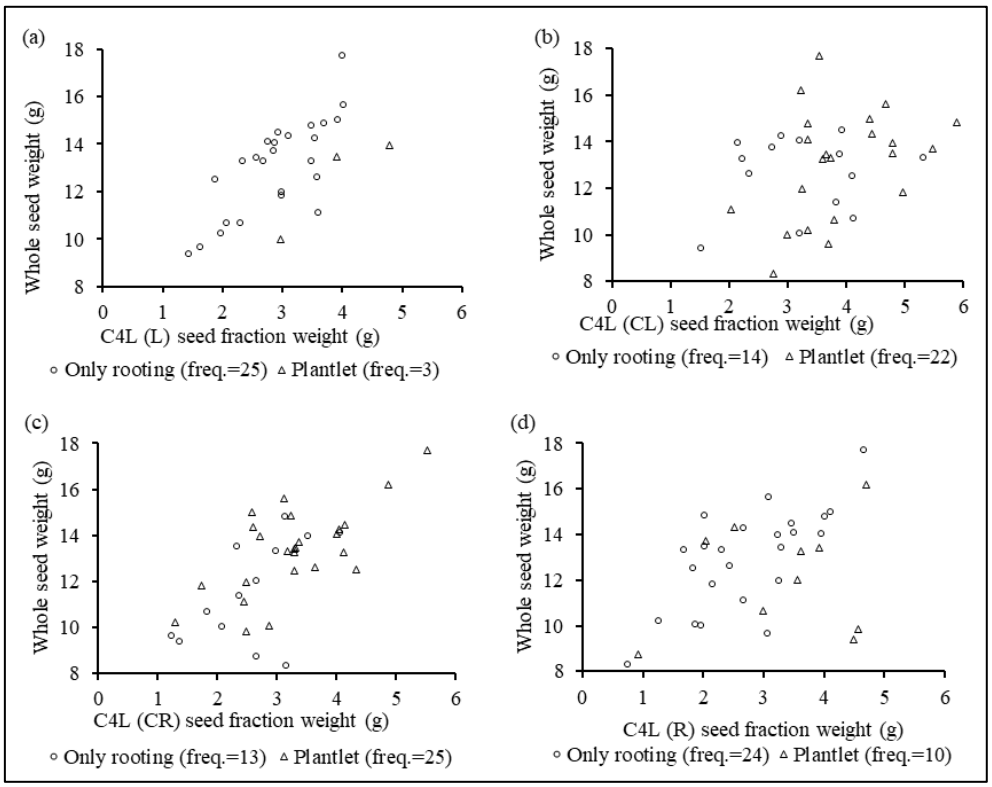


Figure 3.11 Reserve Effect on Sprouting of C4L Seed Fractions; N = 40; (a) C4L(L), (b) C4L(CL), (c) C4L(CR), (d) C4L(R); 4 Seeds Did Not Develop Any Full Plant with All Four Fractions; 16 Seeds Had Only One (Any One) Fraction Developed Full Plant Each; 16 Seeds Had Two (Any Two) Fractions Developed Full Plants Each; 4 Seeds Had Three (Any Three) Fractions Developed Full Plants Each; No Seed Developed Full Plant with All Four Fractions

With C2L2T, the left and right distal portions of the seed exhibited a lower full plant development rate (11 full plants each) (Figure 3.12; Appendix A: A1). The development of full plants was higher with both quarters having the left or right proximal parts. C2L2T (RP) provided the highest number of full plants (19 full plants) among all C2L2T fractions. With C2L2T, there were also four seeds that each demonstrated the ability to provide three full plants with their quartered fractions (Figure 3.11, Appendix A: A1).

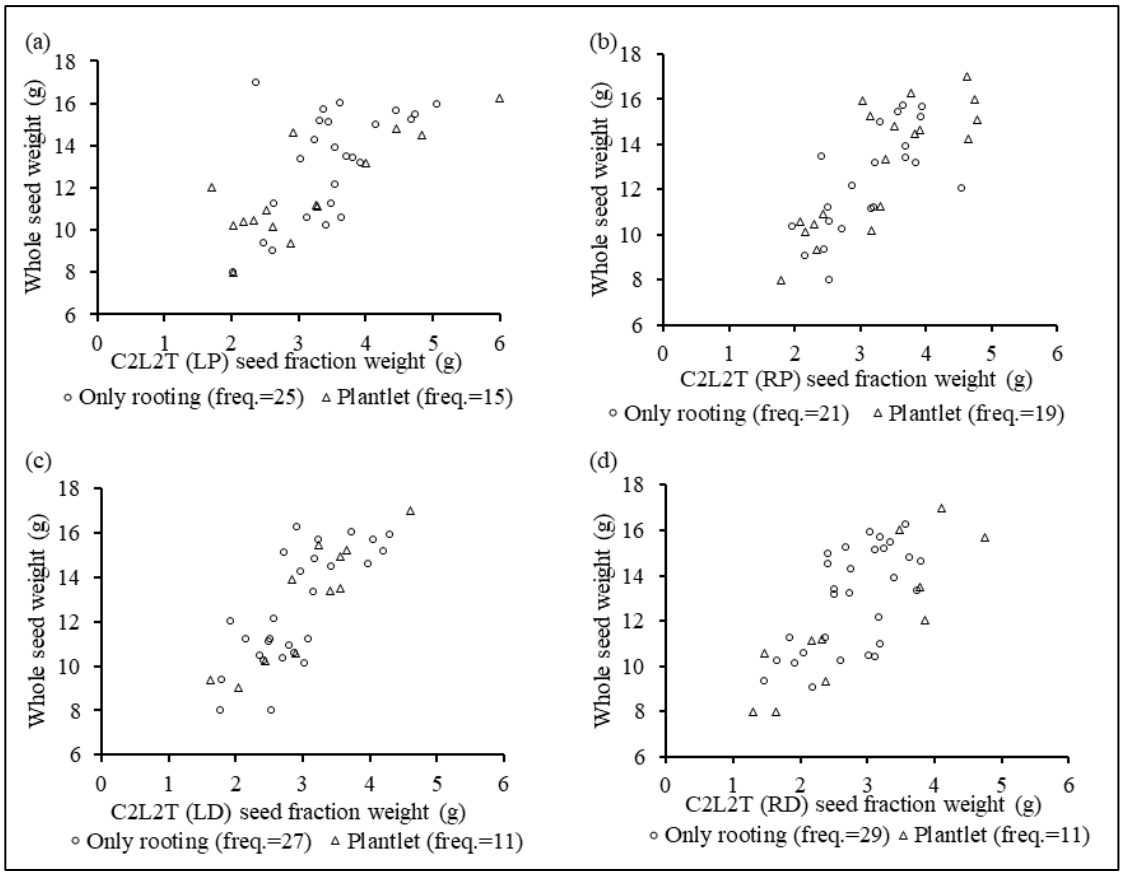


Figure 3.12 Reserve Effect on Sprouting of C2L2T Seed Fractions; N = 40; (a) C2L2T(LP), (b) C2L2T(RP), (c) C2L2T(LD), (d) C2L2T(RD); 6 Seeds Did Not Develop Any Full Plant with All Four Fractions; 16 Seeds Had Only One (Any One) Fraction Developed Full Plants Each; 14 Seeds Had Two (Any Two) Fractions Developed Full Plants Each; 4 Seeds Had Three (Any Three) Fractions Developed Full Plants Each; No Seed Developed Full Plant with All Four Fractions

In several cases, shoots developed from the cut (cotyledonary) surfaces first, instead of root development prior to growth of the shoot. In other words, cut seeds exhibited a unique ability where the shoot emerged before root development (Figure 3.13). This could be evidence of the induction of growth beyond normal seed germination, which always has radicle protrusion followed by growth of plumule.

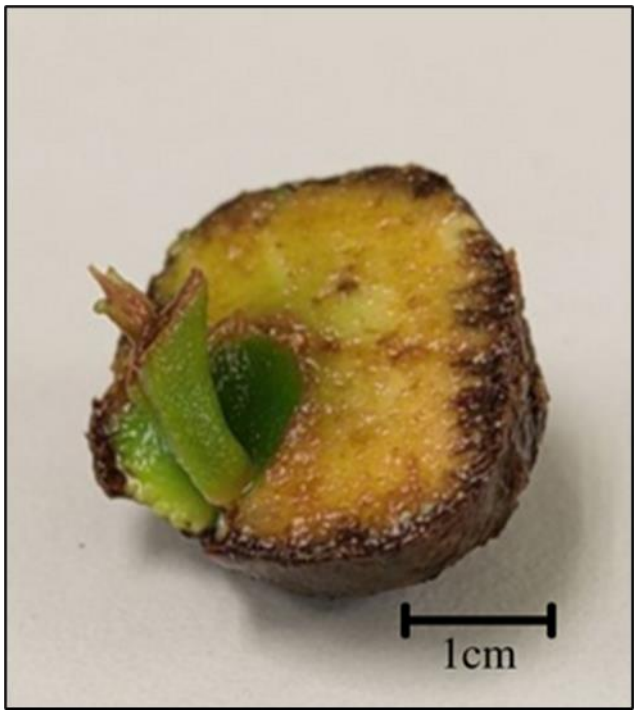


Figure 3.13 Shoot Initiation before Rooting from Seed Fraction

For the seed fractions that did not develop full plants, most of them rooted haphazardly from the cotyledonary surface (Figures 3.8 – 3.9 and 3.11 – 3.12). These roots were fine adventitious roots and were different from the robust radicles (Figure 3.14).

For halved seeds, a sprouting rate (full plant regeneration rate + rooting rate not accompanied by shoot emergence by the end of the study period) of 1.98 was achieved when the seeds were subjected to treatment C2L (Table 3.2; Appendix A: A1). This sprouting rate was almost double that of the intact seeds having a sprouting rate of 0.93. The sprouting rate of 1.98 recorded following C2L did not differ significantly from that demonstrated by the seeds subjected to treatment C2T (1.95).

With seed quartering method, all four separated parts of most seeds demonstrated protrusion of roots but the ability to develop into full plant was limited with some seed fractions. Although the rate of full plant regeneration for C2L2T was lower than that of C4L, seeds that were cut according to C2L2T possessed a higher total sprouting rate (Table 3.2). Seeds subjected to treatment C2L2T demonstrated the highest total sprouting rate (3.95) among all cut seeds.

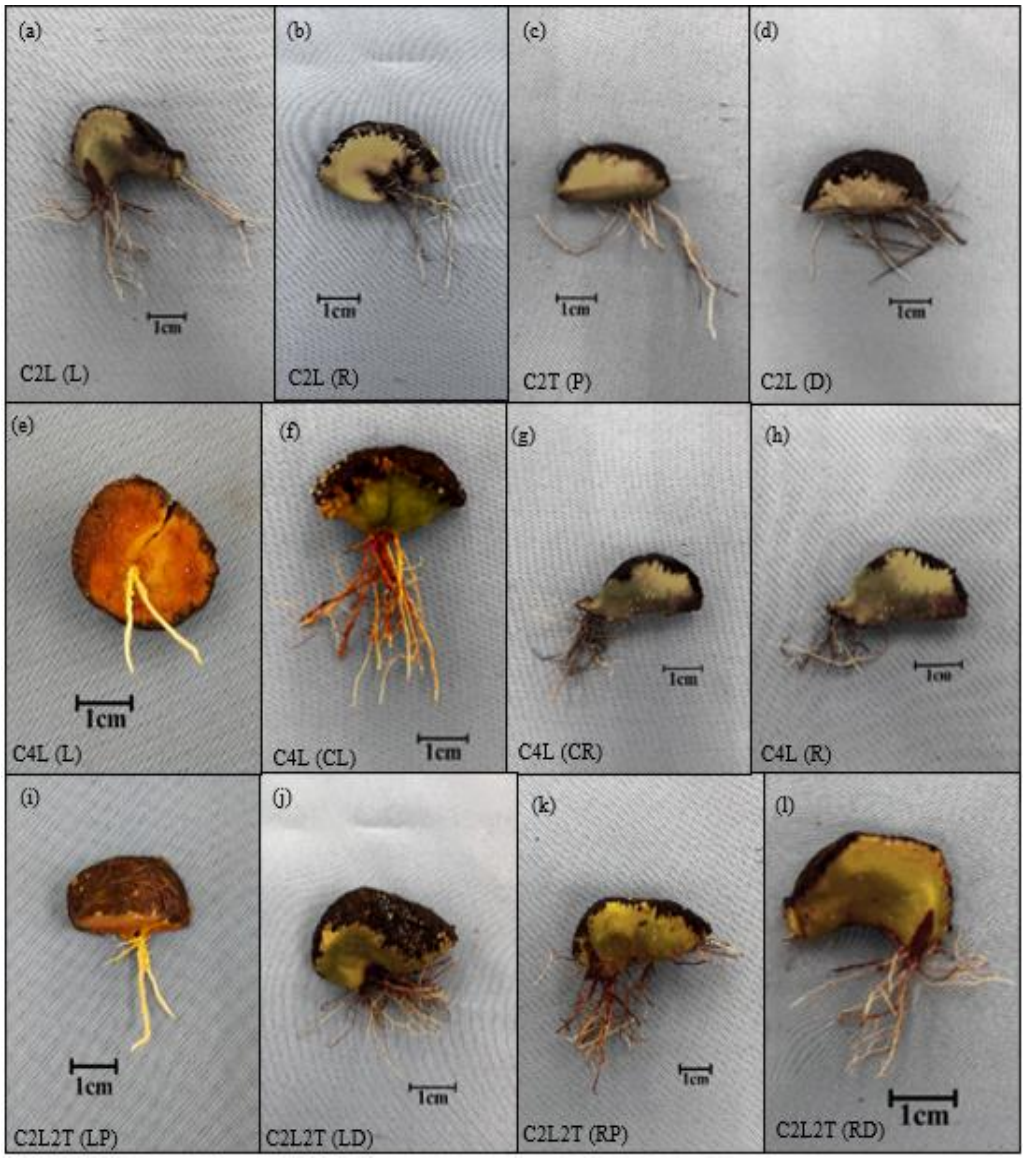


Figure 3.14 Adventitious Root Development from Seed Fractions that Was Not Followed by Shoot Development; (a) C2L(L), (b) C2L(R), (c) C2T(P), (d) C2T(D), (e) C4L(L), (f) C4L(CL), (g) C4L(CR), (h) C4L(R), (i) C2L2T(LP), (j) C2L2T(LD), (k) C2L2T(RP), (l) C2L2T(RD)

A few seed fractions did not sprout throughout the study period of up to four months. Most of the non-sprouting cut seeds remained alive with minimal microbial contamination throughout the study period. Then, these "non-viable" seed fractions turned rotten and were discarded accordingly.

3.3.4 Sprouting Time

This section reports the sprouting time of intact and cut seeds. The intact seeds and fractioned seeds that formed full plants in this study generally sprouted with root

development first in less than 20 days (Figure 3.15, Appendix A: A1). The roots were mostly robust, indicating possibly the growth of radicle or embryonic roots. Intact seeds generally exhibited faster sprouting, particularly in the emergence of primary roots, compared to fractioned seeds. For the cut seeds, it was noted that the shoot development times varied within the treatment group. In general, seeds that were cut into two portions, the shoot emergence was faster for halved seeds obtained with longitudinal cutting (C2L), as compared to seeds that were halved transversely (C2T). For quartered seeds, C4L fractions exhibited faster shoot emergence than those subjected to the C2L2T treatment. In several cases, seeds required more than three months to initiate shoot development, although rooting occurred much earlier in less than one month. A particularly notable observation was with the C4L (CR) treatment, where shoot emergence was visible after 114 days (Figure 3.15). While some seed fractions took a prolonged time to develop their shoots, the median result for shoot emergence for all seed fractionation treatments was 15.00 days, and the mean shoot development time was 20.43±16.35 days.

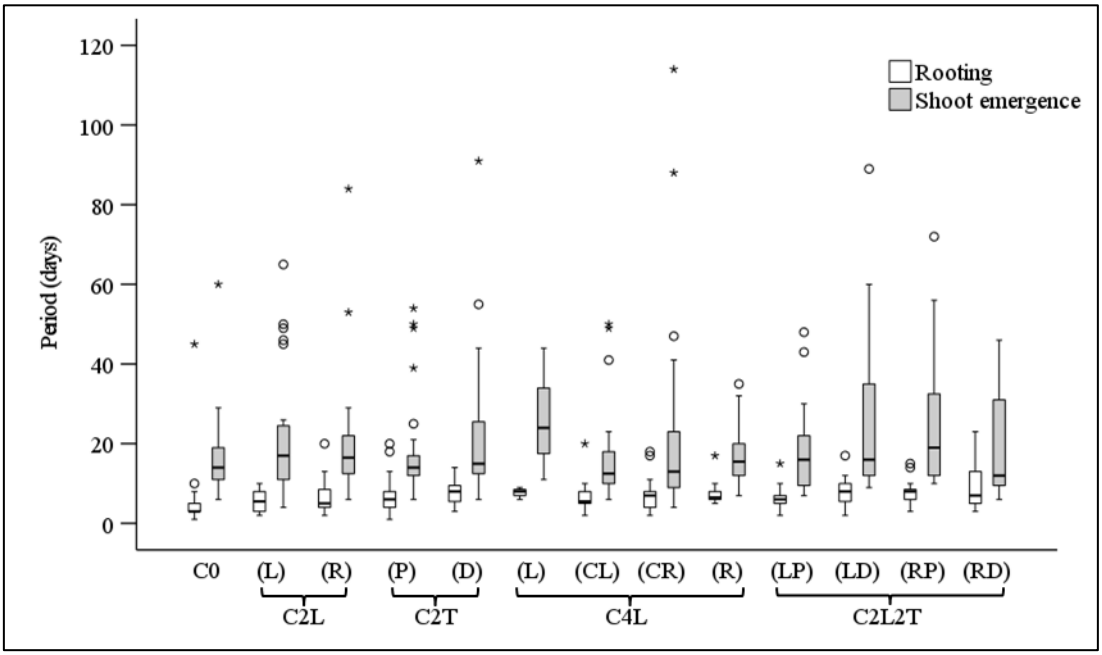


Figure 3.15 Quartiles of Rooting and Shoot Emergence Periods of Seed Fractions

For the small number of seed fractions that did not demonstrate growth of shoots in this study, they generally took a longer time to develop their adventitious roots compared to primary root development in whole intact seeds (Figure 3.15). Therefore,

the rooting period had a median value of 6.00 days. The mean time to rooting was 6.79±4.38 days.

3.3.5 Resprouting

The resprouting potential of *S. malaccense* seed fragments was also proven possible in this study. Table 3.3 shows the seed fragment weight and re-rooting rates across different treatments after their initial full plants and portions of the cotyledon attached to them were cut off. The number of seed fragments used to study the resprouting potential of *S. malaccense* seeds was inconsistent, depending on the earlier full plant regeneration rates, and the weight of seed fragments also varied. Resprouted seed fragments that were initially subjected to Treatment C0 had the highest fragment weight, followed by fragments originally subjected to seed halving treatments, while the fragments left behind by the quartered seeds had the smallest weight values.

Table 3.3
Re-rooting Rate in Relation to Seed Fragment Weight after Removal of Full Plant and a Part of Cotyledon Attached to it

Treatment	N	Seed fragment weight (g)*	Re-rooting rate
C0	35	4.2458 ±1.8301	0.82
C2L(L)	24	3.878 ± 1.6305	0.83
C2L(R)	26	3.2603±1.8249	0.89
C2T(P)	26	2.8029±1.4475	0.81
C2T(D)	14	2.9136±1.267	0.86
C4L(L)	3	2.4514±.52187	0.67
C4L(CL)	18	2.0639±0.7521	0.78
C4L(CR)	16	2.0202±1.1395	0.88
C4L(R)	8	1.9929±0.8361	0.88
C2L2T(LP)	13	2.0257±1.1987	0.77
C2L2T(LD)	8	1.9433±1.0139	0.75
C2L2T(RP)	15	2.3399±1.0796	0.93
C2L2T(RD)	9	1.729±0.5512	0.78

* mean±SD

S. malaccense seed fragments showed a remarkable capacity to resprout following excision of the initial full plant. The seed fragments showed a high possibility for new adventitious root development from the cotyledon, but no shoot regeneration was obtained in this resprouting study. The resprouting rates were mostly above 0.8 (Table 3.3; Figure 3.16; Appendix A: A2). This again proved that the reserve effect hypothesis does not explain the growth potential from the cotyledonary well; small

fragments following seed quartering could also resprout well. In this study, fragments of C4L(L) after removal of their initial full plants had the lowest resprouting rate (Table 3.3).



Figure 3.16 Re-rooting after Removal of Full Plant and a Part of Cotyledon Connected to it on the Regenerated Seed Fraction

In terms of resprouting time, some seed fragments resprouted in a week (Figure 3.17; Appendix A: A2). Others generally resprouted within one month. The results obtained showed that seed fragments with a larger remaining cotyledon did not necessarily resprout the fastest.

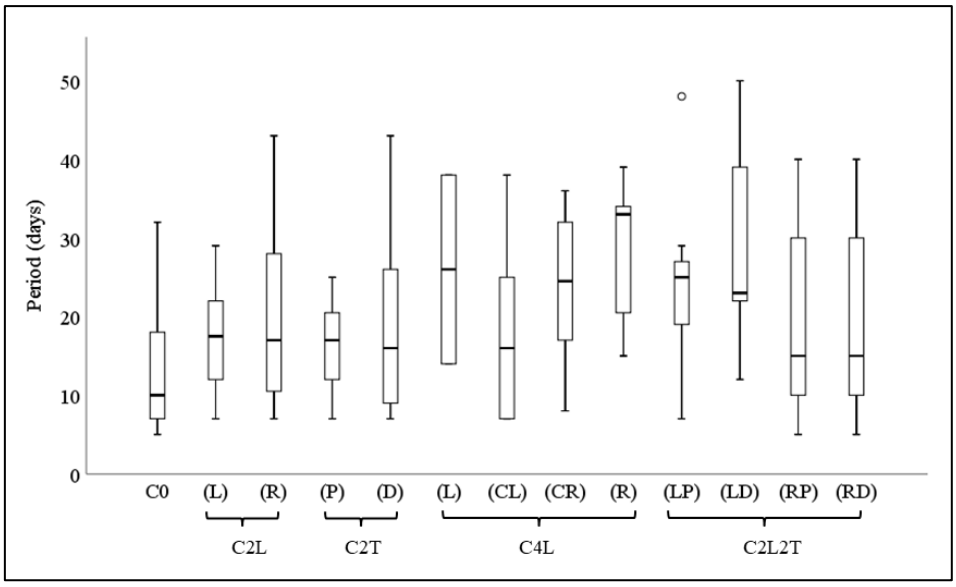


Figure 3.17 Quartiles of Re-rooting Time After Removal of Full Plant and a Part of Cotyledon Connected to it on the Regenerated Seed Fraction

3.4 Discussion

The present study presented the capacity of *S. malacccense* seeds to develop root and shoot after fractionation. It showed that the fractions of *S. malaccense* seeds possessed a remarkable capacity for regeneration of full plants, which could be a potential alternative for the propagation of the species in both the natural environment and for the horticultural industry.

Past studies suggested that seed cutting may have lowered the number of reserves, which may have made it insufficient for full plant development (Teixeira & Barbedo, 2012). A study by Delgado et al. (2022) also suggested that the size of seeds and the number of cuts applied to seeds may have an impact on the regeneration success of cut seeds. In addition, the authors mentioned that the small size of the embryonic root-shoot axis makes it less possible for the cuttings to coincide over that axis every time a cut is made. However, the previous study managed to show that the potential for entire plant regeneration remained consistent regardless of these factors. Seeds with up to 75% of their cotyledon removed, such as those subjected to C4L and C2L2T treatments, produced multiple plants with their separated fractions in this study with *S. malaccense*, proving that the seeds are not dependent on a large reserve for successful full plant regeneration. In this study, seeds cut into quarters also exhibited high total sprouting rates, with C2L2T achieving the highest sprouting rate at 3.95. These results suggest that the morphometrics of the seed fraction were not a limiting factor for full

plant regeneration success of this species. This finding contradicts the traditional view that larger seed reserves are necessary for successful germination (Harms & Dalling, 1997).

It was an important observation that the cut seeds were able to have more than one full plant growing from a seed following the fractionation of a seed into separate portions. The study exhibited the embryogenic competence of *S. malaccense* seed as it shows that it is possible for cut seeds to give rise to more than one full plant from each seed, with each of the shoots having its own root system. Seeds submitted to quartering treatments demonstrated the ability to produce more than one plant per seed and some could even provide up to three plants per seed despite reduced reserves. This is probably because of the presence of non-differentiated meristematic tissue in the seed cotyledon that could give rise to new embryos (Joshi et al., 2006)

The seed demonstrated another unique ability, where the shoot was able to grow from an adventitious root system. This experiment indicated that shoot development is possible even without the plumule-radicle axis and proved that cotyledons may differentiate into regenerative cell leading to full plant development. It is noteworthy because some seeds exhibited shoot initiation prior to its root development, which is not common for seeds and this also does not happen in true seed germination. This case was probably due to "traumatic" injury caused on the cotyledon of the seed, i.e., seed cutting treatments. This, somehow, also proves the seed to be totipotent and also shows the unique capabilities of cell in *S. malaccense* seed to undergo division and differentiation for the formation of full plants (Baskin & Baskin, 2025).

In this study, the fastest to sprout generally involved primary roots or radicles. Adventitious roots were generally slower to develop compared to primary roots. In terms of sprouting time, intact and fractioned seeds that developed full plants generally rooted in less than 20 days. However, some seed fractions took a longer time to sprout. The difference in sprouting period indicates that the seed reserves are not immediately exhausted. As mentioned by Delgado et al. (2010), such seeds do not lose their reserves in simultaneous sprouting, instead saving them for sequential sprouting. Additionally, according to Teixera and Barbedo (2012), this behaviour is likely due to the fact that cutting the seed into fractions can allow the fractions to grow from those where they have already begun to sprout, as well as sprouting from the other parts. Even after a long period of time, the cut seeds may still have the growth potential, which could have been stimulated earlier but yet to be displayed. In addition, the seed fractions having a

difference in sprouting time could be indicative of whether they are a zygotic type that originates from the plumule-radicle axis or an adventitious type.

The present study also analyzed the resprouting capacity of *S. malaccense* fragments after full plant development. The results showed that the resprouting was also not affected by reserve or weight of seed fragments. Having a large seed reserve does not necessarily mean that the seed fragment will root faster. Furthermore, the ability of the seed fragments to resprout was limited to producing new adventitious roots. This is yet to be studied as to whether it is linked to signaling or other molecular studies.

3.5 Conclusion

The objective to evaluate the relationship between reserve reduction and the sprouting potential of *S. malaccense* seed fractions was successfully achieved. The findings demonstrated that the reserve effect hypothesis was not applicable to this species; statistical analysis revealed that significant reductions in seed size and fresh weight did not hinder sprouting success or the development of full plants. Regardless of the weight reduction, even the smaller seed fractions demonstrated rapid sprouting and successful development into full plants. The study also identified C4L and C2L2T as the most efficient propagation treatments. While the C2L and C2T methods yielded a maximum of 2 plants per seed, the C4L and C2L2T treatments exceeded this, yielding up to 3 full plants from a single seed. In addition to full plant regeneration success from seed fractions, the high resprouting rates obtained after removal of the full plants from the fractions confirmed the growth potential from the cotyledonary surfaces of *S. malaccense* seeds.

CHAPTER 4
REGENERATION FROM SEED FRACTION UNDER DIFFERENT LIGHT CONDITIONS

4.1 Introduction

The previous work showed that quartered seeds of *S. malaccense* could also develop entire plants in vivo despite losing their differentiated embryonic axes. The partial loss of reserve could have activated the relatively undifferentiated cells in the somatic tissues of cotyledons, leading to the development of an embryonic cell and, ultimately, an entire plant (Alonso et al., 2019; Bidabadi & Mohan Jain, 2020). The ability of the seed fractions to form full plants resulted in an almost double full plant regeneration rate for this woody fruit tree species, compared to intact seeds. In contrast, the whole intact seeds had a maximum full plant development rate of 1.

However, while the potential for seed fragmentation in plant propagation has been discovered, little is known about the exogenous factors that influence the regeneration success of the fragmented seeds. External factors such as light, temperature, and humidity play a vital role in the growth and development of plants. Light, in particular, plays an important role in the early development of plants (Suzuki et al., 2010; Lee et al., 2016). Different plant species vary in their light requirements for their physiological responses, including sprouting and germination processes. Some seeds require light to germinate, while others require a complete absence of light (Ferreras et al., 2025; Pirola et al., 2021). The effects of light on seeds can vary based on the light intensity and duration, leading to different morphological responses.

In a forest environment, the light conditions are not static but rather dynamic and are constantly changing. Various factors, such as the structure of the canopy, the time of day, and the weather conditions, can cause this variability. These changes in light availability can significantly impact various aspects of the forest ecosystem, including the regeneration of both intact and fragmented seeds, mostly in the form of partially predated or damaged seeds. When a seed is injured, for example, the type imposed by a predator, its ability to regenerate and grow into a healthy plant may be compromised. The amount of light it receives during this critical stage can either help or hinder its growth, and therefore, it is essential to understand how changes in light

availability in the forest can impact the regeneration of seeds, including the partially predated seeds.

In laboratories or greenhouses, the use of artificial lights like light-emitting diodes (LEDs) enables studies for sprouting from seeds and plant cultivation in a controlled environment. LED lights offer several benefits compared to conventional light sources, as they are more affordable, cool, and controllable (Zhang et al., 2020; Paradiso & Proietti, 2022). The use of LED lights is versatile, where specific light wavelengths can be manipulated and related to certain plant morphogenesis, including sprouting from seeds (Massa et al., 2010) . Several reports have confirmed reliable results on seed germination and cultivation of different species under LED illumination (Singh et al., 2015; Berkovich et al., 2017; Lazzarin et al., 2020; Rahman et al., 2024).

Aside from external cues like light, another important aspect of seed germination is the interplay between light conditions and the plant cells. Previous studies have primarily focused on the macroscopic outcomes of regeneration, such as sprouting rates and seedling development, but not the microscopic factors behind the regeneration. Several studies have reported a link between cell expansion and elongation, and light conditions (Falcioni et al., 2019; Stoynova-bakalova et al., 2002). These studies exhibited expansion of cell surface area in developing embryos, while cell expansion was affected by lighting. Prolonged exposure to intense light can lead to photoinhibition, where excessive light can damage the seedling's cellular structures and suppress mitotic activity, which will affect the overall growth and development (Goh et al., 2012; Stoynova-bakalova, 2004; Suzuki et al., 2010). The inhibitory effect of continuous illumination on germination is known as the high-irradiance response (Mancinelli & Rabino, 1978; Schopfer & Plachy, 1993; Pons, 2000). According to a study on in vitro propagated *Arabidopsis* cotyledon induced in darkness, cell division was stimulated alongside exogenous application of hormones in the absence of light (Stoynova-bakalova, 2004).

With lighting effects in mind, the current study aimed to understand the potential of seed fragmentation for *S. malaccense* propagation in vivo, devoid of the application of hormones. The experiment reported in this chapter addressed the second objective of the research work, Objective 2: to determine the influence of light conditions on regeneration from quartered seeds. Understanding how plants perceive and respond to light quality and intensity can lead to innovative strategies for optimizing resource use in a controlled environment for propagation of *S. malaccense* through the seed

fractionation technique.

4.2 Materials and Methods

This section describes the materials applied in the study and explains the procedures of experimentation in detail.

4.2.1 Test Material

Due to seed size differences, unsynchronized fruit development, availability and predation of seeds by borers, *S. malaccense* fruits were collected from various trees in different localities, including Port Dickson, Masjid Tanah, Merlimau, and Kampung Seri Mendapat (N 2° 13' – 2° 30', E 101° 48' - 102° 28'), from the end of September to early November 2022. It was needed to obtain 240±60 intact seeds for this study.

Unlike the preceding experiment, only half-ripe fruits were collected from the standing trees for this experiment. The fruits were segregated by labeling plastic bags according to the tree from which they were collected. After collecting the fruits, they were brought to the Plant Science Laboratory at Universiti Teknologi MARA, Jasin Campus (N 2° 13' 16.446", E 102° 27' 8.262") on the same day or the following day. The fruits were incubated under ambient conditions in the laboratory for one week until they were fully ripe, with the entire pericarp showing bright red colour. The temperature and relative humidity of the laboratory were 28±2 °C and 55±5%, respectively.

4.2.2 Study Location

The research was also conducted at the Plant Science Laboratory of Universiti Teknologi MARA (UiTM), Jasin Campus.

4.2.3 Experimental Procedure

The experimental procedure in this section consisted of a series of steps, including test material preparation, seed weighing, seed cutting, sprouting in different light conditions. Each stage was carried out carefully to ensure accurate data collection and analysis for the study.

4.2.3.1 Seed Extraction and Selection

Ripe fruits were sorted out, and their seeds were extracted accordingly. The seeds in this study were also washed with running tap water to remove any remaining fruit pulp. After washing, the seeds were air-dried for 2 hours on paper towels. Then, the seeds were numbered in grid containers (Figure 4.1).



Figure 4.1 Seeds Arranged in a Grid Box and Labelled for Seed Identification

4.2.3.2 Seed Morphometrics

The length and breadth of the seeds were measured using a Vernier caliper to the nearest 0.01 mm. Each seed was also weighed using the analytical balance with an accuracy of 0.0001 g.

4.2.3.3 Medium Preparation

Sand was also used as a medium in tests on the sprouting potential of fractions

of *S. malaccense* seeds under different light conditions in this study. Sand was sieved to obtain that sized 0.2-2.0 mm (ISTA, 2019). Then, the sieved sand was also washed thoroughly with slow-running tap water, and dried completely under the sun, followed by cooling to room temperature before use. In each plastic container measuring 14.5 x 9.5 x 6.5 cm, 500 g dry sand was moistened evenly with 80 mL tap water prior to sowing of the seed fractions.

4.2.3.4 Seed Fractionation

For this study, the seed cutting treatments of C4L and C2L2T were exclusively applied due to their good performance in producing a higher number of full plants in the preceding experiment. For Treatment C4L, a sharp knife was also used to cut the seed longitudinally into approximately equal quarters. For Treatment C2L2T, the seeds were each cut into half longitudinally, followed by a transversal cut on each longitudinally halved fraction. After fractionation, the fragmented seeds were sprouted in moist sand in the boxes and labelled accordingly. The seed fractions were monitored for sprouting under three different light conditions.

4.2.3.5 Sprouting Procedure under Different Light Conditions

The sprouting process of the labelled cut seeds of two subgroups (C4L and C2L2T) was carried out using a randomized complete block design with seeds originating from different localities applied in four replicates (Table 4.1). After seed cutting, the seed fragments were sown at a depth of 10-12 mm and covered with moistened sand. No surface sterilization was required for the seed fractions. Likewise, the plastic containers with sprouting cut seeds were also closed with their lids to create a suitable environment for sprouting.

The sprouting of the seed fractions was studied under three different light conditions, respectively, at ambient temperature in the laboratory (Table 4.1). For sprouting under LED, the seed fractions in plastic containers were placed on a shelf at approximately 30 cm beneath a white fluorescent LED tube of 4’ in length (Philips®). Sprouting under LED was carried out at a 16/8h light/dark photoperiod. With a HOBOWARE® datalogger, the seed fractions in the boxes received a rather constant light intensity of 555 lux when the LED was switched on for 16 hours daily according

to the set timer (Figure 4.2). The light intensity devoid of LED from 11 p.m. to 7 a.m. in the following morning was 11.8 lux.

Table 4.1
Experimental Treatments for Studying Regeneration from Seed Fraction under Different Light Conditions

Seed Fractionation	Light condition for sprouting of cut seeds
C4L	LED Natural light Dark
C2L2T	LED Natural light Dark

For sprouting under natural light conditions, the seed fractions in plastic containers were placed near the windows. The light intensity varied according to time of day as well as weather conditions, whether sunny, cloudy, or rainy, as shown by the standard error bars for mean light intensity at different times in Figure 4.2.

For sprouting in the dark, the seed fractions in the plastic containers were placed on a bench in the laboratory and completely covered with blackout cloth to prevent light from the surrounding reaching the sprouting seed fractions. A constant 11.8 lux light intensity was recorded beneath the blackout cloth (Figure 4.2).

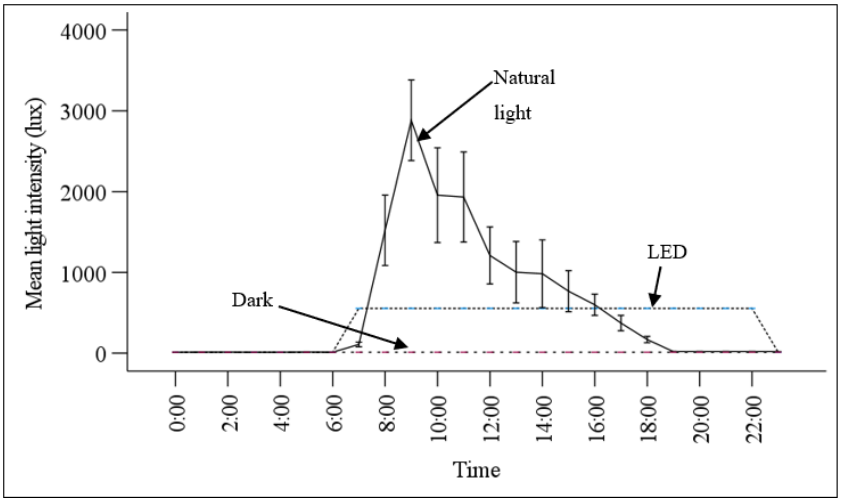


Figure 4.2 Light Intensities for Sprouting under LED, Natural Light, and in the Dark as Measured by a HOBOWARE® Datalogger; Bar Indicates Standard Error of Mean

With the containers closed with their lids throughout the sprouting experiment, the sand medium generally remained moist while a suitable amount of water was sprayed onto the medium using a hand spray bottle when and as necessary. Dead seed fractions were also recorded and discarded immediately. Like the preceding experiment,

the cut seeds in this study were also recorded for their time to rooting and full plant development.

4.2.4 Data Collection

4.2.4.1 Whole Seed Size and Weight and Seed Fraction Weight

Each numbered whole seed was weighed using the analytical balance. Then, the seeds were each measured for length and diameter using a Vernier caliper. After fractionation, each seed fraction was weighed accordingly using the analytical balance before it was sown into the moistened sand.

4.2.4.2 Sprouting Rate and Time

Seed fractions in the containers were examined for sprouting under their respective light conditions. Each container was opened and recorded for any sprouted cut seeds on alternate days. A seed fraction was considered to have sprouted when a root measuring at least 2 mm was noticeable. After rooting, the seed fractions were also monitored further for the growth of their shoots. In view of the hypogeal sprouting of this woody fruit species. The growth of the shoot was recorded only when the epicotyl had elongated to at least 10 mm in length. The first two tiny leaves of about 2 mm each were distinguishable at this stage, confirming the growth of a shoot.

For sprouting in the dark, the full plants developed were removed from the containers after data recording and transferred to other boxes containing similar sprouting medium and placed near the window to receive natural light. This was carried out to observe the de-etiolation of the full plants. All transferred full plants were labelled accordingly.

Sprouting of seed fractions was carried out for a period of 180 days after no new growth of the full plant or rooting was recorded for a consecutive 30 days. The full plant regeneration rate, rate of only rooting success (with no shoot development), and total sprouting rate for each treatment in Table 4.1 were calculated using the formula in Bányai & Barabás (2002):

$$Rate = \left(\frac{\sum_{i=1}^{k} n_i}{N}\right) \tag{4.1}$$

where,

n_i = number of sprouted experimental units (whole seeds or seed fractions, respectively) on the i^{th} day (not the accumulated number)

N = total number of experimental units

k = last day of the sprouting procedure

Each seed fraction was also recorded for the period taken for root and shoot development, where applicable.

4.2.4.3 Full Plant Development

To observe the morphological changes in response to different light conditions, visual assessments were performed. The morphological assessment was done by observing the difference in colour of cotyledon, hypocotyl, and young leaves, and overall morphology of the full plants.

4.2.5 Statistical Analysis

Whole seed attributes of length, diameter, and weight were each subjected to test of homogeneity followed by descriptive analysis and analysis of variance (ANOVA). Seed fraction weight data were also handled similarly, while mean separation was performed using Tukey's Honestly Significant Difference (HSD) test.

Full plant regeneration rate and total sprouting rate as affected by the combined effect of seed fractionation and sprouting under different light conditions, as in Table 4.1, were respectively subjected to an independent-samples Kruskal-Wallis test. To determine the statistical differences between two independent treatments, Mann-Whitney U tests were carried out accordingly. Lastly, the times to first root development and growth of the shoot from the seed fractions were described using box plots. All statistical analyses were performed using SPSS Version 28.

4.3 Results

4.3.1 Whole Seed Size and Weight

The length, diameter, and weight of whole seeds were recorded before experimentation and subjected to ANOVA to ensure homogeneity of the seed size and weight used for the experimentation. The mean length of intact seeds was 18.56±7.56 mm, while the mean diameter was 23.48±8.12 mm (Table 4.2; Appendix B). The mean weight of all intact seeds used in this study was 13.3729±6.0524 g, showing moderate variability (Appendix B).

Table 4.2
Whole Seed Morphometrics and Weight across Treatments

Light condition	Seed fractionation	N	Length (mm)*	Diameter (mm)*	Weight (g)*
LED	C4L	40	19.11±7.64	24.03±8.5	14.3781±6.1016
	C2L2T	40	18.39±7.43	23.17±8.23	13.1034±6.5901
Natural light	C4L	40	17.91±7.88	22.92±8.86	13.3219±6.6064
	C2L2T	40	18.01±7.84	23.12±7.63	13.0806±5.3546
Dark	C4L	40	18.02±7.6	23.06±7.88	12.7202±5.4448
	C2L2T	40	19.9±6.86	24.61±7.5	13.6327±6.0536
Grand mean			18.56±7.56	23.48±8.12	13.3729±6.0524
P-value			>0.05	>0.05	>0.05

* mean±SD

4.3.2 Seed Fraction Weight

The mean seed fraction weight varied across treatments, as summarized in Table 4.3 (Appendix B). The C4L fractions had more uniform weight, whereas the C2L2T fractions exhibited greater variability. The cutting method itself could have influenced how the reserves were distributed among the seed fragments during the seed cutting procedure. The intact seeds varied in weight, and thus, this affected the size of seed fractions as expressed in fraction weight.

Table 4.3
Seed Fraction Weight across Treatments

Light condition	Treatment	Cut seed fraction	Seed fraction weight (g)
LED	C4L	C4L(L)	3.6228±0.5621 abc
		C4L(CL)	3.8372±0.4307 ab
		C4L(CR)	3.1060±0.4677 abcde
		C4L(R)	3.0881±0.3336 abcde
	C2L2T	C2L2T(LP)	3.9471±0.6893 a
		C2L2T(LD)	2.3804±0.2592 e
		C2L2T(RP)	3.2065±0.2799 abcde
		C2L2T(RD)	2.4702±0.2029 de
Natural light	C4L	C4L(L)	2.8878±0.4277 abcde
		C4L(CL)	3.3983±0.1593 abcde
		C4L(CR)	2.9000±0.2337 abcde
		C4L(R)	3.1335±0.0873 abcde
	C2L2T	C2L2T(LP)	3.2692±0.3601 abcde
		C2L2T(LD)	2.6710±0.2493 cde
		C2L2T(RP)	2.4425±0.4729 de
		C2L2T(RD)	3.6811±0.2974 abc
Dark	C4L	C4L(L)	3.0424±0.8980 abcde
		C4L(CL)	2.8836±0.4562 abcde
		C4L(CR)	3.3299±0.1698 abcde
		C4L(R)	3.2698±0.2803 abcde
	C2L2T	C2L2T(LP)	3.4120±0.3440 abcde
		C2L2T(LD)	2.7429±0.1604 bcde
		C2L2T(RP)	3.5365±0.7777 abcd
		C2L2T(RD)	3.2755±0.4775 abcde

* mean±SD; means having similar letter are not significantly different at P=0.05 by Tukey's HSD test.

4.3.3 Sprouting Rate under Different Light Conditions

The seed fractions developed into full plants at different rates under the different light conditions as studied. Both C4L and C2L2T, respectively, had a full plant regeneration rate of above 1, indicating the feasibility of cutting a seed into separate fractions for acquiring more planting materials (Figure 4.3a; Appendix B). However, the Kruskal-Wallis test showed that there was a statistically significant difference in the full plant formation rate across treatments ($p < 0.05$). It was apparent that more seed fractions were induced to develop full plants when sprouted in the dark, with longitudinal fractions obtained following C4L being better than the quarters of C2L2T for the entire plant regeneration (Figure 4.3a). With dark sprouting, cut seeds obtained with the C4L procedure had the highest mean full plant formation rate of 1.8±0.1826, which was close to double the maximum regeneration rate of 1 achievable by the intact

seeds. One of the seeds subjected to C4L even developed four separate full plants with its four separate fractions, suggesting that the dark sprouting of C4L is likely applicable for propagation purposes. Under the same dark-sprouting condition, C2L2T had a full plant formation rate of 1.625±0.1708, which was statistically comparable to C4L sprouting in the dark.

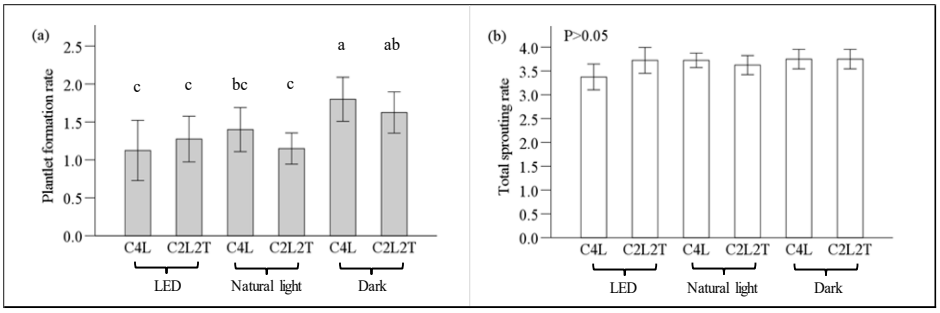


Figure 4.3 Sprouting of C4L and C2L2T under Different Light Conditions; (a) Full Plant Formation Rate; (b) Total Sprouting Rate; Means having Similar Letter in (A) are Not Significantly Different At P=0.05 by Mann-Whitney U-Tests

With natural light sprouting near the windows in the laboratory, the seed fractions of C4L again proved that they were capable of developing more full plants than the quarters of C2L2T sprouted under similar light conditions (Figure 4.3a; Appendix B). On the other hand, both C4L and C2L2T sprouting under 16/8h photoperiod provided by LED had the lowest full plant regenerations (Figure 4.3a; Appendix B).

Despite significant differences in the entire plant formation rates, most remaining seed fractions of both C4L and C2L2T rooted, while the mitotic cells in the cotyledonary fractions were yet to become regenerative for the growth of shoots within the study period (Figure 4.3b; Appendix B). Thus, all treatments studied, as in Table 4.1, recorded high total sprouting rates; there was at least a total sprouting rate of 3.375, as demonstrated by the cut seeds of C4L sprouting under LED, while others had higher total sprouting rates ranging from 3.625 to 3.75, which were almost four-fold as compared to a maximum sprouting rate of 1 achievable by whole seeds. Statistically, the studied treatments did not incur any significant difference in the total sprouting rate (full plant regeneration rate + fraction rooting rate but yet to develop their shoots; $P > 0.05$) (Figure 4.3b).

4.3.4 Photomorphogenesis of Full plant

Seed fractions and full plants sprouting under different light conditions exhibited light-mediated development in response to photomorphogenesis (Figure 4.4). When sprouting under LED or natural light, the cotyledons of seed fractions were dark green in colour while their newly emerged shoots had a normal appearance of light green epicotyls, each with two dark reddish small leaves (Figures 4.4a and 4.4b). In contrast, skotomorphogenesis was observed for the cotyledons of the seed fraction sprouting in the dark. The cotyledons were light yellowish (Figure 4.4c). The small leaves were pale pinkish and their shoots exhibited etiolation phenotype, which was seen as also pale yellowish and elongated hypocotyls (Figure 4.4c). Nonetheless, the transition from skotomorphogenesis to photomorphogenesis made the young plants recover from etiolated growth, turning greenish with normal appearance after being exposed to natural light near the windows of the laboratory.

For the rooted fractions, there was no obvious difference in root morphology (Figure 4.4d – 4.4f). The roots were likely adventitious type, which also likely developed from the cotyledonary surfaces.

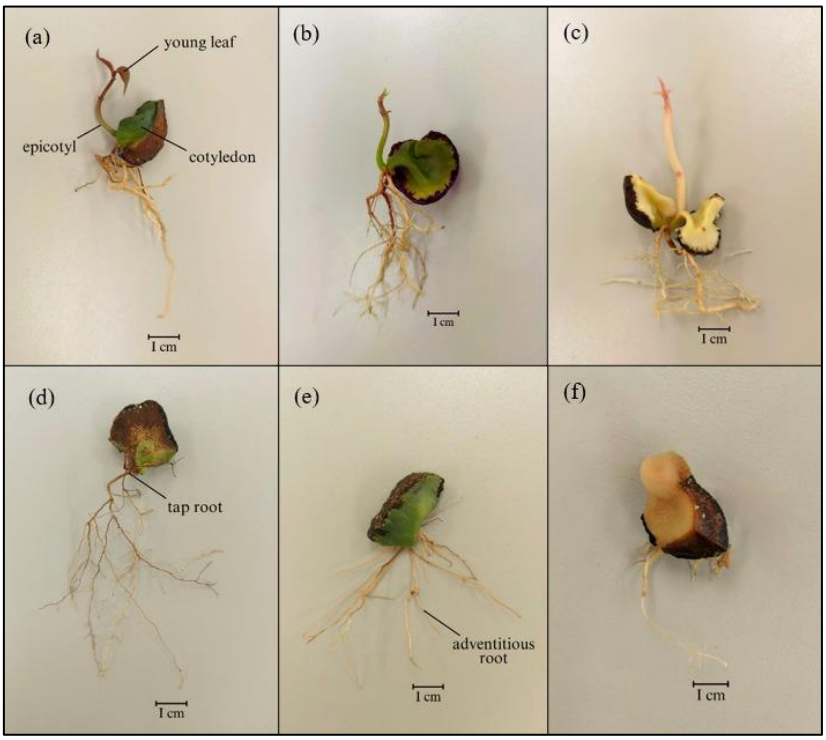


Figure 4.4 Full Plant Developed with (a) C2L2T (RP) Under LED; (b) C4L (CR) with Natural Light; (c) C4L(R) in the Dark; Rooting Success with (d) C4L(R) Under LED; (e) C2L2T (LP) with Natural Light; (f) C2L2T (RD) in the Dark

4.3.5 Sprouting Time

In the event of full plant regeneration, the seed fractions generally had root growth in less than 20 days, except for the few C4L(L) sprouting under LED (Figure 4.5a; Appendix B). The growth of their shoots followed in the next one to three weeks, culminating in the development of entire plants. However, there were possibilities for a long delay in the growth of shoots from the rooted cut seeds; some exhibited their shoot development even after 4 months, regardless of the seed fractionation method and light intensity provided during sprouting (Figures 4.5a and 4.5b; Appendix B). In other words, the sprouting of seed fractions in the dark was appropriate for the production of more full plants, but such a propagation technique did not necessarily achieve full plant development within a shorter time.

For the seed fractions that could root but were yet to develop their shoots, they showed a much wider range of rooting period as indicated by the large quartiles and whiskers in Figure 4.5c (Appendix B). Some rooted fast within several days, but some took more than 4 months to root, while these seed fractions remained alive with very minimal microbial infection until the end of the study period of 180 days, despite having no surface sterilization procedure prior to sowing in the moistened sand.

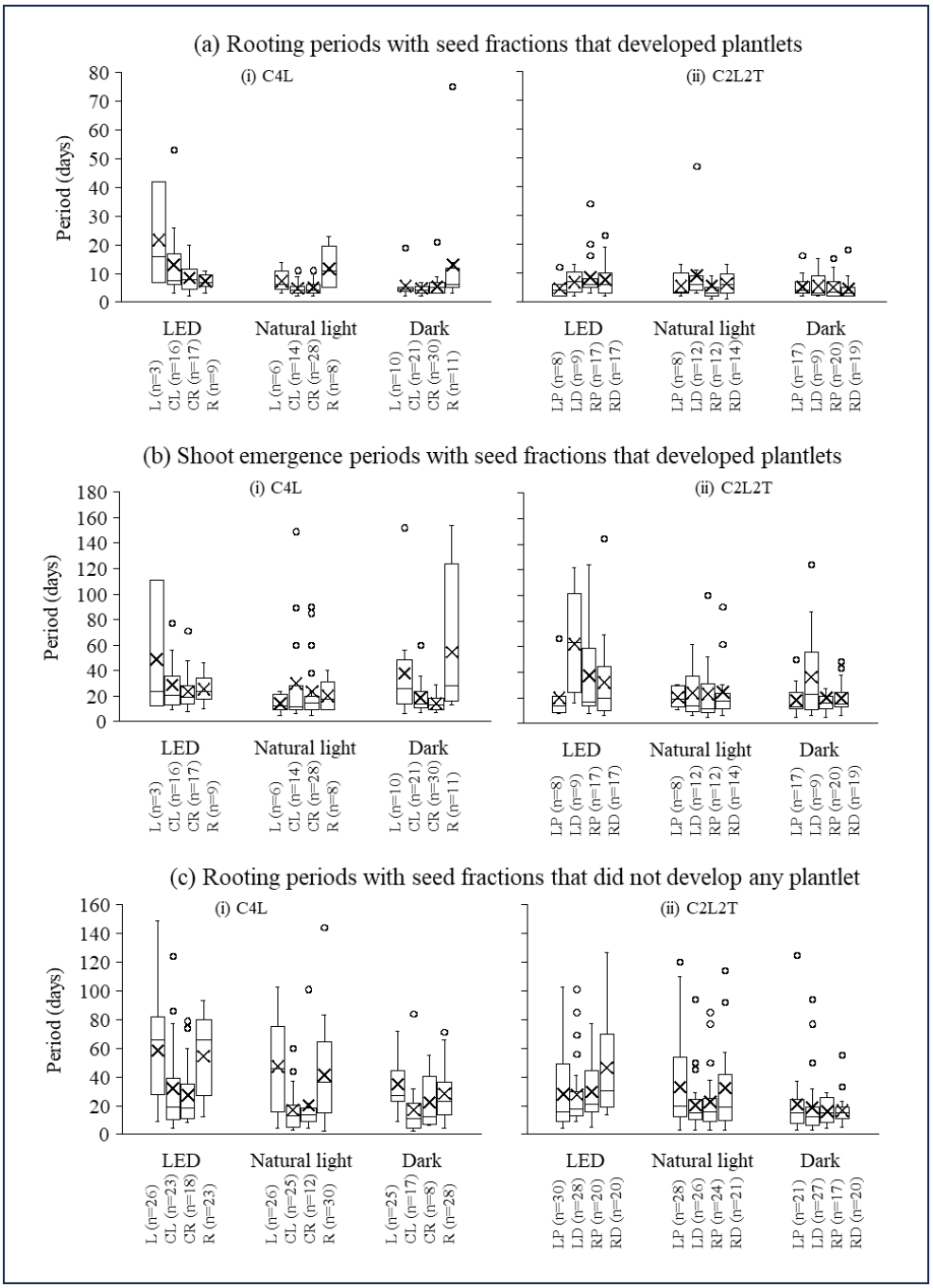


Figure 4.5 Sprouting Period Quartiles and Whiskers of C4L and C2L2T Seed Fractions Under Different Light Conditions; X Indicates Mean Value; O Indicates Outlier

4.4 Discussion

The response of seeds to different light conditions is an important area of study in plant biology, particularly in understanding how this factor could influence germination and subsequent seedling development. During germination, phytochromes mediate between environmental stressors and germination by signaling hormonal cues

that control the process. It has been reported that phytochromes mediate germination responses on the basis of the red/far red ratio occurring during seed development (Franklin & Whitelan, 2005; Cocco et al., 2022) This is possibly due to the phytochromes' properties, sensitivity to environmental stimuli/stressors, and their direct/indirect ability to influence germination. Phytochromes can be found in their inactive red-absorbing form or in their active far-red-absorbing form. This is because these phytochromes are well known for directly regulating the germination responses to light, but particularly for acting indirectly by regulating the conversion of inactive hormones into bioactive ones, hence, inducing the sprouting.

The current study showed that the sprouting of cut seeds of *S. malaccense* was influenced by different light conditions. The seed fractions were able to sprout, but it was found that there were significant differences in the full plant formation rate across treatments. Both the spectral quality and light intensity could have affected the sprouting of *S. malaccense* seed fractions in the current work, in view of the significant differences in the entire plant regeneration rates with constant 16/8h light/dark under the household cool white LED, fluctuating natural light in accordance with weather conditions, and total darkness. The study demonstrated that the number of seed fractions that developed shoots under LED light was lower than the number of seed fractions that sprouted under the other two light conditions. The seed fractions showed high-irradiance response (HIR), and this condition may have reduced the development potential from the cotyledonary surfaces (Mancinelli & Rabino, 1978; Schopfer & Plachy, 1993). As a result, there was a smaller number of shoot development incidences for light-induced seeds. Therefore, seed fractions from both treatments of C4L and C2L2T achieved a higher full plant formation rate when left in darkness.

The photoreceptor in the plants functions to optimize photosynthetic performance and overall fitness throughout their lifespan. Through these mechanisms, plants acclimate to surrounding light conditions by adjusting their morphology to maximize light harvesting (Paradiso & Proietti, 2022). This study has shown variations in the morphogenesis of plants grown under different light conditions. These adaptations are important for their survival. For instance, the light-induced plants showed shorter epicotyl growth, due to photoinhibition in response to prolonged and excess light absorption (Goh et al., 2012). This is because of the existence of photoreceptors in the shoots (Lee et al., 2016). The cells respond differently to too intense or too long illumination at different growth stages or, simultaneously, under

varied circumstances, for example, upon injury with predation or deliberate incision carried out on an intact seed (Nameth et al., 2012).

Concerning the regenerative potential, growth and development in any cell, specific lighting would be beneficial, while others could reverse the responses. For instance, the blue light emitted by the LED lamp has been reported to suppress mitotic cell activity (Suzuki et al., 2010). The suppressed mitotic activity under LED illumination, as suggested by Suzuki et al. (2010), may not entirely be true in the case of fragmented seeds of *S. malaccense*, as the seed fragment could sprout well under LED, at least with rooting from the cut surfaces.

The findings of this investigation revealed that the cotyledon of *S. malaccense* was pale yellowish in color (Figure 4.4f), similar to other dark-induced seeds of other species in previous studies (Suzuki et al., 2010; Lee et al., 2016). In the absence of light, the shoot also becomes etiolated and demonstrates a symptom of a lack of chlorophyll. It is also referred to as the shade avoidance syndrome (Franklin & Whitelan, 2005; Repeat, 2020; Wang et al., 2020). Thus, a dark-induced plant of *S. malaccense* exhibited greater epicotyl elongation compared to those grown under light. In a natural environment, this elongation is a survival strategy that allows the newly developed plants to reach light sources more effectively once they emerge from the soil. These symptoms were, however, reversible, as the etiolated plants recovered and turned green colour when they subsequently received natural light near the windows of the laboratory after removal from the dark sprouting conditions. The ecological implication of this finding is significant because seeds that can sprout in dark conditions may have a competitive advantage in environments where light availability is limited or fluctuates. This adaptability is important for the species' survival in a diverse and changing environment.

The sprouting time is influenced by the abiotic competitive environment (high light versus low light), with some species germinating earlier in resource-limited (low light) settings (Zhang et al., 2014). The limited exposure to light in the dark sprouting of seed fractions with reduced mass may have created a condition of low resource availability within the seed fragments. Zhang et al. (2014) stated that there was a correlation between germination time and seed mass in their study in the Tibetan Plateau in China. In other words, this lacking condition with regard to light as an essential resource in the life of a plant could have created a sense of urgency that induced the development of the shoot, as it had limited resources to sustain itself over a prolonged

period of time. This is especially true for seed species that are adapted to low-light conditions, such as those that naturally grow under a forest canopy, like *S. malaccense*.

The current findings enhanced knowledge on how light conditions affect the regeneration of seed fragments for improved propagation efficiency, particularly for species with uncertain seed availability following predation, for example, *S. malaccense.* The implications of this research encompass not only advancing cost-effective plant propagation practices but also contributing to the understanding of cotyledonary fragment responses to different light conditions.

4.5 Conclusion

The objective to determine the influence of light conditions on the regeneration of quartered *S. malaccense* seeds was achieved. The results demonstrate that light conditions significantly affected the capacity for full plantlet development, with dark sprouting at ambient temperature yielding the highest number of entire plants compared to LED or natural light conditions. In darkness, the longitudinal cut seeds resulted from C4L, especially the C4L(CL) and C4L(CR) seed fragments, showed a relatively higher capability of forming full plants. Furthermore, the dark-induced plant's ability to de-etiolate and acclimate to the changing light conditions determined the competitive capability of this technique for the propagation of this woody plant species. The cut seed sprouting technique used in this study has the potential to guide planting material production, as well as conservation efforts. The findings of this study will help other researchers, plant scientists, and even nurserymen to understand the light requirement of the propagation of seed fractions of this fruit species. The perseverance of regenerative cells in the cotyledonary fractions in darkness, as evidenced by inconsistent and even long delayed sprouting periods, calls for additional research in order to facilitate mass production of planting materials through dark sprouting of cut seeds. The subsequent plant performance should also be further studied.

CHAPTER 5
PLANT GROWTH PERFORMANCE FOLLOWING IN VIVO REGENERATION FROM SEED FRACTIONS

5.1 Introduction

The quality and morphology of fruits and seeds are fundamental for successful plant propagation, yet these traits can exhibit significant variability. The resulting morphological characteristics, which can be profoundly influenced by differing ecological conditions and genetic differences among individual plants, are critical for a species' reproductive strategy (Wulff, 1986; Michaels et al., 1988). For instance, environmental conditions such as soil composition, nutrient availability, light exposure, and local pest pressure can directly affect the maternal plant. These factors can lead to observable variations in fruit size, shape, and seed viability (Moreira et al., 2021; Vidaković & Poljak, 2024; Shahbazian et al., 2025). These morphological variations in turn determine critical aspects of the plant's life cycle, including germination success, and subsequent growth of seedling development (Yanlong et al., 2007; Liu et al., 2015).

The early seedling development is a vulnerable phase for most plant species; therefore, a vigorous seedling is more likely to thrive. A seedling is considered vigorous if it shows rapid and uniform germination under a wide range of conditions (Sivasubramaniam & Selvarani, 2012; Mondo et al., 2013; Mahender et al., 2015; Maulana et al., 2021). Rapid germination and shoot development are important as they help the plant cope with environmental stress (Mahender et al., 2015). A seedling that emerges earlier will typically have a more robust development than those that experience a delay in emergence (Mondo et al., 2013). Another trait that is closely related to early seedling vigor is stem diameter, leaf area per seedling, and height of the seedling (Mondo et al., 2013). These characteristics are highly associated with the seedling's competitive ability for resources like sunlight, which is critical for photosynthesis.

For the monoembryonic seeds, the seed contains only one zygotic embryo that will germinate into a single seedling. In other angiosperms, a single seed can develop multiple embryos, which then give rise to multiple seedlings. This occurrence is referred to as polyembryony. While most citrus species are well known for having

polyembryony, some species in the families of Myrtaceae and Clusiaceae have been reported to show regenerative capacities from cotyledonary surfaces of seed fragments, analogous to polyembryony (Sivasubramaniam & Selvarani, 2012; Nacata & de Andrade, 2020; Tsan, 2023).

The multiple plants from a seed can consist of all zygotic seedlings, all nucellar plants or a combination of both types. To identify the type of regenerated plants, researchers use various identification methods. One of these methods is morphological characterization. Morphological identification relies on observable physical traits, or phenotypes, such as a seedling's height, leaf size, and stem diameter (Kishore, 2016). While this approach can provide a quick assessment, its findings are often debated among scientists. On the other hand, molecular techniques are used for genetic similarity analysis for the multiple plants regenerated from a single seed. These include studies with molecular markers such as random amplified polymorphic DNA (RAPD) analysis, simple sequence repeat, and inter-simple sequence repeat-polymerase chain reaction (ISSR-PCR) (Liu & Yang, 2012; Ochoa et al., 2012; Rohela et al., 2013; Prakash et al., 2016). RAPD and ISSR are the most commonly used molecular marker techniques to study the genetic similarity of the mother plant and offspring because no prior knowledge of the genome sequence is required. These techniques will detect polymorphism between the regenerated full plant and the mother plant. These are the recent, more advanced, and reliable methods to study gene expression.

To understand the above factors on the regeneration of *S. malaccense* from seed fractions, this chapter describes the maternal environmental effects on the morphological characteristics of fruits and seeds from two locations, followed by an analysis of the regeneration success of seed from these locations following longitudinal seed quartering (C4L). The findings from these analyses were then discussed to understand the seed source factor for effective propagation strategies. Following regeneration from these seed fractions, the growth performance of the plants was also assessed using key metrics such as height and leaf area. This question revolves around the seed-seedling hypothesis, which states that larger seeds are more likely to produce vigorous seedlings compared to smaller ones (Green & Juniper, 2004, 2004a; Quero et al., 2007). The rationale behind this is the abundance of stored reserves in larger seeds, which are critical for supporting seedling establishment and development, especially in conditions where environmental resources are scarce. This experiment was in line with Objective 3, which was to assess the growth performance of plants regenerated from

quartered seeds in expressing polyembryony. The research was guided by two primary hypotheses. First, it was hypothesized that significant differences in fruit and seed morphology would be observable for samples taken from two different locations, a result of the maternal effects of varying environmental conditions. The second hypothesis was that seed fragments with a greater initial mass would produce more vigorous plants with greater height and leaf area. Comprehending these intricate relationships is critical for the development of robust and efficient propagation protocols tailored for this specific species.

5.2 Materials and Methods

This section details the materials used and the procedures to assess the plant growth performances in plug trays containing peat moss following regeneration from *S. malaccense* seed fractions. This experiment aimed to identify the potential of clonal planting material production for this fruit tree species.

5.2.1 Test Material

S. malaccense fruits were collected from two different locations in January 2024: Merlimau and Muar (Table 5.1). The sampled trees were located on two different private properties. The study sites were chosen based on the presence of flowering and fruiting individual trees, habitat type, range of climatic conditions, accessibility, and landowner consent or a combination of these factors.

S. malaccense tree in Merlimau, Malaysia (2.05° N, 102.42° E) was located in an open, sun-exposed urban environment (Figure 5.1). The tree was situated in an unshaded area, adjacent to a house and a paved road. It had a single, well-defined trunk with a broad, dense, and rounded canopy. The tree was healthy and fully foliated. In such an open environment, the tree had minimal canopy competition from surrounding vegetation.

Table 5.1
Locality Information for Seed Sources for Studying Growth Performances Following Regeneration from C4L Seed Fractions

Location	Coordinate	Land use
Merlimau	2.05° N, 102.42° E	Private property planted with fruit trees for personal consumptions.
Muar	2.06° N, 102.58° E	Home garden with fruit tree and forest trees at vicinity.



Figure 5.1 The Tree in Merlimau where Seeds were Collected for Studying Growth Performances Following Regeneration from C4L Seed Fractions

S. malaccense tree in Muar, Malaysia (2.06° N, 102.58° E) was located in a shaded, semi-forest environment (Figure 5.2). Unlike the tree in Merlimau, this tree was surrounded by taller, mature vegetation, including a large tree that imposed a significant amount of shade on this *S. malaccense* tree at some hours daily. The tree in Muar had a multi-stemmed structure, with several trunks emerging from the base. Its canopy was less dense compared to the tree from Merlimau, suggesting that it had adapted to or been influenced by light competition. The surrounding environment was characterized

by limited direct sunlight and competition from neighboring plants.



Figure 5.2 The Tree in Muar, where Seeds were Collected for Studying Growth Performances Following Regeneration from C4L Seed Fractions

In each location, 45 half-ripe fruits were harvested from a single tree. The fruits were stored in labelled plastic bags and transported to the Plant Science Laboratory, as described in 3.2.1(Test Material). Upon arrival at the laboratory, the half-ripe fruits were also allowed to fully ripen for a week before experimentation, as described in 4.2.1.

5.2.2 Study Location

The research work on the assessment of the plant growth performances following regeneration from cut seeds was conducted at the Plant Science Laboratory of Universiti Teknologi MARA (UiTM), Jasin Campus.

5.2.3 Experimental Procedure

The section explains procedures for assessing plant growth performances, starting from the measurement of fruit and seed morphometrics, fruit and seed weight, seed fractionation, sprouting, plant transplanting, and growth performance recording.

5.2.3.1 Fruit and Seed Morphometrics

After incubation at ambient temperature in the laboratory, 40 intact and fully ripe fruits were selected randomly. Each fruit was labelled for traceability throughout the study. The morphometric and weight data of fruits were taken before seed extraction. Fruit length and diameter were measured with a Vernier caliper. Fruit length was determined by placing the caliper's jaws along the longitudinal axis from the fruit stalk scar to the basal part of the fruit. The fruit diameter was measured by placing the caliper's jaws at the widths of the fruit perpendicular to the longitudinal axis. The first measurement was taken at the fruit's widest point, and the second was taken perpendicular to the first. The average of these two measurements was calculated and used for all subsequent statistical analyses. All measurements were recorded in millimeters (mm). On the other hand, the weight of each fruit was recorded accordingly using an analytical balance.

The seeds were manually removed from each fruit by using a sharp knife, taking care of traceability, and also not to injure the seeds. After that, the seeds were thoroughly washed under slow-running tap water, air dried for 2h on a paper towel, followed by measurements of their length and diameter using the Vernier caliper, and weight measurement using the analytical balance.

5.2.3.2 Medium Preparation

Sand sized 0.2 to 2.0 mm was washed, sun-dried, and used as a cut seed sprouting medium in this study, similar to medium preparation in preceding experiments.

5.2.3.3 Seed Fractionation

Each seed was quartered longitudinally by using a sharp knife, i.e., subjected to seed cutting treatment C4L. Then, the seed fractions of C4L(L), C4L(CL), C4L(CR), and C4L(R) were sprouted and assessed for their plant growth performance. Each treatment was replicated four times, each with 10 seed fractions.

5.2.3.4 Seed Fraction Sprouting in the Dark

Seed fraction sprouting was also carried out based on a completely randomized design in labelled containers measuring 14.5 x 9.5 x 6.5 cm each in moistened sand medium. Containers were also placed under blackout clothes on the laboratory bench for dark sprouting purposes. The temperature and relative humidity of the laboratory were also 28±2 °C and 55±5%, respectively, during the experimentation. The sprouting seed fractions were also taken care of similarly to ensure sufficient water and oxygen requirements. The sprouting of the seed fractions was also carried out for a period of 180 days after no new rooting or full plant development was recorded for a consecutive 30 days.

5.2.3.5 Regenerated Plant Transplanting and Growth Performance Assessment

Full plants successfully developed from seed fractions in the dark were assessed for their growth performances. Each plant with a shoot length of approximately 2 cm was carefully taken out of the dark sprouting container. The tagged plants were transferred to plug trays (170×170×120 mm) with peat moss medium.

Following transplantation, the plants underwent a one-week acclimatization period on a laboratory bench first to prevent transplanting shock. Subsequently, they were moved to the corridor of the laboratory to receive partial sunlight exposure. This provided a natural light environment conducive to photosynthesis while avoiding the stress of direct, high-intensity sunlight.

The plants were watered with a quarter-strength Hoagland solution every two days. Plant growth performance was systematically assessed after a period of two months. The primary growth parameters measured were plant height and leaf area, which served as key indicators of plant vigor and development.

5.2.4 Data Collection

Data collection was performed at specific intervals throughout this experiment. Morphometric and qualitative data were collected to assess both the initial characteristics of the seed fractions and the subsequent plant growth and development. All measurements related to morphometric data, regeneration success, and plant growth (e.g., height, leaf area) were collected from these individual units. This was to ensure that the statistical analyses accurately reflected the characteristics and performance of each unique seed fraction, providing a thorough finding about the regenerated plants from seed fractions.

5.2.4.1 Initial Fruit and Seed Measurements

Prior to seed fractionation and sprouting, 40 intact fruits in four replicates from each location were randomly selected and tagged for morphometric analysis. The length (mm) and diameter (mm) of each fruit were measured using a Vernier caliper. Then, each tagged fruit was also weighed using an analytical balance. After the fruits were cut and the seeds were extracted from the fruits, the seeds were also measured for their morphometric parameters (length and diameter) and weight.

5.2.4.2 Sprouting Rate

Throughout the experiment, observations were made every two days to record the time of root emergence and shoot development. The full plant regeneration rates, rates of rooted seed fractions not followed by shoot development, and the total sprouting rates (rate of full plant development + rate of only rooting not accompanied by shoot development) were calculated according to the formula in Bányai & Barabás (2002):

$$Rate = \left(\frac{\sum_{i=1}^{k} n_i}{N}\right) \tag{5.1}$$

where,

n_i = number of sprouted experimental units (whole seeds or seed fractions, respectively) on the i^{th} day (not the accumulated number)

N = total number of experimental units

k = last day of the sprouting procedure

5.2.4.3 Plant Growth Performance

All plants raised in peat moss supplied with quarter-strength Hoagland nutrient solution were assessed for growth performance two months after planting (transfer to plug trays). The plant height, measured in cm, was taken from the root collar to the apical point of the main stem in its form using a measuring tape or ruler, whichever applicable (Figure 5.3a). Meanwhile, the area of each leaf of each plant was estimated using a transparent plastic sheet marked with 1x1 cm square grids (Figure 5.3b). Then, the total leaf area of each plant in cm^2 was calculated accordingly.



Figure 5.3 Measuring the Plant Growth Performance by Using (a) a Ruler to Measure the Plant Height From the Root Collar to the Apical Tip of the Main Stem; (b) a Transparent Plastic Sheet with 1x1 cm Square Grids over a Leaf

5.2.5 Experimental Design and Statistical Analysis

The experiment was set up in a completely randomized design with four treatments, i.e., C4L(L), C4L(CL), C4L(CR), and C4L(R), each was replicated four times. There were 10 seed fractions per replicate. Each seed fraction was treated as a single unit within a replicate. Statistical analyses were performed using SPSS. To analyse the morphological attributes and weight of fruits and seeds obtained from Merlimau and Muar, the data were subjected to the Mann-Whitney U tests. A Mann-Whitney U test was selected to compare the two independent groups of fruits and seeds

from each location because the data were not normally distributed. Seed fraction weight data were analysed using the Kruskal-Wallis test. All statistical analyses were performed at a significance level of α=0.05. Full plant development rates, rates of rooting with no shoot development, and death rates for each location were summarized and presented using a chart.

To illustrate the plant growth performances, plant height was plotted against the time taken for shoot (full plant) development from the seed fractions, in accordance with the locality of acquisition of fruits (seeds) to explain the possible growth of clonal plant with delayed full plant development. Likewise, total leaf area was also plotted against the time taken for shoot (full plant) development from the seed fractions, in accordance with the locality of acquisition of fruits (seeds). To test the reserve effect hypothesis, plant height and total leaf area of the plant were, respectively, plotted against the seed fraction weight, in accordance with the locality of acquisition of fruits (seeds).

5.3 Results

This section reports the morphological attributes of fruits and seeds from two different locations, i.e., Merlimau and Muar, their seed fraction weight, developmental outcomes, and subsequent plant growth performances following regeneration from seed fractions.

5.3.1 Fruit and Seed Attributes

A significant difference was found in the morphology of fruits harvested from the two different locations. Fruits collected from Merlimau were significantly larger in length, breadth, and weight compared to those from Muar (Table 5.2; Appendix C). The mean weight of fruits in Merlimau differed by 14.86 g; these fruits were heavier than those obtained from Muar.

In contrast to the fruit morphology, all morphological attributes of seeds from both locations exhibited no significant difference. The seeds also did not differ significantly in their weight despite the fruit sources from these two different locations (Table 5.2; Appendix C).

Table 5.2
Morphometrics and Weight of Fruits and Seeds Used for Studying Growth Performances Following Regeneration from C4L Seed Fractions

	Parameter	Merlimau	Muar	P-value*
Fruit attributes	Length (cm)	58.90±6.38 (42.70-68.00)	53.62±3.55 (47.45-63.00)	<0.05
	Breadth (cm)	47.41±6.36 (30.30-61.10)	43.05±4.10 (35.00-52.00)	<0.05
	Weight (g)	64.74±11.26 (44.56-110.07)	49.88±12.62 (29.33-76.94)	<0.05
Seed attributes	Length (cm)	24.14±2.88 (17.45-29.40)	24.03±2.78 (18.80-34.00)	>0.05
	Breadth (cm)	28.64±3.00 (22.00-35.50)	27.69±3.59 (19.30-37.00)	>0.05
	Weight (g)	11.27±3.36 (5.21-19.82)	11.54±4.02 (4.89-26.31)	>0.05

N=40 for each location; mean±SD; figures in parentheses are minimum-maximum values;
* within row is calculated with Mann-Whitney U test.

5.3.2 Seed Fraction Weight

The analysis of seed fraction weight, categorized by location and fraction type, is summarized in Table 5.3 (Appendix C). The seed fractions were not statistically different in weight ($P > 0.05$), but the standard deviation values for means of weight across different fraction types from the two different localities were high. This means that the fractions had varied sizes, probably attributed to the varied seed sizes, as well as some inconsistencies in the seed fractionation procedure.

Table 5.3
Weight of Seed Fraction Subjected to C4L Seed Fractionation

Fraction type	Merlimau	Muar	P-value*
L	2.4672±0.9587 (0.7881-4.1127)	3.0329±1.0818 (1.3144-6.3560)	0.064
CL	2.7450±1.1082 (1.1339-6.8100)	2.6096±1.1100 (0.7873-5.3672)	
CR	2.6221±0.9927 (1.2945-6.3484)	2.8411±1.1599 (1.3134-6.5659)	
R	2.3020±1.0066 (0.7823-5.2564)	2.7287±1.2369 (0.9836-7.3733)	

N=40 for each location; mean±SD; figures in parentheses are minimum-maximum values;
* calculated with Kruskal-Wallis test

5.3.3 Sprouting from Seed Fractions

Figure 5.4 reveals the frequencies of full plant development for *S. malaccense* seed fractions according to fruit sources of Merlimau and Muar, categorized by seed fraction types of L, CL, CR, and R. The centre fractions of CL and CR were better in regeneration of full plants as compared to the leafmost (L) and rightmost (R) fractions (Appendix C), regardless of the sources of test materials or the maternal environment. Nevertheless, fractions obtained from the fruits harvested in the housing area of Merlimau had a relatively higher full plant regeneration performance, while the effect of environmental stresses on such a seed fragment regeneration potential is yet to be studied. With the 40 fruits and 40 seeds from Merlimau, the seed fractions provided 91 full plants in total, while a similar number of fruits and seeds from Muar regenerated 80 full plants with their quartered fractions. The number of full plants was relatively higher compared to that obtained with dark sprouting in the preceding chapter (72 full plants) (Appendix B), indicating that the environment of the mother plant, for example, the nutrition, could also affect the regeneration potential of the seed fractions. This aspect of the study awaits further investigations.

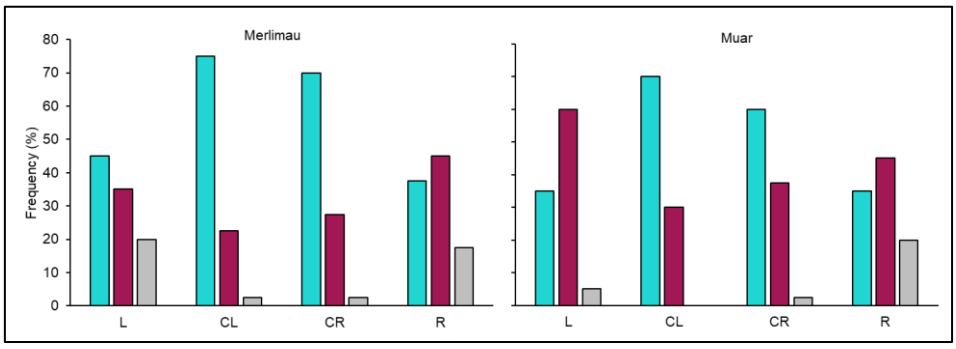


Figure 5.4 Frequencies of Full Plant Development (Cyan Bars), only Rooting Success (Dark Red Bars), and Death (Grey Bars) by Seed Sources

Figure 5.5 presents the regenerative capacity of *S. malaccense* seed fractions, showing that all four fractions from the same single seed were capable of developing into full plants. This observation was obtained with seeds from both Merlimau and Muar and strongly supports the quantitative data presented in Figure 5.4, which revealed a high frequency of successful full plant development across all seed fragment types (L, CL, CR, and R).

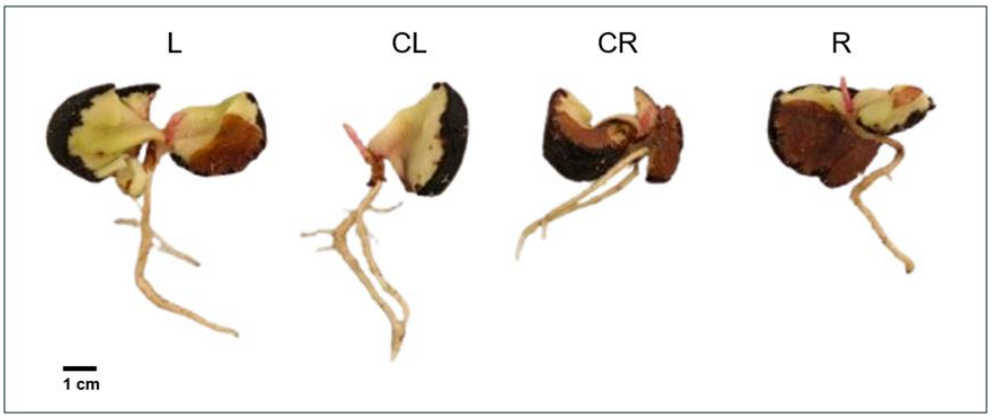


Figure 5.5 Full Plant Development from All Four Fractions of a Single Seed

Figure 5.6 shows that the time for seed fractions to develop into full plants and roots varied between the two locations. For the seeds obtained from Merlimau, many seed fractions of CL and CR sprouted faster, while there were also fractions that showed delayed rooting and growth of shoots (Appendix C). On the other hand, their rightmost (R) fractions took a much longer time to root and develop full plants. Seed fractions that developed adventitious roots generally took a longer time for rooting as compared to those that successfully developed full plants.

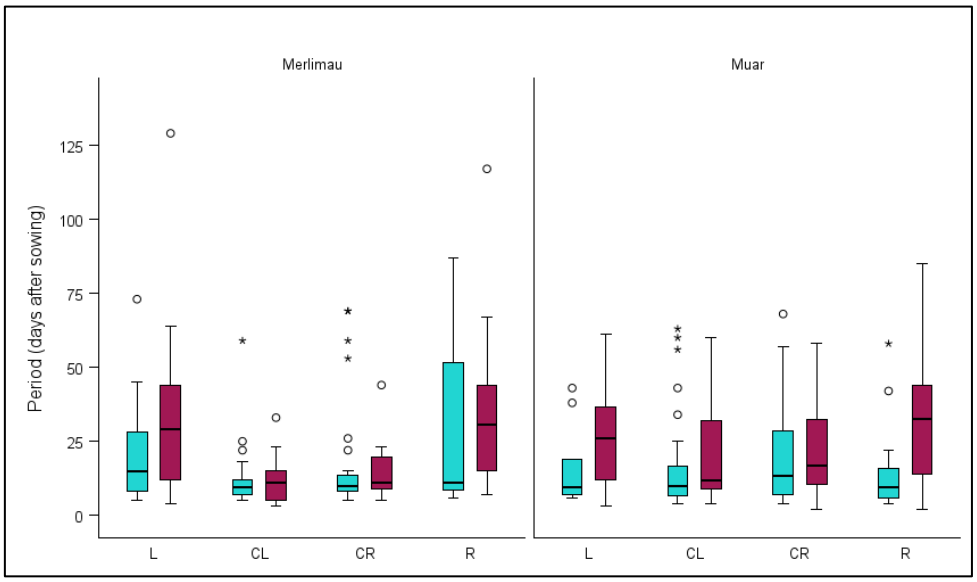


Figure 5.6 Full Plant Development Time for Fractions that Could Develop Both Their Roots and Shoots (Green Bars), and Rooting Time for Fractions that Showed Only Rooting Success (Dark Red Bars) by Seed Sources; Circles and Asterisks Represent Mild and Extreme Outliers, Respectively

For the seeds originating from Muar, their fractions generally showed less consistent sprouting time, especially for rooting time (Appendix C). While these

fractions provided fewer full plants compared to the fractions of seeds from Merlimau, the longer sprouting or full plant regeneration time could indicate that more fractions from Muar had adventitious growth.

5.3.4 Plant Growth Performance

When raised in moist peat moss with quarter-strength Hoagland nutrient solution in plug trays, the full plants developed earlier from the seed fractions showed superior growth characteristics in terms of height and leaf development as compared to the plants that showed delayed sprouting (Figure 5.7). Figure 5.8 depicts plant growth performance after two months, examining the relationships between developmental parameters (time for shoot development) and subsequent growth metrics (height and leaf area) (Appendix C). There was a correlation between the time taken for shoot development from seed fractions and subsequent plant growth. The seed fractions that initiated the growth of shoots rapidly produced taller plants with larger leaves (greater leaf area) by two months after transfer to plug trays. Furthermore, the data also suggests a possible influence of the seed source, as plants originating from Merlimau generally exhibited faster shoot development and better growth performances as compared to those from Muar. However, regeneration from seed fractions of *S. malaccense* was again proven uncorrelated to the reserve effect hypothesis. In Figure 5.9, larger seed fractions did not necessarily produce larger plants (Appendix C). Some small seed fractions could regenerate large plants.

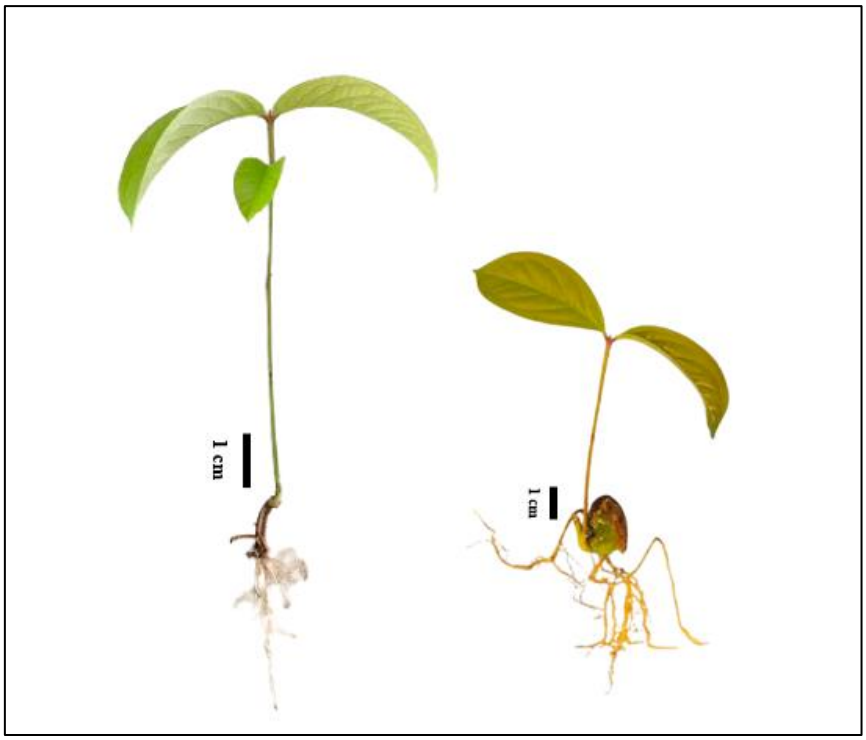


Figure 5.7Plants at Three Weeks After Transfer to Peat Moss in a Plug Tray; The Larger Plant Originated from a Fraction That Regenerated at 4 Days After Sowing (Left), and the Smaller Plant Originated from a Fraction that Regenerated at 38 Days After Sowing (Right); the Plants Showed Obvious Differences in Plant Height and Leaf Size Due to Regeneration Time from Seed Fractions

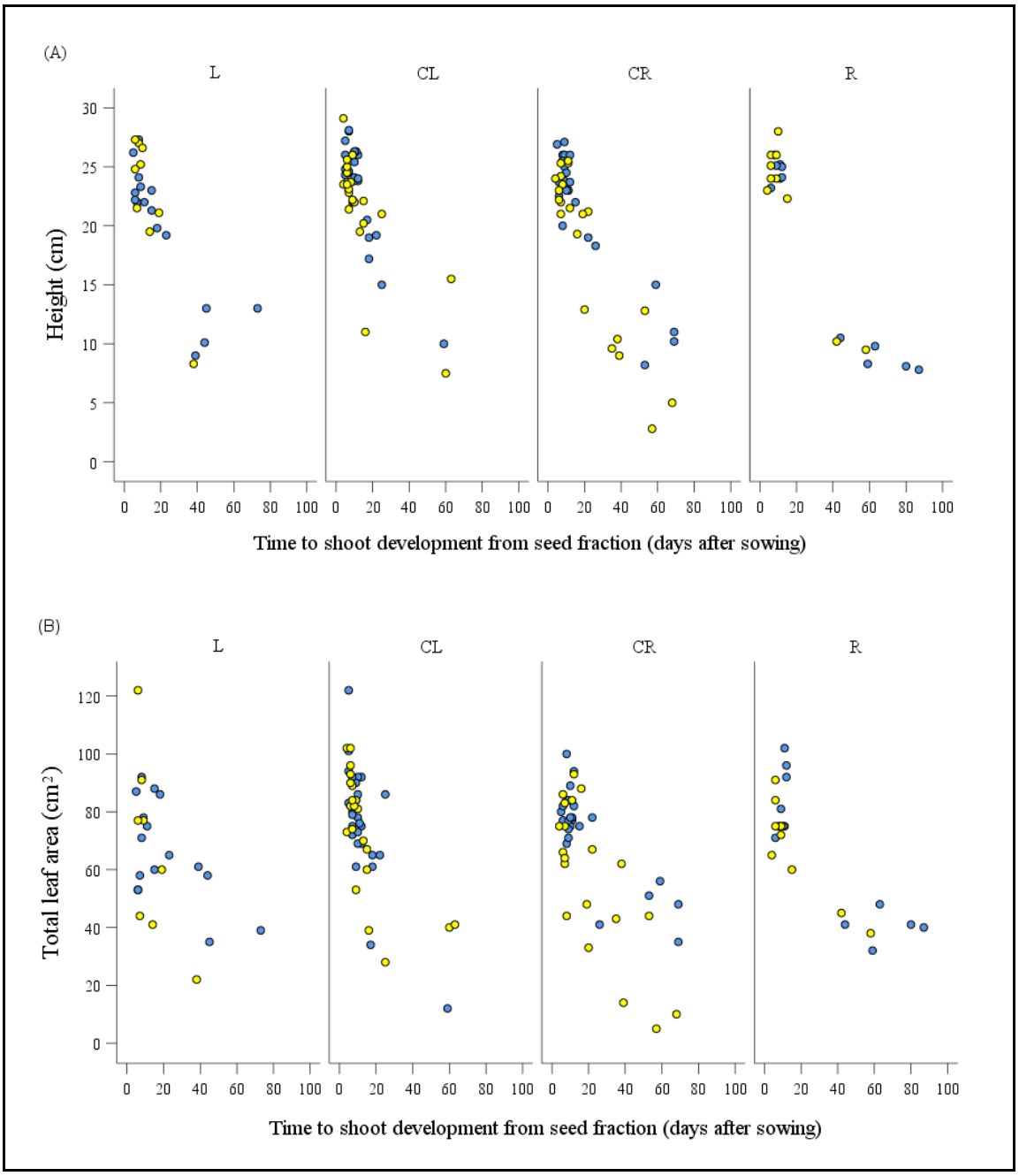


Figure 5.8 Height (a) and Leaf Area (b) of Plants After Two Months Against Time for Shoot Development from Seed Fractions; Blue and Yellow Circles Represent Seeds Collected From Merlimau and Muar, Respectively

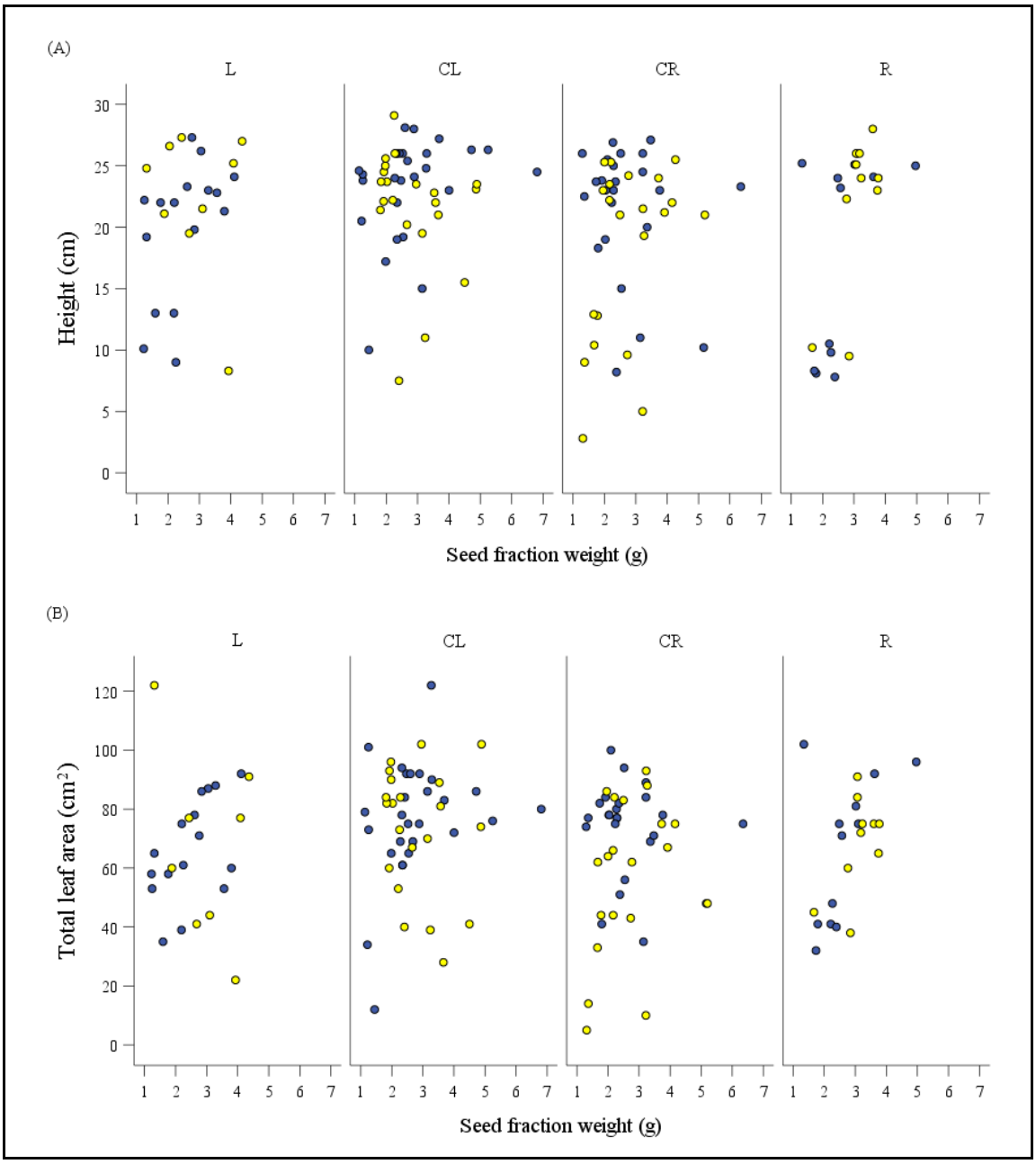


Figure 5.9 Height (a) and Leaf Area (b) of Plants After Two Months Against Seed Fraction Weight; Blue and Yellow Circles Represent Seeds Collected from Merlimau and Muar, Respectively

5.4 Discussion

The study investigated the fruit collected from two sites, namely Merlimau and Muar. The study demonstrated a significant maternal effect on fruit morphology, with Merlimau fruits being significantly larger in length, breadth, and weight compared to those from Muar ($P<0.05$). This is likely due to the more favorable environmental conditions of one mother plant compared to the other. Surrounding maternal growing

conditions include greater sunlight exposure, water availability, and potentially better soil conditions (Gonçalves et al., 2023; Vidaković & Poljak, 2024; Shahbazian et al., 2025).

To illustrate the differences of the mother trees in this study, *S. malaccense* tree in Merlimau was situated in a private property, growing at a distance from other crops, and it was taller than the building next to it. In contrast to open growth conditions, *S. malaccense* plant in Muar was surrounded by taller vegetation, including mangosteen trees and other forest species, and was slightly shorter than its neighboring trees. It is possible that the surrounding taller trees in Muar shaded *S. malaccense* tree, limiting its access to direct sunlight to some extent. Consequently, this reduced sunlight availability may have caused the tree to prioritize resource allocation towards survival mechanisms rather than fruit development, potentially affecting fruit characteristics.

Furthermore, there is a noticeable difference in the environment of the two mother plants. The soil surface around the mother plant in Merlimau was clear of weeds, while the plant in Muar was surrounded by the growth of various weed species. This obvious environmental condition may have contributed to the observed morphological differences in the fruits. This is because certain weed species are known to produce allelochemicals, which are secondary metabolites that can inhibit the growth and development of nearby plants. These chemicals can disrupt physiological processes in neighboring plants, such as cell division, nutrient uptake, photosynthesis, and flowering, etc. (Putnam & Duke, 1978; Shirgapure & Ghosh, 2020). This might have caused the reduction in fruit size and overall yield. Since the tree in Merlimau experienced less competition from surrounding weeds, this might explain the more favorable fruit characteristics it produced compared to the tree in Muar. While the site-specific differences between Merlimau and Muar were statistically significant ($P < 0.05$), the exact factors remain unclear as soil nutrient profiles and microclimatic data were not quantified. These factors are only proposed as potential factors to the observed variance. However, similar findings by previous researchers have been attributed to maternal environments (Nguyen et al., 2021; Moreira et al., 2021; Vidaković & Poljak, 2024; Shahbazian et al., 2025).

Although there were differences in fruit size, seed attributes (length, breadth, and weight), and seed fraction weight were not significantly affected by the differences between the two locations of maternal sources. This stability likely contributed to the consistently high frequency of successful full plant development observed across seed

fractions with different origins as studied. In the current study, *S. malaccense* seeds exhibited relatively uniform size despite variations in fruit size. The stability in seed weight also contributed to the stability in seed fraction weight, showing no significant difference across treatments. This suggests that seed size in *S. malaccense* was a highly stable trait, potentially influenced more by genetic factors than by the maternal environment (Michaels et al., 1988; Gonçalves et al., 2023).

The study recorded a high frequency of successful full plant development across both locations. This uniformity in plant development, despite the seeds' origins, may be due to the optimal conditions provided during the sprouting process. Sprouting the seed fragments in the dark is believed to contribute to the consistent sprouting rates. However, while overall plant development was consistent, there were also variations in rooting success and instances of plant death, indicating that there were other factors influencing the regeneration process.

The analysis of plant performance provided insights into the subsequent growth of the regenerated full plants. The results implied that seed fractions that exhibited early sprouting produced taller plants with larger leaves. Some authors suggested that early shoot emergence is one of the indicators of plant vigor (Mondo et al., 2013; Mahender et al., 2015). Similar results were reported by Sivasubramaniam & Selvani (2012) on the polyembryonic *Syzygium cumini* seeds. Among the two factors (time to develop shoot and seed fraction weight) in the current study, the most compelling finding was that early shoot development time appeared as a stronger predictor of plant size than initial seed fraction weight. A seed fragment that quickly establishes a shoot could rapidly begin photosynthesis and become independent of its initial reserves, potentially outperforming a heavier seed fragment that is slower to initiate growth. As indicated by Finch-Savage and Bassel (2016), seedlings that emerge later after sowing will inevitably be smaller at any given point in time compared to seedlings that germinate quickly. This highlights the importance of physiological vigor and the efficiency of early developmental processes. When the lighter seed sprouts and develops a shoot within a week, while the heavier one takes two weeks, the early-sprouting seedling has a week's head start in terms of photosynthetic capacity and resource acquisition, potentially leading to a larger plant.

On the other hand, there was no obvious correlation between seed fraction weight and plant growth parameters (height and leaf area). Instead of the reserve effect hypothesis, this finding aligns with general totipotency principles; the seed fractions,

regardless of size (reserves), had the potential to develop full plants (Green & Juniper, 2004a; Souza & Fagundes, 2014; Loayza et al., 2015; Velasquez-Rosas et al., 2017). *S. malaccense* seed fractions may have the ability to initiate sporophytic apomixis, as is well known for citrus species. Such asexual growth will lead to the formation of nucellar or adventitious embryo(s), usually following certain stimuli (Souza-Pérez and Speroni, 2017; Xu et al., 2021; Ren et al., 2024). This could bring about multiple embryos that develop into full plants, which are likely genetically identical to the mother plant. In the current research work, and as also reported in the preceding chapter, sprouting in the dark could be advantageous for the development of more full plants from the seed fractions. Other evidence on the possible adventitious embryo development from the *S. malaccense* seed fraction in this study included the need for the newly developed tender plants to acclimatize in the laboratory bench for one week. Otherwise, these newly formed smaller plants died, as found in preliminary work prior to actual experimentation. In addition, these smaller plants might still be vulnerable after transplanting, therefore, increasing their mortality. More care was given to these smaller plants to ensure some data could be collected from them.

In the absence of a genetic fidelity study, an initial assumption can be drawn regarding the genetic origin of the plants regenerated from seed fractions based on their observable traits. In this matter, seed fractions that sprouted earliest (in less than 20 days) might be zygotic, primarily because they exhibited a superior phenotype, characterized by greater height and larger leaf area. Conversely, plants with a less vigorous phenotype, which sprouted later (after 20 days), might be nucellar. However, a conclusive determination based solely on these morphological observations remains a challenge due to conflicting perspectives within the scientific literature. Some research supports the idea that zygotic seedlings, being sexually produced, are the first to germinate and are often the most vigorous (Kishore, 2016). This is attributed to the fact that sexual recombination introduces a diverse range of genotypes, which, while capable of producing both vigorous and less vigorous offspring, can also lead to the emergence of superior individuals (Maulana et al., 2021). On the other hand, other studies present an equally compelling argument that nucellar seedlings, resulting from asexual reproduction, tend to be more vigorous when a vigorous or superior genotype is applied for propagation purposes. This perspective is based on the idea that nucellar embryos are genetically identical to the mother plant, thereby consistently expressing favorable maternal traits. This genetic homogeneity, free from the random

recombination associated with zygotic development, is believed to translate into superior and more uniform early growth. For the multiple plants developed from a seed following fractionation, future studies on the genotypes of these regenerated plants are needed to fully understand the relationship between genetic origin, vigour, and overall growth performance.

5.5 Conclusion

The objective to assess the growth performance of plants regenerated from quartered seeds in expressing polyembryony was partially achieved. Statistical analysis of the growth parameters showed that the timing of shoot emergence was the determinant of subsequent plant vigor. Early growth of full plants from longitudinally cut fractions of C4L can be an indicator of plant vigor for selection of planting material Plantlets that emerged earlier demonstrated significantly superior growth performance, characterized by increased plant height and larger leaf dimensions, compared to late-emerging plantlets. This correlation remained consistent regardless of the initial fragment mass, further confirming that growth performance is independent of the amount of reserve tissue in the quartered seeds. While the phenotypic growth performance of the regenerated plantlets has been established, their specific genetic relationship remains unconfirmed. In addition, this research also provided a probability of maternal effect on the morphological characteristics of fruits and seeds. The environmental conditions and genetic makeup of the mother plant may have affected the traits of the fruits and seeds. Nevertheless, further research is needed to quantify the maternal details and their effects on the fruit and seed phenotype. Further research should also be conducted to identify the plant genotypes to explore the production of genetically uniform planting materials with favorable characteristics.

CHAPTER 6
REGENERATION FROM PREDATED SEEDS

6.1 Introduction

In the past collections of *S. malaccense* fruits for experimentation, several important observations were made. Fruits harvested from different trees exhibited distinct characteristics that were likely influenced by their unique environments. One important observation was the presence of a high number of seedless fruits from certain trees, a condition considered a normal physiological phenomenon for *S. malaccense*. Yusnita et al. (2017) previously reported that it is not uncommon for an entire tree to produce fruits devoid of viable seeds for this species. This phenomenon urges nurseries to consider such natural variation when assessing seed availability and planning propagation strategies. From the marketing point of view, *Syzygium* is also reported for conjoined fruits and seeds. This physiological disorder, characterized by the disorganized proliferation of meristematic tissues, results in the development of flattened, ribbon-like, or malformed fruits and seeds (Singh & Kumar, 2023). While this condition is not uncommon in other plant species, its presence in *S. malaccense* is particularly noteworthy, as it makes the affected fruits unfit for commercial markets due to their abnormal development.

In addition to these developmental anomalies, variations in seed quality were observed, specifically in the incidence of predated seeds. *Syzygium* species are prone to predation. The ecological conditions surrounding the mother tree are responsible for the occurrence of fruit and seed predation, as the intensity of plant-animal interactions is often affected by the demography of surrounding vegetation that hosts and discourages the population growth of the predators (Euler et al., 2014). A few authors have reported that these observed traits, especially the seedless fruits and susceptibility to predation, are influenced by the unique environmental conditions of the mother plants (Bonfil, 1998; Euler et al., 2014; Moreira et al., 2021; Nguyen et al., 2021). This phenomenon is known as the maternal effect (Valencia-Díaz & Montaña, 2005).

Even though seed predation may look like a threat, it is actually an ecological process that causes pressure on plant species. This constant pressure has caused the evolution of various adaptive traits that enable seeds to tolerate damage from predators

(Janzen, 1969; Rosenthal & Kotanen, 1994). While extensive seed predation can harm or even kill seeds, some predators only cause partial damage (García-Hernandez et al., 2023).

When the seeds are partially predated by herbivores, the embryos may remain viable, allowing the remaining seed fragments to produce functional plants. This resilience has been observed in several genera (Dalling et al., 1997; Vallejo-Marín et al., 2006; Ceballos et al., 2016; García-Hernandez et al., 2023). Most of these species have the same common trait, which is having large seeds.

The regeneration success of a partially damaged seed largely depends on how much of it is damaged and exactly where that damage occurs (Hou et al., 2010; Bartlow et al., 2018; Perea et al., 2018). Large-seeded species often tolerate predation well. enabling successful sprouting despite significant damage (Bartlow et al., 2018). This is because larger seeds typically contain more substantial reserves and, as Perea et al. (2018) suggested, a relationship often exists between seed size and embryo size. This implies that larger seeds may harbor larger embryos, which could reduce the probability of total embryonic damage from partial predation, thus significantly increasing the seeds' tolerance to damage. The position of the embryo within the seed can also vary among species, potentially influencing its vulnerability to predation. While the remaining reserves in partially predated large seeds can certainly provide enough resources for initial sprouting, there's an ongoing discussion about whether these reserves are sufficient for subsequent plant growth.

The young plants may have a better chance of survival if the damage is only on the surface or does not hit vital parts of the embryo. Various studies have highlighted the benefits of reserve quantity on overall seedling development (Foster, 1986; Harms & Dalling, 1997; Chen et al., 2025). Losing essential reserves such as carbohydrates, proteins, and fats can negatively affect not only germination but also subsequent seedling emergence and early growth performance (Perea et al., 2011; Bartlow et al., 2018).

While large seeds often seem beneficial, it is important to understand that research findings on their advantages in predation are not always consistent. Some studies present contradictory findings, suggesting that the relationship between seed size, predation, and seedling success is complex and influenced by multiple ecological factors (Moles & Westoby, 2004). These factors could include the specific predator involved, the environmental conditions post-predation, and the overall plant community

dynamics. Therefore, while seed size is a significant adaptive trait, its advantages against predation are not universal.

In some cases, partial predation has been reported to actually accelerate seed sprouting. This might occur because the partially damaged seed rapidly produces photosynthetic tissues, essentially trying to make up for the initial loss of its stored energy reserves. Previous experiments involving artificially damaged seeds support this statement, showing relatively high regeneration rates and successful full plant establishment (Loayza et al., 2015; Prataviera et al., 2015). This strongly suggests another potential tolerance mechanism against seed predation.

The different tolerance mechanisms to predation in seeds are particularly important given the types of predators. For instance, the insect *Anselmella* is known to induce seed-like galls within *Syzygium* fruits (Juniper & Britton, 2010; Liao & Yang, 2018; Lin et al., 2018; Reghunath & Raju, 2020). Another pest, *Curculio c-album*, burrows into seeds, and its larval feeding leads to internal decay (Dodiya et al., 2023). This concealed damage often results from insects laying eggs (oviposition) during the fruit's early development, when the fruit is small and softer (Reghunath & Raju, 2020). Once hatched, the larvae can burrow directly into the developing seeds, feeding and growing within. As the fruit matures, its outer appearance remains unblemished, showing no external signs of the internal infestation. Consequently, fruits that appear perfectly healthy from the outside may contain seeds severely compromised or completely consumed by larval activity, causing regeneration failure (Dodiya et al., 2023).

Given these challenges, this study aimed to investigate the predation severity in the naturally insect-damaged or infested *S. malaccense* seeds, and its relationship with plant regeneration potential from these predated seeds to fulfil the last objective of this research work. This study also assessed the subsequent growth performances of the plants regenerated from the predated seeds.

6.2 Materials and Methods

This section outlines the materials utilized in the study. Explanations on the methods used to conduct the experiment effectively are also provided in this section.

6.2.1 Test Material

The study of natural seed predation in *S. malaccense* was a combination of observational and experimental data collection, similar to the procedures carried out by Wenny (2000), as the nature and extent of seed damage were dictated by wild biotic interactions rather than predetermined experimental treatments. For the observational records, site attributes, and the conditions of fruits and seeds collected from various locations throughout the research period, from November 2021 to July 2024, were examined carefully. The extent of damages on the fruits and seeds was described. The extent of seedless fruits was also described. While this approach lacked the controlled manipulation of a laboratory experiment, it provided a more ecologically authentic representation of the species' natural predation information.

For collecting the experimental data regarding the sprouting potential from naturally predated seeds, *S. malaccense* fruits collected from two trees in Merlimau, Malacca (2.15ºN, 102.42ºE) in January 2024 were used. These fruits had the highest number of predated fruits and seeds. The trees were in the compound of a private residence with no nearby bushland. All samples of fruit were collected directly from the trees, while there were three partially predated fruits collected from the ground. The fruits were then brought to the Plant Science Laboratory. The healthy and predated fruits were labelled accordingly with the aid of different grid containers.

6.2.2 Study Location

The research was conducted at the Plant Science Laboratory of Universiti Teknologi MARA (UiTM), Jasin Campus.

6.2.3 Experimental Procedure

The procedures of fruit measurements and predation assessments, seed measurements and predation assessments, sprouting and subsequent growth performance assessment of the resulting young plants are explained below.

6.2.3.1 Fruit Measurement and Predation Assessment

The fruits were grouped into damage categories based on visual assessment. Each fruit was visually inspected for external signs of damage, such as bite marks, punctures, or burrowing holes, and the type of predator was noted where identifiable (e.g., insect borers, small animals). The fruits were labelled for identification, and their fruit predation severity was recorded using the scales of no predation, up to 25% fruit flesh loss, >25%-50% fruit flesh loss, >50%-75% fruit flesh loss, and >75% fruit flesh loss, respectively. Then, the fruits were measured for their length, diameter, and weight, or leftover length, diameter, and weight, where applicable, with the aid of the Vernier caliper and analytical balance.

6.2.3.2 Seed Extraction, Measurement, and Predation Assessment

After recording the data regarding the fruits, seeds were extracted from the fruits individually in accordance to fruit numbering and cleaned individually using slow-running tap water. The loss of seed mass following predation was recorded as no predation (intact seed), up to 25% seed mass loss, >25%-50% seed mass loss, >50-75% seed mass loss, and >75% seed mass loss, respectively. The symptoms and causal agents were recorded, where identifiable.

The seeds, either intact or predated, were measured for their size (length and diameter), or leftover size as applicable. The seed length was measured from hilum to the distal side of the seed using the Vernier caliper, and the seed diameter was the perpendicular measurement, similar to procedures explained in preceding chapters.

6.2.3.3 Seed Trimming and Weighing of Seed Fractions

For each labelled seed, damaged parts of the seeds were removed by knife, leaving only the undamaged/intact portion of the seeds. The seeds were positioned where the hilum was upward, and the pit was determined as the right part of the seeds before cutting off the damaged seed portions. The remaining undamaged part of each seed was labelled accordingly as left half C2L(L), right half C2L(R), proximal half C2T(P), distal half C2T(D), longitudinal leftmost quarter C4L(L), longitudinal central left quarter C4L(CL), longitudinal central right quarter C4L(CR), longitudinal

rightmost quarter C4L(R), left proximal quarter C2L2T(LP), left distal quarter C2L2T(LD), right proximal quarter C2L2T(RP) or right distal quarter C2L2T(RD). After removal of damaged parts, the labelled seeds were weighed using an analytical balance.

6.2.3.4 Sprouting of Seed Fractions after Removal of Predated Portions

The labelled fractions with no surface sterilization were sown randomly in moistened soil sized 0.2-2.0mm in plastic boxes 17.5 x 12.5 x 6.5 cm (ISTA, 2019). While the damaged part of any seed was found randomly, there was no proper replication and no experimental design for the sprouting study for each type of seed fraction after removal of the damaged part.

Seed fractions were sprouted in the dark. The rate and period of sprouting were recorded. Sprouting was recorded when the radicle of at least 2mm had formed. Then, the rooted seed fractions were observed for subsequent shoot development. Sprouting was terminated at 60 days after sowing when no new root or shoot development was observed for a consecutive 30 days.

6.2.3.5 Transplanting and Growth Performance Assessment

Following shoot emergence, the newly developed plants were also acclimatized for a week to obtain a shoot length of at least 2 cm before transplanting into plug trays (170x170x120 mm) filled with moist peat moss. The plants were also grown with quarter-strength Hoagland solution. The survival rates of the young plants were recorded by six weeks after transfer to the plug tray. The height, stem diameter, number of leaves, and total leaf area of each young plant were recorded.

6.2.4 Data Collection

6.2.4.1 Fruit and Seed Inspection, Selection, Morphometric and Weight Measurements

For each fruit collection during the periods ranged from November 2021 to July 2024, the number of predated fruits and seeds, and the number of seedless fruits were

recorded on the following days upon arrival in the laboratory. For the current study on regeneration from predated seeds, the fruit and seed length, diameter and weight were recorded.

6.2.4.2 Sprouting of Predated Seeds

Sprouting of predated seeds (after removal of the damaged seed portions) was carried out in moistened sand medium, similar to the sprouting procedures carried out in preceding experiments. The seed fractions were each recorded for the time for emergence of roots when at least a root of 2 mm was observed, and time for subsequent shoot emergence. Seed fragments (after removal of damaged portions) that died or failed to sprout were also recorded.

6.2.4.3 Assessment of Growth of Regenerated Plants

After six weeks, the growth performance of plants was determined by measuring their height, stem diameter, number of leaves, and total leaf area of each plant.

6.2.5 Statistical Analysis

To assess differences in the morphometric parameters and weight of fruits and seeds among groups of varying predation severity, an independent-samples Kruskal-Wallis test was employed for each parameter. Subsequently, Mann-Whitney U tests were performed to identify significant differences between any two independent groups. The relationship between a seed's reserve availability (fraction weight after removal of damaged/predated portions) and its sprouting potential was described. Finally, descriptive analyses were used to present the survival rates and growth performances of plants that developed from the different seed fractions following the removal of their damaged/predated portions. All statistical analyses were performed using SPSS Version 29.

6.3 Results

6.3.1 Predation of Fruits and Seeds Collected from Various Locations

6.3.1.1 Fruits and Seeds from Sepang, Selangor

S. malaccense tree in Sepang, Selangor (N 2°38'56", E 101°35'39), was in a private property surrounded by durian (*Durio zibethinus*) trees. Fruits collected from Sepang in the end of November 2021 were mostly healthy but partially predated by small animals of squirrels and bats. As the fruits were fully ripe and had fallen from the tree, they were soggy. Therefore, fruit size and weight measurements could not be recorded.

With a total of 334 fruits collected, the seeds were carefully taken out from the soggy fruits (Table 6.1; Appendix D: D1). Each fruit contained a seed. All the seeds were found free from predation by visual observation. A total of 300 seeds were packed loosely in plastic bags for transfer to the laboratory in UiTM, Jasin Campus, and used for experimentation as described in Chapter 3.

6.3.1.2 Fruits and Seeds from Port Dickson

A total of 104 fruits collected from Port Dickson (N 2° 31' 37", E 101° 48' 25") in October 2022 were mostly healthy. The tree was located in a gated house at a suburban area, planted at the backyard of the house. There were no taller crops surrounding the trees. However, 44% of the fruits obtained were seedless fruits (Table 6.1; Appendix D: D1). Fruits containing seeds were used for experimentation, as described in Chapter 4.

Table 6.1
Predated Fruits, Seedless Fruits and Predated Seeds by Locality During the Research Work Period

Fruit collection date	Location	Fruit mass lost to predator(s)					Number of seedless fruits	Seed mass lost to predator(s)				
		Nil	up to 25%	>25-50%	>50-75%	>75%		Nil	up to 25%	>25-50%	>50-75%	>75%
Nov-21	Tanjung Sepat, Selangor, Malaysia (2°38'56"N, 101°35'39E) – 1 tree	334	0	0	0	0	0	334	0	0	0	0
Oct-22	Port Dickson, Negeri Sembilan, Malaysia (2° 31' 37"N, 101° 48' 25") – 1 tree	104	0	0	0	0	46	58	0	0	0	0
Nov-22	Kampung Seri Mendapat, Malacca, Malaysia (2° 13' 5"N, 102° 27' 29"E) – 5 trees	252	0	0	0	1	93	154	5	0	0	1
Nov-22	Merlimau, Malacca, Malaysia (2° 8' 59"N, 102° 25' 34"E) – 2 trees	202	3	2	1	2	163	39	1	1	0	6
Nov-22	Masjid Tanah, Malacca, Malaysia (2° 21' 35"N, 102° 5' 54"E) – 1 tree	36	0	2	4	2	15	21	0	1	0	7
Apr-23	Masjid Tanah, Malacca, 1 tree	23	0	1	2	0	7	19	0	0	0	0
Jul-23	Kampung Seri Mendapat – 3 trees	55	0	0	1	0	19	36	0	1	0	0
Aug-23	Kampung Seri Mendapat – 1 tree	58	0	0	0	0	0	58	0	0	0	0
Dec-23	Kampung Seri Mendapat – 1 tree	16	0	0	0	0	1	15	0	0	0	0
Jan-24	Kampung Seri Mendapat – 1 tree	49	0	0	0	0	20	29	0	0	0	0
Jan-24	Merlimau – 3 trees	249	9	9	1	4	90	137	3	10	17	16
Jan-24	Muar, Johor, 1 tree (2° 0' 18"N, 102° 44' 1"E)	45	0	0	0	0	5	40	0	0	0	0
Jul-24	Muar, Johor	35	0	0	0	0	12	23	0	0	0	0

6.3.1.3 Fruits and Seeds from Kampung Seri Mendapat, Malacca

There were six trees in Kampung Seri Mendapat, Malacca (N 2° 13' 51", E 02° 27' 29"), where fruits were collected for experimentation in Chapter 4. In November 2022, Tree 1 (N 2° 13' 51", E 02° 27' 29") in the compound of a private property in the village, surrounded by horticultural and landscape crops, produced fruits. A total of 71 fruits with no sign of predation externally were collected, but most of the fruits were seedless. Only 17 seeds were obtained in this fruit collection exercise, and the seeds were used for experimentation as mentioned above (Appendix D: D1). Later, the tree was cut, and the fruit source was lost.

Tree 2 (N 2° 13' 6", E 102° 27' 20") in Kampung Seri Mendapat was located in the backyard of a village house. This tree was a single tree there and far from other vegetation. A total of 61 seeds were collected from this tree in November 2022 (Appendix D: D1). There were 18 seedless fruits (29.5%). A few fruits had very minor signs of predation, while one fruit was severely predated, exposing its seed. Fruit predation could be caused by small animals.

Tree 3 (N 2° 13' 8", E 102° 27' 33") in Kampung Seri Mendapat was located at the backyard of another house in the village. The tree was surrounded by other vegetations including rambutan (*Nephelium lappaceum*) and rose apple (*Syzygium jambos*) trees. In November 2022, only 3 (12.5%) out of 24 fruits collected were seedless fruits, while other fruits were free from predation and used for experimentation in Chapter 4 (Appendix D: D1). Later in July 2023, only 7 intact fruits were obtained while one intact fruit contained a seed predated by borer within (>25%-50% seed mass loss), exhibiting the fact that internal seed damage was possible despite no predation signs on the fruit. No seedless fruits were found for this batch of fruits (Appendix D: D1).

Tree 4 (N 2° 13' 1", E 102° 27' 15") in Kampung Seri Mendapat was planted in the compound of a village house amongst crops like banana trees (*Musa* spp.), vegetables, and other fruit trees. All 66 fruits collected in November 2022 were free from predation, but 18 fruits (27.27%) were seedless (Appendix D: D1). The remaining seeds were used for experimentation in Chapter 4. Later in July 2023, more than half of the fruits collected (59.4%) were seedless (Appendix D: D1). These seeds were used for some preliminary work of growth performance of the entire plants regenerated from longitudinally quartered (C4L) fractions.

Tree 5 (N 2° 13' 3", E 102° 27' 9") in Kampung Seri Mendapat was located at roadside. There were also other crops planted nearby, such as rambutan (*Nephelium lappaceum*) and bitter beans (*Parkia speciosa*). In November 2022, July 2023, and December 2023 fruit collections, no fruit or seed predation was recorded, and all fruits contained single seeds each, except for one seedless fruit collected in December 2023 (Appendix D: D1). However, this tree had consistently shown low fruiting.

Tree 6 (N 2° 13' 5", E 102° 27' 29") in Kampung Seri Mendapat was only approximately 10 m away from Tree 1 mentioned above. Fruits were collected from Tree 6 in August 2023 and January 2024. The fruits showed no fruit or seed predation. There were two conjoined fruits collected in August 2023, each with two conjoined seeds (Figure 6.1; Appendix D: D1), while 20 out of 49 fruits collected in January 2024 were seedless fruits.

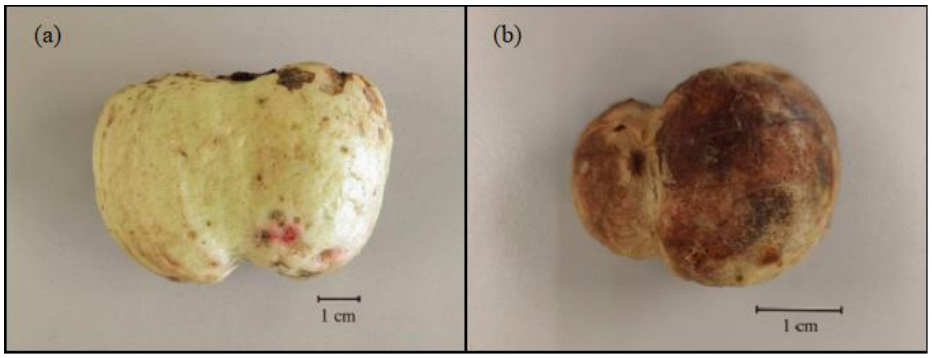


Figure 6.1 Conjoined (a) Fruit and (b) Seed from Kampung Seri Mendapat Tree 6

6.3.1.4 Fruits and Seeds from Merlimau, Malacca

There were three trees in Merlimau, Malacca (N 2° 8' 59", E 102° 25' 34"), where the fruits were collected for experimentation. Tree 1 and Tree 2 were within the same property, approximately 30 m apart. Tree 1 was planted in front of the property facing the roadside, while Tree 2 was located in the backyard of the house, where the owner did some farming and the tree was surrounded by crops like rambutan, jackfruits (*Artocarpus heterophyllus)*, mulberry (*Morus* spp.), etc., as well as adjacent to a small oil palm (*Elaies guineensis)* area.

In Merlimau, Malacca, Tree 1 produced a significant number of seedless fruits. In November 2022, 86.49% of the fruits collected were seedless. This incidence was observed again in January 2024 (65.17% seedless fruits).

Fruits and seeds from Tree 1 had some predation signs. In November 2022, 6 out of 148 fruits were predated (Appendix D: D1). The seeds obtained from this fruit collection exercise were used for experimentation in Chapter 4. In January 2024, 22 out of 150 fruits were predated, and the number of predated seeds was 31. The predations were probably caused by insect pests, such as borers and small animals. The fruits harvested in January 2024 were used to determine the sprouting performance of predated seeds below, since there was a variation of predated seeds; some seeds lost partial seed mass, while others had multiple tunnels caused by borers.

Tree 2 in Merlimau also showed a high rate of seedless fruits, with 56.45% of fruits collected in November 2022 being seedless (Appendix D: D1). However, out of 60 fruits collected in January 2024, only 30% of the fruits were seedless (Appendix D: D1). Predation on fruits from this tree was present but less severe compared to fruit predation occurrence on Tree 1. In November 2022, two fruits out of 62 from Tree 2 were predated, and two seeds also showed damage caused by insect borers. In the January 2024 fruit collection, there was only one predated fruit out of 60, with no recorded seed predation. This indicates that the predation on Tree 2 might be less consistent. The fruits collected from this tree were used in experiments in Chapters 4 and 5.

Tree 3 (N 2° 8' 58", E 102° 25' 32") in Merlimau was located near an ungated house in a suburban area. Just like most other *S. malaccense* trees in this research work, the tree was surrounded by other fruiting crops. The fruits from this tree were harvested in January 2024. There were 14 seedless fruits out of 29 collected fruits, indicating again a high level of seedless fruits was common (Appendix D: D1). The fruits from this tree did not exhibit any predation signs. However, there was a high level of seed predation within fruits, with 13 predated seeds found, attributed mostly to borer larvae. Fruits that contained seeds were used in the experiment on regeneration from predated seeds below.

6.3.1.5 Fruits and Seeds from Muar, Johor

The tree in Muar, Johor (N 2° 0' 18", E 102° 44' 1"), was planted in the backyard of a private property surrounded by taller trees. There was also a forest nearby. At about 13 m from this tree, there was another *S. malaccense* tree planted beside the house. However, in both fruit collections in January and July 2024, only one tree was fruiting,

and the fruits showed no fruit or seed predation. Despite the lack of predation, the reproductive output of the tree was still limited by the occurrence of seedless fruits. In the January 2024 collection, 5 out of 45 fruits were seedless (Table 6.1; Appendix D: D1). This trend continued in the July 2024 collection, where 12 out of 35 fruits were seedless (Table 6.1; Appendix D: D1). The fruits collected in January 2024 were used for experimentation described in Chapter 5.

6.3.2 Fruit Size, Weight and Predation

Table 6.2 depicts the size and weight of the fruits grouped by the fruit predation category. A total of 38.24% fruits examined displayed no visible signs of damage like punctures or scars, while only a small proportion of fruits (11.77%) showed severe flesh loss exceeding 75% due to predation (Appendix D: D2). There was a statistically significant difference in weight and length, with fruits exhibiting minimal or no visible damage generally being heavier and longer compared to those with severe predation ($P < 0.05$). For example, the undamaged fruits in the Nil category had an average weight of 67.77 ± 15.37 g, which was significantly higher than the 51.31 ± 12.64 g average for fruits with over 75% predation (Appendix D: D2). Interestingly, fruits with up to 25% predation exhibited the highest average weight, at 82.75 ± 13.89 g. These results suggest that the animals could have favoured larger fruits. However, fruits with severe damage were significantly the smallest (Table 6.2; Appendix D: D1).

Table 6.2
Fruit Predation Severity

Flesh loss estimate	Frequency	%	Fruit weight (g)#	Fruit length (mm)#	Fruit diameter (mm)#	Remarks
Nil	24	52.17	70.17±12.51a (51.19-98.68)	57.45±4.81a (43.0-66.0)	48.54±4.38a (40.0-57.3)	Intact fruit by naked eyes
Up to 25%	9	19.57	79.46±16.33a (53.10-106.25)	58.34±4.78a (48.3-65.0)	49.82±6.41a (39.8-59.7)	A few visible holes in flesh that were caused by insects or loss of <25% flesh due to herbivores, some with partial seed exposure
26-50%	9	19.57	70.93±29.30a (50.35-138.85)	53.82±5.92b (47.2-67.0)	48.31±4.41a (43.3-57.8)	Loss of up to half of the flesh to herbivores, partial seed exposure/ predation
51-75%	0	-	-	-	-	-
>75%	4	8.70	51.31±12.64b (40.31-65.31)	51.18±2.72b (48.7-55.0)	45.96±2.35a (42.5-47.6)	>75% loss of flesh to herbivores with some rotting flesh, partial seed exposure/ predation

N=46
# mean±SD; figures in parentheses are minimum-maximum values
Means having similar letter within column are not significantly different at P=0.05 by Mann-Whitney U tests.

The study observed a range of herbivoral damage to *S. malaccense* fruits, from no visible damage to over 75% flesh loss. Figure 6.2 exhibits an example of fruit with no visible signs of damage. Meanwhile, Figure 6.3(A-D) shows fruit predation ranging from 25% to more than 75% mass loss.

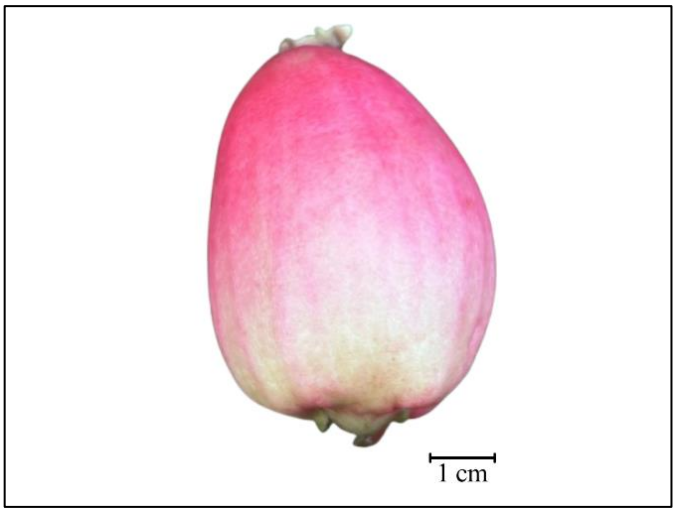


Figure 6.2 Unpredated Fruit

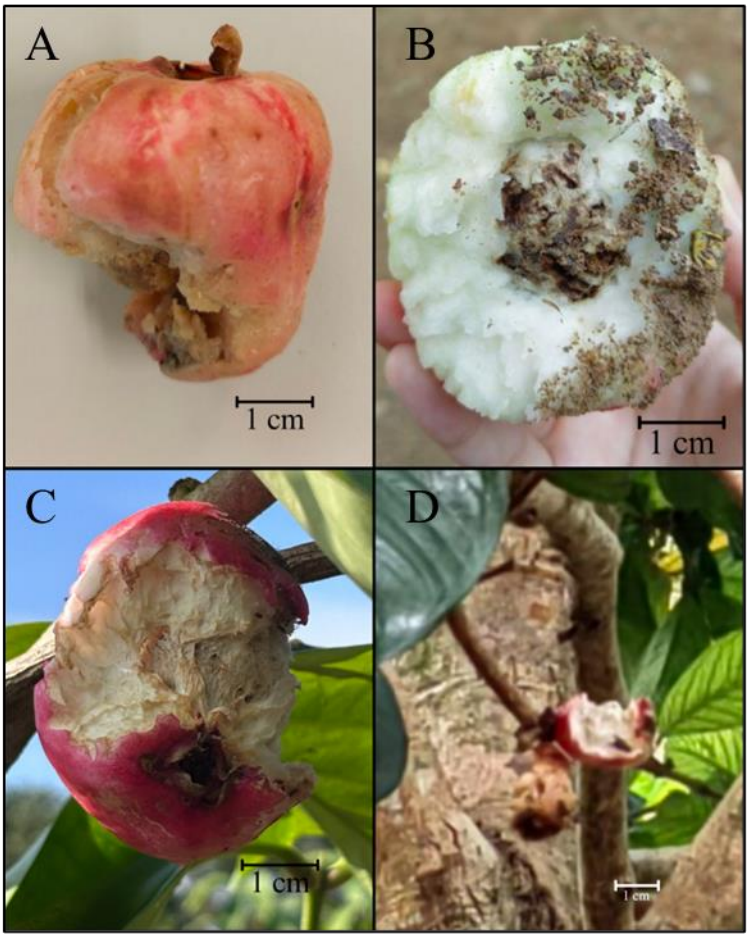


Figure 6.3 Visual Representations of Herbivory Damages On *S. Malaccense* Fruits; (a) Up To 25% Predation: Partially Eaten Fruit; (b) >25-50% Predation: Half Eaten Fruit With Minimal Damage on the Seed Found on the Ground; (c) >50-75% Predation: Severely Damaged Fruit Still Attached to the Tree; (d) a Fruit with >75% Flesh Loss on the Tree

There were black-colored insects surrounding the fruits (Figure 6.4). The morphology of the insect is consistent with that mentioned as *Anselmella* spp.

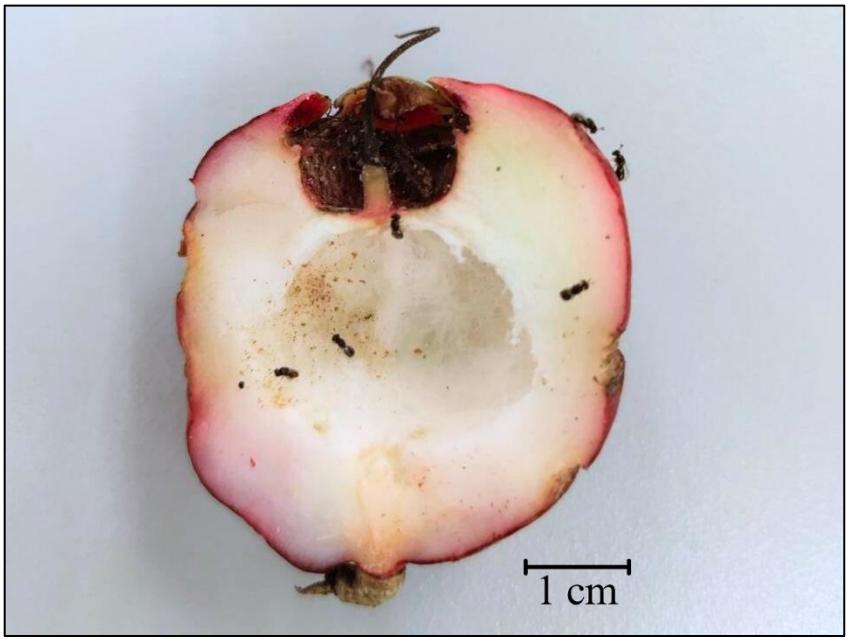


Figure 6.4 Insects from Probably the Genus *Anselmella* found Surrounding the Fruit

6.3.3 Seed Size, Weight, and Predation

Table 6.3 exhibits the varying size (length and diameter) and weight of the seeds based on the predation group (Appendix D: D2). There was no significant difference in size and weight of seeds that experienced no predation and up to 25% predation. The fruits that experienced more than 75% predation contained seeds with significantly smaller weight compared to the other seeds.

Table 6.3
Seed Predation Severity according to Fruit Predation Groups

Fruit predation group	Seed weight (g)#	Seed length (mm)#	Seed diameter (mm)#	Frequency by seed predation severity*				
				Nil	Up to 25%	26-50%	51-75%	>75%
Nil	14.27±3.32a (10.42-22.15)	25.74±2.40a (21.0-30.5)	30.17±2.56a (25.9-36.4)	-	1	3	9	11
Up to 25%	15.65±3.91a (11.17-22.14)	26.76±2.59a (24.0-32.2)	31.05±3.06a (26.8-35.9)	-	-	3	3	3
26-50%	10.66±3.68b (6.25-18.24)	23.88±3.09ab (20.0-28.6)	27.76±3.09b (23.2-33.5)	-	1	2	4	2
>75%	6.20±2.13c (3.32-7.86)	19.83±2.41b (16.4-22.0)	23.43±3.69b (18.6-27.3)	-	1	2	1	-

mean±SD; figures in parentheses are minimum-maximum values.
Means having similar letter within column are not significantly different at P=0.05 by Mann-Whitney U-tests.
* Based on seed mass loss estimate in the cavity for seed within fruit; up to 25% seed predation was mainly attributed to consumption by small animals; >20% seed predation was mostly caused by larval infestations.

The result found varying degrees of damage to the seeds (Table 6.3: Appendix D: D2). While some intact fruits showed severe seed predation, the amount of seed loss generally increases with the level of fruit predation (Figure 6.5). For the predated seeds, the granivore(s) did not show a specific preference for certain seed parts; there were different types of larval predation, with some seeds losing some portions, while other seeds had multiple tunnels caused by the larvae (Figure 6.6).



Figure 6.5 Intact Fruit with a Severely Predated Seed

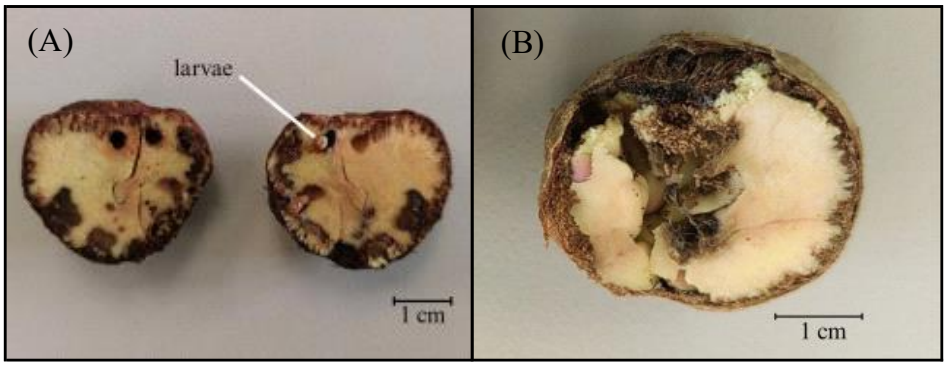


Figure 6.6 Larval Infestation in Seeds; (a) Holes in the Seeds due to Larvae Boring into the Seed; (b) Rot due to Insect Infestation

6.3.4 Sprouting of Predated *S. malaccense* Seeds

Seed fragments after removal of the damaged/predated portions showed both rooting and development of full functional plants (Figure 6.7). Moreover, the seed fraction also exhibited varied development rates (Table 6.4). The central left C4L(CL) and central right C4L(CR) fractions had the highest rooting success, at 92.86% and 100%, respectively (Appendix D: D2). Shoot development, however, varied: CL fractions reached 71.43% success, while CR fractions achieved 83.33% (Appendix D: D2).

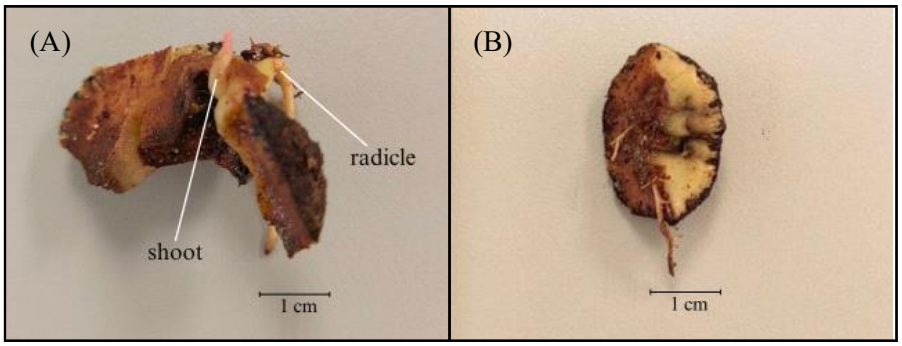


Figure 6.7 (a) Full plant; (b) Rooting from Predated Seeds (after Removal of the Damaged Seed Portions)

Table 6.4
Sprouting Rate and Full Plant Regeneration Rate from Predated Seed (After Removal of Damaged Seed Portions)

Seed fraction type	Frequency	Percent	Fraction weight (g)	Rooting rate (%)	Full plant development rate (%)
C2L(L)	14	29.79	6.2049±3.8579 (1.3602–13.5686)	92.86	71.43
C2L(R)	12	25.53	4.9152±2.6456 (1.3042-9.9496)	100	83.33
C4L(CR)	1	2.13	3.6925	100	0
C2L2T(RP)	2	4.26	2.1665±0.0045 (2.1633-2.1696)	100	50
C2L2T(LD)	1	2.13	2.9311	100	100
C2L2T(RD)	1	2.13	2.7606	100	0
Whole seeds bored by larvae (>75% seed predation)	16	34.04	14.6923±3.3296 (10.7430-22.1513)	0	0

Mean±SD

Other seed fractions, such as the left distal quarter C4L(LD) and right distal quarter C4L(RD), also regenerated successfully with 100% rooting and shoot development. However, these findings were based on single samples. Nevertheless, predation severity impacted seed fragment sprouting significantly. Seeds with over 90% mass loss failed to sprout. The fragments rotted after a short period.

Table 6.5 exhibits the varying periods of rooting and full plant development from fragments of *S. malaccense* after removal of the damaged/predated parts. The earliest root emergence was 2 days, exhibited by fragment C2L(L) and C2L(R), while rooting and shoot formation from these seed fractions were observed within 1 month (Appendix D: D2).

Table 6.5
Rooting and Full Plant Development Time of Predated Seeds (after Removal of the Damaged Seed Portions)

Seed Fraction Type	Period (days after sowing)#	
	Rooting	Shoot (full plant) Development
C2L(L)	7.08±6.92 (2-28)	12.00±5.08 (5-20)
C2L(R)	4.08±1.83 (2-8)	9.20±3.05 (5-15)
C4L(CR)*	17	-
C2L2T(RP)	12.00±8.49 (6 & 18)	28*
C2L2T(LD)*	5	10
C2L2T(RD)*	12	-

mean±SD; values in parenthesis are minimum-maximum values; * single plant.

6.3.5 Plant Growth Performance from Predated Seeds

Growth of the regenerated plants, assessed six weeks after transplanting into moist peat moss in plug trays, varied significantly among seed fractions (Table 6.6). Plants from C2L(L)and C2L(R) fractions showed similar heights (16.91 ± 4.48 cm and 17.50 ± 5.12 cm) and stem diameters (2.98 ± 0.15 mm and 2.98 ± 0.04 mm) (Appendix: D2). However, C2L(R) fractions had a slightly larger leaf area (81.33 ± 24.32 cm^2) compared to C2L(L)fractions (78.00 ± 43.65 cm^2) (Appendix D: D2).

In contrast, single plants from C2L2T(LD) and C2L2T(RP) fractions showed no mortality and superior growth. They reached greater heights (e.g., 22.1 cm for C2L2T(LD)) and had larger total leaf areas (97.00 cm^2 for C2L2T(RP)).

Table 6.6
Performance of Plants Regenerated from Predated Seed (after Removal of the Damaged Seed Portions)

Seed fraction type	Mortality (%)	Height (cm)#	Stem diameter (mm)#	Number of leaves#	Total leaf area (cm^2)#
C2L (L)	20	16.91±4.48 (10.5-24.3)	2.98±0.15 (2.70-3.20)	6.38±1.77 (4-9)	78.00±43.65 (20-140)
C2L (R)	40	17.50±5.12 (12.5-25.0)	2.98±0.04 (2.90-3.00)	6±0	81.33±24.32 (62-127)
C2L2T (RP)*	0	16.1	3.00	6	97.00
C2L2T (LD)*	0	22.1	4.00	6	81.00

mean±SD; values in parenthesis are minimum-maximum values; * single plant.

6.4 Discussion

The study exhibited a varying morphological condition of fruits and seeds of 13 different *S. malaccense* trees. The high variability in the rate of seedless fruits, even between closely located trees, for example, Merlimau Tree 1 and Tree 2, is a major concern because it will cause a lack of planting materials. The seedless fruits are possibly related to pollination effectiveness, which could be influenced by a lack of suitable pollinators or their activity, environmental stressors like nutrient deficiency, or the genetic factors within the mother plants (Bonfil, 1998; Valencia-Díaz & Montaña, 2005; Euler et al., 2014; Moreira et al., 2021; Nguyen et al., 2021).

Another type of anomaly found during fruit collection was the tree bearing conjoined fruits and seeds. This malformation is called fasciation, where fusion happens at two growing points. This could happen due to several natural factors like pressure,

crowded stands of plants, or insect attacks (Gorter, 1965). In this study, conjoined fruits were found in two trees, one from Kampung Seri Mendapat, and the other from Merlimau, Malacca. The cause of this phenomenon was not clear, since these trees were not growing in a crowded stand and there were no signs of fruit or seed predation at both times of fruit availability.

The results also show that *S. malaccense* fruits and seeds experienced varying degrees of predation, from minor herbivory to severe larval infestations. For instance, some trees in Merlimau and Kampung Seri Mendapat had obvious predation. Observation from Kampung Seri Mendapat Tree 3 was interesting because it showed that internal damage to the seed was possible in the absence of external signs of fruit predation. This finding is important as there might be a potential underestimation of predation rates if assessment is based solely on the fruit damage, and it suggests the presence of boring insects that can access the seed without causing evident fruit injury.

Additionally, the obvious increase in predation on fruits and seeds from Tree 1 in Merlimau between November 2022 and January 2024 shows the dynamic nature of the fruits/seeds and ecological relationships, which can be impacted by seasonality of pest populations or other ecological factors.

Based on observations, there were two main types of fruit damage. The first was biting scars on the fruit surface, which were probably caused by monkeys, squirrels, or bats. The second was larval infestation. Upon inspection of the damaged fruits, several small, dark-colored insects were found moving around the fruits. Additionally, some fruits showed no visible signs of predation despite significant internal seed damage. This hidden damage was a direct consequence of insect oviposition occurring during the early stages of fruit development, when the fruit tissues were softer and more permeable. Once hatched, the larvae of these insects, probably from the genus of *Anselmella*, as indicated by the visual evidence, immediately fed on the seed, making tunneling activities within the developing seeds and the surrounding fruit pulp. This internal feeding allowed the larvae to grow and develop while being completely encapsulated by the maturing fruit. As the fruit progressed through its developmental stages, its outer pericarp hardened and maintained an intact appearance. This mechanism aligns with previous documentation regarding insects like *Anselmella*, which induces seed-like galls, and *Curculio c-album*, known for burrowing into seeds and causing internal decay that renders fruits unmarketable (Dodiya et al., 2023).

Despite partial predation, these seeds retained their ability to regenerate,

showing the species' tolerance to predation. The success of sprouting in predated seeds varied based on the extent and location of damage. Seed fractions like the C2L(L)and C2L(R) showed high rooting and shoot development rates, indicating that partial predation does not prevent a seed from growing. Past studies suggest that large reserves offer higher tolerance, allowing viability despite damage (Bonfil, 1998; Khan, 2004; Fukumoto & Kajimura, 2014). However, it was observed that some seeds with slightly larger reserves failed to sprout and sustain growth. For instance, the smaller C2L2T(RP) fraction left behind after cutting off the predated seed part sprouted, but the larger C4L(CR) fraction failed to grow. Also, despite having more seed reserves, some plants regenerated from the C2L(L) and C2L(R) fractions did not survive after predation.

Plants grown from partially predated seeds showed varying growth performances. Certain plants, such as those regenerated from C2L2T(LD) and C2L2T(RP), showed superior height, leaf area, and survival rates. This demonstrates that even small seed portions can support vigorous growth under favorable conditions.

The ability of *S. malaccens*e to sprout and produce functional plants from partially predated seeds may serve as an adaptive strategy to ensure population survival when facing predation. From another perspective, these findings also contribute to efficient planting material production in nurseries, as it is reasonable to use not only intact seeds but also predated ones.

6.5 Conclusion

The findings of this study emphasized the ecological challenge to the reproductive success of *S. malaccense*, which is localized but impactful predation and the occurrence of seedless fruits. While the impact of fruit and seed predation varied significantly across the studied sites, suggesting its influence could be dependent on local environmental conditions, the consistent and high rates of seedless fruits observed in some locations are a major concern for the species' reproductive output. This study suggests that partially predated seeds are not fatal but can be a valuable resource for efficient planting material production in nurseries and conservation efforts, especially for rare and often seedless plant species like *S. malaccense*. Therefore, the findings showed that the objective to investigate the plant regeneration potential from naturally insect-damaged or infested seeds was achieved.

CHAPTER 7
GENERAL DISCUSSION

7.1 Introduction

The current research involved a few experiments in order to understand the regenerative capabilities of *S. malaccense* seeds, particularly when subjected to fragmentation and natural predation, and to understand the factors influencing the sprouting ability and the subsequent growth performance of the resulting full plants. This study has offered useful information, contributing to both the understanding of seed regeneration and the propagation of recalcitrant and large-seeded species like *S. malaccense.* The information was gathered by exploring the morphometric and reserve effects, the influence of varied light conditions, the cellular mechanisms on the cotyledonary surfaces behind the regeneration process, and the dynamics of the growth performances of the regenerated plants. The research also assessed the impact of natural predation on the regeneration of the seed fragments and their growth performance. This study collectively presents findings for novel, efficient, and affordable propagation strategies that can be directly translated into nursery practices.

7.2 Reserve Effect Hypothesis

The evaluation of the relationship between reserve reduction (size and fresh weight) and the regenerative potential of *S. malaccense* seed fractions confirms that initial seed mass is not a primary limiting factor for sprouting from seeds of this fruit species. Despite a significant reduction in fresh weight through systematic longitudinal and transverse sectioning, quartered fragments retained a robust capacity for successful plantlet development. The findings of this study proved that sprouting of *S. malaccense* seeds contradicted traditional beliefs regarding the need for intact seeds for successful germination and seedling development. According to past literatures, larger seeds are said to have a significant advantage due to their substantial nutrient reserves, supporting early seedling establishment (Foster, 1986; Harms & Dalling, 1997; Khan, 2004; Hou et al., 2010; Chen et al., 2025). However, the recent findings for *S. malaccense* have shown the contrary. The results align with research by Moles and Westoby (2004,

2006), suggesting that the size of reserves may be secondary to the presence of high-density regenerative cells within the cotyledonary tissue.

Moreover, rather than only one seedling produced per seed, three or four times more full plants can be produced from a single seed when the seed is cut into separate fractions. This is proven by the maximum full plant regeneration rate of 1.5, which was achieved when a seed was cut longitudinally into halves (C2L) or quarters (C4L), a difference to the maximum full plant regeneration rate of 1 from intact seeds. It is advantageous as it enables the production of more planting materials from a smaller number of parental seeds, which helps to conserve the valuable germplasm and reduces the cost of fruit/seed collection.

The varied sprouting times observed in fractionated seeds, with some taking significantly longer periods to initiate shoot development, indicated that the delayed full plants may be an adventitious type, whose development was induced by environmental stimuli. Although not a favourable aspect, the clonal planting material production could be a strategic advantage. Instead of a single, synchronous production of planting materials, different sprouting times and emergence can also be scheduled for a more consistent availability of planting material over an extended period, reducing peaks in production. This can enable flexible management, reducing the effort of labor and resources. Moreover, it can also lower the risk in case an initial batch fails; later-sprouting seed fractions can serve as a backup, ensuring continuous production. This flexibility, where reserves are not immediately exhausted but saved for sequential sprouting, provides a natural buffer against environmental fluctuations or initial propagation setbacks.

Although the resprouting in the experiment was mostly restricted to the development of adventitious roots and not full plants, it nevertheless indicates a strong means of survival. This result demonstrates the ability of *S. malaccense* seeds to tolerate damage. For example, if the young plants encounter injury during handling, or due to pest infestation, or through stress, it might still be recoverable as the root systems may redevelop (Bellingham & Sparrow, 2000). Also, if future research can induce shoot regeneration from resprouted roots, it may be possible to even further multiply from initial fragments. This natural flexibility in compensating for biomass loss shows a robust adaptive strategy that is useful for plant propagation (Green & Juniper, 2004).

7.3 Light Effect for Inducing Full Plant Development from Seed Fractions

The investigation into exogenous environmental factors identified light as a primary regulator of regeneration in fractionated *S. malaccense* seeds. Results indicated that environmental manipulation of light regimes significantly impacted the efficiency of plant production, offering precise control for plant propagation. The most interesting result was that dark was the best condition to produce full plants from *S. malaccense* seed fractions. Longitudinal quartered seeds (C4L) in particular showed the highest entire plant formation rate in the dark (1.8 ± 0.1826), which was a higher rate compared to that obtained with the same types of seed fractions in Experiment 1 above. It exhibited almost a two-fold increase in full plant regeneration rate as compared to the maximum rate of perfect 1 obtained from whole seeds. In the dark induction of full plant development, it was presumed that the undifferentiated meristematic cells within the cotyledons were activated with environmental stimuli, for example, darkness, and this leads to *de novo* embryo formation or the proliferation of pre-existing, but latent, embryonic structures (Joshi et al., 2006). This contradicts the common assumption that light is beneficial for germination and early development, since the cotyledons of *S. malaccense* are green in colour. This preference for sprouting in dark conditions aligns with the natural ecology of *S. malaccense*, which seeds typically germinate under a dense forest canopy, where light penetration is minimal.

The study also demonstrated light-mediated morphological responses, with dark green growth under LED light, normal green growth in natural light, and etiolated growth (pale, elongated shoots) in dark-induced plants regenerated from the seed fractions. However, these etiolated plants were proven to be reversible, recovering normal morphology and turning green upon subsequent exposure to natural light. Etiolation, though seemingly undesirable, serves as a survival strategy that elevates rapid shoot elongation to reach light sources (Repeat, 2020; Paradiso & Proietti, 2022). This enables the production of large batches of fragmented seeds in dark conditions to maximize planting material numbers, then transfer them to illuminated environments such as shaded greenhouses for hardening. In addition, shade-avoidant species like *S. malaccense,* when cultivated in crowded monoculture, will not affect the fitness of the newly developed plants. This suggests that shade avoidance species have a major fitness benefit in crowded conditions (Franklin & Whitelan, 2005). However, these shade avoidance plants have elongated stems, which increase the risk of stem breakage (Wang

et al., 2020).

This finding has established a specific seed fraction sprouting protocol that is useful and can be implemented by nurseries, agronomists, or plant scientists. This specific dark-sprouting protocol for *S. malaccense* seed fractions can help maximize the number of entire plants. This might involve placing containers under blackout clothes to induce dark conditions for sprouting. This propagation method promotes energy efficiency. By strategically implementing dark periods during sprouting, growers can potentially reduce their reliance on artificial lighting, leading to significant cost savings and environmental benefits.

7.4 Growth Performance of the Regenerated Plants

The evaluation on the growth performance in expressing polyembryony was also achieved. The findings managed to show a strong positive correlation between early shoot emergence time and subsequent plant vigor (height and leaf area). Plants that initiated early shoot growth generally produced taller plants with larger leaves after two months. This finding is useful for a simple yet highly effective screening protocol for high-quality planting materials. Plants that emerge and develop shoots quickly can be prioritized for transplanting and further cultivation, as they are inherently more vigorous and likely to perform better. This allows for the selection of superior planting materials, resulting in a more uniform and higher-quality batch of planting materials. Moreover, resources like space, nutrients, and labor can be preferentially allocated to these high-performing plants, maximizing return on investment. This aligns with the principle that early vigor is a key determinant of overall plant performance (Finch-Savage & Bassel, 2016).

Furthermore, the observed differences in plant growth performance based on the mother plant's origin (Merlimau vs. Muar) highlight the importance of seed sourcing. Despite uniform seed morphology, C4L seed fractions from fruits collected Merlimau in January 2024 developed more full plants (90 full plants from 40 whole seeds), in comparison to those from Muar collected at the same time, i.e., January 2024 (81 full plants from 40 whole seeds). With a similar dark sprouting condition, the 40 whole seeds collected from Port Dickson, Kampung Seri Mendapat, Masjid Tanah, and Merlimau earlier in October and November 2022, and subjected to C4L, produced 72 full plants. This was probably due to the environmental conditions where the mother

plants were grown (Vidaković & Poljak, 2024). Even though the origin of mother plant does not directly affect seed size, it can influence seed quality, nutrient reserves, or even epigenetic programming, which in turn affects offspring vigor (Gutterman, 1980; Finch-Savage & Bassel, 2016). Identifying and prioritizing seeds from mother plants in optimal growing conditions, for example, ample sunlight, nutrient-rich soil, etc., can lead to more vigorous seeds that produce more full plants from seed fractions. For species like *S. malaccense*, understanding these maternal effects can also inform breeding programs aimed at developing superior cultivars for propagation.

While the initial reserve quantity was not a limiting factor for sprouting, the finding demonstrated the possibility of adventitious embryony from seed fractions of *S. malaccense* (Green & Juniper, 2004; Loayza et al., 2015; Souza & Fagundes, 2014; Velasquez-Rosas et al., 2017). There was no obvious relationship between seed fraction weight and the growth performances of plants regenerated from seed fractions. Genetic fidelity analysis is needed to determine whether the smaller plants are clonal or adventitious type, while larger plants, on the other hand, are zygotic type.

7.5 Regeneration from Naturally Predated Seeds

Aside from studying the regeneration ability by deliberate seed cutting, this research work also provided insights into the species' tolerance to predation in nature. The study revealed that *S. malaccense* fruits and seeds suffered from varying degrees of predation, including hidden internal predation caused by insect pests like seed borers. Fruits that appeared healthy could contain severely compromised seeds due to larval boring and feeding. A similar finding can be found within other *Syzygium* species (Xiao et al., 2006; Liao & Yang, 2018; Lin et al., 2018; Reghunath & Raju, 2020; Dodiya et al., 2023). The investigation on the plant regeneration potential from naturally insect-damaged or infested seeds was successfully achieved as the result showed partially predated seeds, even those with severe loss of reserve, maintained a high potential to sprout and could produce functional plants. This is a significant consideration for species with limited seed availability, seedless fruits, or irregular fruiting patterns, like *S. malaccense*.

Salvaging predated seeds helps to save costs by making the best use of the harvested material as well as avoiding the need for huge and often expensive seed collection procedures. Moreover, utilizing damaged seeds promotes a more resource-

efficient approach for germplasm collection and planting material production, thereby reducing pressure on natural populations by making the most of available resources.

The collective findings from the study provide a view of the regenerative capacity of *S. malaccense* and a solid foundation for its successful propagation through seed fractionation techniques. This research strongly advocates for a comprehensive protocol for *S. malaccense* using seed fragmentation. Specifically, the protocol should include: (1) strategic seed sourcing: prioritizing fruits from vigorous mother plants grown in optimal environments; (2) saving predated seeds: identifying and utilizing viable portions of partially predated seeds; (3) controlled seed fragmentation: systematically cutting seeds into quarters, especially by longitudinal seed quartering (C4L), to maximize the number of propagules per seed; (4) optimize dark sprouting: initiating sprouting of fragmented seeds in dark conditions to enhance full plant formation rates; (5) staged light acclimatization of the possible clonal plants regenerated from seed fractions: transferring the etiolated plants from darkness to light suitable for greening and hardening; (6) early vigor selection: implementing a screening process to prioritize plants with rapid shoot emergence for further cultivation; (7) precision nutrient management: adjusting nutrient delivery in raising plants regenerated from seed fractions.

Beyond *S. malaccense*, the concepts covered in this research could potentially be applied to other large-seeded, recalcitrant, or difficult-to-propagate Myrtaceae species, and even other plant families exhibiting similar regenerative ability. This research contributes significantly to providing a robust method for *ex situ* conservation of threatened or endangered species by maximizing planting materials from limited seed resources. Propagation of plant species through seed fragmentation offers a novel, potentially more efficient and sustainable alternative to traditional vegetative propagation methods, particularly for species where vegetative propagation by natural methods is difficult, as some seed fractions are believed to develop clonal planting materials.

While promising, the implementation of these strategies in a commercial setting will require addressing certain practical challenges and warranting further research. While this study assessed the growth of plants regenerated from seed fractions for only two months, long-term field performance of plants originated from fragmented or predated seeds, especially their fruiting capacity and overall tree health, needs to be evaluated. This would confirm their suitability for commercial orchards. Moreover, a

deeper investigation into the biochemical and hormonal pathways triggered by seed fractionation and dark conditions that lead to *de novo* embryo formation and shoot development would provide a more fundamental understanding, potentially leading to even more refined propagation methods.

CHAPTER 8
CONCLUSION AND RECOMMENDATION

8.1 Conclusion

This study has successfully demonstrated that the seeds of *S. malaccense* possess a capacity for regeneration, even when subjected to physical damage, such as fragmentation. This finding is important given the challenges associated with the propagation of this species, including the physiological phenomenon of seedless fruits and susceptibility to natural predation, which can make viable seeds rare.

The study on the regeneration capacity *S. malaccense* seeds has broadened the understanding of the reserve effect of *S. malaccense*. The reserve effect hypothesis, which suggested a direct correlation between seed reserve mass and regenerative success, does not apply to *S. malaccense*. Smaller fractions of quartered seeds were proven capable of producing full plants. Subsequently, the seed fractions also exhibited resprouting after removal of the newly formed full plant, although the seed is monoembryonic.

Subsequent study on the effects of light conditions on entire plant regeneration from quartered seeds showed that seed fractions had enhanced regeneration rates with dark sprouting. This result provides a highly efficient propagation strategy. The information has given insights into the strategy to produce more planting materials with a cost-saving approach.

In the subsequent growth of plants regenerated from seed fractions, results indicated that early sprouting was related to larger plants in terms of height and total leaf area. These plants are likely zygotic type. On the other hand, plants regenerated later from seed fractions were smaller. These smaller plants are yet to be studied as whether they are true-to-type adventitious or clonal type.

Aside from countering scarce resources of propagation material by fragmentation in vivo method, this research has demonstrated another ability of this species; naturally predated seeds still retained a capacity for regeneration and produced full functional plants. This finding suggests that nurseries can optimize available resources, including the predated seeds.

By understanding and utilizing the regenerative ability of these seeds, being

intact following fractionation or predated, propagators can overcome the natural limitations of low seed availability, thereby establishing a more reliable and efficient method for producing planting materials. It is hoped that the foundation of this study will generate new perspectives on the unconventional method of planting material production.

8.2 Recommendation

To further understand *S. malaccense* regeneration from seed fractions or predated seeds, and improve its practical application, several areas of future research are recommended. First and foremost, the study should be made on environmental factors beyond light. Future research should investigate other exogenous factors, such as nutrition and pollination, and their combined effects on the regeneration of fragmented seeds. Understanding these interactions could optimize propagation protocols for different environmental conditions.

Aside from that, a detailed analysis of the hormonal and biochemical changes occurring within the cotyledonary tissues during regeneration from seed fractions would provide a deeper understanding of the cellular mechanisms activated by fragmentation. This could help in identifying the specific signaling pathways that trigger the formation of new embryonic cells. Further studies to identify the biochemistry aspects of in vivo sprouting of the seed fractions should be done in the future to add knowledge regarding the role of hormonal changes that lead to the induction of shoot and root development from cotyledons. Last but not least, the genetic analysis of plants regenerated from seed fractions should be carried out to confirm if the plants developed from cut seeds are genetically identical to the mother plant.

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APPENDICES

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AUTHOR'S PROFILE



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LIST OF PUBLICATIONS:

Nur Marini Fatini, M. A., Tsan, F. Y., Siti Maslizah, A. R., & Yusuf, C. Y. L. (2024). Propagation of *Syzygium malaccense* through seed fractionation technique. *Food Research*, *8*(4), 43–50. https://doi.org/https://doi.org/10.26656/fr.2017.8(S4).8

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