AVOCADO SEED PHYSIOLOGY ASPECTS

By

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DECLARATION

I, **MAMOUN AHMED ARABI ABDALLA**, declare that the research reported in this thesis, except where otherwise indicated, is my original work. This thesis has not been submitted for any degree or examination at any other university.

Mamoun Ahmed Arabi Abdalla

August 2021

I certify that the above statement is correct.

Prof. Isa Bertling

Supervisor

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PUBLICATIONS AND CONFERENCE PRESENTATIONS

All chapters of this work, except chapter 1, are intended for publication, and as a result, are written in the form of manuscripts. Due to the time limit, only chapter 6, is published, while chapter 3 is currently under review. Others will be submitted to relevant journals.

Published papers

Arabi, M.A. and Bertling, I., 2019, November. *Moringa oleifera* leaf extract affects avocado (*Persea americana*) seed phenolics, sugars and starch as well as germination. In: *II International Symposium on Moringa. Acta Horticulturae,* 1306: pp. 185-192. (This paper was also presented at the II International Symposium on Moringa)

Conference presentation

Seed germination of 'Hass' and 'Fuerte' avocado is affected by seed coat thickness and seed coat phenolic compounds. presented at the Combined Congress Conference as an oral presentation, Cape Town, January 2018.

DEDICATION

This thesis is dedicated to:

The soul of my father, to my mother, brothers and sisters with all my love.

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PREFACE

The research contained in this dissertation was completed by the candidate while based in the Discipline of Horticultural Science, School of Agricultural, Earth and Environmental Sciences of the College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Pietermaritzburg, South Africa. This thesis is a compilation of manuscripts where individual chapter is an independent article introduced disjointedly. Hence, some repetition between individual chapters has been inevitable.

GENERAL ABSTRACT

The avocado seeds/seedling is needed as rootstock for other economic trees and loss of tress stand in orchids after establishment is of great commercial loss in avocado orchids around the worldwide and South Africa. The aim of this study was to evaluate and compare avocado seeds development of various seeds ages by investigating seeds germination percentage over three generations, as there is little information on avocado seeds growth and development, despite the importance of the seeds in avocado propagation. Seed harvesting was carried out over various developmental stages, from early fruit development to two-year-old seeds (Generation 1, 24 to 29 months after full bloom MAFB). Seed from current season (Generation 2, 12 to 17 MAFB) and newest seeds (Generation 3, 0 to 5 MAFB) of two cultivars ('Hass' and 'Fuerte') was analysed. Seed of three generations were analysed: 'Hass' Generation 1seed (seed from the oldest, commercially over-mature, fruit full bloom in July/ August 2017); Generation 2 (full bloom in July/ August 2018) and Generation 3 (full bloom in July/August 2019). Similarly, 'Fuerte' fruit of three generations were compared: from the avocado fruit, (Generation 1, full bloom in June/July 2017), to Generation 2 (full bloom in June/July 2018) to Generation 3 (full bloom in June/July 2019). Seed were extracted from fruit to determine seed parameters, such as germination percentage, seed viability, seed moisture content and seed respiration rate. Further, seed physiological parameters, such as cotyledonal sugars and starch concentrations, seed coat phenolic compound concentrations and polyphenol oxidase (PPO) concentrations were determined. Anatomical features of the seed coat, such as seed coat thickness and seed coat ultrastructure were also observed. In both cultivars, the germination percentage was higher in Generation 2, 12 to 18 MAFB), than in Generation 1, 24 to 29 MAFB) seed from June to September. From October to November Generation 3 (0 to 5 MAFB) had a higher germination percentage than Generation 2. Seed viability was higher in Generation 2 of both cultivars and lower for the Generation 1; similar results were found for the germination percentage, with seed from Generation 2 having a higher germination rate than seed from the Generation 1. Seed viability differed significantly between seed age, and the interaction between generations and months was statistically significant ($P < 0.001$). The seed collected from fruit of the Generation 2 of both cultivars had a slightly higher moisture content and a higher germination percentage than the Generation 1. Seed moisture content ranged between 54.5 and 62.1 % in 'Hass' (Generation 2 seed age 12 to 15 MAFB), harvested in June to September, while the Generation 1 seed age 23 MAFB) seed had a lower moisture percentage (39.2%) in June. 'Hass' seed of (Generation 3 seed age 4

MAFB) harvested from October to November had a higher seed moisture than seed from (Generation 2,15 MAFB). 'Fuerte' seed showed a similar pattern with the highest moisture percentage (60.5%) in July and the lowest in June (33.2%). (Generation 2' seed age 13MAFB seed had higher moisture percentages than Generation 1 from June to September, and (Generation 3, 3 MAFB) had higher moisture percentage than Generation 2. Seeds respiration rate, determined following fruit harvest, decreased over the time. Generally, Generation 2 respired more than the Generation 1, from June to September. From October to November 2019 the Generation 3 respired more than Generation 2 seed. The respiration rate of seed extracted from June to September 2018 Generation 2 declined rapidly. The Generation 3 (collected October to November 2019, seed age 4 to 5 MAFB) were characterized by a higher respiration rate than seed of Generation 2, seed age 16 to 17 MAFB; therefore, younger seeds generations respired more than older ones. It is concluded that the contribution of seeds respiration rate to avocado whole fruit respiration decreases with development over the time. The ability of the avocado seed to germinate quickly and produce seedlings is dependent on the carbohydrate reserves in the cotyledons, which make up the bulk of the avocado seed. In seed coats of both cultivars, phenolic concentrations inhibited seed germination of Generation 1, probably due to the higher level of phenolic concentrations in older seed coats. Seed coats generally contained high amounts of phenolics (2.3 mg GAE*g^{-1} DM for 'Hass' and 2.02 mg GAE* g^{-1} DM for 'Fuerte'). Seed extracted from Generation 1 fruit in June to September 2018, had a higher amount of seed coat phenolics than those from Generation 2 fruit. In fruit from October to November 2019 the Generation 3 seed coat had lower phenolic concentrations than Generation 2 seed coats, confirming that older seed coats contain more phenolics than younger seed coats. Germination percentages of Generation 3 seed were higher than those of Generation 2 seed. The high phenolic concentration in the seed coats seems to be aligned with the seed turning dark brown upon maturation, probably due to sufficient oxygen present in the fruit to allow phenolic oxidation of the seed coat; the seed coat becoming entirely brown and very thin, could, therefore, be used as an indication that the fruit has reached physiological maturity. Seed at this stage of maturation are, however, characterized by a low germination percentage, possibly due to the seed coat phenolic compounds interfering with germination. This is supported by the positive correlation between lower seed coat phenolic compound concentration and higher seed germination rate for both cultivars ($r = 0.11$, $P < 0.61$). Seed coat thickness of Generation 1 (24 to 28 MAFB) and Generation 2 (12 to 16 MAFB) 'Hass' seed coats differed, with

the younger seed generation displaying thicker seed coats than the older ones (0.51 *versus* 0.11 mm, respectively). In 'Fuerte', in June and July older seed coats Generation 1, 24 to 25 MAFB, respectively) were thicker than Generation 2 (12 to 13 MAFB) (0.46 and 0.15 mm, respectively. There was, however, negative relationship between seed coat thickness and germination percentage (r = -0.11). Polyphenol oxidase (PPO) and phenolic concentrations of avocado seed coats were also investigated in the seed coat of Generation 1and Generation 2 'Hass' and 'Fuerte' seed. Polyphenol oxidase (PPO) and phenolic concentrations of the avocado seed coats of the two avocado cultivars of Generation 1, 27 to 26 MAFB) and Generation 2, 15 to 14 MAFB) respectively, seed coats were investigated. During the colder (winter) season (June-August), Generation 1, fully mature 'Hass' seed coats showed higher polyphenol oxidase (PPO) concentrations than seed coats from the Generation 2. From October to November the Generation 3 seed coat also had a lower PPO concentration than those of Generation 2. Generation 2 'Hass' seed coats had relatively low PPO concentrations in June /July, when fruit were 12 to 14 MAFB, but PPO concentrations increased thereafter and remained at a higher-level until October/ November. Generation 1 'Fuerte' seed coat had a similar PPO concentration during all investigated months. Phenolic compounds were present in seed coats of both avocado cultivars, with seed coats of older seeds containing a much higher phenolic concentrations than the seed coats of the newer generation. The seed (cotyledons plus embryo) sugar profile was dominated by the C7 sugar perseitol, followed by the C6 sugar, sucrose, while mannoheptulose and glucose were present in very small amounts. Perseitol was present in in both cultivars with 14 months-old 'Hass' (September) cotyledons containing 9.8 $mg*g^{-1}$ DM and 15-months-old 'Fuerte (September) containing 10.3 $mg*g^{-1}$ DM. Avocado cotyledons were found to also be a large starch source, probably providing carbohydrates for seed development and germination. The Generation 2, 14 to 15 MAFB) of 'Fuerte' and 'Hass' had a higher starch concentration than the Generation 1, 26 to 27 MAFB) and similarly, Generation 3, 4 to 5 MAFB, respectively, had higher starch concentration than Generation 2 for both cultivars, indicating the use of this carbohydrate reserve to sustain embryo development. The highest concentration of starch in 'Hass' seeds was detected in August as 88.8% of seed DM (Generation 2, seed age 13 MAFB), while for 'Fuerte' seed the highest starch concentration was in August at 90.5% of seed DM (Generation 2, 14 MAFB). Starch seems, therefore, more related to avocado seed development than to avocado fruit growth and development. Delaying fruit harvest to October (seed age 16 to 18 MAFB) allows seed to fully mature and to continue accumulating sugars and starch. To improve

percentage and velocity of germination, seeds were soaked in various concentrations of aqueous moringa leaf extract (MLE, 0, 2.5, 5.0 and 7.5 % w/v) over different periods (0, 10, 30 or 120 minutes). Younger seed were stronger affected by the increasing MLE concentration. Soaking in 2.5% MLE tended to enhance the germination percentage more so than the other MLE concentrations. The lowest germination percentage was determined for seeds soaked in 7.5% MLE for 120 minutes, indicating that younger seed (from10 to 12 months after fruit set 'Fuerte' fruit harvested April to June) should be used as 'nurse seed'. Overall, this study revealed that avocado seed germination and development do not coincide with the commercial fruit harvesting period, the avocado fruit needs 15 to 18 months to change from its flowering blooming period to a full harvest, and seed age12 MAFB can germinate for both cultivars. The study further confirmed perseitol as the dominant free storage sugar that assists in seed development, while starch is also an important energy provider for the developing embryo.

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CHAPTER SIX

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GENERAL INTRODUCTION

Avocado (*Persea americana* Mill.) is an evergreen, subtropical fruit originating from an area including the current countries of Guatemala and Mexico, as well as other parts of Central America. Avocado belongs to the family Lauraceae, a family containing mostly woody species distributed worldwide in tropical and subtropical areas (Rohwer, 1993). *Persea americana* is a very variable species, encompassing different ecological races (Knight, 1999). It is an important tropical fruit that is rich in fibre, B vitamins and saturated as well as unsaturated fatty acids, carbohydrates and other nutritional compounds (Bahru *et al*., 2019).

'Hass' and 'Fuerte' are worldwide the most important avocado cultivars grown commercially, these cultivars belong Mexican race (Bender, 2012). Three botanical varieties (races) of avocado are widely recognized: the Mexican, the Guatemalan and the West Indian race (Berg and Ellstrand, 1986; Berg, 1992; Lavi *et al*., 2003). Avocado seeds appearance differs between races, the seed of the Guatemalan race is usually smaller than that of the two other race and the fruit have a thick skin to protect the pulp and seeds (Bergh and Ellstrand, 1986). The West Indian race is characterized by a generally large seeds and the seeds is not tightly attached to the mesocarp (Wood, 1984). The Mexican race has large to very large seeds, and the seeds is not tightly attached to the pulp, and fruit skin is thin (Mhameed *et al.*, 1997; Scora *et al.*, 2002).

The avocado mesocarp generally encloses a large seed (approximately 20 - 60 g) that plays an important role in fruit growth and development; seedless fruit also exist. Seeded fruit are, however, always bigger than seedless ones (Lovatt, 1990). Commonly, the avocado seeds accounts for 16% to 20 % of the total mass of the avocado fruit (Dabas *et al*., 2019). Avocado seeds contain various components that can be advantageous during early seedling development, such as the starch reserves, which constitute between 60% to 75% of the dry mass of the avocado seeds, depending on cultivar. The seeds, or more specifically the seed coat, also contains polyphenols which possess antioxidant activity (Kahn, 1987; Wang *et al*., 2010; Orhevba and Jinadu, 2011). Avocado seeds represent, therefore, an important carbohydrate and starch reservoir and contain high levels of fatty acids and nitrogen sources for the embryo. The seeds also contain low concentrations of glycosides, saponins, carnitine and sterols (Jimenez-Arellanes *et al*., 2013). Seeds, moreover, also contain the common C6 carbohydrate storage form starch, as well as the soluble sugars sucrose and glucose; additionally, the C7 sugars mannoheptulose and perseitol are

present, a feature common in plants that produce these less-common carbohydrates (Häfliger *et al*., 1999; Liu *et al*., 2002; Tesfay *et al.*, 2012). Furthermore, the high cytokinin to abscisic acid ratio found in avocado seeds is the most likely trigger of the long-lasting viability of the seed coat (Cutting and Bower, 1989).

The sugar profile of avocado seeds and/or seedlings grown in light *versus* dark differs (Kazama *et al*., 1978). In avocado, the sugar alcohol perseitol has been reported to be the most likely reserve or storage carbohydrate (Cowan, 2004). This function of perseitol was supported by Tesfay *et al.* (2012), who demonstrated that this sugar is predominantly stored in avocado seeds tissue. Previous studies have also suggested that starch and sucrose play important roles as energy sources in avocado seeds tissue (Bertling and Bower, 2005; Tesfay *et al.*, 2012).

The large carbohydrate reservoir in the cotyledons acts as a valuable carbon source for seeds growth and development (Jimenez *et al.,* 2013). During seeds development, reserve C7 sugars (particularly perseitol) are found in greater abundance than other sugars in the cotyledons. Cowan *et al*. (1997) investigated the solute transport to the avocado seeds during fruit growth and development and Moore-Gordon *et al*. (1998) demonstrated active solute transport via plasmodesmata.

Fruit is divided into two types according to its respiratory pattern close to maturity, namely, non-climacteric and climacteric fruit. The non-climacteric pattern shows no peak in ethylene and CO_2 evolution during fruit development and maturation, while climacteric fruit are characterized by an increased production in CO_2 and in ethylene during the maturation process. This higher gas production is followed by a decline and CO_2 release (Gardjito *et al*., 2015). Avocado fruit follows a higher climacteric pattern than most other fruit (Bower and Cutting, 1988). In the young avocado seeds, however, the respiration rate is higher than in the mature avocado seeds. The seeds do not seem to exhibit a climacteric pattern, once the fruit is harvested, avocado seeds respiration simply decreases after harvest; therefore, the ratio between the respiration rate of the avocado fruit and that of the seeds changes during fruit development, with the contribution of seeds respiration to entire fruit respiration decreasing over the growing season (Zauberman and Schiffmann, 1972).

The avocado seeds are surrounded by a seed coat, which is used as an internal characteristic, indicative of the fruit's quality and harvest maturity. The seeds are enveloped by two integuments,

which develop into a seed coat, but it is difficult to distinguish these two fused layers and, therefore, these are simply termed "the seed coat". The thickness of these integuments varies with seeds development, constituting 4.5% of the total fresh mass of the immature fruit; as the seed coat shrinks, gradually drying up; this 'dried seed coat' is used as a measure of the horticultural maturity of the fruit (Blumenfeld and Gazit, 1970; 1971). Previous studies have shown that the seeds of late-harvested avocado fruit tends to germinate inside the fruit, so that this radicle protruding into the mesocarp possibly becomes the reason of avocado fruit drop (Gazit and Blumenfeld, 1970).

Several studies have reported that there is a high antioxidant activity present in various plant parts (Ramarathnam *et al*., 1995), such as the seeds of avocado (Soong and Barlow, 2004), the fruit of the avocado (Bertling *et al*., 2007) and pineapple fruit (*Ananas comosus*) (Gardner *et al*., 2000), the root of carrot (*Daucus carota*) (Ghasemzadeh *et al.,* 2012), the tubers of potato (*Solanum tuberosum*) (Akrimi *et al*., 2020) and tobacco (*Nicotiana tabacum*) (Zygadlo *et al*., 1994). Antioxidant compounds exist in the seeds of many tropical and subtropical plants, such as mango (Puravankara *et al.,* 2000), guava (*Psidium guajava*) and papaya (*Carica papaya*) (Norshazila *et al*., 2010). In avocado, the antioxidant activity in the seeds is higher than in the fruit pulp (Alagbaoso *et al*., 2015). Kristanty *et al*. (2014) reported that the avocado seeds contain a high level of phenolic compounds, contributing majorly to seeds antioxidant activity.

Avocado seeds have been studied as a traditional medicine and as a food source, due to their therapeutic properties and high nutritive content (Ranade and Thiagarajan, 2015). Many medicinal effects of avocado seeds, including hypoglycaemic, hypotensive, anti-viral, analgesic and anti-inflammatory effects, have been described by Imafidon and Okunrobo (2009). Some studies also suggest avocado seeds to be a source of anti-cancer, anti-hypertensive, anti-diabetic, hypo-cholesterolemic, as well as insecticidal and antimicrobial compounds (Dabas *et al.*, 2013; Calderón *et al*., 2016; Soledad *et al.*, 2021).

The avocado seeds also act as a potential water reservoir during fruit development (Wolstenholme and Whiley, 1999). Avocado fruit contain a single seed, and there is a positive correlation between seeds and fruit size, with larger seeds generally enclosed by larger fruit. The seeds affect avocado fruit features (shape and size) and processes (growth and maturation) during different stages of development (Cannel, 1985; Wolstenholme, 1985; Cowan *et al*., 2001). Indications are, that

during fruit development, the seeds have a certain dominance over other parts of the avocado fruit, successfully competing for available water and nutrients (Moore-Gordon *et al*., 1998; Cowan *et al*., 2001).

The purpose of the seed coat seems to be that of a trafficking route control for solute movement between the mesocarp and seeds through its plasmodesmata (Moore-Gordon *et al*., 1998; Crawford and Zambryski, 1999; Botha *et al*., 2000; Van Bel, 2003). The function of the seeds as a non-endospermic storage organ at maturity (Bewley and Black, 1994) points out that the seeds regulate biochemical and physiological processes in the avocado fruit during maturation and development (Gillapsy *et al*., 1993). As avocado fruit development progresses, seeds moisture declines in the early-maturing 'Fuerte' seeds more so than in the late-maturing 'Hass' seeds (Kalala *et al.,* 2005). The avocado seeds are recalcitrant (Egli, 1990; Wolstenholme and Whiley 1999); therefore, the seeds cannot be stored for long periods, the seeds can lose its viability.

The typical feature of recalcitrant seeds, instant germination, results in a short seeds storage life (Walters *et al.,* 2013). While the seeds can act as a water reservoir during fruit growth and development, the mesocarp, on the other hand, controls water availability and solute transport from the tree to the seeds (Moore-Gordon *et al*., 1998; Cowan *et al*., 2001). Water, moving between the seeds and the mesocarp, is the vehicle that carries essential nutrients, and other compounds to maintain plant tissue functions (Welbaum and Bradford, 1990). This movement requires the diffusion of solutes from the embryo via the surrounding seeds coat and mesocarp of fruit. The embryo controls the solute movement from the surrounding tissues into the seeds (Singh, 1953); therefore, plasmodesmata need to carry molecules/solutes between the mesocarp and seeds and *vice versa* (Moore-Gordon *et al.,* 1998; Crawford and Zambryski, 1999; Botha *et al.,* 2000; Van Bel, 2003).

Seeds are main storage tissues for nutrients to supply the embryo with compounds necessary for development, maturation and germination (Bewley and Black, 1994).

While avocado seed development has been investigated for more than half a century, still little is known about general avocado seed physiology, although the seed is very important in the production of commercial avocado trees. A healthy nurse seed is double grafted with a rootstock and scion to ultimately produce a healthy seedling for a high yielding clonal tree that produces high quality fruit.

Research Aim

The overall aim of this study was to investigate seed germination and development in relation seed maturity of seeds extracted from either avocado fruit of the Generation 1, Generation 2 and Generation 3. Findings of the study will provide further knowledge on avocado seed development and will be useful to the avocado nursery industry, where a strong, vigorously growing 'nurse seed' is required as part of the double-grafting method to establish clonal trees. Therefore, the aim of this study was to evaluate avocado seeds of various stages of maturity of two cultivars, 'Hass' and 'Fuerte', with respect to their physiological characteristics, as well as seed their germination features and early seedling development.

Significance of Research

In certain avocado-producing conturies seeds of the avocado cultivars 'Hass' and 'Fuerte' are used as 'nurse seed' to produce commercial trees by grafting clonal (genetically identical) rootstocks onto seedlings (genetically variable) derived from 'Hass' and 'Fuerte' fruit/seeds. The shoot of the 'nurse seedling' is therefore carrying the clonal rootstocks. The scion (cultivar) is then also grafted onto this clonal rootstock, which devolop in own roots. When the propagated avocado tree leaves the nursery, a girdle is placed around the seedling rootstock, so that the young avocado tree planted in the orchard only consists of the clonal rootstock and the clonal cultivar.

Research Objectives

The study aimed at, firstly, advancing the basic knowledge of avocado seeds physiology and, secondly, determine the optimal time for avocado nurseries to harvest fruit/seed to be used as 'nurse seed' in propagation. Results from the study will be vital to the local avocado industry and for word-wide expansion of avocado production. to examine avocado seeds performance in terms of germination, seeds age and quality, early seedling growth and development of 'Hass' and 'Fuerte' avocado seeds.

The specific objectives of the study were:

1. To determine the seed germination percentage and describe the early development of two generations of 'Hass' and 'Fuerte' avocado seeds. To evaluate maturity parameters of avocado seeds by comparison of such parameters with the germination of seed from varying fruit ages

2. To determine the concentration of total phenolic compounds in the avocado seed coat as well as determining seed coat thickness of the two avocado cultivars in relation to seed germination percentage. To gain a deeper understanding of the involvement of phenolics in avocado seed germination by determining the polyphenol oxidase (PPO) concentration of the avocado seed coat in relation to seed germination

3. To determine the sugar concentrations present in the avocado seed (cotyledons) in order to establish whether avocado seed development is aligned with seed sugar and/or starch concentrations, seed respiration rate, moisture content and germination percentage. It was further attempted to determine, if C6 and C7 sugar and/or starch concentrations in the avocado cotyledons are aligned with seed respiration and seed germination percentage

4. To evaluate morphological characteristics of the avocado seed coat surface as distinctive features for identification of the fate of the avocado seed coat from immature to mature seeds, and to compare the inner (between seed coat and seed) and the outer (between seed and mesocarp) seed coat surfaces using scanning electron microscopy (SEM). To evaluate the effect of various concentrations of methanolic moringa leaf extracts (MLE) combined with different soaking durations on growth and development of 'Fuerte' avocado seed extracted from fruit of different ages on seed germination

Outline of the dissertation

The dissertation is, hence, made up of seven chapters:

Chapter 1: General Literature Review

Chapter 2: Seed Germination of 'Hass' and 'Fuerte' Avocado as Affected by Seed Coat Thickness and Seed Coat Phenolic Compounds

Chapter 3: Seasonal Variations in Avocado Seed Viability and Respiration Rate Affect Germination Percentage

Chapter 4: Seasonal Alterations in Polyphenol Oxidase Activity and Phenolic Compounds of The Avocado Seed Coat Over Several Seed Generations

Chapter 5: Phenolic Compound Concentrations and Ultrastructural Changes of the Seed Coat Throughout Avocado Seed Maturation

Chapter 6: *Moringa oleifera* Leaf Extracts Affect Avocado (*Persea americana* Mill.) Seed Phenolics, Sugars and Starch, as well as Germination

Chapter 7: General Discussion, Conclusion and Outlook

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CHAPTER ONE

GENERAL LITERATURE REVIEW

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1.1. Introduction

Avocado (*Persea americana* Mill.) belongs to the Lauraceae family of tropical and Mediterranean trees and shrubs. As typical of many evolutionary old species (Rohwer, 1993), avocado contains a single, rather large seeds (Blumenfeld and Gazit, 1974) (approximately 20 to 60g, depending on cultivar), encompassing the two large cotyledons and the embryo. Seeds commonly also store carbohydrates, as well as nutrients, to provide the growing embryo with its 'first food' (Mauseth, 1988). Generally, non-edible seeds, like that of avocado, contain high levels of bioactive compounds, particularly those with antioxidant activity (Vinha *et al.*, 2013; Mensah *et al*., 2015).

Avocado seeds, therefore, is a rich source of various phytochemicals, such as phenolic compounds. Wang *et al*. (2010) analysed the fruit phenolic distribution, finding 64% of the total phenolics in the avocado seeds (combining seed coat, cotyledons and embryo), 13% in the pulp and 23% in the peel. Mainly due to these phenolics, seeds and peel of avocado contribute 57% and 38% to the antioxidant capacities of the entire fruit, respectively (Wang *et al*., 2010).

Carbohydrates are sources of energy and used as building blocks in plant biosynthetic reactions. The avocado seeds contain certain polyhydroxy-aldehydes (aldoses), ketones (ketoses), alcohols and acids, that fulfil certain mechanisms (Cowan, 2004). Carbohydrates exist in varying amounts in avocado seeds, depending on cultivar; however, the carbohydrate in the seeds is mainly made-up of sucrose and certain forms of starch (Liu *et al.*, 2002). This is particularly important, as avocado belongs to a group of plants producing scarce amounts of C6 carbohydrates (Häfliger *et al.*, 1999). The avocado seeds, however, plays a major role as a C6 sink/storage organ, affecting fruit growth and development; this is unusual, as, in avocado, fruit physiological processes, such as fruit development and maturation, are largely involving the C7 sugar metabolism (Duffus and Duffus, 1984). The avocado seeds also have two fused integuments, making up the seed coat (Blumenfeld and Gazit, 1970; Gazit and Blumenfeld, 1974). Several authors stated that plant hormones in the seeds play an important role in mobilizing resources used in fruit development (Martens *et al*., 1994). Blumenfeld and Gazit (1974) suggested that growth-promoting substances can be synthesized in seeds tissues; therefore, seeds are important in the prevention of fruit abscission and in fruit set as sources of growth regulators. Gibberellins, auxins and cytokinin's are involved in the continued cell division and expansion of avocado fruit tissue until fruit harvest, as well as in fruit ripening after harvest.

When the avocado fruit is still commercially immature (exceeding a certain amount of water or having not accumulated a certain oil percentage in the mesocarp), the seed coat is light, almost transparent, relatively thick and succulent. During seeds maturation, this structure shrivels and browns (due to oxidation of the phenolics), as the fruit nears maturity. The avocado seed coats have an inner integument, called tegmen, and an outer integument, the testa (Storey, 1973; Corner, 1976). When the avocado fruit has reached maturity, the seed coat has died, which is visible by its dark-brown colour and the complete loss of succulence, making it very thin (Blumenfeld and Gazit, 1971). The appearance of this thin, brown seed coat has been used as an indication of avocado fruit maturity (Erickson, 1966).

Generally, the seed coat is considered as a primary defensive tissue of the seeds, providing protection against environmental stress and biological attacks. It provides the embryo and seeds components attached with a physical and/ or chemical barrier to unfavourable conditions, thereby protecting the seeds against infection and deterioration caused by microorganisms (Bass, 1980; Priestley, 1986).

The avocado seeds contain various minerals, such as sodium, potassium, calcium, zinc, iron, manganese and magnesium. Seeds of unripe avocado fruit contain a higher concentration of zinc and potassium and a lower level of sodium compared with seeds of ripe avocado fruit (Alagbaoso *et al*., 2015). The amount of minerals, such as calcium, magnesium and potassium, found in mature avocado seeds are less in seeds of Mexican cultivars than in those of the Guatemalan race; however, avocado seeds generally contains relatively low amounts of calcium and magnesium, slightly higher amounts of phosphorus and high amounts of potassium (Haas, 1951). Similar to avocado seeds, the rather large mango seeds were found to also contain sodium, calcium, magnesium, iron, potassium, zinc, and copper, these minerals are essential for plant establishment and growth development (Fowomola, 2010). In general, seeds germination under a particular condition and season is determined by the interaction between the seedling's growth and dormancy-releasing factors, which are all influenced by light, phytohormones, water, temperature, nutrients, moisture and other cues, particularly mechanical, cues (Bareke, 2018).

Fresh and dry mass of the avocado seeds from one cultivar, grafted onto different rootstocks, can differ, even, if the fruit was grown under similar orchard conditions (Bender *et al.,* 2012); the nutrient composition of such fruit can also differ. Although the calcium and magnesium

concentrations of the seeds are relatively low, these concentrations decrease severely, as the fruit matures. Such losses may have implications on seeds viability, and particularly on seeds vigour; these will be important considerations, when the seeds are used as 'nurse seed' (Van der Walt and Witkowski, 2017).

The seed inside the avocado fruit influences the fruit's growth rate, its shape and size as well as its maturation. Avocado fruit maturation is characterized by the rapid accumulation of oil in the mesocarp, preceded by seed coat shrivelling and discontinuation of the transfer of substances between the seed coat and the fruit mesocarp (Blumenfeld and Gazit, 1974). 'Fuerte' avocados can remain attached to the tree for 10 to 15 months after full bloom, before horticultural maturity is reached (Blumenfeld and Gazit, 1974). 'Hass' avocados also remain on the tree for 13 to 18 months after fruit set, before reaching horticultural maturity (Barmore, 1976).

Commercial avocado seeds are relatively important to produce quality of the trees grown in the nursery, to determines the success of a grafting (Ahsan *et al*., 2019). Trees that received poor or incorrect treatment in the nursery will lag in the orchard. Is very important to use healthy seed to be rootstock (Robles, 2010).

1.2. Physiology of the Recalcitrant Avocado Seeds

Generally, seeds can be divided into two broad categories: orthodox and recalcitrant. Orthodox seeds can be dried to a low water content (<7%) with little effect on seeds viability (Pritchard *et al*., 2004), in contrast, recalcitrant seeds do not even tolerate drying to a water content as high as 20% to 30%. Because desiccation-sensitive seeds have to be stored at higher humidity, they progress towards germination when stored under wet conditions; they are, therefore, difficult to store for longer periods and are usually only stored short term.

Recalcitrant seeds, as well as orthodox seeds, can be stored under low temperature to maintain viability. When dried, recalcitrant seeds of tropical and subtropical species are stored short term, as it generally loses viability quickly. Hydrated storage is used for long term conservation and for storage of genetic material of plants producing recalcitrant seeds (Berjak and Pammenter, 2003).

Another difference between recalcitrant and orthodox seeds is that recalcitrant seeds can germinate, despite not having completed seeds maturation; such seeds can germination without

provision of additional water supply within the immature fruit (Finch-Savage and Clay, 1994). Therefore, the level of recalcitrance of a certain seed is related to how far maturation and germination have progressed before seeds drying. Orthodox seeds, on the other hand, can only germinate after complete (5-6% moisture) desiccation (McDonald, 2004). Such seeds can remain on the plant for an extended period without germination (Barbedo *et al*., 2013).

Most tropical seeds are recalcitrant, *i.e*., they cannot be stored for long periods of time without losing viability (Pammenter and Berjak, 1999). Popular tropical plants that produce recalcitrant seeds are avocado, cocoa, mango, litchi and the rubber tree. For seeds production and seeds storage, recalcitrance is a negative property, since recalcitrant seeds cannot be stored at low RH, making them prone to micro-organism attack (Farnsworth, 2000; Mngomba *et al*., 2007). Orthodox seeds, however, can be stored for months and even years, while recalcitrant seeds are characterized by short life spans of only days to months, or, for temperate species, perhaps a year or two, as long as such seeds will tolerate low (not sub-zero, but less than 10 °C) temperatures (Chin and Roberts, 1980; Shih *et al*., 2008).

Avocado seeds are recalcitrant, that is, they die after losing a certain percentage of water, approximately between 20% to 50%, of their fresh mass; therefore, avocado seeds cannot tolerate long periods of storage (Kozlowski and Pallardy, 2002; Ernst *et al*., 2013; Walters *et al*., 2013).

1.3. Development and Maturation of the Avocado Seeds

Seeds maturation is considered complete, when storage compounds have accumulated in the seeds and seeds moisture (water content) has decreased. Hormonally, at this stage, ABA levels have increased, and desiccation tolerance and primary dormancy have occurred. Abscisic acid plays an important role in the induction of seeds dormancy during seeds maturation and in the maintenance of the dormant state. The role of ABA as an inhibitor of seeds germination and a trigger that maintains seeds dormancy has been reviewed by several researchers, such as Kucera *et al*. (2005).

Being able to alter the timing of seeds development and maturation can represent an evolutionary advantage, as fast-forwarding these stages allows plants to cope with unfavourable environmental conditions; alternately, plants can interrupt their life cycle and resume growth, when favourable

conditions resume (Bewley, 1997; Bentsink and Koornneef, 2002). Avocado seeds maturation is required for seed development, which included reserve oil accumulation, embryo growth cease, tegument (seed coat) differentiation. seed maturation and it is expressed the most in late development (Petrollini, 2012).

Seeds morphology, physiology and maturation vary greatly between species, so does the accumulation of sugars and starch in various seeds tissues (Vicente and Carbonero, 2005). There is great variability between plants in terms of seeds physiology and maturation, seeds morphology, accumulation of various compounds, such as oils, seeds storage proteins, sugars, and starch, in the various tissues of the seeds. In some plants, seeds maturation is not even a mandatory step in the plant's life cycle (Pedreschi *et al.*, 2019).

Seed quality is a "multi-faceted concept", including physical purity, genetic quality, vigour, ability to germinate, uniformity in size, as well as freedom from seed impurities and diseases (Basra, 2006). Seed quality is determined by the total of several endogenous and exogenous factors that influence seed development and maturation. The last two stages of such seed's development encompass the embryo maturation stage, when the embryo increases in mass, and the desiccation stage, characterized by a marked decrease in seed water content (Harada, 1997). During seed maturation significant changes in embryo size and mass occur, due to accumulation of storage reserves. Seed quality is the "potential performance of the seeds established at physiological maturity" (Hilhorst and Toorop, 1997). In different plant species, maximum seed quality is, therefore, attained at different times during seed maturation (Demir and Samit, 2001).

Generally, in dormant seeds, ABA levels are high and GA levels are low (Lefebvre *et al*., 2006). Under normal germination conditions, GA synthesis starts shortly after seeds imbibition, which is essential for the rupture of both, testa and endosperm (Debeaujon and Koornneef, 2000; Lee *et al*., 2002). Following imbibition, ABA levels drop rapidly, and the radicle starts to emerge (Lopez-Molina *et al*., 2001; Muller *et al.*, 2006; Bethke *et al.*, 2007), while GA levels generally increase during the germination process (Skubacz and Daszkowska, 2017).

1.4. Phenolics and Maturation of Avocado Seeds

The seed of the avocado contains higher concentrations of phenolics and other antioxidants than the mesocarp (Wang *et al*., 2010). Antioxidants are a group of substances that significantly delay

or prevent the oxidation of oxidizable substrates, thereby decreasing tissue damage caused by oxidative stress (Kurutas, 2015). Phenolic metabolites protect the seeds against biological and environmental stresses; therefore, the avocado seeds contain high levels of phenolic compounds (Kristanty and Suriawati, 2014). Various other phytochemicals are also present in avocado seeds, such as phytosterols, fatty acids, triterpenes, proanthocyanidins and furanoic acids (Ding *et al.*, 2007; Leite *et al.,* 2009).

While the basic structure of phenolic compounds is simply an aromatic ring with a hydroxyl group attached, phenolics can also be highly complex structures, such as tannins and lignins (Harborne, 1994). Phenolic compounds can play a role as antioxidants through their redox properties, when acting as reducing agents, but they can also be hydrogen donors, singlet oxygen quenchers or metal chelators (Kasote *et al.*, 2015).

Phenolic compounds in foods are considered important in the human diet, preventing diseases associated with oxidative stress (Dykes and Rooney, 2007). The addition of phenolic compounds, extracted from plant material, can also be used to replace synthetic antioxidants, thereby increasing the food's nutritional quality and shelf-life (Sang *et al*., 2002). The commercial development of plants as sources of antioxidants (mostly phenolic compounds), to benefit human health and food preservation, has gained attention (Hajaji *et al*., 2010; Win *et al*., 2011). Phenolic phytochemicals are the most abundant groups of plant metabolites and form an important part of the human diet (Bravo, 1998). Epidemiological and biomedical studies indicate that consumption of fruit and vegetables, rich in polyphenols, may help to protect the human body against many chronic diseases, such as diabetes, cancer and cardiovascular ailments (Scalbert *et al*., 2005).

As the avocado fruit requires a long developmental period of six to more than twelve months from flowering to maturity (Scora *et al*., 2002), it is likely that the fruit is exposed to environmental stresses during this extended period. The fruit, and particularly the seeds, therefore, benefit from higher phenolic concentrations, as these acta as antioxidants, able to protect fruit and seeds from biotic and abiotic stresses. Phenolics are commonly synthesized as the plant develops, but their biosynthesis can be up regulated in response to microorganism infection, wounding and extreme temperatures (Naczk and Shahidi, 2006). When the plant identifies an attack, defence mechanisms in the plant are induced, both, at the distant, uninfected tissues and at the site of initial infection (Matern and Kneusel, 1988).

Plants produce phenolics and accumulate these in the subepidermal layers of the tissues exposed to stress and pathogen attack (Clé *et al.,* 2008). These phenolic compounds are the substrate of the browning reaction in plant tissue, when oxygen and phenolic substances react in the presence of the normally compartmented polyphenol oxidases (Chandra *et al.,* 2013).

1.5. Avocado Seed Germination

Seed germination is a complex physiological development stage that starts with water uptake by imbibition of the dry seeds and is completed when the embryo commences its expansion growth; this is visible as the protrusion of the radicle through the seed coat (Hilhorst, 1995). Germination results in the formation of the seedling; it is also the stage of reactivation of metabolic processes in the seed and ends with the emergence of the radicle and the plumule. During avocado seed germination the embryo emerges between the large cotyledons and penetrates the seed coat (Nonogaki *et al.,* 2010).

Plants have evolved several strategies to regulate germination; many seeds undergo a dormancy period during which germination is restricted, even under conditions that are normally favourable for germination (Bewley and Black, 1994). Dormancy is an ecological tool, allowing seeds to germinate in a specific season or at a specific time suitable for seedling growth and development. Seed germination and dormancy are controlled by a hormonal balance, particularly a certain GA to ABA ratio (Seo *et al.,* 2009). Gibberellins induce germination and release dormancy, while ABA is involved in the induction and maintenance of seed dormancy. Levels of GA and ABA are determined by the biosynthetic rate of their precursors and/or their release from conjugates (Seo *et al*., 2006; Yamaguchi *at el.,* 2007). Due to a certain endogenous GA/ABA ratio, seed dormancy blocks seed germination under otherwise favourable germination conditions; however, for uniform germination to occur, seeds need to have a low level of dormancy (Tuan *et al*., 2019).

Avocado fruit contain a single large seed of one embryo surrounded by the seed coat; the embryo consists of two large, starchy cotyledons with a centrally attached, very small, embryonic axis (Blumenfeld and Gazit, 1974). In the early development of the fruit, a gelatinous endosperm, as well as an embryo, are distinguishable; however, this small endosperm disappears completely at the final seed development stage; avocado seeds are, therefore, considered non-endospermic (Sedgley, 1979). During the late seed development stages, the vascular connection between the

mesocarp and the seed has disappeared (Blumenfeld and Gazit, 1974). When the seed has reached maturity, no further water can enter into the embryo, and the seed has no defence mechanism against dehydration (Adjei *et al*., 2011). Avocado seeds, therefore, lose viability during storage relatively quickly and the seed needs to be planted shortly after separation from the mesocarp to allow radicle protrusion (Adjei *et al*., 2011).

The avocado seed is one of the non-edible parts of the fruit, hence, it is usually discarded as residue(Bahru *et al*., 2019). Avocado seeds are used in propagation as nurse seed and thus, need to germinate with ease and uniformly. This germinating seed develops into a seedling onto which a clonal rootstock bud is grafted; subsequently, different scion cultivars are grafted onto the rootstock of the nurse seed/rootstock combination (Barriento *et al*., 1998). In nursery production the use of such a nurse seed is important for ease of commercial production of trees having both, a clonal rootstock and clonal scion cultivar. The nurse seed is subsequently removed. Such grafting is also important to improve avocado production to develop uniform tolerance of the young trees to soil stress, resistance to disease and to increase final tree productivity (Schaffer *et al*., 2013).

Commercial nurseries producing young avocado trees have, in the past, used numerous management tools to achieve uniform and rapid seed germination; these treatments include cutting the tip of the cotyledons (Johnston and Frolich, 1956), removing of the seed coats (Eggers, 1942) and puncturing the seed coat (Martinez *et al*., 1969). While non-uniformity of avocado seeds used as rootstocks would assist in selecting rootstocks adapted to different soil conditions, producing uniform avocado orchards requires clonal propagation (Ben-Ya'acov, 1985). It is, however, difficult to propagate and produce avocado vegetatively. When a certain avocado cultivar is grafted onto a seedling rootstock, this seed is taken from commercial cultivars "mother" tree of a known cultivar. When this seed develops into a seedling, a selected rootstock cultivar is grafted onto this seedling-rootstock combination, and, onto this combination, the commercial cultivar is inserted (double-grafting technique) (Ben-Ya'acov and Michelson 1995).

1.6. The Embryo of Avocado Seeds

The avocado fruit develops a single, large seeds consisting of two large cotyledons, while the radicle and plumule remain very small, and even at seeds maturity together only weigh

approximately 0.1g (Blumenfeld and Gazit, 1970). The tissue surrounding embryo and cotyledons, the fused integuments, influence seeds size (Berger *et al*., 2006). This seed coat is derived from the maternal tissue of the ovule (Ingram, 2010). The embryo's growth is supported by the surrounding cotyledons, from which it extracts minerals and carbohydrates for its growth and development (Sfaxi-Bousbih *et al.,* 2010). Generally, the developmental stage of the embryo is aligned with seed germination percentage, a mature embryo can germinate better than an immature one (Bridgen, 1994; Carimi *et al*., 1998).

Generally, dicotyledonous seeds consist of two cotyledons, an embryo and two fused integuments, making up the seed coat. The embryo tissues are differentiated into radicle and plumule. The radicle gives rise to the root of the plant and plumule tissue becomes the shoot of the emerging seedling.

Avocado embryos are surrounded by the two large cotyledons and covered by seed coat (Sánchez-Romero *et al*., 2007). The anatomy of the embryo of avocado seeds is characterised by a vascular system of several strands running in groups parallel in the cotyledons. These vascular tissues merge into single bundle and supply the radicle and hypocotyl with minerals and other supplies (Schroeder, 1958).

1.7. Seed Quality

A mature embryo is a prerequisite for a high-quality seed. Seed quality is, however, more than a mature embryo, it is a combination of physiological, physical, pathological and genetic seed qualities. Only a high-quality seed is able to produce a good crop and guarantees rapid plant development, even under adverse environmental conditions. Other factors, such as soil fertility, agronomic practices, rainfall, and pest control can, however, also effect crop performance (Rao *et al.,* 2017). Seed quality is characterized by certain measurable physical features, such as seed purity, seed viability, mechanical damage to the seed and freedom from pest and disease (seed health); however, seed vigour is also an essential component (Perry, 1980). Seed quality is defined as "the physiological and genetic purity of seeds" (De Geus *et al.,* 2008). To achieve genetic purity, highly homozygous parental lines need to be selected that, when crossed, are not carrying characteristics undesirable to the hybrid (Erickson and Atnaseo, 2011). Genetically pure seeds will produce a seedling and, finally, tree that is similar to the mother plant in all characteristics.

These quality features are important to increase crop yield or resistance to diseases or other quality characteristics (Elias *et al.*, 2012).

Other researchers (Moterle *et al*., 2011) have defined seed quality as the sum of many individual components, such as the seed's genetic quality (capacity to perform vital functions related to germination), while Bishaw *et al.* (2012) characterised physical purity as "seeds germination velocity and percentage, vigour, uniform size and health" (freedom from disease). Generally, poor quality seeds exhibit a low germination rate, poor emergence and seedling growth, reduced viability, and poor tolerance to sub-optimal conditions (Bedi and Basra, 1993), while high-quality seeds are those that have the ability to germinate and produce normal seedlings under a wide range of environmental conditions (Finch-Savage and Bassel, 2016). A universal test of seed quality is the germination percentage of a group of seeds or seed lots. According to ISTA (1987), "the standard germination test is a universal test for seed quality and evaluates the potential of seeds to germinate under an ideal set of conditions". A high germination percentage does, however, not necessarily result in fast and regular emergence, or in a vigorous stand under field planting conditions (Delouche and Baskin, 1973). The two most important indicators of seed quality are germination capacity ("germination percentage of seeds that would normally germinate under optimal conditions" (Domin *et al.,* 2020)), and seed maturity, the accumulation of starch and protein, seed germination and vigour increase gradually, and reach a maximum at physiological maturity (Salisbury and Ross, 1999; Qun *et al*., 2007).

Seeds vigour is "the sum total of those properties of the seeds that determine the potential level of activity and performance of the seeds during germination and seedling emergence" (Perry, 1978, 1980). This physiological quality is determined by the seed's genetic constitution, as well as several endogenous and external factors, occurring during seed development on the plant, at harvest or during storage (Tekrony, 2003; Powell *et al*., 2005).

1.7.1. **Seed Viability**

A viable seed is a seed that has the potential to germinate, to emerge and to form a normal, healthy seedling under suitable environmental conditions. Viability is *"*the percentage or proportion of viable seeds in a seed lot" (Sawma and Mohler, 2002). Seed viability tests are important to determine the quality of seeds, which has a major impact on the quality of the plants arising from

such seeds. Seed viability and seed vigour are physiological aspects of seed quality, where the loss in seed vigour precedes the loss in viability (Marcos, 1998).

Testing of seed viability is commonly carried out using the tetrazolium (TTZ) test; in this test respiring tissues are stained due to the formation of a formazan complex by transfer the reactions catalysed under the action of dehydrogenase enzymes; this test is generally considered as the most accurate method of determining seed viability. The TTZ test biochemically differentiates between viable tissues and dead tissues and is based on the relative respiration rate of such tissues, when in a hydrated state. Seed viability is then interpreted according to the tissue staining pattern of the embryo and the intensity of tissue coloration (Copeland and McDonald, 1995). Viability testing with tetrazolium (TTZ) is always preceded by germination testing to be able to develop a relationship between seeds viability and germination, characteristics important for testing seed viability and quality of seeds before sowing (ISTA, 1985). The estimate of the viability through the germination test takes several days, while the TTZ test is a quick test, determining seed quality within hours (ISTA, 1996).

When dried seeds are imbibed, cell membranes lose the ability to keep the solutes inside the cells; therefore, dried seeds are characterised by leakage of solutes, such as sugars, ions, amino acids and organic acids, into the imbibing medium (Bewley and Black, 1985; Copeland and McDonald, 1995; Sørensen *et al*., 1996). It is important to store the seeds under favourable conditions to avoid desiccation beyond a certain point, so that the seeds will not lose their ability to germinate.

1.7.2. **Seed Vigour**

Seeds vigour affects the recommendation for seeding rates and is, together with the germination test, the standard test for describing seeds lots (Steiner, 1990). Copeland and McDonald (1995) emphasized that seeds vigour tests can also have an important role in production and marketing decisions. Seeds vigour describes the seed's germination behaviour under optimal conditions, or identifies, if seeds from different sources portray similarly high levels of germination (Perry, 1980). Under more stressful conditions, however, the same seeds may have different abilities to grow into a plant, due to differences in seeds vigour (Finch and Bassel, 2016). Seeds vigour can be considered as the potential performance of the seed's viability in agricultural practice, and this potential is determined by genetic and environmental components (Hodgkin and Hegarty, 1978).

Seed vigour is “the combination of seed features potentially determining rapid and uniform seedling germination under different environmental conditions” (ISTA, 1995). The definition accepted by ISTA (1987) as seeds vigour is “the sum of those seeds properties that determine the potential level of activity and performance of the seeds during germination and seedling emergence” (Perry, 1978). There is no single factor defining seed vigour; seed vigour is a concept related to certain characteristics of seed performance; these include uniformity and rate of germination, as well as seedling growth and the ability of seeds to emerge under unfavourable environmental conditions; seed vigour also includes the ability of seeds to germinate and emerge after storage (Finch and Bassel, 2016).

1.8. Avocado Seed Quality

Knowledge of the seed quality of a certain seed lot allows the seed user to achieve the goal of establishing highly uniform seedlings and of increasing the yield of horticultural and agronomical crops under a variety of growing systems (Matthews *et al*., 2012). Generally, the seed’s potential to establish a seedling and for the seedling to grow into a healthy plant cannot be determined until germination has occurred (McDonald *et al*. 1993).

Avocado seeds size, an important indicator for seedling growth, affects the germination rate. Deb and Sundriyal (2017) demonstrated that medium-sized avocado seeds germinate better than larger seeds, with the lowest germination rate found in small-sized seeds. Larger seeds are, therefore, preferred, when used as ‘nurse seeds’ (Castro, 2017). Such seeds are important in avocado propagation, where the clonal rootstocks are grafted onto a seedling, derived from this ‘nurse seed’. This seed provides the initial root system; hence, it must be a healthy, vigorously growing seed. If avocado trees are to be propagated continuously throughout the year, seed storage is necessary, particularly, when the fruit production of grafted trees does not match the fruit harvesting season (Castro and Fassio, 2013; Ernst *et al.*, 2013).

The avocado seed contains a large carbohydrate reservoir and provides, therefore, a good carbon source for microbial growth. In addition, avocado seeds are characterised by a high level of fatty acids and also contain nitrogen and phosphorus sources (Jimenez *et al*., 2013). The quality of avocado trees to generate sufficient propagation material to use as grafting to produce to high quality avocado seedlings (Sawma and Mohler, 2002). Healthy seed and high-quality avocado

seeds contain certain anti-nutritional components, such as hydrocyanic acid, cyanogenic glycosides, condensed polyphenols and some tannins (Schmidt and Hebbel, 1986).

1.8.1. **Avocado Seed Storage**

Seed storage is necessary for avocado propagation, as the nurse seed, extracted from mature fruit, is not available throughout the year and the fruit harvesting season may not match with the tree propagation timing (Castro and Fassio, 2013). Storage of avocado seeds is, however, problematic, as the seeds are recalcitrant and do not tolerate losing 20-50% of their fresh mass; therefore, seeds cannot be stored over long periods (Kozlowski and Pallardy, 2002; Ernst *et al*., 2013; Walters *et al*., 2013). The lifespan of avocado seeds differs with genotypes but lies between 5 to 15 months under refrigeration (between 4.4 and 9°C) (Ernst *et al.,* 2013).

1.8.2. **Avocado Seed Coat Development**

The avocado seeds coat is developing from the two integumental tissues of the ovule; this seed coat is the outside layer of the seed, providing protection by covering it during development and seed maturation. The seed coat plays an essential role in various processes, such as nutrition of the growing embryo, mechanical and chemical protection of the seed, as well as maintenance of seed dormancy, seed dehydration, imbibition and, finally, seed germination (Boesewinkel and Bouman, 1995). Generally, the seed coat develops first, followed by growth of the cotyledons/endosperm and, lastly, the embryo develops (Weber *et al*., 2005). The seed coat acts as an intermediary layer between the embryo and the mesocarp; therefore, a properly functioning seed coat is essential for normal fruit development, particularly, as it contains a high concentration of plant growth regulators (Blumenfeld and Gazit, 1971; Cowan *et al.,* 1997).

In avocado, the seed coat develops relatively early and, therefore, plays a very important role in fruit and seeds development; firstly, by controlling active transport through the plasma membrane, secondly, by passive transport through the plasmodesmata (Lucas *et al*., 1993; Morris, 1996).

Generally, the seed coat surrounds the cotyledons and the embryo; it acts as a mechanical protection layer and can sometimes be involved in seed dispersal and in controlling germination (Ohto *et al*., 2009). The seed coat is also the first line of defence against external factors, as a channel for transmitting cues from the environment outside the seeds to conditions in the interior

of the seeds. A further function of the seed coat is to be the primary layer controlling movement between the embryo and the outside of the seeds (Radchuk and Borisjuk, 2014).

Generally, the seeds develop from the fertilized ovule and, depending on the stage of development, are usually composed of three structures. These constitute, firstly, the embryo, arising from fertilization of the egg cell by one of the pollen tube nuclei; secondly, the nutritive tissue of the cotyledons or the endosperm, generated by the fusion of two polar nuclei of the embryo sac with the other sperm nucleus, and, lastly, a protective seed coat (testa), derived from the inner, outer or both ovular integuments (Bradford and Nonogaki, 2009). Some mature seeds may also include funicular tissue or layers of the nucellus and/or the endosperm (Fahn, 1990). During seed coat development many histological changes take place. Periclinal and anticlinal cell divisions, combined with cell enlargements, allow for the growth of the seed coat. At seed maturity, however, much of the integumental tissue has degenerated and is absorbed by other tissues (Fahn, 1990).

The seed coat is tightly attached to the seed and characterised by relatively small and tightly packed parenchyma cells. Besides the two integuments, several layers of parenchyma exist between these two tissue layers; collectively these three cell types make up the pachychalazal (seed coat) tissue, which, in avocado, increases in thickness, as the parenchyma cells continue to divide in mature seeds (Moore- Gordon, 1997).

Blumenfeld and Gazit (1971) had reported two layers of the avocado seed coat, in addition to the embryo and the young ovule. In mature avocado seeds this seed coat is dark brown and thin, while it is light-coloured and thick in immature seeds. Tomer *et al.* (1980) observed that the avocado seed coat of the 'Fuerte' and 'Ettinger' cultivar became brown and thin, as well as that the small ovule was shrivelled, when the seed was mature. In olive, a fruit with a single seed similar in structure to the avocado, the mature seeds are characterized by a thick and brown seed coat, while the endosperm remains white and surrounds the embryo (Zienkiewicz *et al.*, 2011).

1.8.2.1. **Phenolic Compounds in Avocado Seed Coats**

The seed coat is the seed's primary defence against harsh environmental conditions. A hard seed coat protects the seeds from mechanical stress, microorganism invasion, as well as temperature and humidity fluctuations (Mohamed *et al.*, 1994). The seed coat differentiation involves cellular

changes over the seed's growth and development period and ends in seed coat death (Haughn and Chaudhury, 2005). Phenolics, the main category of secondary compounds in vascular plants (Lemma *et al.*, 2019), are known as inhibitors of seed development (Schmauch and Grubb, 1954). Phenolic compounds also play important roles in resistance to pests and pathogens (Li *et al.*, 2010; Silva *et al*., 2006), plant growth and development, reproduction, and in pigment and lignin biosynthesis (Boudet, 2007); phenolics are also involved in plant allelopathy (Boudet, 2007).

In plants, phenolic compounds are manufactured through the shikimic acid pathway. Phenolic compounds in the seed coat contribute to seeds hardness. During germination, the seed coat also protects the seeds from water stress and electrolyte leakage (Mohamed *et al*., 1994). Furthermore, phenolic compounds in the seed coat play a vital role in seed longevity (defined as 'the total time span during which seeds remain viable', Sano *et al*., 2016), and phenolics in the seed coat reduce water loss as well as increase mechanical resistance of seed (Mohamed *et al*., 1994).

The avocado exocarp and seed coat contain high levels of phenolic compounds, acting as inhibitors of protein oxidation, as well as lipid and pigment deterioration; therefore, it is unsurprising, that the avocado seeds of the 'Hass' and 'Fuerte' cultivar have been found to contain a higher level of phenolic compounds than the avocado pulp (Rodríguez *et al*., 2011).

Polyphenol oxidases (PPOs) are ubiquitous enzymes and involved in defence mechanisms against pathogens and herbivores, but their biological function is not fully elucidated (Zhang and Sun, 2020). The oxidation of phenolics results in the formation of quinones; these can be polymerized resulting in browning of tissues. These PPOs are present in the thylakoids of chloroplasts (Mayer and Harel, 1979) as part of the plant's defence mechanism, assisting the seed in case of attack from insects and pathogens. These enzymes are, further, involved in free radical scavenging and biosynthesis of various plant compounds (Constabel *et al*., 2000; Fuerst *et al*., 2014).

As enzymes catalysing the browning reactions of phenolics to benzoquinones are also present in fruit tissues, browning of fruit is a common phenomenon in injured tissues and of special commercial importance to the plant food industries, because such browning reduces product quality. Due to the general occurrence of phenolics, it is assumed, that they play a role in biochemical protection of sensitive plant developmental stages against environmental stress and microbes (Aniszewski *et al.*, 2008).

Polyphenol oxidases can be arranged into three groups, according to their reaction mechanisms and substrate specificity: (1) Catechol oxidases that oxidise o-diphenols to o-diquinones (EC 1.10.3.2), (2) laccases that oxidise p-diphenols to p-diquinones (EC 1.10.3.1), (3) tyrosinases (in animals) or cresolases (in plants and microbes) that are PPO-type enzymes with an additional function for hydroxylation of monophenols to o-diphenols (EC1.14.18.1) (Mayer, 1987; Aaiszewski *et al*., 2008). In intact plant tissues, polyphenol oxidases, respectively the catecholase oxidases that oxidise o-diphenols to o-diquinones, are widely found to be located in the thylakoid membranes of the plastids, as first described by Arnon (1949).

The carbohydrates broken down during germination will be transported to the embryo to supports its growth. PPO probably plays an important role in degradation of this food source during embryo respiration. The PPO has a higher activity in embryos, when germination starts, as there is a positive correlation between the activity of this enzyme complex and the respiration rate (Stiles, 1960). This PPO activity increases to its highest level during the germination process, this may indicate that the PPO enzyme plays an important role in seed germination.

1.9. Seed Germination of Avocado

The development of the seed, from ovule fertilization to physiological maturity, is divided into four phases: (I) cell division, (II) cell expansion, (III) reserve accumulation resulting in seed mass increase and (IV) seed moisture loss, resulting in desiccation. During most of these developmental periods, seed moisture remains high, seed moisture loss only occurs at the end of maturation, when changes in cell structure organization occur, as well as increases in enzyme synthesis in preparation for germination. Recalcitrant seeds, such as avocado, however, usually do not display a clear transition period between maturity and germination due to the relatively large amount of water present in the seeds (Bareke, 2018).

Seed germination is one of the most important phases in a plant's life cycle and strongly affected by environmental conditions. The early, crucial events during germination consist of imbibition, followed by mobilization (translocation and hydrolysis) of seed storage reserves to supply metabolic processes with energy, organic carbon and nitrogen, as well as minerals (Bewley and Black, 1978). Certain enzymatic processes in the avocado cotyledons convert the storage sugar perseitol into the transport sugar mannoheptulose (Tesfay *et al*., 2012). Through respiration, the

embryo receives energy that can be used in different anabolic pathways, assisting in its growth (Bishnoi *et al.*,1993).

Generally, seeds contain carbohydrate reserves, as well as lipid, protein and other compounds that are broken down into building blocks used during germination to synthesize substrates and supply energy for the early growth and development of the seedling. Seed carbohydrates are mobilized to support germination and seedling growth (Ziegler, 2017).

Oily seeds contain storage lipids derived from the carbohydrates synthesized by the mother plant and transported to the seeds. During seed germination, one of the major end-products of lipid storage breakdown are sugars. Thus, the sugar concentrations in seed tissue are considered to play an important regulatory role, during both, lipid breakdown and lipid accumulation (Borek *et al*., 2015). In oil seeds, a large conversion of triacylglycerol to sugar occurs after seed germination (Kornberg and Beevers, 1957). Carbohydrates of oily seeds may be manufactured from the oils by β-oxidation of fatty acids to acetate or the conversion of acetate to malate through the glyoxylate cycle, followed by reversal of gluconeogenesis from fatty acids (Cioni *et al.*, 1981). The sugars produced are transported into the seedling, where they support growth and development (Stumpf *et al*., 1980). Tesfay (2009) reported that in avocado, the oily seeds also produce carbohydrates upon germination.

During germination and seedling growth, cell division and cell enlargement require transportation of respiratory substrates, in the form of soluble sugars and low molecular mass proteins (such proteins are rarely used in respiration in protein-rich seeds during germination), from seed storage organs to the site of growth (Bewley and Black, 1994). Germination rate and seedling growth are affected by storage conditions. Seeds of many species lose their viability following short periods of storage, ultimately dying (Mohamed *et al*., 1994; Berjak and Pammenter 2008); therefore, in such seeds, the ability of healthy seeds to germinate can be negatively affected by the storage condition (Wawrzyniak *et al*., 2020).

1.9.1. **Environmental Effects on Avocado Seed Germination**

Seed germination requires suitable conditions, which include availability of water, oxygen, and a certain temperature regime (Walker and Simmons, 1987). Avocado seeds are used in the propagation of rootstocks as ‘nurse seeds’ to supply the clonal rootstock bud and developing

seedling with water and carbohydrates, during the development of the root system of the grafted rootstock, which will subsequently be grafted onto a clonal scion cultivar (Castro and Fassio, 2013; Ernst *et al*., 2013).

Mature and immature avocado fruit contain the embryo inside the single seed, which is surrounded by a thick or a thin seed coat; the embryo consists of two starchy cotyledons with a centrally attached, very small, embryonic axis (Blumenfeld and Gazit, 1974). Therefore, germination of avocado seeds is the result of the extension of the embryonic axis due to the embryo's growth and radicle emergence through the micropyle (Moore-Gordon, 1997). According to Nonogaki (2014), germination will take place when the active embryo radicle is able to emerge and extend through the seed coat.

Seed germination percentage varies with seed source, parental nutrition, seed maturity and environmental condition during development (Chaisurisri *et al*., 1992). Tropical conditions of high temperature and relative humidity during the final stages of seed development and maturation can present serious seed production problems, as they result in rapid loss of seed viability (Delouche, 1980).

1.9.1.1. **Influence of Temperature and Relative Humidity on Avocado Seed Germination**

Avocado seeds are recalcitrant (Berjak and Pammenter, 2013); storage conditions, therefore, seriously affect seedling emergence, growth and development (Hartmann *et al*., 2002). Storage under low temperatures and high RH allows seedlings to grow and develop as a result of an active metabolism and a high seed moisture content (Vinha *et al.*, 2013).

For most crop species, seed germination increases over a certain temperature range; this increase in temperature affects the growth and development of the species. In general, species distribution and survivability of individual plant species are also affected by ambient temperature, because of the relationship between germination, temperature and dormancy (Arana *et al.*, 2016). Temperature is also the most important variable affecting seed germination rate (Roberts, 1988; Milbau *et al*., 2009).

Germination is affected by temperature in three primary ways, through ambient moisture, hormone production and activity, and general enzyme activity. In seeds germination, water is

needed for imbibition. A warmer environment increases evaporation and decreases soil moisture and relative humidity, which negatively affects germination (IPCC, 2007). Two major hormone groups play regulatory roles in germination: abscisic acid (ABA) and gibberellins (GAs).

If a certain temperature range is not achieved or is exceeded, enzymes necessary for while germination may become inactive (Peterson *et al*., 2007). When the temperature requirement of a certain species is not met, subsequent effects of temperature on hormone and enzyme activity will significantly affect germination (Belderrok, 1968). Some crop species are, however, able to germinate well under lower temperature (e. g. *Pisum sativum*). The ambient temperature is, therefore, one of the most important environmental factors (George, 1967) influencing the induction of seed dormancy during seed development (Olsson and Mattson, 1976), maintaining the dormant stage (Strand, 1980), as well as overcoming seed dormancy to allow germination (Weisner and Grabe, 1972). While hormonal and environmental conditions affect the development of physical structures of the seed, such as the seed coat, they can also affect germination. The seed coat does, however, not only present a physical barrier to germination, but also contains phytochemicals, such a phenolics, that hinder germination, while simultaneously protecting the seed (Sarkar and Shetty, 2014).

1.10. Respiration Rate of Avocado Seeds

Respiration is a catabolic process by which organic material (carbohydrates, organic acids, proteins and fats) is broken down primarily into water and carbon dioxide, while releasing energy. During this process in fruit, O_2 is used metabolically, CO_2 is released (Kader, 2002). The respiration rate of a commodity is a good indicator of the metabolic activity of its tissues and, thus, is useful to determine the potential storage life of fresh fruit and vegetables, as it will not be possible to store fruit with a high respiration rate for an extended period (Hardenburg *et al.,* 1986), unless the respiration rate is modified (Kader, 2002).

The respiration of avocado seeds, after being separated from mature fruit, decreases during storage; avocado seeds, extracted from immature fruit, respire more so than mature seeds (Zauberman and Schiffmann, 1972). Therefore, the respiration rate of the avocado fruit and the avocado seed changeduring fruit development, with a higher respiration rate of the young avocado seed from fruit harvested early in the season compared with a lower respiration rate of mature

seed harvested later in the season. In the process of respiration, the C6 and C7 sugars, substrates of respiration for producing energy, are depleted, with C7 sugar concentrations reduced more slowly than those of C6 sugars during the process of seed germination (Liu *et al*., 1999).

1.11. Sugar Concentrations in Avocado Seeds

The rather large avocado seed requires a high amount of energy to be produced, this energy must be provided by carbohydrates derived from photosynthesis (Wolstenholme, 1986). From a physiological and biochemical perspective, the avocado fruit is different from most others, as it predominantly contains seven carbon (C7) sugars, particularly mannoheptulose and the related C7 sugar alcohol, perseitol, as forms of non-structural carbohydrates. These C7 sugars are present in all tissues and organs, even in the seed and peel of the avocado fruit (Liu *et al.,* 1999a, 1999b). The C7 sugars, D-mannoheptulose and perseitol, were the dominant soluble sugars detected in avocado seeds, while the mesocarp and exocarp of avocado had particularly high levels of the C7 sugar mannoheptulose (Bertling and Bower, 2005).

Furthermore, different to the occurrence of C7 sugars in the mesocarp and exocarp, the seeds seem to be a pool of perseitol, with this sugar possibly acting as the storage sugar. Perseitol plays an important role in seed development, seemingly acting as an energy reserve for seed development, as the highest level of this sugar was found in seed tissue, when compared with otherorgans of the tree (Liu *et al.,* 1999); seemingly, both, C6 and C7 sugars are important in avocado fruit growth and development.

The non-osmotic characteristic of perseitol, as a sugar alcohol, indicates its potential function as a storage molecule in avocado (Liu *et al*., 2002). This theory of perseitol as a major carbon reserve compound in the seeds was confirmed by Bertling and Bower (2005).

The sugar profile of avocado seedlings differs in light- versus dark-grown plants (Kazama *et al*., 1978). The C6 sugars are present in dark-grown avocado seeds with the energy for growth supplied by C6 carbohydrates, while light-exposed seedling will use the C7 pathway (Tesfay, 2009). The avocado seeds have been reported to contain large amounts of the C7 sugar perseitol, and this sugar has been postulated to be the storage form of D-mannoheptulose. This is supported by the finding that the avocado seeds, or rather the two large cotyledons, contain perseitol, while the predominant soluble carbohydrate in the seeds is D-mannoheptulose (Tesfay *et al.*, 2010).

Both sugars, D-mannoheptulose and perseitol are found in and are exuded from the cut seedling's stem, demonstrating that both sugars are xylem-transportable (Liu *et al*., 2002). Perseitol is the major C7 sugar in the avocado seed and is transported from the seed into the developing seedling, resulting in D-mannoheptulose, unlike perseitol, not being present in large amounts in avocado seeds (Tesfay *et al.*, 2010).

The total sugar content is relatively low in avocado fruit, and, as the season advances and the fruit become more mature, the percent total sugar in the fruit decreases (Church and Chase, 1920-1921; Biale and Young, 1971). According to Church (1921-1922), this decrease in sugars is almost as good an indication of maturity as the increase in oil concentration during fruit development. Both alterations appear to be closely related to fruit maturation.

Bean (1958) tested the sugar variation in a 'Zutano' and a Mexican seedling. In both plant materials, higher amounts of sugar in the early growth stages than in the later stages were discovered. Hatton *et al*. (1964) described the inconsistency in the amounts of sugar present at maturity. Haas (1937) also reported that there is a variation between cultivars in sugar content in the avocado mesocarp. More sugars were found in the apical, stem half of avocado fruit than in the basal half.

1.12. Starch Concentration in Avocado Seeds

Starch is the most common form in which plants store carbohydrate reserves, with the main storage tissues and stored amounts of starch differing between species (Mesa, 2015). Starch is a natural biopolymer of glucose (Lacerda *et al*., 2014). Starch is also an important resource for various industries and can be obtained from fruit, seeds, roots and stem tubers. The most common sources of commercial starch are cereals, such as corn (fruit) and wheat (seeds), as well as roots (cassava) and stems (potato) (Lacerda *et al*., 2014; Khlestkin *at al*., 2018).

Carbohydrate storage is the result of photosynthetic activity in plants; therefore, the total carbohydrates synthesized are determined by the plant's photosynthetic activity, while the carbohydrate distribution between vegetative and reproductive tissues is dominated by source-sink relationships (Oliveira and Priestly, 1988). Starch can be synthesized in specialized plastids (amyloplasts in storage organs), while in leaves starch is produced in the chloroplast (Keeling and Myers, 2010). Therefore, these plastids contain three enzymes: ADP-glucose pyrophosphorylase,

starch synthase, and starch-branching enzyme (Martin and Smith, 1995). Starch is the main carbohydrate storage form in plants. It is renewable, abundant, non-toxic, easy to extract, and convertible into different carbohydrates by chemical and biochemical process (Bello *et al*., 2010). In avocado seeds, the starch content can reach up to 75 to 80% of its dry mass, depending on cultivar (Orhevba *et al*., 2011; Maryam *et al.*, 2016).

Starch can, chemically, be derived from two glucan polymers, which are amylopectin and amylose. Amylose influences the packing of amylopectin in the crystallites and the grouping of the crystalline lamella within starch granules. This is important for properties related to water uptake, such as swelling and gelatinization; another factor that defines certain starch properties is the length of the glucose chains in the amylopectin molecule (Copeland *et al.*, 2009), which differs between species (Singh *et al*., 2010). Low molecular mass of amylopectin with long, branched chains simplify the formation of the amylose-lipid helicoidal complex (Li *et al*., 2008).

1.13. Conclusion

The avocado seeds of the cultivars ‘Hass’ and ‘Fuerte’ are commonly used as ‘nurse seeds’, for clonal rootstock production. Onto this ‘nurse seed’-rootstock plant, clonal cultivars are grafted. The avocado ‘nurse seed’ also assists in the development of a strong initial root system, so that the young, grafted plant leaves the nursery to produce a healthy avocado tree. Nurseries grafting avocado trees are, therefore, interested in a healthy, vigorous ‘nurse seed’. Avocado seeds of the most commonly grown cultivars, ‘Hass’ and ‘Fuerte’ contain the common C6 carbohydrate storage forms sucrose (little free glucose or fructose) and starch, as well as the C7 sugars mannoheptulose and perseitol, the latter in greater concentration.

Avocado seeds mature after the seed coat has shrivelled and turned brown, and phenolic compounds are concentrated in the seeds coat. These phenolics inhibit germination in mature seeds, while the immature seeds are able to germinate easily. When using a ‘nurse seeds’ for grafting, a vigorous and high-quality seeds is needed; these features exist in immature seeds and are important aspects for the avocado nursery industry. One of the treatments to enhance avocado seed germination is *Moringa oleifera* leaf extract applied to seeds to enhance and fast-forward germination.

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CHAPTER TWO

SEED GERMINATION OF 'HASS' AND 'FUERTE' AVOCADO AS AFFECTED BY SEED COAT THICKNESS AND SEED COAT PHENOLIC COMPOUNDS

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Abstract

The avocado seed consist of two large cotyledons and the embryo. This structure is surrounded by a two-layered seed coat, derived from the integuments. Throughout seed development, seed coat thickness changes, possibly affecting seed germination. The seed coat of avocado serves to protect the seed from harsh environmental conditions. Similarly, phenolic compounds, present in the seed coat, might affect germination, and guard the seed from fruit browsers, while also delaying seed germination. Avocado seed coat thickness and the seed coat phenolic concentration were, therefore, monitored over time in 'Hass' and 'Fuerte' avocado seeds. Seed germination percentage, seed coat phenolic compound concentration, seed coat thickness and seed viability of 'Hass' and 'Fuerte' fruit were determined to provide further insights into the role of the total phenolic concentration in the seed coat and in seed development of avocado. Three generations were present in the experimental orchard: Generation 1 (full bloom in July/August 2017), Generation 2 (full bloom in July/ August 2018) and Generation 3 (full bloom in July/August 2019). Seed coats of the Generation 1 seeds of both cultivars had the highest phenolic concentrations ('Fuerte' and 'Hass' with 2.02 and 2.3 mg GAE* g^{-1} DM, respectively), with a seed coat thickness of 0.15 mm ('Fuerte') to 0.11 mm ('Hass'). The germination percentage was generally low (lowest value 23.75% for 'Fuerte' and 31.25% for 'Hass') from June to September. Generation 2 seed coats tended to contain lower levels of phenolic compounds and were thicker in 'Fuerte' (0.31mm) than in 'Hass' (0.51 mm). Generation 2 seeds had a higher germination percentage (52.5% to 67.5%, in 'Fuerte' and 'Hass', respectively) than Generation 1. Seed germination was greater from October to November in Generation 3 and phenolic compound concentrations were low, with thicker seed coats than those of Generation 2 seed. There was a negative correlation between the concentration of phenolic compounds and the germination percentage in Generation 3 seeds of both cultivars, 'Fuerte' ($r = 0.11$, $P > -0.26$) and 'Hass' ($r = 0.16$, $P > 0.69$). Moisture content of the seeds were aligned with the germination rate, younger generations had higher level than old ones. A positive relationship ($r = 0.71$) between germination rate and seed moisture content was observed for both cultivars. Seed viability, determined as germination percentage and via the tetrazolium chloride (TTZ) test, tended to be higher in Generation 2 compared with Generation 1 seeds; this was the case for both cultivars. Generation 1 seeds, however, had low viability ('Fuerte' 26.25% and 'Hass' 31.25%). Seed coat thickness differed between Generation 2 and Generation 1. Generation 2 'Hass' seed coats were

significantly thicker than those of Generation 1 ($P < 0.001$, 0.51 and 0.11 mm) in June and August, respectively. In 'Fuerte', Generation 1 seed coats were thinner than Generation 2 one's (0.15 and 0.39 mm, in June and July, respectively). There was a negative correlation between seed coat thickness and germination rate ($r = 0.11$, $P < -0.28$) over the generations. Generation 2 had a thicker seed coat and greater germination rate than the Generation 1 seeds, which had a thinner seed coat and a lower germination percentage. Generation 2 seed coats were characterized low phenolic concentrations, with the germination rate of such seeds being high, probably due to this lower phenolic concentration. It seems, therefore, that immature seeds should be used by the avocado nursery industry, as such seeds were characterised by lower phenolics and greater germination percentage.

Keywords: Seed coat thickness, germination percentage, phenolic compounds, tetrazolium test, avocado, 'Hass', 'Fuerte'

2.1. Introduction

Although avocado fruit physiology is well-researched, little is known about avocado seed physiology. Avocado contains a relatively thick seed coat, derived from the two integuments surrounding the ovary. Seed coats generally play an important role in seed development by controlling seed germination, dormancy, and embryo development (Moise *et al*., 2005). The interactions between the exterior environment and the internal structures of the seeds can be modified by the seed coat (Sano *et al*., 2016). It also transfers gases and water between the embryo and the environment during the germination process (Souza and Marcos-Filho, 2001). A further, very important function of the seed coat is to supply nutrients to the embryo during seed development (Chen *et al.*, 2015).

A thin seed coat is likely to lose viability more quickly than a thicker seed coat (Mandizvo and Odindo, 2019). Thick seed coats serve as a mechanical protection layer against chemical, physical and biological damage to the embryo. Such seed coats often contain a layer of sclerenchyma cells, which can be variable in shape and size (Esau, 1977; Metcalfe and Chalk, 1979). When the seeds reached maturity, the seed coat is relatively thick, and can be differentiated into three regions: A middle seed coat layer or mesotesta, an outer seed coat layer or exotesta and inner seed coat layers or endotesta (Cutler *et al*., 2007). The seed coat provides a physiological connection between the

mesocarp and seeds (Moore-Gordon, 1997). The seed of the avocado consists of two fleshy cotyledons, as well as the hypocotyl, plumule and radicle, with the entire structure surrounded by two fused integuments (Cummings and Schroeder, 1942); when the seeds approach maturity, no endosperm remains in the cotyledons (Lackey, 2010).

Generally, the seed coat is a primary defence barrier of the seeds against environmental stress, such as temperature and humidity extremes. This tissue also protects seeds from various stress factors, with this protection often brought about by phenolics found in the seed coat. These phenolic compounds also protect seeds from electrolyte leakage and seeds dehydration (Mohamed *et al*., 1994). Seed coat development can be affected by environmental conditions through their influence on plant hormones, which may also change the permeability for water and the thickness of the seed coat (Gray and Thomas, 1982; Gurerman, 1982).

The seed coat also limits germination, not only due to the resistance it provides to water and oxygen flow. It also simply provides a mechanical resistance to radicle protrusion, hampering easy germination. The germination inhibiting properties of the seed coat are also positively related to the colour of the seed coat; the seed coat is most commonly brown due to the phenolic compounds present (Debeaujon *et al*., 2000). Several studies have also established the importance of seed coat colour in seeds or seedling water uptake and early protein synthesis (Kantar *et al*., 1996; Bewley, 1997). The seed coat is also a barrier to gas exchange and water absorption during germination (Souza and Marcos, 2001). During development, the oxygen level in the outer layer of the seed coat declines, suggesting that oxygen from the surrounding air is restricted from entering the seeds (Borisjuk and Hardy, 2009). A further, special function of the seed coat is to control the supply of nutrients to the embryo during seed development and germination (Chen *et al*., 2015).

Seed maturation is an important stage of seed development. Certain compounds accumulate in the embryo, while others participate in the formation of the protective seed coat layer; together, these processes lead to seed dormancy (Gutierrez *et al*., 2007). Seed maturation is critical for seed development, particularly for embryo morphogenesis (Harada, 1997). Previous studies investigating embryo development in avocado fruit reported that seed storage products accumulate at the last developmental stage, seed maturation (Sanchez-Romero *et al.,* 2002; Peran-Quesada *et al*., 2005).

During avocado fruit development, the seed coat loses its succulence, indicating the cessation of the seed's influence on fruit growth and maturation; the shrivelling of the seed coat has, therefore, been strongly connected with fruit growth processes and fruit maturation (Blumenfeld and Gazit, 1970; 1974).

Viable seeds must be alive to have the potential to germinate, when inducive environmental conditions prevail. Measuring seed viability is important to gage its value as a 'nurse seed', as a seed may be viable, but not able to germinate, because of some physical and/or chemical inhibitors (Basra, 2006; Copeland and McDonald, 2012). Copeland and McDonald (1997) reported that a viable seed is characterised by high enzyme activity, a feature aligned with easy seed germination.

Seed moisture strongly influences the keeping quality of seeds in storage. In addition, seed moisture plays an important role in seed quality, in seeds stored at high moisture content the losses could be very rapid due to fungal growth (Doijode, 2001); A low seed moisture content is required for seed storage, reducing contamination by storage pests and diseases and increasing the shelf life of the seeds (Harrington, 1972).

Phenolic compounds in seeds may be responsible for the preservation of seed viability and physiological dormancy prior to germination. Phenolic compounds found in the seeds can act as inhibitors of seed germination (Inacio *et al.*, 2013); they can reduce the germination rate by inhibiting peroxidase activity, allowing for reactive oxygen species to oxidize phenolic compounds, a process essential to breaking the thick seed coat and allow for easy emergence of the seedling (Kong *et al*., 2008).

Phenolic compounds are natural secondary metabolites present in plants cells, they are also responsive to environmental factors, defend injured, wounded plants and are beneficial in the defence against pest and diseases (Kefeli *et al*., 2003). The seed's cotyledons contain large amounts of reserve substances, such as starch, proteins and carbohydrates. The seed coat, which plays a pivotal role as a protective layer of the cotyledons, contains a high concentration of phenolic compounds (Troszynska *et al*., 1997; Shahidi *et al.*, 2001; Dueñas *et al.*, 2003). In avocado, the seed coat also has high levels phenolic acids, as well as other organic acids, flavonoids, catechins and phenolic alcohol derivatives (Figueroa *et al.*, 2018); in particular, there

is evidence of specialised phenolic compounds and of tannin accumulation (Moore-Gordon, 1997).

The browning of the seed coat, as the seeds matures, indicates the oxidation of phenolic compounds. These secondary metabolites, manufactured via the shikimic acid pathway, differ severely in complexity and function, being involved in plant allelopathy (Boudet, 2007) and pest and disease resistance (Silva *et al*., 2006; Li *et al.*, 2010). Phenolic compounds also play important roles in plant growth and reproduction. Certain phenolic compounds and their derivatives are, further, known as inhibitors of seeds germination (Schmauch and Grubb, 1954).

The objective of the present experiment was, therefore, to determine the concentration of total phenolic compounds in the avocado seed coat, as well as determining seed coat thickness in two avocado cultivars. It was also attempted to relate seed coat thickness to seed germination; additionally, seed viability and germination were investigated over the same period, to determine, if seed viability, as characterized by the tetrazolium test (TTZ), can be related to seed coat thickness.

2.2. Materials and Methods

2.2.1. Origin of Seeds

Seeds used in this study were extracted from fruit obtained from a commercial orchard in the KwaZulu-Natal Midlands (30°16-E and 29°28-S, South Africa). 'Hass' and 'Fuerte' avocado fruit of three generations (G1, G2, G3), with full bloom in June/July ('Fuerte') and July/August ('Hass') 2017, 2018, 2019, respectively, were collected from the same trees carrying, at one stage, up to three generations at one time. Fruit collected were of uniform appearance and size, characteristic for the generation. Samples were collected monthly from June to November.

Three generations of seeds were analysed: 'Hass' avocado fruit (termed Generation 1), collected over a six-months period from 24 to 29 months after full bloom (MAFB) until November 2019. These fruits were commercially harvested in August 2018. Additionally, the following year's fruit (full bloom in July/August 2018 (Generation 2)) were harvested 12 to 17 MAFB, commercially harvested in July/August 2019 were sampled. Thirdly, newly developing fruit, with full bloom in July/August 2019 (Generation 3, 0 to 5 MAFB) were collected. In 'Fuerte', similarly, three generations were collected: the oldest avocado seed Generation 1 (full bloom in June/July 2017,

24 to 29 MAFB), these fruits were commercially harvested in June/July 2018, Generation 2 (full bloom in June/July 2018, 12 to 17 MAFB), these fruits were commercially harvested in June/July 2019, and Generation 3 (full bloom in July 2019), these fruits were commercially harvested in June/July 2018. Generation 3 was removed from the tree (9 to 10 MAFB) prior to commercial harvest for both cultivars. All fruit were removed from trees and immediately transported to laboratory.

Seeds were extracted from fruit after they had ripened to eating softness (finger-feel). The seeds were removed from the mesocarp, dried with tissue and planted into containers filled with 2cm agar- to determine the seed germination percentage. For further seed coat analyses the seed coat was peeled off from the seeds and oven-dried at 70°C for 24h.

2.2.2. **Phenology of avocado fruit during sample collation period**

The phenological cycle of avocado fruit of two cultivars (‘Hass’ and ‘Fuerte’) has been described on the Everdon farm, Howick, KwaZulu-Natal, RSA (Kaiser 1994; Whiley and Wolstenholme 1990). For ‘Hass’ fruit flowering starts from August to October and commercial harvest will take place in the following year from June/July. Seeds collected in September readily germinate. In ‘Fuerte’ the flowering time extends from June/July to October and fruit can be collected for use as ‘nurse seeds’ in the same year but harvesting of such fruit will only occur in the following year (Kaiser 1994; Whiley and Wolstenholme 1990).

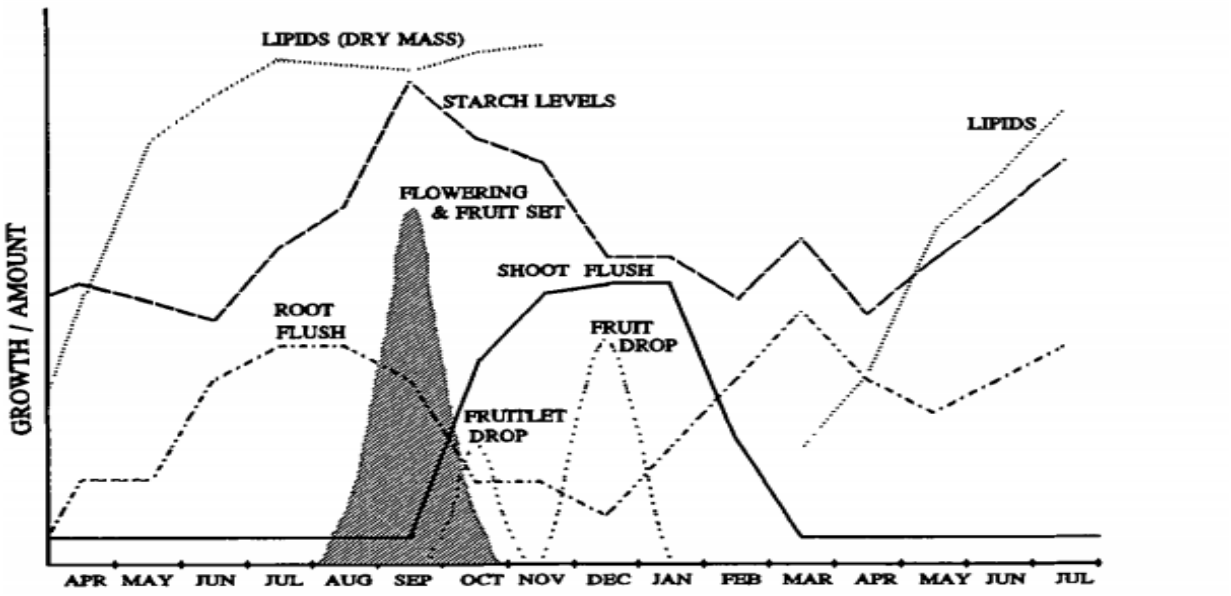

Figure 1. Phenological cycle of the 'Hass' avocado cultivar indicating time of flowering and fruit set as well as vegetative and reproductive growth at Everdon (Howick) (Source: Kaiser, 1994)

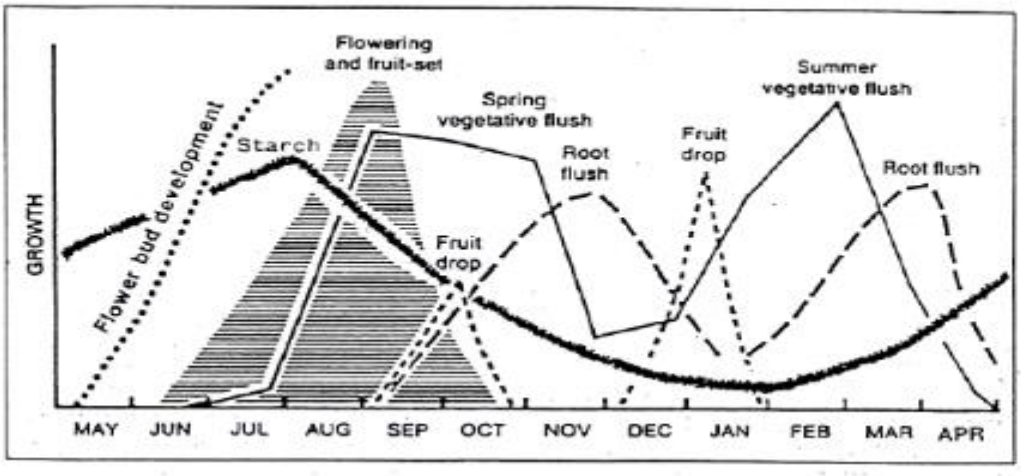

Figure 2. Phenological growth cycle of 'Fuerte' indicating the relationship between vegetative and reproductive growth at Everdon (Howick) (Source: Whiley and Wolstenholme, 1990).

2.2.3. **Germination Percentage**

Two-litre plastic containers were filled to a depth of 2cm with agar (12 g L^{-1}, ACE, South Africa); three seed were placed onto the cooled agar and the containers covered with aluminium foil to allow germination. For both cultivars, 'Hass' and 'Fuerte', germination percentage, seed coat

thickness, seed coat phenolic concentration and seed viability were investigated. Seed were arranged in a completely randomized factorial design with four replications. Containers were placed in a growth room at 25°C for 23 days. Germination percentage was determined weekly, measured as plumule emergence.

2.2.4. **Seed Coat Thickness Measurement**

Seed coat thickness was measured weekly over a period of six months, from June to November, using digital callipers (Absolute Digimatic, Mitutoyo, Japan). Freshly harvested fruit were cut open, the exocarp and mesocarp removed, so that only the seed remained, allowing the seed coat to be separated from the seed, so that seed coat thickness could be determined.

2.2.5. **Determination of Phenolic Compounds in Avocado Seed Coats**

A Folin-Ciocalteau method, described by Blainski *et al.* (2013), was used to determine the phenolic compound concentration of the seed coats. Freeze-dried samples (0.5g DM) were ground and homogenized in 10mL 80% ethanol, vortexed for 30min and left at room temperature (25±1°C) for 30min to extract the phenolic compounds. The mixture was then centrifuged, and the supernatant filtered through Whatman® no. 1 filter paper. The filtrate was centrifuged again in a bench-top centrifuge (PLC – 05 Centrifuge PLC Gemmy, Berlin, Germany) at 4000*g* for 10min to remove further fine precipitates. Then, an amount of 2.5mL extract was transferred into a test tube and mixed with 0.1mL 0.2N Folin-Ciocalteau reagent (Sigma–Aldrich/Fluka, St Louis, MO, USA). A subsample (5mL) of this solution was kept for 30min at room temperature. This was followed by addition of 2.0mL 75g L^{-1} sodium carbonate (Na_2CO_3) and samples were allowed to stand for 5min. The samples were then placed into the dark at room temperature for 2h. Thereafter, the absorbance of the samples was measured at 760nm against ethanol as a blank using a UV–Vis spectrophotometer SP 8001 (Metertech Inc., Taipei, Taiwan). Phenolic compound concentrations were calculated using gallic acid as a standard and expressed as mg GAE*g^{-1} DM.

2.2.6. **Seed Viability Determination**

The tetrazolium (TTZ) test was used to determine seed viability. Seeds were incubated in a 1% (w/v) aqueous solution of tetrazolium chloride at 25°C and stored for 24h. The reduction of the TTZ solution to a highly coloured, reddish end-product by NADH-dependent reductases was used

to determine seed viability as the ability to respire, according to Berridge *et al*. (1996). In this assay, seed tissue that produces an intense, red colour are regarded as healthy tissue, while dead tissue does not stain or stains abnormally. Seed viability was measured as the percentage of cotyledon area that turned red.

2.2.7. **Determination of Seed Moisture Content**

The moisture percentage of avocado seed were determined as described by Kaiser (1994). Avocado seeds extracted from 'Hass' and 'Fuerte' fruit were dried to a constant mass at 75C°. Moisture content was determined as follows (Khir *et al*., 2011):

$$MC = \frac{W_F - W_D}{W_D} \times 100$$

Where, MC = % moisture content, W_F = initial fresh mass and W_D = dry mass.

2.2.8. **Statistical Analysis**

Data were subjected to analysis of variance (ANOVA), using GenStat statistical analysis software (version 18.2; VSN International, Hemel Hempstead, UK), to determine significant differences in germination percentage, phenolic compound concentration, seed coat thickness and seed viability. Means were separated using Fisher's Protected Least Significant Difference test, when seed parameters showed differences at $P \leq 0.001$.

2.3. Results

2.3.1. **Germination Percentage**

In both cultivars, Generation 1 had the lowest germination percentage from June to September (Fig. 1a and 1b), with the lowest germination percentage (23.75%) determined for 'Fuerte' seed in August compared with 'Hass' (31%) seeds (Fig. 1a and 1b).

'Hass' seed, Generation 1, showed a lower germination percentage from June to September than Generation 2. The lowest germination percentage (31%) was recorded in July for Generation 1; Fig. 1a). Generation 2 of 'Hass' seed showed higher germination rates than Generation 1from June to September (Fig. 1a). From October to November the germination rate of the Generation 3 was greater than that of Generation 2 seed (Fig. 1a). Seeds extracted from 'Hass' avocado fruit

of the Generation 2 were able to germinate (from June to September) (Fig. 1a and 1b). When comparing cultivars, Generation 2 had a greater germination percentage than the Generation 1 (Fig. 1a).

'Fuerte' avocado seed of Generation 1 showed lower germination rates than Generation 2, from June to September, while, from October to November, the germination rate of Generation 2 was lower than that of Generation 3 (Fig. 1b). The highest germination percentage was that of Generation 2 'Fuerte' seeds (52. 2% and 58%) in September and October, respectively (Fig. 1b). Similarly, 'Fuerte' seeds of Generation 2 showed a greater germination percentage than Generation 1 from June to September (Fig. 1a).

Avocado seeds of both cultivars showed that the older Generation 1 had a lower germination percentage than Generation 2 from June to September; the youngest generation, Generation 3, seeds had a greater germination rate than seeds from Generation 2, from October to November (Fig. 1a and 1b). From thereon, Generation 2 seeds had lower amounts of phenolics ($P < 0.001$) and a higher viability (TTZ test) ($P < 0.001$), aligned with a greater germination percentage ($P < 0.001$) than that of Generation 1.

For both cultivars, the oldest generation, Generation 1, had a lower germination rate than Generation 2; similarly, Generation 2 had a lower germination percentage than the newer Generation 3 ($P < 0.001$; Fig. 1a and 1b).

Statistical analysis showed significant differences between three seed generations for both cultivars with respect to germination percentage ($P < 0.001$; Fig. 1a and 1b); the interaction in germination percentage between seed generation and month was also significant ($P < 0.001$).

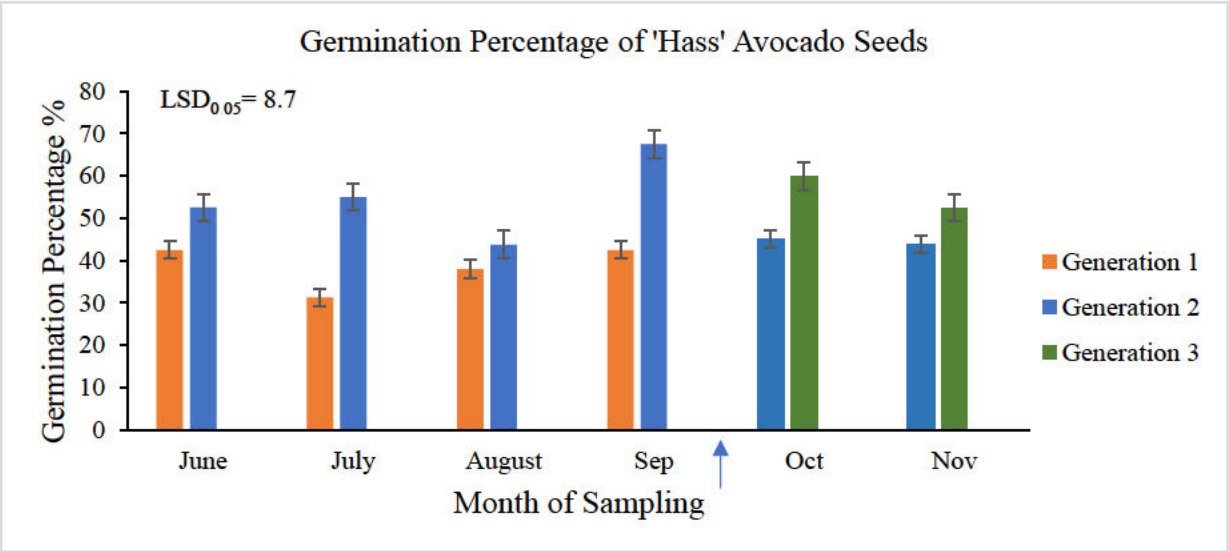

Figure 1a. Germination percentage (%) of three different 'Hass' avocado seed generation): Generation 1 (full bloom in July/August 2017), Generation 2 (full bloom in July/August 2018) and Generation 3 (full bloom in July/August 2019). $LSD_{0.05}$ is least significant difference at $P \leq 0.001$. The arrow indicates the month, when the oldest fruit were collected, and the new generation (full bloom in July/August) had reached harvestable size.

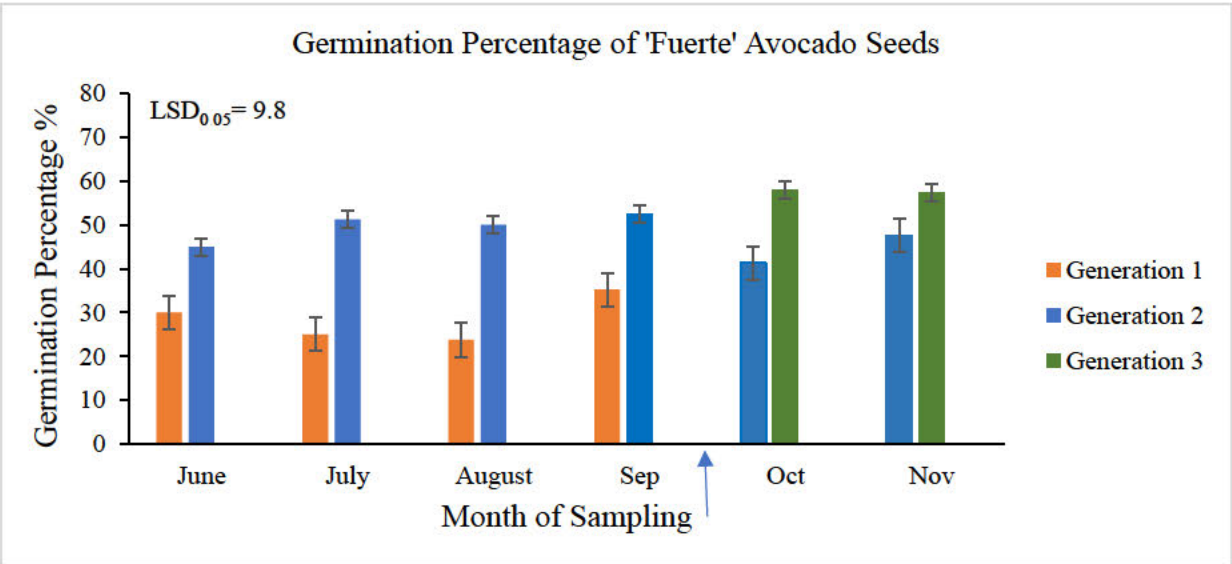

Figure 1b. Germination percentage (%) of three different 'Fuerte' avocado seed generations: Generation 1 (full bloom in June/July 2017), Generation 2 (full bloom in June/July 2018) and Generation 3 (full bloom in June/July 2019). $LSD_{0.05}$ is least significant difference at $P \leq 0.001$. The arrow indicates the month, when the oldest fruit were collected, and the new generation (full bloom in June/July) had reached harvestable size.

2.3.2. **Phenolic Compounds**

In both cultivars, ‘Hass’ and ‘Fuerte’, the phenolic concentrations in the seed coats were significantly higher in Generation 1 than in Generation 2 from June to September. Generation 2 had greater phenolic concentrations than Generation 3 (Fig. 2a and 2b). The avocado seed coats of both cultivars of Generation 1, from June to September, were brown, while the Generation 2 and Generation3 seed had white and thick seed coats. Comparing phenolic concentrations between the generations of sampling, ‘Hass’ and ‘Fuerte’ seed coats had low values in June; these rose towards July and remained relatively constant until September. From October onwards, seed coat phenolics decreased in Generation 3 seed coats (from 4 to 5 MAFB); these seed coats had, however, a lower phenolic concentration than those of Generation 2.

The concentrations of phenolic compounds in Generation 1 seed coats were greater (from June to September) than in Generation 2 seed coats, while from October to November Generation 3 seed had lower phenolic concentrations than Generation 2 (Fig. 2a and 2b).

The phenolic concentration in seed coats also differed between seed generations: A greater phenolic concentration was detected in ‘Hass’ seed Generation 1 than in ‘Generation 2’, and phenolic concentrations were low in Generation 3 compared with Generation 2 (Fig. 2a). Similarly, the ‘Fuerte’ seeds of Generation 1 showed a greater concentration of phenolic compounds than the Generation 2; further, Generation 3 had lower phenolic concentrations than Generation 2 (Fig. 2b). There were not significant differences between Generation 1 seeds and Generation 2 seeds for both cultivars with respect to monthly phenolic concentrations (Fig. 2a and 2b). The interaction between generation and harvesting month was, however, highly significant ($P < 0.001$).

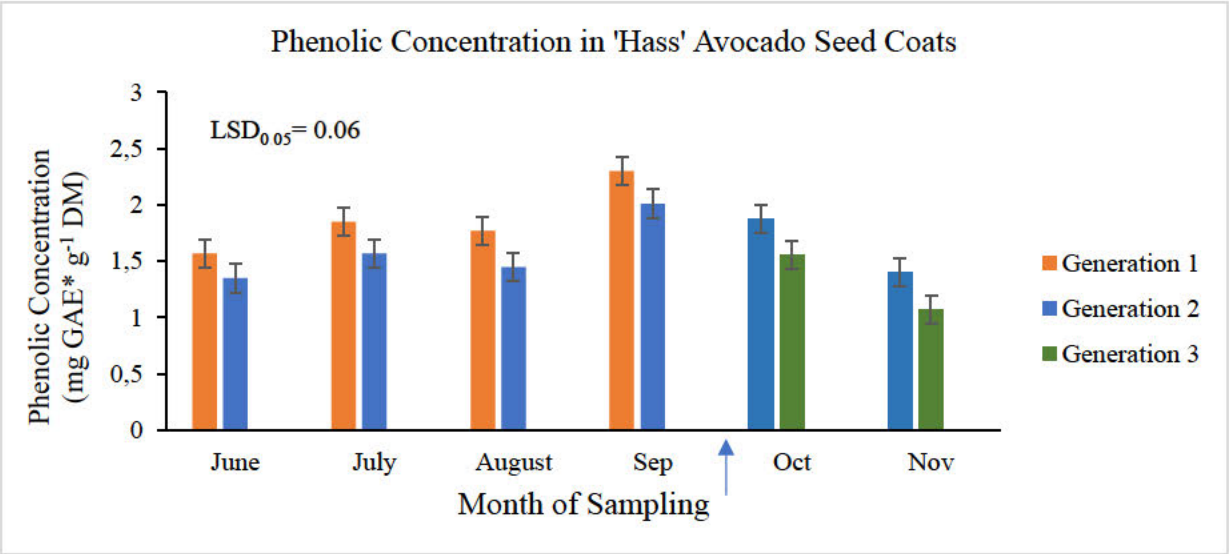

Figure 2a. Pattern of phenolic compound concentrations of three developmental generations of 'Hass' seed coats: Generation 1 (full bloom in July/August 2017), Generation 2 (full bloom in July/August 2018) and Generation 3 (full bloom in July/August 2019). The arrow indicates the month, when the oldest fruit generation was Harvested, and the new generation (full bloom in July/August) had reached harvestable size.

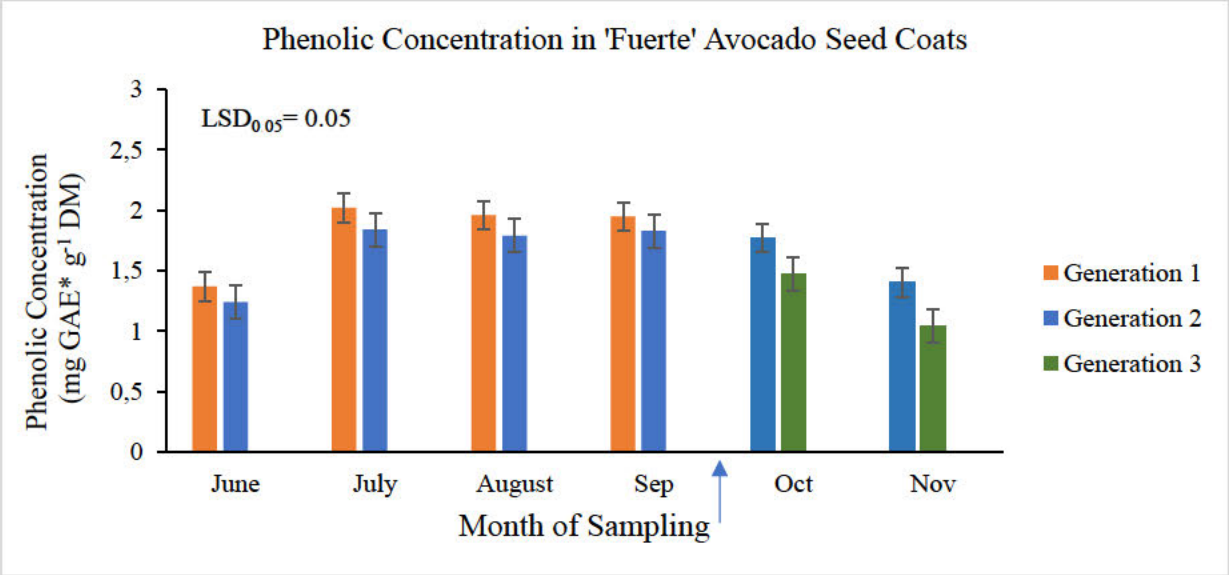

Figure 2a. Pattern of phenolic compound concentrations of three developmental generations of 'Fuerte' seed coats: Generation 1 (full bloom in June/July 2017), Generation 2 (full bloom in June/July 2018) and Generation 3 (full bloom in June/July 2019). $LSD_{0.05}$ is least significant difference at $P \leq 0.001$. The arrow indicates the month, when the oldest fruit were harvested, and the new generation (full bloom in June/July) had reached harvestable size.

2.3.3. **Seed Coat Thickness of Avocado Seeds**

There were significant differences in seed coat thickness between the two avocado cultivars (Fig. 3a and 3b). In ‘Hass’ seeds the thinnest seed coat (0.11 mm) was measured in the oldest generation, Generation 1, in June, while Generation 2 had the thickest seed coat (0.51 mm) in August (Fig. 3a). In ‘Hass’ seeds, the seed coat of Generation 1 seeds was thinner from June to September than the seed coat of Generation 2 seeds, and from October to November Generation 2 seed coats were thinner than those of Generation 3 (Fig. 3a).

The thickest seed coat (0.46 mm) was that surrounding the ‘Fuerte’ seed Generation 1 in August, while the thinnest seed coat (0.15 mm) was observed surrounding ‘Fuerte’ Generation 1 seed in June (Fig. 3b). In ‘Fuerte’, the thickest seed coat was that of Generation 2 with 0.39 mm in July and the thinnest seed coat was determined in the same generation with a diameter of 0.19 mm in June (Fig. 3a). In Generation 2 ‘Fuerte’ seeds, the seed coat thickness increased in June and July, thereafter, it dropped significantly (August), and seed Generation 1 had a thicker seed coat than Generation 2 (Fig. 3b). From October to November the Generation 3 ‘Fuerte’ seed had thinner seed coats than Generation 2 (Fig. 3b).

In this study, Generation 1 had thinner seed coats than the Generation 2 and Generation 2 had thicker seed coat than Generation 3 for both cultivars (Fig. 3a and 3b). Germination percentage was, however, not influenced by the thicker seed coat, as Generation 2 seed had a significantly ($P < 0.001$) higher germination percentage than Generation 1 and Generation 3 also had same trend (Fig. 1a and 1b; Fig. 3a and 3b)

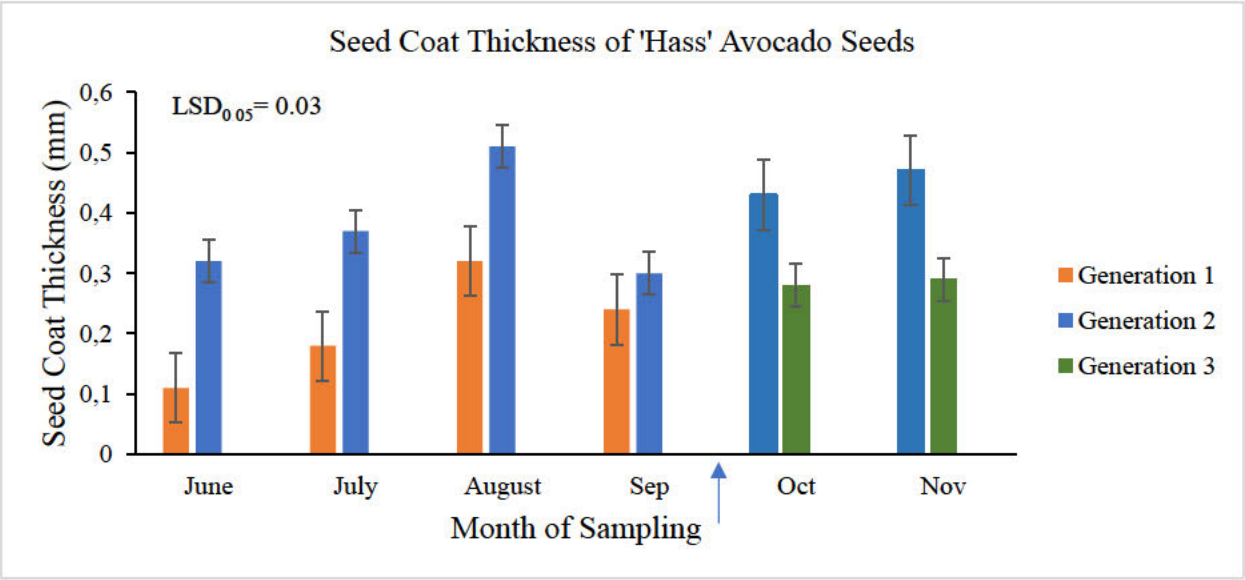

Figure 3a. Seed coat thickness of three 'Hass' seed generations: Generation 1 (full bloom in July/August 2017), Generation 2 (full bloom in July/August 2018) and Generation 3 (full bloom in July/August 2019). $LSD_{0.05}$ is least significant different ($P \leq 0.001$). The arrow indicates the month, when the oldest fruit were collected, and the new generation (full bloom in July/August) had reached harvestable size.

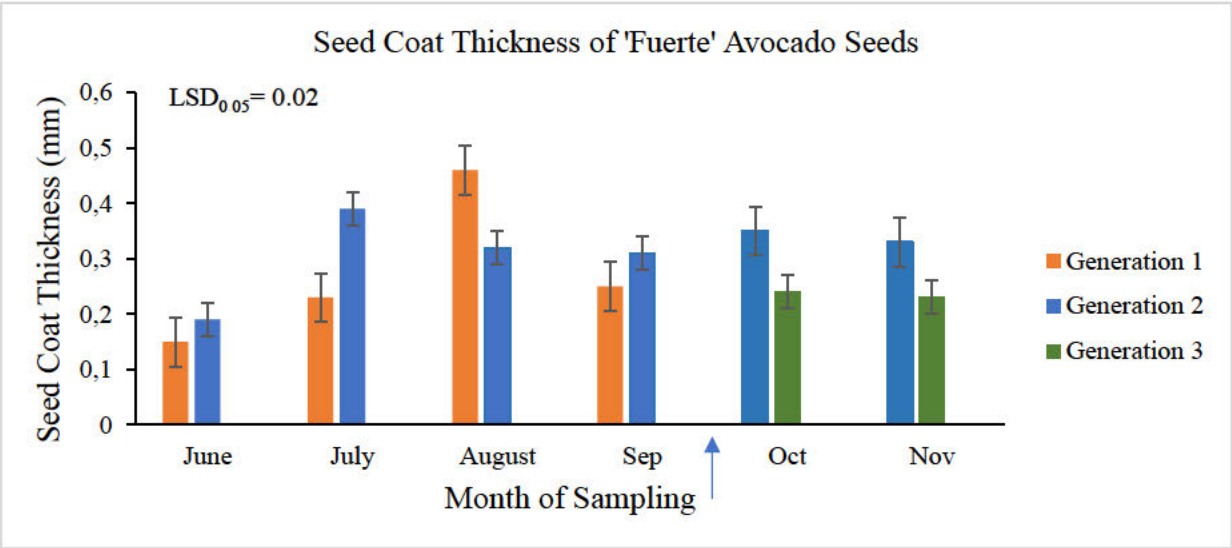

Figure 3b. Seed coat thickness of three 'Fuerte' seed generations: Generation 1 (full bloom in June/July 2017), Generation 2 (full bloom in June/July 2018) and Generation 3 (full bloom in June/July 2019). $LSD_{0.05}$ is least significant different ($P \leq 0.001$). The arrow indicates the month, when the oldest fruit were collected, and the new generation (full bloom in July/July) had reached harvestable size.

2.3.4. **Viability of the Avocado Seeds**

Seed viability, as determined by the TTZ test, showed that Generation 1 seed of both cultivars were lower in viability than Generation 2 (Fig. 4a and 4b). Generation 2 seed were highly viable from June to September, significantly more so than Generation 1 ($P < 0.001$). 'Hass' seeds Generation 1 showed a lower viability than the Generation 2 from June to September and from October to November Generation 2 had lower viability than generation 3 (Fig. 4a), with the highest viability found in Generation 3 (61.25%) in September (Fig. 4a).

From October to November Generation 2 'Fuerte' seeds displayed lower seed viability (26.25%) in July compared with Generation 3 seeds (with 52.5% to 66.25% viability) (Fig. 4b). The highest viability of 'Fuerte' seeds was 66.25% in November of the newest seed (Generation 3) (Fig. 4a).

Avocado seeds, Generation 2, of both cultivars ('Hass' and 'Fuerte') showed higher seed viability than Generation 1 from June to September. Generation 2 seeds germinated better than Generation 1 seeds, Generation 3 seeds germinated greater than those from Generation 2 (Fig. 4a and 4b). Seed viability of Generation 3 was high in October and November, with 66.25% and 60.0% germination, respectively (Fig. 4a and 4b).

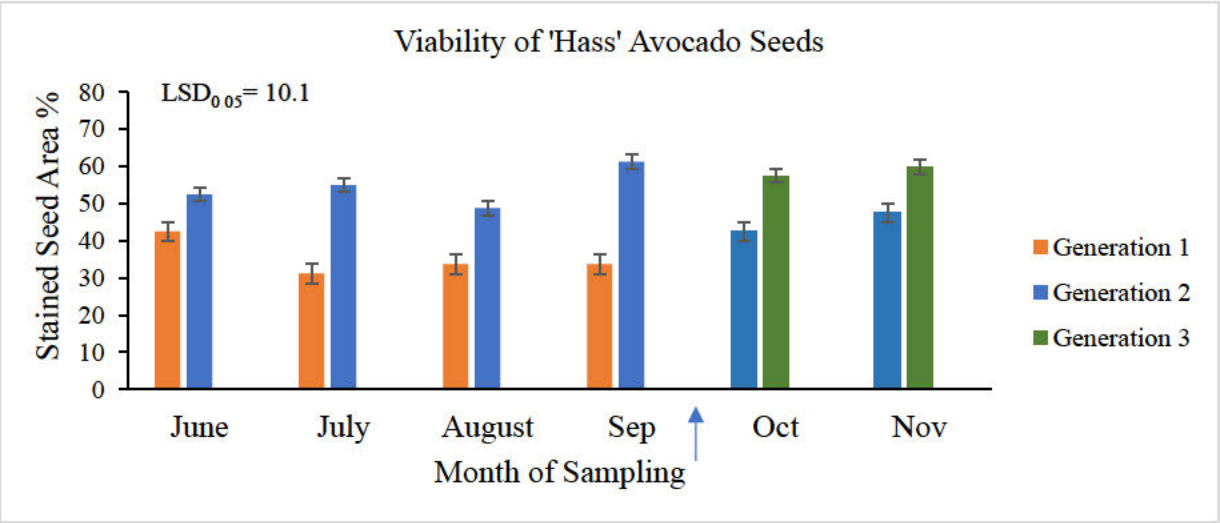

Figure 4a. Seed viability % of avocado 'Hass' seeds as determined by the Tetrazolium Test (TTZ), % means the percentage of cotyledon area that turned red over the three developmental generations: Generation 1 (full bloom in July/August 2017), Generation 2 (full bloom in July/August 2018) and Generation 3 (full bloom in July/August 2019). $LSD_{0.05}$ is least significant different ($P \leq 0.001$). The arrow indicates the month, when the oldest fruit were collected, and the new generation (full bloom in July/August) had reached harvestable size.

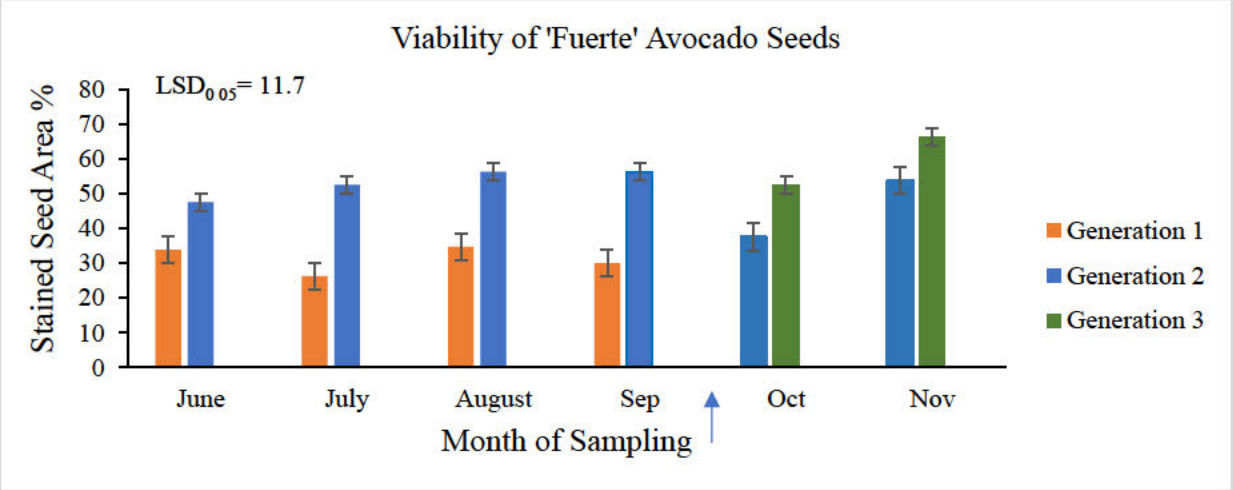

Figure 4b. Seed viability % of avocado 'Fuerte' seeds as determined by the Tetrazolium Test (TTZ) % means the percentage of cotyledon area that turned red over the three developmental generations: Generation 1 (full bloom in June/July 2017), Generation 2 (full bloom in June/July 2018) and Generation 3 (full bloom in June/July 2019). $LSD_{0.05}$ is least significant different ($P \leq 0.001$). The arrow indicates the month, when the oldest fruit were collected, and the new generation (full bloom in June/July) had reached harvestable size.

2.3.5. **Moisture Content of Avocado Seeds**

A greater seed moisture content was aligned with a greater germination percentage and a higher viability, so that a lower moisture content was associated with a lower viability and a lower germination rate in both cultivars (Fig. 5a and 5b). Generation 2 seeds of both cultivars (collected 12 to17 MAFB) had a slightly higher moisture content and a higher germination percentage than Generation 1 seeds (24 to 29 MAFB), and Generation 3 (0 to 5 MAFB) had higher moisture content than Generation 2 (Fig. 5a and 5b; Fig. 1a and 1b). The moisture content ranged between 54.5 and 62.1% in 'Hass' seeds, Generation 2, harvested in June to September, while Generation 1 had a comparatively lower moisture percentage (39.2%, Fig. 5a). 'Hass' seeds of Generation 3, harvested from October to November, had a higher moisture content than seeds of Generation 2 (Fig. 5a).

Younger generations of avocado seeds always had a higher moisture content than older ones, for both cultivars (Fig. 5a and 5b). The overall highest moisture content was found in 'Hass' seeds in September (62.1% in Generation 2), with the lowest seed moisture (39.2%) detected in Generation 1 June seeds (Fig. 5a). 'Fuerte' seeds showed a similar pattern with the highest moisture (60.5%) detected in July seeds of Generation 2, while the lowest seed moisture content was recorded in June seeds (33.2%, Generation 1) (Fig. 5b). Statistical analysis showed significant differences in moisture content between the three seed generations for both cultivars ($P = 0.046$, Fig. 5a and 5b).

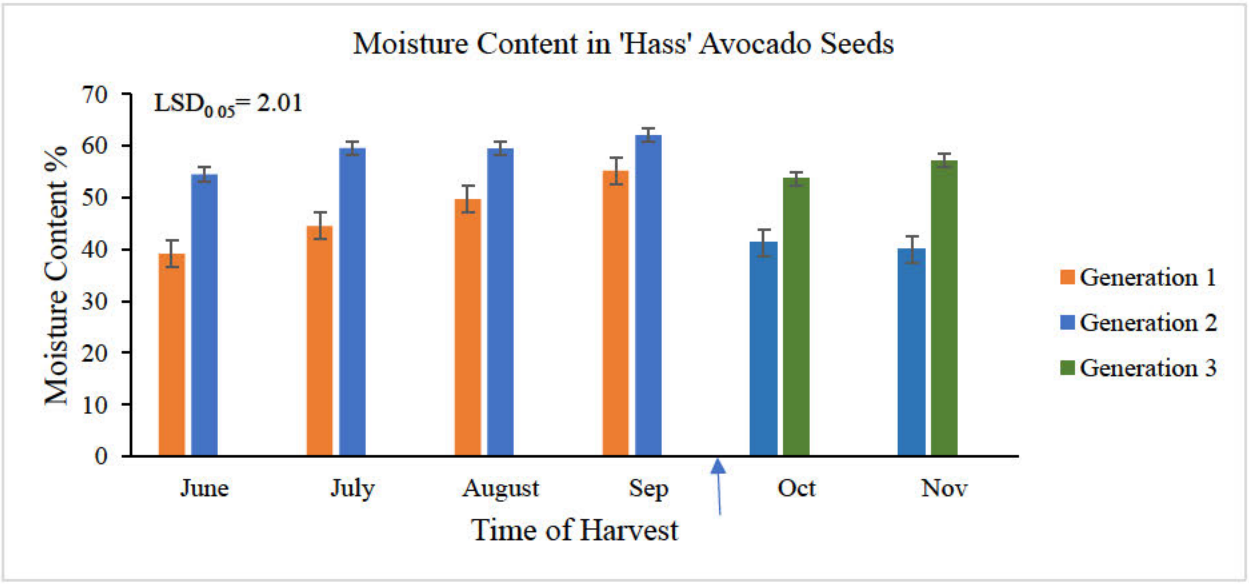

Figure 5a. Effect of seed generation on moisture content of three 'Hass' avocado seed generations: Generation 1 (full bloom in July/August 2017), Generation 2 (full bloom in July/August 2018) and Generation 3 (full bloom in July/August 2019). The arrow indicates the start of a new season, with Generation 1 removed from the tree and a new generation (Generation 3) present on the trees. Error bars above the columns denote least significant difference ($LSD_{0.05}$).

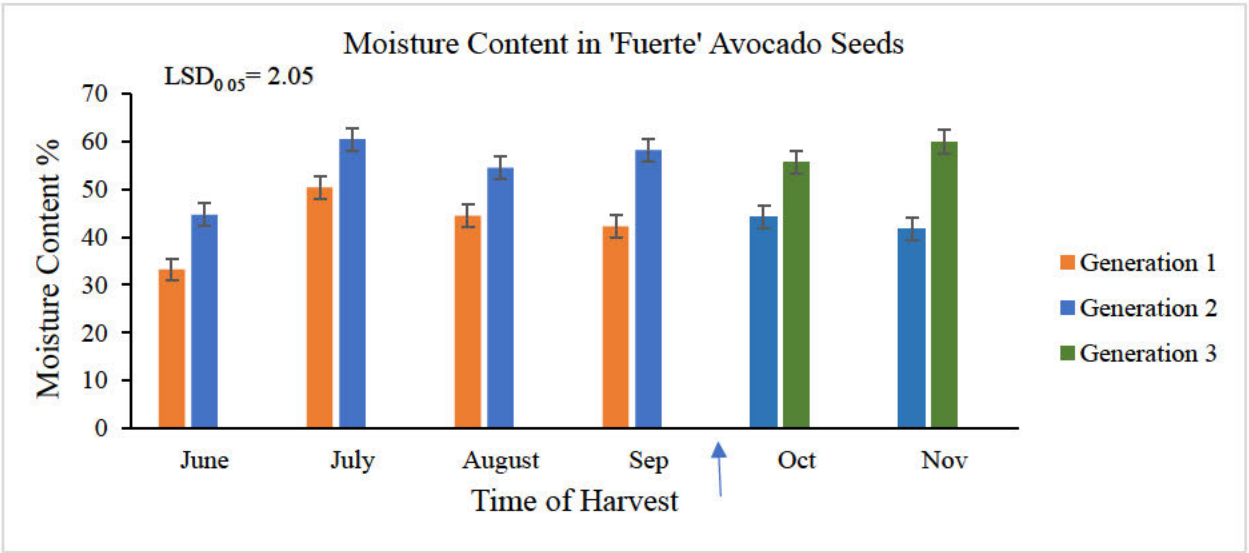

Figure 5b. Effect of seed generation on moisture content of three 'Fuerte' avocado seed generations: Generation 1 (full bloom in June/July 2017), Generation 2 (full bloom in June/July 2018) and Generation 3 (full bloom in June/July 2019). The arrow indicates the start of a new season with Generation 1 removed from the tree and a new generation (Generation 3) present on the trees. Error bars above the columns denote least significant difference ($LSD_{0.05}$).

2.4. Discussion

2.4.1. Germination Percentage

Delayed germination of seeds poses difficulties to the avocado nursery industry, as these seeds need to establish quickly to allow grafting of the rootstock onto this 'nurse seed' (Geering, 2018). Avocado seed germination was affected by seed maturity, with differences in seed germination percentage of both cultivars, depending on the stage of seed development (Fig. 1a and 1b). Avocado seeds used as 'nurse seed' need to produce a strong seedling, to be able to graft the clonal rootstock onto. Generally, avocado seeds are genetically highly variable in characteristics, such as resistance to diseases and also the ability to produce a strong root system (Bergh *et al.*, 1976).

In this study, it was shown that a high seed germination percentage of both cultivars ('Hass' and 'Fuerte') was positively affected by the seed's physiological age and the time of seed harvest (Fig. 1a and 1b). Burgos and Ledbetter (1993) reported that immature apricot (*Prunus armeniaca* L.) seed cultured *in vitro* had a higher germination percentage than mature seeds. Similarly, seed germination percentages differed between avocado seeds of the three generations (Generation 1, 24 to 29 MAFB, Generation 2, 12 to 17 MAFB and Generation 3, 0 to 5 MAFB) as well as between month of fruit age; therefore, seed germination was affected by the developmental stage of the seeds. The Generation 1 avocado seeds (24-29 MAFB) had a lower germination rate than Generation 2 (14-16 MAFB) (Fig. 1a and 1b), possibly due to the higher phenolic concentrations in the seed coat of older seed inhibiting germination (Fig. 2a and 2b). This is supported by Singh and Singh (2018) who reported that a high phenolic concentration in guava (*Psidium guajava*) seeds may inhibit or reduce guava seed germination. The Generation 3 fruit collected from October to November (4 to 5 MAFB) were extracted from immature fruit but were very suitable as 'nurse seed' (germination percentage: 'Hass' 60% to 52.5% and 'Fuerte' 58%to 57.5%). This fruit is far from commercial harvest, occurring 9 to 10 MAFB, indicating that nurserymen do not need to wait until fruit maturity in June/July in the following year to collect 'nurse seed'.

During maturation of the avocado seeds, the seed coat turns dark brown and becomes thin; the browning of the seed coat by phenolics is used as an indicator of horticultural fruit maturity and of avocado seed maturity (Juarez-Escobar *et al.*, 2021). Generally, the interaction between the embryo and seed coat phenolics determines, whether a seed will germinate (Debeaujon *et al.*,

2007). In this study, the Generation 1 seeds of both cultivars had a lower germination percentage than the Generation 2 seeds during the entire study period (Fig. 1a and 1b). Kalala *et al*. (2005) reported a decline in seed tissue water potential with increasing maturity of 'Fuerte' and 'Hass', while the germination rate was low. Therefore, it is likely that the germination percentage of Generation 2 seeds was greater (Fig. 1a and 1b) due to the higher seed moisture content. Kalala *et al.* (2005) demonstrated that the water potential of younger seed tissue is higher than of the older avocado seeds and seed germination percentage is aligned with a higher moisture content. It also demonstrates that, as a recalcitrant seed (Berjak and Pammenter, 2013), avocado does not tolerate drying out, as this reduction in seed moisture content reduces the seed's ability to germinate (Bewley and Black, 1994).

2.4.2. **Phenolic Compounds**

Phenolic concentrations play an important role in seed germination and affect the ability of seeds to be permeable to water and gases (Siddiqui and Khan, 2010). The concentration of phenolics in the Generation 1 seed coat affected germination negatively; the germination percentage was lower in Generation 1 seeds than in Generation 2 seeds. The Generation 3 seeds had lower phenolic concentrations and a greater germination percentage than Generation 2. Generally, a lower germination percentage was aligned with a higher phenolic concentration, and, *vice versa*, a lower phenolic concentration in Generation 2 and Generation 3 was aligned with a high germination percentage (Fig. 2a and 2b). Avocado seed coat death, as visible by browning, is aligned with the destruction of channels of water movement and general communication pathways between the embryo, the seed coat and the external environment (Blumenfeld and Gazit, 1971).

Seeds impermeable to water and gas exchange are not allowing radicle protrusion through the seed coat, thereby resisting seed emergence (Asiedu *et al*., 2000). Seed permeability to gases and water seems correlated with seed coat colour, with brown-coloured seeds unable to germinate due to high phenolic concentrations (Fengshan *et al*., 2004). In line with this, phenolics have been found to inhibit germination in corn (*Zea mays* L.) (Krogmeier and Bremner, 1989) and sorghum (*Sorghum bicolor* L.) (Isidori *et al.,* 2005); generally, seed coat colour is an important external factor assisting in imbibition, coloured seed coats have higher phenolic concentrations and, thereby, lower seeds germination (Serrato *et al.,* 1993; Wolf and Fiske, 1995; Tyler, 1997).

Kristanty *et al*. (2014) reported that avocado seeds contain high levels of phenolic compounds, when the avocado seeds have reached 'full maturity'; these seeds were found to have the lowest germination percentage. In our study, both cultivars (Fig. 2a and 2b) showed higher phenolic concentrations in Generation 1 avocado seeds from June to September compared with Generation 2; these Generation 1 seeds had a lower germination percentage. Generation 2 seeds had a lower seed coat phenolic concentration aligned with better germination (Fig. 1a, 1b and Fig. 2a, 2b). There was a negative correlation between the concentration of phenolic compounds in the seed coat and the germination percentage ($r = 0.11$, $P > -0.26$). Generation 1 seed coats had higher concentrations of phenolics and a lower germination rate than the younger Generation 2. These findings are in line with Maga and Katz (1978), who reported that the presence of phenolic compounds in the seed coat is aligned with decreased germination of legume seeds. The higher phenolic concentrations of Generation 1 avocado seed coat, in both cultivars, during July to September, affected the germination percentage of the seeds negatively and resulted in a lower germination rate of the seeds, compared with Generation 2 seeds and Generation 3 seeds which had lower phenolic concentrations and a higher germination percentage than Generation 1 seeds (Fig. 2a and 2b).

Generation 1 seeds of both cultivars showed no physiological activity, the seed coat was brown, the tissues had seemingly died, hence, reserves or biological activity in the seed did not contribute to the germination process anymore. These results confirm reports by López-Amorós *et al*. (2006) that younger legume seeds have a lower phenolic concentration than mature seed.

The seed coat of the Generation 2 of both cultivars had a lower phenolic concentration than that of the Generation 1, from June to September (Fig. 2a and 2b, $P < 0.001$); from October to November the seed coat of Generation 3 had less phenolics than that of Generation 2 (Fig. 2a and 2b). Generation 2 contained a lower phenolic concentration from June to September than Generation 1 and Generation 3 contained a lower phenolic concentration from October to November that Generation 2, for both cultivars (Fig. 2a and 2b). This is in line with Muscolo *et al*. (2001) who reported that the phenolics concentrated in the mature seed coat contribute to the inhibition of germination through inhibition of enzyme activities.

'Hass' seed coats of the Generation 3 contained low levels of phenolic compounds (1.07 mg GAE* g^{-1} DM) in November, similar to 'Fuerte' seed coats, while the highest phenolic

concentrations in ‘Hass’ seed coats were found in September (2.3 mg GAE* g^{-1} DM) in the Generation 1 (Fig. 2b). In these Generation 1 avocado seeds the germination percentage was lower than in the Generation 2 seeds, possibly because of the higher concentration of phenolic compounds in the older seeds (from June to September). A high phenolic concentration in the seed coat has been postulated to play an important role in maintaining seed dormancy (Come, 1968). On the other hand, when lower concentrations of phenolic compounds are present in the avocado seed coat, the embryo can easily germinate; this may play an important role in controlling seed dormancy, affecting plant hormone activity, such as those of gibberellins and cytokinin’s (Dziewanowska and Lewak, 1975).

2.4.3. **Seed Coat Thickness of Avocado Seeds**

Seed coat of the ‘Hass’ avocado (Generation 2) was thicker (between 0.11 and 0.51 mm; $P < 0.001$; Fig. 3a) than Generation 1 seed coats from June to September. This Generation 2 seed coat was also thicker than that of the Generation 3 from October to November ($P < 0.001$; Fig. 3a). Generation 1 ‘Fuerte’ seed coats differed significantly in thickness between months, ranging from 0.15 to 0.46 mm ($P < 0.001$) in June *versus* August (Fig. 3b). From October to November Generation 3 had thinner seed coats than Generation 2 ($P < 0.001$; Fig. 3a).

The germination percentage was higher in both cultivars in Generation 2 than Generation 1, when the seed coats were thicker (Fig. 3a and 3b). Contrary results were reported by Traveset *et al.* (2008), who found the seed coat thickness of some species of the Oleaceae family affected germination rate, with thinner seed coats aligned with a higher germination percentage.

Mohamed *et al.* (1994) and Blumenfeld and Gazit (1974) reported that the seed coat of avocado seeds of different cultivars (‘Fuerte’ and ‘Ettinger’) were thick, white and fleshy in the early development stage. As the fully mature avocado seed coat contains various components, such as phenolics, particularly tannins, flavonoids, catechins and other polar compounds, seed coat is thick (Figueroa *et al.*, 2018), and germination is hindered. Contrary results were found in Fig. 3a and 3b with differences in seed coat thickness between generations; Generation 2 younger seeds had thicker seed coats than Generation 1 older seed was thin. After the seed reaches maturity, the seed coat begins to darken and shrivels until the seed coat dies. At this stage the seed is completely mature, and the seed coat is unable to transfer substances between the embryo and the mesocarp.

The lack of this 'transportation layer' might affect the transport of gases and water during the germination process negatively. This could possibly explain, why the mature seed, with a non-functional seed coat, had a lower germination rate (Fig. 3a and 3b).

In mature seeds the transfer of water to the embryo is almost impossible, as the seed coat is dead. Kalala *et al.* (2005) reported that the water potential of younger seed is higher than that of older avocado seed; therefore, the immature seed coat was thicker than the fully mature seed coat (Fig. 3a and 3b).

This study found seed coat thickness of the avocado seed, over various months, to be aligned with the germination percentage; seed coats of Generation 2 were thicker and had a higher germination rate in both cultivars. During seed development, up to the time when the seed became mature, the seed coat became thinner, and seeds had a lower germination percentage (Fig. 3a and 3b). From October to November the Generation 3 had thinner seed coats than the Generation 2 seeds. At this stage, Generation 2 seeds had matured. Statistical analysis of seed coat thickness of 'Hass' seed coats showed a significant difference between months and generations ($P < 0.001$).

'Hass' seeds the thickness of this tissue varied between Generation 2 and Generation 1 seeds, 0.51 mm and 0.32 mm, Fig. 3b) respectively. The seed coat of Generation 2 was thicker in 'Hass' Generation 2 than in the Generation 1, from June to September (Fig. 3b). Avocado seed coat thickness in Generation 2 and Generation 1 'Fuerte' seeds ranged between (0.39 mm to 0.25 mm, Fig. 3a) respectively.

The germination percentage of Generation 2 was greater than that of Generation 1 one's, possibly because of the lower concentrations of phenolic compounds. Younger generation had always thicker seed coat than older ones.

These results, therefore, agree with Erickson (1966), who reported that the seed coat is a thick, white and fleshy layer, when the avocado fruit is young, also indicating that the seed coat thickness will decrease with increasing seed maturity.

2.4.4. **Seed Viability of Avocado Seeds**

When the seeds of both cultivars ('Hass' and 'Fuerte'), after removal of the pericarp, were soaked in TTZ solution for 24h staining of the different seed generations varied. The TTZ test reflects

respirational activity in tissue; all seeds tissues which respire are able to germinate; dead tissue does not stain read as there is no respiration (Ellis, 2013). Results showed visible staining of the cotyledons, while the embryo was too small to determine its colour. The statistical analysis between the two cultivars and generations revealed no significant effect of generation on seed viability, but in Generation 2 better alignment of the TTZ-stained area and the germination percentage was discovered (Fig. 4a and 4b), indicating higher viability of these Generation 2 seeds than that of Generation 1 seeds.

Generation 1 seeds had a lower viability than Generation 2 seeds from June to September, while, from October to November, Generation 3 had a higher seed viability than Generation 2. This was the case for both cultivars (Fig. 4a and 4b), indicating a trend of a certain physiological age being better for seed germination and development (Fig. 4a and 4b). During germination, the food stored in the cotyledons of the avocado seeds is released by anaerobic respiration. This aerobic respiration is made possible by dehydrogenases, tested in the TTZ assay, which stimulate chemical processes that stain tissues under aerobic conditions in viable seeds (Chiu *et al*., 1995). Non-viable seeds do not stain due to denaturation of oxidoreductases and the loss of the ability to reduce TTZ to the red formazan.

Seed viability of 'Hass' and 'Fuerte' seeds of Generation 2 was higher than that of the Generation 1 from June to September. Based on the germination test conducted on the seeds, germination of avocado seeds for both cultivars ('Hass' and 'Fuerte') were high in Generation 2 from June to September (Fig. 4a and 4b). Bhattara *et al*. (2009) confirmed that the germination of younger tomato seed is higher than that of older seeds. The Generation 2 of both cultivars had a greater viability and germination was greater compared with Generation 1 seeds; the Generation 3 had also a greater viability and a greater germination rate than Generation 2 for both cultivar (Fig. 4a and 4b). Mangena and Mokwala (2019) indicated that high germination rates in soybean (*Glycine max* L.) *in vitro* depend largely on seed viability during seed development. Results depicted in Fig. 4a and 4b are in alignment with Parreñode *et al.* (2011) who reported that seed viability of fully mature jatropha (*Jatropha curcas* L.) seeds was lower compared with immature (younger) seeds, which had a higher viability.

3.4.5. **Moisture Content of Avocado Seeds**

It was observed that seed moisture percentage was higher in seeds from the younger Generation 2 compared with older Generation 1 (Fig. 5a and 5b). Similar to this study, Adams and Rinne (1980) indicated that seed maturation and development are associated with an overall loss in seed moisture. Usually, young seeds have a higher moisture content than old seeds. In this experiment, Generation 2 seeds had higher moisture content than the Generation 1, and from October to November the youngest Generation 3 seeds had a higher moisture content than those from Generation 2 (Fig. 5a and 5b).

Dabas *et al*. (2013) investigated seed moisture content of mature 'Hass' and 'Fuerte' avocado seeds and determined a percentage of 50.2 and 54.0, respectively. Similarly, in this study, Generation 1 'Fuerte' seeds ranged in moisture content between 41.0 and 58.6% (Fig. 5b), while 'Hass' seed recorded between 42.1 and 61.0% moisture (Fig. 5a).

A decrease in moisture content during avocado fruit ripening was reported by Olaeta *et al*. (2007). The findings of the present study confirm this, as the oldest seed had a lower moisture content than youngest seed. Additionally, in the present study, from October to November, the older seed of both cultivars, 'Hass' and 'Fuerte', had a lower moisture content than seed coming from the newest generation (Fig. 5a and 5b).

In general, seed moisture content decreases, as seeds approach maturity; however, the amount of water that remains in the seeds is relatively high throughout most of its maturation, because water is transferring nutrients and other solutes from the plant to the developing seeds (Bareke, 2018). Osuna-Garcia *et al.* (2010) also reported that during avocado fruit maturation, the oil concentration increases in the fruit; this is associated with an accumulation in fruit dry matter and a decrease in fruit moisture content. The presence of water, furthermore, plays an important role in the variation in maturity of avocado fruit (Bower *et al.*, 2007). There also is a negative relationship between 'Hass' seed moisture and seeds oil content (Avhad and Marchetti, 2015).

Analysis of variance showed significant differences in seed moisture content of different generations, and significant differences in seed moisture between months. There also was a statistically significant ($P < 0.001$) interaction between avocado cultivar and month of fruit

harvest in seed moisture. El Balla *et al.* (2011) reported that the seed moisture content in okra cultivars decreased significantly with seed maturity. On the other hand, in both avocado cultivars, Generation 1 had a lower moisture percentage than Generation 2. Germination percentage and moisture content were significantly affected by cultivar at seed maturity. There was, in both cultivars, a significant, positive correlation between seed moisture content and germination percentage ($r = 0.71$). It is, therefore, unsurprising, that seed moisture content has been described as a very important factor in the ability of the avocado seeds to germinate (Bower and Cutting, 1988); however, seed water content is not the only factor important in seed germination.

2.5. Conclusion

Germination percentage of the two avocado cultivars, 'Hass' and 'Fuerte', was observed over three seed generations. Germination of seeds differed significantly between the youngest generation of and older generations. Generation 2 of both cultivars were more viable (germinated better), and seeds stained red, compared with Generation 1 (lower viability and germination percentage). Seed moisture content was also related to germination, as higher and lower moisture concentrations resulted in higher and lower germination, respectively.

In 'Hass' seeds, however, the seed coat of Generation 2 was thicker, and the germination percentage was greater than in Generation 1. In 'Fuerte', the seed coat investigation showed that seed coat thickness was not involved in the lower/higher germination percentage of Generation 1 *versus* avocado seeds Generation 2. Therefore, this study points out that the thicker seed coats of Generation 2 are aligned with a greater germination percentage in 'Hass, but not in 'Fuerte'. A thicker seed coat possibly contains a higher concentration of compounds that foster germination, so that seeds with thicker seed coats will germinate faster, possibly also due to a faster imbibition.

From the present study it can be concluded that phenolic compounds could be used as a protective tool to suppress the germination processes of avocado seeds; however, the germination process of avocado seeds is modified by the phenolics they contain. The changes in phenolic concentrations observed seem to be affected by seed age and seed maturity. In both cultivars, Generation 1 seed coats had a higher phenolic concentration than Generation 2 seed coats and Generation 3 seed coats had higher level of phenolics than those of Generation 2, and these high phenolic concentrations could have inhibited seed germination of mature seeds.

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CHAPTER THREE

SEASONAL VARIATIONS IN AVOCADO SEED VIABILITY AND RESPIRATION RATE AFFECT GERMINATION PERCENTAGE

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Abstract

There is limited information on growth and development of the avocado seed, despite its common as a 'nurse seeds' in the double grafting techniques to produce commercial avocado trees. This study attempted, therefore, to investigate how avocado seed viability and germination are affected by seed respiration, as well as cotyledonal sugar and starch concentrations. The experiment analysed seed development from early fruit (0 to 5 MAFB) to 2-y-old seeds (24 to 29 MAFB and 12 to 17 MAFB) of two cultivars ('Hass' and 'Fuerte'). In such seeds were parameters, including germination percentage, seed viability as well as seeds respiration rate were investigated; thereafter, internal parameters of seed tissues, such as sugar and starch concentrations, were investigated. Seed germination rates of 'Hass' and 'Fuerte' seeds were evaluated for three seed generations and for different ages within these generation (from early full bloom to very late-hung fruit). Germination percentages were higher in younger than older seed generations and seed viability was aligned with seed germination rate. Seed viability, determined through the germination and the tetrazolium chloride (TTZ) test, was, in both cultivars, lower in the older Generation 1 than in Generation 2, and Generation 2 had also lower viability than the youngest Generation 3; the same trend was found for germination percentage (greater germination in younger seeds). Avocado seed respiration rates of both cultivars were higher in the younger (Generation 2) than in the (older) Generation 1 seeds and respiration was lower in Generation 2 compared with Generation 3. Cotyledonal starch concentrations, in both cultivars, were higher in younger generations than in older ones. Starch concentrations were high in immature seed of both cultivars from June to November, reaching 90% of DM. The avocado seed seems to be able to supply starch for early seed growth and development. Younger seeds of both cultivars had, however, a higher starch concentration than the older, Generation 1 seeds. There was a very strong, positive relationship between seed starch and seed germination ($r = 0.80$). It was found that the seven carbon sugars perseitol and mannoheptulose were predominant amongst all soluble carbohydrates, while only small amounts of sucrose and glucose were present. In the seeds (predominantly consisting of the cotyledons), perseitol was the dominant sugar (9.8 and 10.3 mg g^{-1} DM in 'Hass' and 'Fuerte', respectively). Mannoheptulose levels were lower than perseitol levels in seeds of both cultivars. The common C6 sugars glucose and sucrose were also detected in seeds, with perseitol being the dominant sugar. Sucrose was present in higher concentrations than glucose, while the C7 sugar mannoheptulose and glucose were present in lower amounts.

Avocado seeds of both cultivars had a high perseitol concentration, which seems to be important as a storage sugar to be used in seed germination. Cotyledonal starch characterised Generation 2 and 3, potentially feeding the new seedling, assisting in a fast germination. In both cultivars, the young, Generation 3, seeds contained the highest starch concentration 0 to 5 MAFB and germinated better than seeds from both older generations. It seems that a high starch concentration and a high perseitol and mannoheptulose concentration are aligned with the higher germination percentage in younger seeds of both cultivars.

Keywords: Carbohydrates, avocado seed age, germination, tetrazolium test, respiration, cotyledons, generation, sugar

3.1. Introduction

Seed respiration is a sign of an actively metabolizing seed. This respiration rate dramatically increases during the first minutes of seed imbibition, taking up oxygen in germination phase I (Kollofel, 1967). The respiration rate slows during germination phase II and oxygen concentrations are at a constant level, until the germination process is completed, and the radicle emerges (stage III) (Bewley and Black, 1978). The initial water uptake by the seed allows the occurrence of increased metabolic activity. Seed respiration increases very quickly, following imbibition (Hourmant and Pradet, 1981). Most seeds undergo oxidative respiration to complete the germination process (Al-Ani *et al.,* 1985; Corbineau and Côme, 1995). The respiration in germination phase I and II is due to the breakdown of compounds that release energy to sustain the developing seedling (Hourmant and Pradet, 1981). Generally, respiration rates increase as imbibition proceeds and reach a peak prior to radicle emergence. The protrusion of the embryo through seed structures surrounding the embryo sac is considered as the final stage in germination (Weitbrecht *et al*., 2011; Bewley *et al*., 2013). The respiration rate increases again after the radicle has emerged (Woodstock and Grabe, 1967; Ibrahim *et al*., 1983; Dahal *et al*., 1996).

In respiration the respiratory substrates are broken down to release energy; respiration substrates are carbohydrates, lipids and proteins; additionally, biochemical energy is produced in the form of ATP (Dahal *et al*., 1996). The major energy source used for plant biological activities is ATP; this ATP increases in the seed immediately after imbibition (Perl, 1986). Together with the

energy-carrying molecule NADPH+H, ATP is also needed to produce proteins that support embryonic growth, thereby allowing the completion of germination (Bewley *et al*., 2013).

Avocado seeds are storing food reserves as carbohydrates, proteins and lipids; these reserves are used to maintain seedling development. Avocado seeds also contain oil and protein, these are the main reserve compounds in the seed which provide sources of nitrogen, energy and carbon during seedling growing (Orhevba and Jinadu, 2011). The mobilization of reserve substances during germination is required in order to provide the embryo with food, fuelling germination (Vinha *et al*., 2013).

Avocado seeds are recalcitrant (Berjak and Pammenter, 2013); therefore, the seed dies, if between 20 to 50% of its moisture is lost, impacting seed viability and seedling emergence (Hartmann *et al.,* 2002). Avocado seeds can, consequently, not tolerate long storage periods, as they will dry out (Kozlowski and Pallardy, 2002; Ernst *et al*., 2013; Walters *et al*., 2013). During fruit and seed development, the healthy seed coat, surrounding the seed, allows the flow of solutes and gas exchange between the seed and the mesocarp through plasmodesmata (Moore-Gordon *et al*., 1998; Crawford and Zambryski, 1999; Van Bel, 2003). Avocado seeds are non-endospermic, their main carbohydrate storage is found in the cotyledons at seed maturity (Kalala *et al.,* 2005), indicating that the energy for biochemical and physiological processes during maturation and development arises from the cotyledons (Bewley and Black, 1994). Also, avocado seeds contain polyphenols and other antioxidant compounds that are more prevalent in seeds than in the mesocarp (Soong and Barlow 2004; Wang *et al.,* 2010). These phytochemicals could have enhanced seed germination as sources of nutrients and as phytochemicals that can control diseases (Lillehoj *et al.,* 2018).

The avocado seeds have a large carbohydrate reservoir in their cotyledons; thus, these structures contain valuable carbon sources for seedling growth and development (Jimenez *et al.,* 2013), as visible in the large starch concentration of avocado seeds, ranging between 30 to 75% of its dry mass (Olaeta *et al*., 2007; Orhevba and Jinadu, 2011).

As various factors seem to interact in avocado seeds germination, the aim of this study was to align seed sugar and starch concentrations, as well as seed moisture content, with seed respiration and seed germination percentage. This would help in elucidating what contribution the various

carbohydrates make to avocado seed development. The concentrations of seed sugar, starch, as well as the seed respiration rate of two avocado cultivars ('Hass' and 'Fuerte') were determined over a six-month period. It was also attempted to align the germination percentage with seeds viability, evaluated using the tetrazolium test (TTZ).

Seed germination of three generations of avocado seeds were investigated in this study. Younger generations always had a higher seed viability, respiration rate, and higher C7 and starch concentrations. This study analysed three generations to confirm that younger seeds germinate easier and faster than older seeds, starch sugars concentrations as well as respiration rate are contributed to germination percentage in younger avocado seeds.

3.2. Materials and Methods

3.2.1. Origin of Seeds

Seeds used in this study were extracted from fruit obtained from a commercial orchard in the KwaZulu-Natal Midlands (30°16-E and 29°28-S, South Africa). 'Hass' and 'Fuerte' avocado fruit of three generations (G1, G2, G3), with full bloom in June/July ('Fuerte') and July/August ('Hass') 2017, 2018, 2019, respectively, were collected from the same trees carrying, at one stage, up to three generations at one time. Fruit collected were of uniform appearance and size, characteristic for the generation. Samples were collected monthly from June to November.

Three generations of seeds were analysed: 'Hass' avocado fruit (termed Generation 1), collected over a six-months period from 24 to 29 months after full bloom (MAFB) until November 2019. These fruits were commercially harvested in August 2018. Additionally, the following year's fruit (full bloom in July/August 2018 (Generation 2)) were harvested 12 to 17 MAFB, commercially harvested in July/August 2019 were sampled. Thirdly, newly developing fruit, with full bloom in July/August 2019 (Generation 3, 0 to 5 MAFB) were collected. In 'Fuerte', similarly, three generations were collected: the oldest avocado seed Generation 1 (full bloom in June/July 2017, 24 to 29 MAFB), these fruits were commercially harvested in June/July 2018, Generation 2 (full bloom in June/July 2018, 12 to 17 MAFB), these fruits were commercially harvested in June/July 2019, and Generation 3 (full bloom in July 2019), these fruits were commercially harvested in June/July 2018. Generation 3 was removed from the tree (9 to 10 MAFB) prior to commercial

harvest for both cultivars. All fruit were removed from trees and immediately transported to laboratory.

Seeds were extracted from fruit after they had ripened to eating softness (finger-feel). The seeds were removed from the mesocarp, dried with tissue and planted into containers filled with 2cm agar- to determine the seed germination percentage. For further seed coat analyses the seed coat was peeled off from the seeds and oven-dried at 70°C for 24h.

3.2.2. **Parameters Determined**

3.2.2.1. **Seed Germination Tests**

Fruit of both cultivars and of three generations were harvested and kept at room temperature until fruit started to soften (hand feel). Seeds were extracted from the mesocarp and placed into plastic containers (Length x Width x Height: 13 x 13 x 8.5cm), filled with agar (12g L^{-1}, ACE, South Africa) to a depth of 2cm. Containers were covered with aluminium foil to allow germination and were placed into a growth chamber at 25°C for 23 days. Germination percentage was determined weekly by plumule emergence (Porceddu *et al*., 2016). The containers were arranged in a completely randomized factorial design with three replications. The experiment was repeated monthly over six months (June to November).

3.2.2.2. **Seed Viability Determination**

Seeds were incubated in a 1% (w/v) aqueous solution of tetrazolium (TTZ) chloride (Sigma–Aldrich/Fluka, St Louis, MO, USA); the TTZ solution was prepared by adding 1g 2,3,5-triphenyltetrazolium chloride powder to 100mL distilled water. Avocado seeds were soaked in this solution for 24h. The production of the highly coloured, reddish formazan end-product was used to determine seed viability according to Berridge *et al*. (1996). The seed colour differentiation of tissues followed the criteria established by França Neto (1994), where intense red colour indicated healthy, respiring tissue, while deteriorated tissue lacked this red colour, indicating non-respiring, dead tissue. The percentage cotyledon area that turned red was, hence, recorded immediately.

3.2.2.3. **Determination Seed Respiration Rate**

The amount of CO_2 released by the seed was measured using an infrared gas analyser (model F-950 Three Gas Analyzer, Felix instrument Inc., Camas, WA, USA). Individual seeds were incubated in a 1 L plastic container for 60 min. The headspace CO_2 concentration was recorded as the hourly respiration rate considering seed mass, seed volume, free space in the jar and the ambient CO_2 concentration. The CO_2 production of each seed was calculated using Eq. (3), according to Kassim *et al.* (2013).

$$CO_2 \text{ evolution} = \frac{NetCO_2}{1000} \quad X \quad Headspace\ X \quad \frac{1000}{m} \; X \frac{60}{t}$$

Where:

Net CO_2 = seeds CO_2 - ambient CO_2 (mL)

Headspace = container volume – fruit volume (mL)

m = sample mass (g)

t = duration of incubation.

3.2.2.4. **Concentrations of Sugars in Avocado Cotyledons**

The concentrations of avocado seed (embryo plus cotyledons) sugars were determined according to Liu *et al*. (1999) with alterations according to Tesfay *et al*. (2010) using an isocratic HPLC system (LC –20AT; Shimadzu Corp., Kyoto, Japan) equipped with a refractive index detector (RID-10A; Shimadzu Corp., Kyoto, Japan). Freeze-dried material (0.20g DM) was mixed with 10mL 80% (v/v) ethanol and homogenized for 1min. Thereafter, the mixture was incubated in an 80°C water bath for 1h and kept at 4°C overnight. On the following day, after centrifugation at 12000*g* for 15min at 4°C, the supernatant was filtered through glass wool and taken to dryness in a Genevac personal evaporator (EZ-2.3, SP Scientific, Genevac Ltd, Ipswhich, England). Dried samples were re-suspended in 2mL ultra-pure water and centrifuged at 10000*g* for 5min. Samples were filtered through 0.45µm nylon syringe filters, before being injected into the isocratic HPLC system (Tesfay *et al*., 2010). Samples were separated using a gel column connected to a refractive index detector. Individual sugar concentrations were determined by comparison with authentic standards.

3.2.2.5. **Starch Concentrations in Avocado Cotyledons**

Avocado seed starch was determined as described by Raigond *et al*. (2015). Samples (0.2g DM) were homogenized and extracted in 80% ethanol overnight, before being centrifuged at 10000*g* for 5min. The supernatant, containing the free sugars, was collected and the extraction was repeated twice, using 30mL 80% ethanol to remove any free sugar residues from the pellet. The two supernatants were combined, and the sample extracts made up to a final volume of 100mL with de-ionised water. To allow colour development, 50µL sample and 900µL distilled water was boiled in presence of 2mL anthrone-sulphuric acid reagent (0.1g anthrone (Sigma–Aldrich/Fluka, St Louis, MO, USA) in 100mL chilled, concentrated sulphuric acid) for 8min. Samples were then cooled to room temperature and absorbance was recorded at 620nm. The starch concentration was calculated by comparison with a glucose standard curve.

3.2.3. **Statistical Analysis**

Analyses of variance were performed using GenStat (version 18.2; VSN International, Hemel Hempstead, UK). Means were separated using Fisher's Protected Least Significant Difference, when seeds showed significant effects on measured parameters at $P \leq 0.001$.

3.3. Results

3.3.1. **Germination Percentage**

'Hass' and 'Fuerte' seeds showed different germination percentages from June to November (Fig. 1a and 1b). Seeds of the older Generation 1 of both avocado cultivars displayed a lower germination percentage from June to September than Generation 2. From October to November the germination percentages were lower in Generation 2 than Generation 3 (Fig. 1a and 1b).

The germination percentage of Generation 2 'Hass' seeds, harvested from June to September, ranged between 49.5 and 67.5%, while seed from Generation 1 had a significant lower germination percentage, ranging between 34.5 to 52.3%. Similarly, 'Hass' seeds from Generation 2, harvested in October and November, had a lower percentage than seeds from Generation 3(0-5 MAFB). Seeds of commercially mature 'Hass' fruit of Generation 2 had a lower germination percentage compared with the younger Generation 3 (Fig. 1a). The lowest germination percentage was in Generation 1 in June (34.5%). Generation 1 'Hass' seeds (24 to 29 MAFB) had a lower

germination percentage than seeds from the Generation 2 (12 to16 MAFB) from June to September, when this fruit had reached commercial maturity. In ‘Hass’ seeds the highest germination percentage was detected in September (67.5%) in Generation 2 (Fig. 1a).

Generation 1 ‘Fuerte’ seeds (23 to 28 MAFB) had a lower germination percentage than seeds from Generation 2 (13-15 MAFB) from June to September, when the latter fruit had reached commercial maturity. In ‘Fuerte’ seeds the germination percentage of Generation 2 ranged between 45.0 and 58.6% (12 to 18 MAFB), when fruit were harvested between June to September; ‘Fuerte’ seeds from August fruit had a significantly higher germination rate (66.8%, 14 MAFB) than seeds from October to November, which recorded only between (41.25 to 47.5%, 17 to 18 MAFB) germination (Fig. 1b).

Over this specific period Generation 3 ‘Fuerte’ seeds had a greater germination percentage than Generation 2 (Fig. 1b). Seeds from the Generation 3 ‘Fuerte’ fruit, harvested from October to November (0 to 5 MAFB) displayed a higher germination percentage than Generation 2 seeds (Fig. 1b). Altogether, the germination percentage of ‘Hass’ seeds was generally higher than that of ‘Fuerte’ seeds ($P < 0.001$).

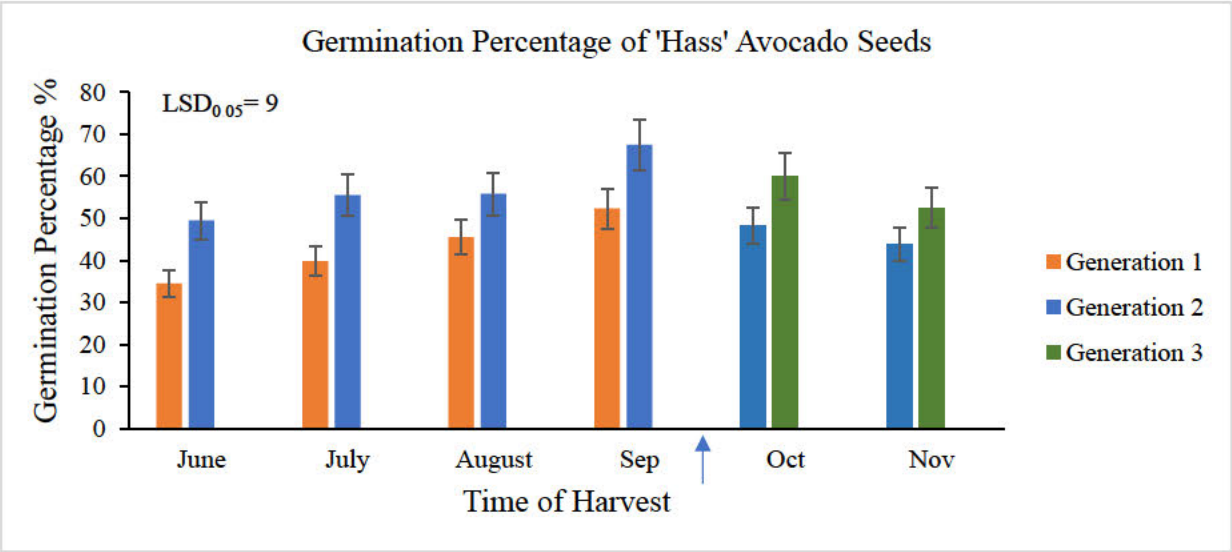


Figure 1a. Germination percentage of three generations of 'Hass' avocado seeds: Generation 1 (full bloom in July/August 2017), Generation 2 (full bloom in July/August 2018) and Generation 3 (full bloom in July/August 2019). The arrow indicates the start of a new season with Generation 1 removed from the tree and a new generation (Generation 3) present on the trees. Error bars above the columns denote least significant difference ($LSD_{0.05}$).

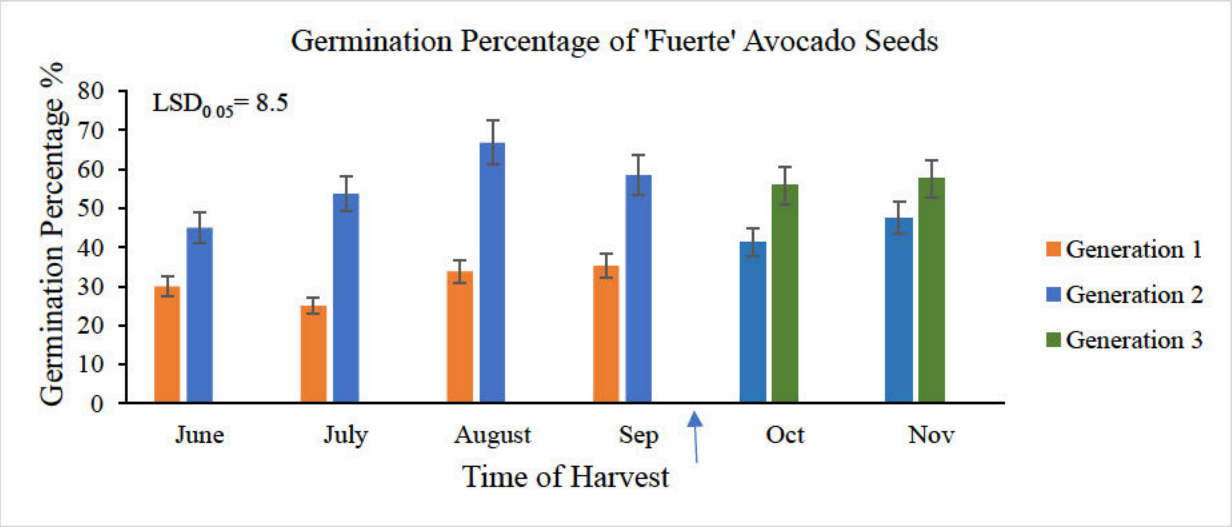


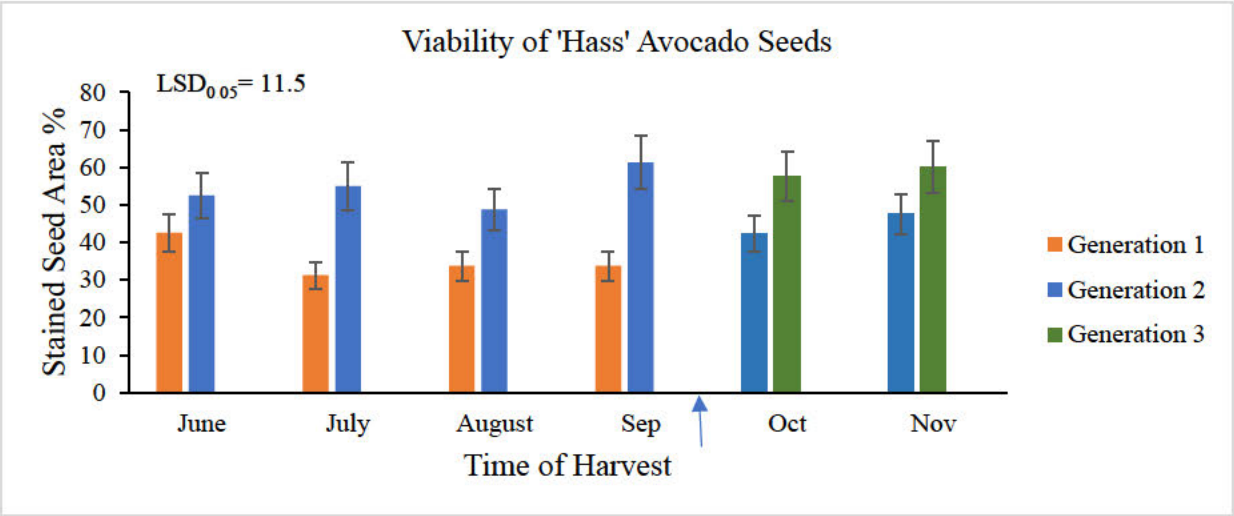
Figure 1b. Germination percentage of three generations of 'Fuerte' avocado seeds: Generation 1 (full bloom in June/July 2017), Generation 2 (full bloom in June/July 2018) and Generation 3 (full bloom in June/July 2019). The arrow indicates the start of a new season with Generation 1 removed from the tree and a new generation (Generation 3) present on the trees. Error bars above the columns denote least significant difference ($LSD_{0.05}$).

3.3.2. **Viability of Avocado Seeds**

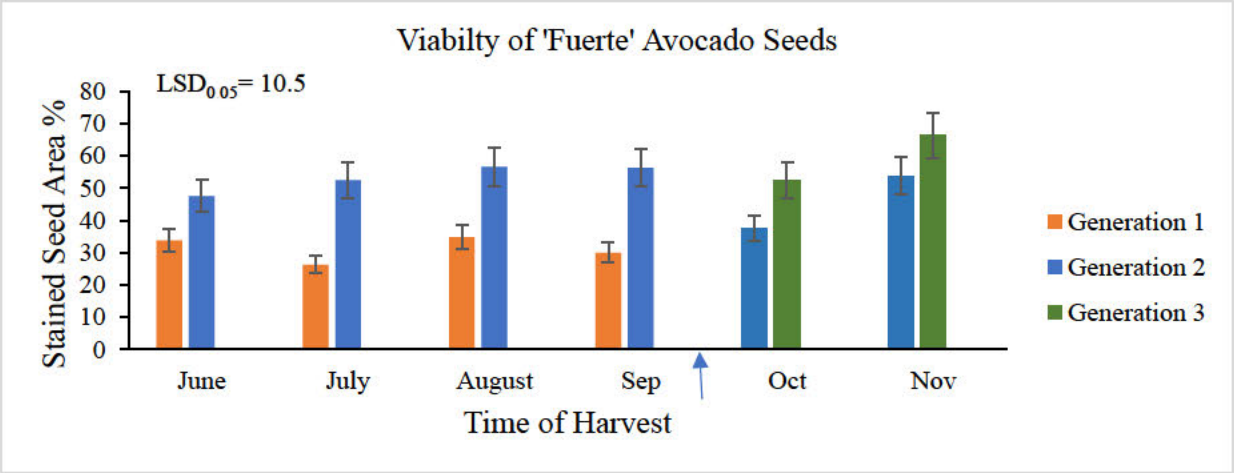
Seed viability and germination of avocado seeds were following a similar trend in both monitored cultivars. In both cultivars, seeds of Generation 1, 24 to 28 MAFB, had a lower seed viability than Generation 2 (12 to 18 MAFB) from June to September (Fig. 2a and 2b). Seeds from Generation 2 of both cultivars had a lower viability from October to November than Generation 3 (0 to 6 MAFB, for both cultivars) (Fig. 1a, and 1b, Fig. 2a and 2b).

In 'Hass' seeds, viability of the oldest generation (Generation 1) declined from June onwards, remaining at similar levels (50-60%) until the last observation, in September (Fig. 2a). 'Fuerte' seeds of the oldest generation (Generation 1) (24 MAFB) also had a viability of around 30% in June to September (31.25%; 27 MAFB, Fig. 2b).

Analysis of variance of the relationship of seed viability and seed generations (for both cultivars) showed statistical significance ($P < 0.001$). Generation 1 seeds were generally less viable than those of Generation 2; the former, Generation 2, seeds had the highest viability (61.25% for 'Hass' and 56.5% for 'Fuerte') (Fig. 2a and 2b) from June to September. Seeds from 'Hass' and 'Fuerte' Generation 3 were highly viable from October to November (60.0% and 66.25%) compared with Generation 2 (47% and 53%) (Fig. 2a and 2b).



Figures 2a. Viability (%) of 'Fuerte' avocado seeds as determined by the Tetrazolium Test (TTZ), % means the percentage of cotyledon area that turned red over the three generations periods: Generation 1 (full bloom in June/July 2017), Generation 2 (full bloom in June/July 2018) and Generation 3 (full bloom in June/July 2019). The arrow indicates the start of a new season with Generation 1 removed from the tree and a new generation (Generation 3) present on the trees. Error bars above the columns denote least significant difference ($LSD_{0.05}$).



Figures 2b. Viability (%) of 'Fuerte' avocado seeds as determined by the Tetrazolium Test (TTZ), % means the percentage of cotyledon area that turned red over the three generations periods: Generation 1 (full bloom in June/July 2017), Generation 2 (full bloom in June/July 2018) and Generation 3 (full bloom in June/July 2019). The arrow indicates the start of a new season with Generation 1 removed from the tree and a new generation (Generation 3) present on the trees. Error bars above the columns denote least significant difference ($LSD_{0.05}$).

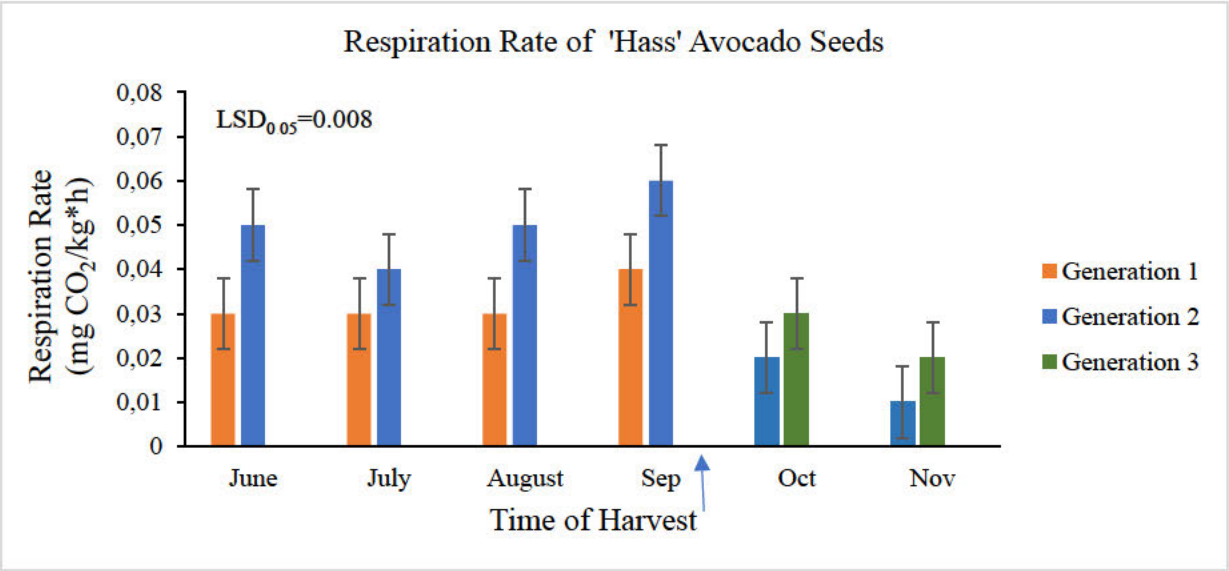
3.3.3. **Respiration Rate of Avocado Seeds**

The seed respiration rate of the two cultivars tended to decrease over time; generally, seeds of Generation 1 respired less than seeds of Generation 2, with a significant drop-in respiration rate from September to October ('Hass', Generation 2) or October to November ('Fuerte') in Generation 2 as well as Generation 3 (Fig. 4a and 4b).

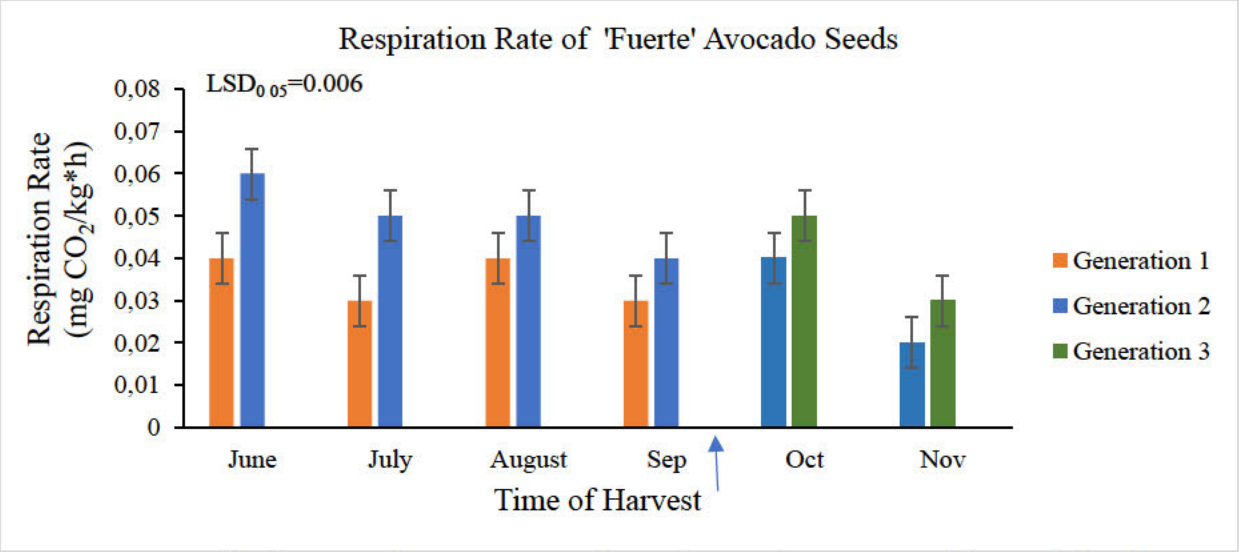
'Hass' seed respiration rates declined rapidly with fruit age, dropping from the highest point in September (0.06 mg CO_2/kg*hr, in Generation 2) to the lowest respiration rate in November (0.01 to 0.02 mg CO_2/kg*hr in Generation 3; Fig. 4a).

'Fuerte' seeds had a relatively high respiration rate in June, declining consistently until November for all generations. The respiration rate of 'Fuerte' seeds of Generation 1 dropped from 0.04 mg CO_2/kg*hr in June to 0.03 mg CO_2/kg*hr in September; the lowest 'Fuerte' respiration rate was detected in November (0.01 mg CO_2/kg*hr; Fig. 4b); similarly, a lower seed respiration rate was detected in November in Generation 3 seed than in October. At the time, when commercial harvest usually starts, in June or July, the respiration rate of 'Fuerte' seeds was high (0.06 mg CO_2/kg*hr in Generation 2, dropping to 0.04 mg CO_2/kg*hr in September.

Statistical analysis revealed a significant difference ($P = 0.06$) between the three seed generations for both cultivars. The interaction between seed generation and month was also significant ($P < 0.001$).



Figures 3a. Respiration rate of 'Hass' avocado seeds at various stages of the seed development over three generations: Generation 1 (full bloom in July/August 2017), Generation 2 (full bloom in July/August 2018) and Generation 3 (full bloom in July/August 2019). The arrow indicates the start of a new season with Generation 1 removed from the tree and a new generation (Generation 3) present on the trees. Error bars above the columns denote least significant difference ($LSD_{0.05}$).



Figures 3b. Respiration rate of 'Fuerte' avocado seeds at various stages of the seed development over three generations: Generation 1 (full bloom in June/July 2017), Generation 2 (full bloom in June/July 2018) and Generation 3 (full bloom in June/July 2019). The arrow indicates the start of a new season with Generation 1 removed from the tree and a new generation (Generation 3) present on the trees. Error bars above the columns denote least significant difference ($LSD_{0.05}$).

3.3.4. **Sugar Concentrations in Avocado Seeds**

Analysis of carbohydrates by reversed phase HPLC in the avocado seeds, consisting of the embryo plus, mainly, the cotyledons revealed that the major sugars present in this tissue included sucrose, glucose and very low amounts of mannoheptulose, while perseitol was the predominant sugar (Fig. 4 to 7).

3.3.4.1. **Sucrose Concentration in Avocado Seeds**

The sucrose concentrations present in avocado seeds differed during the study period for both cultivars (Fig. 4a and 4b). Generation 1 (24 to 28 MAFB) 'Hass' avocado seeds had a slightly greater sucrose concentration than Generation 2 seeds (12 to 18 MAFB). The highest sucrose concentration in 'Hass' seeds was detected in September (4.8 mg g^{-1} DM). In Generation 1 'Hass' seeds, the lowest and highest sucrose concentrations were 4.3 mg g^{-1} DM and 4.8 mg g^{-1} DM, respectively (Fig. 4a).

The amount of seeds sucrose was higher than that of glucose and mannoheptulose ($P = 0.039$). In 'Hass' seeds Generation 2 the concentration of sucrose was relatively constant over the entire observation period, with 4.5 mg g^{-1} DM the highest and 3.9 mg g^{-1} DM as the lowest concentration (Fig. 4a). Similarly, in 'Fuerte' seeds, sucrose tended to be higher in Generation 1, from June to September, than in seeds from Generation 2. The highest sucrose concentration was detected in September (5.1 mg g^{-1} DM, Fig. 4b) in 'Fuerte' seeds. From October to November, Generation 2 seeds tended to contain higher sucrose concentrations than Generation 3 (0 to 6 MAFB, Fig. 4b). In 'Fuerte' seeds sucrose concentrations were higher in Generation 1 than Generation 2 from June to September, with levels of sucrose ranging, in Generation 1, between 4.3 to 5.1 mg g^{-1} DM. Generation 2 seeds recorded between 4.5 mg g^{-1} DM in September and 3.9 mg g^{-1} DM in June; from October to November the sucrose level tended to be higher in Generation 2 compared with Generation 3 (Fig. 4b). Statistically, there was no significant difference in sucrose concentration between generations during seasons and for seeds of the two cultivars (Fig. 4a and 4b).

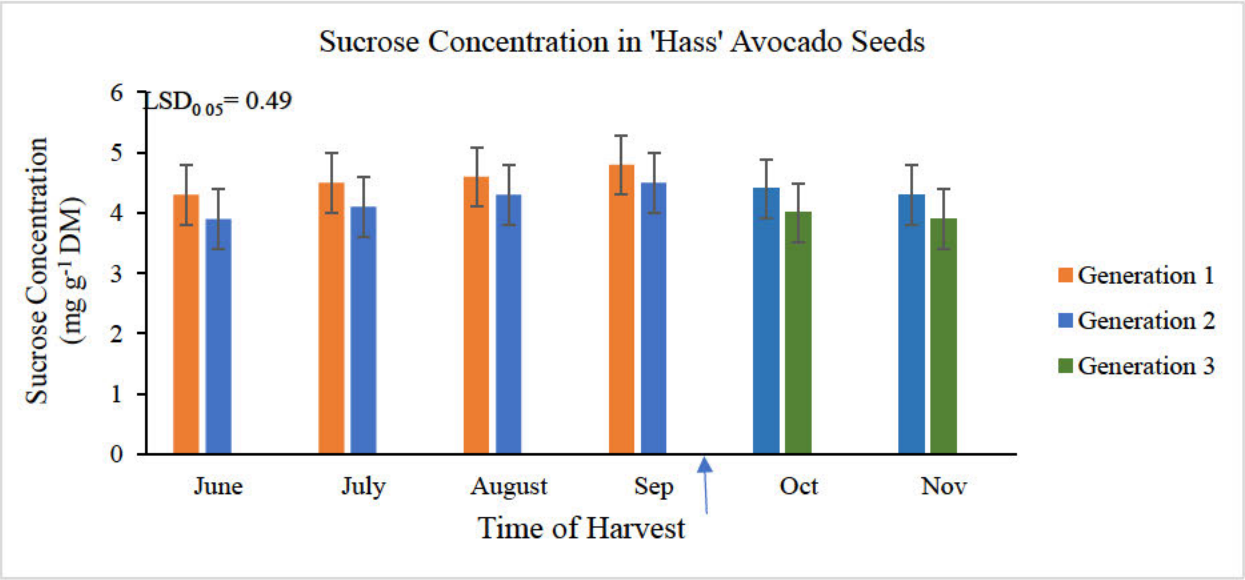


Figure 4a. Sucrose concentration of 'Hass' avocado seeds over three generations: Generation 1 (full bloom in July/August 2017), Generation 2 (full bloom in July/August 2018) and Generation 3 (full bloom in July/August 2019). The arrow indicates the start of a new season with Generation 1 removed from the tree and a new generation (Generation 3) present on the trees. Error bars above the columns denote least significant difference ($LSD_{0.05}$).

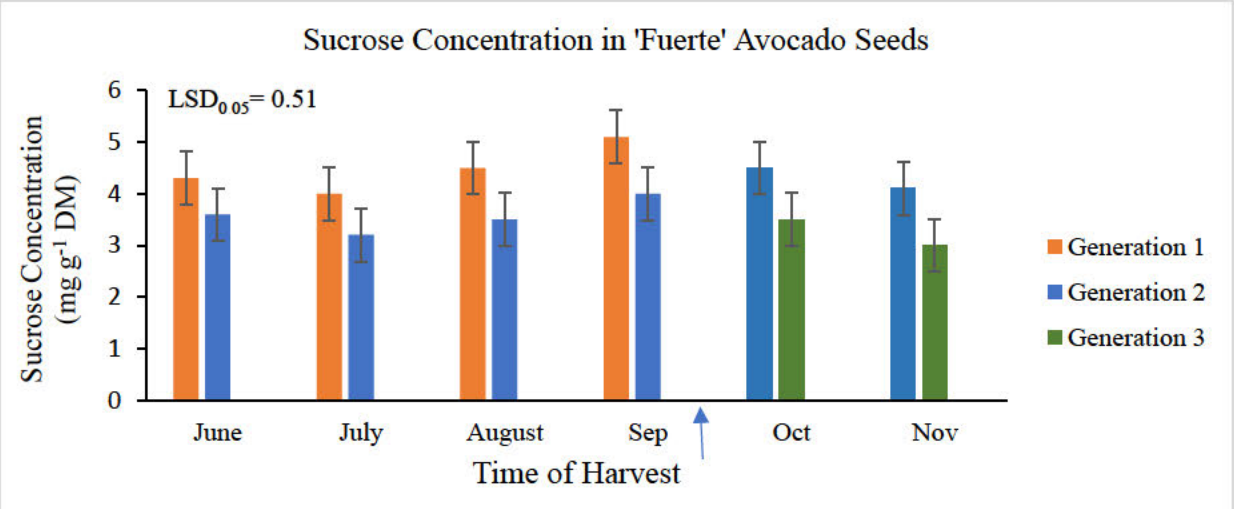


Figure 4b. Sucrose concentration of 'Fuerte' in avocado seeds over three generations: Generation 1 (full bloom in June/July 2017), Generation 2 (full bloom in June/July 2018) and Generation 3 (full bloom in June/July 2019). The arrow indicates the start of a new season with Generation 1 removed from the tree and a new generation (Generation 3) present on the trees. Error bars above the columns denote least significant difference ($LSD_{0.05}$).

3.3.4.2. **Glucose Concentration in Avocado Seeds**

The glucose and sucrose concentration in seeds of both cultivars ('Hass' and 'Fuerte') was always higher in the older seed generations than in the younger generations (Fig. 4a, 4b and Fig. 5a, 5b). From June to September 'Hass' seeds of Generation 1 contained greater concentrations of glucose than the Generation 2. From October to November the concentration of glucose in 'Hass' seed of Generation 2 was higher than in the Generation 3 (Fig. 5a). In Generation 1 glucose increased monthly until August, from its lowest value in June, then declining to the September value. In Generation 2 'Hass' seeds, the glucose concentration was detected as 0.6 to 1.2 mg g^{-1} DM). A new generation (Generation 3) was present in October that had a lower seed glucose concentration compared with Generation 2 (Fig. 5a).

In 'Fuerte' seeds of Generation 1 the glucose concentration was higher than in seeds of Generation 2 from June to September. In October and November, the glucose concentration was greater in Generation 2 than in Generation 3 (Fig. 5b). The concentration of glucose in 'Fuerte' seeds in Generation 1 ranged between (0.9 to 1.3 mg g^{-1} DM) in June and July. Generation 2 of 'Fuerte' seeds had glucose concentration from 0.6 to 1.4 mg g^{-1} DM between June and November (Fig. 5b).

Generally, the concentrations of glucose in avocado seeds were low in both cultivars (Fig. 5a and 5b). The highest glucose concentrations detected in 'Hass' seeds were found in August (1.4 mg g^{-1} DM), and in 'Fuerte' in November (1.4 mg g^{-1} DM) (Fig. 5a and 5b). There were significant differences between months and seed generations ($P < 0.001$).

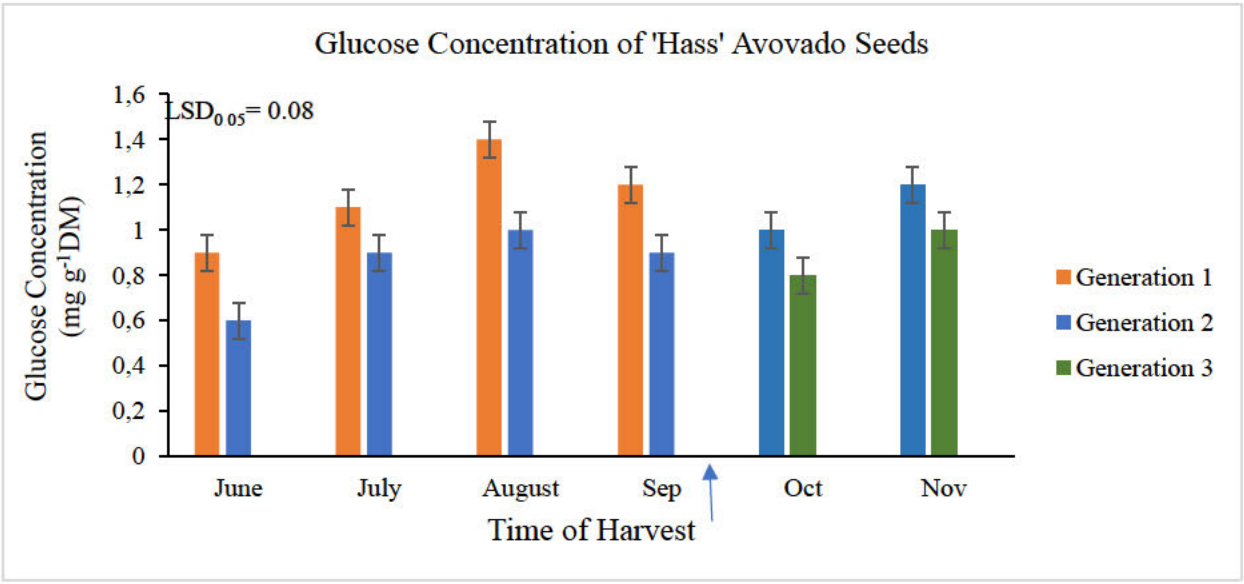


Figure 5a. Glucose concentration in 'Hass' avocado seed over three generations: Generation 1 (full bloom in July/August 2017), Generation 2 (full bloom in July/August 2018) and Generation 3 (full bloom in July/August 2019). The arrow indicates the start of a new season with Generation 1 removed from the tree and a new generation (Generation 3) present on the trees. Error bars above the columns denote least significant difference ($LSD_{0.05}$).

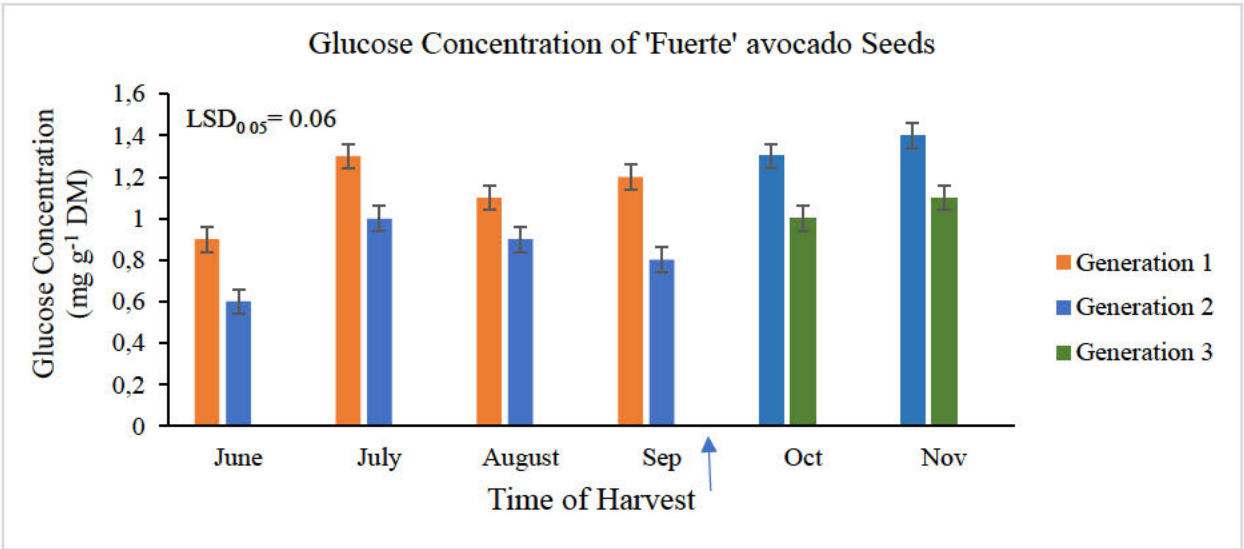


Figure 5b. Glucose concentration in 'Fuerte' avocado seed over three generations: Generation 1 (full bloom in June/July 2017), Generation 2 (full bloom in June/July 2018) and Generation 3 (full bloom in June/July 2019). The arrow indicates the start of a new season with Generation 1 removed from the tree and a new generation (Generation 3) present on the trees. Error bars above the columns denote least significant difference ($LSD_{0.05}$).

3.3.4.3. **Mannoheptulose Concentration in Avocado Seeds**

The concentrations of mannoheptulose and perseitol were always greater in younger seed generations than in older ones. In both cultivars and at all times, seed mannoheptulose concentrations were lower compared with perseitol concentrations (Fig. 6a, 6b and 7a, 7b).

In 'Hass' seeds the concentration of mannoheptulose was lower in Generation 1 than in generation 2, from June to September. From October to November Generation 2 had lower mannoheptulose concentration than the Generation 3 (Fig. 6a). In 'Hass' seeds, mannoheptulose concentration in Generation 1 ranged between 0.9 to 1.4 mg g^{-1} DM in June and August, and for Generation 2 the concentration ranged between 0.7 to 1.8 mg g^{-1} DM in November and July, respectively (Fig. 6a). From October to November the mannoheptulose concentration were lower in Generation 2 compared with Generation 3. This generation recorded 1.2 to 1.01 mg g^{-1} DM. In Generation 2 levels of this sugar dropped consistently to the lowest level in November (Fig. 6a).

In 'Fuerte' seeds Generation 2 higher mannoheptulose concentrations were detected than in the older Generation 1 from June to September. From October to November the mannoheptulose concentrations were higher in Generation 3 compared with Generation 2 (Fig. 6b). The mannoheptulose concentration ranged in the oldest generation (Generation 1) between (0.9 mg g^{-1} DM to 1.6 mg g^{-1} DM) in June to September, and Generation 2 seeds contained between 0.9 to 1.9 mg g^{-1} DM in October and August, respectively. Generation 3 had greater mannoheptulose concentrations than Generation 2 (Fig. 6b). The lowest concentration was present in 'Fuerte' seeds in June and July (Fig. 6b). Concentrations were lower again in October and November. There were significant differences in mannoheptulose values between months and seed generations ($P= 0.047$).

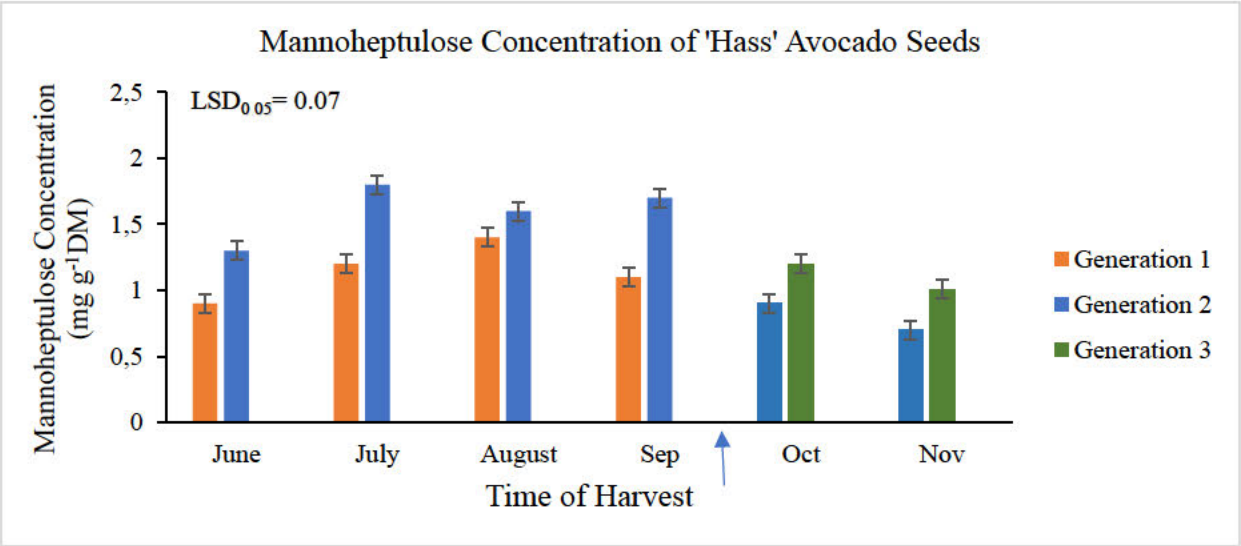


Figure 6a. Mannoheptulose concentration in 'Hass' an avocado seed over three generations: Generation 1 (full bloom in July/August 2017), Generation 2 (full bloom in July/August 2018) and Generation 3 (full bloom in July/August 2019). The arrow indicates the start of a new season with Generation 1 removed from the tree and a new generation (Generation 3) present on the trees. Error bars above the columns denote least significant difference ($LSD_{0.05}$).

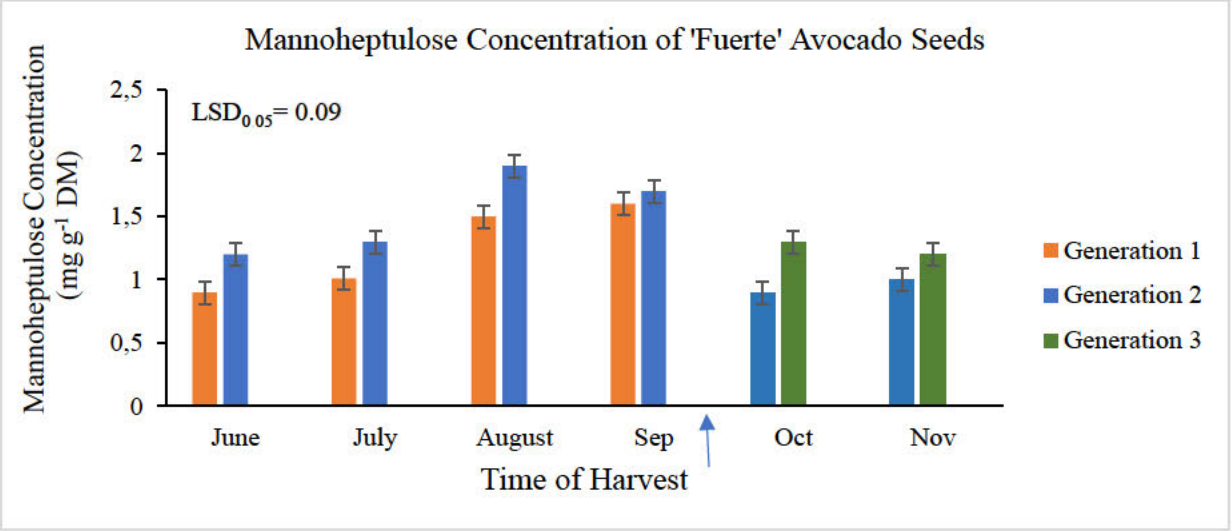


Figure 6b. Mannoheptulose concentration in 'Fuerte' an avocado seed over three generations: Generation 1 (full bloom in June/July 2017), Generation 2 (full bloom in June/July 2018) and Generation 3 (full bloom in June/July 2019). The arrow indicates the start of a new season with Generation 1 removed from the tree and a new generation (Generation 3) present on the trees. Error bars above the columns denote least significant difference ($LSD_{0.05}$).

3.3.4.4. **Perseitol Concentration in Avocado Seeds**

The C7 sugars perseitol and mannoheptulose were the dominant sugars in 'Hass' and 'Fuerte' avocado seeds. Perseitol was the most-prominent sugar in both cultivars, with concentrations exceeding that of mannoheptulose and the measured C6 sugars (Fig. 4 to 7). There was a slight difference between generations in perseitol concentration present in 'Hass' and 'Fuerte' seeds (Fig. 7a and 7b), with lower perseitol concentrations in Generation 1 seeds than in Generation 2 seeds from June to September. Generation 2 seeds had lower perseitol concentrations than Generation 3 in both cultivars from October to November (Fig. 7a and 7b). The highest concentration of perseitol was detected in 'Hass' seeds Generation 2, reaching 9.8 mg g^{-1} DM in September, with the lowest concentration in Generation 1 seeds (6.9 mg g^{-1} DM) in October (Fig. 7a). Generation 1 'Hass' seed had, similar to 'Fuerte' lower perseitol levels than Generation 2 from June to September, similarly from October to November Generation 3 seeds had greater perseitol concentrations than Generation 2 (Fig. 7a and 7b).

In Generation 1 'Fuerte' seeds slightly lower perseitol concentrations were detected than in Generation 2 seeds. From June to September, there were small differences in perseitol concentration between generations from October to November. Generation 3 had higher perseitol concentrations than Generation 2 (Fig. 7b). The highest concentration of perseitol in 'Fuerte' Generation 2 seed was 10.3 mg g^{-1} DM in September, with the lowest amount as 6.8 mg g^{-1} DM) in October (Fig. 7b). In Generation 1 the highest amount of perseitol (9.1 mg g^{-1} DM) was found in September and the lowest amount was found in October (6.8 mg g^{-1} DM; Fig. 8b). Altogether, there were significant differences in perseitol concentration between months and seed generations ($P < 0.001$).

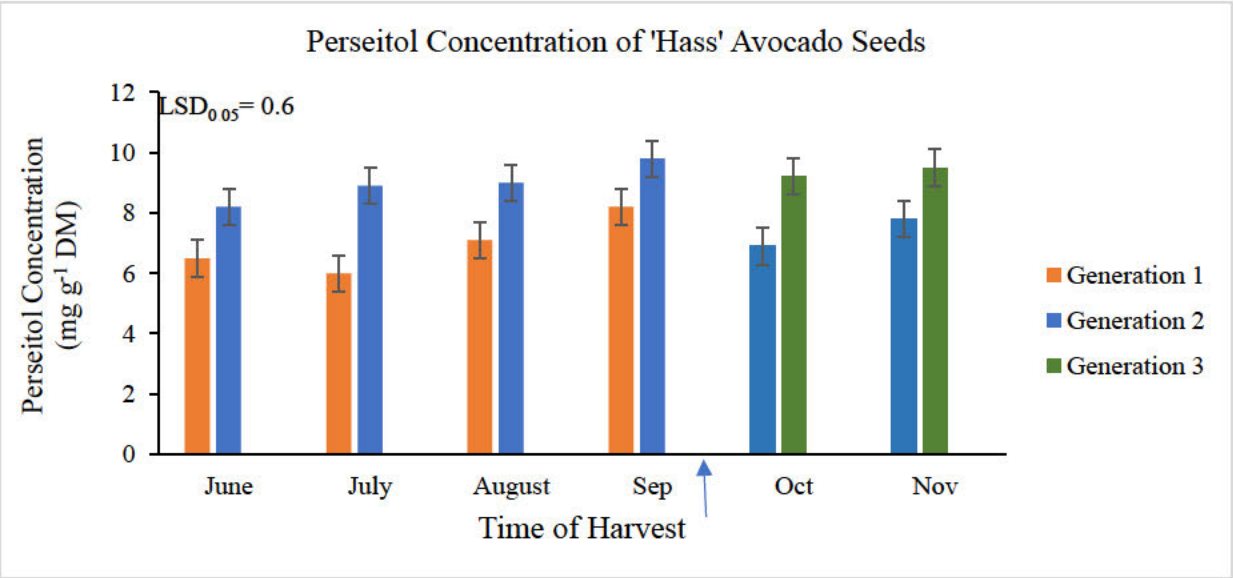


Figure 7a. Perseitol concentration in 'Hass' avocado seeds over three generations: Generation 1 (full bloom in July/August 2017), Generation 2 (full bloom in July/August 2018) and Generation 3 (full bloom in July/August 2019). The arrow indicates the start of a new season with Generation 1 removed from the tree and a new generation (Generation 3) present on the trees. Error bars above the columns denote least significant difference ($LSD_{0.05}$).

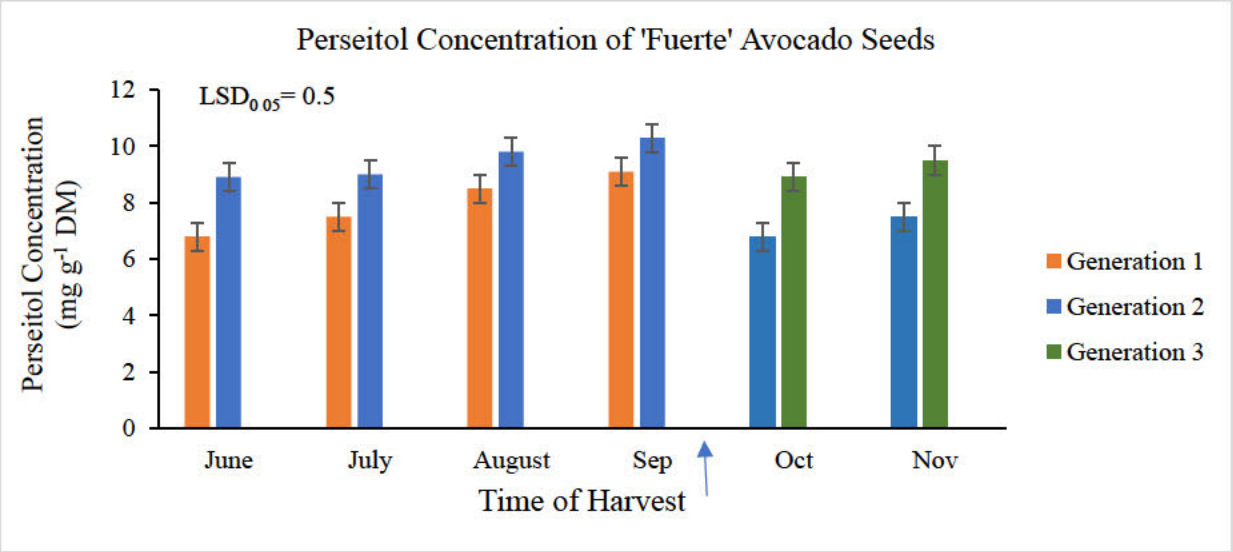


Figure 7b. Perseitol concentration in 'Fuerte' avocado seeds over three generations: Generation 1 (full bloom in June/July 2017), Generation 2 (full bloom in June/July 2018) and Generation 3 (full bloom in June/July 2019). The arrow indicates the start of a new season with Generation 1 removed from the tree and a new generation (Generation 3) present on the trees. Error bars above the columns denote least significant difference ($LSD_{0.05}$).

3.3.5. **Starch Concentration in Avocado Seeds (Cotyledons)**

Very high starch concentrations (88.8% and 90.5% DM) were found in all avocado seeds in 'Hass' and 'Fuerte' (Fig. 8a and 8b, respectively), youngest seeds had the highest starch concentration than older seeds. Generation 1 (24 to 28 MAFB) had lower starch concentrations than Generation 2 seeds (12-18 MAFB) from June to September. The highest starch concentration in Generation 1' 'Hass' seed was detected in August (69%), while the lowest starch concentration in this generation was present in June (52.3%). Starch concentrations in Generation 1 were relatively low in June and increased till August, declining towards September (Fig. 8a). In Generation 2 the highest starch concentration in 'Hass' seed was found in Generation 2 (12 to 18 MAFB), as well as in Generation 3 in October (0 to 6 MAFB). From October to November the starch concentration was higher in Generation 3 than in Generation 2, 12 to 18 MAFB (Fig. 8a and 8b). Generation 3 'Hass' seeds had, similarly, higher starch concentrations than Generation 2 (Fig. 8a).

In 'Fuerte' seeds the highest starch concentration in Generation 1seeds (26 MAFB) was found in August (70.2%), while the lowest starch concentration was present in September (27 MAFB, 58.2%, Fig. 8b). In 'Fuerte' seeds the starch concentration was highest in August (90.5%) for Generation 2 (12-17 MAFB). Starch concentrations in Generation 2 seeds ranged between (90.5 to 52.8%) in August and November, respectively (Fig. 8b). Generally, starch concentrations of both cultivars were low in June, increasing until August and then declined over the subsequent seed's development (Fig. 8a and 8b).

Concentrations of starch in 'Fuerte' seeds were lower in Generation 1 compared with Generation 2 from June to September (Fig. 8b). In October and November there were significant differences between Generations 3 and Generation 2, with Generation 3 (0 to 6 MAFB) having higher starch concentrations than Generation 2 in both cultivars. Generally, seeds starch concentrations were higher than those of soluble sugars; there was a significant difference between seeds starch concentration and seeds sugars in 'Fuerte' ($P = 0.043$) over the entire observation period. Similarly, 'Hass' seeds had higher starch than C6 and C7 sugar concentrations during the observed period ($p = 0.049$). In this experiment, starch concentration was significantly ($P < 0.001$) affected by cultivar and sampling month. Statistically, there were significant differences in starch concentrations between generations over the time for seeds of both cultivars ($P < 0.001$).

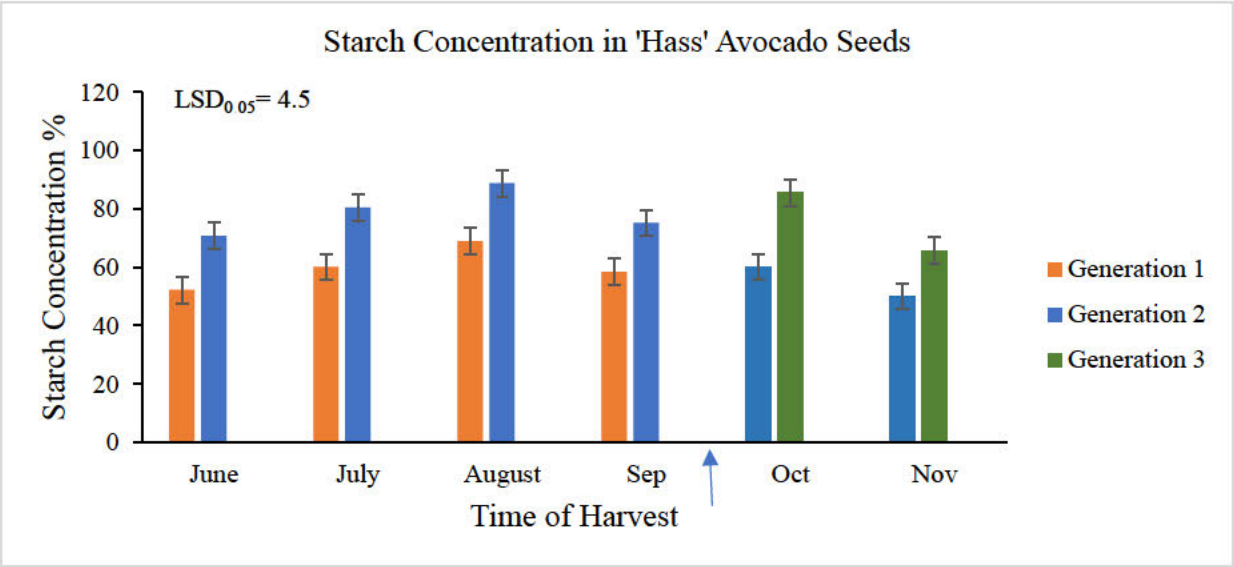


Figure 8a. Starch concentration (% DM) in 'Hass' an avocado seed over three generations: Generation 1 (full bloom in July/August 2017), Generation 2 (full bloom in July/August 2018) and Generation 3 (full bloom in July/August 2019). The arrow indicates the start of a new season with Generation 1 removed from the tree and a new generation (Generation 3) present on the trees. Error bars above the columns denote least significant difference ($LSD_{0.05}$).

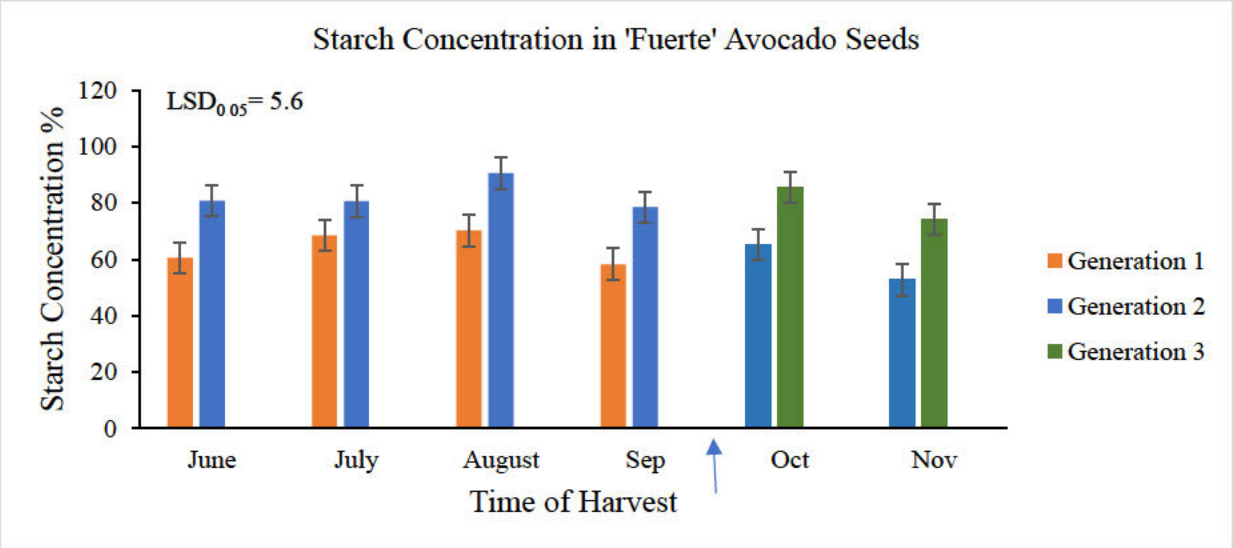


Figure 8b. Starch concentration (% DM) in 'Fuerte' an avocado seed over three generations: Generation 1 (full bloom in June/July 2017), Generation 2 (full bloom in June/July 2018) and Generation 3 (full bloom in June/July 2019). The arrow indicates the start of a new season with Generation 1 removed from the tree and a new generation (Generation 3) present on the trees. Error bars above the columns denote least significant difference ($LSD_{0.05}$).

3.4. Discussion

3.4.1. Avocado Seed Germination Percentage

Generally, germination of seed requires exposure to a warm environment with a high moisture content (Walck *et al*., 2001). Therefore, the avocado seeds begin germination, similar to any seeds in the plant kingdom, by water uptake in imbibition, followed by emergence of the radicle from the moistened and raptured seed coat (McGill *et al.*, 2018).

The germination rate of avocado seeds for both cultivars ('Hass' and 'Fuerte') was higher in seeds of Generation 2 (12 to 18 MAFB), from June to September, compared with Generation 1 (24 to 28 MAFB, Fig. 1a and 1b). When comparing cultivars, 'Hass' seeds showed a greater germination percentage than 'Fuerte' seeds ($P < 0.001$). It was also noticed that from October to November avocado seeds of Generation 2 (12 to 18 MAFB) showed a lower germination percentage than the Generation 3 seeds (0 to 6 MAFB, Fig. 1a and 1b). This could have possibly been due to the higher phenolic concentration in the seed coat of Generation 1 seeds; age might have also damaged enzymes involved in germination (Singh *et al.*, 2017) Seemingly, C6 sugars, more so than C7 sugars, are important in the early development of the seed/ seedling (Fig. 1a and 1b), confirming findings by Tesfay *et al*. (2012). In addition, in both cultivars, Generation 2 seeds showed higher germination percentages than Generation 1 until September; thereafter, when the seeds had reached maturity, the germination percentage declined. These findings agree with Blumenfeld and Gazit (1974), who reported that when the avocado seed has reached maturity, the germination rate decreases. Mature avocado seeds seemed to have been exposed to oxidation, which could have resulted in an oxidative destruction of compounds necessary for germination, ultimately resulting in the lower germination of older seeds (Olaeta *et al*., 2007).

In both cultivars, there were significant differences in seed germination between generations throughout the observation period ($P < 0.001$). Younger seeds seemed to be able to germinate better than older seeds, Generation 1 seeds had a lower germination rate than Generation 2 and Generation 3 seeds performed better than Generation 2 seeds (Fig. 1a and 1b); similarly, in all three generations, seeds from the younger generations germinated better than from those of the older generations. This was true over all three generations. Blumenfeld and Gazit (1970) reported on the influence of seed on fruit development. These authors stated that the high cytokinin activity present in the seed coat, during the early stages of fruit development, enhanced germination. The

seed coat had a high cytokinin activity during early stages of maturity and enzyme activity decreased when seeds approached maturation. Our findings agree with the above-mentioned authors, as younger seed generations were very active (germination capacity according to the TTZ test, Fig. 2a and 2b) with a greater germination percentage in Generation 2 seeds compared with the older Generation 1. Blumenfeld and Gazit (1974) reported that enzyme activity decreases during avocado seed maturation, and the seed coat is in control of germination by transporting soluble compounds between the embryo and the pericarp. These authors' investigations agree with the presented study, as, in both cultivars, older seeds had a lower germination percentage than younger seeds (Fig. 1a and 1b). As the seed coat of both cultivars was dead in older seeds (Fig. 3a and 3b, see 2.3.3), it had lost its capability of providing a link between seed and mesocarp. This agrees with previous studies that the avocado seed coat is a mechanical barrier for water absorption into the seeds; hence, its presence slows down the germination process (Whiley and Whiley, 2005).

Starch is the major carbohydrates source for germination; seeds with higher germination percentage (young seeds in October) had already accumulated significant starch amounts that seemed able to drive germination (Fig. 1a, 1b and Fig. 8a, 8b). Germination was better in younger seeds which had higher starch concentrations. Old seeds did not have sufficient starch reserves to drive a fast germination.

3.4.2. **Viability of Avocado Seeds**

The viability test performed on seeds of 'Hass' and 'Fuerte', is a biochemical test that measures the effect of respiration, whereby the tetrazolium (TTZ) salt (2, 3, 5-triphenyl tetrazolium chloride) is changed into a coloured product, formazan, staining the entire seeds tissue red. Results of this seed viability test are commonly expressed as the proportion of stained seed tissue (Tekrony and Egli, 1997). Copeland and McDonald (1995) reported that the TTZ test can be successfully used to distinguish between healthy seeds, staining the seed tissue completely and abnormal seeds, staining it only partially.

Avocado seed viability of the older generation was found to be consistently lower when compared with the younger generation (Fig. 2a and 2b). This is in line with findings on the germination percentage, using the TTZ test, of Jatropha (*Jatropha curcas*) seeds (Parreño-de Guzman *et al.*,

2011), also an oil-containing seeds of a perennial species, where seed viability was moderately higher in younger compared with older seeds.

Seeds of both cultivars had a higher viability percentage in Generation 2 seeds than in Generation 1 seeds, from June to September (Fig. 2a and 2b). Seeds with high viability (TTZ test) also showed higher germination percentage compared with seeds of lower viability. There, hence, was a positive correlation between seed viability and germination percentage ($r = 0.85$); (Fig. 1a, b and 2a, b). From October to November, a higher viability of both cultivars in Generation 3 seeds was again aligned with a higher germination rate. The results of the current study agree with the results by Mangena and Mokwala (2019) that poor/lower seed viability is associated with lower seed germination. In this study, lower seed viability was mirrored by lower seed germination rates and higher seed viability was aligned with a higher germination percentage (Fig. 1a, 1b; Fig. 2a, 2b).

Analysis of variance of the relationship between seed viability and seed generation (for both cultivars) showed statistical significance for 'Hass' ($P = 0.75$) and 'Fuerte' seeds ($P = 0.84$). Seed viability differed between generations, both cultivars had a higher seed viability in Generation 2 than Generation 1, from June to September, while from October to November seeds were more viable in Generation 3 (Fig. 2a and 2b). The results from both cultivars, at different stages of maturity, showed that viability decreased gradually with maturity, and, at maturity, seeds were characterized by lower viability and a lower germination rate than at the early seed development stage. These differences in seed viability and germination percentage could be explained by the higher viability of younger compared with older seeds and ae characteristic for recalcitrant seed (Berjak and Pammenter, 2013).

Seed germination and viability largely depend on the availability of resources, such as carbohydrate reserves, nutrients and water. There were significant differences with respect to seed viability between the cultivars and seed generations ($P < 0.001$, Fig. 2a and 2b). It is, therefore, likely that the reduced amount of starch and of the C7 sugar perseitol in older seeds (Fig. 8a, 8b and Fig 7a, 7b), was due to these carbohydrate reserves being used up to maintain the metabolism of the embryo. Seeds also differed in moisture content (Fig. 3a, 3b), with the lower moisture content of older seeds possibly resulting in physical dormancy, as Baskin (2003) reported that, generally, seeds of lower moisture content are prone to such physical dormancy.

3.4.3. **Respiration Rate of Avocado Seeds**

The respiration rate of avocado seeds decreased during development. Seed respiration of both cultivars, 'Hass' and 'Fuerte', declined rapidly in seeds from Generation 2 (June to November), with Generation 1 seeed respiration remaining consistently lower than that of seeds from Generation 2 (Fig. 3a and 3b). Zauberman and Schiffmann (1972) found, similarly, that younger avocado seeds had a higher respiration rate than older ones. The contribution of seed respiration to entire fruit respiration is, therefore, also higher in young fruit and was found to decrease with fruit development over time (Fig. 3a and 3b) The detected change in avocado seed respiration between seed generations, therefore, confirms findings on changes in fruit respiration throughout fruit development (Zauberman and Schiffmann (1972). In this study, in both cultivars, the seed respiration rate of Generation 2 was lower than that of the youngest Generation 3 from October to November (Fig. 3a and 3b). Miller *et al*. (1983) found, similarly, that older soybean seeds had a lower respiration rate than younger ones.

Generally, the respiration rate of seeds and their O_2 consumption are excellent indicators of an active metabolism, aligned with seed germination; seeds with lower respiration rate do not germinate rapidly **(**Patanè *et al*. 2006). In the present study, Generation 1 avocado seeds had a lower respiration rate and a lower germination percentage than Generation 2 (Fig. 3a and 3b). Respiration rate is also a useful indicator, when evaluating seed vigour in different plants (Zhao and Zhong, 2012). Avocado seed respiration and germination percentage decreased in response to seed maturity in both cultivars (Fig. 1a, 1b and Fig. 3a, 3b), possibly due to reduced seed vigour. A positive correlation was observed between respiration rate and germination percentage in younger avocado seeds, Generation 2 and Generation 3 seeds had a higher respiration rate and a higher germination percentage than Generation 1 and Generation 2, respectively. Dahal *et al.* (1996) similarly reported that there is, generally, a positive correlation between germination percentage and respiration rate in tomato seeds (*Solanum lycopersicum*) during seed development.

Analysis of variance showed a significant difference in seed respiration rate between cultivars, and there, also, was a significant difference in seed respiration between months of a specific generation ($P < 0.001$). Zauberman and Schiffmann (1972) stated that the high avocado seed respiration rate after harvest was not a result of the separation of the seed from the tree. The

respiration rate was generally slightly higher in younger seeds than in older seeds. Similarly, in this study, younger seeds of both, 'Hass' and 'Fuerte', were also characterised by a higher respiration rate (Fig. 3a and 3b).

3.4.4. **Sugars Concentration in Avocado Seeds (Cotyledons)**

During seed development of 'Hass' and 'Fuerte' seeds, four major sugars were detected, the C7 sugars perseitol and mannoheptulose, as well as the common C6 sugars (glucose and sucrose) (Fig. 6, 7 and Fig. 4, 5). Amongst these, sucrose is the dominant C6 sugar, while perseitol is the dominant C7 sugar. Tesfay *et al.* (2012) postulated that the C7 sugars are the most important carbohydrate players in seed and fruit development, while the present results show sucrose to be present in higher amounts than mannoheptulose. Perseitol was, however, clearly the dominant free sugar in avocado seeds. Additionally, the findings of Tesfay *et al*. (2012), Liu *et al*. (2002) and Landahl *et al*. (2009) that mannoheptulose and perseitol exist in older and younger avocado tissues (in seed, mesocarp and exocarp) were confirmed. The present results also clearly demonstrate seed age affects the seed sugar concentration, with C6 sugars present in higher concentration in older seeds and C7, particularly perseitol, being higher in younger seeds.

Soluble sugars and starch are the predominant storage and reserve carbohydrates available for plant growth, energy and maintenance (Daie, 1985; Dey and Dixon, 1985). Liu *et al.* (2002), as well as Tesfay *et al.* (2012), suggested that avocado plants use perseitol mainly as a storage carbohydrate, while the purpose of mannoheptulose is to transported C7 sugars in the plant.

Thus, despite perseitol and mannoheptulose being dominant sugars in avocado flowers, fruit and leaves (Tesfay *et al.*, 2012), only perseitol was found in large amounts in seeds (Fig. 7a and 7b). Mannoheptulose is, possibly, only the transport vehicle of C7 sugars, being converted into the C7 storage form, perseitol, in the seeds. Liu *et al.* (2002) also detected both C7 sugars in avocado fruit, while Woldu and Tsigie (2015) found both C7 sugars in avocado seeds.

Sugars in avocado tissues are known to play an important role in source-sink relationships or as sugar reserves (Tesfay *et al*., 2012), being transferred through vascular tissues (Liu *et al*. 2002). Liu *et al*. (1999) reported that, in avocado plants, the direction of carbohydrate transport depends on carbohydrate use and mobilization. The C7 sugars could play a vital role in carbon allocation

processes in tissues and, hence, in sink establishment. This could explain why high concentrations of C7 sugars were found in younger seeds, where a rapid rate of cell division results in high demands for energy to fuel seed metabolism and enzyme activities. All avocado seeds contained sucrose and perseitol, whether the seeds were old or young, with the highest perseitol concentration found in the younger seeds; these seeds consisted predominantly of the cotyledons. Glucose and mannoheptulose concentrations, on the other hand, were found to be low in avocado seeds (Fig. 5a, 5b and Fig. 6a, 6b). Young, developing avocado seeds were seemingly supplied with C7 carbohydrates, particularly perseitol, as well as C6, particularly sucrose. Similarly, the C6 storage carbohydrate, starch, increased in concentration during seed development. Sugar metabolism in the seed *versus* the mesocarp seems to differ, as C7 sugars, particularly mannoheptulose, are present in high amounts in the mesocarp (Tesfay *et al*., 2012). The C6 sugar sucrose is present in amounts similar to perseitol in the seeds (Fig. 4a, 4b and 5a, 5b 5 *versus* Fig. 6a, 6b, and 7a,7b). The seeds also contain large energy resources in the form of starch (Fig. 8a and 8b), probably fuelling germination together with perseitol. This indicates, that in avocado seeds the C6 as well as the C7 sugar metabolism are in operation.

Tesfay *et al*. (2012) confirmed that *D*-mannoheptulose is the predominant sugar in the avocado exocarp, while the seeds contained less *D*-mannoheptulose; moreover, these authors reported perseitol to be present at the lowest concentration in the avocado mesocarp, however, it is the dominant sugar in avocado seed tissue. These findings agree with the current study, as mannoheptulose was present at low concentrations in avocado seeds of both cultivars (Fig. 6a and 6b), while perseitol concentrations were higher than those of other sugars in both, 'Hass' and 'Fuerte' seed tissues (Fig. 7a and 7b). The soluble sugar profile found in avocado seeds (Fig. 4 to 7) revealed sucrose, *D*-mannoheptulose and glucose to be present at lower levels, while perseitol was found at a higher concentration. In both cultivars, younger seeds had a higher germination percentage than older seeds (Fig. 1a and 1b); the C7 sugar concentrations followed the same tendency, with higher amounts of such sugars in younger seeds than of C6 sugars (Fig. 6a, 6b and Fig. 7a, 7b), indicating that perseitol, and possibly starch, drive germination.

The presence of C7 sugars in different tissues of avocado fruit and seeds seems to indicate that C7 sugars can be synthesized in various avocado tissues (Liu *et al*., 1999). Mesocarp and seed sugars (C7 and C6) declined when fruit reached maturity; however, mannoheptulose remained

higher in mesocarp than in seed tissue, while the seed perseitol concentration reached lower levels when seeds approached maturity (Tesfay *et al*., 2012). Low seed perseitol levels in older seeds were aligned with a lower germination percentage of such seeds (Fig 1a, 1b and Fig. 7a, 7b), indicating the importance of perseitol in this process. Seemingly the sugar metabolism of the seeds uses different pathways to the mesocarp, with sucrose being supplied to the seeds from the mesocarp to the seeds (Cripps and Cowan, 2000), but seeds are mainly operating a C6 metabolism, containing high amounts of sucrose and starch (Fig. 5a, 5b and Fig. 8a, 8b).

The two dominant C7 sugars, perseitol and mannoheptulose, were found to be present in all older and younger avocado seeds, confirming reports by Liu *et al*. (2002) and Landahl *et al*. (2009). The dominance of perseitol, among the soluble sugars, in the avocado cotyledons has been reported at physiological maturity of the avocado (Tesfay *et al*., 2012). Figure 7a and 7b demonstrate that the perseitol concentration in the cotyledons does not fluctuate significantly from month to month, even differences in C7 sugar concentration between the three generations of seeds were not significant. The importance of the typical avocado sugar, perseitol, was previously reported by Liu *et al.* (2002), and its presence as the major soluble sugar in avocado seeds has been confirmed (Fig. 7a and 7b); levels of this sugar were relatively high in 'Hass' and 'Fuerte' seeds over the entire observation period. These results imply that seeds/ cotyledons store perseitol, as in both cultivars seed tissue contained higher amounts of this sugar than of any other analysed sugar (Fig. 4 to 7).

3.4.5. **Starch Concentration in Avocado Seeds (Cotyledons)**

Starch isolated from avocado seeds can be characterised as a light-brown powder with a characteristic scent and a smooth texture (Chel *et al.,* 2016). Avocado seeds are a potential starch source, reportedly containing around 30% to 74% starch, depending on cultivar (Olaeta *et al*., 2007; Orhevba and Jinadu, 2011). In this study, the seed starch concentration ranged between 52.3 and 88.8 % in Generation 2 from 'Hass', while Generation 1 seeds recorded a starch percentage between 52.8 and 69% (Fig. 8a). Similarly, 'Fuerte' seeds (Generation 2) ranged in starch percentage between 52.0 and 90.5%, while it ranged in the Generation 1 from 58.2 to 70.2% (Fig. 8b). This indicates that younger seeds have the potential to accumulate higher starch amounts than older seeds. These findings agree with Orhevba and Jinadu (2011), as well as with

Rivera-González *et al*. (2019), that the avocado seed is a starch-rich tissue; therefore, it could be potentially used as a commercial starch source.

The highest starch concentration was detected in June (90.5%) (Fig. 8b) in Generation 2 'Fuerte' seeds, while the highest amount of starch in 'Hass' seeds was detected in August (88.8%), both in Generation 2 (Fig. 8a). The fact that 'Hass' is a later-maturing cultivar than 'Fuerte' implies that the timing of the "starch peak" in seeds could be an indication of the time of fruit maturity. The amount of starch was relatively higher in Generation 2 seeds for both cultivars than in Generation 1 seeds. This indicates that this starch can be used as a carbon source in seeds to produce free sugars, as the avocado seeds can contain more than 80.1% starch (Fig. 8a and 8b; Maryam and Kasim, 2016).

Statistical analysis showed starch concentrations and germination percentage to have a significant, positive correlation ($r = 0.80$, $P < 0.001$), and Generation 3 seeds were characterised by a higher starch concentration aligned with a higher germination percentage (Fig. 1a, 1b and 8a, 8b). There was a strong, positive correlation between seeds starch content and germination rate ($r = 0.80$).

During the avocado seed growth period, the younger seed derives its energy requirements from seed carbohydrate reserves, found, particularly, in the cotyledons (Chong *et al*., 2002). The C6 carbohydrates were present in significant amounts in Generation 1 avocado seeds, while perseitol and mannoheptulose were found at lower concentrations. In Generation 2 seeds, however, perseitol and sucrose were present in high concentrations (Fig. 7 and 4); similarly, the storage carbohydrate starch was predominantly detected in Generation 2 seeds. Starch concentrations decreased with physiological maturity (Fig. 8a and 8b); older seeds had lower starch concentration than newer seeds. These findings agree with reports by Liu *et al*. (1999) that the seed starch concentration is higher in younger seeds.

Liu *et al*.'s (1999) findings are similar to the present study, in that younger seeds had a higher starch concentration and perseitol was the dominant sugar in such seeds (Fig. 7 and 8). This suggests that starch, as well as perseitol, are indeed avocado storage carbohydrates, accumulating in sink/ or reserve tissues. This role is further confirmed by the drop in perseitol and starch concentration in seed cotyledons during the early germination process (Tesfay *et al*., 2012).

Tesfay *et al*. (2012) further reported that the avocado embryo contained equal of amounts of fructose, glucose, sucrose, perseitol and *D*-mannoheptulose, probably to assist the seed in germination. The first carbohydrates for germination are released through the breakdown of starch. All these soluble carbohydrates, present in avocado seeds, assist in seed germination; therefore, immature/ younger seeds had higher levels of these carbohydrates than mature seeds, aligned with a higher germination percentage of young seeds. This also confirms that stored starch is produced in high amounts in the cotyledons (Schnarrenberger and Oeser, 1974), making avocado seeds a potential, alternative carbohydrate source for starch powder (Maryam and Kasim, 2016).

3.5. Conclusion

The avocado seed germination percentage is affected by seed generations and cultivar; however, germination of the older Generation 1 was lower than that of seeds from the relatively younger Generation 2. The youngest seeds (G2 and G3) of both cultivars contained more than 90% starch during early development. A deeper investigation into germination percentage-related parameters (cotyledon sugar and starch concentrations) did relate these carbohydrates to germination. Seed viability was also related to germination, as higher and lower viability resulted in higher and lower germination, respectively; therefore, avocado seed germination in relation to seed viability percentage should be investigated.

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CHAPTER FOUR

SEASONAL ALTERATIONS IN POLYPHENOL OXIDASE ACTIVITY AND PHENOLIC COMPOUNDS OF THE AVOCADO SEED COAT OVER SEVERAL SEEDS GENERATIONS

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Abstract

The avocado seed coat is an important tissue of avocado seeds, with functional properties including the protection of the seed from pests and diseases. The objectives of the present investigation were to determine the enzymatic activity of polyphenol oxidase (PPO) and the concentration of phenolic compounds present in the avocado seed coat, as PPO and phenolics can provide plants a protection against pathogens and general adverse environmental conditions. This study analysed phenolics and PPO activity in seed coat tissue, in an attempt to correlate these phenolic characteristics with seed germination during seed development. The polyphenol oxidase (PPO) activity and phenolic concentration in the avocado seed coats of two avocado cultivars ('Hass' and 'Fuerte') from Generation 1, Generation 2 and Generation 3 seeds were investigated from June to November. Generation 1 'Hass' seed coats had higher PPO activity than those of Generation 2 from June to September, while, as the seasons progressed (October to November) PPO activity was lower in Generation 3 than Generation 2 seed coats. Generation 2 'Hass' seed coats had a low PPO activity in the first two months (June /July), but the activity increased thereafter and remained at a higher-level during October and November. Generation 1 'Fuerte' seed coats had a similar PPO activity during all observed months. Phenolic compounds in the seed coat of Generation 1 seeds exhibited higher phenolic concentrations than those of Generation 2 seeds during the entire observation period. Results showed a positive correlation ($r = 0.42$, $P < 0.001$) between phenolic compound concentration and PPO activity in the seed coat. It can, therefore, be concluded that, as the seeds progresses through the season, PPO activity remains similar, while the concentration of phenolic compounds increases in Generation 1 avocado seed coats, therefore, possibly inhibiting germination. In Generation 2 seed coats PPO activity and phenolic compound concentrations were lower than in Generation 1. Monthly germination percentages of Generation 2 seeds were higher than those of Generation 1 seeds, possibly due to the lower phenolic concentration and the lower PPO activity in the seed coat of Generation 2 seeds. In both cultivars, from October to November, Generation 2 seed coats had higher level of PPO activity and phenolic compound than Generation 3 seed coats. Seemingly, older generations always had greater amount of PPO and phenolic concentrations in the observation period. Generation 3 seeds had a higher germination rate than Generation 2 seeds, possibly because of lower PPO activity and phenolic concentration in these seed coat. In conclusion, the higher phenolic concentration and higher PPO activity in the Generation 1 avocado seed coats of both

cultivars could have contributed to inhibiting seed germination. Generation 2 seed coat had a lower phenolic concentration, combined with lower PPO activity, thereby enhancing germination.

Keywords: Polyphenol oxidase, phenolic compounds, 'Hass', 'Fuerte', germination, seed coat, generations, avocado

4.1. Introduction

Phenolic compounds are substances commonly occurring in plants and are characterised by an aromatic ring with one or more hydroxyl groups. It is estimated, that about 8000 phenolic compounds exist in the plant kingdom (Marinova *et al.*, 2005); amongst these are simple phenols, tannins, coumarins, phytosterols, flavonoids, lignins, lignans as well as benzoic and cinnamic acid (Ding *et al*., 2007; Leite *et al.*, 2009; Khoddami *et al.*, 2013). Phenolic compounds have wide biochemical activity and function, such as to act as antioxidants and to modify gene expression in plant cells (Sulaiman and Balachandran, 2012). Aliyu *et al.* (2009) and Okpuzor *et al.* (2009) confirmed that phenolics are the largest phytochemical group contributing to the plant's or plant parts' antioxidant activity. As the largest group of phenolics, flavonoids play an important role as antioxidants (Kähkönen *et al.*, 1999); this is due their ability to scavenge free radicals, such as the reactive oxygen species (ROS), the hydroxyl radical and the superoxide radical. Recently, phenolic compounds have attracted the attention of researchers, as they can protect humans from excessive ROS exposure, thereby reducing the risk of many diseases (Halliwell, 1996).

Phenolics are a group of secondary metabolites synthesized via the shikimate and phenylpropanoid pathway, in response to certain ecological or physiological stimuli (Tomas and Espin, 2001; Dicko *et al*., 2002; Balasundram *et al*., 2006). Phenolics are important compounds that inhibit oxidation and reduction reactions in plant cells. Environmental conditions play a very important role in affecting the phenolic concentration in fruit, vegetables, and seeds. Soil composition (organic matter, presence of minerals) and water/ irrigation also have an effect on the phenolic concentration and the PPO activity of plants and/ or plant products. A high concentration of phenolic compounds can also cause physiological disorders during plant development (Tomas and Espin, 2001). As major enzymes involved in chemical reactions, PPOs and their activity and concentration can change during fruit maturation, particularly influencing enzymes involved in the quality of products (Thé *et al*., 2001).

In avocado, higher levels of phenolics and phenolic enzymes are present in the seeds than in the avocado pulp; phenolic compounds have, reportedly, excellent health benefits (Soong and Barlow, 2004). Phytochemical products, particularly present in the avocado seed coat, are antidiabetic, insecticidal, and possess blood pressure-reducing function (Anaka *et al*., 2009; Imafidon and Okunrobo, 2009).

The phenolic concentration also varies with cultivar, stage of maturity and environmental condition (Tesfay *et al.,* 2010; Wang *et al.,* 2010). Some phenolic compounds are highly toxic, adversely affecting certain living organisms and being involved in growth, pigmentation and pathogen resistance (Davidenko *et al*., 2004; Lattanzio *et al*., 2008).

Polyphenol oxidases (PPOs) are one the most-important biomolecules in avocado seeds, catalysing phenolic oxidations. Generally, seeds have an enzyme (PPO)-based, biochemical defence system present in the seed coat. This defence system against phenolic oxidation represents the main mechanism of seed longevity and seed survival (Fuerst *et al.,* 2014). A positive correlation between phenolic concentration and PPO activity has been reported in crocus (*Crocus sativus*) seeds (Esmaeili *et al*., 2017); seasonal alterations in phenolic concentrations have been studied in various seeds, including apple (Burda *et al.,* 1990). Season and developmental stage can affect the activity of PPO enzymes, as well as the accumulation of phenolics in plants. Various PPO enzymes are part of a plant's defence mechanism, providing barriers against pathogen and insect attacks. Phenolics and PPOs not only scavenge free radicals but are also involved in the biosynthesis of other compounds that are part of the enzymatic browning reactions in injured fruit tissue (Constabel *et al*., 2000; Minatel *et al*., 2017).

Polyphenol oxidases are present in the thylakoids of chloroplasts (Mayer and Harel, 1979). These enzymes are commonly present in vegetables and fruit and are important in these industries. As PPOs are responsible for browning reactions following tissue injury (Chazarra *et al*., 1997), their activity results in tissue browning (Martin-Diana *et al*., 2005). Van Rooyen and Bower (2003) reported that the pre-harvest concentration of phenolics and the activity of PPOs determine the browning potential of avocado mesocarp.

Avocado seeds have a higher PPO concentration than the avocado pulp (Soong and Barlow, 2004; Wang *et al*., 2010). During the process of avocado fruit maturation, PPO enzyme activity varies.

The activity of the enzyme is higher in the early maturation stages; similarly, immature seeds are also characterized by a higher PPO enzyme activity than mature seeds (Vanini *et al*., 2010).

Phenolic compounds may also contribute to certain functions; such compounds include anthocyanins and other flavonoids, providing colour or phenolic acids, altering taste (Santiago *et al*., 2000; Boudet 2007; Khoddami *et al.*, 2013). The reduction in phenolic concentration during fruit development may be due to oxidation of phenolics by PPOs (Dabas *et al*., 2011).

Polyphenol oxidases (PPOs) are produced in the thylakoid membranes and combine in the cytoplasm with the phenolic substrates; PPOs and phenolics are present in different cell compartments. Upon cell injury, membranes are broken down and allow the contact of PPOs with phenolics, resulting in browning (Bower and Cutting, 1988). Polyphenol oxidases (PPOs) catalyse the oxidation of phenolic compounds to quinones; this is the initiation of oxidative browning in plant tissues due to spontaneous polymerization of such quinones to certain brown pigments (Constabel and Barbehenn, 2008). These PPO enzymes catalyse both, the oxygen (O_2) -dependent hydroxylation of the monophenols or o-diphenols and the o-diphenols oxidation of o-quinones (Lin *et al*., 2010).

Due to the importance of phenolics and PPO in the development of avocado seeds, the objectives of the present study were, to determine the phenolic concentration and the PPO activity in seed coats of 'Hass' and 'Fuerte' avocados and to elucidate, whether there is a correlation between PPO activity and phenolic compound concentration and, if phenolic concentration and/or PPO activity are linked to germination of avocado seeds of various ages.

4.2. Materials and Methods

4.2.1. Seed Sampling

Seeds used in this study were extracted from fruit obtained from a commercial orchard in the KwaZulu-Natal Midlands (30°16-E and 29°28-S, South Africa). 'Hass' and 'Fuerte' avocado fruit of three generations (G1, G2, G3), with full bloom in June/July ('Fuerte') and July/August ('Hass') 2017, 2018, 2019, respectively, were collected from the same trees carrying, at one stage, up to three generations at one time. Fruit collected were of uniform appearance and size, characteristic for the generation. Samples were collected monthly from June to November.

Three generations of seeds were analysed: 'Hass' avocado fruit (termed Generation 1), collected over a six-month period from 24 to 29 months after full bloom (MAFB) until November 2019. These fruits were commercially harvested in August 2018. Additionally, the following year's fruit (full bloom in July/August 2018 (Generation 2)) were harvested 12 to 17 MAFB, commercially harvested in July/August 2019 were sampled. Thirdly, newly developing fruit, with full bloom in July/August 2019 (Generation 3, 0 to 5 MAFB) were collected. In 'Fuerte', similarly, three generations were collected: the oldest avocado seed Generation 1 (full bloom in June/July 2017, 24 to 29 MAFB), these fruits were commercially harvested in June/July 2018, Generation 2 (full bloom in June/July 2018, 12 to 17 MAFB), these fruits were commercially harvested in June/July 2019, and Generation 3 (full bloom in July 2019), these fruits were commercially harvested in June/July 2018. Generation 3 was removed from the tree (9 to 10 MAFB) prior to commercial harvest for both cultivars. All fruit were removed from trees and immediately transported to laboratory.

4.2.2. **Avocado Seed Extraction**

Harvested avocado fruit were kept at room temperature until fruit started to soften. Upon softening, avocado seeds were separated from the fruit. After the seeds were extracted from the mesocarp, the seed coat was peeled off the seeds. Seed coats were then oven-dried for 24h at 75°C.

Finally, the seed coats from each sample were ground into a fine powder, to be used for phenolic analysis. In the same material PPO analysis and phenolic analysis were carried out, according to Van Lelyveld *et al.* (1984).

4.2.3. **Determination of Polyphenol Oxidase Activity in Avocado Seed Coat Tissue**

Polyphenol oxidase (PPO) activity was assayed as described by Van Lelyveld *et al.* (1984) with modification by Tesfay *et al*. (2011). The crude enzyme extract (100 mL) was added to a mixture of 1.45mL 10mM acetate buffer (pH 5.0) and 1.45mL 20mM 4-methyl-catechol. The absorbance was recorded at 420nm against a reagent blank using a UV–Vis spectrophotometer SP 8001 (Metertech Inc, Taipei, Taiwan). Polyphenol oxidase activity was expressed as the change in optical density at 420nm (U PPO/kg^{-1} DM seed coat tissue).

4.2.4. **Determination of Phenolic Compounds in Avocado Seed Coat Tissue**

The phenolic concentration of avocado seed coats was determined using a Folin-Ciocalteau method described by Blainski *et al.* (2013). Ground samples (0.5g DM) were homogenized in 10mL aqueous ethanol (80%) at room temperature; then 5mL sample solution was kept for 30min at room temperature (25±1°C), before samples were filtered through Whatman No.1 filter paper. Thereafter, samples were centrifuged at 20 000*g* for 10min, the supernatant removed, and 2.5mL supernatant mixed with 0.1mL 0.2N Folin-Ciocalteau reagent (Sigma-Aldrich/Fluka, St Louis, MO, USA) for 5min. The reaction was terminated using 2.0mL 75g L^{-1} sodium carbonate solution. The sample mixture was then vortexed and incubated at room temperature (25±1°C) for 2h. The absorbance was read at 760nm against a reagent blank using a UV-Vis spectrophotometer SP 8001 (Metertech Inc., Taipei, Taiwan).

4.2.5. **Statistical Analysis**

Data were analysed by one-way analysis of variance and results were considered statistically significant at $P \leq 0.001$. Statistical analyses were made using GenStat (version 18.2; VSN International, Hemel Hempstead, UK). The values of all parameters were subjected to statistical analysis. Significant differences among treatments were evaluated by the least significant difference (LSD) test (Gomez and Gomez, 1984).

4.3. Results

4.3.1. **Polyphenol Oxidase (PPO) Activity in Avocado Seed Coat Tissue**

For both cultivars, ‘Hass’ and ‘Fuerte’, the activity of PPO appeared to increase slightly in Generation 1 seed coats from June to September compared with Generation 2. From October to November PPO was lower in Generation 3 seed coats than in those of the Generation 2. After October, PPO increased in Generation 2 seed coats, now being greater than that of the new seed Generation 3. Seed coats of Generation 2 contained higher PPO activity than those of Generation 3. This was the case for both cultivars (Fig. 1a and 1b).

The PPO activity in ‘Hass’ seed coats was higher from July to September in Generation 1 seeds (0.44 to 0.50 U/kg^{-1} DM) than in June; in the following months, PPO activity was higher in Generation 1 compared with Generation 2 seed coats (June to September). The new generation

of avocado fruit (Generation 3), present from October onwards, had a lower seed coat PPO activity than Generation 2 seed coats (Fig. 1a). The PPO activity in 'Hass' seed coats of Generation 2 increased from June to August (Fig. 1a). In 'Fuerte' seed coats the highest activity of PPO was present in Generation 1 in August and September (0.47 to 0.49 U/kg^{-1} DM) (Fig. 1b). From June to September, 'Fuerte' Generation 1 seed coats showed higher PPO activity than Generation 2 seed coats; however, there were younger, Generation 3 seeds, sampled from October to November and these 'newest' seed coats had a lower PPO activity than those of Generation 2 ($P < 0.001$; Fig. 1b).

In both cultivars, seed coats of Generation 1 had a higher PPO activity from June to September compared with the one-year-younger seed coats (Generation 2). The specific enzyme activity seemed to remain at a higher level in the older seed coats compared with the younger seeds (Figure 1a and 1b). For both cultivars, seed coat PPO activity differed significantly between generations and months ($P < 0.001$) (Fig. 1a and 1b).

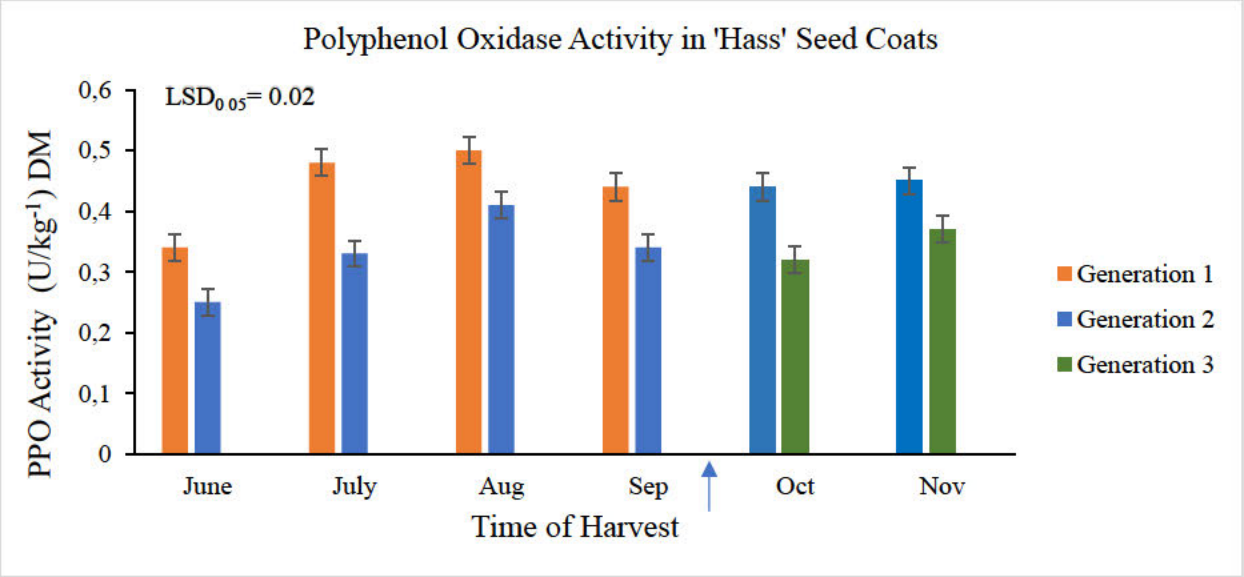

Figure 1a. Polyphenol oxidase (PPO) activity of the 'Hass' avocado seed coats from June to November; Generation 1 (full bloom in July/August 2017), Generation 2 (full bloom in July/August 2018) and Generation 3 (full bloom in July/August 2019). $LSD_{0.05}$ is least significant difference ($P \leq 0.001$). The arrow indicates the time, when the oldest fruit were collected, and a new generation (Generation 3) started to grow to harvestable size.

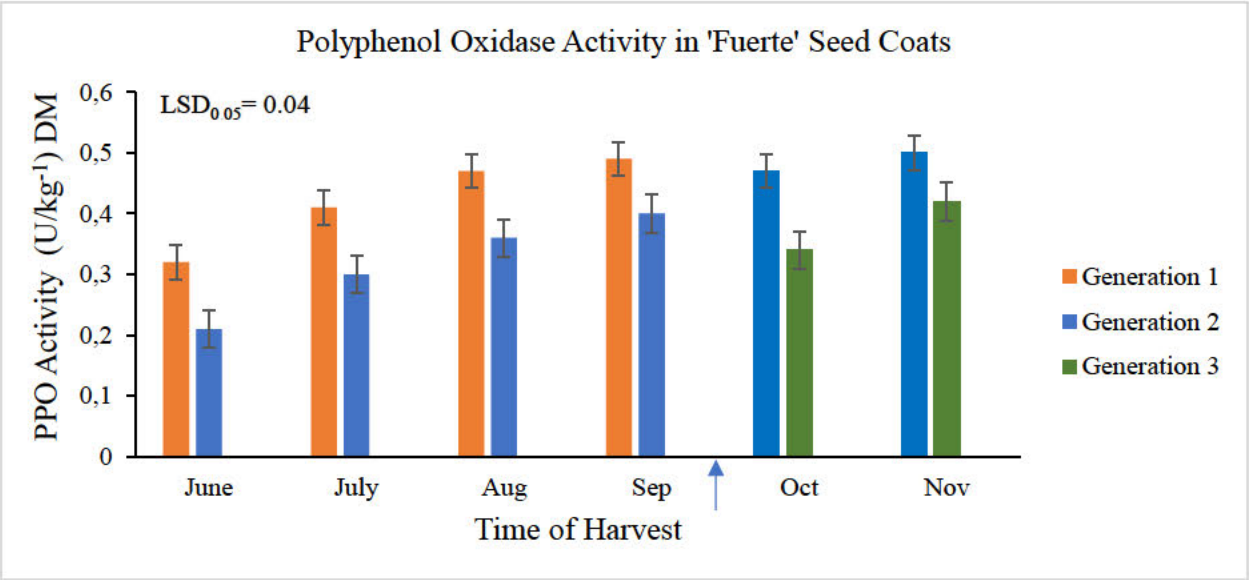

Figure 1b. Polyphenol oxidase (PPO) activity of the 'Fuerte' avocado seed coats from June to November; Generation 1 (full bloom in June/July 2017), Generation 2 (full bloom in June/July 2018) and Generation 3 (full bloom in June/July 2019). $LSD_{0.05}$ is least significant difference ($P \leq 0.001$). The arrow indicates the time, when the oldest fruit were collected, and a new generation (Generation 3) started to grow to harvestable size.

4.3.2. **Phenolic Concentrations in Avocado Seed Coats**

The concentration of phenolic compounds in 'Hass' and 'Fuerte' seed coats followed a pattern similar to PPO activity (Fig. 2a and 2b). The concentrations of phenolic compounds in the seed coats differed significantly ($P = 0.08$) between the two cultivars (Fig. 2a and 2b). Differences between generations of one cultivar were also significant ($P < 0.001$).

In both cultivars, the Generation 1 seed coat had higher phenolic concentrations compared with the Generation 2 seed coat from June to September; similarly, from October to November the phenolic concentrations were lower in Generation 3 seed coats than in the Generation 2 seed coats.

In 'Hass' seed coats of Generation 1, phenolic concentrations were highest in September (2.3 mg GAE* g^{-1} DM). The highest phenolic concentration in Generation 2 was found in October (2.5 mg GAE* g^{-1} DM) (Fig. 2a). In 'Fuerte' seed coats the phenolic concentration was highest in September (2.1 mg GAE* g^{-1} DM) in Generation 1. In Generation 2 seed coats, the highest phenolic concentration was detected in November (2.0 mg GAE* g^{-1} DM) (Fig. 2b), coinciding

with the maturation of the seeds. The lowest concentrations of phenolic compounds was found in 'Hass' seed coats of Generation 2 in June (1.2 mg GAE* g^{-1} DM) and in November of Generation 3 (1.2 mg GAE* g^{-1} DM) (Fig. 2a); similarly, the lowest phenolics concentrations in 'Fuerte' seed coats were detected in June and July in Generation 2 seed coats (1.3 mg GAE* g^{-1} DM and 1.1 mg GAE* g^{-1} DM, respectively) and in November in Generation 3 seed coats (1.28 mg GAE* g^{-1} DM; Fig. 2b) .

There were significant differences in seed coat phenolics between seed generations and between harvesting months in both, 'Hass' and 'Fuerte' seed coats ($P < 0.001$) (Fig. 2a and 2b).

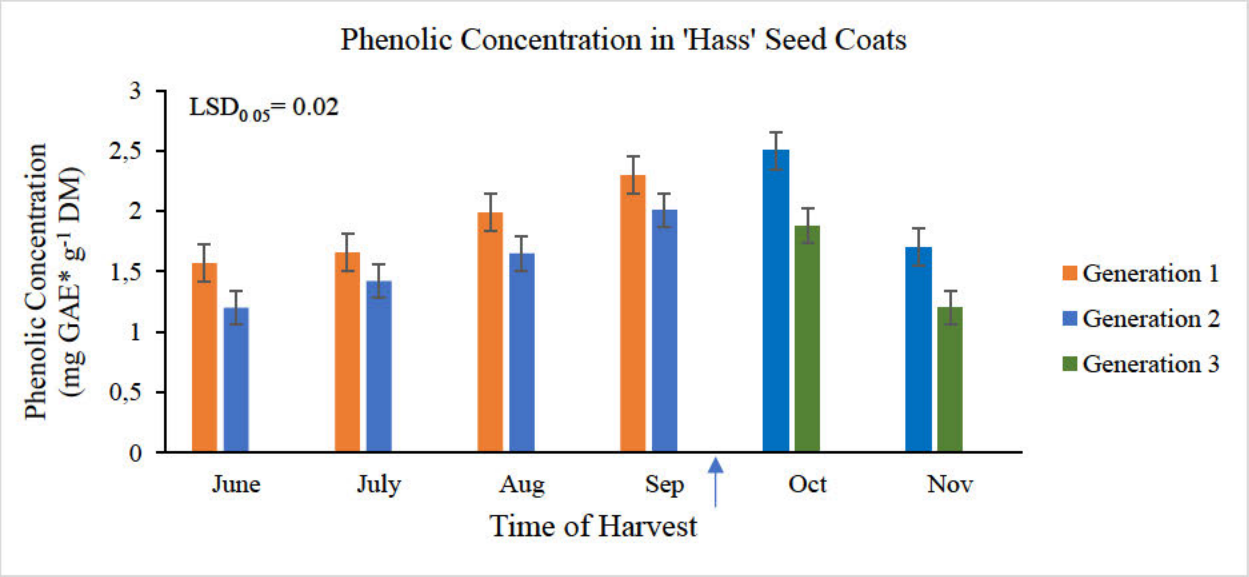

Figure 2a. Phenolic compound concentrations of 'Hass' avocado seed coats from June to November; Generation 1 (full bloom in July/August 2017), Generation 2 (full bloom in July/August 2018) and Generation 3 (full bloom in July/August 2019). $LSD_{0.05}$ is least significant difference ($P \leq 0.001$). The arrow indicates the time, when the oldest fruit were collected, and a new generation (Generation 3) started to grow to harvestable size.

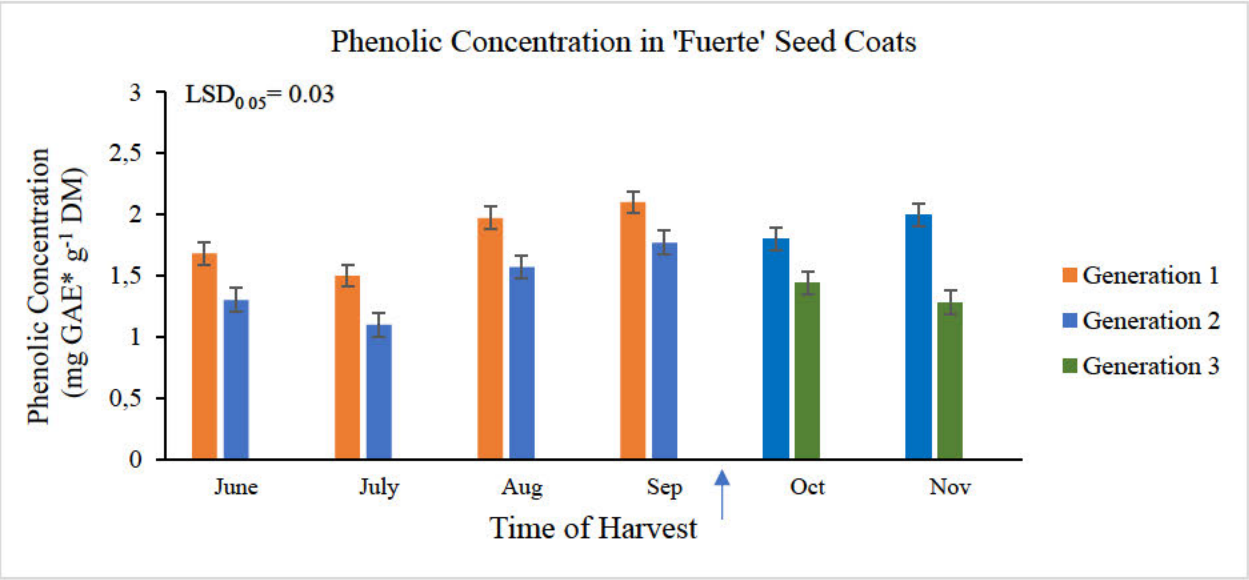

Figure 2b. Phenolic compound concentrations of the 'Fuerte' avocado seed coat from June to November; Generation 1 (full bloom in June/July 2017), Generation 2 (full bloom in June/July 2018) and Generation 3 (full bloom in June/July 2019). $LSD_{0.05}$ is least significant difference ($P \leq 0.001$). The arrow indicates the time, when the oldest fruit were collected, and a new generation (Generation 3) started to grow to harvestable size.

4.4. Discussion

4.4.1. Polyphenol Oxidase (PPO) Activity in Avocado Seed Coats

The enzyme PPO oxidizes phenolic compounds and is present in most plant tissues. Generally, the phenolic concentration is primarily degraded by PPOs (Thypapong *et al*., 1996). The phenolic compound concentration is also affected by ecological conditions, geographical location of plants and climate (Dinçer *et al*., 2013). Seed coat tissues of a certain cultivar and species differ in the composition of various phytochemicals, such as phenolics, tannins and flavonoids, as well as in PPO enzyme activity (Figueroa *et al.,* 2018). Soliva-Fortuny *et al.* (2002) and Quevedo *et al.* (2011) reported that polyphenol oxidase (PPO) activity plays an important role in the oxidation of polyphenolic compounds responsible for enzymatic browning in various vegetables and fruit. This enzymatic browning does not only lead to colour change, but also results in organoleptic alterations, nutritional losses and antioxidant degradation (Zhang *et al*., 2017; Tinello and Lante, 2018). Fig. 1a and 1b show differences between Generation 2 and Generation 1seed coats in PPO

activity in avocado seed coats from June to September. In Generation 3, PPO activity was lower than in Generation 2 from October to November, for both cultivars (Fig. 1a and 1b).

Activity of PPOs was significantly different between seed coats of Generation 1 seeds and Generation 2 seeds ($P < 0.001$), as well as between cultivars ($P < 0.001$). The interaction between sampling months indicates that differences in PPO activity, caused by seed age, were dependent on the time seeds were collected. Van Rooyen and Bower (2002) explained that PPO in avocado is a membrane-bound enzyme, and that membrane stability decreases, when the seed approaches maturity. Therefore, due to membrane instability, oxidation of phenolics by PPOs will occur. Fig. 1a and 1b demonstrate that Generation 2 seed coats had lower PPO activity than the Generation 1 seed coat. Results also showed that the older avocado seed coat had a higher PPO activity than the younger ones. These findings are in line with those by Vanini *et al.* (2010) who reported that the PPO activity in the avocado fruit increased, as it reached the ripening and fruit maturity stage. Similar results were reported in avocado seed by Gomez-Lopez (2002) who reported high PPO activity in avocado seed, while avocado pulp has a low PPO activity. The PPO activity of the avocado pulp has been reported to be lowest in the outer pulp, close to the blossom end, and this activity gradually increases towards the inner pulp close to seed coat; however, PPO activity in the pulp of seedless avocados of various cultivars is very low (Hershkovitz *et al.*, 2009); these finding indicate the importance of seed tissue in PPO activity and that PPO activity in the seed coat is aligned with fruit PPO activity.

In this study, it was observed that higher PPO activities were present in Generation 1 seed coat than in Generation 2 seed coats (Fig. 1a and 1b), seemingly inhibiting seed germination (see 2.3.1., Fig. 1a and 1b). It was found, however, that, in both cultivars, PPO activities were higher in Generation 1 seed coats than in Generation 2 seed coats, with Generation 2 seeds displaying a higher germination percentage than Generation 1 seeds (see 2.3.1., Fig. 1a and 1b). This seems not surprising, as carbohydrates reserves are broken down in the seed tissues and passed onto the embryo to be used for its growth and development (Taneja and Sachar, 1974; Rao and Deosthale, 1987). Possibly, PPOs play a role in the degradation of carbohydrates used by the embryo for respiration (Kabar and Kocaqaliskan, 1990). In fact, the high PPO enzyme activity in the embryo supports the positive correlation between respiration rate and activity of these enzymes (Stiles, 1960). The seed coats of both cultivars showed PPO activity throughout the observation period

(Fig. 1a and 1b); this supports the theory that PPOs are involved in seed coat browning (Toledo and Aguirre, 2017).

Vamos (1981) reported that PPO enzyme activity differs between plants, cultivars, tissues and even cell types. Studies on interactions between PPO enzymes and factors involved in the germination percentage, such as temperature, salinity, soil structure and various environmental conditions, showed that the relationship between PPO enzyme activity and germination is rather complex (Kocacaliskan *et al*., 1995). In this study, it was found that PPO have greater activity in Generation 1 than in Generation 2 seed coats and Generation 3 had lower PPO activity than Generation 2 (Fig. 2a and 2b), demonstrating that the maturity stage of the seed affects PPO activity. Similarly, PPO activity was found to be greater in germinating barley, bean, chickpea, and wheat seed than in the equivalent dormant seeds (Kocacaliskan *et al.*, 1995). The higher germination percentage in younger seed was coupled with a low PPO activity, while older seed were characterised by higher PPO activity.

Generally, the PPO concentration in seed coats was low, with the highest level of PPO activity being 0.50 U/kg^{-1} DM in August in 'Hass' seed coats of Generation 1. In 'Fuerte' seed coats the highest PPO activity was also detected in the older Generation 1 (in September) seed coats (0.49 U/kg^{-1} DM). In addition to physiological age, environmental conditions and agricultural practices, such as fertilization, irrigation and salt stress, also affect PPO activity (McNabnay *et al*., 1999; Rodriguez *et al*., 2000).

These differences in PPO concentration maybe due to the oxygen volume able to penetrate the older seed coat and allowing higher O_2 coming into that tissue (Gordon, 1980), as the seed coat sits tightly between the seed and the mesocarp. It seems, therefore, that, once the embryo enters into germination, oxygen needs to be able to enter into the seeds, so that PPO can catalyse the breakdown of the seed coat and the loosening of the seed coat from the seeds.

4.4.2. **Phenolic Concentrations in Avocado Seed Coats**

Phenolics are important phytochemicals affecting plant growth and development, as well as human and animal well-being. Phenolic compounds are secondary metabolites synthesized in plants; they fulfil important functions in photosynthesis and in seed growth and development.

Such compounds are often produced by injured plants in response to environmental factors and disease infestation (Kefeli *et al*., 2003). Phenolics can improve plant adaptation to abiotic stress conditions, such as water and cold stress, as well as to biotic stresses, such as infection by microorganisms (Shetty, 2004).

The findings of the present study confirm that the avocado seed coat contains variable amounts of phenolic compounds, with the phenolic concentration depending on seed maturity. These findings agree with Figueroa *et al*. (2018), who reported that different amounts of phenolics can be found in avocado seed coats at different stages of seed maturity.

In this study, Generation 1 seed coats were found to contain higher phenolic compound concentrations than Generation 2 seed coat from June to September. Generation 3 had, similarly, a lower seed coat phenolic concentration than Generation 2 in October and November (Fig. 2a and 2b). Similarly, it was observed in orange and mandarin seeds that fully mature seeds had a higher phenolic compound concentration than seeds of immature seeds (Inan *et al.*, 2018).

The avocado seed coat has been reported as a source of phenolic compounds, acting as antioxidants; it also contains many other antioxidant compounds, such as carotenoids and ascorbic acid. All these antioxidants play an important role in seed health (Dabas *et al.*, 2013). Attention on phenolic compounds is increasing due to their ability to inhibit avocado seed germination, despite seeds being mature and able to germinate. Singh and Singh (2018) reported that high phenolic compounds in seeds may inhibit seed germination or reduce the seed germination rate. In this study, it was found that the higher phenolic concentration in the older Generation 1 seed coats was aligned with a lower seed germination percentage, while a lower phenolic concentration and higher germination rate were found in Generation 2 and Generation 3 seed/ seed coats (Fig. 2a and 2b, see, 2.3.1, Fig. 1a and 1b). Tesfay *et al.* (2010) similarly observed that in the seeds of the 'Hass' cultivar, phenolic concentrations increase with maturation.

In the present study, Generation 1 seed coats had a high concentration of phenolics in both cultivars (Fig 2a and 2b). In these seeds, the germination percentage was low, possibly because the seed coat containing high amounts of phenolics, hindering germination. These seeds had difficulties to emerge, possibly as they contained a significantly ($P < 0.001$) higher amount of phenolics compared with the other two generations.

The activity of PPOs and the presence of phenolic compounds in avocado seeds/ seed coats could have a protective function against stress conditions, as these enzymes provide resistance to damage from the harmful influences of phenolics on seed germination and seedling growth. The high phenolic concentration in avocado seed coats could be exploited as a natural product, that could potentially be used to prevent the risk of certain diseases.

4.5. Conclusion

The objective of this study was to evaluate the polyphenol oxidase activity (PPO) and phenolic concentration in different physiological stages of avocado seed coat development of two cultivars, 'Hass' and 'Fuerte'. Despite the importance of PPO enzymes and phenolics in seed coats, there have only been limited studies on the parameters in avocado seeds. In this study the development of seed coats was followed by comparing three generations of two cultivars over a certain development period. Different PPO levels were discovered between generations, when PPO increased, or decreased, phenolic concentrations also increased or decreased.

Phenolic compound metabolism is driven by the activity of PPO enzymes; when the seed coat has a high PPO concentration, such as in Generation 1 seed, the phenolic concentration was also high in these tissues and seed germination was low. The PPO concentration and phenolic compound levels present a positive correlation ($r = 0.42$, $P < 0.001$), with both, PPO and phenolic compound concentration highly affected by seed maturity stage. The concentration of PPOs was lower, when the phenolic concentration was lower, too, and seed germination was higher in seed from Generation 2 and Generation 3. The lower level of phenolics in mature seed coats suggests the importance of phenolic compounds in germination, seemingly the higher phenolic concentration in Generation 1 coats resulted in a lower germination rate. In addition, the higher concentration of phenolics in Generation 1 seeds can be due to oxidation by PPO. Investigating PPO activity in seeds coats of various seeds ages might reveal a correlation between higher PPO activity in older seeds, as visible in this study in the higher phenolic concentration of older seed coats. These phenolics were possibly involved in inhibiting germination of older seeds. Altogether, it is recommended to use younger seed as 'nurse seed' when propagating avocado, as such seeds are able to germinate easier.

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CHAPTER FIVE

PHENOLIC COMPOUND CONCENTRATIONS AND ULTRASTRUCTURAL CHANGES OF THE SEED COAT THROUGHOUT AVOCADO SEEDS MATURATION

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Abstract

Phenolic compounds can interfere with seed germination in a positive or negative manner. In avocado, both, the seed and the seed coat, contain phenolic compounds. To investigate, if the phenolics in the seed coat act as a barrier to germination, or, if the phenolic concentration present is unrelated to seed germination, phenolics of the seed coat were determined. Additionally, the structure of the seed coat was viewed under a scanning electron microscope (SEM), to determine, if seed coat ultrastructure and seed coat phenolics are related. Two cultivars ('Hass' and 'Fuerte') and two fruit generations, the generation of fruit enclosing a 'young' seed (Generation 2), as well as an older, 'late-hung' generation (Generation 1), carrying the previous year's commercial harvest, were used in the present study. Ultra-structural changes and phenolic concentrations of the seed coat of these two fruit generations were compared. In both cultivars, phenolic concentrations in the seed coat of Generation 1 fruit were higher than in Generation 2 seed coats. Seeds from the older generation had a lower germination percentage, coupled with a higher phenolic concentration, in both cultivars. High phenolic concentrations could, therefore, be involved in inhibiting seed germination of Generation 1 seeds in both cultivars. Generation 2 seed coats had a lower amount of phenolics, and their seed coats were thin. The seed coat tissue facing the cotyledons (inner layer), as well as the seed coat tissue facing the mesocarp (outer layer), were examined using scanning electron microscopy (SEM). The surface morphology differed between the two sides and seed coat generations: Generation 1 of both cultivars had more sclerified cell walls than Generation 2. Seed coats of Generation 2 (both cultivars) had a reticulate and thin cell wall shape. The radicle of these seeds easily penetrated the seed coat. The seed coat was, hence, not a physical hinderance to seed germination, but a physiological barrier must have existed, possibly in the form of phenolics, as the ability of the radicle to emerge was aligned with the phenolic concentration of the seed coat, not the physical, appearance (thicker seed coat of older seeds, Generation 1, thinner seed coats of younger seeds, Generation 2).

Keywords: Avocado seed coat, ultrastructure, scanning electron microscopy, phenolic compounds, seed coat, germination percentage, seed maturation

5.1. Introduction

In dicotyledons, the seed coat is the primary barrier of the seed, affording protection against adverse environmental conditions that could result in a loss of solutes due to leaching (Radchuk and Borisjuk, 2014). The seed coat, furthermore, protects the seeds from invasion by microorganisms and from mechanical and environmental stress, particularly from temperature and humidity extremes (Rao *et al*., 2017). Phenolic compounds accumulate in the layers of the seed coat (Preet and Punia, 2000) and play an important role in the chemical and physical defence of the seed and the embryo, when exposed to environmental stress (Troszynska *et al.,* 2002). These phenolic compounds are responsible for the above-mentioned protection by the seed coat (Mohamed *et al.,* 1994).

The seed coat is a multifunctional tissue that also plays an important role in embryo nutrition during seed maturation (Bewley and Black, 1994; Mohamed *et al*., 1994; Bradford, 2002). The seed coat also exerts certain germination-limiting actions, as it is commonly impermeable to water and gases for a long period, thereby providing mechanical resistance to radicle emergence. This inhibition has been positively correlated with dark seed coat colour due to the phenolic compounds found in the seed coat tissue (Debeaujon *et al*., 2000), present in seeds of various crops, such as olive (*Olea europea*), sorghum (*Sorghum bicolor*), grape (*Vitis vinifera*) and bean (*Phaseolus vulgaris*) (Moure *et al*., 2001).

Many morphological features of the seeds can be used for systematic identification and assist in the understanding of the evolutions of plant species (Buss *et al.,* 2001; Zhang *et al*., 2005; Gontcharova *et al*., 2009). Generally, the morphological features of seeds and the texture of seed coats differ between cultivars of the same species (Chowdhury and Buth, 1970; Al-Gohary and Mohamed, 2007).

Among the many roles and functions of the seed coat are preservation and protection of the seed, and, thereby, the embryo - against attack by pests and diseases. Further, the seed coat protects the seed against mechanical injuries and regulates gaseous exchanges (O_2 and CO_2) between the seed, and, therefore, the embryo, and the external environment (Souza and Marcos, 2001).

Seeds, which can be stored in a dry state (less than 10% moisture content), are known as orthodox seeds (King and Roberts, 1979). Seeds, which die after losing between 20–50% moisture, and,

therefore, do not tolerate long storage periods, are known as recalcitrant seeds; avocado seeds fall into the latter grouping (Kozlowski and Pallardy, 2002; Ernst *et al*., 2013; Walters *et al*., 2013). The number of species known to produce recalcitrant seeds is increasing (Tweddle *et al*., 2003), with many important food plants, such as mandarin (*Citrus reticulata*) (Khan *et al*., 2002), avocado (*Persea americana*) (Raja *et al*., 2001), mango (*Mangifera indica*) (Lizada, 1991; Girija and Srinivasan, 2000) and cocoa (*Theobroma cacao*) (Li and Sun, 1999; Liang and Sun, 2002) identified as recalcitrant. As such seeds easily lose viability due to water loss, the seed coat plays a pivotal role in protecting the seeds against such moisture loss. During the second phase of seed development, seed maturation, seed moisture naturally declines (Gutierrez *et al*., 2007). Hanson (1984), therefore, observed that the term 'desiccation-sensitive' characterizes recalcitrant seeds, as seed viability is lost, once seeds are dried beyond a certain moisture threshold.

Avocado seeds of the Mexican and Guatemalan botanical varieties (races) may be kept under refrigeration between 8 to 15 months (at 90% RH and 5.5 °C) (Halma and Frolich, 1949). The lifespan of seeds from different avocado cultivars ranges between 5 and 15 months, when stored under refrigeration (4.4°C to 9°C) (Ernst *et al*., 2013). Castro and Fassio (2013) indicated that avocado seeds of different genotypes can be stored for two months at 6°C and 80% RH, with subsequent successful germination.

Numerous studies on seed development have shown that the transition from a permeable to an impermeable seed coat, coincides with the decline in seed moisture during the maturation/ drying phase of seed development (Jaganathan, 2016). In general, embryo tissues are synthesized during morphogenesis (maturation stage), while cotyledonal tissue is produced during embryogenesis (Vieitez and Barciela, 1990); similarly, storage products, such as proteins and oils, tend to accumulate during the maturation processes prior to and in preparation for seed dormancy. The variation in seed coat morphology and in embryo growth may affect seed size; it is thought that the degree to which the seed coat can expand, influences the embryo size during seed development (Wang and Hedley, 1993).

Phenolic compounds are one of the most-widely distributed secondary products in the plant kingdom; many of these compounds play very important ecological and physiological roles, being involved in the regulation of stress responses and in redox reactions (Curir *et al*., 1990; Takahama and Oniki, 1992; Delalonde *et al.,* 1996). These phenolics also protect the seed against pests and

diseases (Nagarathna *et al*., 1993, Dübeler *et al*., 1997). Phenolic compounds are, further, important for seed germination, as they inhibit enzymes, such as amylase, protease, and lipase (Joshi, 2018), and give seed coats the brown colour (Lu *et al*., 2018). Generally, the phenolic metabolism and, therefore, the presence of a certain phenolic concentration, is controlled by the activity of certain enzymes, such as peroxidases, polyphenol oxidases, and phenylalanine ammonia lyases (Smith *et al*., 1998).

The avocado seed coat could act as a source of phenolic compounds, which have been postulated to interfere with germination and water uptake (Rosero *et al*., 2019). In various species a positive correlation between seed coat colour and the presence of phenolic compounds exists (Debeaujon *et al*., 2000). Phenolic compounds and their derivatives have been long known as inhibitors of seed germination (Schmauch and Grubb, 1954). In avocado seeds, phenolic compounds occur predominantly in the seed coat (Figueroa *et al*., 2018), visible in the phenolic oxidation. Once the seed coat is exposed to oxygen, the brown external seed colour results (Gausseres *et al*., 1997; Duenas *et al.,* 2004) due to the formation of phenolic oxidation products (Dabas *et al*., 2011). Previous studies reported that 'Hass' and 'Fuerte' avocado seeds contain considerably more phenolics compounds than the avocado pulp (Rodríguez *et al*., 2011); hence, the avocado seed coat turns brown, and the pulp turns dark because of high phenolic concentrations in these tissues (Tesfay *et al.,* 2010; Wang *et al.,* 2010). Phenolic compounds are also present as anthocyanins and phenolic acids, the latter are involved in browning reactions (Santiago *et al*., 2000; Boudet, 2007; Khoddami *et al.,* 2013). There are many reports which demonstrate that seeds, covered by lightly coloured seed coats, germinate better than those with dark-coloured seed coats (Wyatt, 1977; Powell, 1989; Kantar *et al.,* 1996).

Seed coat morphology is usually influenced by external environmental cues, transmitted through the exocarp and mesocarp onto the developing avocado seeds (Barton, 1965; Rivera *et al.,* 2017; Munhuweyi *et al.,* 2020). Ultrastructure and micro-morphology of seeds can assist in the classification and taxonomy of seed plants and plays an important role in the botanical classification system of avocado (Ross *et al*., 1993; Abraham *et al.,* 2018). Many SEM studies have demonstrated a variation in seed coat cells, such as size, type, length and density (Juan *et al.,* 2000; Segarra and Mateu, 2001), and particularly in seed coat tissues between similar species (Zainhasan and Lester, 1990; Karam, 1997; Khalik, 2013). Scanning electron microscopy (SEM)

gives a deeper insight into the morphological features of seed coat tissues and is an important tool in plant taxonomy (Brisson and Peterson, 1976; 1977). The method also allows for easy ultra-structural observation of the surface features of small fruit and seeds (Crawford and Evans, 1978; Hauptli *et al.*, 1978; Crow, 1979).

Scanning electron microscopy studies of seed coat morphology have revealed ultrastructural features, including the type of cell arrangement, cell shape and distribution of papillae in the outer layer of seed coats (Clark and Jernstedt, 1978; Canne, 1979; Carolin, 1980). Seed coat features differ between tissues of the different species in composition, as well as in concentration of phenolics. A mature seed coat, as visible by its brown colour, indicates a mature embryo, as the seed coat matures prior to the embryo (Weber *et al.*, 2005).

With the assistance of SEM, seed characteristics, mainly outer seed coat features, have been described; these features are important in the evolutionary development and diversification of a species (Karihaloo and Malik, 1994; Segarra and Mateu, 2001). The ultrastructural pattern of the seed coat, identified using SEM, can be used to differentiate between different species (Barthlott, 1981; Yoshizaki, 2003).

The main aim of this study was to determine the phenolic compound concentration of the seed coat of two generations of two avocado cultivars, 'Hass' and 'Fuerte'. To determine possible differences in phenolic concentration in the seed coat of Generation 1seeds and Generation 2 seeds; potential differences in phenolic concentrations could possibly be aligned with differences in avocado seeds germination. To further investigate structural barriers of germination, seed coats were viewed under SEM to confirm avocado seed coat microstructure, and to identify variations between this structure of Generation 1 and Generation 2 avocado seed coats.

5.2. Material and Methods

5.2.1. Origin of Chemicals

All chemicals were obtained from Sigma-Aldrich®, Saarchem®, Fluka®, or Glycoteam GmbH.

5.2.2. Harvesting of Seeds

Avocado seeds used in this investigation were obtained from fruit of a commercial orchard at Bounty Farm, in the KwaZulu-Natal Midlands (30°16-E and 29°28-S, South Africa). Avocado

seeds of two cultivars ('Hass' and 'Fuerte') from fruit of two generations were used in this study. Fruit from these two generations of typical size and appearance for the generation, were collected monthly, from June to November. Seeds of two fruit generations were analysed: 'Generation 1' 'Hass' avocado seeds with full bloom in July/August 2017, collected 24 to 29 months after full bloom (MAFB) until November 2018 and the following year's fruit, with full bloom in July/August 2018 (Generation 2, collected 12 to 17 MAFB)). In 'Fuerte' Generation 1 seeds (full bloom in June/July 2017) and Generation 2 seeds (full bloom in June/July 2018) were investigated. Fruit of both cultivars were commercially harvested in Jun/July 2018. Fruit was removed from trees and immediately transported to laboratory.

Green mature fruit of both cultivars were harvested and kept at room temperature until fruit started to soften. Then, the seed was extracted from the mesocarp, and the seed coat was peeled off the seed and dried for 24h at 75°C. For phenolic analysis the seed coats were ground into a fine powder, while for SEM observation, cut pieces of the dried seed coat where used.

5.2.3. **Determination of Phenolic Compounds in Avocado Seed Coats**

The phenolic concentration of avocado seed coats was determined using a Folin-Ciocalteau method described by Blainski *et al.* (2013). Ground samples (0.5g DM) were homogenized in 10mL aqueous ethanol (80%) at room temperature; then 5mL sample solution was kept for 30min at room temperature (25±1°C), before samples were filtered through Whatman no.1 filter paper. Thereafter, samples were centrifuged at 20 000*g* for 10min, the supernatant removed, and 2.5mL supernatant mixed with 0.1mL 0.2N Folin-Ciocalteau reagent (Sigma-Aldrich/Fluka, St Louis, MO, USA) for 5min. The reaction was terminated using 2.0mL 75g L^{-1} sodium carbonate solution. The sample mixture was then vortexed and incubated at room temperature (25±1°C) for 2h. The absorbance was read at 760nm against a reagent blank using a UV-Vis spectrophotometer SP 8001 (Metertech Inc., Taipei, Taiwan).

5.2.4. **Scanning Electron Microscopic (SEM) Image Analysis of Avocado Seed Coats**

For SEM analysis avocado seed coat samples were dissected into small pieces (10 x 10 mm) using scissors and fixated in 3% glutaraldehyde for 1 to 2min, before being washed with 0.1M sodium cacodylate buffer, followed by dehydration using ethanol (80%) for 1h to reduce the water present in the samples. These samples were, after removal from the ethanol solution, viewed under SEM

(Zeiss EVOLS15) (Quanta 200; FEI, Hillsboro, OR, USA), according to Talbot and White (2013). Samples were mounted onto aluminium stubs with two-sided carbon tape and sputter-coated with gold-palladium. Then, three spots per sample were observed under the SEM. Every seed coat sample was evaluated for diagnostic seed coat inner-side (layer facing cotyledons) and outer-side (layer facing mesocarp) surface features.

5.2.5. Statistical Analysis

Data were analysed by one-way analysis of variance and results were considered statistically significant at $P \leq 0.001$. Statistical analyses were made using GenStat (version 18.2; VSN International, Hemel Hempstead, UK). The values of all parameters were subjected to statistical analysis. Significant differences among treatments were evaluated by the least significant difference (LSD) test (Gomez and Gomez, 1984).

5.3. Results

5.3.1. Phenolic Concentrations

The phenolic concentration of the avocado seed coat varied between cultivars ('Hass' and 'Fuerte'). Phenolics extracted from Generation 1 seed coats of both cultivars were higher (2.03 mg GAE* g^{-1} DM for 'Hass' and 1.98 mg GAE* g^{-1} DM for 'Fuerte') than in Generation 2 seed coats (1.29 mg GAE* g^{-1} DM and 1.27 mg GAE* g^{-1} DM, for 'Hass' and 'Fuerte', respectively; Fig. 1a and 1b). The overall highest phenolic concentration was determined in 'Hass' seed coats in September, Generation 1. Comparing the phenolic concentration over the entire observation period, the highest phenolic concentration was recorded in Generation 2 (September, Fig. 1a). 'Fuerte' seed coats contained the highest phenolic concentration slightly earlier, in August (Generation 1), while the highest phenolic concentration in 'Fuerte' seed coats was detected in Generation 2 (September, Fig. 1b).

In both cultivars phenolic concentrations decreased during the last two months of the investigation (November, December) (Fig. 1a and 1b) in both seed coat ages and cultivars investigated. Phenolic concentrations were lowest in June and November in seed coats of both cultivars.

Comparing the two ages of avocado seed coats, Generation 1 seeds had greater phenolic compound concentrations, while Generation 2 seed coats had significantly ($P < 0.001$) lower phenolic concentrations. Significant differences were also found between month ($P < 0.001$) for both cultivars.

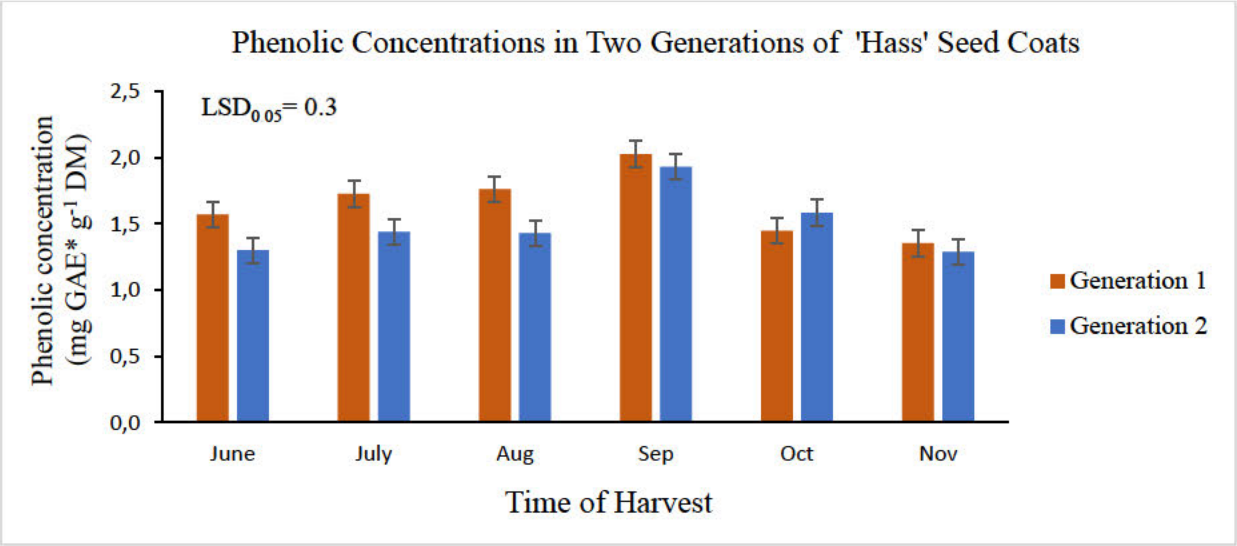

Figure 1a. Concentration of phenolic compounds in the seed coat of 'Hass' avocado over two generations: Generation 1 (full bloom in July/August 2017), and Generation 2 (full bloom in July/August 2018), from early fruit set to very late-hung fruit.

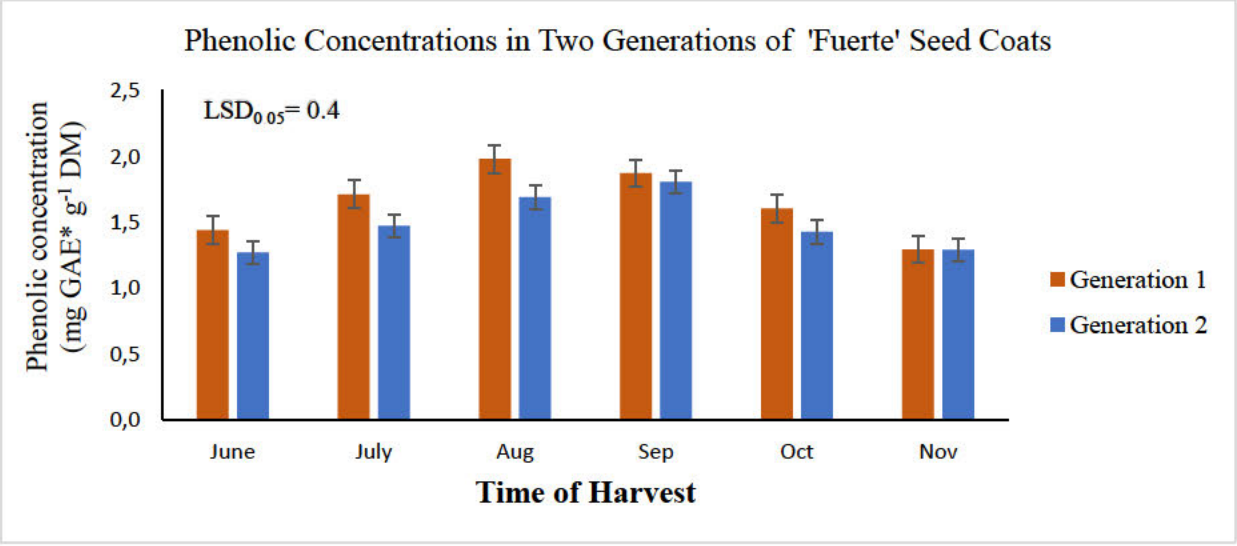

Figure 1b. Concentration of phenolic compounds in the seed coat of 'Fuerte' avocado over two generations: Generation 1 (full bloom in June/July 2017), and Generation 2 (full bloom in June/July 2018), from early fruit set to very late-hung fruit.

5.3.2. **Scanning Electron Microscopy (SEM)**

Scanning electron micrographs revealed differences between seed coats of the two cultivars, as well as between the outer (layer facing mesocarp) and the inner (layer facing cotyledons) seed coat of both cultivars (Fig. 2a and 3b). 'Hass' seed coats contained a distinct outer and inner cell layer in Generation 2 seeds (Fig. 2a, Plate A and B, respectively), while Generation 1 seed coat cells differed in size and ultrastructure (Fig. 2b, Plate C and D).

In 'Hass' Generation 2 seed coat, the outer seed coat consisted of several layers of wax coverings (Fig. 2a, Plate A), while the inner seed coat had thinner wax layers (Fig. 2a, Plate B). In Generation 1 'Hass' seed coats, the outer layer seemed to be ruptured and the structures of the inner side had disintegrated (Fig. 2b, Plate C and Fig. 2b, Plate D).

Seed coat thickness of 'Hass' fruit Generation 1 ranged between 0.11 mm and 0.32 mm (Fig. 3b, see 2.3.3); seed coats of such fruit were yellow to brown in colour and thin with a higher phenolic concentration than Generation 2 seed coats. In Generation 2 seed coat thickness ranged between (0.30 mm and 0.51 mm, Fig. 3b, see 2.3.3); these seed coats were white in colour and thick, with a low phenolic concentration (Fig. 2a and 2b, see 2.3.2). During the early stages of seed development, the Generation 2 'Hass' outer seed coat layer (facing the mesocarp) were covered with wax, while the inner layers (facing cotyledons) were less defined. Generation 2 seed coats of 'Hass' seeds (outer layer) showed disintegrated structures, leaving gaps between cells (Fig. 2b, Plate. C). In Generation 1 seeds of 'Hass' seed coats the inner cell layers appear less structured, giving a 'smooth surface' impression (Fig. 2b, Plate D). The cells of the outer avocado seed coat wall of both cultivars had a higher phenolic concentration (Fig. 2a and 2b), seemingly protecting seed and embryo against pest and disease invasions and harsh environmental conditions.

In 'Fuerte' seed coats the appearance of the outer and inner wall differed significantly between months, resulting in a distinguishable, taxonomic variation between the two sides of the seed coat. Differences were also found in seed coats of the two seed generations, with a visible difference in cell shape. The morphology of the Generation 1 and Generation 2 seed coat of both cultivars was compared in sections of the seed coat through SEM, where the inner and outer sides of seed coat were observed.

In ‘Fuerte’ Generation 2 seed coats, the inner cell layer seems to be covered by a thicker wax layer (Fig. 3a, Plate F), compared with Generation 2 ‘Hass’ seed coats; ‘Fuerte’ seed coats showed distinct outer cell features and the inner seed coat was covered by a thick wax layer (Fig. 2b, Plate E and Fig. 2b, Plate G).

Difference between seed coats of the two seed generations seem not obvious in the outer layers/ sides of the Generation 1 and Generation 2 ‘Fuerte’ seed coat, while the inner seed coat surfaces seemed to differ (Fig. 3a and 3b, Plate G, E). The inner seed coat of Generation 2 ‘Fuerte’ seed had several wax layers, while the inner Generation 1 seed coat was thinner and showed signs of degeneration (Fig. 3b, Plate. F, H).

A B

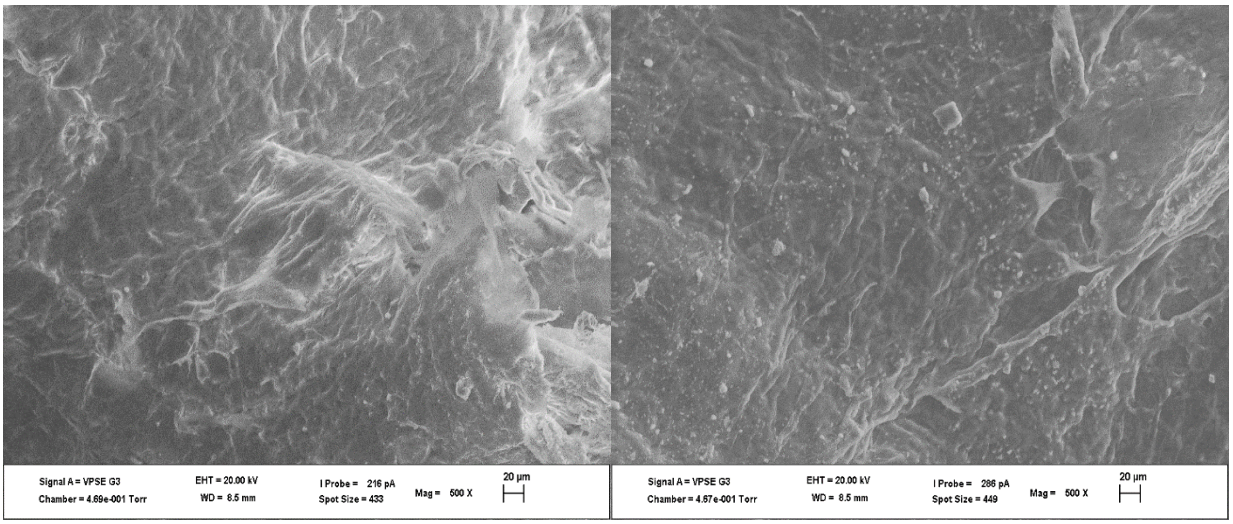

Figure 2a. Scanning electron micrographs of ‘Hass’ seed coat cells Generation 2 (full bloom in July/August 2018); two sides of the seed coat were observed: (A) electron micrograph of the outer seed coat (facing the mesocarp) (B) electron micrograph of the inner seed coat (facing the cotyledons).

C D

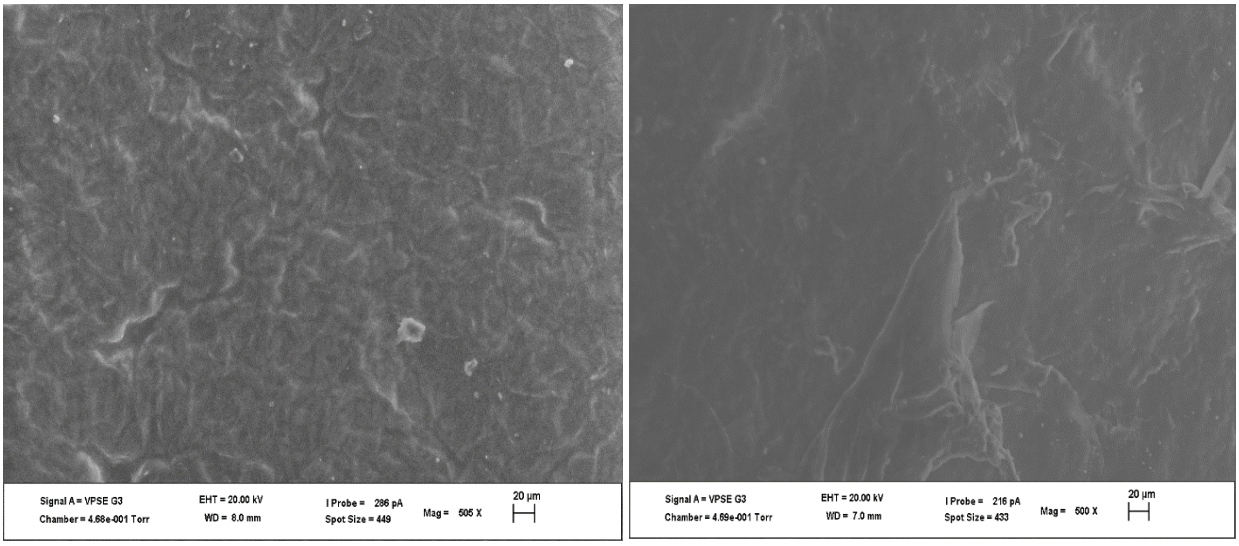

Figure 2b. Scanning electron micrographs of 'Hass' seed coat cells Generation 1 (full bloom in July/August 2017); two sides of the seed coat were observed: (C) electron micrograph of the outer seed coat (facing the mesocarp) (D) electron micrograph of the inner seed coat (facing the cotyledons).

E F

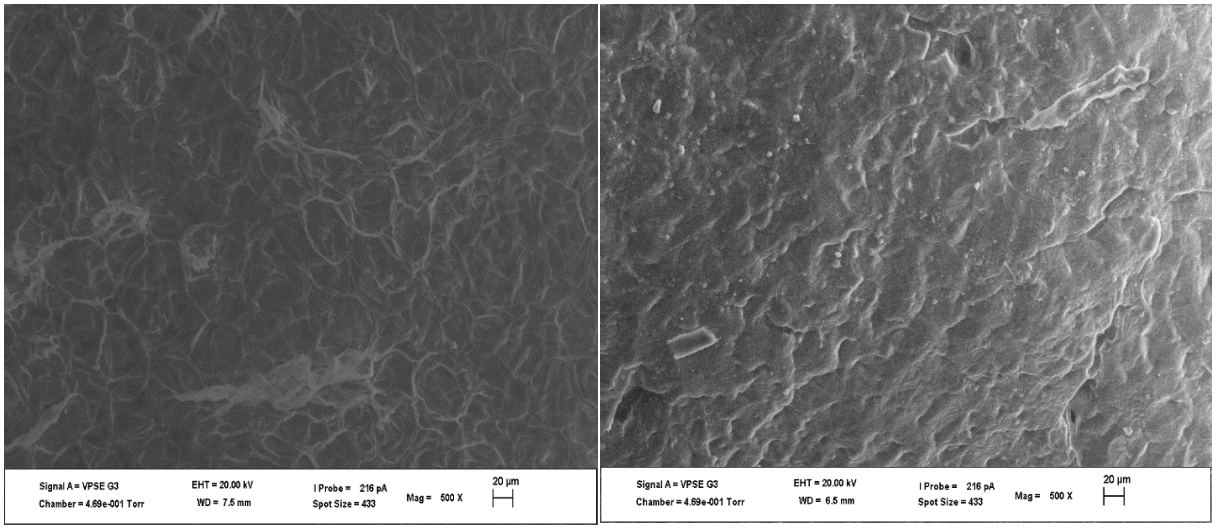

Figure 3a. Scanning electron micrographs of 'Fuerte' seed coat cells Generation 2 (full bloom in June/July 2018); two sides of the seed coat were observed: (E) electron micrograph of the outer seed coat (facing the mesocarp) (F) electron micrograph of the inner seed coat (facing the cotyledons).

G H

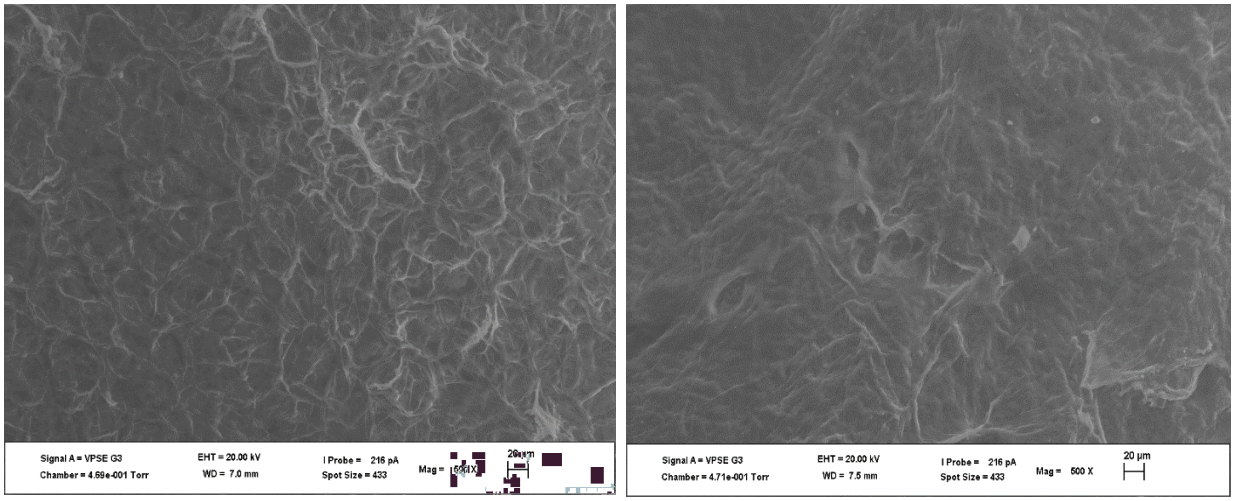

Figure 3b. Scanning electron micrographs of 'Fuerte' seed coat cells Generation 1 (full bloom in June/July 2017); two sides of the seed coat were observed: (G) electron micrograph of the outer seed coat (facing the mesocarp) (H) electron micrograph of the inner seed coat (facing the cotyledons).

5.4. Discussion

5.4.1. Phenolics in Avocado Seed Coats

Phenolic compounds are produced as a plant response to defend injured or infected areas and to protect cells from the spread of micro-organisms (Kefeli *et al.,* 2003). Higher phenolics present in older seed coats, therefore, protect seed and embryo for a long time. Early germination of the avocado seed was seemingly inhibited (Fig. 1a and 1b, see, 2.3.1) by this high concentration of phenolic compounds ('Hass' seed coat 2.03 mg GAE* g^{-1} DM, Fig. 1a; 'Fuerte' seed coat 1.98 mg GAE* g^{-1} DM, Fig. 1b), which has been reported to reduce germination (Chalker-Scott and Fuchigami, 2018). Zhou *et al.* (2010) reported that the phenolic concentration of the seed coat of legumes was responsible for the decreased germination rate when wild soybean (*Glycine soja*) **s**eed approached maturity.

Dabas *et al*. (2013) reported that the avocado seed coat is rich in phenolic compounds. This was confirmed, as the avocado seed coat contained about 2mg GAE*g^{-1} DM. Singh *et al*. (2017) reported that the phenolics in seed coats of soybeans (*Glycine max*) ranged between 1.57 and 5.57 mg GAE/g^{-1} DM.

The Generation 1 avocado seed coat contained a higher concentration of phenolic compounds than the Generation 2 seed coat; this was the case for both cultivars (Fig. 1a and 1b). This higher phenolic concentration in the seed coat of Generation 1 seeds could have inhibited seed germination (see 2.3.1, Fig. 1a and 1b). Similarly, Singh and Singh (2018) reported that the high phenolic compounds in guava (*Psidium guajava*) seeds may inhibit or reduce seed germination.

Phenolic concentrations were higher in seed coats of Generation 1 than of Generation 2, with Generation 1 seeds characterised by a thin and brown seed coat. Generation 2 seed coats, however, were thick and white with a low concentration in phenolic compounds (2.3.3, Fig. 3a and 3b), as previously described by Blumenfeld and Gazit (1971). Generally, peel (exocarp) and seeds of 'Hass' and 'Fuerte' fruit contain considerably higher amounts of phenolic compounds, more so than the avocado pulp (Rodríguez *et al*., 2011). In both avocado cultivars, seeds had significantly higher levels of phenolic compounds than the pulp, confirming various other reports (Torres *et al*., 1987; Soong and Barlow, 2004; Wang and Bostic, 2010). Increasing attention has been drawn to the phenolic compounds in the avocado seed, due to their ability to inhibit avocado seed germination of the fully mature seed (Kristanty *et al*., 2014). The avocado seed coat of the Generation 2 fruit was, in both cultivars, found to also contain low phenolic concentrations; a higher germination percentage was aligned with these lower phenolic seed coat concentrations.

Kosińska *et al*. (2012) reported that the phenolic concentration in 'Hass' and 'Fuerte' avocado seeds are higher than in the peel, when the fruit is completely mature. For both cultivars, 'Hass' and 'Fuerte', the phenolic concentrations were found to be significantly different between the two generations ($P < 0.001$). In 'Hass' seed coats phenolics were highest in September in Generation 1 fruit (2.03 mg GAE* g^{-1} DM) (Fig. 1a), while for 'Fuerte' the highest seed coat phenolic concentration was determined one month earlier, in August. A similar pattern was determined for Generation 1 'Fuerte' seed coats, containing 1.98 mg GAE* g^{-1} DM (Fig. 1b) and in Generation 2 'Fuerte' seed coats the highest amount of phenolics was 1.81 mg GAE* g^{-1} DM in September (Fig. 2b).

López-Cobo *et al*. (2016) reported that the phenolic concentration in avocado seed coats increased up to the fruit maturity stage and phenolic concentrations are higher in overripe fruit and seeds than in unripe fruit. Similarly, in the present study, the phenolic concentration of the older generation 1 seed coat was higher than in the younger seed coat (Generation 2, Fig. 1a and 1b).

This could possibly explain the long lifespan of the mature seeds, as phenolics can protect the seeds from adverse environmental conditions and against pathogen attack, keeping the seeds in a healthy state. Oliviera *et al*. (2015) also reported that phenolic compounds are biologically active substances, able to be prevent pathogen attack.

5.4.2. **Ultrastructure of Avocado Seed Coats**

Variations in seed morphology can occur in terms of shape, size and surface features, with such variations described for the morphology of the legume seeds, where the hilum, micropyle and raphe region can be differentiated (Boesewinkel and Bouman, 1984).

Many previous studies have employed SEM to characterize the structure of seed coats of species, such as bush plum (*Carissa spinarum*), stranglevine (*Cynanchum acutum*) dogbane (*Leptadenia arborea*) (Rugenstein and Lersten, 1981; Behnke and Barthlott, 1983; Watanabe *et al*., 1999), and common bean (*Phaseolus vulgaris*) (Yeung and Cavey, 1990). The use of electron microscopy has also played an important role in elucidating plant systematics. In some legumes, SEM examination of the seed coat demonstrated that the seed coats vary in morphological features between legumes species (Gopinathan and Babu, 1985).

Scanning electron microscopy has assisted in describing seed coat characteristics and in understanding of the seed micro-structure. Generation 1 seed coats were characterised by a rather thin, dark seed coat, composed of small vacuolate cells, resulting in a stable structure under vacuum, allowing fast preparation for SEM examination (Ghosh *et al.*, 2009). The morphology of avocado seed coats from Generation 1 of both cultivars revealed that these seed coat walls were rather thin with smaller, tightly packed cells. Cells were relatively smaller in Generation 1 seed coats than in seed coats of Generation 2. Cells of younger, Generation 2 seed coats were characterised by variation in surface appearance, visible as large cells and accompanied by a thick seed coat (Fig. 2 and 3). Seed coat micro-topography, therefore, varied between seed generations (Fig. 3a and 3b, see, 2.3.3).

During seed development, the seedcoat plays an important role, as it is water-permeable and allows gas exchange (Haughn and Chaudhury, 2005). In this study, the ultrastructure of the seed coats of the two cultivars differed substantially between Generation 1 and Generation 2. Structures also differed between the seed coat surface attached to the seed (inner layer) from the

seed coat surface attached to the mesocarp (outer layer). Certain structural patterns of the seed coats were specific to a certain seed coat age (Fig. 2b and 3b), so that these seeds could be separated according to generation by structural features.

The outer layer of Generation 1 ‘Hass’ seed coats was typified by small cells (small vacuoles) that had begun to degenerate at this late maturation stage (Fig. 2b, Plate C). The inner layer of ‘Hass’ seed coats Generation 1 was dark (Fig. 2b, Plate. D). Cell walls are likely to contribute to the mechanical strength of the seed coat due to the sclerified, dead tissues (Fig. 2b, Plate C).

The outer layers of the seed coats of Generation 2 ‘Hass’ seeds were thick and smooth during early seed development (Fig. 1a, Plate. A and B). ‘Fuerte’ seed coats were relatively thin in Generation 1, more so than those of Generation 2 seed coats. Chuang and Heckard (1972) reported differences in seed coat morphology in various species. As the seed coat cells of Generation1 seed generally form a thin layer, cells become reticulate and have a waxy texture. The thickness of the ‘Fuerte’ and ‘Hass’ seed coats also varied, with Generation 2 seed coat thickness being between 0.46 and 0.51 mm, while Generation 1 seed coats were much thinner (between 0.15 and 0.11 mm) (Fig. 3a and 3b, see 2.3.3). These findings agree with Cummings and Schroeder (1942), who reported that the thick, immature seed coats of avocado adhere to the seed, so that they are difficult to remove from the seed, as experienced for ‘Hass’ and ‘Fuerte’ seeds (Fig. 3a and 3b, see 2.3.3).

5.5. Conclusion

This study demonstrates that Generation 1 ‘Hass’ and ‘Fuerte’ seed coats had a high concentration of phenolic compounds. These seed coats were thin and had a high phenolic concentration that seemed to inhibit germination. Seed coats of Generation 2 were thicker in ‘Fuerte’ and ‘Hass’ seed than their Generation 1 counterparts. The seed coat of Generation 2 of both cultivars had lower phenolic concentration and a thicker seed coat. From SEM images of the surface structure of the outer and inner side of the avocado seed coat, it becomes clear that Generation 2 seed coats are thicker, waxier and whiter, with a lower phenolic concentration, possibly due to a higher water content. Generation 1 seed coats were brown and thin, with a higher phenolic concentration. These seed coat characteristics are likely to contribute to, if not being the reason for, the difference in germination percentage in avocado seed, as the younger generations always had a higher germination rate than older generations.

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CHAPTER SIX

MORINGA OLEIFERA LEAF EXTRACTS AFFECT AVOCADO (PERSEA AMERICANA MILL.) SEED PHENOLICS, SUGARS AND STARCH, AS WELL AS GERMINATION

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Abstract

Avocado (*Persea americana* Mill.) seedlings, obtained from commercial avocado seeds, are used as 'nurse seed' to graft the clonal rootstock, that is subsequently combined with the cultivar. A healthy nurse seed, growing into a vigorous seedling is, therefore, of paramount importance. This experiment was carried out on avocado seeds of different maturity stages to determine, if moringa leaf extract (MLE) application can enhance seed germination. The experiment was laid out in a completely randomized factorial design with MLE applied at various concentrations (0, 2.5, 5.0 and 7.5 % w/v MLE) over different periods (0, 10, 30 or 120 minutes) of soaking. Thereafter, seeds were placed onto agar to monitor germination and radicle development daily. Additionally, phenolic compounds and starch, as well as sugar concentrations were determined after the 2-hour soaking period. Seed germination occurred from 18 to 23 days; this event was not influenced by MLE application. There is a significant positive relationship between Low seed coat phenolics were aligned with a higher germination percentage, ($r= 0.23$), ($p <0.008$), particularly at MLE 2.5% and 5.0%. Cotyledonal sugar and starch concentrations were neither affected by MLE concentration nor by soaking duration. Application of MLE at high concentration (7.5% w/v) had an inhibitory effect on germination and resulted in lowering cotyledonal starch levels. Germination percentage was positively influenced by all MLE applications, but phenolic compounds, sugar and starch concentrations were neither affected by MLE concentration nor soaking time. The MLE effect on germination velocity subsided with seed age (lowering from April to May to June). Application of 2.5 or 5% MLE could, therefore, be developed as a viable pre-treatment of nurse seed in the avocado industry, particularly for seed from the early season.

Keywords: Seed maturity, soaking time, cracking, cotyledons

6.1. Introduction

Avocado (*Persea americana* Mill.) seeds are used as 'nurse seed' and the clonal rootstock is grafted onto these, which, in turn, has the clonal cultivar grafted onto them. This seed also assists in the development of a strong initial root system of the tree that leaves the nursery (Castro and Fassio, 2013; Ernst *et al*., 2013). To produce a healthy avocado tree that leaves the nursery, a healthy, vigorous nurse seed, is therefore, important. Such vigour could be supplied by moringa leaf extracts (MLE), as moringa leaves contain various phytochemicals (proteins, fibre, carotenes, choline, thiamine, nicotinic acid, amino acids) and various minerals (Bau *et al*., 1994; Sarkar and

Peace, 1994), that, together with other compounds found in moringa leaves (zeatin, ascorbate, phenolic compounds, carotenoids, calcium and potassium), could act in enhancing seed germination and initial seedling growth (Fuglie, 1999; Basra *et al.*, 2011). Many of the phytonutrients in MLE act as antioxidants and are found in chloroplasts and other cellular compartments; these compounds are crucial to defend plants against oxidative stress (Noctor and Foyer, 1998). The aim of this study was, therefore, to evaluate the effect of various concentrations of methanolic moringa leaf extracts (MLE) combined with different soaking durations on growth and development of 'Fuerte' avocado seeds.

6.2. Materials and Methods

The investigation was carried out at the University of KwaZulu-Natal, Pietermaritzburg during April, May and June 2019. 'Fuerte' seeds used in this study were extracted from fruit obtained from a commercial orchard in the KwaZulu-Natal Midlands (30˚6_E and 29˚28_S, South Africa). The green, mature fruit were kept at room temperature until fruit started to soften (tested by gently pressing the fruit), so the seed could be extracted. Experiments were carried out on 100 fruit per month and results averaged over the 3-month period.

6.2.1. Preparation of Moringa Leaf Extract (MLE)

Moringa oleifera leaf powder was obtained from Run-X KZN (Pietermaritzburg, South Africa) and stored in paper bags at room temperature. Dried moringa leaf powder (100g) was soaked in 1L methanol at room temperature (20 ± 2 C) for 24 hours with occasional shaking. The extract (MLE) was filtered through Whatman No.1 filter paper and the following treatments applied to the seed: soaking seeds in three concentrations of MLE (2.5, 5.0 and 7.5% of MLE, in addition to the control (soaking in distilled water) and soaking for three different durations (10, 30 and 120 minutes).

6.2.2. Germination Percentage

The avocado seeds were placed into plastic containers with agar (12 g L^{-1}, ACE, South Africa) and covered with aluminium foil to allow germination. The treatments were arranged in a completely randomized factorial design with three replications. Containers were incubated in a

growth room at 25°C for 23 days. Measurements recorded were days to germination and observed seed cracking, seed coat phenolic, sugar and starch concentrations in the seed cotyledons.

6.2.3. **Determination of Phenolic Compounds in Avocado Seed Coat**

The concentration of phenolic compounds was determined using a Folin-Ciocalteau method (Blainski *et al.*, 2013), after soaking, prior to germination. The extracts (0.5 g ground sample in 80% acetone) and take (5ml) from solution and kept for 30 min at room temperature, the solution filtered through Whatman No.1 filter paper. Subsequently, samples were centrifuged at 20000 *g* for 10 min, then 2.5 mL of the extract was mixed (0.1 ml) with 0.2 N Folin-Ciocalteau reagent (Sigma–Aldrich/Fluka, St Louis, MO, USA), for 5 min, followed by addition of 2.0 ml 75 g L^{-1} sodium carbonate. The absorbance of the samples was measured at 760 nm against a reagent blank using a UV–Vis spectrophotometer SP 8001 (Metertech Inc, Taipei, Taiwan) after 2 h incubation at room temperature.

6.2.4. **Determination of Sugar Concentration in Avocado Cotyledons**

The concentrations of sugars were determined according to Liu *et al*. (1999). Briefly, freeze-dried material (0.05 to 0.10 g) was mixed with 10 ml 80% (v/v) ethanol and homogenized for 1 min. Thereafter, the mixture was incubated in an 80°C water bath for 60 min and then kept at 4°C overnight. On the following day, after centrifugation at 12000 *g* for 15 min at 4°C, the supernatant was filtered through glass wool and taken to dryness in a vacuum concentrator. Dried samples were re-suspended in 2ml ultra-pure water and centrifuged at 10000 *g* for 5 min. Samples were filtered through 0.4 µm nylon syringe filters and injected into an isocratic HPLC system (Tesfay *et al*., 2010).

6.2.5. **Starch Determination in Avocado Cotyledons**

Cotyledonal starch was determined according to Raigond *et al.* (2015). Samples (0.2g DM) were extracted in 80% ethanol overnight before centrifugation at 10000 *g* (5 min). The supernatant, containing free sugars, was collected and the pellet re-extracted twice. Both supernatants were combined, and the sample made up to a final volume of 100 ml with deionised water. For colour development, 50 µl sample and 900 µl distilled water were boiled in presence of 2 ml anthrone-sulphuric acid reagent (200 mg anthrone in 100 ml chilled concentrated sulphuric acid, Sigma–

Aldrich/Fluka, St Louis, MO, USA) for 8 min, the samples were cooled to room temperature and the absorbance recorded at 620 nm. The starch concentration was calculated by using a glucose standard curve.

6.2.6. Statistical Analysis

Analyses of variance (ANOVA) were performed using GENSTAT (version18.2; VSN international, Hemel Hempstead, UK). Least significant difference was used to compare means of traits with $p \leq 0.05$ regarded as significant. The values of all parameters were subjected to statistical analysis (Gomez and Gomez, 1984). The 'F' test was applied to assess the significance of each treatment at the 5% level of probability ($P \leq 0.05$).

6.3. Results and Discussion

6.3.1. Germination Percentage

The germination percentage (GP) of 'Fuerte' avocado seed was significantly ($P < 0.001$) affected by applying different MLE concentrations and different soaking times during seed age (Figure 1). In seed collected in April and June, the GP decreased with an increase in MLE concentration (from 2.5 or 5% to 7.5%); similarly, increasing the soaking time (from 10 or 30 min to 120 min) in April significantly ($P < 0.001$) increased GP. The younger seed (harvested in April) was stronger affected by the increased MLE concentration. Extending the soaking time of May seed increased GP only when using 2.5% MLE. In June, the 2.5% MLE only had a tendency to enhance the GP over the other MLE concentrations. Overall, the highest GP (66.9%) was found in seeds harvested in June soaked in 2.5% MLE. The lowest GP (21.07%) was determined for April seeds soaked in 7.5% MLE concentration. During the experimental period (over the months April, May and June) a higher GP was found in June than in April and May seed at all soaking times and MLE concentrations compared with the control. Extending the soaking duration to 120 min in 7.5% MLE resulted in a reduction in GP (Figure 1). It is likely that MLE enhanced seed germination due to the growth-promoting plant hormones present in MLE (Yasmeen *et al.*, 2013; Rehman *et al*., 2014); however, when the soaking time was extended to 120 min, germination decreased when using the high (7.5%) MLE concentration. This, again, could be a hormonal effect, with the high cytokinin's and GAs present in the MLE exerting an inhibitory effect on germination (Blumenfeld and Gazit, 1970).

While in the young seed (harvested in April) the response to soaking time and MLE concentration differed significantly between treatments ($LSD_{0.05}$= 5.6), this variation was reduced in the following months. Seeds from fruit picked in June had the highest GP, possibly due the relatively advanced maturity, allowing the nutrients and vitamins in the MLE (Begum *et al.,* 2009) to assist in seed germination. During soaking in MLE, certain compounds present in MLE (potassium, nitrogen, calcium and ascorbate) could have become available to the embryo (Farooq *et al.,* 2010). These nutrients and phytochemicals could have enhanced germination (observed as seed cracking and radicle emergence) and the subsequent development of the seedling. The long soaking time (120 min) had an inhibitory effect on GP (Figure 1), possibly due to anaerobia setting in over that extended soaking period, hindering germination (Hala *et al*., 2017).

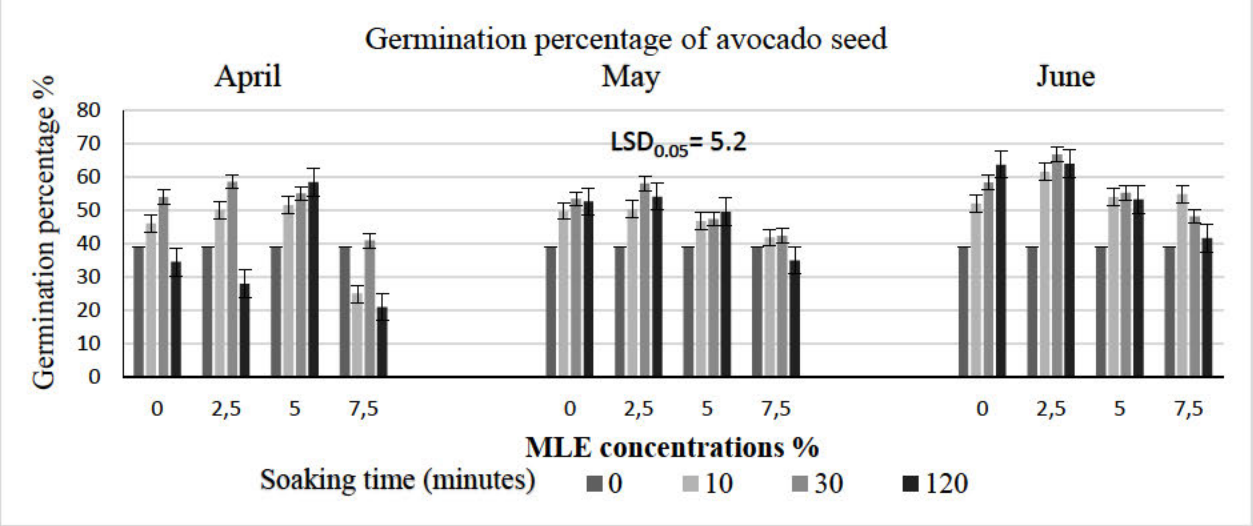

Figure 1. Effect of different concentrations of MLE and different soaking times on the germination percentage of 'Fuerte' avocado seed

6.3.2. Phenolic compound Concentration in Avocado Seed Coats

In avocado seeds, phenolic compounds occur predominantly in the seed coat (Figueroa *et al.,* 2018). Phenolics are known to either hinder or promote germination (Williams and Hoagland, 1982). A tendency towards a higher phenolic concentration in seed coat tissue was noted in the 7.5% MLE treatment in fruit harvested in April. There was no significant effect of soaking time on phenolic concentration (Figure 2). The interaction between MLE concentration and soaking time was significant; there also was a significant difference in seed coat phenolic concentration

between months, ($P < 0.001$). Seed coat phenolic concentrations were generally low (2.07 to 0.87 mg g^{-1}DM), with the highest amount found after soaking seeds in 7.5% MLE for 120 min. The 7.5% MLE treatment and 120min soaking time had the highest phenolic concentration and the lowest germination percentage (compare Figure 1 and Figure 2), suggesting that the phenolics present in the seed coat may contribute to inhibiting germination.

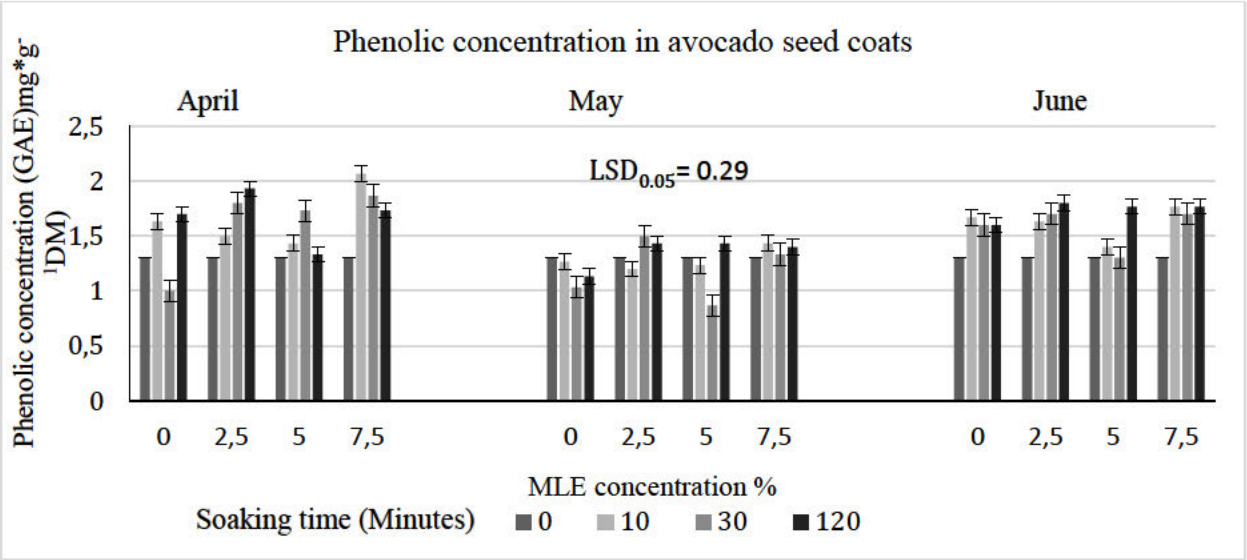

Figure 2. Effect of MLE and soaking time on phenolic compounds in "Fuerte" avocado seed coats

6.3.3. **Starch Concentration in Avocado Seeds**

Starch is a common food reserve in seeds; it is distributed equally between cotyledons and the embryonic axis (Dkhil and Denden, 2010). Avocado seed, consisting mainly of the two large cotyledons, has been reported to contain about 74% starch, depending on the avocado cultivars (Orhevba and Jinadu, 2011), while, on the other hand, an amount of 30% has been claimed (Olaeta *et al*., 2007). In our study avocado seed contained between 61.8% and 33.8% starch (Fig. 3). Soaking seeds in various MLE concentrations did, not show a clear tendency on seed starch. The highest starch concentration for treated seed was detected in June, before soaking and after 10 min soaking time at 5% MLE concentration. The amount of starch was relatively high in April with control (65.5%), the percentage of starch in June was (58.4%) and the lowest amount was in May (34.1%) (Fig. 3). This study found the 7.5% and the 120min MLE treatment to result in a

low amount of starch; the highest starch concentration was detected following 10- and 30-min soaking (Fig. 3). The starch extract was brown due to the presence of phenolic compounds, in line with Chandra *et al.* (2013) who discovered high amounts of the phenolic 3,4- dihydroxy phenylalanine in avocado seeds. The statistical analysis showed MLE concentrations and different soaking times to have a positive, significant effect on starch concentration compared with control.

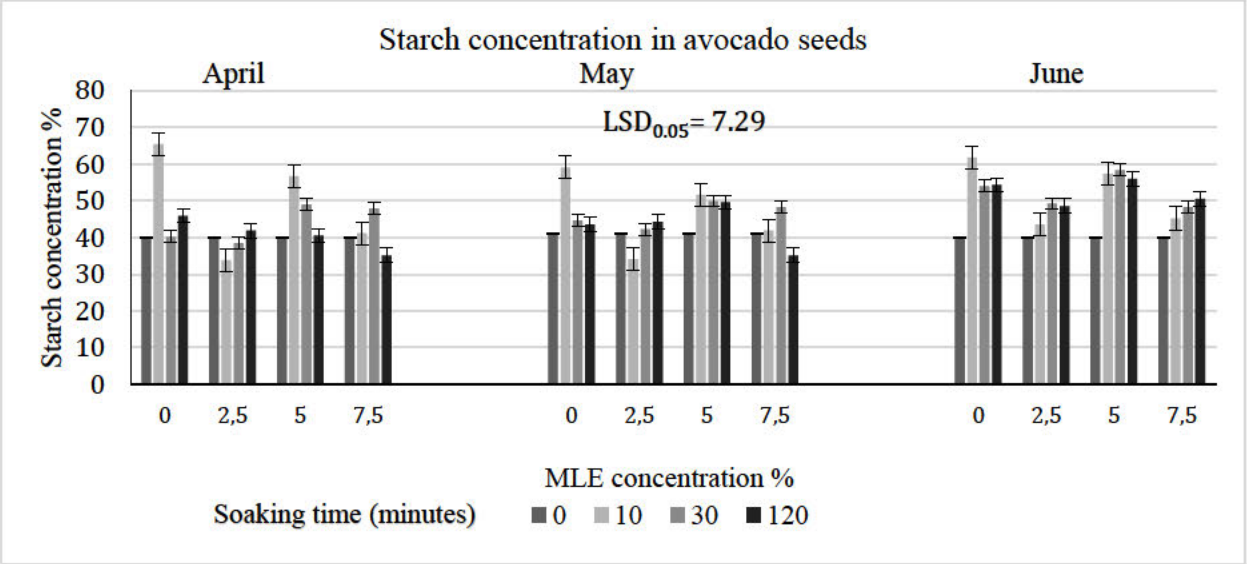

Figure 3. Effect of MLE concentration and soaking time on starch concentration in 'Fuerte' avocado seeds

6.3.4. **Sucrose Concentration in Avocado Seeds**

The sucrose concentration (3.5 to 5.5 mg g^{-1} DM, Figure 4) in avocado seeds was higher than the glucose and mannoheptulose concentrations, indicating the potential influence of sucrose in avocado seed development (Liu *et al.,* 1999). Sucrose may act as the transport sugar in avocado seed, while the C7 sugars possibly play a major role in seed development.

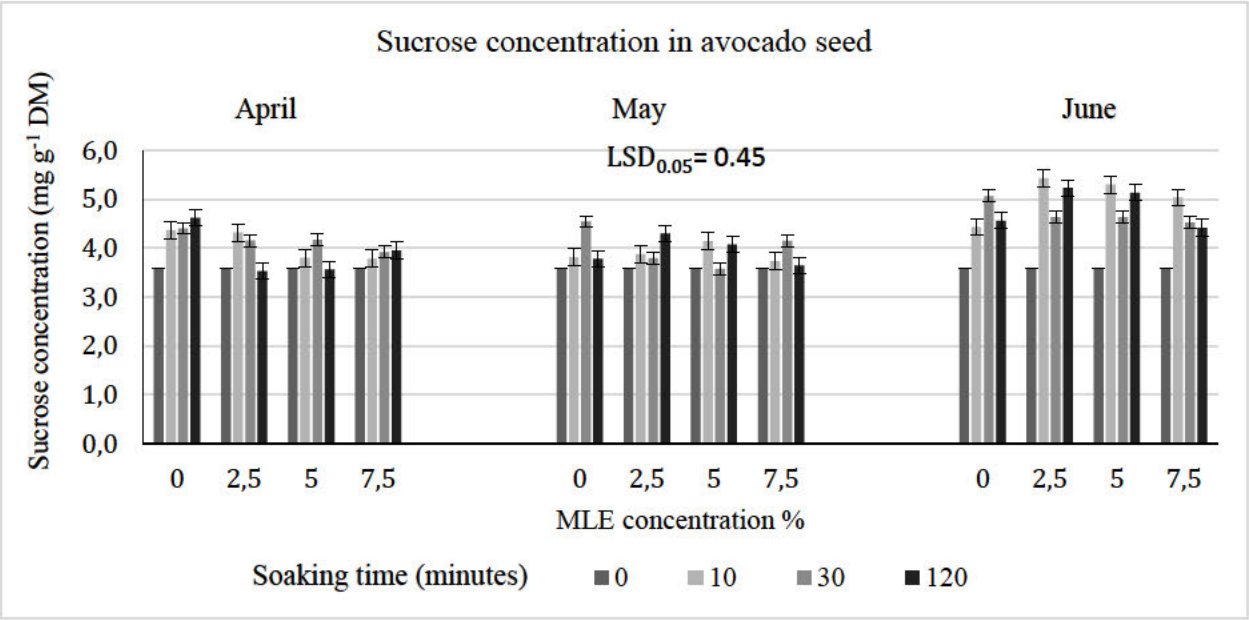

Figure 4. Sucrose concentration in 'Fuerte' avocado seed treated with different concentrations of MLE for different soaking durations

6.3.5. **Glucose Concentration in Avocado Seeds**

The analysis of variance showed that the glucose concentration of avocado seeds was not significantly affected, neither by MLE concentration nor by soaking time (data not shown); therefore, the glucose concentration seems not related to avocado seed germination percentage. Similarly, soaking time did not significantly affect the glucose concentration; thus, the MLE concentrations used, and the extent of the soaking time did not have any positive effect on seed germination ($p > 0.05$) of 'Fuerte' avocado seeds.

6.3.6. **Mannoheptulose Concentration in Avocado Seeds**

The mannoheptulose concentration found in 'Fuerte' seeds was generally low (1.79 to 1.21 mg g^{-1} DM) compared with other tissues reported to contain low concentrations in mesocarp (Tesfay *et al.,* 2010). This mannoheptulose concentrations was not affected by MLE treatment, with no significant difference between seed treated with different MLE concentrations for various soaking times ($p < 0.01$) (data not shown).

6.3.7. **Perseitol Concentration in Avocado Seeds**

Perseitol in the avocado seeds was present at higher amounts than any other sugar (Figure 5), confirming results by Liu *et al.* (1999) and Tesfay *et al.* (2012). Perseitol concentrations in the seed after soaking in MLE at various concentrations and for various times were positively affected by all MLE treatments; however, these elevated perseitol concentrations did not fast-forward germination, ($r = 0.52$), ($p \leq 3.38$) and there is no significant positive relationship between perseitol concentration and germination percentage (Figure 1). The pool of the dominant sugar, perseitol, was not reduced by any of the treatments. Statistically, there were no significant differences between MLE treatments, but between these treatments and the control.

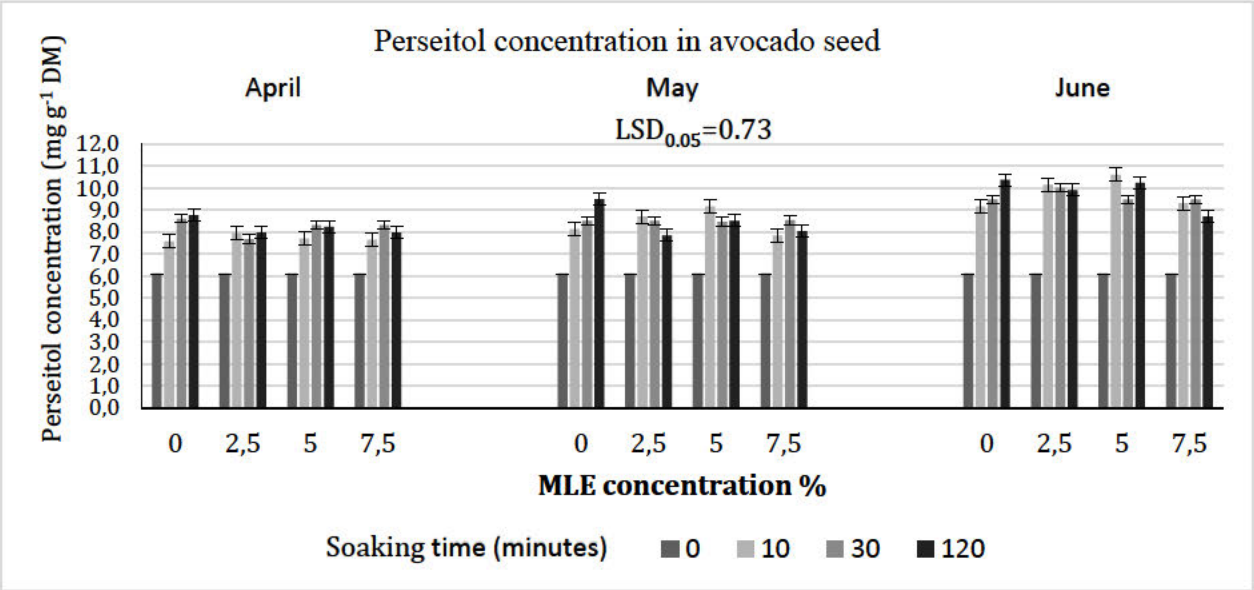

Figure 5. Perseitol concentrations in 'Fuerte' avocado seeds treated with different concentration of MLE at different soaking durations

6.4. Conclusion

Moringa oleifera leaf extract applied as a soaking treatment to 'Fuerte' avocado seeds was not able to enhance the germination percentage, although an improvement of germination factors has been reported in other crops. In fact, germination percentage decreased after 120 min of soaking and application of 7.5 % MLE.

A deeper investigation into germination-related parameters (sugars, starch) did not relate to percentage germination. Phenolics, however, were related to germination, as high MLE concentrations resulted in high phenolics and lower germination percentage. The additional layer covering the seed due to moringa application might have inhibited germination.

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CHAPTER SEVEN

General Discussion, Conclusion and Outlook

Avocado is an evergreen fruit grown worldwide; as a subtropical crop, it is grown in the Limpopo and Mpumalanga provinces in the North-East of the country, but production has also expanded into KwaZulu-Natal and the Eastern and Western Cape provinces (up to 33 °S). The species is very variable, consisting of different ecological races. Avocado fruit have become popular worldwide due to their nutritional value, as they are a source of healthy fats as well as minerals, vitamins and special carbohydrates (Majid *et al*., 2020). The seed of the avocado is non-edible and is usually discarded (Adaramola *et al.,* 2016). Avocado seeds are, however, used as 'nurse seed' for clonal rootstock production, as the clonal rootstock is grafted onto this seedling rootstock (Reeksting *et al*., 2014). This 'nurse seed' also assists in the development of a strong initial root system of the tree that leaves the avocado nursery to produce a healthy avocado tree (Ernst *et al*., 2013). Nurseries grafting avocado trees are, therefore, interested in a healthy, vigorous 'nurse seed'. Avocado 'nurse seed' is used as part of the double-grafting method to establish orchard trees (Fassio *et al.,* 2016).

This thesis described experiments using avocado fruit of three generations to determine the most-suitable seed age at which seeds could best be used as 'nurse seed'. Whitsell (1989) reported that physiological age of avocado seed is very important to determine, as different seed ages germinate at different speeds and are, therefore, more or less suitable for grafting the rootstock onto.

In this study it was demonstrated that the seeds extracted from fruit of two cultivars 'Hass' and 'Fuerte' of three generations were analysed; Generation 1, over-mature fruit, 24 to 29 months after full bloom (MAFB), Generation 2, mature fruit, 12 to 17 MAFB and Generation 3, newest fruit, 0 to 5 MAFB, all generation were found in same trees. Germination percentages in Generation 1 was lower compared with Generation 2 and 3, from June to September and Generation 3 the youngest seeds from October to November. Older seeds had more phenolics and PPO may be effect germination.

The present work was, therefore, aimed at determining the quality of avocado seed of the 'Hass' and 'Fuerte' cultivar, over three successive generations. Characteristics of seeds of several generations, as well as germination characteristics of such seed and their physiological parameters were analysed throughout seed development.

The dissertation is, hence, made up of six chapters:

Chapter 1: General Literature Review

Chapter 2: Seed Germination of 'Hass' and 'Fuerte' Avocado as Affected by Seed Coat Thickness and Seed Coat Phenolic Compounds

Chapter 3: Seasonal Variations in Avocado Seed Viability and Respiration Rate Affect Germination Percentage

Chapter 4: Seasonal Alterations in Polyphenol Oxidase Activity and Phenolic Compounds of The Avocado Seed Coat Over Several Seed Generations

Chapter 5: Phenolic Compound Concentrations and Ultrastructural Changes of The Seed Coat Throughout Avocado Seed Maturation

Chapter 6: *Moringa oleifera* leaf extracts affect avocado (*Persea americana* Mill.) seed phenolics, sugars and starch, as well as germination

Following a review (**chapter 1**) on avocado seed development and seed physiology, **chapter 2** describes the germination percentage of two cultivars, 'Hass' and 'Fuerte', as observed over three seed generations. Germination of seeds differed significantly between the Generation 1 and Generation 2. Seed of Generation 2 and 3 of both cultivars were more viable (higher germination percentage) and seeds were viable (stained red using the TTZ test) compared with seeds Generation 1 (lower viability and lower germination percentage). Generation 3, similarly, had higher viability and germinated better than Generation 2 in both cultivars. The seed coat of Generation 2 of both, 'Hass' and 'Fuerte' seeds was thicker, and the germination percentage was higher than that of older generations. Therefore, this study points out that the thicker seed coats of Generation 2 are aligned with a higher germination percentage. A thicker seed coat possibly contains more compounds, but not phenolics that contribute to a faster germination, so that seeds with thicker seed coats germinated faster. Phenolic compounds could have been involved in suppressing germination of the avocado seed; germination of avocado seeds seems to be modified by the phenolics contained in the seed coat. A higher phenolic concentration Generation 1 seems aligned with a lower germination percentage, while seed coat Generation 2 with lower phenolic concentrations germinated better. Generation 3 seed coats had a lower phenolic concentration than Generation 2 seed coats and this lower phenolic concentration resulted in a higher

germination percentage. Throughout seed development a higher germination percentage was recorded in younger seeds.

In chapter 3, starch and soluble carbohydrate concentrations of the seed were analysed. The avocado seed Generation 2 of both cultivars contained about 90% starch. A deeper investigation into germination percentage-related parameters (cotyledon sugar and starch concentrations) revealed that these cotyledonous sugar and starch concentrations are related to the germination rate. Younger seed generations had higher starch concentrations and higher C7 sugar concentrations coupled with a higher germination percentage than the older generations. Avocado seed contains C6 and C7 reserve carbohydrates to maintain its development; these carbohydrates are possible energy sources for the germination process. The dominance of the C7 sugar perseitol and the high starch concentration in younger avocado seed tissue indicates their importance in early the avocado seed development, while the C6 sugars are present in higher concentrations in older seed. Seed moisture content, also investigated in this chapter, was related to germination percentage, as higher moisture content resulted in higher germination in younger seed. A lower seed moisture content in older seed was, on the other hand, aligned with a lower germination percentage. This could possibly explain why the mature, high moisture-containing seed had a higher germination percentage; therefore, avocado seed germination in relation to seed moisture percentage, should be investigated.

In chapter 4, polyphenol oxidase activity (PPO) and phenolic concentration of different physiological ages of avocado seed coats were evaluated throughout avocado fruit development. In this study the development of seed coats was followed by comparing three generations of two cultivars over a certain developmental period. Although PPO concentrations were different between generations, decreases and/or increases of phenolic and PPO concentrations occurred in the same months. Phenolic compound metabolism is driven by the activity of PPO enzymes; when seed coats had a high PPO concentration, such as in ‘over-mature’ seed, the phenolic concentration was also high and seed germination was lower than that of ‘mature’ seed. The seed coat concentration of PPOs was lower when the phenolic concentration was lower, too. Seed germination was higher in seeds from the Generation 2 than that of Generation 1 The lower level of phenolics in mature seed coats suggests the importance of phenolic compounds in germination, seemingly the higher phenolic concentration in over-mature seed coats resulted in a lower

germination rate. In addition, the higher concentration of phenolics in Generation 1 seed could be due to phenolic oxidation by the polyphenol oxidase (PPO) enzyme, which showed higher concentrations during seed development; therefore, it will be crucial to investigate PPO activity in seed coats of various maturities in the future.

In chapter 5, scanning electron microscopy (SEM), a tool used to determine the ultrastructure of biological samples in order to identify and characterize plant tissues, was employed to examine seed coat characteristics and to reveal the seed micro-structure. In avocado seed coats Generation 1 of 'Hass' and 'Fuerte' a high concentration of phenolic compounds was detected. Generation 1 seed coats were characterised by a rather thinner, dark seed coat, composed of cells with small vacuoles, while seed coats Generation 2 of both cultivars had lower phenolic concentrations and a thicker seed coat. The SEM images of the outer (layer facing mesocarp) and inner (layer facing cotyledons) side of the avocado seed coats clearly depicted that the younger seed coat is covered by a thicker and white wax layer, due to higher moisture/water content and other compounds as well as much lower phenolic concentrations, while in older seeds the cell walls of the seed coats are thin and brown, due to higher phenolic concentrations.

In chapter 6, it was demonstrated that soaking seeds in *Moringa oleifera* leaf extracts (MLE) can enhance avocado seed germination. Seeds were soaked in various concentrations of MLE (0, 2.5, 5.0 and 7.5 % w/v MLE) over different periods (0, 10, 30 or 120 min). The younger seed was affected more so by the increased MLE concentration; the 2.5% MLE concentration tended to enhance germination percentage over other MLE concentrations, and the lowest germination percentage (GP) was determined for seeds soaked in the 7.5% MLE concentration. Extending the soaking duration to 120 min in 7.5% MLE resulted in a reduction in GP. Soaking in MLE enhanced seed germination, probably due to the growth-promoting plant hormones present in MLE. *Moringa oleifera* leaf extract, applied as a soaking treatment to 'Fuerte' seeds, was not able to enhance the germination percentage, although an improvement of germination parameters has been reported in other crops. A deeper investigation into germination-related parameters (sugars, starch) revealed that the concentration of these compounds does not relate to percentage germination. Phenolics, however, were related to germination, as high MLE concentrations resulted in high phenolics and lower germination percentage. The soaking process could have added a 'moringa layer' covering the seed that might have inhibited germination.

Overall, this thesis has demonstrated that the timing, when a 'nurse' seed is extracted from, the avocado fruit is important for seed industry, it is uncertain, at which exact time the seed should be extracted. This timing of fruit, and, therefore, seed harvest, affects the germination percentage. While the seed needs to be mature enough to provide sufficient energy/ resources for the growth and development of the rootstock that is grafted onto it, earlier harvest could be beneficial from a grafting timing perspective. Future studies could explore the most suitable seed harvesting time and investigate, why germination of the 'nurse seed' is better at certain times, by following the concentration changes in carbohydrates and oils of the avocado seed over the seasons. The use of tools to analysis seed coat features, such as SEM, may provide an understanding of the interaction between cultivars and the environmental variables in relation to seed quality and seed coat appearance, as the seed coat structure is important to allow germination to occur.

The timing of optimal avocado seed germination and commercial fruit harvest do not coincide. Avocado nurseries must collect fruit from commercial orchards long before commercial fruit maturity. The seed germinates better, while fruit have not reached harvest maturity; therefore, 'nurse seed' should be harvested and planted, before the avocado fruit is ready to be harvested.

Seeds from previous season fruit do not germinate as well as seeds from commercially immature fruit. It should be investigated, if removing the seed coat from the older seed will enhance germination, as the seed coats contains several compounds that could inhibit germination. Alternately, younger seeds from commercially immature fruit should be used as 'nurse seed, as their seed coat is rich in minerals, enzymes and other compounds able to easily transfer solutes that stored in the cotyledons to the embryo. Younger seeds have higher water content, that assist seed germinate immediately after removed it from fruit, these seed easy to loss viability if kept long time after harvest. The Moisture content of the seed (cotyledons) seems to be associated with avocado seed quality. Moisture content of the seed is, in a way, a resource for the developing embryo, if it is able to extract the water from the seed; seed moisture content decreased during the observation period, so maybe the lack of moisture in later stages disallowed germination, despite the embryo actually being ready to germinate. That would be an interesting study.

The moringa leaf extract (MLE) applying in different avocado seed ages results in a significant increase in germination percentage in young avocado seeds. It would be interesting to investigate, if treating seeds with moringa extracts has different effects at different seed ages. Further it would

be worth investigating, which compounds in the moringa extracts positively affect seed germination. Seed age alters the response of the avocado seed to moringa extracts; therefore, further research should determine, which moringa concentrations best invigorate seedling growth at what seed ages. The MLE solution may well have more so an effect on older seed that does not germinate easily, possibly providing plant hormones, to kick-start seed development. Combining moringa with other germination enhancing treatments, might also have beneficial effects, as moringa contains plant growth substances that might boost germination. This will be particularly important for organic food production, where the use of artificial growth regulators is prohibited.

In this study, we investigate two cultivars (‘Hass’ and ‘Fuerte’) are most important avocado cultivars growing in KwaZulu- Natal South Africa, use those cultivars for grafting to produce good guilty of fruit. ‘Hass’ is early cultivar and growing in wide area ‘Fuerte’ is late cultivar the commercial harvest time for avocado in June/July for ‘Hass’ and July/ August for ‘Fuerte’. The main factor effect germination percentage is concentration of phenolics and PPO activity on seed coat its inhibited germination for both cultivars, younger seeds always had lower amount of phenolics and PPO and had grater germination rate during observation period. The suitable age for ‘nurse seed’ industry to use younger seeds 12 MABF and 5 to 6 MAFB. Phenolics and PPO concentration are act as inhibiter for may seed exp a guava, Orange and legumes, it’s good to use immature/younger seeds for nursery.

Understanding the seed physiological (respiration, carbohydrates, oil usage), biochemical (effect on enzymes necessary for germination), and molecular processes (process of transcription, replication and translation of the genetic material from the parent to new generations) would also be useful to identify genes aligned with production of a strong, healthy seed that can be used as a ‘nurse seed’. These strong and healthy seed will form the basis for establishing avocado orchards, so that strong trees develop which, in turn, will produce high quality fruit; thereby increasing avocado production worldwide and providing nutritious food for a growing world population.

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