FLOWERING, FRUIT GROWTH AND INTRACELLULAR STORAGE COMPONENT FORMATION IN DEVELOPING *MORINGA OLEIFERA* LAM. SEED AS INFLUENCED BY IRRIGATION

by

Quintin Ernst Muhl

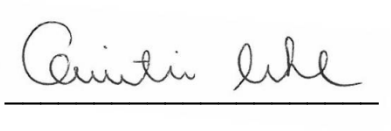
Submitted in partial fulfilment of the requirements
for the degree PhD Horticultural Science
In the Faculty of Natural and Agricultural Sciences
University of Pretoria
PRETORIA

Supervisor:	Prof. E. S. du Toit
Co-supervisors:	Prof. J. M. Steyn
	Prof. Z. Apostolides

June 2014

DECLARATION

I, the undersigned, hereby declare that the thesis submitted herewith for the degree PhD Horticultural Science at the University of Pretoria, contains my own independent work and has not been submitted for any degree at this or any other tertiary institution.



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ABSTRACT

Moringa oleifera has become increasingly popular as an industrial crop in recent times due to its multitude of useful attributes as a water purifier, nutritional supplement and biofuel feedstock. Besides this, *Moringa oleifera* has also demonstrated high tolerance to sub-optimal growing conditions, particularly towards drought. As a result, the current and anticipated cultivation areas of this tree are in medium to low rainfall areas. To what extent this drought tolerance was at the expense of reproductive development remained unclear. This study therefore aimed to assess the effect of different irrigation rates on flowering, fruit growth and storage compound synthesis of *Moringa oleifera*. Established *Moringa oleifera* trees were subjected to three different irrigation treatments simulating total annual rainfall

amounts of 900 mm/annum (900IT), 600 mm/annum (600IT) and 300 mm/annum (300IT). Irrigation was administered through surface drip irrigation, while semi-weekly soil water measurements using a neutron probe were performed at several depths to ascertain differences between treatments. After having exposed trees to the different irrigation rates for nine months, the treatment effects were assessed. Firstly, individual inflorescences from each treatment were tagged during floral initiation and monitored throughout until fruit set. Flower bud initiation was 65.3% higher at the 300IT and 4.6% higher at 600IT compared to the 900IT. Fruit set however, was 22.0% lower for the 300IT and 4.4% lower for 600IT, compared to the 900IT. Floral abortion, reduced pollen viability as well as moisture stress in the style were contributing factors to the reduction in fruiting/yield observed at the 300IT.

Subsequently, microscopic studies of developing seed were performed to better comprehend storage compound biosynthesis and accumulation throughout seed growth. From these studies, the endosperm was found to be nuclear, becoming cellular at a fruit diameter of ±6 mm from the micropylar side towards the developing embryo. At a fruit diameter of ±8 mm the cellular endosperm had covered the entire inner integument, which coincides with the developing embryo reaching the globular stage. Cotyledon development commenced at a fruit diameter of ±12 mm and continued up until ±24 mm. At the end of this phase the cotyledons had filled the entire seed coat, while the unicellular epidermal layer of the inner integument remained distinctly visible between the cotyledons and the testa. Fruit growth measurements throughout the first 60 days after flowering (DAF), revealed a significant reduction in fruit growth rates and final fruit size with decrease in irrigation rate. The number of mature fruit, average seed count and time to maturity however,

increased with the rate of irrigation. Average seed mass also increased from the 300IT to the 600IT, but decreased again between the 600IT and the 900IT.

As photosynthesis is central to plant growth and development, the extent to which the irrigation treatments affected photosynthesis and ultimately tree performance had to be assessed. Photosynthesis measured during the vegetative, flowering and fruit development stages revealed not only a reduction in photosynthetic activity throughout the growing season, but also with reduction in irrigation rate. Lower photosynthetic rates were primarily as a result of stomatal (conductance and SI) and non-stomatal (possible RuBP regeneration, ATP synthesis and mesophyll conductance) limitations, while the decreases observed throughout the growing season were as a result of diminishing leaf chlorophyll concentrations.

Harvested fruit were categorized according to their diameter (Ø0 mm - 28 mm) at 2 mm increments and the compositional changes monitored at each irrigation treatment. Starch was the first to accumulate during the initial histo-differentiation phase (Ø0 mm - 12 mm), while both oil and protein levels remained comparatively low. During the subsequent expansion phase (Ø12 mm - 24 mm) however, stored starch was remobilized and used in oil biosynthesis, thereby reducing the starch content percentage. Most of the oil and protein reserves were synthesized during this phase. As fruit reached their final maturation phase (Ø24 mm - 28 mm), the average oil content percentage was 24.8%, while the protein content percentage was 24.7% and the starch content percentage was 8.8%. The different irrigation treatments did not influence the final content percentage of the seed components as much as it did affect the time and rate of their synthesis throughout seed

development. By using both light and electron microscopy in conjunction with histochemical staining techniques, the intracellular locality of storage compounds as well as the initiation of their synthesis could be determined. The storage compound detecting stains were; Sudan III (oil), Light Green SF (protein), Orange G (protein) and Periodic Acid-Schiff's reagent (starch). During early seed development (fruits of 8 mm in diameter), starch was synthesized by the plastids and transiently stored in the cell periphery. Protein and oil bodies were synthesized in association with the endoplasmic reticulum and finally stored in the centre of cotyledonous cells. Protein and oil body formation only commenced in significant amounts inside cotyledons at a fruit diameter of ±14 mm. Intracellular protein bodies were largest at between ± 5 µm to 8 µm in diameter while oil bodies ranged from ± 0.2 µm to ± 1 µm in size. Identification of numerous intracellular compounds using histochemical staining proved very effective. This prompted further investigation into whether storage compound content could be quantified from stained sections using digital imaging software. The percentage stain coverage area calculated using Adobe® Photoshop® was compared to the content of the same compound determined analytically at the corresponding developmental stage. The best correlation between measured seed storage compound and stain coverage was observed for the protein and oil detecting stains, Orange G and Sudan III throughout seed growth. A stage specific factor based on seed mass was however necessary in order to estimate storage compound content (g) from image analysis results.

Conclusively, results from this study suggest that moderate water stress prior to flower development encourages floral initiation. Irrigation should however be resumed once flowering has commenced to ensure good pollination, fruit set and

yield. Between the fruit diameters of 12 - 24 mm, the majority of storage reserves were synthesized and as a result this stage of fruit development was found most susceptible to water stress. Increased irrigation rates not only shortened the time to storage compound synthesis initiation but also increased their synthesis rate. Reduced irrigation in contrast, delayed the onset of oil biosynthesis, and as a result starch levels continued to increase reaching much higher levels prior to its remobilization during oil biosynthesis. The highest oil content percentage and seed mass at maturity were measured at the intermediate irrigation treatment. Along with decent flowering and fruit set, 600 mm/annum appeared to be the most suitable irrigation/rainfall amount for *Moringa oleifera.* This irrigation amount was low enough to initiate floral initiation, but also sufficient to sustain the subsequent seed developmental processes. Digital image stain quantification proved reasonably effective for simple comparisons between storage compound contents at different developmental stages. Differences between storage compound contents of the same developmental stage from different irrigation treatments were however, not as clearly distinguishable using this method. Although the success of digital image analysis might be stain dependant, it certainly is a cost effective technique for compound content estimation.

This study enabled the identification of sensitive stages throughout fruit/seed development, by providing detailed insight into oil, starch and protein biosynthesis. Ultimately these findings provide current and prospective Moringa growers with recommendations to ensure suitable site selection as well as irrigation management guidelines.

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It is with the Watchword for 2014 “But as for me, it is good to be near God” Psalm 73.28, that I want to sincerely thank my Lord and Saviour for being my source of strength and blessing me in countless ways.

RESEARCH OUTPUTS

To date, the research outputs from this study are as follows:

Paper Presentation:

DU TOIT, E.S., MUHL, Q.E., STEYN, J.M. & APOSTOLIDES, Z. (2012) Bud Development, Flowering and Fruit Set of *Moringa oleifera* Lam. (Horseradish Tree) as Affected by South African conditions. 109th Annual International Conference of the American Society for Horticultural Science, Miami, Florida, USA.

MUHL, Q.E., DU TOIT, E.S., STEYN, J.M. & APOSTOLIDES, Z. (2012) Bud Development, Flowering and Fruit Set of *Moringa oleifera* Lam. (Horseradish Tree) at Three Irrigation Levels. Departmental Post-graduate symposium. Post-graduate Centre, University of Pretoria, Pretoria.

Poster Presentation:

MUHL, Q.E., DU TOIT, E.S., STEYN, J.M. & APOSTOLIDES, Z. (2011) Anatomical, Histological, and Histochemical Changes in Developing Seed of *Moringa oleifera* Lam. (Horseradish tree) Under Three Irrigation Levels. 108th Annual International Conference of the American Society for Horticultural Science, Hilton Waikoloa Village, Kona, Hawaii, USA.

Peer-reviewed Article Publications:

MUHL, Q.E., DU TOIT, E.S., STEYN, J.M. & APOSTOLIDES, Z. (2013) Bud Development, Flowering and Fruit Set of *Moringa oleifera* Lam. (Horseradish Tree) as Affected by Various Irrigation Levels. *Journal of Agriculture and Rural Development in the Tropics and Subtropics*, 114, 79-87.

MUHL, Q.E., DU TOIT, E.S., & STEYN, J.M. (2014) Irrigation amounts affect the compositional changes of *Moringa oleifera* seeds throughout different developmental stages. *International Journal for Agriculture and Biology*, 16, 201-206.

CONTENTS

LIST OF ABBREVIATIONS

ABF : aniline blue fluorochrome

ANOVA : analysis of variance

c_a : ambient CO_2 concentration

c_i : intracellular CO_2 concentration

cm : centimetre

CO_2 : carbon dioxide

CoA : coenzyme A

DAF : days after flowering

ER : endoplasmic reticulum

FAA : Formaldehyde-acetic acid alcohol

g : gram

GWh : gigawatt hour

H_2SO_4 : sulphuric acid

H_3BO_3 : boric acid

hexose P : hexose phosphate

IT : irrigation treatment

ITs : irrigation treatments

K_3PO_4 : potassium phosphate

LSD : least significant difference

µg/ml : microgram per millilitre

$\mu mol.m^{-2}.s^{-1}$: micromole per square meter per second

m : meter

M : mole/molar

ml : millilitre

mm : millimetre

$mol.m^{-2}.s^{-1}$: mole per square meter per second

nm : nanometre

O_2 : oxygen

OsO_4 : aqueous osmium tetroxide

PAS : Periodic Acid-Schiff reagent

PGR : plant growth regulators

PKM1 : Periyakulam 1

PKM2 : Periyakulam 2

RER : rough endoplasmic reticulum

RGB : red:green:blue colour ratio

rpm : revolutions per minute

SD : standard deviation/ stomatal density

SEM : Scanning Electron Microscopy

SI : stomatal index

TAG : triacylglycerol

TAGs : triacylglycerols

TEM : Transmission Electron Microscopy

triose P : triose phosphate

V_T : volume soil

V_w : volume water

µm : micrometre

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CHAPTER 1

LITERATURE STUDY

1.1 Background

Climate change, increasing food prices and the production of biofuel from food sources have been identified as the three key factors that affect food security and malnutrition (Harvey and Pilgrim, 2011). In addition to global climate change, dwindling water resources and the degradation of agricultural land will further reduce food production, especially in Sub-Saharan Africa. Global warming, food production and fresh water resources are highly interlinked and consequently affect one another (Oki and Kanae, 2006, Solomon *et al.*, 2009). The increasing global demand for natural resources such as energy, food and water have led to escalating food and fuel prices as well as ever increasing food shortages (Tirado *et al.*, 2010, Trostle, 2010, Trostle *et al.*, 2011). Rising food prices, also have the potential to exacerbate food insecurity (Rosen and Shapouri, 2008). As one of 193 member states of the United Nations, South Africa has vowed to meet the 2015 Millennium Development Goals aimed to reduce the number of people living in hunger by 2015 (Khan and Hanjra, 2009).

Although the extent global warming and climate change on future water resources are still uncertain, the ever increasing demand for water and the scarcity of this commodity may even surpass the challenges faced by global warming (Vörösmarty and Sahagian, 2000).

Agriculture and food production accounted for 90% of all fresh water consumption during the previous century (Shiklomanov, 2000), with an estimated global population growth of 50% by 2050, the demand for water is expected to double (Watkins, 2009).

Energy is essential for the growth and development of the local as well as the international economy (Goldemberg, 1995). While the current production of biofuel is still relatively low, the global demand is continuously increasing, as numerous countries have pledged to blend fossil fuels with fuel from renewable sources over time. During 2007, 62 billion litres of biofuel were produced worldwide, which accounts for 1.8% of the global transportation fuel needs. Biodiesel production contributed 10.2 billion litres to this figure during 2007 (Ajanovic, 2011). The European Union under the "EU 2020" strategy aim to replace 10% of all transportation fuel with biofuel by 2020 (Özdemir *et al.*, 2009). But current biofuel production is still either directly, or indirectly dependant on food crops (Ajanovic, 2011). Despite the fact that the increasing demand for biofuel could raise food prices, the higher food prices in turn could also result in nutrient deficiencies within poorer communities as they opt for a less-balanced diet, consuming primarily inexpensive staple foods (Tirado *et al.*, 2010).

The South African Government, specifically the Department of Minerals and Energy have published a White Paper on Energy Policies in 1998. This document was revised in 2003 to make provision for the introduction and promotion of renewable energy sources such as biodiesel (DME, 2003).

According to this document, renewable energy sources should contribute approximately 4% (10 000 GWh) towards the total annual energy consumption by 2013. Should South Africa meet these targets, the estimated job creation could be as much as 142 919 jobs according to Von Malititz and Brent (2008).

Only 11% of the 13 billion hectares of land available on earth is suitable for crop production, of this a mere 1% (14 million hectares) was being used for the cultivation of biofuels in 2006. Twenty-one percent of land on earth has been classified as unsuitable for crop production because of amongst others, low soil fertility, drought and other environmental and climatic conditions. Given that most of the land suitable for cultivation is already being used for food production, ideally biofuel crops should be cultivated on land unsuitable for food production (Pimentel *et al.*, 2008). According to Pingali *et al.* (2008) the production area for cereal crops in Southern Africa could be increased by 53% if these areas could be irrigated. The assumption could thus be made that the other environmental conditions could be considered conducive for crop production. Since South Africa is a water scarce country (Molobela and Sinha, 2012), these increases in production figures will largely remain unachievable, unless there is a crop that would be able to grow under dryland conditions.

The current global challenge is thus to ensure food security, sequestrate greenhouse gases and meet the growing demand for fuel. Besides these global challenges, the continent of Africa faces additional challenges such as malnutrition, poverty, disease, water scarcity and large areas of sub-optimal agricultural land (Tirado *et al.*, 2010).

A potential solution to this and the other challenges is the planting of multipurpose energy producing trees, which have the potential to address the above-mentioned concerns. Trees have the ability to improve the environment through, CO_2 sequestration, remediation of erosion by reducing soil run-off and as well as improving the micro-climate through re-forestation. Trees generally require less climatic stability, are able to grow in less hospitable environments and produce yields for several years after planting.

Oil producing tree crops are more cost effective, given that they do not have to be replanted every season. The initial plantation establishment costs might be to some extent higher, but the running costs will be far less compared to annual commercial field crops.

There is a tree with numerous attributes that can help address several of the above-mentioned challenges, it is called *Moringa oleifera*, commonly known as the “miracle tree”. *Moringa oleifera* is considered as one of the most useful tropical trees in the world, due to its uses as nutritious food, fuel, medicine, water purifier and fodder amongst others (Foidl *et al.*, 2001, Palada and Chang, 2003).

Moringa oleifera has been identified by Padulosi *et al.* (2011) as one of the worlds "underutilized species” in their book “Underutilized Species and Climate Change: Current Status and Outlook”. They define “underutilized species” as a “species whose potential to improve people’s livelihoods as well as food security and sovereignty, is not being fully realized because of their limited competiveness with commodity crops in mainstream agriculture”.

They continue to state: “Another excellent hardy crop is the tree Moringa (*Moringa oleifera*), the “wonder tree,” which as well as its drought-resistance trait also has leaves of high nutritional content”.

According to Emongor (2009): “More research on the agronomy of this crop (*Moringa oleifera*) is needed, in order to reap its benefits as food and food ingredients, potential production of antioxidants and pharmaceutical products for local and international markets, water purification, and livestock and fish feeds.”

Despite *Moringa oleifera* being grown throughout the world including the continent of Africa, the limited scientific data that is currently available on its cultivation could be the reason for the absence of large-scale commercial *Moringa oleifera* plantations. However, prior to the cultivation thereof under reduced soil water levels, the possibility of reduced fruit set and yield needs to be quantified.

As one of 13 species within the *Moringa* genus and member of the monogeneric *Moringaceae* family, *Moringa oleifera* Lam. is the most common, versatile and extensively utilized species (Ramachandran *et al.*, 1980, Jahn, 1988). Besides numerous country-specific vernacular names such as ‘drumstick tree’, ‘horse radish tree’ or kelor tree, various *Moringa oleifera* cultivars such as ‘Jaffna’, ‘Chauakacheri Murunga’, ‘Chem’, ‘Kadu’, ‘Palmurungai’, ‘Periyakulam 1’ (PKM 1) and ‘Periyakulam 2’ (PKM 2) have been identified (Tsaknis *et al.*, 1998, Anwar and Bhanger, 2003). The varieties are distinguishable by fruit size, while the high-yielding, long-fruit, Indian variety ‘Periyakulam 1’ (PKM 1) was used throughout this study.

Although *Moringa oleifera* is currently found in tropical regions throughout Africa, South-East Asia and South America, its origins lie in the sub-Himalayan regions of north-western India (Jahn, 1988). *Moringa oleifera* is a fast-growing, perennial, medium-sized tree reaching a maximum height of between 7-12 m (Figure 1.1). Trees also produce a tuberous taproot, enabling them to be cultivated under a wide range of annual rainfall (250 mm to 1500 mm). *Moringa oleifera* favours hot and humid environments and is thus found throughout a range of ecological zones from dry savannah to rainforests. Temperature governs their natural distribution as they are able to tolerate light frost but are susceptible to severe frost (Foidl *et al.*, 2001, Council, 2006).

The trees remain evergreen under tropical environments, while being deciduous in temperate climates. Depending on the climate, trees either flower biannually or throughout the year, producing creamy-white zygomorphic flowers. Flowers are insect pollinated and produce light green, slender, three lobed, pendulous, longitudinally furrowed and angled fruit. Although numerous authors refer to the Moringa fruit as pods, this term is technically incorrect, as Moringa does not belong to the Fabacae family. Once mature, fruit dry out and turn brown, splitting along the three seams revealing anything between 12 and 35 winged seeds. Seeds are round, covered by a semi-permeable three-winged seed coat as illustrated in Figure 1.2 (Ronse Decraene *et al.*, 1998, Folkard *et al.*, 1999, Foidl *et al.*, 2001, Council, 2006)

Figure 1.1 *Moringa oleifera* tree at the Hatfield Experimental Farm (University of Pretoria).

Figure 1.2 *Moringa oleifera* seed.

Moringa oleifera is one of the most versatile plants on earth as virtually the entire tree can be utilized, which makes it of great economic value (Anwar *et al.*, 2007). Not only, is virtually the entire tree edible, but it is also highly nutritious with above-average levels of carbohydrates, protein, minerals and vitamins, making it the most nutritious among all tropical vegetables (Mughal *et al.*, 1999, Fuglie, 2001, Council, 2006).

Moringa oleifera also has numerous medicinal properties (Anwar *et al.*, 2007) such as, antitumor (Makonnen *et al.*, 1997), antipyretic (Oliveira *et al.*, 1999), antiulcer (Pal *et al.*, 1995), antispasmodic, diuretic (Cáceres *et al.*, 1992), antihypertensive (Faizi *et al.*, 1998), cholesterol lowering (Ghasi *et al.*, 2000), antioxidant (Siddhuraju and Becker, 2003), hepatoprotective, antibacterial and fungicidal activities (Ruckmani *et al.*, 1998).

Moringa oleifera seeds have an oil content percentage of between 35-40%. This oil has been tested and found suitable for the production of biofuel, in the form of biodiesel (Rashid *et al.*, 2008, Rashid *et al.*, 2011) as well as for the making of cosmetics and soaps (Ramachandran *et al.*, 1980).

Moringa oleifera seed powder can also be used as a water purifier due to its coagulating and antimicrobial properties, which has the ability to reduce waterborne bacteria (Jahn, 1988, Folkard *et al.*, 1999, Ferreira *et al.*, 2011).

The majority of current first-generation biofuels are produced from food crops such as maize, sugarcane, rapeseed and soybean (Ajanovic, 2011). To ensure future food

security, an increasing proportion of non-food feedstock should be used in future biofuel production. *Moringa oleifera* is quite unique in this regard as its oil producing seed can be used for biofuel production and water purification, while its nutrient-rich leaves can be used as a nutritional supplement. Given the correct management and sustainable harvesting, *Moringa oleifera* can potentially be a multipurpose tree, contributing towards various very diverse industries.

1.2 Aim of the study

This thesis aims to determine the feasibility of cultivating *Moringa oleifera* under reduced irrigation or rain fed production systems and to what extend this will lead to yield reductions. In addition, a thorough understanding of the deposition of seed storage components and how these might be affected by reduced soil water levels, needs to be established. The prospect and need for further studies in this field on emerging crops such as *Moringa oleifera* is strengthened by the following quote:

“The understanding of seed metabolism and especially the accumulation of storage products is of major economic importance and could be a key factor for sustainable agriculture” (Baud *et al.*, 2002).

Prior to the establishment of commercial *Moringa oleifera* plantations, for the production of seeds, leaves, oil, etc., comprehension of several cultivation aspects is essential. One of the key factors influencing any plant growth, and especially *Moringa oleifera*, due to its prevalence in tropical climates, is soil moisture. The main objectives of this study were thus:

- To evaluate the effect of three irrigation rates on flowering, pollination and consequent seed production. Furthermore, the identification of an irrigation regime that would favour flower initiation and fruiting.
- To determine how *Moringa oleifera* fruit growth and development is affected by various rates of irrigation.
- To determine when oil, protein and starch are formed during seed development, as well as the effect which irrigation had on the biosynthesis of these compounds.
- To quantify to what extent reduced plant water availability affects photosynthesis and ultimately yield in *Moringa oleifera*.
- To localise the synthesis of oil, protein and starch in the seed cotyledons with the aid of histochemical staining.
- To determine whether commercial photo-editing software such as Adobe® Photoshop® could be used to quantify the amount of stain through colour selection within sectioned seed and how this would compare to regular analytical analysis results.

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CHAPTER 2

BUD DEVELOPMENT, FLOWERING AND FRUIT SET OF *MORINGA OLEIFERA* AS AFFECTED BY VARIOUS IRRIGATION RATES

2.1 Summary

Moringa oleifera is becoming increasingly popular as an industrial crop due to its multitude of useful attributes as a water purifier, nutritional supplement and biofuel feedstock. Given its tolerance to sub-optimal growing conditions, most of the current and anticipated cultivation areas are in medium to low rainfall areas. This study aimed to assess the effect of various irrigation rates on floral initiation, flowering and fruit set. Three treatments namely; a 900 mm (900IT), 600 mm (600IT) and 300 mm (300IT) per annum irrigation treatment were administered through drip irrigation, simulating three total annual rainfall amounts. Individual inflorescences from each treatment were tagged during floral initiation and monitored throughout until fruit set. Flower bud initiation was 65.3% higher at the 300IT and 4.6% higher at 600IT compared to the 900IT. However, fruit set was 22.0% lower for the 300IT and 4.4% lower for 600IT, compared to the 900IT. Floral abortion, reduced pollen viability as well as moisture stress in the style were contributing factors to the reduction in fruiting/yield observed at the 300IT. Moderate water stress prior to floral initiation could stimulate flower initiation, this should however, be followed by sufficient irrigation to ensure good pollination, fruit set and yield.

2.2 Introduction

As a member of the *Moringaceae* family, *Moringa oleifera* also known as the miracle, horseradish or drumstick tree, is one of the most useful trees currently found throughout the tropics worldwide (Jahn, 1988). This fast growing, small to medium-sized tree is used as animal forage, source of nutrition, medicine, water purification, cosmetics and even as biofuel (Fuglie, 2001, Anwar *et al.*, 2007, Rashid *et al.*, 2008). Flowers are white to cream coloured, zygomorphic and pollinated by a large number of different insects including lepidopterans (Jyothi *et al.*, 1990, Bhattacharya and Mandal, 2004)(Figure 2.1). The tree bears 20 to 30 cm long fruit that once mature, change colour from green to brown, revealing numerous round or triangular seeds with three papery wings (Folkard *et al.*, 1999). Despite *Moringa oleifera* being cultivated throughout the world including the continent of Africa, the limited scientific data that is currently available on its cultivation could be the reason for the absence of large-scale commercial Moringa plantations.

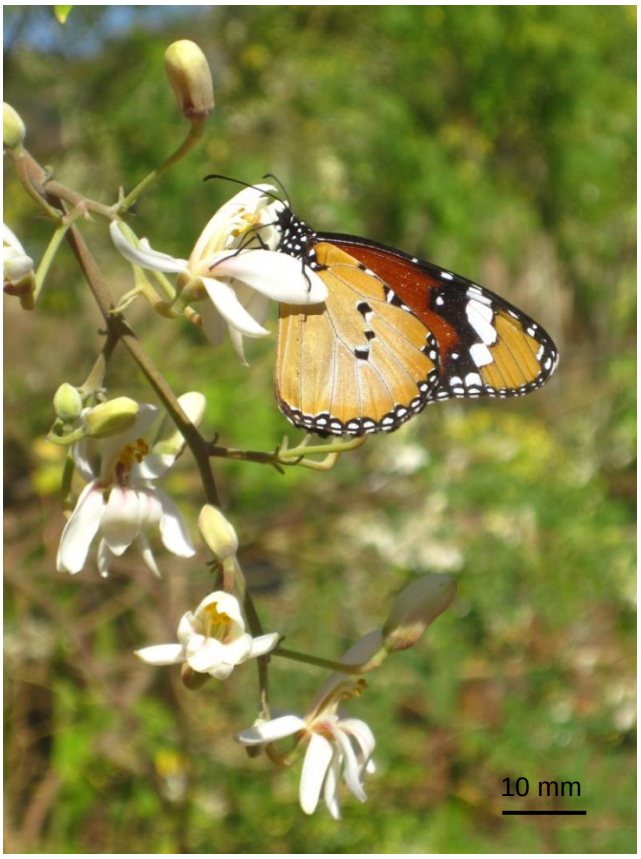


Figure 2.1 Monarch butterfly (*Danaus plexippus*) pollinating a *Moringa oleifera* flower.

Moringa oleifera is known to tolerate sub-optimal growing conditions (Palada and Chang, 2003). However, prior to the cultivation thereof under reduced soil water levels, the possibility of reduced fruit set and yield needs to be quantified. Due to the potential uses of *Moringa oleifera* fruit as a food/fuel source, the main objective of this study was thus to evaluate the effect of three irrigation rates on flowering, pollination and consequent seed production. Furthermore, the identification of an irrigation regime that would favour flower initiation and fruiting was attained.

2.3 Materials and Methods

Trials were conducted on six-year-old *Moringa oleifera* trees at the field trial section on the Hatfield Experimental Farm of the University of Pretoria (25°45'S, 28°16'E) at an altitude of 1372 m above sea level and an average annual rainfall of 674 mm. Trees for the purpose of this trial were grown from PKM1 variety seeds, sourced in India and transplanted into the field in a single row, spaced ±2 m apart. Trees were then divided into three groups of four trees each, while a single guard tree separated treatments. Each of the three groups was subject to a different irrigation treatment. Irrigation water was applied through a surface drip irrigation system at three rates. Three dipper lines were installed at the 900IT, two dripper lines at the 600IT, while the 300IT had a single dripper line at the base of the tree trunks. The in-line dripper spacing was 30 cm, with a continuous application rate of 2.1 litres/hour/dripper. According to Palada and Chang (2003) the minimum annual rainfall requirement for *Moringa oleifera* is 250 mm/year. The three administered irrigation rates were thus based on the minimum (300 mm/year)(300IT) amount for the tree, average (600 mm/year)(600IT) annual rainfall for the research site and a higher (900 mm/year)(900IT) treatment, simulating supplement irrigation under field conditions. The irrigation amounts were administered, simulating total annual rainfall (mm/year).

Plastic sheeting was then placed over the dripper irrigation, underneath the trees covering an area of 4 m on either side of the trunks (Figure 2.2). With this rainfall exclusion method, irrigation could be administered with greater accuracy without having to compensate for rainfall. Organic mulch was placed on top of the plastic sheeting so as not to adversely affect the energy balance of the soil (Figure 2.3). A single neutron probe access tube was installed in each of the three irrigation treatments, while twice-weekly soil water content measurements were conducted using a neutron probe (Campbell Pacific Nuclear, 503DR Hydroprobe) to verify differences in soil water levels between treatments for the duration of the trial. Neutron probe readings were taken at five depths namely 0-200 mm, 200-400 mm, 400-600 mm, 600-800 mm and 800-1000 mm and calibrated against soil samples from each of these depths (Tesfamariam, 2004).

Trees were subjected to the irrigation treatments for nine months prior to the initial floral assessment. Flowering was assessed over two consecutive seasons, in 2011 and 2012. During bud development, four random inflorescences per tree (four trees per treatment), within each of the three irrigation treatments were tagged and monitored until fruit set. Mathew and Rajamony (2004) classified *Moringa oleifera* flowers into seven developmental stages. Besides the seven stages described by Mathew and Rajamony (2004), two additional stages, namely fertilized flower and fruit set were added to total nine developmental stages. The flower classification stages are described and illustrated in Table 2.1.

Figure 2.2 Plastic sheeting placed on either side of the *Moringa oleifera* trees as a rainfall exclusion method.

Figure 2.3 To prevent the plastic sheeting from disturbing the soil energy balance, it was covered with organic mulch.

Table 2.1 Stages of *Moringa oleifera* bud and flower development adapted from Mathew and Rajamony (2004).

Stage		Description	Picture
1	Globular	Buds were greenish and inconspicuous	
2	Slightly globular	The colour changed to light green and bud was bulged. Ridges and furrows visible.	
3	Elongated	Colour became greenish white. The bud enlarged and ridges and furrows more prominent. Tip of the bud became creamy white.	
4	Slightly elongated	Colour creamy white. The bud was enlarged.	
5	Much elongated	Colour yellowish white all over, calyx green. Centre portion bulged out, both ends tapering.	
6	Elongated upper portion roundish	Yellowish white colour. One side of the bud split opened and one of the petals exposed. The smallest petal exposed first.	
7	The flower bud had fully opened	Sepals and petals five, yellowish white. Anthers yellow, dorsifixed filaments were of different lengths, style creamy white and pitted stigma.	
8	Fertilized flower	Senescence of sepals, petals and anthers. Ovary appears swollen.	
9	Fruit set	Sepals, petals and anthers have senesced and visible fruit has been formed.	

2.3.1 Pollen viability test

The *in vitro* pollen germination test was performed during the first flush of flowering in late spring using the hanging drop method (Shivanna and Rangaswamy, 1992). According to Bhattacharya and Mandal (2004) a 10% sucrose solution with a 200 µg/ml boric acid (H_3BO_3) concentration yielded the highest pollen germination percentages for *Moringa oleifera*. Consequently, the abovementioned concentration was used in the pollen germination trials across all three irrigation treatments. Pollen collected from freshly opened flowers within each of the three irrigation treatments was immersed in a drop of the sucrose solution using a needle. Pollen was left to germinate at room temperature (20°C) for two hours. The cover slip containing the pollen was then placed onto a microscope slide and observed under a Leitz Biomed light microscope, while digital pictures were taken using a Canon PowerShot A630 camera. A hundred randomly selected pollen grains per slide were assessed for their viability. Pollen was considered viable once the pollen tube length was equal or greater than the diameter of the individual pollen grains (Rodriguez-Riano and Dafni, 2000).

2.3.2 Fluorescence microscopy

Flower pistils were collected from trees subjected to the three irrigation treatments and prepared for fluorescence microscopy according to Martin (1959). Pollinated pistils were removed from the flower using a scalpel and fixed in FAA (80% ethanol: 37% formaldehyde: 100% acetic acid, in proportions 8:1:1 v/v/v) for 24 hours. After rinsing the pistils in distilled water, they were softened in 5M NaOH solution for 24 hours and again rinsed three times in distilled water. Pistils were then stained in 0.1% ABF (Aniline Blue Fluorochrome) and 0.1 N K_3PO_4 for 24 hours in complete darkness. After incubation, samples were removed and placed in a drop of 50% Glycerin on a microscope slide and

covered with a microscope slide that was gently pressed to flatten the pistil. Samples were then viewed using a Zeiss Inverted Fluorescence microscope, while digital pictures were taken using an AxioCamMR5 Camera.

Data were statistically analyzed using the Statistical Analysis Software (SAS Version 9.2) program for Microsoft Windows, by the Statistics Department at the University of Pretoria. Data were normalized using the Blom method to correct for heteroscedasticity in the data (Blom, 1958). Data were subjected to analysis of variance (ANOVA) using Proc GLM (SAS).

2.4 Results and Discussion

Soil samples were collected from each of the five measurement depths and the sand:silt:clay fractions determined for the respective profile layers, as given in Figure 2.4. The fractional distribution of sand, silt and clay enabled the determination of the soil texture as well as the respective field capacities (m^3/m^3), results of which are illustrated in Figure 2.5. The soil texture varied from a sandy clay loam in the top layer (0-200 mm) to layers of increasing clay-, and decreasing sand content in the mid sections (200-800 mm), back to a sandy clay loam at the bottom of the profile. The soil texture variation throughout the profile was found similar for all trees subjected to the three irrigation treatments.

Neutron probe measurements were calibrated against soil samples from each measurement depth and finally expressed as volumetric water content (θ)(m^3/m^3), which is the volume of water (V_w) within a given volume of soil (V_T) (Brady and Weil, 2002, Tesfamariam, 2004).

$$\theta = \frac{V_w}{V_T}$$

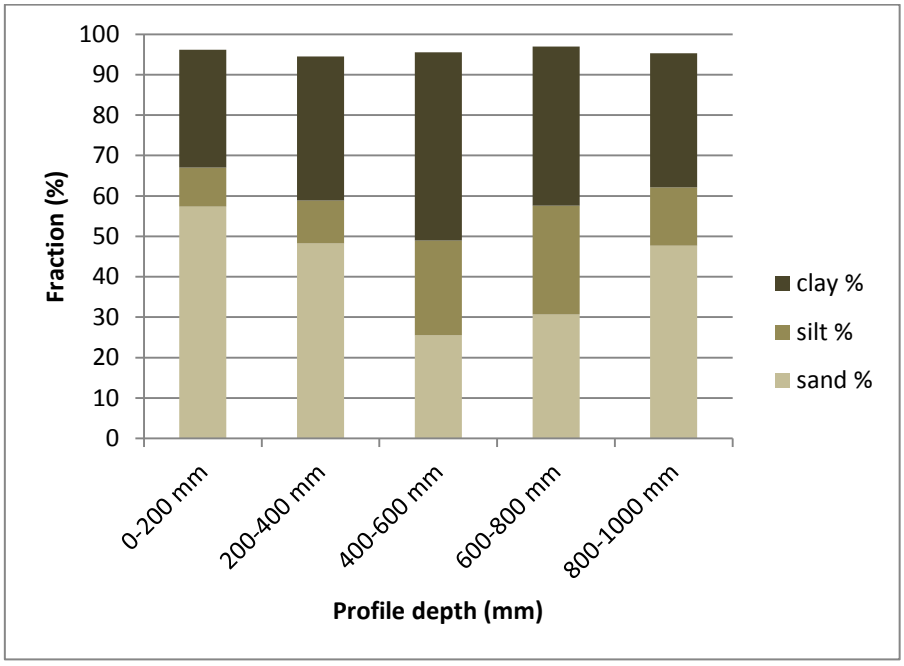


Figure 2.4 The sand, silt and clay factions of the soil layers at different depths at the trial site.

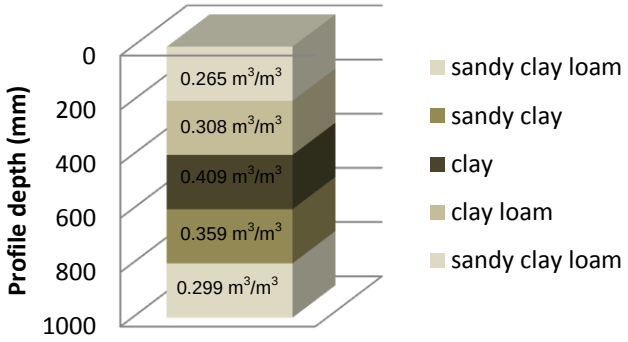


Figure 2.5 Soil texture and the calculated field capacity (m^3/m^3) for each layer throughout the profile.

The volumetric water content (m^3/m^3) for each of the three irrigation treatments (900IT, 600IT and 300IT) at five profile depths (0-200 mm, 200-400 mm, 400-600 mm, 600-800 mm and 800-1000 mm) is given in Figure 2.6. From these measurement results, the treatment effect between the three irrigation treatments could be confirmed.

The soil texture throughout the profile was not homogenous and this would have had an effect of soil water movement. Up to a profile depth of between 400-600 mm, the clay content increased with depth. This would have been conducive to downward and lateral soil water movement, rather than upward (through capillary action) as pore-sizes decreased with depth. This also reduced the probability that dryer surface layers could be moistened from wetter soil lower down in the profile. Throughout the upper three soil layers (0-600 mm) the irrigation treatment effect was more prominent, as soil at the 900IT had the highest volumetric water content amongst treatments. The data given in Figure 2.6 and Figure 2.7, illustrates that the two bottom layers (600-800 mm and 800-1000 mm) had little to no differences in volumetric water content between treatments. This might partially be attributable to the preceding clay layer hindering water movement into the courser-textured clay loam (600-800 mm) and sandy clay loam (800-1000 mm) layers beneath. Based on the findings of a root excavation study, these layers might even lie beyond the root zone and were thus not subjected to water extraction by the roots.

Soil texture variation between the different profile layers also meant different field capacity values for each layer (Figure 2.5). Based on soil water measurement results (Figure 2.6 and Figure 2.7), the 200-400 mm soil layer at the 900IT was nearly at field capacity throughout the trial period. At a depth of 800-1000 mm, however, all three irrigation treatments reached field capacity.

Soil water measurement results from all three irrigation treatments, confirmed substantial differences between them. Therefore, differences observed in flowering, fruiting and storage compound development between treatments could be attributed (or at least partially), to differences in soil water availability.

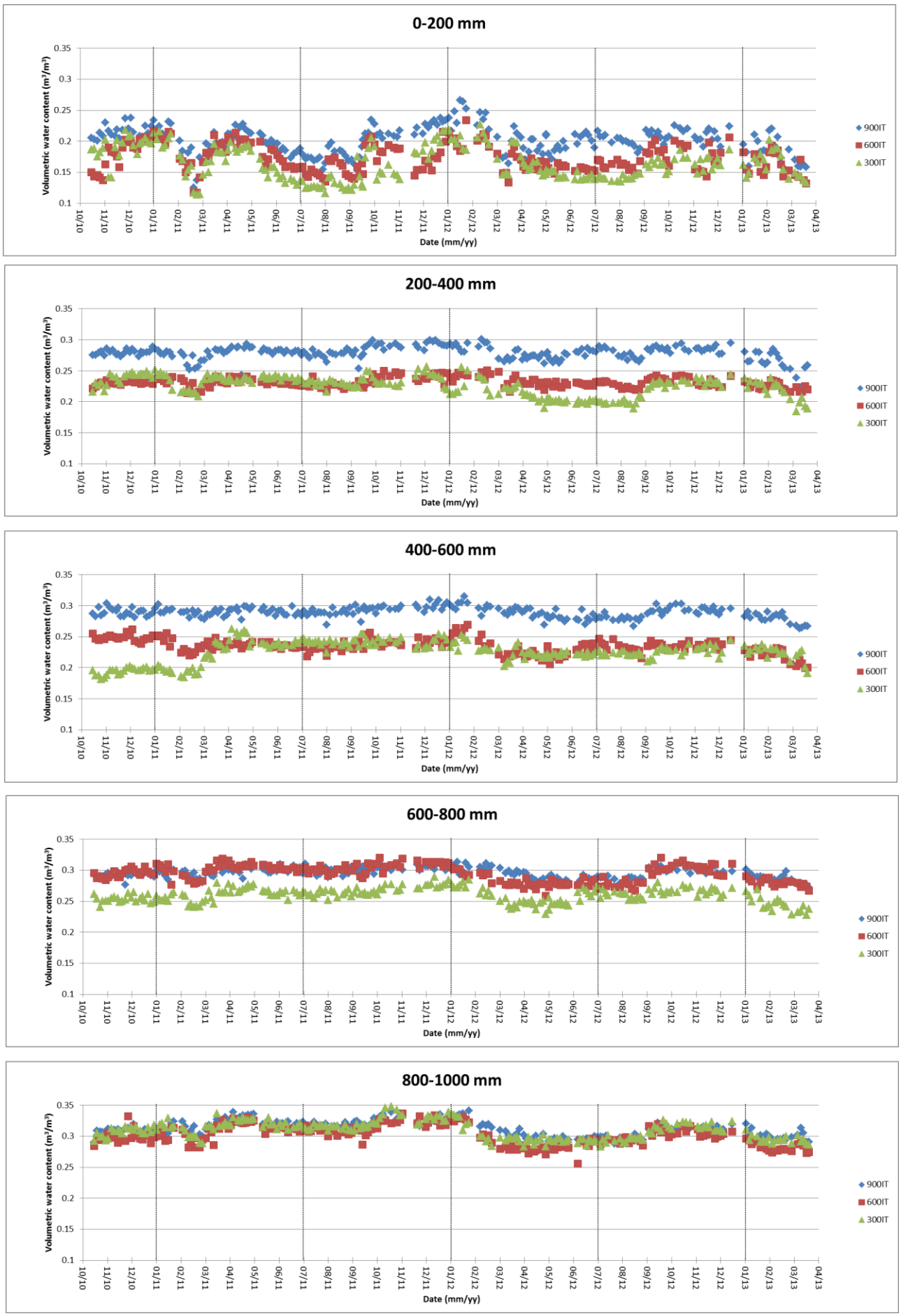


Figure 2.6 Soil volumetric water content (m^3/m^3) at three irrigation rates at five different profile depths.

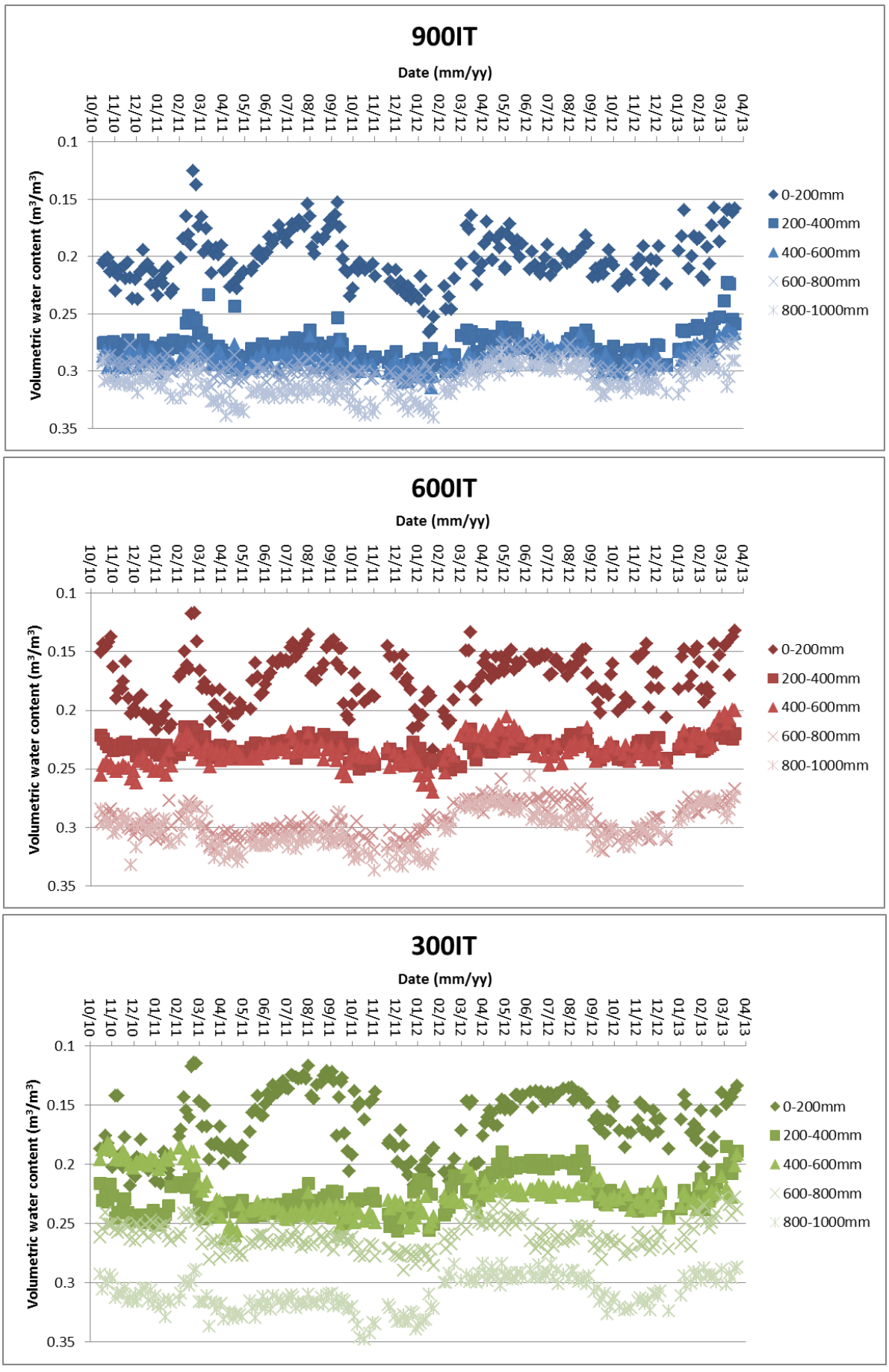
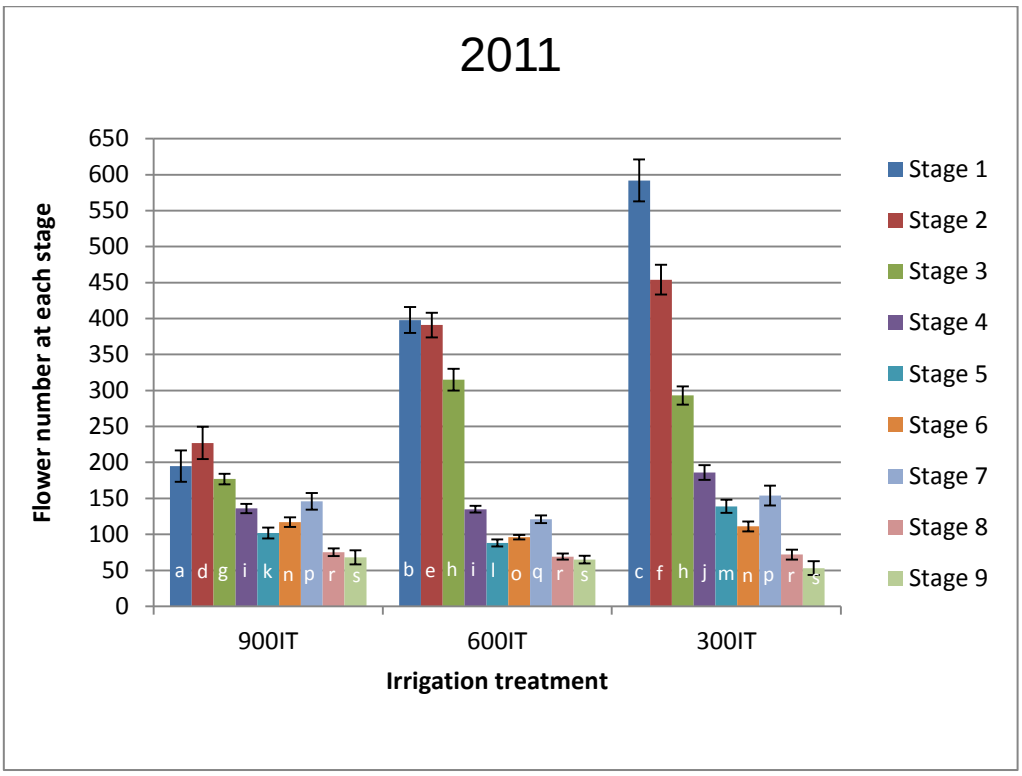


Figure 2.7 Soil volumetric water content (m^3/m^3) at five different profile depths within each of the three irrigation treatments.

Results revealed that the average number of flower buds formed per inflorescence increased with a decrease in irrigation treatment during both the 2011 and 2012 season (Figure 2.8). Monitored inflorescences (16) at the 900IT had a total of 195 initiated flower buds, while the total number of initiated flower buds on the 16 monitored inflorescences at the 300IT was 592 in 2011. In 2012, a total of 248 flower buds were initiated at the 900IT compared to 529 at the 300IT. The decrease in irrigation between the 900IT and 300IT increased flower numbers by 67.1%, during 2011 and 53.1%, during 2012. Instances of increased flower initiation under moderate water stress have also been observed in *Litchi chinensis* (Stern *et al.*, 1993), *Averrhoa carambola* (Salakpetch *et al.*, 1990), *Garcinia mangostana* (Poonnachit *et al.*, 1996), *Anacardium occidentale* (Nambiar, 1977), *Mangifera indica* (Singh, 1977), *Theobroma cacao* (Alvim, 1977) and *Coffea arabica* (Alvim, 1960, Maestri and Barros, 1977, Schuch *et al.*, 1992). Although short periods of water stress seemingly increase flower initiation in certain species, prolonged water stress is reported to induce bud abscission and decreased flowering/fruiting.



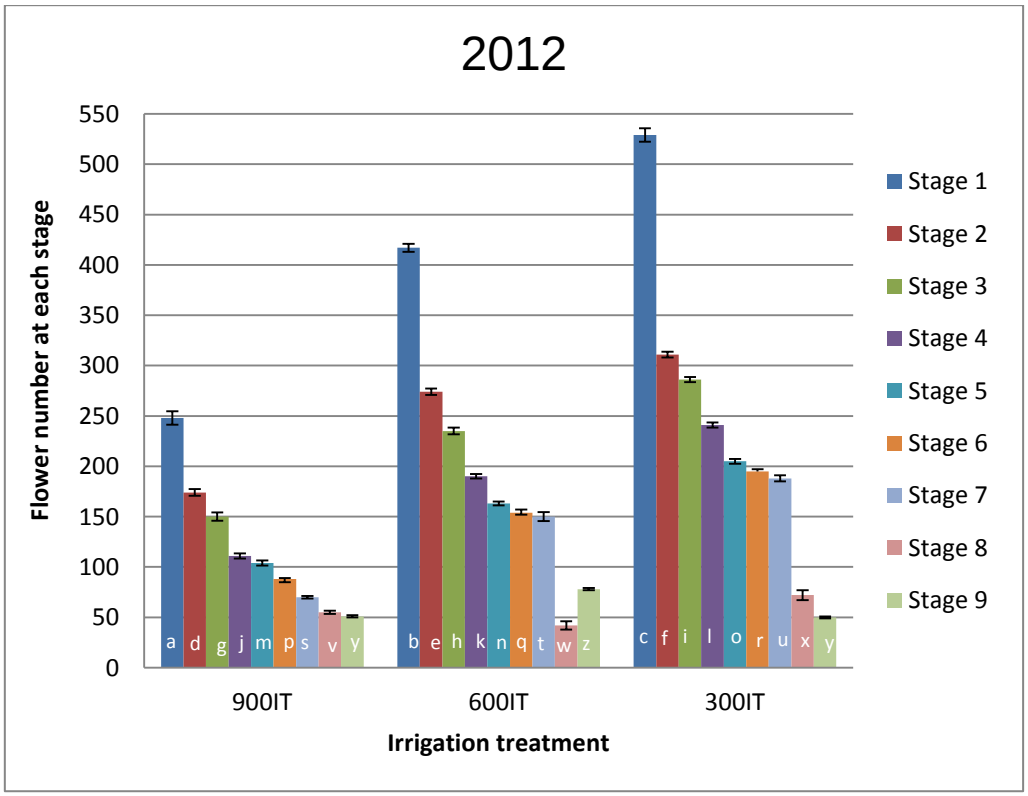
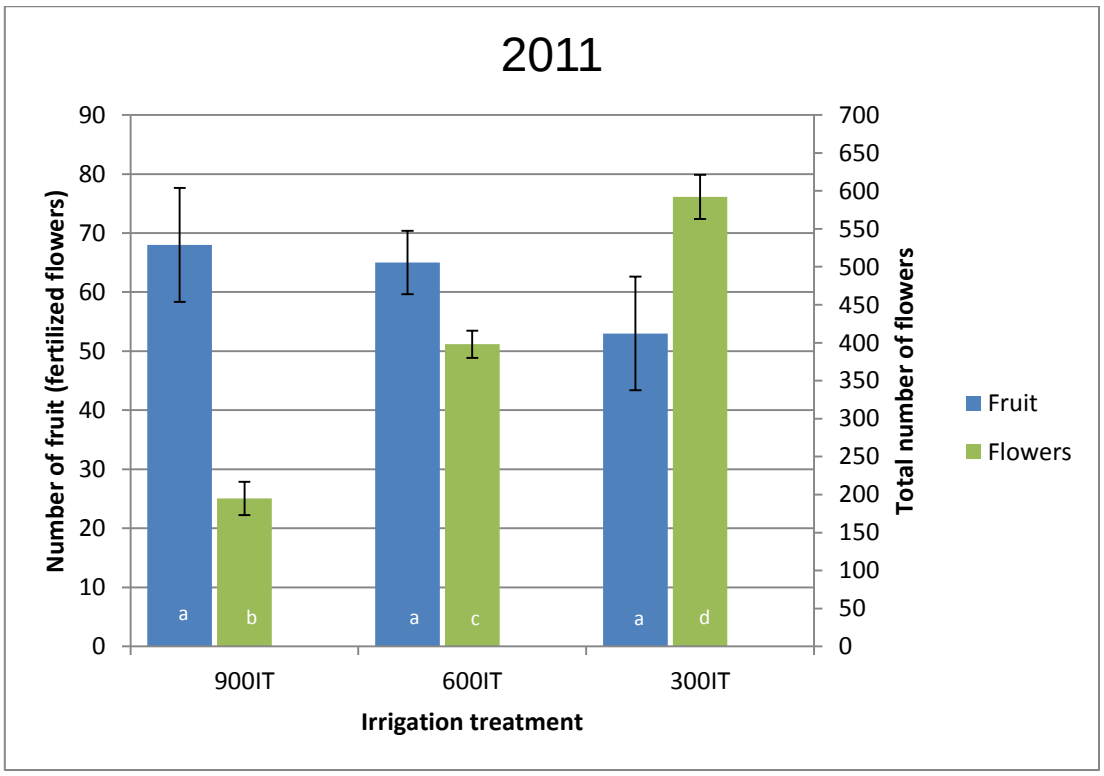


Figure 2.8 Number of flowers at each flowering stage at three irrigation treatments. Vertical bars (±) indicate standard errors of the mean (SEM). Different letters indicate significant differences at P ≤ 0.05 according to the F-test.

Although *Moringa oleifera* flower initiation seemingly benefited from lower irrigation rates (water stress), increased instances of bud abortion as well as a reduction in the number of fertilized flowers were observed with a decrease in irrigation. A total of 68 flowers were fertilized at the 900IT, compared to 65 for the 600IT and 53 of the 300IT during 2011 (Figure 2.9). This is a 4.4% and 22.1% reduction in fertilized flowers between the 900IT and 600IT, as well as the 900IT and 300IT respectively. During 2012, 50 flowers were fertilized at the 900IT, compared to 78 at the 600IT and 51 at the 300IT (Figure 2.9). Between the 900IT and 600IT there was a 35.6% reduction in fertilized flowers, while both the 900IT and 300IT had similar numbers of fertilized flowers. When comparing the bud-to-fruit ratio during 2011, for every 2.9 buds initiated at the 900IT, one fruit was produced, whereas the bud-to-fruit ratio for the 600IT and 300IT was 6.1 and 11.2 respectively. During 2012 however, the bud-to-fruit ratio for the 900IT was 4.9, 5.3 for the 600IT and 10.6 for the 300IT. Thus, despite increased flower numbers at the 300IT, proportionally fewer flowers were fertilized, thereby limiting the yield potential (Paull and Duarte, 2011). The majority of flower abortions at both the 600IT and 300IT during 2011 and 2012 occurred between stage one and four where after flower numbers stabilized and reached similar levels to that of the 900IT (Figure 2.8). Similar observations, where reduced irrigation (moisture stress) initiated flower shedding have been reported by Joseph (2007) in Moringa and other crops such as *Olea europaea* L. (Rapoport *et al.*, 2011) and *Citrus* spp. (Carr *et al.*, 2012). The maximum potential number of seed (yield) is fixed at fertilization (Alqudah *et al.*, 2011). However, further post-fertilization water stress could cause abortion of developing ovules and/or reduce the photosynthetic rate, thereby lowering assimilate supply, which will result in smaller seed (Simpson, 1981) and consequently, lower yield.



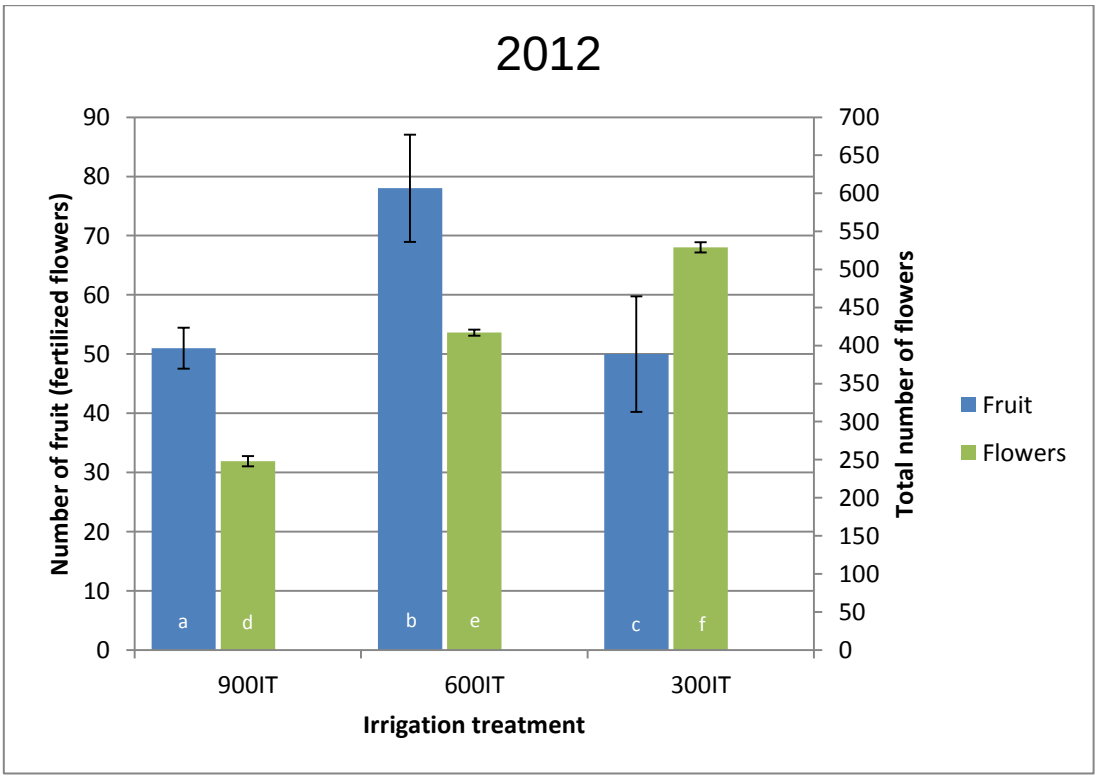


Figure 2.9 Total number of flower buds vs. the number of fruit at each of the three irrigation treatments. Vertical bars (±) indicate standard errors of the mean (SEM). Different letters indicate significant differences at $P \leq 0.05$ according to the F-test.

To ascertain whether reduced fertilization and fruit set was attributable to inferior pollen, pollen viability tests were performed for all three irrigation treatments. Results revealed that pollen viability decreased with a decrease in irrigation amount (Table 2.2). The average pollen viability for the 300IT was found to be 45.4% compared to 57.4% at the 600IT and 76.6% at the 900IT (Table 2.2). Reduced pollen viability at the lower irrigation treatments (300IT and 600IT) possibly explains the poor fertilization and fruit set at these treatments. However, an abundant supply of viable pollen from adjacent well-watered irrigation treatments would rule out this as the sole reason for reduced fruit set (Simpson, 1981).

Table 2.2 *In vitro* pollen germination percentages of *Moringa oleifera* from different irrigation treatments. Different letters indicate significant differences at P ≤ 0.05 according to the F-test.

	Irrigation treatment		
	900IT	**600IT**	**300IT**
Pollen viability (% ± Std Dev)	76.6 ± 3.1[a]	57.4 ± 3.2[b]	45.4 ± 3.1[c]

Flower abortion as well as moisture stress in the style are additional factors that can reduce fruit set (Fang *et al.*, 2010). Flourescence microscopy of the flower styles and ovary was used to detect the presences of pollen tube growth. A significant number of pollen tubes were detected in the styles of flowers from the 900IT, suggesting that conditions in the style were condusive to pollen tube growth. However, pollen tube growth in flowers from the 300IT was severly reduced (Figure 2.10). According to Simpson (1981) and Fang *et al.* (2010), moisture stress in the style contributes significantly to increased instances of flower abortion as fewer pollen tubes reach the ovary. Reduced fruit set observed in the 300IT can thus not merely be attributed to lower pollen viability but also due to moisture stress creating unfavourable conditions within the style for pollen tube growth.

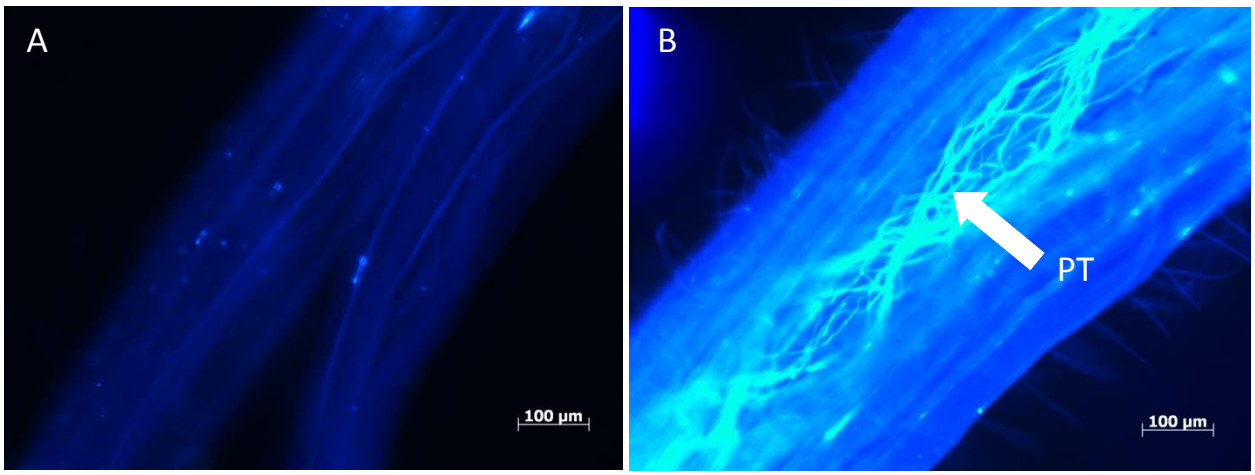


Figure 2.10 Fluorescence microscopy of *Moringa oleifera* styles with and without pollen tube growth. A – 300IT; B – 900IT; PT – Pollen tubes.

2.5 Conclusion

Although low (300IT) irrigation rates resulted in a higher number of flower buds, proportionally fewer flowers were fertilized and produced fruit, which can be attributed to flower abortion caused by lower pollen viability and possibly moisture stress in the styles, inhibiting pollen tube growth and fertilization. The high irrigation rate (900IT) produced fewer flowers. However, a greater proportion were fertilized to produce fruit. Despite *Moringa oleifera* being tolerant to low soil water levels, stress during the reproductive phase can have severe implications on the consequent yield. From an agronomic point of view, the identification of drought sensitive stages in the growth cycle is not only important from a water-use perspective, but also because water is often in short supply. If the aim is thus to maximize yield from limited resources, trees should not be stressed during the reproductive stages. Moderate water stress prior to floral initiation could be beneficial by stimulating increased flower initiation, while ample irrigation thereafter would ensure better fruit set and greater yield.

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CHAPTER 3

EMBRYOGENISIS AND SEED DEVELOPMENT OF *MORINGA OLEIFERA*

3.1 Summary

In order to comprehend storage compound synthesis and accumulation throughout seed growth, the integral processes of embryogenesis and endosperm development need to be described. Microscopical studies of developing *Moringa oleifera* seed have revealed the endosperm to be primarily nuclear, becoming cellular from the chalazal end towards the developing embryo at a fruit diameter of ±6 mm. At a fruit diameter of ±8 mm the cellular endosperm had covered the entire inner integument, which coincides with the developing embryo reaching the globular stage. Cotyledon development commenced at a fruit diameter of ±12 mm and continued up until ±24 mm. It was also during this phase that the majority of storage compounds were synthesized and stored, making it the most sensitive stage to environmental stresses. At the end of this phase the cotyledons had filled the entire space covered by the seed coat, while the unicellular epidermal layer of the inner integument remained distinctly visible between the cotyledons and the testa. The mature seed coat had layers of thickened as well as fibrous cells while being roughly triangular with three wings.

3.2 Introduction

Lately there has been growing interest in *Moringa oleifera*, and more specifically their seed, due to its multitude of uses. Seed can be used as a food/fodder source, for oil extraction, water purification as well as the treatment of various ailments (Oliveira *et al.*, 1999, Fuglie, 2001, Anwar *et al.*, 2007, Santos *et al.*, 2012). Prior to studying the

physiological and biochemical processes during seed growth and development, the post-fertilization anatomy of the endosperm and embryo need to be understood (Bewley and Black, 1994). Due to discrepancies in the literature about the structure of the seed coat, special attention was given to its development and structure. By following seed growth throughout their post-fertilization development, insight into the formation of the various seed structures could be obtained, opposed to only examining seed at maturity. In addition, the effect of environmental stresses during specific developmental stages will be better understood.

3.3 Materials and Methods

Seed from different developmental stages based on the fruit diameter (mm), were randomly sampled from mature *Moringa oleifera* trees growing at the field trial section on the Hatfield Experimental Farm of the University of Pretoria (25°45'S, 28°16'E) at an altitude of 1372 m above sea level with an average annual rainfall of 674 mm.

Seed development throughout the various post-fertilization phases was studied using both dissection and light microscopy, while seed structures are described using Corner (1976a) terminology.

3.3.1 *Light microscopy*

Seed preparation for light microscopy was done according to O'Brien and McCully (1981). After harvesting, seeds were removed from the outer protective pericarp and immediately fixed in FAA (80% ethanol: 37% formaldehyde: 100% acetic acid, in proportions 8:1:1 v/v/v) for 24 hours, before being dehydrated in an ethanol in water series (30%, 50%, 70%, 100%, 100% v/v) for 24 hours at each concentration. Subsequently, ethanol was extracted

through a series of xylene in ethanol concentrations (30%, 50%, 70%, and 100% v/v) and specimens impregnated with paraffin wax (60°C). Embedded seed samples were cut into 10 µm thick sections using a Reichert-Jung semi-thin rotary microtome, and mounted onto microscope slides. After sections had been de-waxed in a series of xylene concentrations, they were stained in both 1% aqueous safranin and 0.5% fast green (95% ethanol) according to O'Brien and McCully (1981).

3.3.2 *Fluorescence microscopy*

Pollen germination, pollen tube growth as well as fertilization were studied using fluorescence microscopy according to Martin (1959). Pollinated pistils were removed from the flowers using a scalpel and fixed in FAA (80% ethanol: 37% formaldehyde: 100% acetic acid, in proportions 8:1:1 v/v/v) for 24 hours. After rinsing the pistils in distilled water they were softened in 5M NaOH solution for 24 hours and again rinsed three times in distilled water. Pistils were then stained in 0.1% ABF (Aniline Blue Fluorochrome) in 0.1 N K_3PO_4 for 24 hours in complete darkness. After incubation, samples were removed and placed in a drop of 50% Glycerin on a microscope slide and covered with a cover slip that was gently pressed to flatten the pistil. Samples were then viewed using a Zeiss Inverted Fluorescence microscope, while digital pictures were taken using an AxioCamMR5 Camera.

3.4 Results and Discussion

Due to the hollow, tubular stigma, several pollen grains were deposited inside the stigma tip (Figure 3.1A). Subsequent pollen tube growth was observed inside the style (Figure 3.1B), all the way down to the ovules (Figure 3.1C and Figure 3.1D).

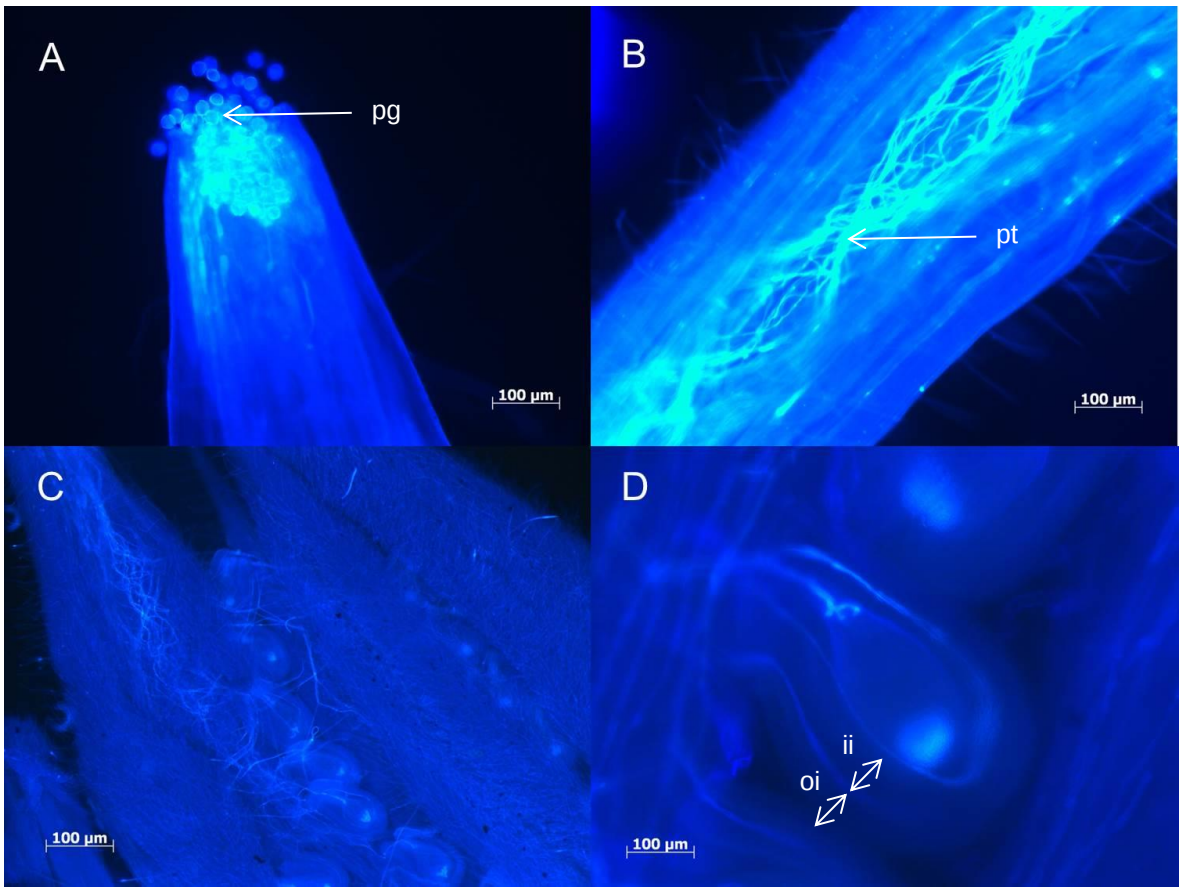

Figure 3.1 A – Pollen grains on the stigma, B – Pollen tube growth through the style, C – Pollen tubes entering the ovary, containing numerous ovules, D – Pollen tubes entering an ovule through the micropyle. pg – pollen grains, pt – pollen tubes, oi- outer integument, ii – inner integument.

The ovules are anatropous and bitegmic with a brightly fluorescing hypostase. Nuclei inside the embryo sac as well as double fertilization were not observed, although the presence of an embryo and endosperm in all investigated young seeds confirmed that the embryo sacs were normal.

The outer integument continued to differentiate into both the inner- (endotesta) and outer layers (exotesta) of the testa, mainly through cell division of the meristematic region in the endotesta up until a fruit diameter of ±4 mm (Figure 3.2). Similar observations were made in the *Arabidopsis* spp. by Nesi *et al.* (2002), Ingouff *et al.* (2006) and Beeckman *et al.* (2000). This was followed by a period of cell enlargement, as most cells seem to have been formed once fruit reached a diameter of ±6 mm (Figure 3.3A and Figure 3.3B).

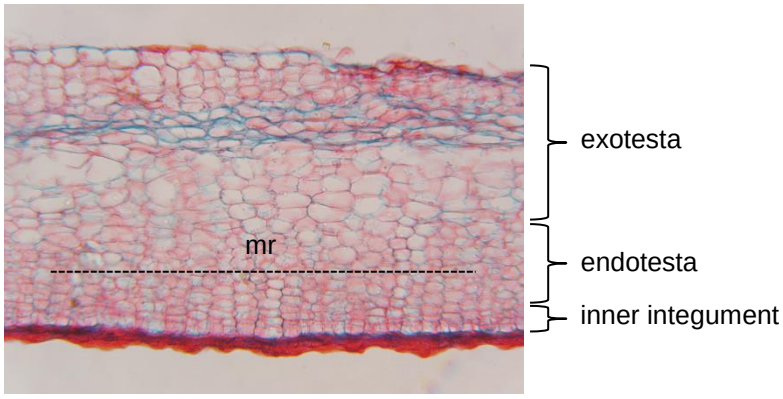

Figure 3.2 Thickening of the ovule wall (testa) at a fruit diameter of 4 mm, through cell division of the meristematic region (mr) giving rise to both the exotesta and endotesta.

Endosperm development in *Moringa oleifera* is initially of the nuclear type (Davis, 1966, Kubitzki, 2003), while cellular endosperm appeared at a fruit diameter of ±6 mm (Figure 3.3B), developing from the chalazal end towards the embryo (micropylar end), covering the entire embryo sac once the embryo had reached the globular stage. The micropyle is principally formed by tissue that is part of the outer integument (Figure 3.4). According to Finkelstein (2010) and Goldberg *et al.* (1994) embryogenesis can be grouped into three distinct phases, namely post-fertilization proembryo formation, cell division/expansion/reserve deposition (globular to cotyledon stage) and lastly maturation/desiccation. At a fruit diameter of ±8 mm, the embryo had reached the globular stage (Figure 3.4), this marked the beginning of the histo-differentiation (embryo differentiation) phase of seed development according to Hartmann *et al.* (2011). It was also during this developmental stage that the epidermal layer of the inner integument was pushed inwards due to significant cell division and enlargement by the outer layer of the inner integument (Figure 3.4). The embryo in a developing *Moringa oleifera* seed, illustrated in Figure 3.4 is enlarged in Figure 3.5. There was no evidence of a suspensor suggesting that it might be absent in *Moringa oleifera*, supporting observations made by Johansen (1950).

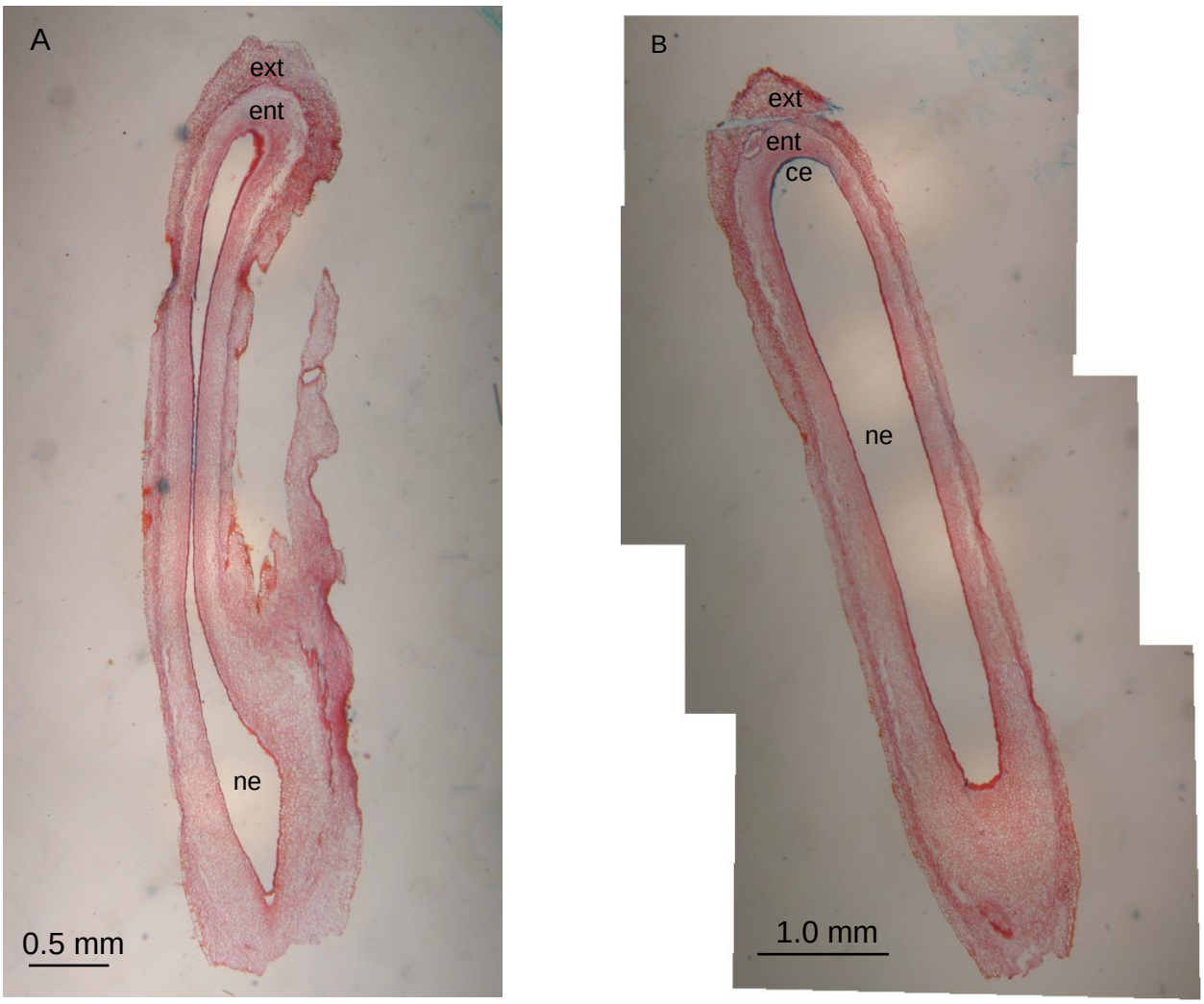

Figure 3.3 Longitudinal section of an immature *Moringa oleifera* seed at fruit diameters of 4 mm (A) and 6 mm (B), demonstrating seed coat development prior to embryogenesis. ext – exotesta, ent – endotesta, ne – nuclear endosperm, ce – cellular endosperm.

The histo-differentiation phase, between a fruit diameter of ±8 mm up until about 12 mm, was when most of the cell division of the embryo took place (Figure 3.6). At the end of this phase (at a fruit diameter of ±12 mm) the individual cotyledons became clearly distinguishable (Figure 3.7). This was followed by a period (between the fruit diameters of ±12 – 24 mm) where the cotyledons increased rapidly both in size and mass, as storage products such as oil, protein and starch were synthesized and deposited inside the cotyledons. These accumulated storage reserves provide emerging seedlings with the necessary resources during seed germination for their growth and development until they become photosynthetically active (Bewley and Black, 1994).

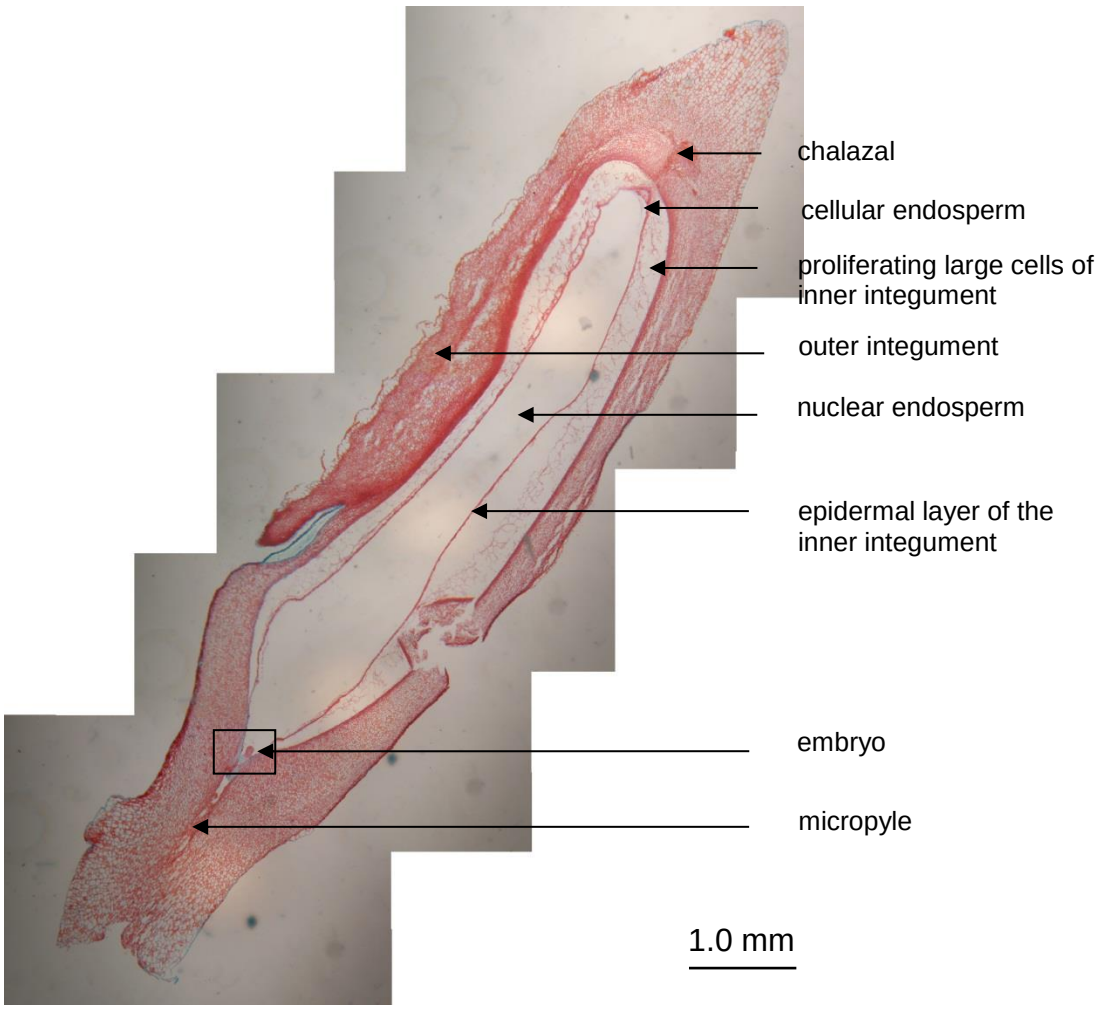

Figure 3.4 Longitudinal section of a *Moringa oleifera* seed during the commencement of embryogenesis at a fruit diameter of 8 mm.

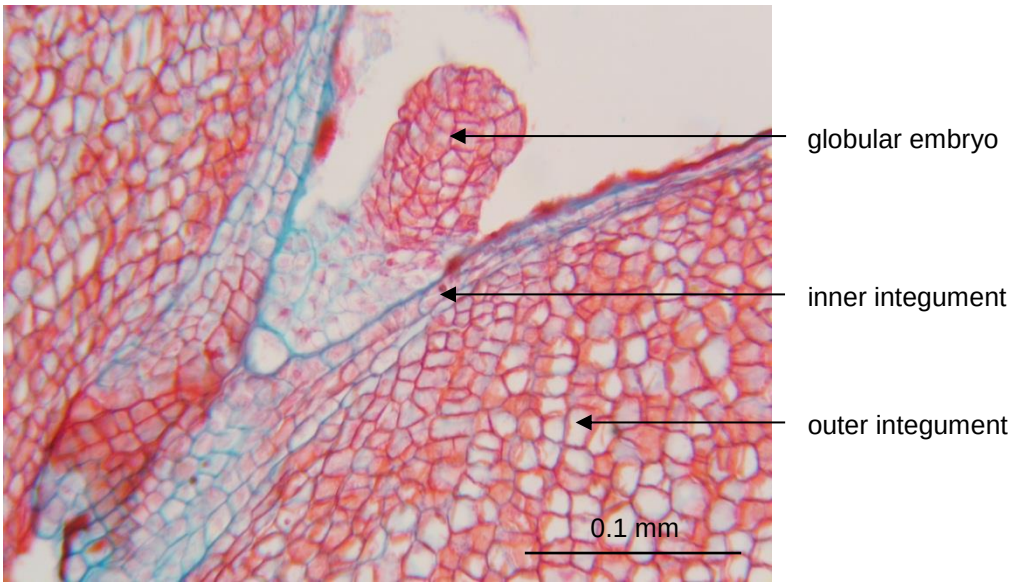

Figure 3.5 *Moringa oleifera* seed embryo at the globular stage.

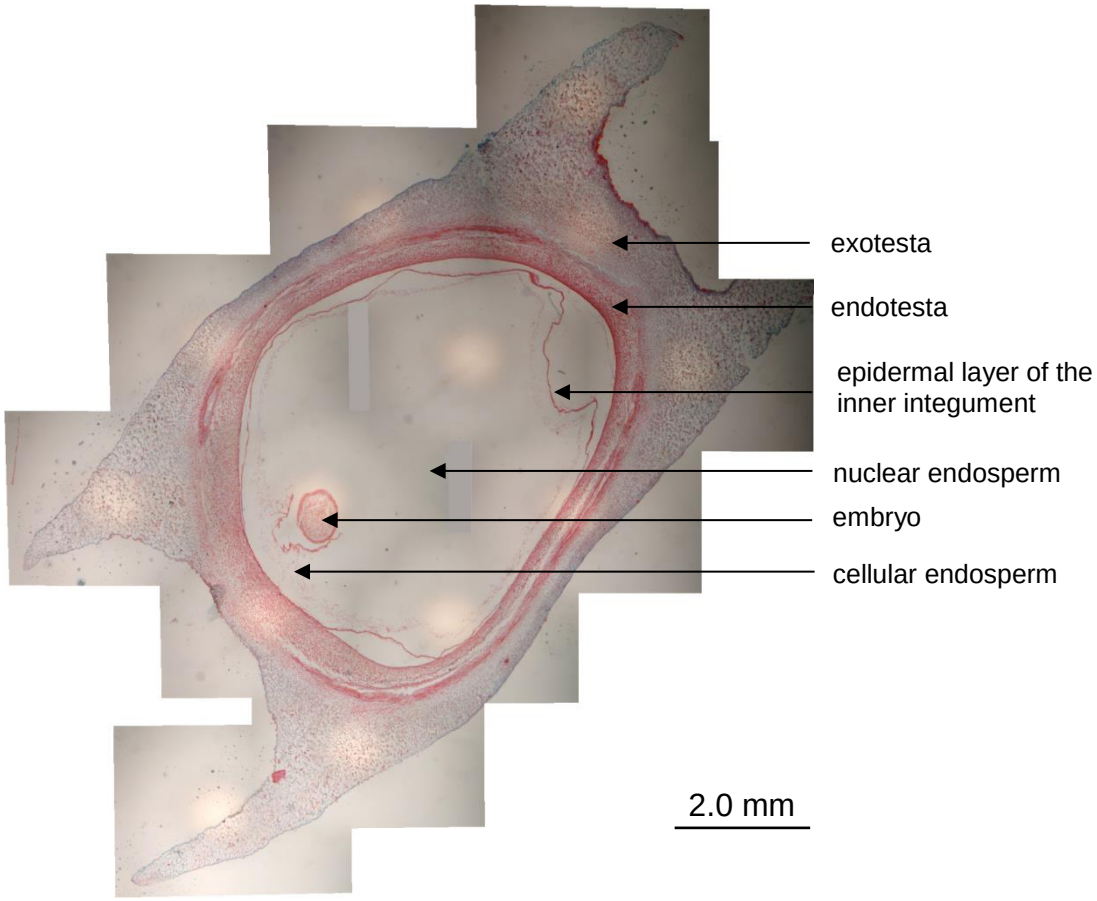

Figure 3.6 Longitudinal section of developing *Moringa oleifera* seed at a fruit diameter of 10 mm.

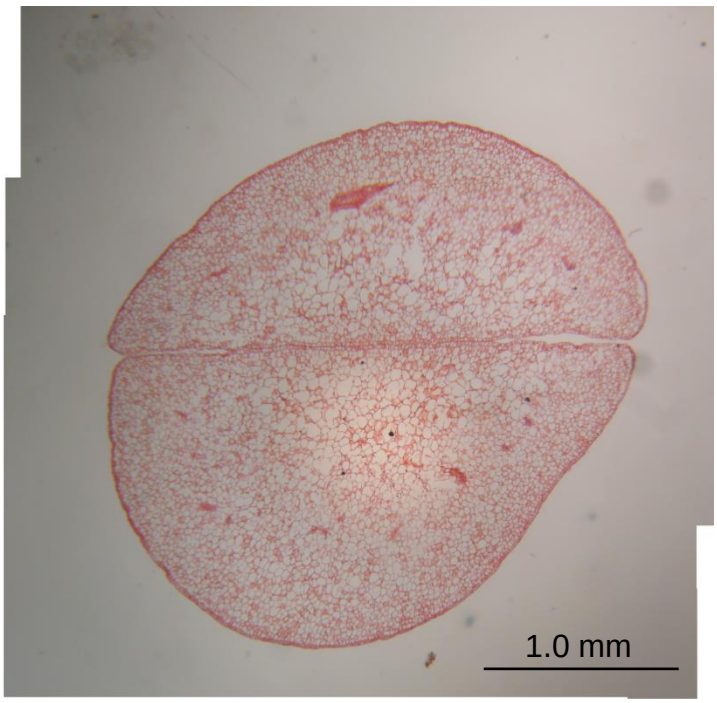

Figure 3.7 Cross-section of developing *Moringa oleifera* seed at a fruit diameter of 12 mm, clearly showing the individual cotyledons.

Cross-sections of developing seed during reserve deposition phase (±12 – 24 mm) are illustrated in Figure 3.8 ③-⑨, at the end of this phase the cotyledons have filled the entire seed, consuming virtually all endosperm. At seed maturity, the epidermal layer of the inner integument was reduced to a thin, but distinct unicellular layer, together with the remaining cell walls of the thin-walled, enlarged tegmen cells crushed by the expanding cotyledons against the testa. Sections stained with Sudan IV have confirmed that both the cotyledons and this epidermal layer contain oil, while the adjacent seed coat, had an oil-free cork-like structure. Once the fruit reached a diameter of approximately 24 mm, the embryo continued to increase in size as its two cotyledons had been fully developed. This also marked the beginning of the maturation phase (fruit diameter 24 mm - 26 mm), which resulted in the hardening of the cotyledons along with the seed coat/testa as illustrated in Figure 3.8 ⑩. It was also during the maturation phase that the cotyledons changed colour from light green to white.

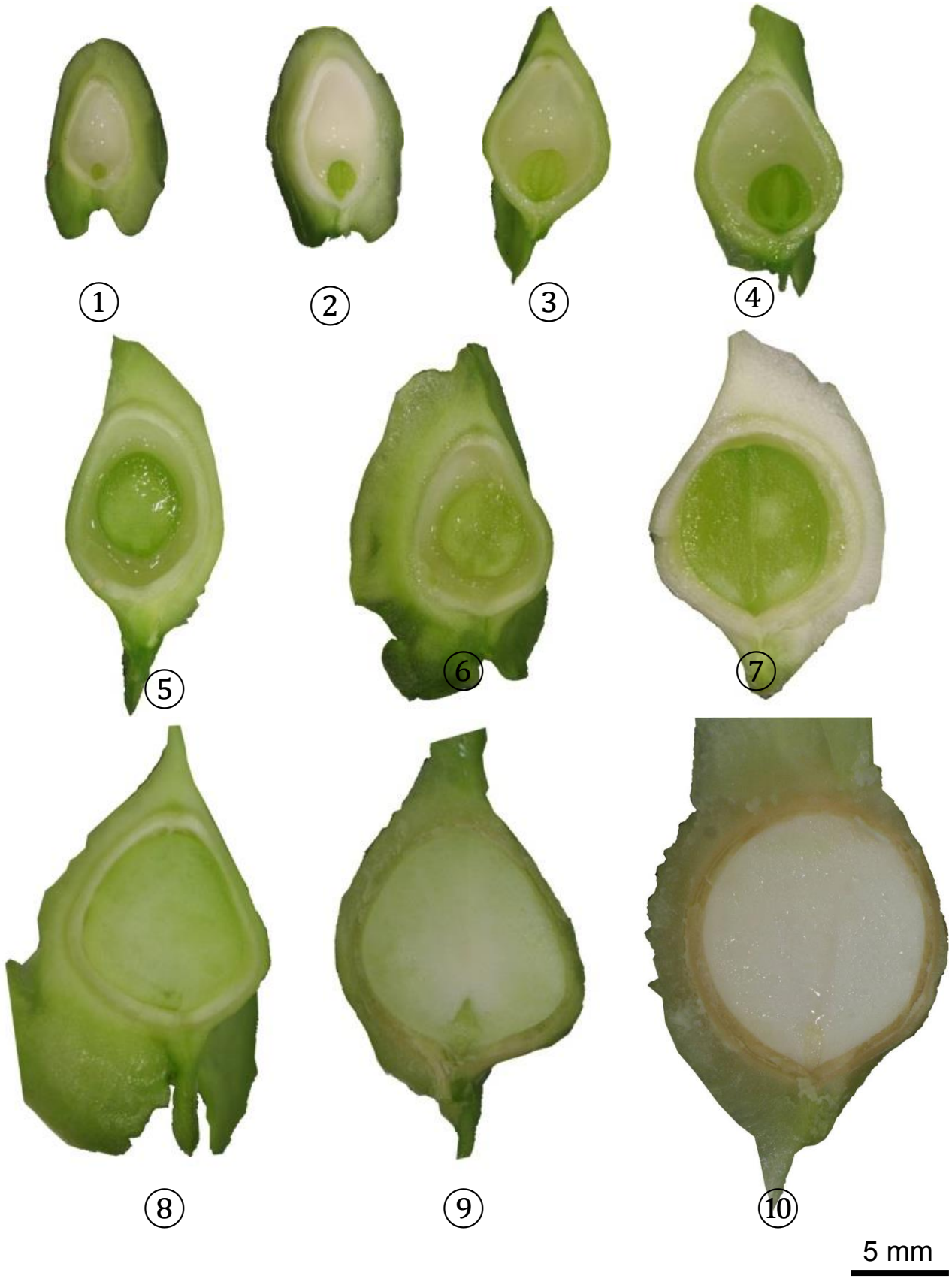

Figure 3.8 Cross-sections of *Moringa oleifera* seed at different stages throughout their development, based on fruit diameter (mm). ① 8 mm ② 10 mm ③ 12 mm ④ 14 mm ⑤ 16 mm ⑥ 18 mm ⑦ 20 mm ⑧ 22 mm ⑨ 24 mm ⑩ 26 mm.

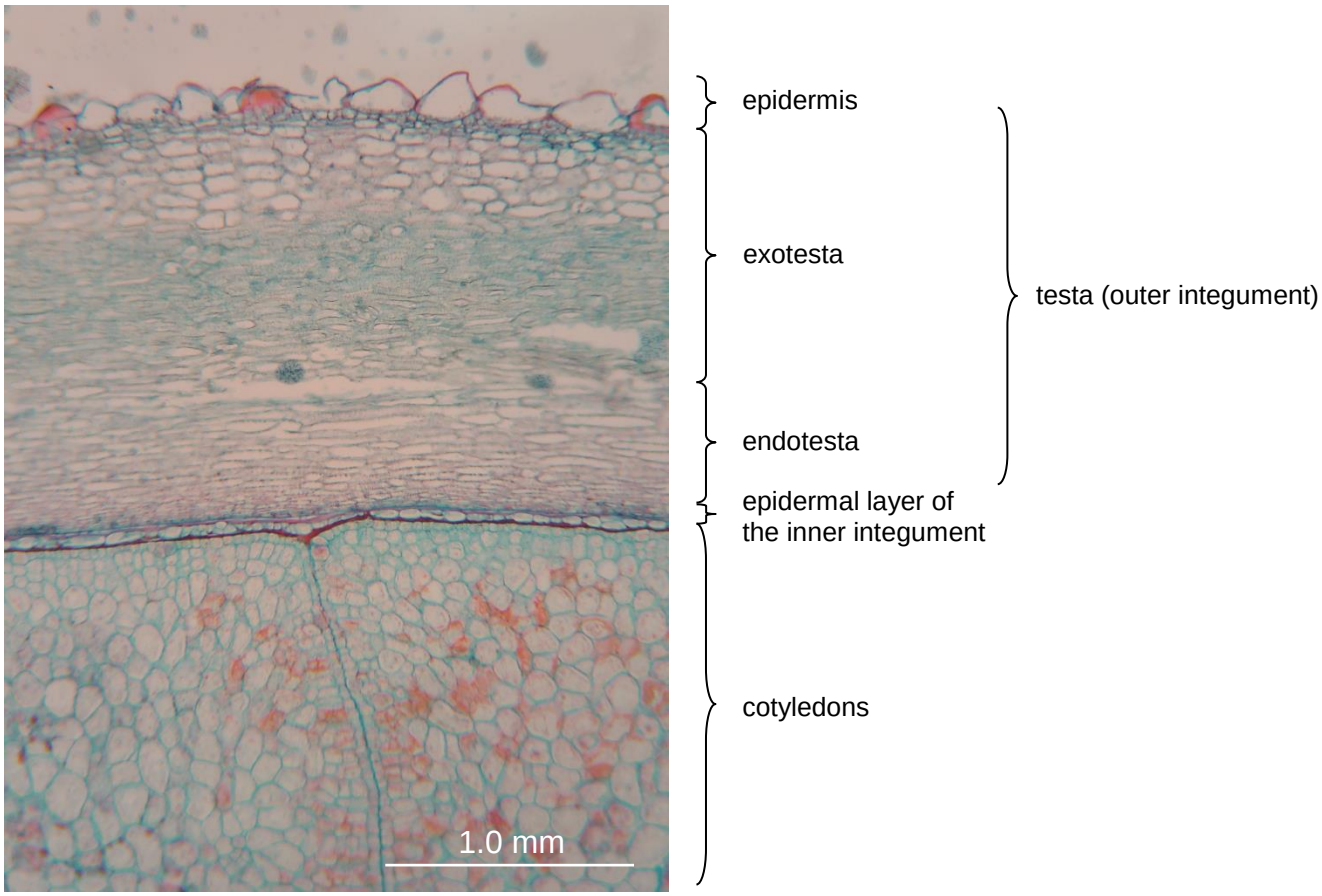

Figure 3.9 Cross-section of the seed coat and cotyledons of a mature *Moringa oleifera* seed.

At maturity, the Moringa seed coat was made up of several distinct layers namely epidermal layer, exotesta and endotesta which originated from the outer integument as well as remnants of inner integument (tegmen) (Figure 3.9). The exotesta's prominent papillate epidermis is followed by one or two layers of very small sub-epidermal cells, which are again followed by much larger but fairly thin-walled reticulate cells (Figure 3.10A). These layers of elongated, thick walled cells have large pits giving the illusion of spaces and extend into the three wings of the seed. The separation of the exotesta and the endotesta is marked by slightly elongated, reticulate cells with intercellular spaces as well as the vascular bundles underlying the seed wings (Figure 3.9 and Figure 3.10B). Figure 3.10C depicts a layer of elongated, thickly reticulate cells of which the inner layers from the previously meristematic region, have been flattened similar to the unrecognizable inner integument. Remnants of the large, thin-walled inner integument can be seen

surrounding the prominent inner epidermis (Figure 3.10D). This observation concurs with that of Corner (1976a), Corner (1976b) however differs from the observations made by Ramachandran *et al.* (1980) and Vaughan (1970), who both identified the unicellular layer as residual endosperm. Cross-sections of the seed at a fruit diameter of 8 mm (Figure 3.4) clearly show an epidermal layer pushed inward by cell enlargement of the inner integument, which was subsequently pressed back against the testa by the growing/expanding cotyledons (Figure 3.9). Furthermore, both the nuclear and cellular endosperm were completely consumed by the growing cotyledons and absent at seed maturity according to these observations. As a result, *Moringa oleifera* seed are non-endospermic.

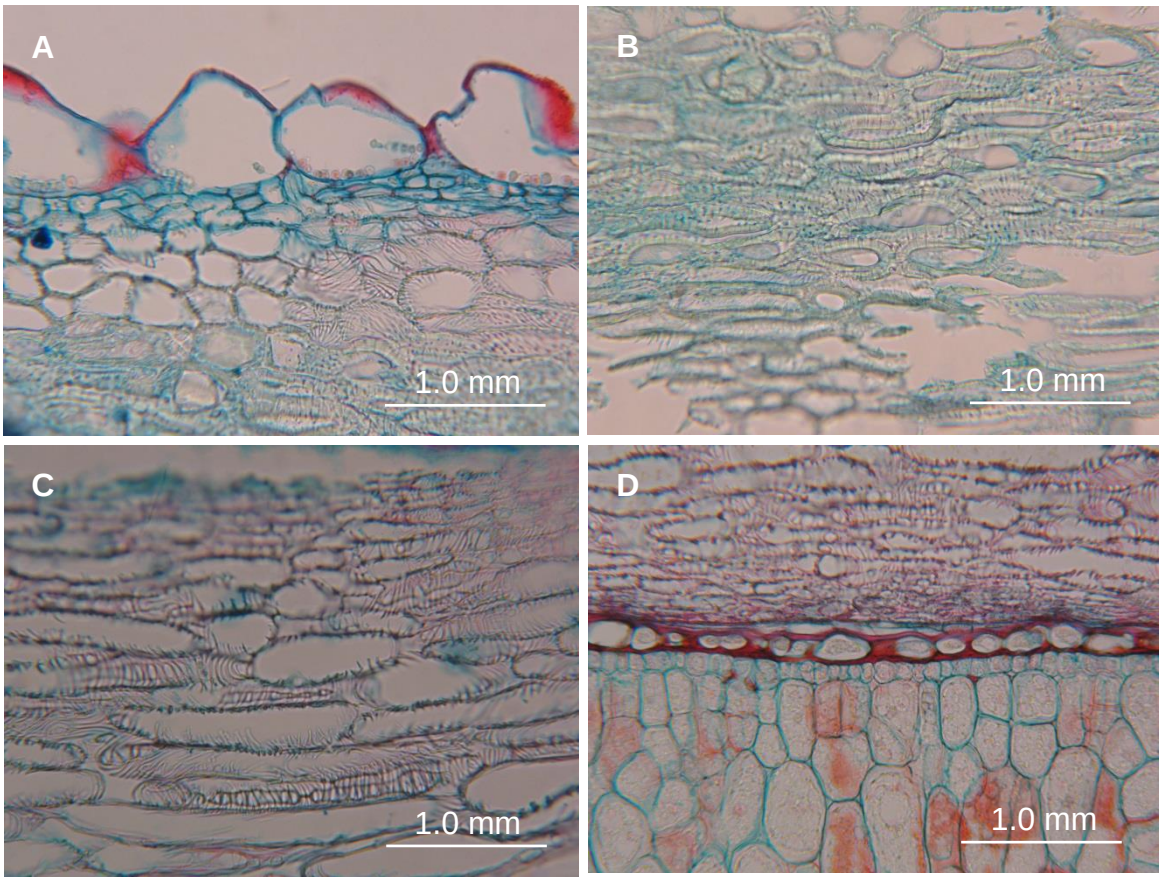

Figure 3.10 Cross-sections though various layers of a mature *Moringa oleifera* testa. A – seed coat epidermal and sub-epidermal layers, B – partial exotesta with thickened cell walls, C – endotesta, consisting of elongated fibrous cells, D – partial endotesta as well as the unicellular epidermal layer of the inner integument, separating the testa and the cotyledons.

At maturity, the average Moringa seed was 10 - 14 mm in diameter, weighed approximately 0.3 g and consisted of the embryo and two cotyledons, both of which were covered by the epidermal layer of the inner integument and enclosed by the more or less triangular seed coat from which three thin wings had developed to between 5 – 25 mm in length. Physical removal of the seed coat at maturity caused the seed coat to separate at the layer of thin-walled cells between the exo- and endotesta. As a result, the endotesta and inner integument remained connected to the cotyledons, together with three furrow lines left by the three vascular bundles.

3.5 Conclusion

Seed growth and storage compound synthesis are fundamental processes affecting both final yield and reproductive potential. Although very little had transpired in terms of seed development by the time fruit had reached a diameter of ±12 mm, the expansion of the cotyledons marked the beginning of reserve deposition. As a result, the growth stages between ±12 mm up until ±24 mm (±30 - 60 DAF) were the most crucial and consequently also the most sensitive to environmental stresses. Moisture stress during these growth stages could negatively affect seed mass and storage compound biosynthesis.

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CHAPTER 4

MORINGA OLEIFERA FRUIT GROWTH AND DEVELOPMENT AS AFFECTED BY VARIOUS IRRIGATION RATES

4.1 Summary

Moringa oleifera has long been recognized as a drought-tolerant tree, however, to what extent this tolerance has been at the expense of fruit growth remains unclear. Especially since fruit/seed are of economic importance, used for nutrition, water purification, cosmetics and fuel. This study therefore aimed to assess the effect of different irrigation rates on fruit growth and development by subjecting trees to three irrigation treatments, namely 900 mm, 600 mm and 300 mm per annum. Fruit growth was assessed during two developmental stages, namely every other day throughout the first 60 days after flowering (DAF) as well as at the end of the growing season when all fruits were harvested. Initial post-fertilization fruit growth demonstrated a significant reduction in fruit growth rates and final fruit size with reduced irrigation. At harvest though, there were no differences in the total fruit number per treatment. The number of mature fruit, average seed count and time to maturity increased with the rate of irrigation. Average seed mass increased from the 300IT to the 600IT, but decreased again between the 600IT and the 900IT. Between the fruit diameters of 12 – 24 mm (±30 – 60 DAF), fruit growth rate and reserve deposition were highest. As a result water stress during these stages could lead to both yield and fruit growth rate reductions.

4.2 Introduction

In order to explain possible yield variation caused by differences in water supply, the growth and development of the reproductive organs need to be thoroughly understood (Barnabás *et al.*, 2008, Alqudah *et al.*, 2011). This chapter therefore aims to illustrate the effect of different irrigation rates on fruit growth and development. *Moringa oleifera* is known to be drought-tolerant and adapted to semi-arid environments (Morton, 1991, Palada and Chang, 2003), however, the effect of water stress on fruit/seed development and yield is still unknown. The extent of the effect that water stress will have on seed development will depend on the developmental stage as well as the severity and duration of the stress (Acosta Gallegos and Kohashi Shibata, 1989, Çakir, 2004). One of the first growth processes affected by water stress is cell expansion (Hsiao, 1973, Farooq *et al.*, 2009), this in turn could lead to a reduction in fruit size and yield. The objective of the study was to establish to what extent irrigation affected post-fertilization fruit length, diameter, mass, growth rate and seed number. In addition, data collected from all fruit at various growth stages could be used to simulate fruit growth. This would allow for future predictions of average seed count, seed mass and maturity level based on the fruit diameter/length.

4.3 Materials and Methods

Trials were conducted on six-year-old *Moringa oleifera* trees at the field trial section on the Hatfield Experimental Farm of the University of Pretoria (25°45'S, 28°16'E) at an altitude of 1372 m above sea level and an average annual rainfall of 674 mm. Trees for the purpose of this trial were grown from seeds sourced in India and transplanted into the field. Trees were then divided into three groups of four trees each. Each of the three groups was subject to a different irrigation treatment. Irrigation water was applied through a surface

drip irrigation system at three rates. According to Palada and Chang (2003), the minimum annual rainfall requirement for *Moringa oleifera* is 250 mm/year. The three administered irrigation rates were thus based on the minimum (300 mm/year)(300IT) amount for the tree, average (600 mm/year)(600IT) annual rainfall for the research site and a higher (900 mm/year)(900IT) treatment, simulating supplement irrigation under field conditions. The irrigation amounts were administered, simulating total annual rainfall (mm/year). Three dipper lines were installed at the 900IT, two dripper lines at the 600IT, while the 300IT had a single dripper line at the base of the tree trunks. The in-line dripper spacing was 30 cm, with an application rate of 2.1 litres/hour/dripper for all three irrigation treatments. Plastic sheeting was then placed over the dripper irrigation, underneath the trees covering an area of 4 m on either side of the trunks. With this rainfall exclusion method, irrigation can be administered with greater accuracy without having to compensate for rainfall. Organic mulch was placed on top of the plastic sheeting so as to not adversely affect the energy balance of the soil. Semi-weekly soil water content measurements were conducted using a neutron probe (Campbell Pacific Nuclear, 503DR Hydroprobe) to verify differences in soil water levels between treatments (measurement results provided in Chapter 2).

Trees were subjected to the irrigation treatments for nine months prior to the initial floral assessment.

The fruit growth and development assessment was performed during two developmental phases. Initially, fruit growth was monitored during the immediate post-fertilization period, while secondly fruits from all developmental stages at all three irrigation treatments were harvested at the end of the growing season. Individual flowers from each of the three irrigation treatments were tagged and monitored every second day, post-fertilization for 60 days after flowering (DAF). During these assessments fruit growth and development was

monitored by measurements of both fruit length and diameter. Fruit length was measured with a measuring tape while a calliper was used to measure fruit diameter. Measurements from all tagged flowers/fruit at the three irrigation treatments were expressed in terms of DAF, for comparison purposes.

At the end of two consecutive growing season (2011 and 2012), all fruit from the three irrigation treatments were harvested, counted (1384 fruit during 2011 and 1429 fruit during 2012) and classified according to developmental stage based on fruit diameter. Fruit diameter was chosen as the best parameter for seed development as this is a non-destructive measurement, which can be performed by growers while the fruit remains attached to the tree. The fruit diameter categories were grouped into 2 mm intervals starting at 8 mm up to 28 mm in diameter (8 mm, 10 mm, 12 mm,… 28 mm). Fruit length from the various diameter categories were measured, while total fruit mass, seed mass and shell (seedcase) mass as well as number of seeds were determined for each irrigation treatment.

Data were statistically analysed using the Statistical Analysis Software (SAS Version 9.2) program for Microsoft Windows, by the Statistics Department at the University of Pretoria. Analysis of Variance (ANOVA General Linear Model) was used to assess if differences between treatments were significantly different ($P < 0.05$). Correlations between fruit diameter (mm) and various other measured parameters (seed count, seed/fruit mass) were determined using regression functions with the highest correlation coefficient.

4.4 Results and Discussion

Fruit growth measurement data of both the fruit length and diameter were plotted against DAF. This representation of data reflects the growth rate. From the growth data represented in Figure 4.1 and Figure 4.2, the effect of the lower irrigation treatments became evident with the decline in growth rates. Similar findings have been reported in *Prunus armeniaca* L. (Torrecillas *et al.*, 2000), *Malus domestica* (Naor *et al.,* 1997) and *Pyrus serotina* Rehder (Caspari *et al.*, 1994). Fruit length increases, illustrated in Figure 4.1, demonstrate the sigmoidal growth pattern of the juvenile *Moringa oleifera* fruit during the first 60 days after fertilization. At 10 DAF the average fruit length of the 300IT was 32.5mm, 58.5mm at the 600IT and 89.3mm at the 900IT. Fruit growth rates were accelerated by the increase in irrigation, especially during the initial 30 DAF. During these 30 days of the trial period, fruit lengths differed significantly between all three ITs. For the subsequent 30 days however, significant differences were only achieved between the 900IT and 300IT. Average growth rates for the entire trial period were 5.3 mm/day at the 300IT, 6.0 mm/day at the 600IT and 6.2 mm/day at the 900IT.

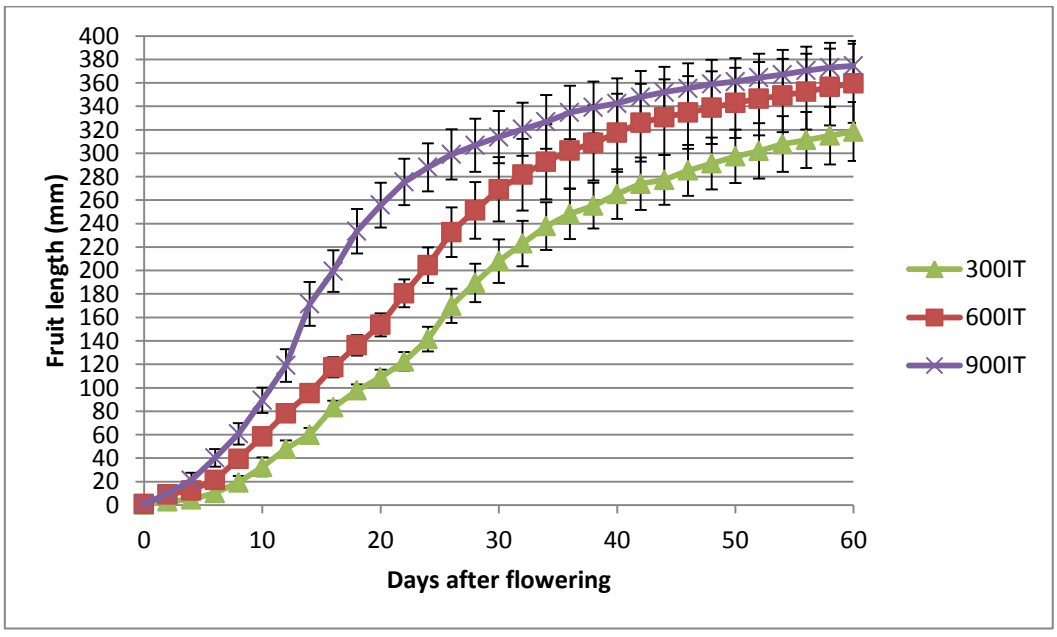

Figure 4.1 Increases in *Moringa oleifera* fruit length at three irrigation treatments over time, expressed in terms of days after flowering (DAF). (900IT – 900 mm/annum, 600IT – 600 mm/annum, 300IT – 300 mm/annum). Vertical bars (±) indicate standard errors of the mean (SEM).

Within the first two DAF, fruits at all three ITs reached a diameter of ±2 mm, at which they remained until approximately 10 DAF, whereafter there was a continuation of linear increases in fruit diameter (Figure 4.2). During this initial lag phase, fruit primarily increased in length. Similarly to the effect that lower irrigation rates had on fruit length, fruit diameter increases were slower and reached a narrower final diameter on average. Average fruit diameter increases for the entire trial period were 0.33 mm/day at the 300IT, 0.42 mm/day at the 600IT and 0.47 mm/day at the 900IT.

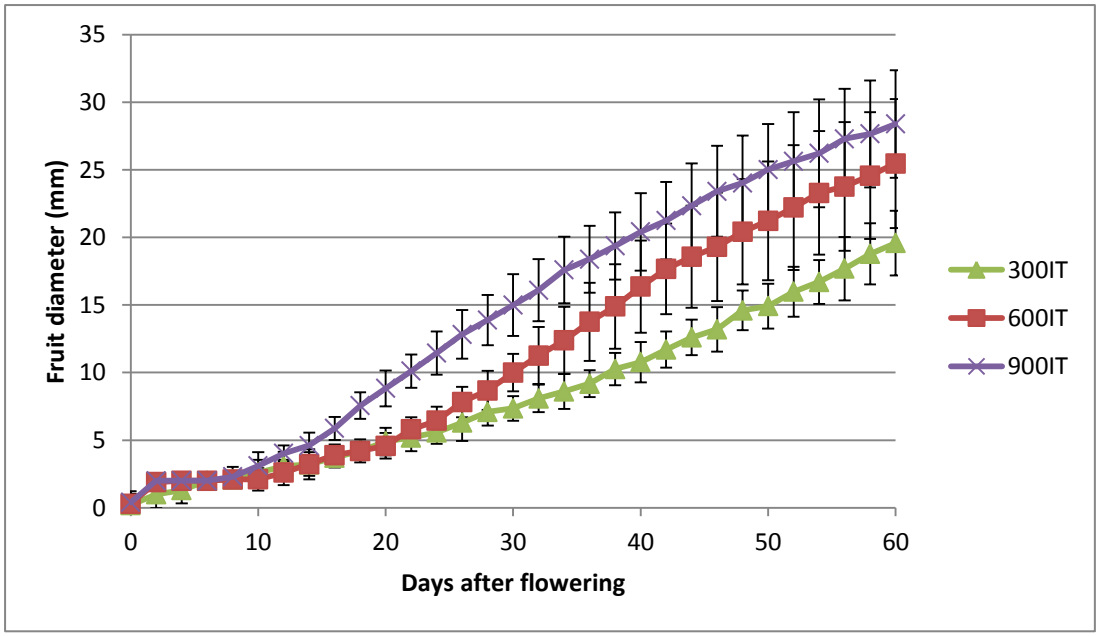

Figure 4.2 Increases in *Moringa oleifera* fruit diameter at three irrigation treatments (900IT, 600IT and 300IT) over time, expressed in terms of days after flowering (DAF). Vertical bars (±) indicate standard errors of the mean (SEM).

Fruit length and diameter increases during the first 60 DAF demonstrated a very similar trend across all three ITs. The irrigation treatments seemingly only affected the onset and growth rate, in other words, the time to reach a certain stage. If the "time factor" is removed by expressing fruit length (x) as a function of fruit diameter (y), as illustrated in Figure 4.3, fruit diameter increased exponentially with an increase in fruit length. Furthermore, the irrigation treatment seemingly had very little effect on the exponential

relationship between fruit length and diameter. However, both fruit diameter and length were lower at 60 DAF, as a result of a reduction in irrigation. The exponential relationship with which fruit diameter (y) increased with fruit length (x) can be expressed with the following functions $y = 1.1956e^{0.0083x}$, $y = 1.2475e^{0.0082x}$ and $y = 1.3816e^{0.0082x}$ for the 900IT, 600IT and 300IT respectively (Figure 4.3).

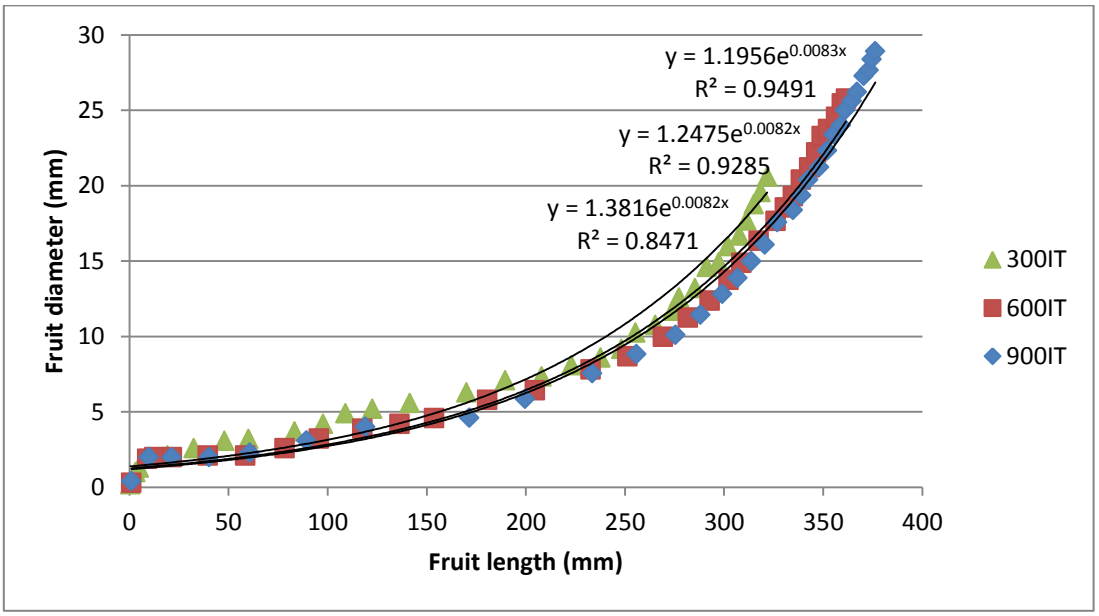

Figure 4.3 *Moringa oleifera* fruit length (mm) expressed as a function of fruit diameter (mm). (900IT – 900 mm/annum, 600IT – 600 mm/annum, 300IT – 300 mm/annum).

During the first 60 DAF, the decrease in irrigation amount clearly restrained the fruit growth rate as well as the final fruit length and diameter. There is however, a clear exponential relationship between fruit diameter and length, which is very similar irrespective of the irrigation treatment. Up until a fruit length of approximately 175 mm (Figure 4.3), the fruit diameter had increased to about 5 mm on average. From the fruit length of about 175 mm until around 350 mm, the fruit diameter had increased on average to about 20 – 25 mm. This suggests that fruit initially increased in length, with very little thickening of the fruit. Once seed filling (reserve deposition) commences, fruit started to thicken and as a result increased the fruit diameter. This observation is illustrated visually in Figure 4.4. Developing immature fruit, illustrated in Figure 4.4A have already reached a significant

length, while remaining relatively slender. Maturing fruit in contrast, as illustrated in Figure 4.4B, have not increased as much in length as they have gained in diameter, with the groves of the individual seeds being clearly visible.

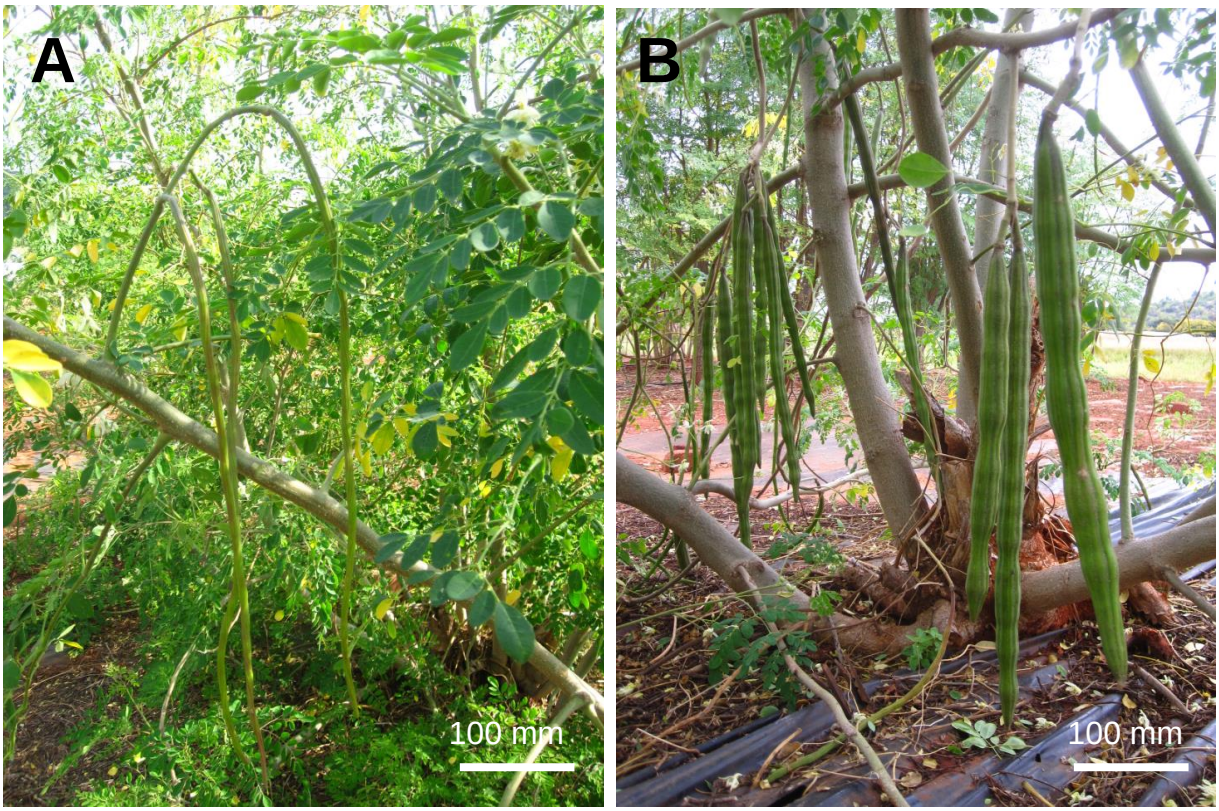

Figure 4.4 *Moringa oleifera* fruit. A – developing fruit; B – mature fruit.

Based on data collected from all harvested fruit (1384 fruit during 2011 and 1429 fruit during 2012), the average total seed count per fruit, based upon their length is illustrated in Figure 4.5. While data distribution is illustrated in Figure 4.5, averages for each fruit length are given in Figure 4.6 demonstrating the positive linear relationship that exists between fruit length and average seed number per fruit. This linear relationship confirms the expectation that the number of seed per fruit generally increases with fruit length/diameter (Figure 4.6 and Figure 4.7). Based on the average seed number per fruit at the various fruit lengths/diameters and the direct linear relationship that exists between these two parameters, seed number (y) could be estimated based on the fruit length (x) using the

function y = 0.0227x + 4.906 and seed diameter (x) using the function y = 0.3861x + 8.4249.

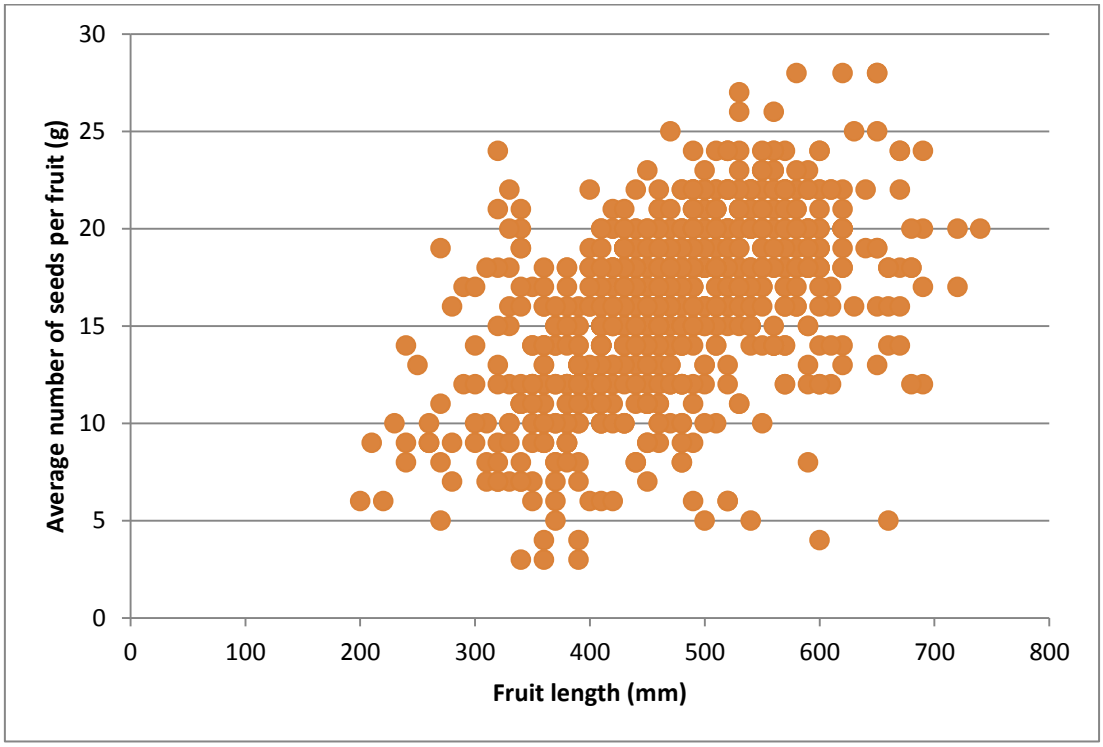

Figure 4.5 Data point distribution of seed number per *Moringa oleifera* fruit as a function of fruit length (mm).

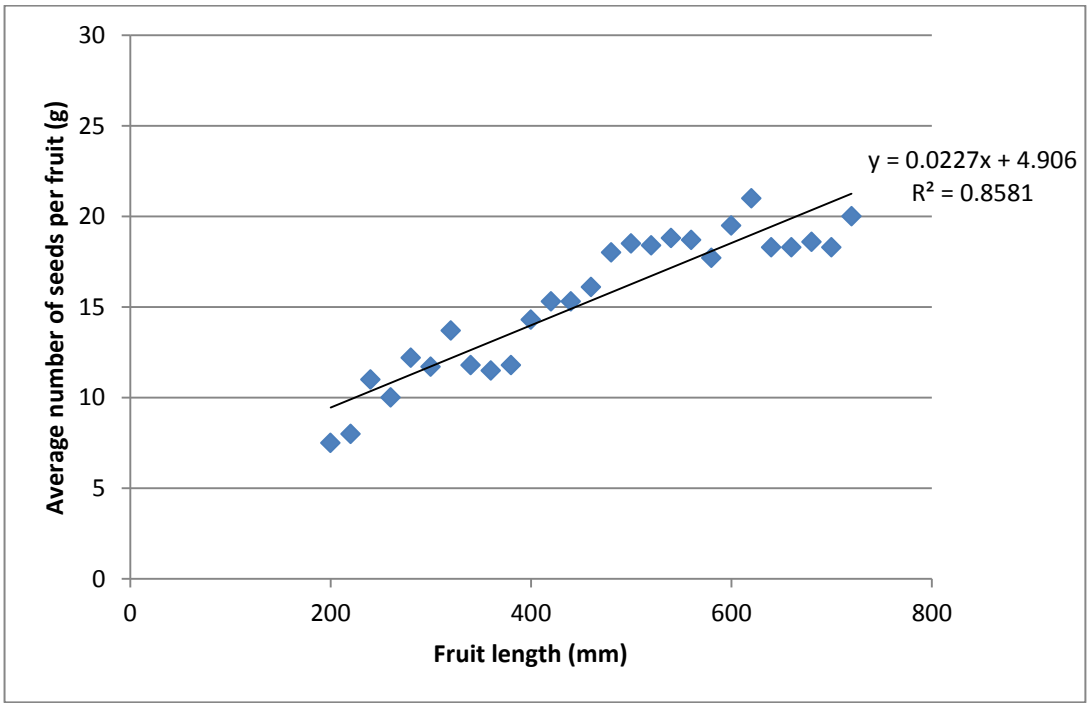

Figure 4.6 Linear relationship between *Moringa oleifera* fruit length (mm) and average seed number per fruit.

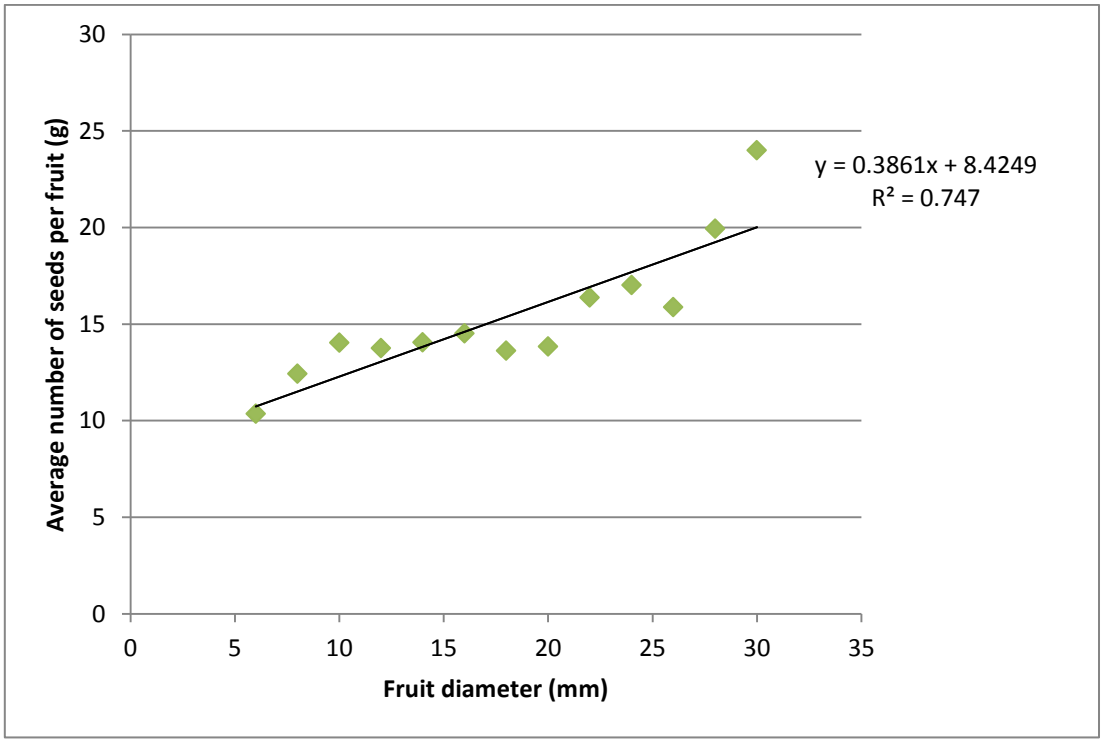

Figure 4.7 Linear relationship between *Moringa oleifera* fruit diameter (mm) and total seed number per fruit.

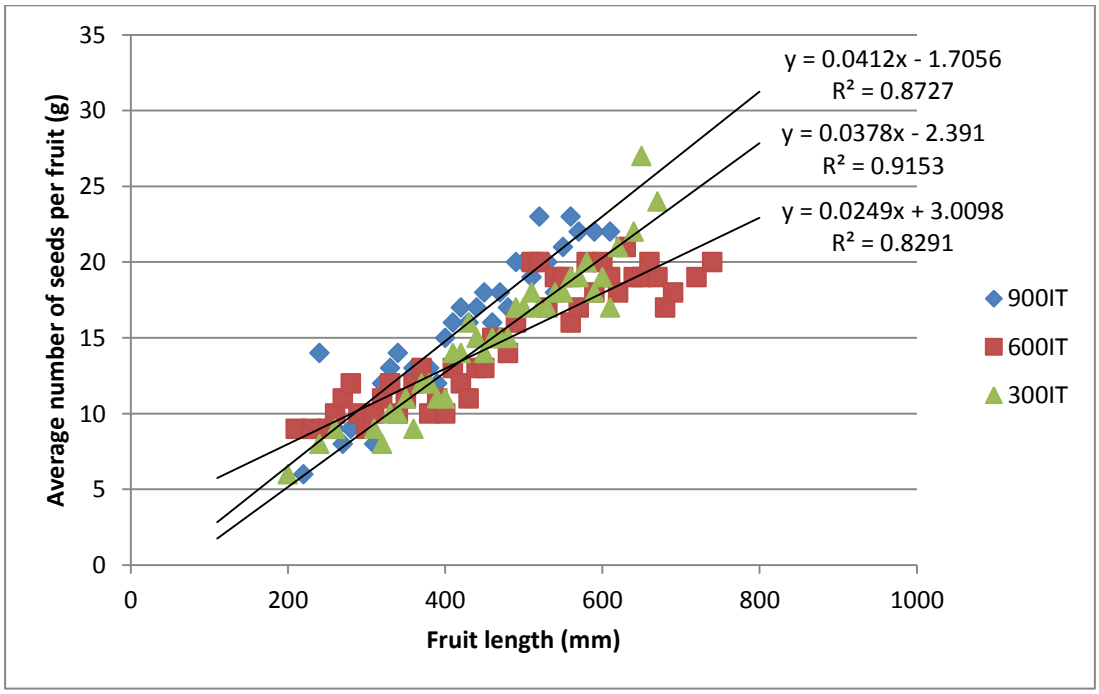

Figure 4.8 Linear relationship between *Moringa oleifera* fruit length (mm) and total seed number per fruit at three ITs. (900IT – 900 mm/annum, 600IT – 600 mm/annum, 300IT – 300 mm/annum).

Despite slight differences in the correlation between seed number and fruit length caused by the three irrigation treatments as illustrated in Figure 4.8, these differences remained insignificant. Fruit (total, shell and seed) mass increases with fruit diameter are portrayed in Figure 4.9.

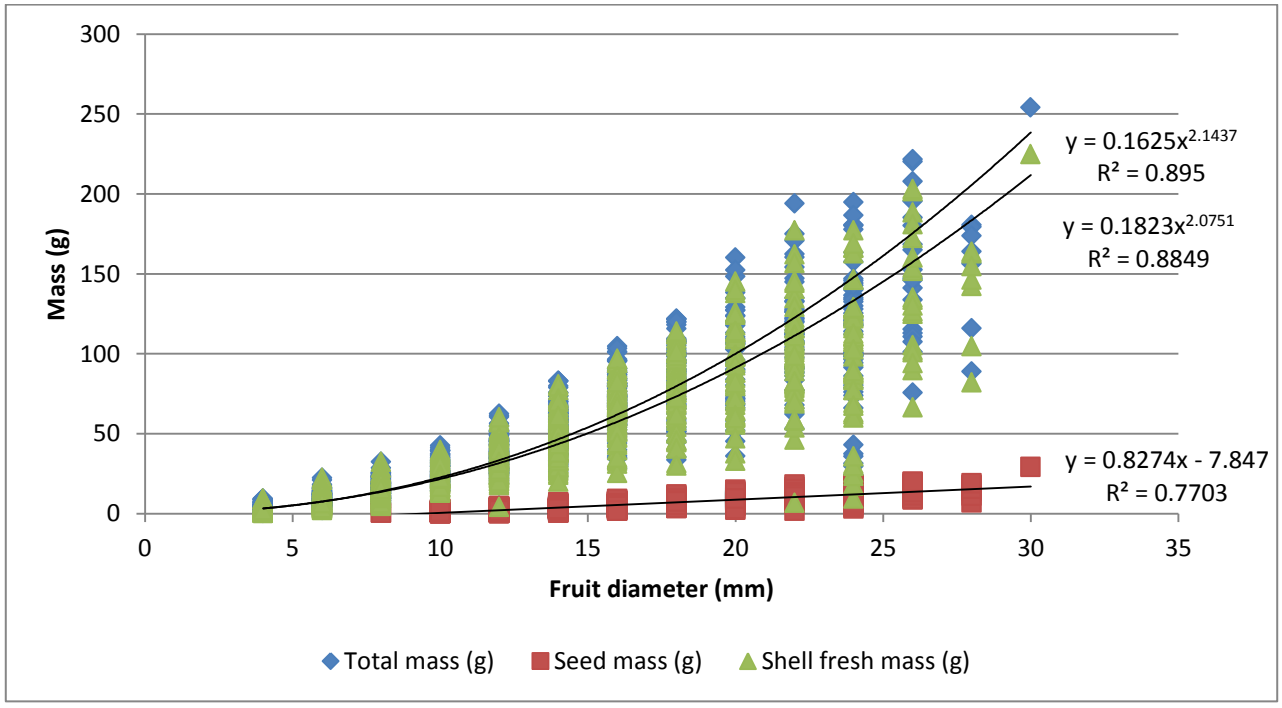

Figure 4.9 Data distribution of *Moringa oleifera* fruit mass (total, shell and seed) (g) as a function of fruit diameter (mm).

Average fruit (total, shell and seed) mass increases are illustrated in Figure 4.10. Linear regression functions best fit the three data sets, with the increase rate of both total and shell mass, being the highest (Figure 4.10). Seed mass also increased linearly with fruit diameter but at a much lower rate. Based on the regression functions given in Figure 4.10, total fresh fruit mass (y) could be estimated based on fruit diameter (x) using the function y = 6.5655x - 34.166. Similarly, y = 5.8747x - 29.199 can be used to estimate shell mass (g) and y = 0.6907x - 4.9666 to determine the approximate seed mass (g). Therefore, by simply measuring the fruit diameter on the tree, both the mean fruit- and mean seed mass can be estimated non-destructively and used in yield predictions.

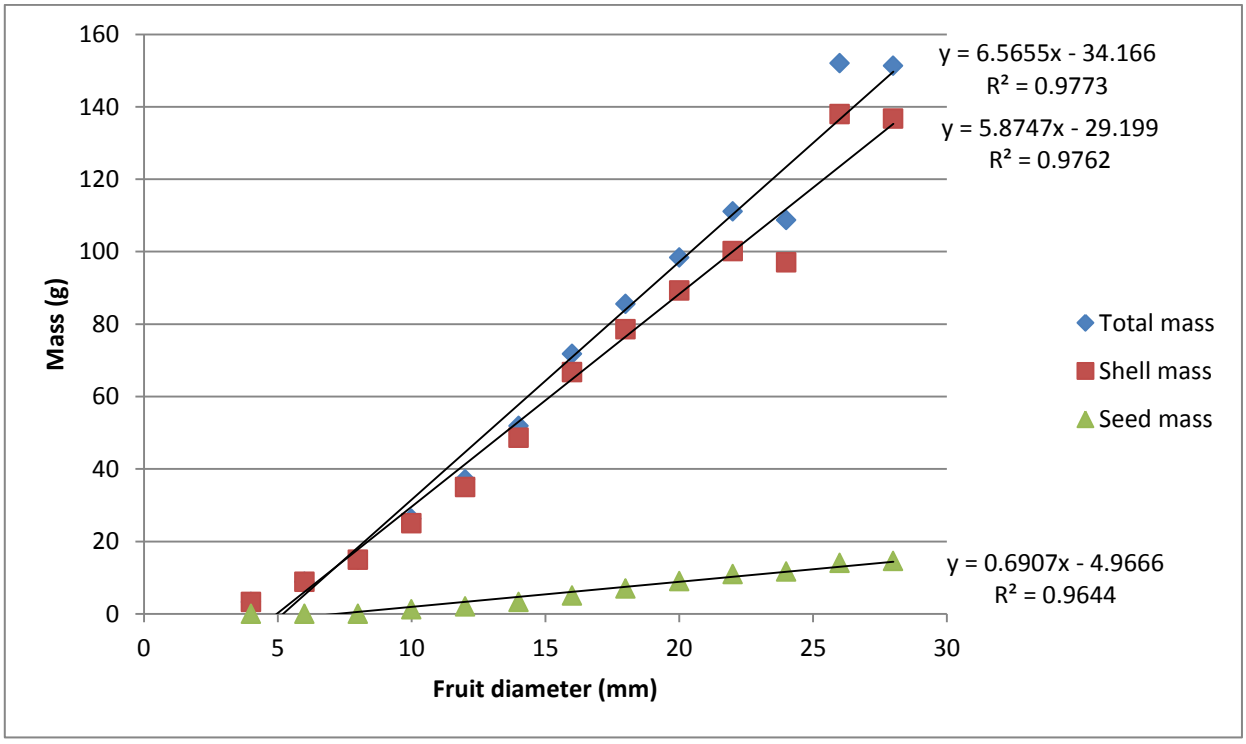

Figure 4.10 Linear relationship between *Moringa oleifera* fruit mass (total, shell and seed) (g) and fruit diameter (mm).

Figure 4.11 illustrates the relative contribution that both the pericarp (outer shell) and seed mass make towards the total fruit mass. The outer shell accounted for an average of 92.9% of the total fresh fruit mass, with the seed mass contributing a mere 7.1% to total fresh fruit mass on average. However, the average seed moisture content was 47.4%, whereas the average shell moisture content was 80.7%. Due to the lower moisture content of the seed, they contribute 17.2% of the total dry mass, while the higher moisture content of the shell reduced the contribution of their mass towards the total dry mass with a considerably larger 82.8%.

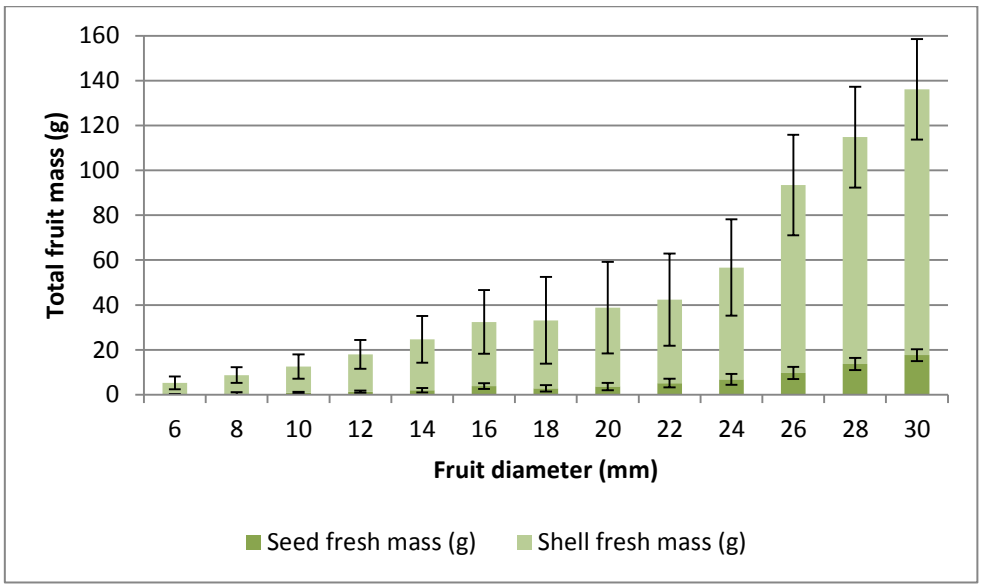

Figure 4.11 Shell and seed mass fractions of average total fruit mass at various diameters. Vertical bars (±) indicate standard errors of the mean (SEM).

The average mass of a single seed increased with the increase in fruit diameter (mm) as illustrated in Figure 4.12 and Figure 4.13. Similar to total seed mass, individual seed mass also increased with fruit diameter (Figure 4.12). Unlike total seed mass though, the gain in mass of individual seed was sigmoidal (Figure 4.13) and not linear.

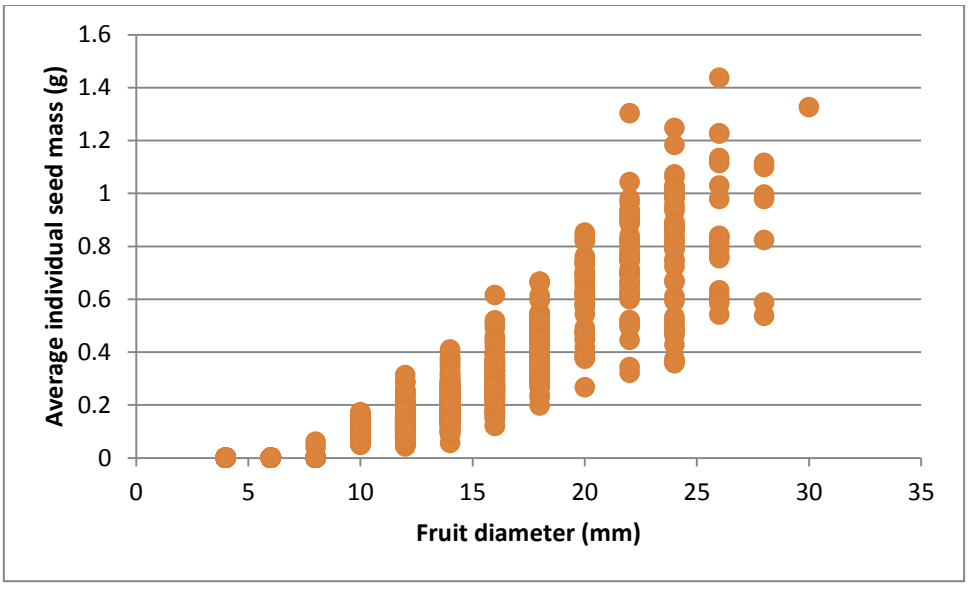

Figure 4.12 Data distribution of average individual seed mass based on fruit diameter (mm) of *Moringa oleifera.*

Based on the average seed diameter (x) values from Figure 4.13, individual seed mass (y) can be estimated using the following sigmoid function $y = -8E\text{-}06x^4 + 0.0004x^3 - 0.003x^2 + 0.0073x$.

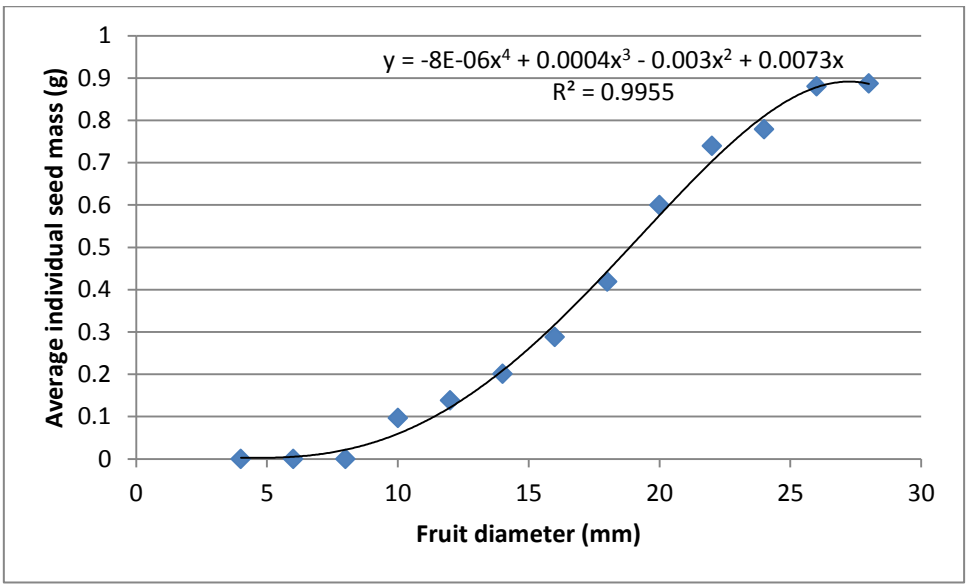

Figure 4.13 Average individual seed mass based on fruit diameter (mm) of *Moringa oleifera.*

Considering the effect that irrigation had on individual seed mass (g) at increasing fruit diameters, the variation between treatments was only slight (Figure 4.14). Average individual seed mass was highest at the 600IT, across all diameter categories, with both the 900IT and 300IT having similar seed masses at any given fruit diameter.

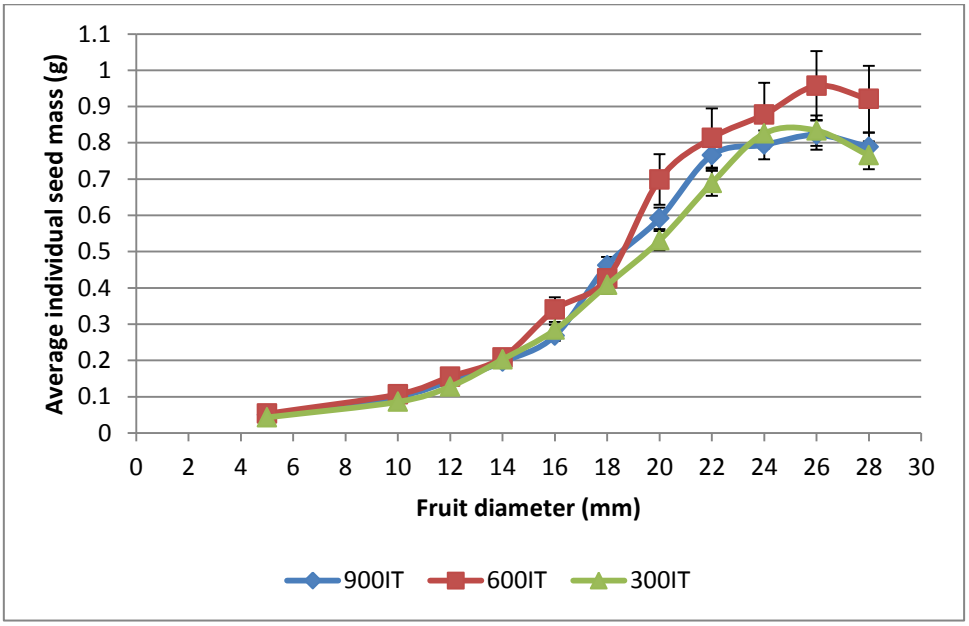

Figure 4.14 Average individual seed mass of *Moringa oleifera* (g) at various fruit diameters (mm) as affected by three ITs. (900IT – 900 mm/annum, 600IT – 600 mm/annum, 300IT – 300 mm/annum). Vertical bars (±) indicate standard errors of the mean (SEM).

Using fruit diameter as an indicator for seed development, the changes in fresh mass (g), dry mass (g) and moisture content (g) at various fruit diameters are illustrated in Figure 4.15. A period of initial mass increase (histo-differentiation) up to a fruit diameter of 12 mm was followed by a period of rapid mass gain as cells expand to accommodate the deposition of synthesized storage reserves. The final stage of maturation commences once seed moisture content starts to decrease (fruit diameter >24 mm) (refer to Chapter 3). The growth period where fruit are between 12 – 24 mm in diameter, is the most susceptible to water stress as this is the period of reserve (oil, starch and protein) deposition (detailed discussion in Chapter 7).

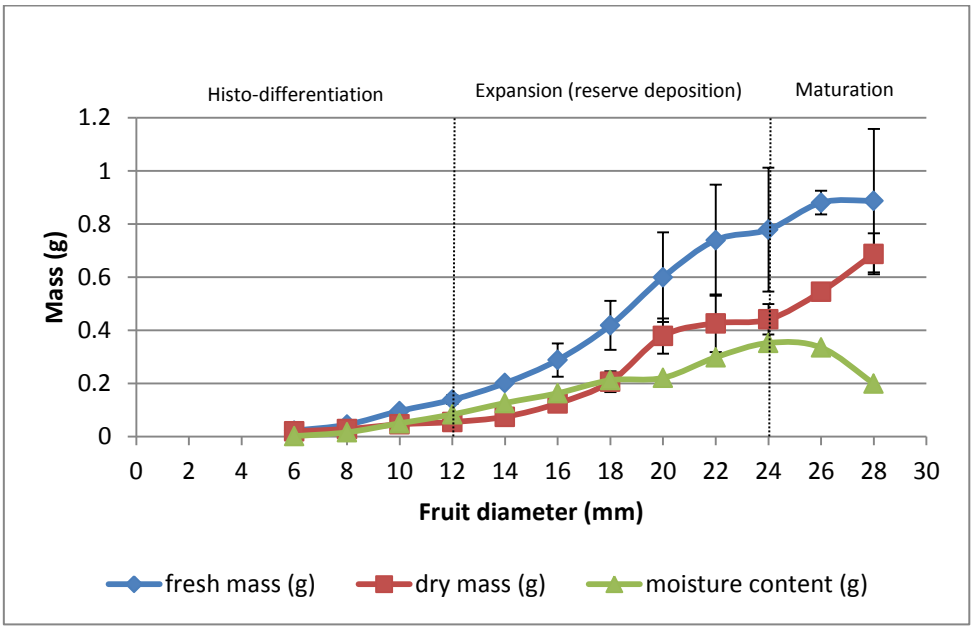

Figure 4.15 Changes in whole-seed fresh mass (g), dry mass (g) and moisture content (g) during development. Vertical bars (±) indicate standard errors of the mean (SEM).

In an attempt to assess to what extent fruit length was affected by the three ITs, all fruits were grouped into fruit length categories of 50 mm increments based on their fruit length at harvest. The total number of fruit within each category of the three ITs were documented and results are illustrated in Figure 4.16. The total fruit count over two consecutive seasons was 901 (446 (2011) + 455 (2012)) for the 900IT, 906 (443 (2011) + 463 (2012))

for the 600IT and 1006 (495 (2011) + 511 (2012)) for the 300IT. Based on the frequency distribution (number of fruit) of each treatment, irrigation did affect final fruit length. At the 900IT the frequency distribution is skewed towards the right, indicating a greater proportion of longer fruit, while relative to the 900IT, the 300IT is skewed towards the left, demonstrating a greater proportion of shorter fruit. The 600IT however, had a lower, symmetrical frequency distribution, signifying a more equal distribution across all fruit length groups.

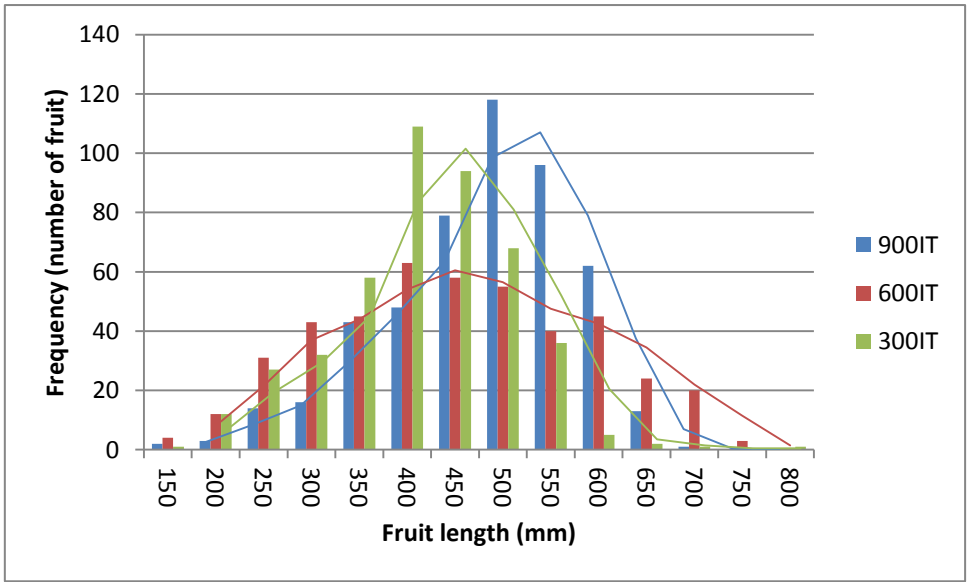

Figure 4.16 Frequency distribution of *Moringa oleifera* fruit as a function of fruit length (mm). (900IT – 900 mm/annum, 600IT – 600 mm/annum, 300IT – 300 mm/annum).

Similarly to fruit length, all fruit were grouped according to their fruit diameter and counted. The fruit diameter frequency distribution of each treatment is given in Figure 4.17. Both the 600IT and 300IT had the highest number of thin fruit, decreasing in numbers as fruit diameter increased. The 900IT in comparison, had the highest number of fruit with an intermediate diameter (14 mm), with decreasing fruit numbers of both slimmer and thicker fruit. This can mainly be attributed to the faster growth rate at the 900IT, whereby more fruit have reached maturity by the end of the growing season, compared to both the 600IT and 300IT.

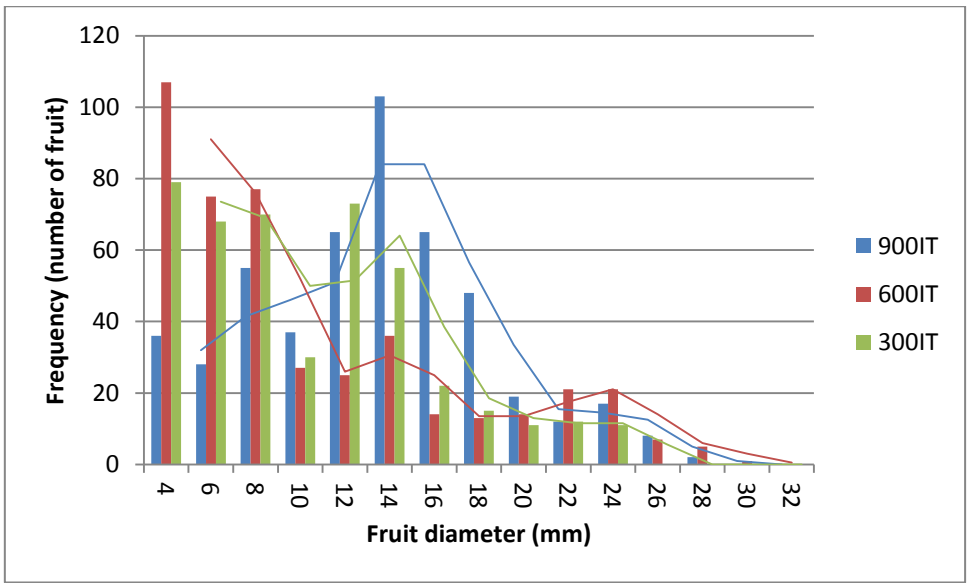

Figure 4.17 Frequency distribution of *Moringa oleifera* fruit as a function of fruit diameter (mm). (900IT – 900 mm/annum, 600IT – 600 mm/annum, 300IT – 300 mm/annum).

4.5 Conclusion

In terms of initial post-fertilization fruit growth, growth rates and final fruit size decreased with the reduction in irrigation. Irrigation did not directly affect yield in terms of fruit numbers as there were similar number of fruit across all treatments. An increased number of mature fruit as well as a greater average seed count per fruit were observed with the increase in irrigation rates, since the time to reach maturity was slightly prolonged, with the reduction in irrigation. Average seed mass increased from the 300IT to the 600IT, but decreased again between the 600IT and the 900IT. The increasing number of abscised flowers with the decrease in irrigation as discussed in Chapter 2, significantly reduced the yield potential. Thus, although *Moringa oleifera* is tolerant to drought, its sensitivity to moisture stress seemingly varies throughout the growing season. The flowering and fruit set stage being more sensitive to drought, opposed to the fruit growth and development, without taking its effect on seed quality into consideration. Considering all the observations made throughout this study, production areas with an annual rainfall amount of ±600 mm/year would be recommended as most suitable for *Moringa oleifera.*

4.6 References

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CHAPTER 5

THE EFFECT OF VARIOUS IRRIGATION RATES ON PHOTOSYNTHESIS, STOMATAL CONDUCTANCE, CHLOROPHYLL CONTENT AND ANATOMICAL STRUCTURE OF *MORINGA OLEIFERA* LEAVES

5.1 Summary

Since photosynthesis is central to overall plant growth, to what extent the photosynthetic rate in *Moringa oleifera* leaves is affected by different irrigation rates needed to be assessed. *Moringa oleifera* trees were grown at irrigation treatments simulating three annual rainfall amounts namely, 900 mm (900IT), 600 mm (600IT) and 300 mm (300IT) irrigation per annum. Photosynthesis measurements were conducted on these trees during three different growth stages (vegetative growth, flowering and fruit development), during the 2011 and 2012 growing seasons. Lower photosynthetic rate was not only observed with a decrease in irrigation treatment but also as leaves aged, with the progression of the growing season. Decreases could not be attributed solely to either stomatal (conductance and SI) or non-stomatal (possible RuBP regeneration, ATP synthesis and mesophyll conductance) limitations within the leaves due to insignificant differences, but were rather a combination of factors with the extent of their contribution varying throughout the growing season. Decreases in photosynthetic rate among treatments were as a result of both stomatal and non-stomatal limitations, while the photosynthetic rate decreases observed throughout the growing season were largely as a result of diminishing leaf chlorophyll concentrations with leaf age.

5.2 Introduction

Plant water availability is an important factor that affects various physiological and metabolic plant processes. Photosynthesis is one such process, which generally decreases with reduced water availability (Flexas and Medrano, 2002, Grassi and Magnani, 2005, Flexas *et al.*, 2009). As plants experience water stress, carbon dioxide (CO_2) diffusion to the chloroplasts is primarily restricted through stomatal closure while other metabolic processes, such as ribulose bisphosphate (RuBP) regeneration and adenosine triphosphate (ATP) synthesis might also be affected (Tezara *et al.*, 1999, Flexas and Medrano, 2002). While photosynthesis is a fundamental process of primary plant metabolism, any reduction in photosynthetic rate will affect photoassimilate partitioning throughout the plant (Chaves *et al.*, 2002, Chaves *et al.*, 2003, Flexas *et al.*, 2004, Lawlor and Tezara, 2009). From an economic perspective, reduced photosynthesis can ultimately be defined in terms of yield reductions (Passioura, 2007). This chapter therefore aims to quantify to what extent reduced plant water availability affects photosynthesis and ultimately yield in *Moringa oleifera*. Since photosynthesis is affected by various physical factors such as stomatal conductance, stomatal index and chlorophyll content, these parameters were also measured to determine how they are affected by irrigation and in turn affect net photosynthesis.

5.3 Materials and Methods

5.3.1 General trial procedures

Trials were conducted on six-year-old *Moringa oleifera* trees at the field trial section on the Hatfield Experimental Farm of the University of Pretoria (25°45'S, 28°16'E) at an altitude of 1372 m above sea level and an average annual rainfall of 674 mm. Trees for the purpose

of this trial were grown from seeds sourced in India and transplanted into the field. Trees were then divided into three groups of four trees each. Each of the three groups was subjected to a different irrigation treatment. Irrigation water was applied through a surface drip irrigation system at three rates. According to Palada and Chang (2003) the minimum annual rainfall requirement for *Moringa oleifera* is 250 mm/year. The three administered irrigation rates were thus based on the minimum (300 mm/year)(300IT) amount for the tree, average (600 mm/year)(600IT) annual rainfall for the research site and a higher (900 mm/year)(900IT) treatment, simulating supplemental irrigation under field conditions. The irrigation amounts were administered, simulating total annual rainfall (mm/year).

Three dripper lines were installed at the 900IT, two dripper lines at the 600IT, while the 300IT had a single dripper line at the base of the tree trunks. The in-line dripper spacing was 30 cm, with an application rate of 2.1 litres/hour/dripper. Plastic sheeting was then placed over the dripper irrigation, underneath the trees covering an area of 4 m on either side of the trunks. With this rainfall exclusion method, irrigation can be administered with greater accuracy without having to compensate for rainfall. Organic mulch was placed on top of the plastic sheeting so as to not adversely affect the energy balance of the soil. Semi-weekly soil water content measurements were conducted using a neutron probe (Campbell Pacific Nuclear, 503DR Hydroprobe) to verify differences in soil water levels between treatments (measurement results provided in Chapter 2).

Trees were subjected to the irrigation treatments for nine months prior to the initial photosynthesis measurements and leaf sampling. Leaf samples were prepared and viewed using both light and electron microscopy techniques to determine the effect of irrigation on leaf thickness, stomatal size and stomatal index (SI).

5.3.2 Light microscopy

Leaflet segments for anatomical examination were randomly collected from the three irrigation treatments and prepared for light microscopy before being imbedded according to methods adapted from O'Brien and McCully (1981). Leaf segments were fixed in FAA (80% ethanol: 37% formaldehyde: 100% acetic acid, in proportions 8:1:1 v/v/v) for 24 hours and dehydrated in a series of increasing ethanol in water concentrations (30%, 50%, 70%, 100% v/v). Samples were subsequently transferred through a series of xylene in ethanol concentrations (30%, 50%, 70%, 100% v/v) and placed into paraffin wax (60°C). Leaf samples were then embedded in paraffin wax. Using a Reichert-Jung semi-thin rotary microtome, 10 µm thick sections were cut and placed onto microscope slides before being de-waxed in a series of xylene concentrations. Sections were stained with both safranin and fast-green before being viewed with a Leitz Biomed light microscope. Photographs were taken digitally using a Canon PowerShot A630 digital camera. These images were subsequently used to measure differences in leaf thickness.

5.3.3 Scanning electron microscopy (SEM)

Randomly collected leaflets from the three irrigation treatments were prepared for SEM according to Coetzee and Van der Merwe (1996). A 3 mm X 5 mm square leaf sample was sectioned from the centre of a pinnule (leaflet) (between the midrib and the pinnule margin) collected from a central pinna. Segment specimens were fixed in 2.5% gluteraldehyde in a 0.075 M phosphate buffer (pH 7.4 - 7.6) for two hours, before being rinsed 3X in the same 0.075 M phosphate buffer (10 min. each). Specimens were then fixed in 0.5% aqueous osmium tetroxide (OsO_4) for two hours and rinsed 3X with distilled water (10 min. each), followed by dehydration of the specimens in a range (30%, 50%,

70%, 90% v/v) of ethanol:water dilutions, followed by three times in 100% ethanol. After dehydration, some of the leaf samples were transferred and dried in a Bio-Rad E3000 critical point drier with liquid CO_2, before being mounted on aluminium stubs and coated with gold in a Polaron E5200C sputter coater. Leaf sections were viewed with a JOEL 840 scanning electron microscope, while photographs were taken digitally.

Initial leaf segments from the three irrigation treatments were examined using SEM for visual representation of the adaxial and abaxial leaf surfaces. Follow-up stomatal count studies however, were conducted using the less time consuming and cost effective method of applying a thin layer of clear nail polish to the abaxial leaf surfaces. Once dry, the film of nail polish was removed and mounted on a microscopy slide in glycerol, which was then viewed using a Leitz Biomed light microscope. Outlines of the stomata as well as the surrounding epidermal cells were clearly visible and could then be counted to calculate the SI (Chen *et al.*, 2001, Miller-Rushing *et al.*, 2009).

5.3.4 Photosynthesis and chlorophyll measurements

Leaf photosynthetic activity was measured using a LI-COR 6400XT portable photosynthesis system (LI-6400XT) at the three irrigation treatments during three physiological stages (vegetative flush, flowering and fruit development) throughout the growing season. Twelve leaves were randomly selected for measurement on each of the 4 trees per treatment.

Leaf chlorophyll measurements were performed using a Minolta SPAD-502 chlorophyll meter (Minolta Camera Co., Ltd., Japan). Chlorophyll measurements (20 per tree) were

taken between the midrib and the leaf margin from leaflets randomly selected from the youngest mature leaf on all (4) trees within each irrigation treatment around midday.

Data were statistically analysed using the Statistical Analysis Software (SAS Version 9.2) program for Microsoft Windows, by the Statistics Department at the University of Pretoria. Data were subjected to analysis of variance (ANOVA) using Proc GLM (SAS).

5.4 Results and Discussion

Difference in irrigation rates did affect the photosynthetic rate both between the different treatments as well as during the various stages throughout the growing season. Despite significant differences in photosynthetic rate between irrigation treatments, there was also a definite decline in photosynthetic rate over the growing season. The extent of the variation in and among trees of the different treatments was however inconsistent. Both during the 2011 and 2012 growing season a decrease in photosynthetic rate ($\mu mol.CO_2.m^{-2}.s^{-1}$) was observed with a decrease in irrigation rate and as the growing season progressed (Figure 5.1 and Figure 5.2).

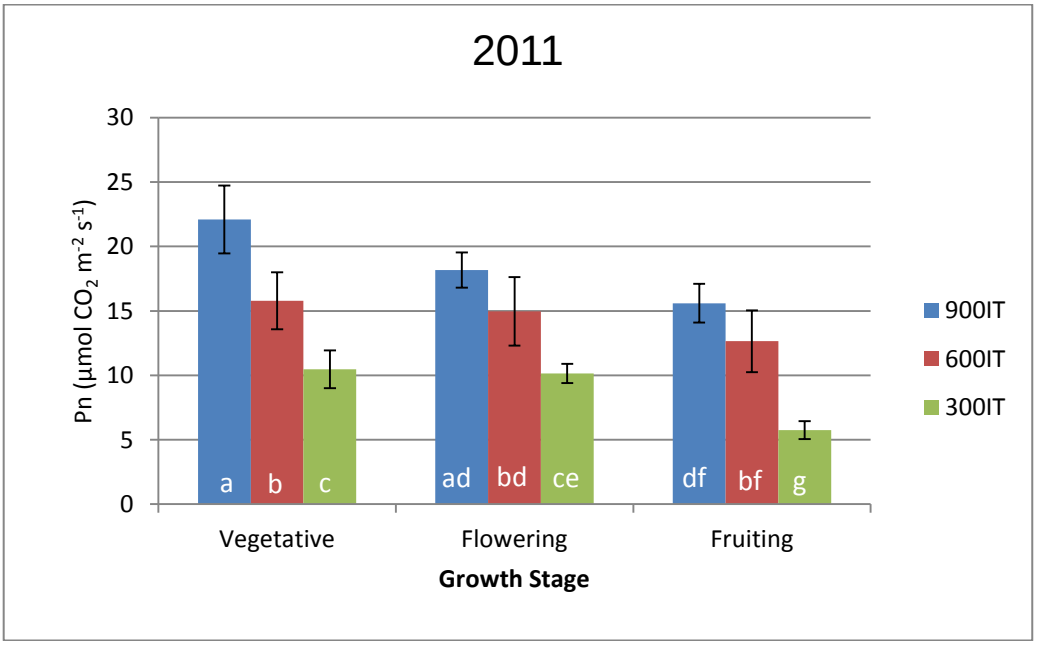

Figure 5.1 Photosynthetic rate (µmol CO_2 m^{-2} s^{-1}) at the three irrigation treatments (ITs) during three stages of the growth cycle throughout the 2011 growing season. Vertical bars (±) indicate standard errors of the mean (SEM). Different letters indicate a significant difference (P<0.05).

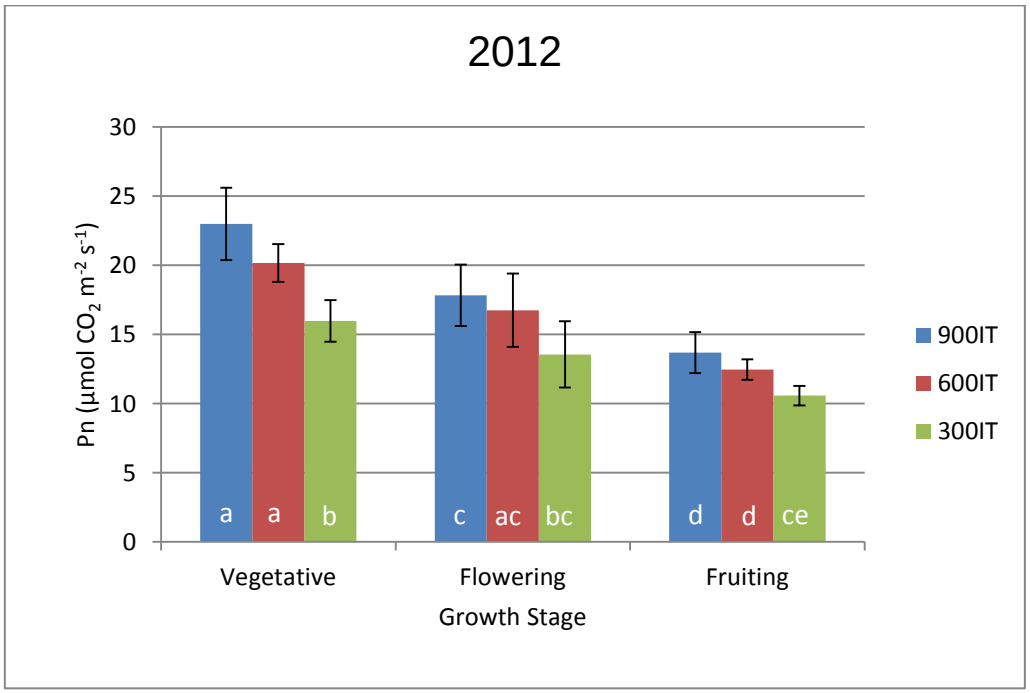

Figure 5.2 Photosynthetic rate (µmol CO_2 m^{-2} s^{-1}) at the three irrigation treatments (ITs) during three stages of the growth cycle throughout the 2012 growing season. Vertical bars (±) indicate standard errors of the mean (SEM). Different letters indicate a significant difference (P<0.05).

Several environmental factors affect stomatal conductance such as irradiance, temperature, leaf water status, relative humidity and leaf age (Farquhar and Sharkey, 1982, Warren, 2006). Generally, there is a strong correlation between leaf conductance and photosynthetic rate, due to the gas (CO_2 and O_2) exchange being a fundamental process during photosynthesis. Stomatal conductance measurements (Figure 5.3 and Figure 5.4) performed across the three irrigation treatments during the same developmental stages, demonstrated a similar trend to those of the photosynthesis measurements (Figure 5.1 and Figure 5.2). However, no clear significant differences in stomatal conductance between treatments could be established for either the 2011 or 2012 growing season, mainly as a result of great variability amongst measurements. This would allude to the fact that stomatal conductance might not be the single contributing factor towards the differences observed in photosynthetic activity between treatments and throughout the growing season. Thus although stomatal conductance was certainly a partial contributing factor to differences in photosynthetic activity, the contribution of additional factors also needed to be explored.

Stomatal conductance is not only affected by stomatal closure but also by stomatal density (SD), as SD is an indicator of stomatal number and not the size of the epidermal cells (Royer, 2001). The SI, rather than SD of the various treatments was determined according to the formula below developed by Salisbury (1927) for comparison, as the SD might be affected by the expansion of the surrounding epidermal cells due to factors such as light, temperature, leaf position and water status (Royer, 2001).

$$SI\ (\%) = \left(\frac{stomatal\ density}{stomatal\ density\ + epidermal\ cells}\right) \times 100$$

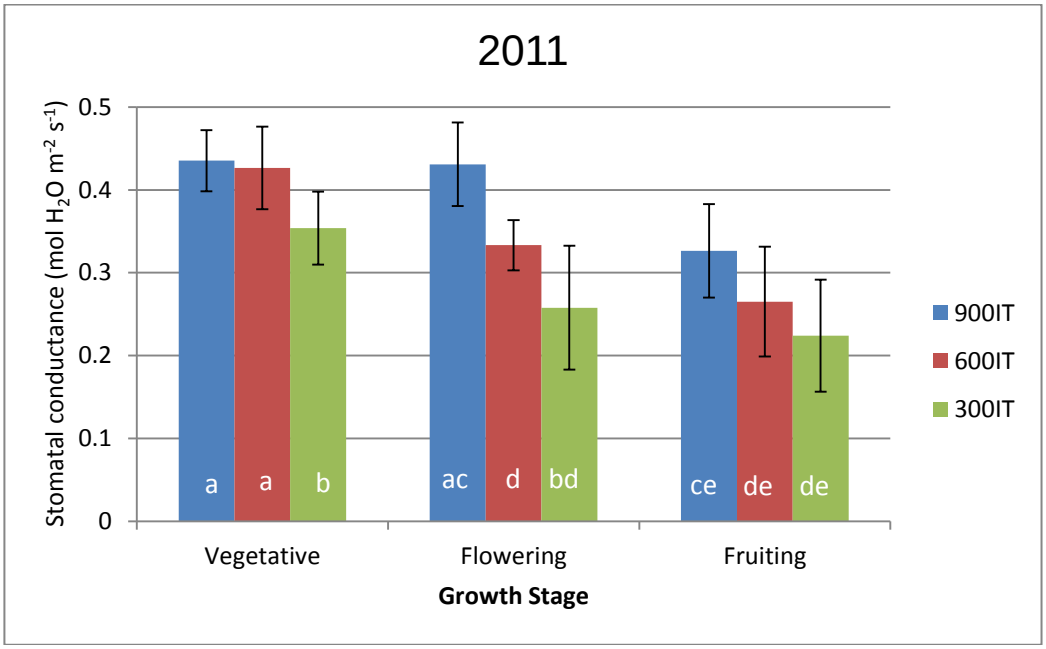

Figure 5.3 Stomatal conductance (mol H_2O m^{-2} s^{-1}) at the three irrigation treatments (ITs) during three stages of the growth cycle throughout the 2011 growing season. Vertical bars (±) indicate standard errors. Different letters indicate a significant difference (P<0.05).

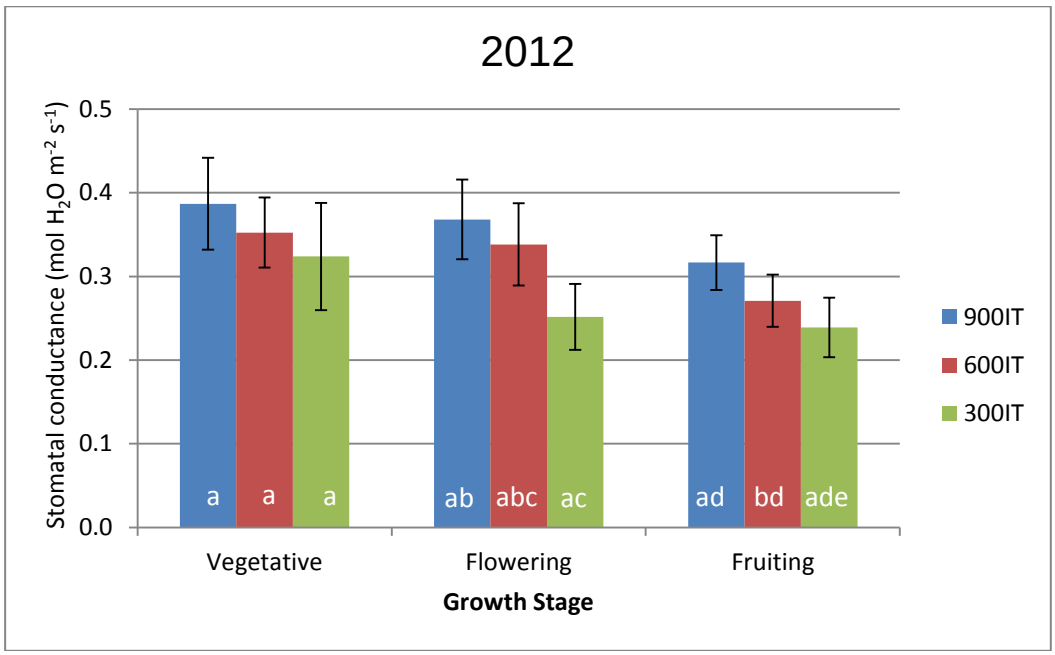

Figure 5.4 Stomatal conductance (mol H_2O m^{-2} s^{-1}) at the three irrigation treatments (ITs) during three stages of the growth cycle throughout the 2012 growing season. Vertical bars (±) indicate standard errors. Different letters indicate a significant difference (P<0.05).

Initially, the adaxial and abaxial leaf surfaces were examined using SEM as illustrated in Figure 5.5 and Figure 5.6 respectively. Since stomata were only found on the abaxial leaf surface, follow-up stomatal count studies were conducted, using the less time consuming and cost effective method of applying a thin layer clear nail polish to the abaxial leaf surfaces. An illustration of the abaxial leaf surface using this method is given in Figure 5.7. Sample images of the abaxial leaf surfaces from the three irrigation treatments are illustrated in Figure 5.8. From these images, the SI at each irrigation treatment could be calculated and results are given in Figure 5.9. SI can be considered a partial contributor to lower stomatal conductance with the decrease in irrigation treatment as there were significant differences between the 900IT and 300IT (Figure 5.9). The SI at the 900IT was significantly higher at 12.5% compared to the 9.5% of the 300IT. The decrease in SI with a reduction in irrigation treatment, lowered the conductance potential thereby indirectly also affecting photosynthesis.

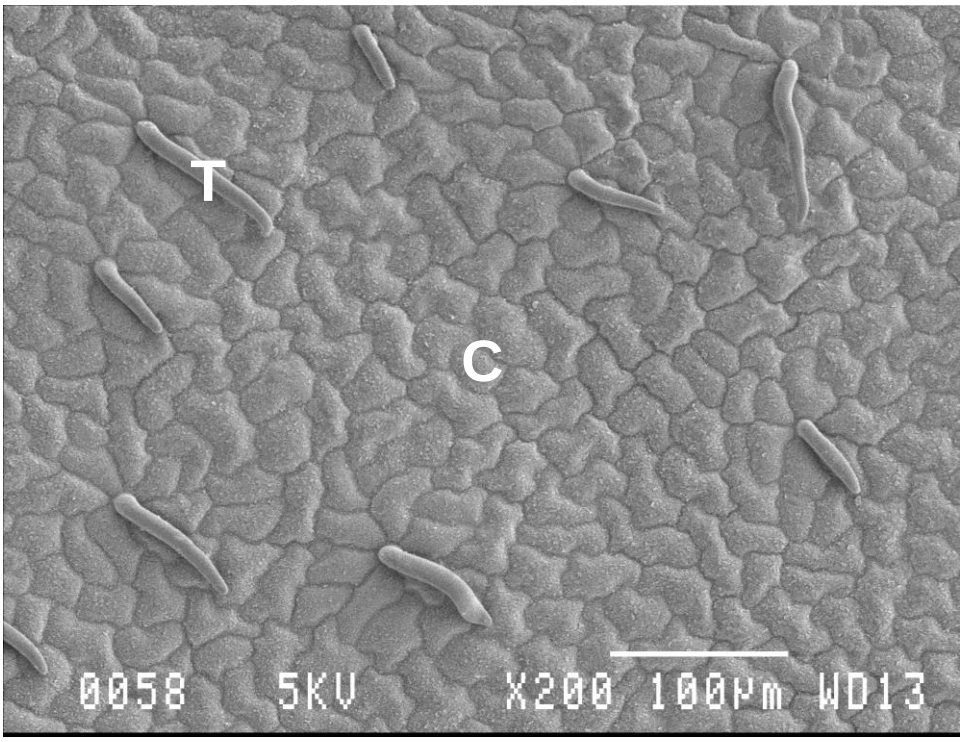

Figure 5.5 Scanning Electron Microscopy (SEM) image of the adaxial *Moringa oleifera* leaf surface. T – trichome, C – cuticle.

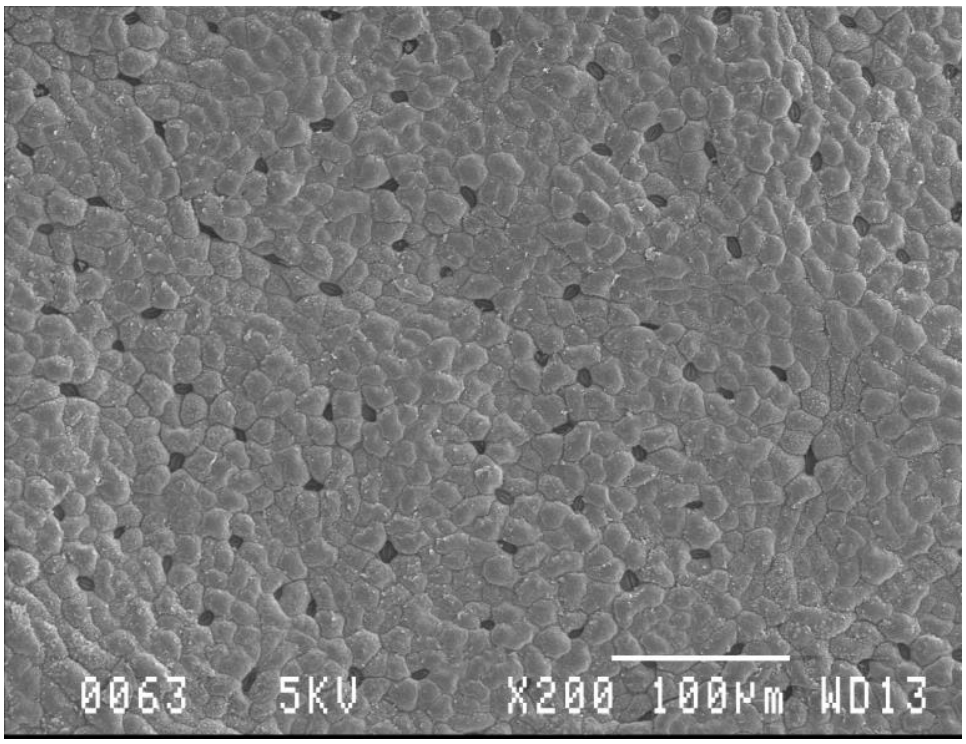

Figure 5.6 Scanning Electron Microscopy (SEM) image of the abaxial *Moringa oleifera* leaf surface.

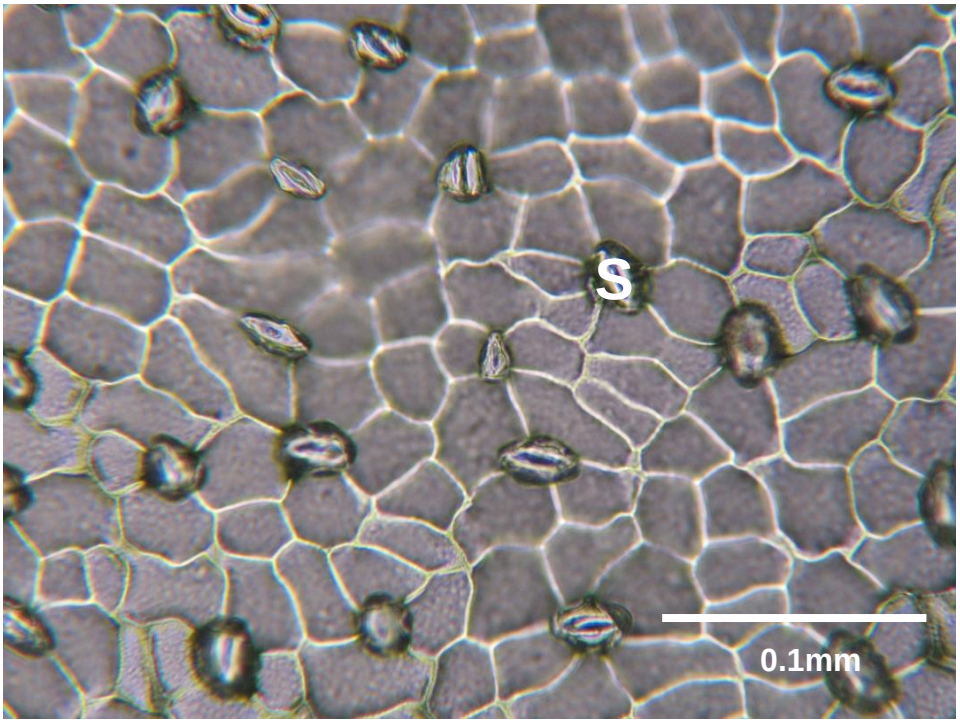

Figure 5.7 Imprint of the abaxial leaf surface of *Moringa oleifera* viewed under a light microscope. S – stomata.

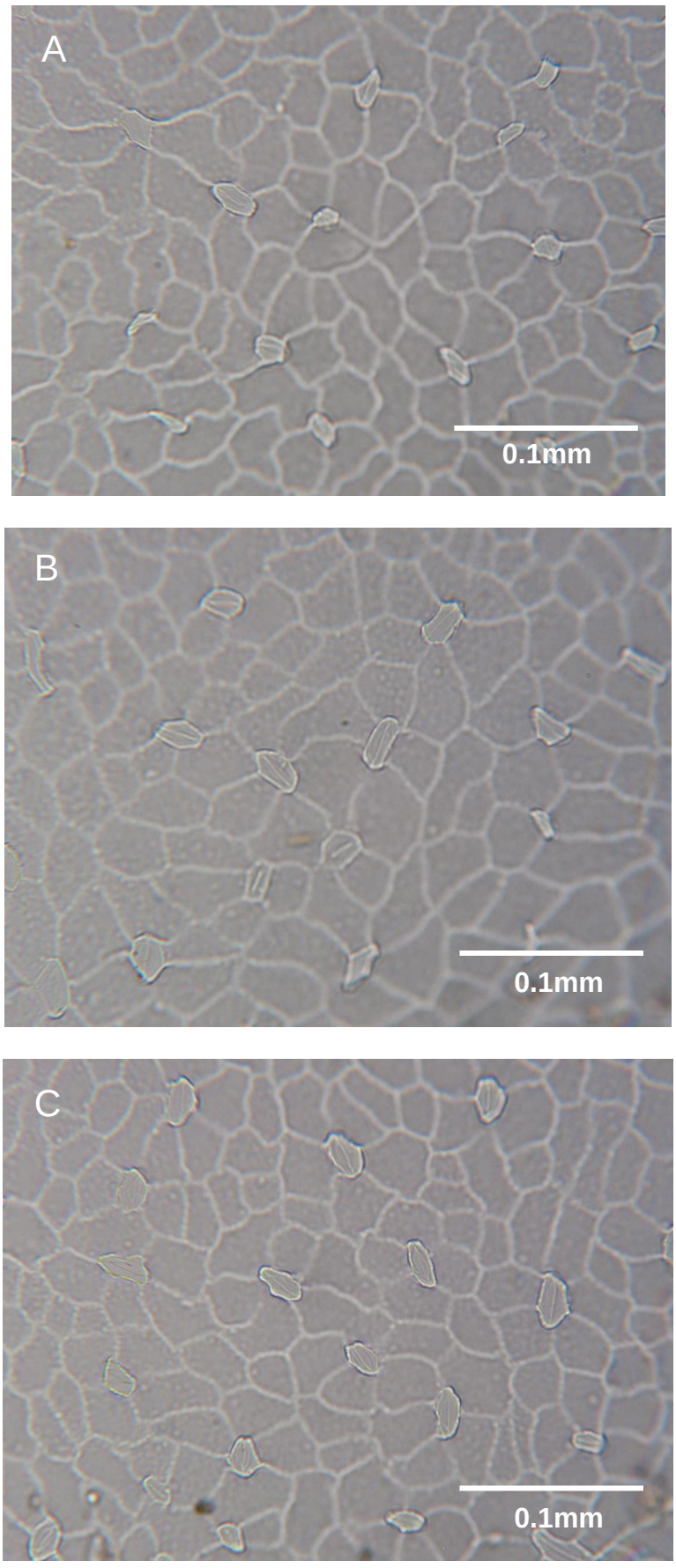

Figure 5.8 *Moringa oleifera* abaxial surfaces of leaves from three irrigation treatments. A – 900IT, B – 600IT and C – 300IT.

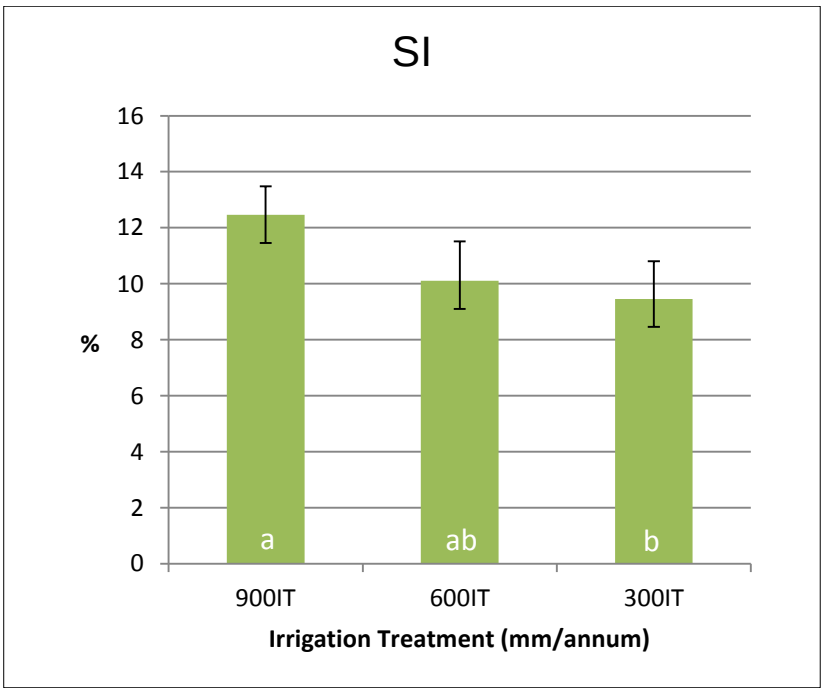

Figure 5.9 Stomatal Index (SI) of *Moringa oleifera* leaves from three irrigation treatments. Vertical bars (±) indicate standard errors. Different letters indicate a significant difference (P<0.05).

Leaf thickness was also affected by the different ITs, as leaf thickness seemingly decreased with a decrease in irrigation. Leaves randomly collected from trees at the 900IT, were on average 0.203 mm thick, while the average leaf thickness at the 600IT was 0.188 mm and 0.156 mm at the 300IT (Figure 5.10). The average leaf thickness was thus reduced by 7.69%, between the 900IT and 600IT and 23.08% between the 900IT and 300IT caused by the decrease in irrigation. According to Búrquez (1987) leaf thickness is directly linked to relative leaf water content, thicker leaves thus being indicative of a higher relative leaf water content. Cutler *et al.* (2006) suggest that well watered plants have larger cells thereby increasing leaf thickness. From Figure 5.10 the longer palisade cells within leaves of the 900IT led to the increased overall leaf thickness when compared to both the lower ITs. Cutler *et al.* (2006) further propose that turgor is easier maintained at low moisture levels by smaller cells, this therefore being a probable reason for the reduction in cell size observed with the decrease in IT.

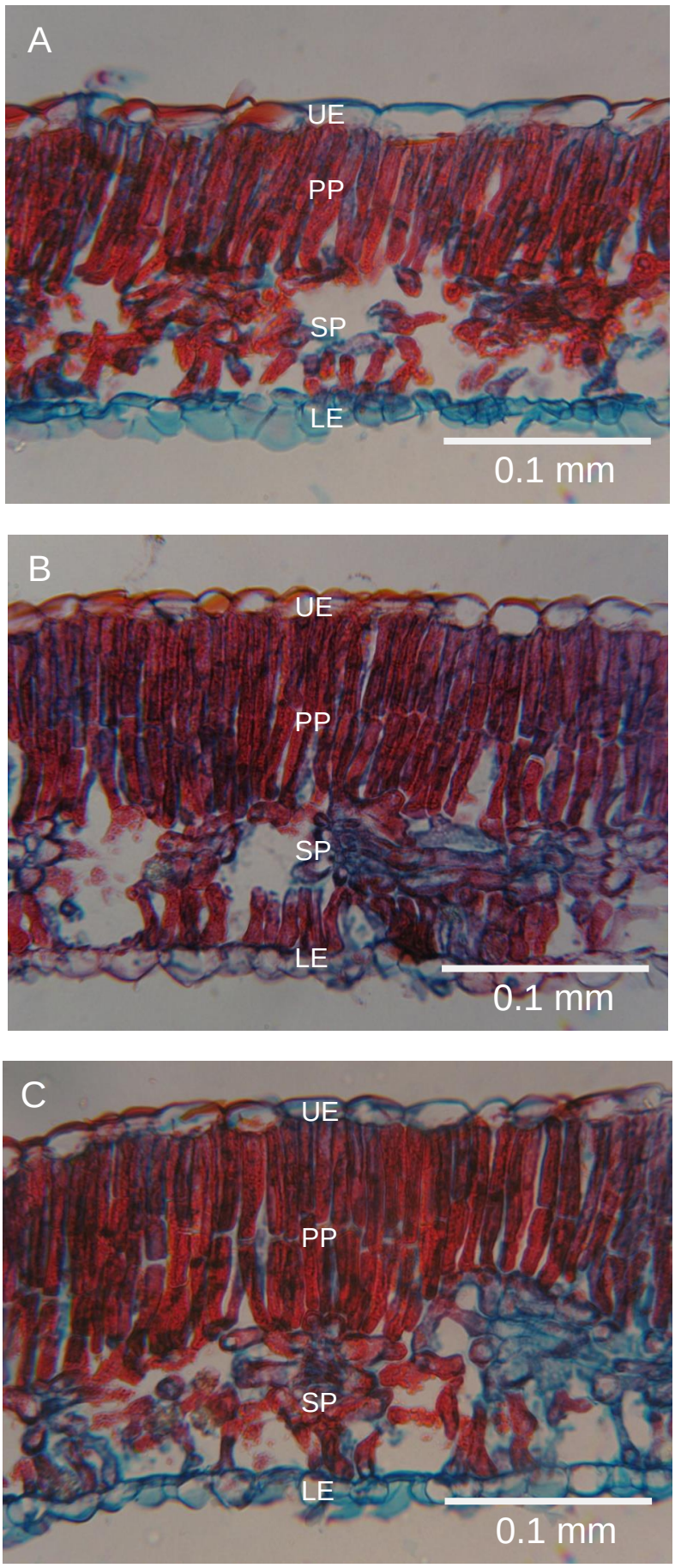

Figure 5.10 Cross-sections of *Moringa oleifera* leaves from three irrigation treatments. A – 300IT, B – 600IT and C – 900IT. UE – upper epidermis, PP – palisade parenchyma, SP – spongy parenchyma, LE – lower epidermis.

Moisture stress might not only have affected photosynthesis through stomatal closure but also the biochemical processes within the leaf, through inhibition of chloroplast activity (Boyer, 1976). According to Ehleringer and Cerling (1995) the ratio of intracellular CO_2 concentrations (c_i) to ambient CO_2 concentrations (c_a) is a measure of gas exchange metabolism efficiency within the leaf. A c_i/c_a ratio equal to one, would imply that a reduction in photosynthetic rate could either be attributed to photosystem limitations or intracellular respiration if CO_2 conductance into the leaf is not the limiting factor. On the contrary, a low c_i/c_a ratio would indicate that photosynthetic rate is not limited by the photosystem but rather by CO_2 uptake, through stomatal closure. The c_i/c_a ratios measured across three irrigation treatments at three growth stages are given in Table 5.1. Based on great variability within the measured data no significant differences could be established between the c_i/c_a ratios. However, from the averages given in Table 5.1 there is a tendency towards increased c_i/c_a ratios with the progression of the growing season, indicating the possible contribution of non-stomatal limitations to photosynthesis.

Table 5.1 The ratios of intercellular to ambient CO_2 concentrations (c_i/c_a) of *Moringa oleifera* leaves at three irrigation treatments at three different growth stages. Different letters indicate a significant difference ($P<0.05$).

	Growth Stage		
	Vegetative	**Flowering**	**Fruiting**
300IT	0.61[a]	0.64[a]	0.74[a]
600IT	0.55[a]	0.63[a]	0.76[a]
900IT	0.51[a]	0.63[a]	0.71[a]

Boyer (1976), Tezara *et al.* (1999), Flexas and Medrano (2002) and Lawlor (2002) have reported non-stomatal limitations under drought stress as contributors to reduced photosynthesis. According to them, drought stress impaired RuBP regeneration as well as ATP synthesis within the Calvin cycle, thereby lowering photosynthetic CO_2 assimilation. Whereas Grassi and Magnani (2005) have found reduced mesophyll conductance to also

significantly lower the net assimilation rate during different stages throughout the growing season. According to Warren (2006) and Kitajima *et al.* (1997) photosynthesis also decreases with leaf age, primarily due to self-shading. Photosynthetic rate is also closely related to the leaf nitrogen content (Field and Mooney, 1986, Evans, 1989), which is again a direct function of the leaf chlorophyll content (Chapman and Barreto, 1997). Leaf chlorophyll content measured using a Minolta SPAD-502 chlorophyll meter, revealed a decrease in chlorophyll content as the growing season progressed. The irrigation treatments however, did not have an effect on the chlorophyll content (Figure 5.11). Decreasing photosynthetic rates with the progression of the growing season could thus also partially be attributed to ageing leaves with decreasing chlorophyll concentrations as well as self-shading (Kitajima *et al.*, 1997).

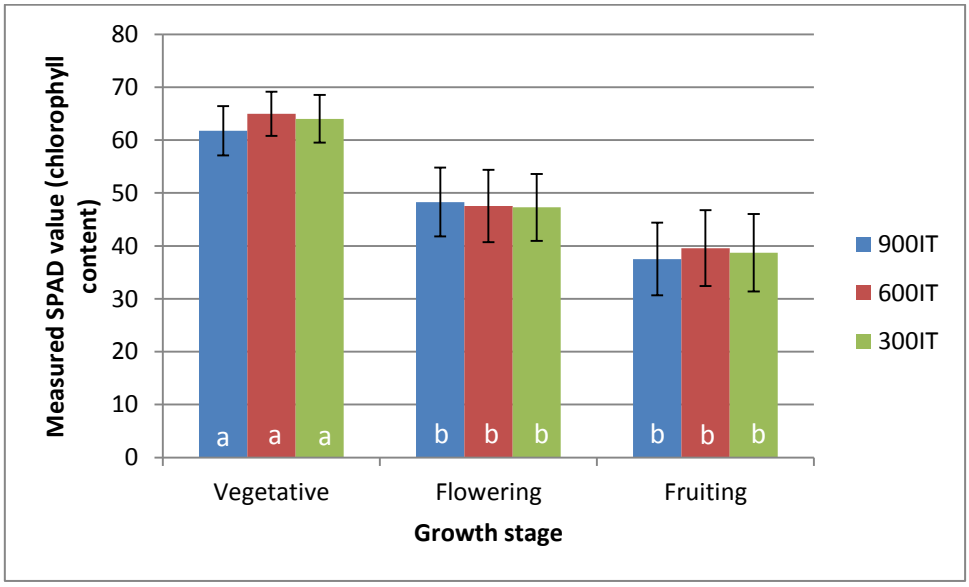

Figure 5.11 Leaf chlorophyll content measured at the three irrigation treatments (IT) at various growth stages. Vertical bars (±) indicate standard errors of the mean (SEM). Different letters indicate a significant difference (P<0.05).

Across all three irrigation treatments, the trees exhibited a rather high photosynthetic rate during the vegetative growth stage. *Moringa oleifera* is an relatively fast growing tree that can reach an average growth rate of 59.0 mm/week under favourable growing conditions

(Muhl *et al.*, 2011). Elevated photosynthetic rates during the vegetative stage would thus be required in order to supply sufficient photo-assimilates to sink organs to sustain such rapid growth.

5.5 Conclusion

From the various measurements collected during this study neither stomatal (conductance and SI) nor non-stomatal limitations (possible RuBP regeneration, ATP synthesis and mesophyll conductance) could be identified as the primary limiting factor for the lower photosynthetic rate observed with the decrease in irrigation. The lower photosynthetic rate between treatments were thus a result of both stomatal and non-stomatal limitations, while the photosynthetic rate decreases observed throughout the growing season were primarily due to the diminishing leaf chlorophyll content. Reduced photosynthesis is thus a combination of both stomatal and non-stomatal limitations with the extent of their contribution varying throughout the growing season.

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CHAPTER 6

INTRACELLULAR HISTOCHEMICAL CHANGES IN DEVELOPING *MORINGA OLEIFERA* SEED

6.1 Summary

In recent times, *Moringa oleifera* has emerged as a new industrial crop, due to its multitude of uses. Many of these uses are products of the Moringa seed, of which their development remains largely unresearched. The use of microscopy and histochemical staining for the identification of storage compounds in developing *Moringa oleifera* seed, not only enabled the determination of compound synthesis initiation but also their intracellular locality. By understanding the storage compound biosynthesis pathways, staining results and compound interaction could be identified. Oil and protein reserves were the two major storage compounds present at seed maturity, while significant amounts of starch were transiently stored in young developing seed. Starch was the first storage compound to accumulate, with its presence being detected in very immature seed (fruit diameter of 8 mm). Having identified plastids as the synthesis and storage site for starch reserves, the presence of starch towards the cell periphery could be explained. While protein and oil were both synthesized in the cell periphery in association with the endoplasmic reticulum, they were subsequently translocated and stored in the vacuole. Protein and oil body formation only occurred in noticeable amounts at a fruit diameter of ±14 mm. Protein bodies were largest at between ±5 µm to 8 µm in size while oil bodies ranged from ±0.2 µm to ±1 µm in size.

6.2 Introduction

Moringa oleifera is increasingly becoming an important industrial crop due to its multitude of beneficial uses and associated products (Foidl *et al.*, 2001, Fuglie, 2001, Anwar *et al.*, 2007). Since many of these products are either directly or indirectly derived from the seed, the onset and locality of storage compound (oil, protein and starch) biosynthesis in developing seed requires thorough understanding. Seeds are net sink organs throughout most of their development and require the import of carbon (sucrose) into the seed tissue where the synthesis of all major storage compounds takes place (Bewley and Black, 1994). According to Weber *et al.* (1997), Murphy *et al.* (1993) and Baud and Lepiniec (2010) the initiation and proportional allocation of the three principal storage compounds (oil, protein and starch) is determined by both genetic and biochemical factors. No information currently exists on the formation and development of oil, protein and starch in *Moringa oleifera* seed. Given the numerous uses for Moringa seeds such as biofuel, food source, water treatment, etc., a better understanding of the storage compound composition at various developmental stages is therefore important (Hills, 2004, Ferreira *et al.*, 2008).

6.3 Materials and Methods

Seeds for this study were randomly collected from mature *Moringa oleifera* trees irrigated with surface drip irrigation at three different rates. The administered irrigation rates were 300 mm/year (300IT), 600 mm/year (600IT) and 900 mm/year (900IT), with four trees per treatment. The in-line dripper spacing was 30 cm, with an application rate of 2.1 litres/hour/dripper. Plastic sheeting was placed over the dripper irrigation, underneath the trees covering an area of 4 m on either side of the trunks and covered with organic mulch.

6.3.1 *Light microscopy*

Due to continuous flower initiation and development, fruit of different developmental stages could be found on the trees throughout the growing season. Ten seeds of each developmental stage were randomly sampled from all three irrigation treatments and prepared for light microscopy according to O'Brien and McCully (1981). After harvesting, seeds were immediately fixed in FAA (80% ethanol: 37% formaldehyde: 100% acetic acid, in proportions 8:1:1 v/v/v) for at least 24 hours, before being dehydrated in an ethanol in water series (30%, 50%, 70%, 100%, 100% v/v) for 24 hours at each concentration. Subsequently, ethanol was extracted from the specimens through a series of xylene in ethanol concentrations (30%, 50%, 70%, 100% v/v) prior to placing them into paraffin wax (60°C). Embedded seed samples were cut into 10 µm thick sections using a Reichert-Jung semi-thin rotary microtome, and placed onto microscope slides before being de-waxed in a series of xylene concentrations. The histochemical staining of the sectioned cotyledons differed depending on the compound that was stained for; hence they are individually discussed below. Throughout early seed development the growing cotyledons consume the entire endosperm and subsequently become the storage site for protein, oil and starch reserves. Once stained, sections were viewed with a Leitz Biomed light microscope. Photographs were taken digitally using a Canon PowerShot A630 digital camera.

6.3.1.1 *Starch*

Starch was stained using Periodic Acid-Schiff (PAS) reagent according to Merck (2011). De-waxed sections were rinsed in distilled water for 5 min. and placed into sodium periodate solution for another 5 min. After rinsing sections in distilled water, they were stained with PAS reagent for 15 min. and rinsed with sulfite water (6 min.) followed by distilled water (10 min.). Sulfite water was prepared by adding a mixture of 10 ml sodium

disulfite solution (10 %) and 10 ml of hydrochloric acid (1 mol/l) to 200 ml of distilled water. Finally, sections were stained with Hemtoxylin solution (2 min.) and rinsed with distilled water (3 min.) before being mounted. Polysaccharides stained purple-magenta with PAS.

6.3.1.2 *Oil*

Seed used for the lipid staining could not be embedded using the conventional procedure used for light microscopy as discussed above, given that seed lipids are xylene soluble. Alternatively, under such circumstances fresh seed specimens would merely be sectioned by hand. By using this method section thickness became inconsistent and this affected staining, making different samples incomparable. Moringa seed posed an additional challenge in that they are relatively hard once mature and difficult to section thinly. For this reason and a lack of other suitable methods, the following customized method was developed for *Moringa oleifera* seed to perform oil staining. Seed samples were fixed in FAA (80% ethanol: 37% formaldehyde: 100% acetic acid, in proportions 8:1:1 v/v/v) for 7 - 14 days, followed by 24 - 48 hours in distilled water to remove excess fixative prior to sectioning. Samples were then sectioned using a freeze-microtome and transferred onto microscopy slides prior to staining. Exploratory trials found Sudan III (Sudan red) to be the best lipid stain for *Moringa oleifera* seed, when compared to Sudan IV and Sudan black B. Hence, Sudan III was used for all subsequent intracellular lipid staining according to Culling (1974). Sections were submerged in propylene glycol (2 min.) and then transferred into Sudan III solution (10 min.) Thereafter, excess Sudan III was removed by rinsing sections in two changes of 85% and 50% propylene glycol. Lipids stained red/orange with Sudan III.

6.3.1.3 *Protein*

Intracellular protein was detected by staining seed sections in duplicate using two different protein stains namely, Orange G and Light Green SF according to James and Tas (1984). The same staining procedure was followed for both these stains. After de-waxing, sections were stained in either Orange G or Light Green SF for 30 min., followed by three rinses of 1% acetic acid and two rinses of distilled water. Sections were then briefly rinsed in three changes of *tert*-butanol, followed by two changes in xylene, before being mounted with synthetic resin. Protein stained bright green with the Light Green SF and bright orange with Orange G.

6.3.2 *Transmission electron microscopy (TEM)*

Seeds were selected in the same manner as discussed for light microscopy and then prepared for TEM according to Coetzee and Van der Merwe (1996). A small (1 mm^3) sample was sectioned from the centre of the developing cotyledons and fixed in 2.5% gluteraldehyde in a 0.075 M phosphate buffer (pH 7.4 - 7.6) for two hours, before being rinsed 3X in the same 0.075 M phosphate buffer (10 min. each). Specimens were then fixed in 0.5% aqueous osmium tetroxide (OsO_4) for two hours and rinsed 3X with distilled water (10 min. each), followed by dehydration of the specimens in an ethanol in water series (30%, 50%, 70%, 90% and 100% v/v). Seed samples were then impregnated with 50% Quetol epoxy resin for one hour followed by four hours in 100% Quetol, and then polymerized at 60°C for 39 hours. Sections of ±70 nm thick were cut with a Reichert Ultracut E ultramicrotome and transferred onto grids. Sections were initially contrasted for 10 min. in 4% aqueous uranyl acetate, followed by two min. in Reynolds' lead citrate solution, rinsing sections with water in-between. Seed sections were viewed with a JEOL JEM-2100F transmission electron microscope, while photographs were taken digitally.

6.4 Results and Discussion

Even though seeds are considered sink organs throughout their development, the presence of chloroplasts in developing embryos would make them partially photosynthetically active. This would enable developing embryos to partially contribute to compound (sucrose) development with their own carbon source (Eastmond *et al.*, 1996). An important part of all seed development, is the differentiation of the plastids, as plastids are fundamental during the intracellular compound development process. To date, leaf plastids are better understood given the research attention they received, opposed to seed plastids, which remain largely uninvestigated (Rolletschek and Borisjuk, 2005, Tschiersch *et al.*, 2011). Although photosynthesis in seed chloroplasts can contribute to the overall energy needs during storage compound biosynthesis, their contributions are fairly limited (Ruuska *et al.*, 2004, Tschiersch *et al.*, 2011). Young *Moringa oleifera* seeds are noticeably green (Figure 6.1) due to the presence of well-developed chloroplasts in the cotyledons as illustrated in Figure 6.2.

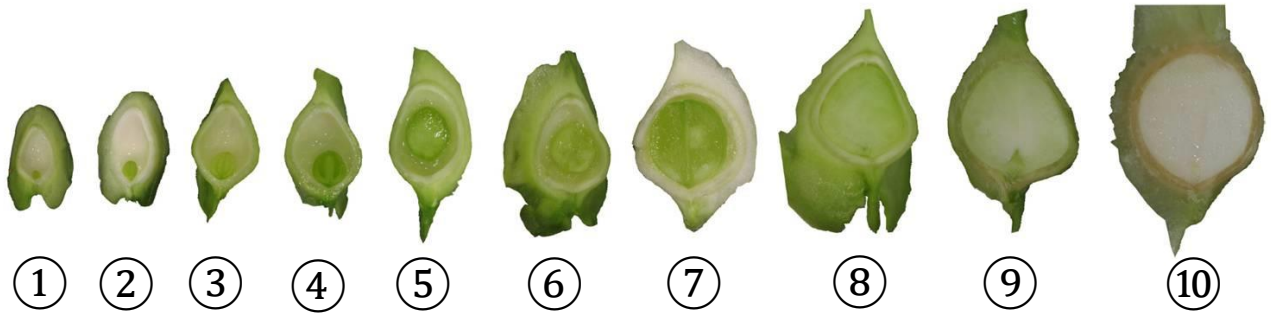

Figure 6.1 Cross-sections of *Moringa oleifera* seed at different fruit developmental stages (①-⑩) based on their diameter (mm). ① 8 mm ② 10 mm ③ 12 mm ④ 14 mm ⑤ 16 mm ⑥ 18 mm ⑦ 20 mm ⑧ 22 mm ⑨ 24 mm ⑩ 26 mm.

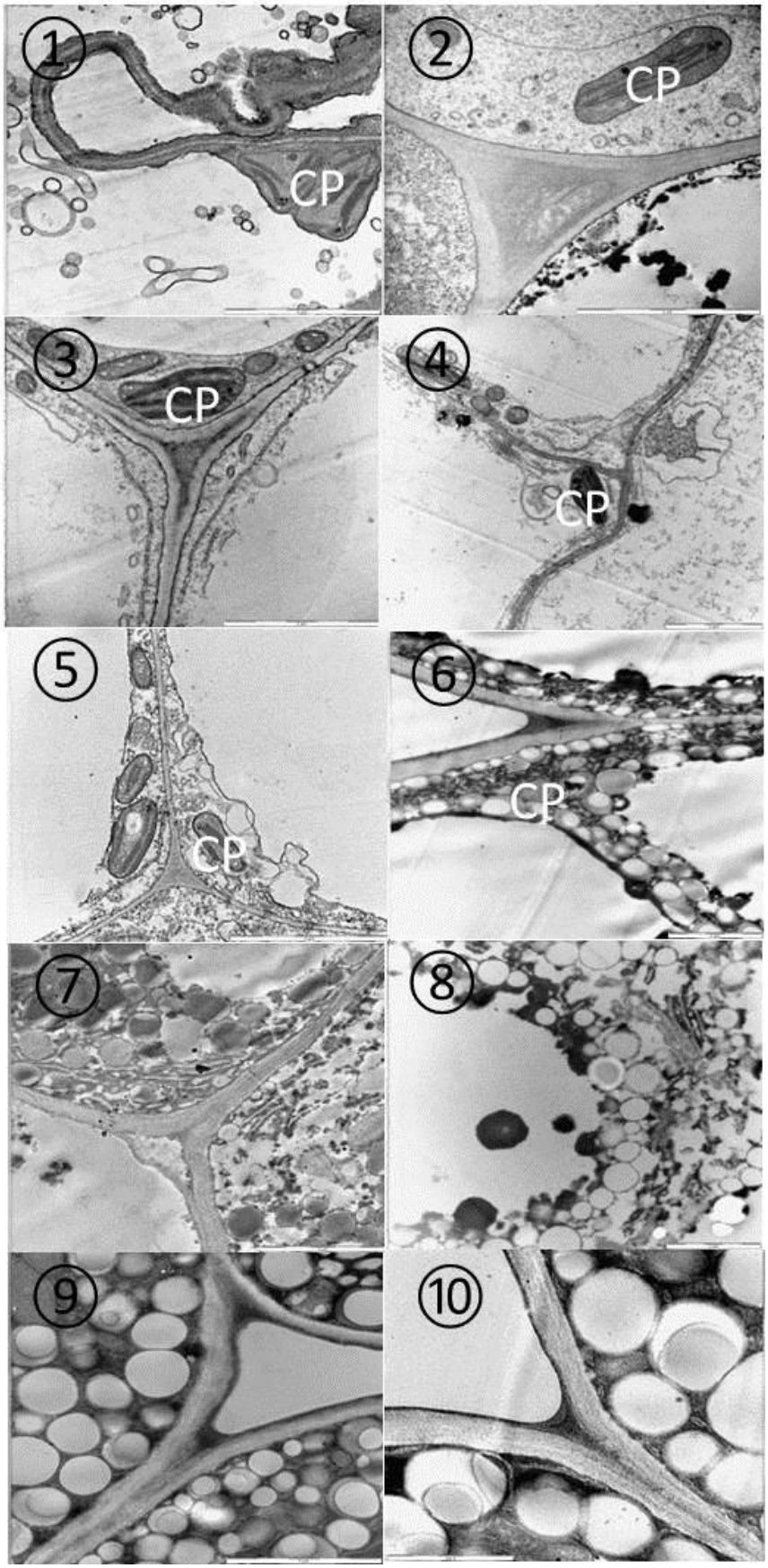

Figure 6.2 TEM cross-sections of developing *Moringa oleifera* seed (cotyledons) at different fruit diameters (mm) to determine the presence of chloroplasts. ① 5 mm ② 10 mm ③ 12 mm ④ 14 mm ⑤ 16 mm ⑥ 18 mm ⑦ 20 mm ⑧ 22 mm ⑨ 24 mm ⑩ 26 mm. CP – chloroplast. The scale bar in each image represents 2 µm.

Throughout the initial developmental phases, up to a fruit diameter of 16 mm, chloroplasts were found to be present in the seed cotyledons. In seeds with a fruit diameter of 18 mm however, chloroplasts started to degenerate (Figure 6.2 ⑥) and at fruit diameters of 20 mm and beyond, they could no longer be clearly identified (Figure 6.2 ⑦-⑩). Furthermore, it was also observed that chloroplasts decreased in size with seed maturity, most likely reducing the photosynthetic ability of the seed. Similarly, Tschiersch *et al.* (2011) have also found seed photosynthetic levels to decrease with maturation in both *Pisum sativum* and *Hordeum vulgare*.

6.4.1 Starch

Throughout early seed development, starch is the main storage compound being synthesized and transiently stored inside the seed plastids. Similar to oil biosynthesis, starch biosynthesis begins primarily with the import of sucrose from the source tissue which is then transformed into either hexose phosphate (hexose P) or triose phosphate (triose P) through glycolysis. Hexose P/triose P is subsequently used during starch/oil biosynthesis. In the case of the cotyledon chloroplasts, some Hexose P/triose P might already be present inside the plastids, while the majority of Hexose P/triose P is transferred from the cytosol into the plastid (Figure 6.3, ①-④) (Bewley and Black, 1994, Martin and Smith, 1995). Both oil and starch biosynthesis share this section of the pathway and are thus essentially in competition for the same carbon source (Murphy *et al.*, 1993). Alternatively, plastids (chloroplasts) found in young developing seed have the potential to produce their own sucrose for starch biosynthesis through photosynthesis, as discussed above. Starch granule formation within a plastid during early seed development is depicted in Figure 6.4.

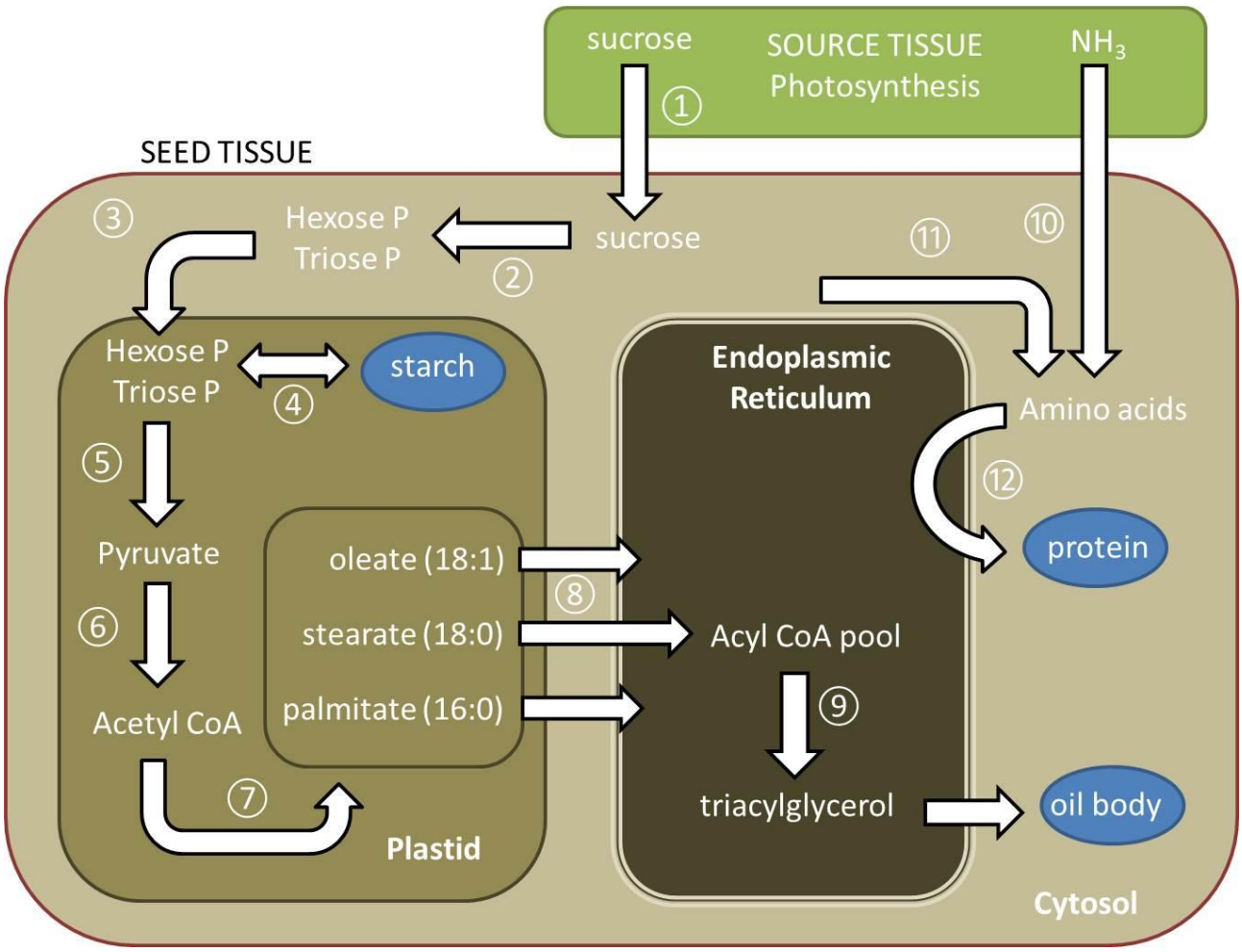

Figure 6.3 Schematic representation of the storage compound (starch, protein and oil) synthesis pathways based on findings by Murphy *et al.* (1993), Bewley and Black (1994), Martin and Smith (1995), Ohlrogge and Browse (1995), Weber *et al.* (2005), Baud and Lepiniec (2010) and Hills (2004) ⑦ FAS – fatty acid synthetase.

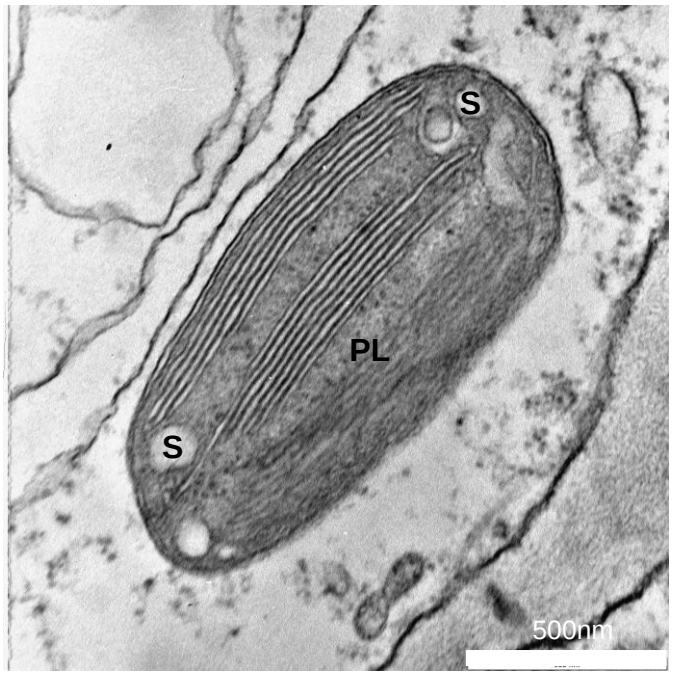

Figure 6.4 Transiently stored starch (S) granules in the plastid (PL) of immature *Moringa oleifera* seed cotyledons at a fruit diameter of 12 mm.

Staining *Moringa oleifera* seed of various developmental stages with PAS reagent revealed that starch did not accumulate in sizable amounts throughout most of the seed's development (Figure 6.5). Results from analytical quantitative analyses reveal that starch levels peaked during the initial development phases and then decreased with maturation (Chapter 7). Unlike protein and oil reserves with large intracellular deposits, starch reserves (stained purple) were rather small and restricted to the cell periphery, which coincides with the location of the plastids.

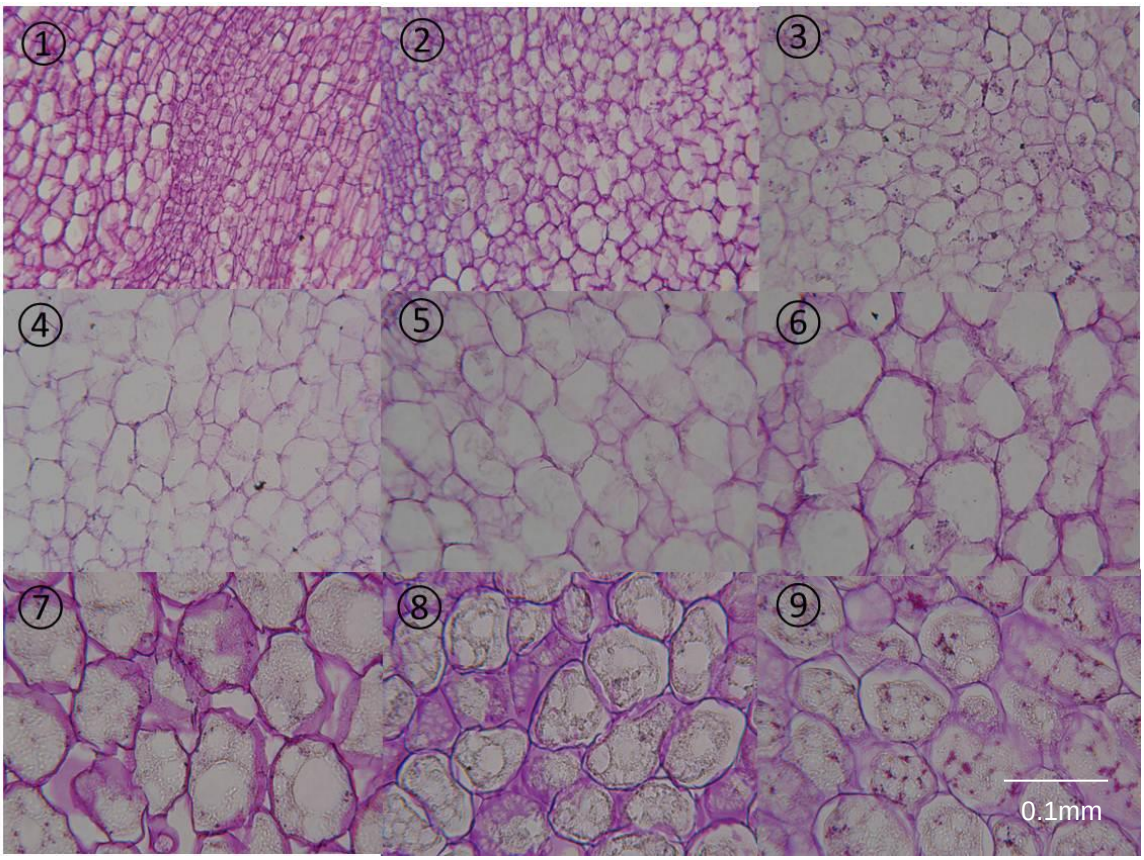

Figure 6.5 Cross-sections of different stages of developing *Moringa oleifera* seed stained with Periodic Acid-Schiff reagent to indicate starch accumulation. ① 8 mm ② 10 mm ③ 12 mm ④ 14 mm ⑤ 16 mm ⑥ 18 mm ⑦ 20 mm ⑧ 22 mm ⑨ 24 mm.

6.4.2 Oil

A significant portion of carbon reserves is stored as triacylglycerol (TAG) in *Moringa oleifera* seed. Triacylglycerols (TAGs) are the most common form of storage lipids found in seed (Murphy *et al.*, 1993). Although the fatty acid compositions of TAGs differ between

plant species, TAG is the most efficient form of energy storage, retaining more than twice the energy on a per-mass basis when compared to starch and/or proteins (Huang, 1992, Bewley and Black, 1994, Ohlrogge and Browse, 1995). Amongst the three major storage compounds (lipids, carbohydrates and proteins), TAGs (lipids) are thus superior, releasing more than twice the amount of energy during oxidation/germination compared to stored carbohydrates and proteins (Graham, 2008).

Oil (TAG) biosynthesis

A schematic representation of the storage compound synthesis pathways is given in Figure 6.3. The subsequent storage compound biosynthesis pathways description is based on Murphy *et al.* (1993), Bewley and Black (1994), Martin and Smith (1995), Ohlrogge and Browse (1995), Baud and Lepiniec (2010), Weber *et al.* (2005) and Hills (2004). Imported sucrose from source tissue is transformed to either hexose phosphate (hexose P) or triose phosphate (triose P) through glycolysis. Hexose P/triose P enter the plastid from the cytosol where they are either transformed into pyruvate or used in starch biosynthesis. During early-mid seed development (Ø<14 mm), quantitative starch and oil analyses revealed relatively high starch levels together with a low oil content, suggesting that initially, hexose P/triose P are primarily used for transient starch synthesis. Throughout further (mid-late) seed development (Ø>14 mm) however, pyruvate is formed using hexose P/triose P from either remobilized starch reserves (Figure 6.3, ④) within the plastid or hexose P/triose P entering the plastid directly from the cytosol (Figure 6.3, ③). Pyruvate is then converted into the fatty acid precursor acetyl-CoA, which is subsequently carboxylated and the fatty acids produced through fatty acid synthetase (Figure 6.3, ⑦). Fatty acids (oleate, stearate and palmitate) are then transferred from the plastid to the endoplasmic reticulum (ER) (Figure 6.3, ⑧), linked with CoA and enter the acetyl-CoA pool to ultimately produce TAG through triacylglycerol biosynthesis (Figure 6.3, ⑨).

Lastly, TAG is released from the ER into the cytosol, where oil storage bodies are deposited (Bewley and Black, 1994, Ohlrogge and Browse, 1995). Figure 6.6 clearly demonstrates oil body formation of the ER in *Moringa oleifera* seed.

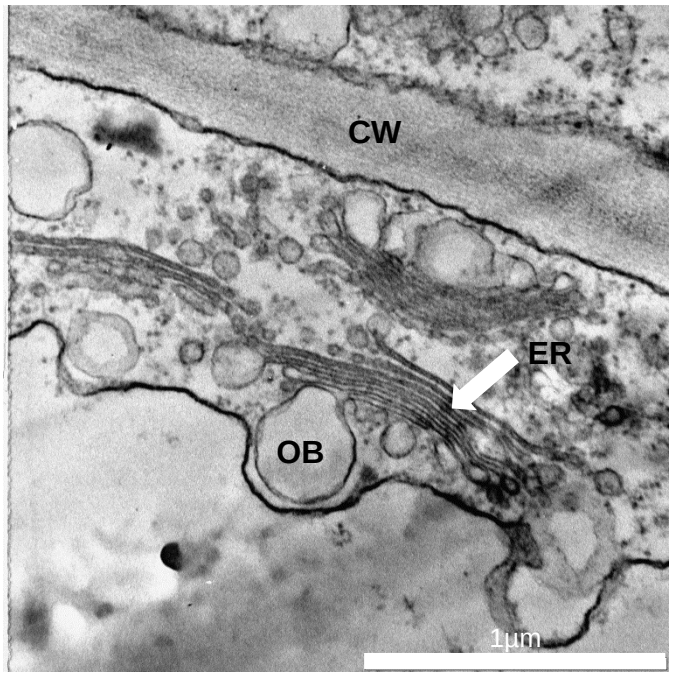

Figure 6.6 Oil body (OB) formation by the intracellular endoplasmic reticulum (ER) in *Moringa oleifera* seed at a fruit diameter of 12 mm. CW – cell wall.

Both the plastids and ER which are central during oil biosynthesis throughout seed development are located inside the peripheral cytoplasm of the cell. Consequently, newly synthesized oil bodies arise from the cell margins and migrate towards the vacuole where they are stored together with protein bodies. This is illustrated in Figure 6.7 which reveals the presence and location of intracellular oil bodies (stained orange) at different developmental stages. Based on the evidence from seed sections stained with Sudan III, no oil was present at a fruit diameter of 8 mm (Figure 6.7, ①) and only gradually increased between the fruit diameters of 10 mm to 16 mm. A closer look at the individual cells during these stages, confirmed that oil bodies originated in the peripheral cytoplasm and filled the cytosol towards the centre (Figure 6.7, ②-⑤). The most substantial increase in oil content was observed between and the fruit diameters of 16 mm and 18 mm (Figure

6.7, ⑤-⑥). The visual observations of oil quantification through staining were comparatively consistent with analytical quantitative analyses results discussed in Chapter 7. Oil bodies varied in size, from a diameter of ±0.2 µm just after synthesis to ±1 µm at maturity.

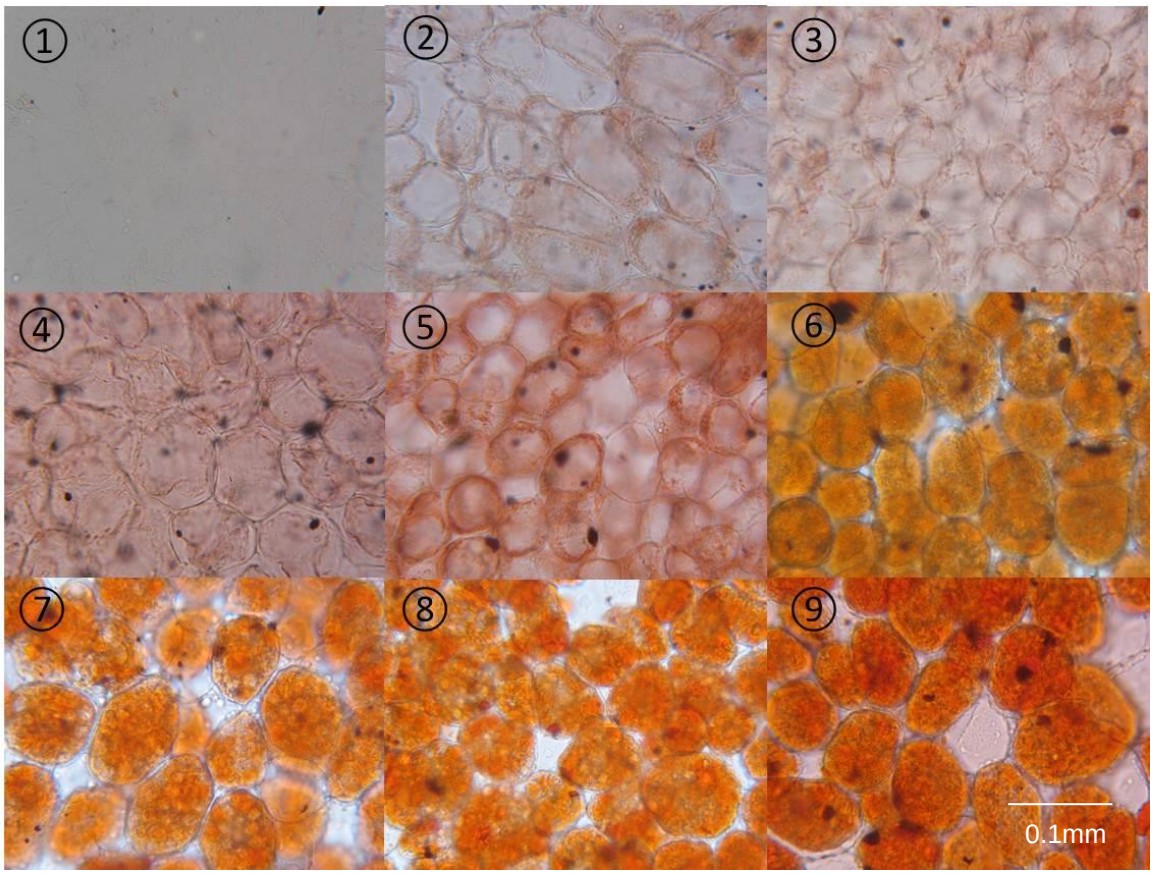

Figure 6.7 Cross-sections of developing *Moringa oleifera* seed stained with Sudan III to indicate oil accumulation. ① 8 mm ② 10 mm③ 12 mm ④ 14 mm ⑤ 16 mm ⑥ 18 mm ⑦ 20 mm ⑧ 22 mm ⑨ 24 mm.

6.4.3 Protein

Intracellular protein development is largely dependent on the import of both carbon (Figure 6.3, ⑪) and nitrogen (Figure 6.3, ⑩) sources as both these elements form an integral part during amino acid biosynthesis (Golombek *et al.*, 2001, Salon *et al.*, 2001). These amino acids form the basis of all seed storage proteins. However, unlike oil and starch biosynthesis, amino acids are not formed by a single pathway but rather as products from various intracellular processes (Shewry *et al.*, 1995). These amino acids are subsequently

used during protein body biosynthesis to form amino acid chains bound by peptide bonds, which are associated with the lumen of the rough endoplasmic reticulum (RER) (Figure 6.3, ⑫) (Bewley and Black, 1994). The amino acid composition is plant specific and determined by gene-specific transcriptional regulation (Verdier and Thompson, 2008). Following biosynthesis, seed storage proteins are translocated and stored in protein storage vacuoles (Ibl and Stoger, 2012). The identification of storage protein accumulation in developing *Moringa oleifera* seed, was achieved by staining seed in duplicate with protein-staining Light Green SF (Figure 6.8) and Orange G (Figure 6.9). Both the Light Green SF and Orange G stains were relatively consistent and revealed an increase in staining intensity with seed maturity. Based on visual analysis protein development was initiated around the 14 mm fruit diameter stage (Figure 6.8, ④ and Figure 6.9, ④) and continued to increase until seed maturity.

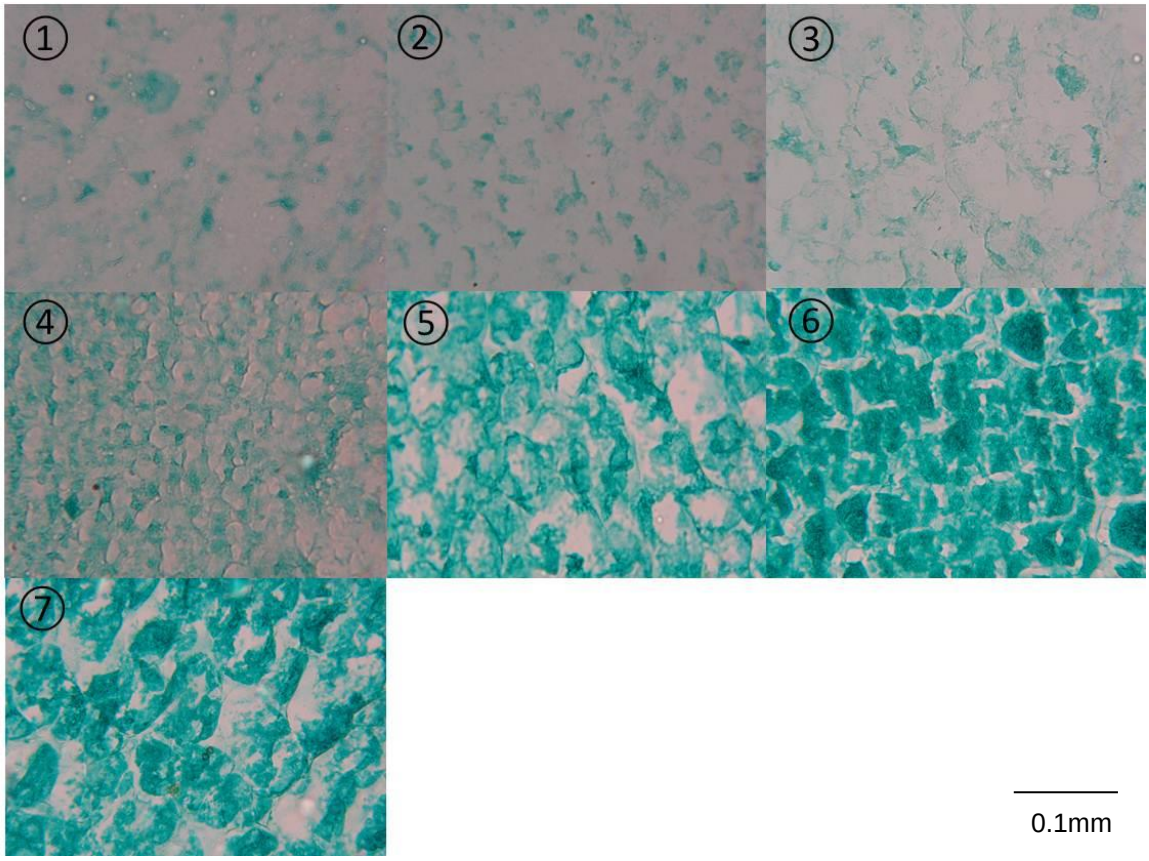

Figure 6.8 Cross-sections of developing *Moringa oleifera* seed stained with Light Green SF to indicate protein accumulation. ① 8 mm ② 10 mm③ 12 mm ④ 14 mm ⑤ 16 mm ⑥ 18 mm ⑦ 20 mm.

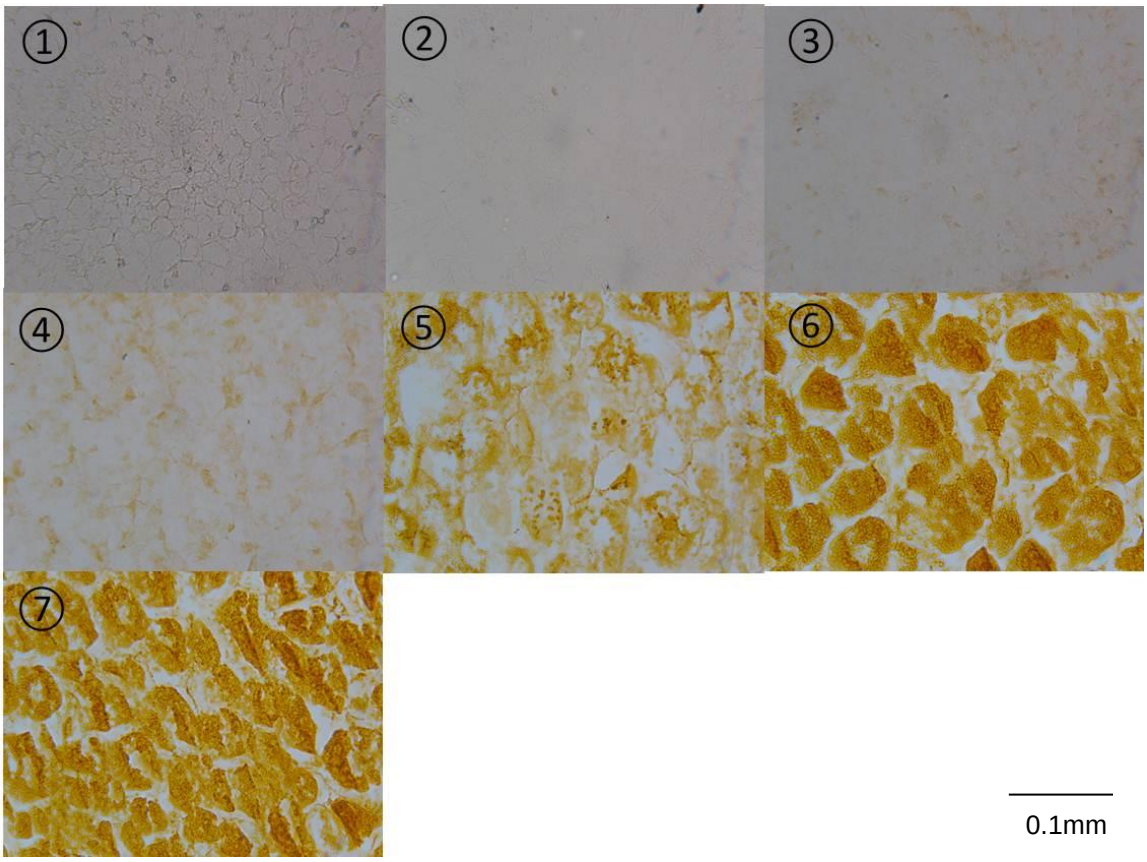

Figure 6.9 Cross-sections of developing *Moringa oleifera* seed stained with Orange G to indicate protein accumulation. ① 8 mm ② 10 mm③ 12 mm ④ 14 mm ⑤ 16 mm ⑥ 18 mm ⑦ 20 mm.

Protein bodies (PB) were the largest intracellular component (Figure 6.10), measuring between ±5 µm to 8 µm in size. Analytical quantitative analyses results revealed that the intracellular protein content percentage progressively increased, peaking at maturity (Chapter 7). This was also apparent from the visual assessment of stained sections.

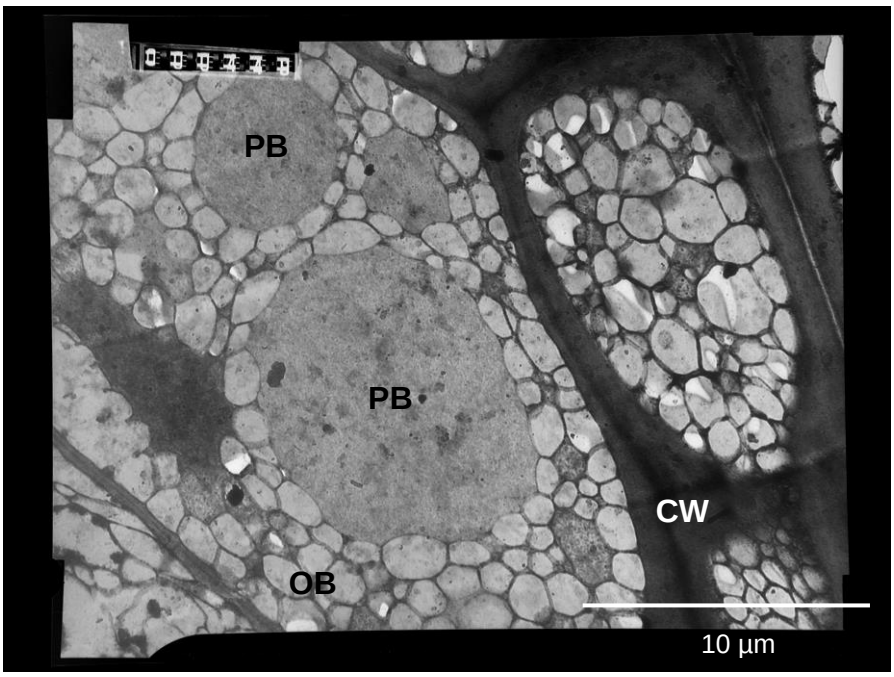

Figure 6.10 Protein bodies (PB) stored in the vacuole of a *Moringa oleifera* seed (cotyledon) cell. CW – cell wall, OB – oil bodies.

6.5 Conclusion

Microscopical analysis (light microscopy and TEM) of developing *Moringa oleifera* seeds provided insight into a facet of Moringa research that thus far has received very little attention. While histochemical staining identified compound synthesis initiation and storage location, TEM studies supported findings that TAG bodies arise from the ER and that starch granules are transiently synthesized and stored inside plastids. At maturity, the two prominent storage compounds found in seeds were the oil and protein bodies. This observation corresponded to that of the analytical quantitative analyses, results of which are discussed in Chapter 7. Furthermore, the presence of chloroplasts during early seed development suggests that seeds are photosynthetically active for at least part of their development. Forthcoming research prospects would be to evaluate whether histochemical seed staining could be used for quantitative compound approximation based on the stain (colour) intensity.

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CHAPTER 7

INTRACELLULAR COMPOSITIONAL CHANGES IN DEVELOPING *MORINGA OLEIFERA* SEED AS AFFECTED BY IRRIGATION

7.1 Summary

The compositional development of *Moringa oleifera* seed across a range of growth stages was monitored at three irrigation treatments, simulating total annual rainfall of, 900 mm, 600 mm and 300 mm/annum over two consecutive growing seasons (24 months). Fruit developmental stages were categorized according to fruit diameter (0 mm – 28 mm) at 2 mm increments. Starch was the first to accumulate during the initial histo-differentiation phase (fruit diameters of 0 mm - 12 mm), while oil levels remained comparatively low. During the subsequent expansion phase (fruit diameters of 12 mm – 24 mm) however, stored starch was mobilized and used in oil biosynthesis, reducing the starch content percentage. The bulk of oil and protein were synthesised throughout this phase with their content increasing sigmoidally. As the seed moisture content decreased during the final maturation phase (fruit diameters of 24 mm – 28 mm), the average oil content percentage reached 24.8%, while the protein content percentage was 24.7% and the starch content percentage was 8.7%. The different irrigation treatments had less of an effect on the final starch, oil and protein content percentages than on the time and rate of their synthesis throughout seed development. Higher irrigation rates principally favoured oil biosynthesis. The highest final oil content percentages were measured at the intermediate irrigation treatment (600 mm/annum), suggesting that both lower and higher irrigation rates could possibly reduce final oil content percentages. The reduction in irrigation amount delayed

the onset of oil biosynthesis and as a result the starch content percentage reached higher levels prior to its remobilization during oil biosynthesis.

7.2 Introduction

The main compounds found in mature *Moringa oleifera* seed are oil, protein and starch (Oliveira *et al.*, 1999, Abdulkarim *et al.*, 2005, Ferreira *et al.*, 2008), with the relative proportion of each component varying slightly amongst the different literature sources. This inconsistency between reported values, can possibly be attributed to varying agro-climatic conditions as well as time of harvest (Singh and Singh, 1992).

Some of the reported oil content percentages are 30.8% ± 2 (mean ± SD)(Abdulkarim *et al.*, 2005), 41.7% (Makkar and Becker, 1997), 34.7% (Duke and Atchley, 1984) 41.2% ± 2.2 (mean ± SD) (Oliveira *et al.*, 1999) and 33.2 to 40.9% (Anwar *et al.*, 2005). Besides Moringa seed oil being a palatable oil (Fuglie, 1999), it also contains all main fatty acids found in olive oil and is therefore comparable to olive oil in terms of quality (Ramachandran *et al.*, 1980, Tsaknis *et al.*, 1998, Tsaknis *et al.*, 1999, Ferreira *et al.*, 2008). The main unsaturated fatty acid found in Moringa oil, is oleic acid ($C_{18:1}$) at 67.9% with the highest saturated fatty acids being palmitic acid ($C_{16:0}$) at 7.8%, stearic acid ($C_{18:0}$) at 7.6%, behenic acid ($C_{22:0}$) at 6.2% and arachidic acid ($C_{20:0}$) at 4.0% (Abdulkarim *et al.*, 2005, Ferreira *et al.*, 2008). Additional characteristics of the oil include its high stability against oxidative rancidity (Lalas and Tsaknis, 2002).

According to Abdulkarim *et al.* (2005) Moringa seed have a crude protein content percentage of 38.3% ± 1.03 (mean ± SD), while Makkar and Becker (1997) reported a protein content percentage of 36.7%, Duke and Atchley (1984) 38.4%, Oliveira *et al.* (1999) 33.3% ± 1.2 (mean ± SD) and Anwar *et al.* (2005) between 28.5 to 34.0%. This is

greater than many other important leguminous crops with average protein content percentage between 18 to 25% (Ferreira *et al.*, 2008). Moringa seed are also known to possess coagulating properties used in water clarification and waste water treatment (Jahn, 1988). Coagulating seed proteins remove organic matter and other compounds through the adsorption and neutralization of charges (Santos *et al.*, 2012).

Similarly to oil and protein content, the reported starch content percentage of *Moringa oleifera* seed varies between different literature sources, some of the reported values are 16.5% (Abdulkarim *et al.*, 2005), 17.8% (Makkar and Becker, 1997), 17.1% (Duke and Atchley, 1984) and 21.1% (Oliveira *et al.*, 1999).

Seed development can be classified into three principle phases. Firstly histodifferentiation, which involves the division, enlargement and differentiation of cells (pre-storage), secondly seed filling during which the storage lipids, proteins and carbohydrates are formed. The final phase is desiccation, once the seed moisture content decreases (Baud *et al.*, 2002, Weber *et al.*, 2005, Dam *et al.*, 2009).

To date, Moringa seed studies have been mainly on the medicinal, nutritional and coagulating properties of the seed (Jahn, 1988, Fuglie, 1999, Anwar *et al.*, 2007), with very little attention to the growth and development of the seeds. The main objective of this study was to determine the feasibility of cultivating *Moringa oleifera* under reduced irrigation or rain fed production systems and to what extent this will affect oil, protein and starch biosynthesis. An additional aim was to determine when oil, protein and starch reserves are formed during seed development, with the objective of identifying sensitive growth stages during which trees should not be stressed.

7.3 Materials and Methods

Due to continuous flower initiation and development, fruit of different developmental stages could be found on the trees throughout the growing season. Six months after the initial flowering, all fruit (regardless of developmental stage) were harvested and grouped according to treatment and developmental stage, based on their fruit diameter. Fruit diameter was chosen as the best parameter for seed development as this is a non-destructive measurement, which can be performed by growers while the fruit remains attached to the tree. After harvesting the fruit, all the seeds were freeze dried for at least 72 hours and ground into a fine powder, which was then used for the different analyses (oil, protein and starch quantification). Cross-sections of seed from various developmental stages, based on fruit diameter are illustrated in Figure 7.1. All quantitative analysis results are expressed as percentages (g/100g) of the dry seed mass.

Figure 7.1 Cross-sections of *Moringa oleifera* seed at different developmental stages (8 mm - 26 mm) based on fruit diameter (mm).

7.3.1 *Assessment of Oil Content*

Oil content percentage determination was performed using a soxhlet extractor fitted with a 500 ml round-bottomed flask and condenser. Ground seed samples were weighed and enfolded into Whatman grade 1 filter paper, placed into cellulose paper extraction thimbles and extracted for 8 hours at 60°C using *n*-hexane (Lalas and Tsaknis, 2002). The solvent (*n*-hexane) was then distilled off to recover the oil. Residual solvent was removed by

heating the flask to 60°C for 60 min. Flasks were weighed to determine the oil content and oil expressed as percentage of original dried sample mass.

7.3.2 *Assessment of Starch Content*

Starch content percentages were determined using a method adapted from Rose *et al.* (1991) by adding 25 ml of 80% ethanol to 0.5 g finely ground seed sample and placed in a water bath at 80 - 90°C for 30 min. Samples were then centrifuged for 10 min. at 2000 rpm and decanted before the process was repeated and followed by overnight drying at 70°C. After removing samples from the oven, 30 ml distilled water was added and mixed before placing them into an autoclave for 2 hours. Once samples have cooled down, the following was added: 2 drops of toluene, 2.5 ml acetate buffer (pH 4.6) and 2.5 ml amyloglucosidase solution. Samples were then placed into a water bath at 55°C overnight, while mixing every 30 min. for the first 2 hours. Subsequently, samples were transferred into 100 ml volumetric flasks, filled to the mark with distilled water, mixed and filtered. One millilitre of filtrate was then transferred into a 50 ml volumetric flask and filled to the mark with distilled water, after which 0.5 ml of that sample solution and 2.5 ml of twice filtered colour reagent were transferred into a test tube and placed in a dark room for 30 min. The reaction was stopped by adding 1 ml of 50% H_2SO_4. Absorbances were read using an Analytikjena Spekol 1300 spectrophotometer at 540 nm and measured against a set of standards.

7.3.3 *Assessment of Protein Content*

Crude protein content percentage was determined using the Dumas method (AOAC, 2002). The quantitative nitrogen content of the ground seed sample was determined using a Leco Nitrogen analyzer (Leco FP-428, Leco Corporation, St Joseph, MI, USA). The crude protein content percentage (%) was determined by multiplying the measured nitrogen content with a conversion factor of 6.25 according to AOAC (2002).

The calorific value (kJ/seed) of oil, protein and starch was calculated by multiplying the average storage compound content per seed (g/seed) for each developmental stage by 37 for oil, 17 for protein and 17 for starch according to Greenfield and Southgate (2003).

Data were statistically analysed using the Statistical Analysis Software (SAS Version 9.2) program for Microsoft Windows, by the Statistics Department at the University of Pretoria. Means and standard errors were calculated using SAS PROC MEANS.

7.4 Results and Discussion

Changes during seed development are twofold, namely physical and biochemical. The former being characterized by changes in seed volume through cell division, enlargement and differentiation, while the latter involves the deposition of storage compounds (oil, starch and protein) (Chung *et al.*, 1995). Before the effect of different irrigation rates on the storage compound biosynthesis can be discussed, the interaction of the various storage compounds throughout seed development needed to be studied.

Starch was the first storage compound to be synthesised, however, the amount fluctuated throughout most of the seed development phase. From the compositional content percentage results illustrated in Figure 7.2, starch was transiently stored at relatively high levels, peaking at 32.1% once fruit reached a diameter of 14 mm. Thereafter the starch content percentage decreased progressively, reaching levels of between 8.5 – 8.8% at maturity. For the first half of the seed development period, starch was the main component present in seed. Similar observations, where starch was transiently stored in seeds have been recorded in *Sinapis alba* (Fischer *et al.*, 1988), *Brassica napus* (da Silva *et al.*, 1997, King *et al.*, 1997) and *Arabidopsis* spp. (Focks and Benning, 1998, Baud *et al.*, 2002).

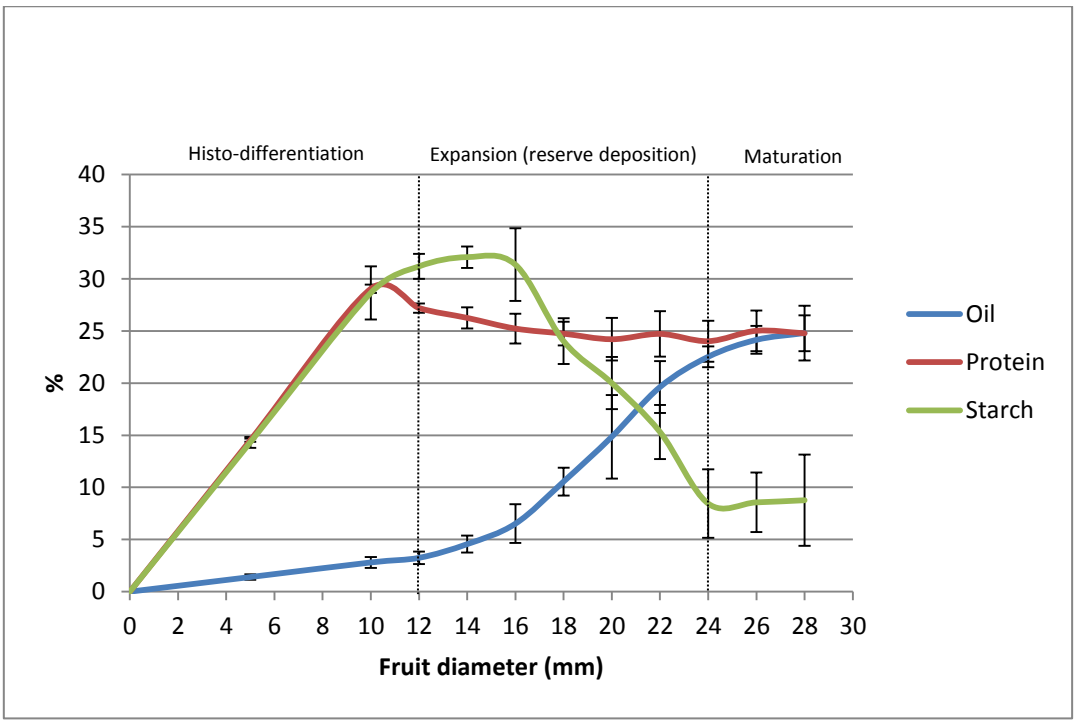

Figure 7.2 Changes in the compositional content percentage (%) of storage compounds in developing *Moringa oleifera* seed, across all three irrigation treatments. Vertical bars (±) indicate standard errors of the mean (SEM).

Previous studies suggested that the initial accumulation of starch in the sink tissue acts as a temporary reserve enabling the regulation and facilitation of growth and biosynthesis of additional storage compounds such as oil and protein (Munshi *et al.*, 1990, Luthra *et al.*, 1991, da Silva *et al.*, 1997, Angeles-Núñez and Tiessen, 2010). Similarly, the starch content percentages decreased after fruit reached a diameter of 14 mm (Figure 7.2), which corresponded with the onset of oil accumulation. As a result, the 72.7% reduction in starch content percentage from when fruit reached 14 mm in diameter to maturity could possibly be attributed to the biosynthesis of oil, as similar observations have been made by Angeles-Núñez and Tiessen (2010) and Luthra *et al.* (1991).

Unlike starch, the oil content percentage increased sigmoidally until reaching an average final oil content percentage of 24.8% at maturity (Figure 7.2). Sucrose entering the developing seed was used for starch, protein and oil biosynthesis. The starch breakdown marked by a decrease in starch content percentage coincided with the increase in oil

content percentage, suggesting that oil was synthesised at least partly by the mobilized starch reserves that were used during triacylglycerol synthesis (Bewley and Black, 1994). Compared to starch or protein, oil has more than twice the stored energy on a per-mass and per-volume basis (Figure 7.3), making it the most efficient form of energy storage (Huang, 1992). Consequently oils are energy intensive to produce, requiring a greater amount of soluble carbohydrates such as sucrose (Baud and Lepiniec, 2010, Rastogi, 2010). As imported sucrose from source tissues (leaves) might be temporarily limited, stored starch is mobilized to support oil biosynthesis (Leprince *et al.*, 2006), causing a decline in starch content percentage.

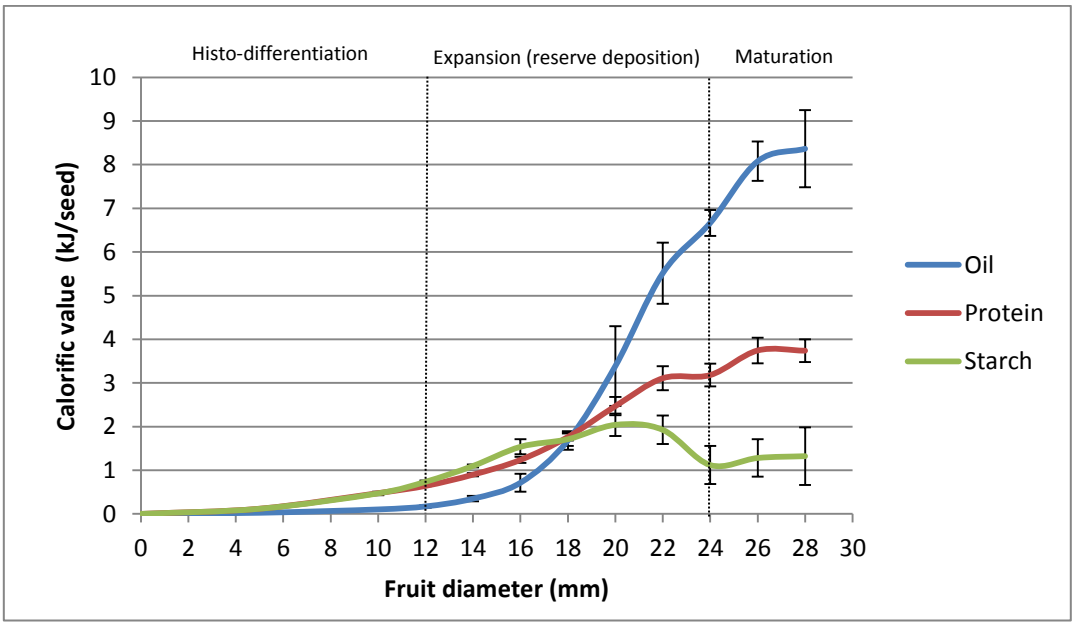

Figure 7.3 Calorific value (kJ/seed) of storage compounds (oil, protein and starch) in the average *Moringa oleifera* seed at different developmental stages, across all three irrigation treatments. Vertical bars (±) indicate standard errors of the mean (SEM).

Protein accumulation remained fairly constant throughout seed development, decreasing slightly from its peak of 29.1% at a fruit diameter of 10 mm to 24.7% at maturity (Figure 7.2). As the individual seed increased in size/mass, so did the protein mass (g). Thus despite a slight decrease in the protein portion (%) (Figure 7.2), the protein mass (g) continued to increase with the increase in seed size as illustrated in Figure 7.4.

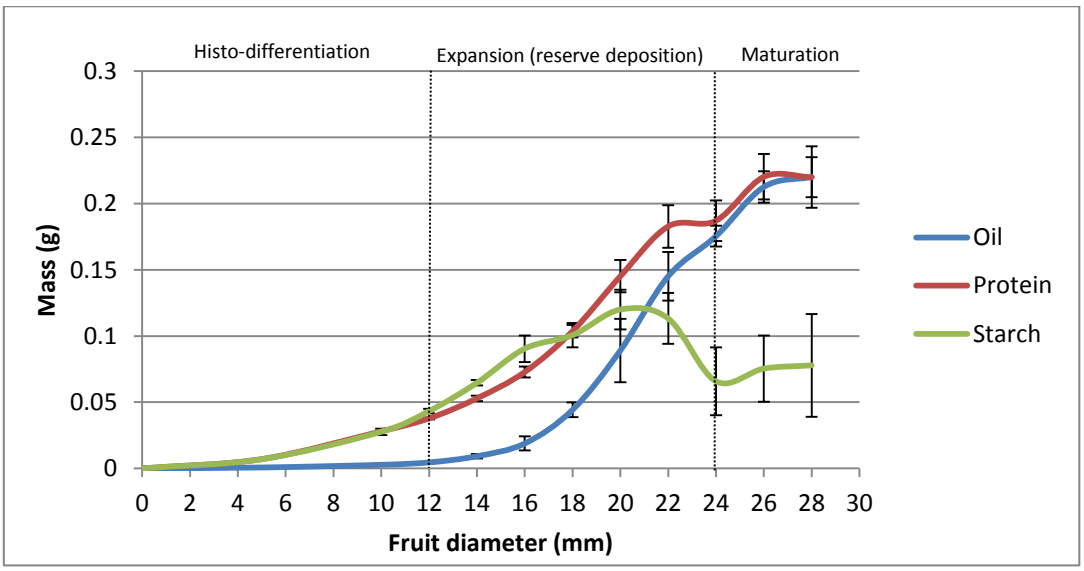

Figure 7.4 Storage compound mass (g) in the average *Moringa oleifera* seed at different developmental stages, across all three irrigation treatments. Vertical bars (±) indicate standard errors of the mean (SEM).

After shedding some light on the compositional changes during seed development, the extent to which the synthesis of starch, oil and protein was affected by different irrigation rates was investigated.

7.4.1 *Starch*

The measured starch content percentages (%) from all three irrigation treatments demonstrated the same tendencies as discussed above, by peaking at a fruit diameter of around 14 mm and then decreasing rapidly until the seed reached maturity. There was however significant variation amongst the different treatments, both in terms of the maximum starch content percentage reached, as well as the onset of starch remobilization to oil (Figure 7.5). A decrease in irrigation amount consistently increased the maximum starch content percentage, with the 900IT having the lowest and the 300IT the highest amount of transiently stored starch. The decrease in irrigation also further delayed the mobilization of stored starch, as the starch content percentage at the low 300IT decreased later compared to the 600IT and even later compared to the 900IT (Figure 7.5). As a result, seed from drought stressed trees typically had a higher starch content percentage

at any stage throughout their development compared to seed from well irrigated trees at the same developmental stage. However the effect of irrigation on starch content percentage cannot be viewed in isolation as oil biosynthesis is at least partly dependent on the mobilization of starch reserves.

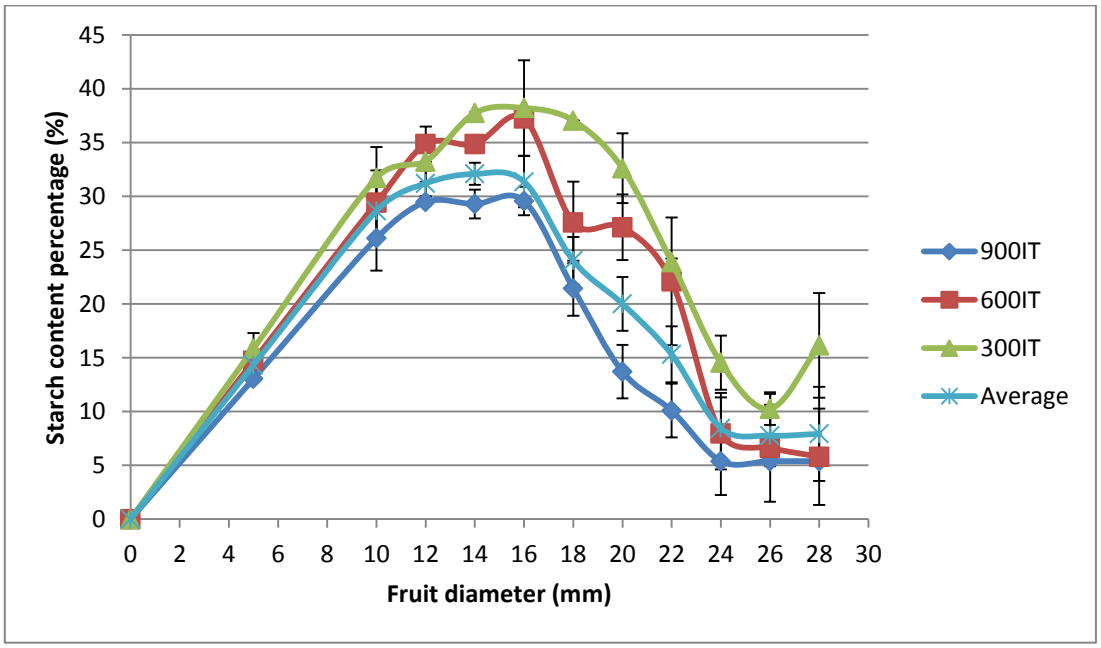

Figure 7.5 Changes in starch content percentage (%) of developing *Moringa oleifera* seed at various developmental stages as affected by three irrigation treatments (IT). 900IT – 900 mm/annum, 600IT – 600 mm/annum, 300IT – 300 mm/annum. Vertical bars (±) indicate standard errors of the mean (SEM).

7.4.2 *Oil*

Results illustrated in Figure 7.6 confirm the interaction between starch and oil, as the degradation of starch reserves coincided (from a fruit diameter of ±14 mm) with onset of oil biosynthesis across all three irrigation rates. Since the decrease in irrigation amount delayed starch remobilization, the commencement of oil biosynthesis was also postponed. With regards to the oil accumulation of the irrigation treatments, they are a direct reflection of starch degradation, as the decrease in irrigation delayed the initiation of oil biosynthesis. Between the three treatments the high irrigation amount (900IT) was initially the most beneficial towards oil biosynthesis, as oil formation commenced at an earlier developmental stage and remained highest throughout most of the developmental stages

amongst the treatments. Subsequently, oil biosynthesis initiation was delayed with the decrease in irrigation treatment.

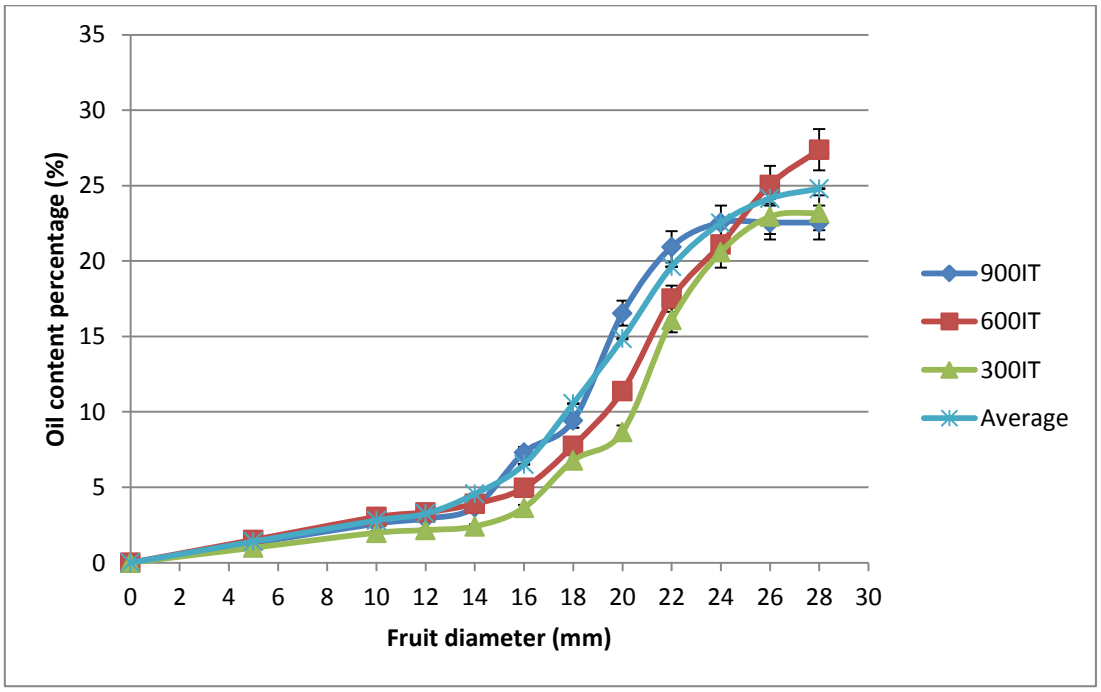

Figure 7.6 Changes in oil content (%) of developing *Moringa oleifera* seed at various developmental stages as affected by three irrigation treatments (IT). 900IT – 900 mm/annum, 600IT – 600 mm/annum, 300IT – 300 mm/annum. Vertical bars (±) indicate standard errors of the mean (SEM).

When comparing oil content percentage (%) at seed maturity, the 600IT yielded the highest average oil content percentage at 27.4%, which was significantly higher compared to the 23.2% at the 300IT and 22.6% at the 900IT (Figure 7.6). Similar observations, where supplementary irrigation has resulted in increased oil yields have been made by Anwar *et al.* (2006). Although oil accumulation seemingly increased progressively with the increase in irrigation rate throughout the initial growth and development phase, it was the intermediate 600IT that had the highest oil content percentage at maturity. This unexpected result, whereby the oil content percentage did not continue to increase with the increase in irrigation could be the result of excessive water at the high 900IT. Although increases in irrigation generally increases the oil yield (Krogman and Hobbs, 1975), an oversupply of irrigation water as well as water stress can however impede oil production

(Mendham and Salisbury, 1995). Reductions in oil yield as a result of excessive irrigation have been reported in *Helianthus annuus* by Mula Ahmed *et al.* (2007) and Yasumoto *et al.* (2011). As the reduction in oil content percentage between the 900IT and 600IT was only slight and no higher irrigation treatments were tested to confirm further reductions in oil yield with an increase in irrigation, other possible contributing factors cannot be excluded.

7.4.3 *Protein*

Seed protein content percentage (%) remained rather consistent, with only a slight decrease throughout seed development across all irrigation treatments (Figure 7.7). Throughout the entire trial period, protein content percentage (%) was lowest for the 300IT, slightly (not significantly) higher for the 600IT and highest for the 900IT. Protein accumulation was seemingly least affected by the reduction in irrigation amount. Similar observations have been made by Alahdadi *et al.* (2011) in *Helianthus annuus*. As a result, higher soil water levels favoured protein formation, even if only by a small margin.

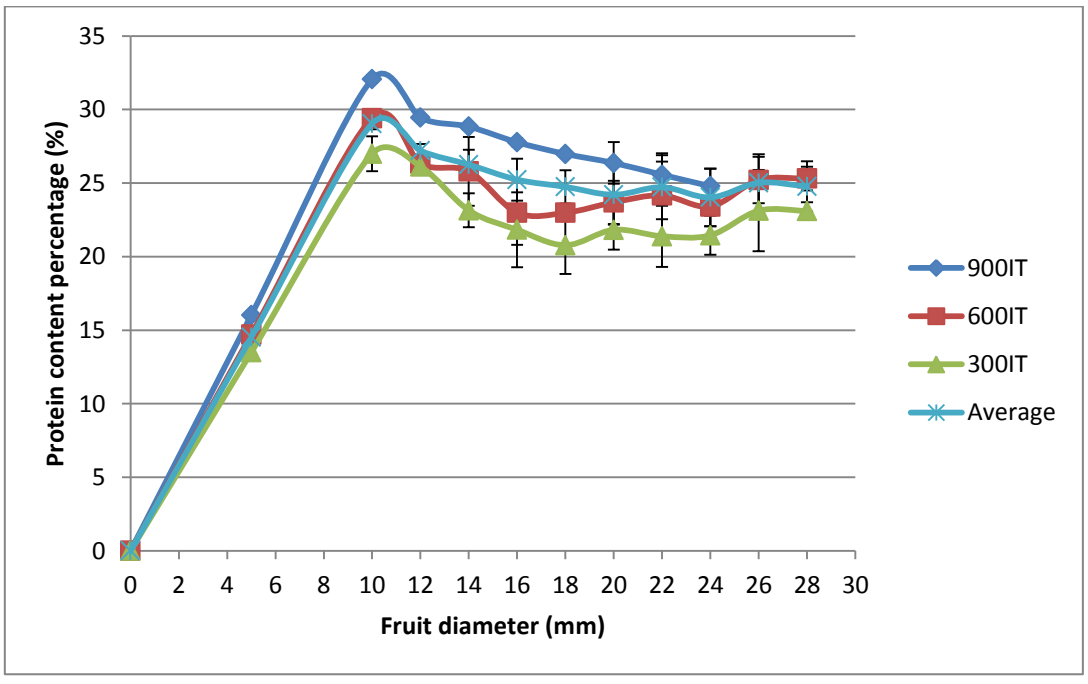

Figure 7.7 Changes in protein content percentage (%) of developing *Moringa oleifera* seed at various developmental stages as affected by three irrigation treatments (IT). 900IT – 900 mm/annum, 600IT – 600 mm/annum, 300IT – 300 mm/annum. Vertical bars (±) indicate standard errors of the mean (SEM).

7.5 Conclusion

Increased irrigation resulted in a shorter oil biosynthesis initiation time and higher accumulation rate. However, despite significant oil content percentage increases from the 300IT to the 600IT, final oil content percentage decreased again between the 600IT and 900IT. An increase in irrigation would almost certainly expedite oil production with higher protein and lower transient starch reserves, while excessive irrigation could result in lower oil content percentages. Reduced irrigation (<600 mm per annum for the current trial site) however, will most likely result in a lower seed oil content percentage. The developmental stage most sensitive in terms of component development to soil moisture stress would be the expansion phase (fruit diameters of 12 mm - 24 mm), since it was during this phase, with the exception of starch, when the majority of storage reserves were synthesized.

7.6 References

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CHAPTER 8

QUANTIFICATION OF INTRACELLULAR SEED COMPOUNDS USING DIGITAL IMAGE ANALYSIS

8.1 Summary

In addition to analytical testing of seed storage compounds, histochemical staining of sectioned seed for the identification of various intracellular compounds has successfully been used in the past. The estimation of storage compound content through the quantification of a specific compound selecting stain through digital image analysis using Adobe® Photoshop® has however not been explored to date and therefore, became the main aim of this study. *Moringa oleifera* seeds of various developmental stages at three irrigation treatments were prepared for microscopical analysis and stained with oil (Sudan III), protein (Light Green SF, Orange G) and starch (Periodic Acid Schiff) detecting stains. The percentage coverage of a specific stain in digital images of sectioned seed was determined using Adobe® Photoshop® and compared to analytical data. Protein stain: Orange G and oil detecting stain: Sudan III demonstrated the best correlation to the measured seed protein and oil content throughout seed development. A stage specific factor based on seed mass was however necessary in order to estimate storage compound content (g) from image analysis results. Differences in storage compound content during the reserve deposition period were found to be significantly different between developmental stages. For simple comparisons between storage compound contents at different developmental stages, image stain quantification proved reasonably effective. Differences between storage compound contents of the same developmental stage from different irrigation treatments were however, not as clearly distinguishable using this method.

8.2 Introduction

The use of histochemical staining of biological material for the identification of various compounds has successfully been used for nearly two centuries (Wick, 2012). Although such stains are best suited for qualitative image analyses, they offer very little quantitative data to perform accurate scientific comparisons. The main aim of this study was thus to determine whether commercial photo-editing software such as Adobe® Photoshop® could be used to quantify the amount of stain through colour selection within a slide and how this would compare to regular analytical analysis results. An additional aim was to determine if differences between developmental stages and irrigation treatment could be statistically confirmed once stained sections have been quantified successfully. Commercial photo-editing software has in the past been used for the quantification of histological samples, predominantly in the medical field of histopathology (Dahab *et al.*, 2004, Lahm *et al.*, 2004), although its application in plant sciences has been limited to date.

8.3 Materials and Methods

Established *Moringa oleifera* trees growing on the Hatfield Experimental Farm of the University of Pretoria were divided into three groups of four trees each, each group being subject to a specific irrigation rate. The irrigation rates administered were; 300 mm/year (300IT) which is about the minimum amount to sustain tree growth (Palada and Chang, 2003), 600 mm/year (600IT) the average annual rainfall for the research site and 900 mm/year (900IT), simulating supplement irrigation under field conditions. The irrigation amounts were administered, simulating total annual rainfall (mm/year). Semi-weekly soil water content measurements using a neutron probe (Campbell Pacific Nuclear, 503DR Hydroprobe) were conducted to verify differences in soil water levels between treatments (measurement results provided in Chapter 2).

8.3.1 *Light microscopy*

Due to continuous flower initiation and development, fruit of different developmental stages could be found on the trees throughout the growing season. Ten seeds of at least nine developmental stages were randomly sampled from all three irrigation treatments and prepared for light microscopy according to O'Brien and McCully (1981). After harvesting, seeds were immediately fixed in FAA (80% ethanol: 37% formaldehyde: 100% acetic acid, in proportions 8:1:1 v/v/v) for at least 24 hours, before being dehydrated in an ethanol in water solution series (30%, 50%, 70%, 100%, 100% v/v) for 24 hours at each concentration. Then, ethanol was extracted from the specimens through a series of xylene in ethanol concentrations (30%, 50%, 70%, 100% v/v) prior to placing them into paraffin wax (60°C). Embedded seed samples were cut into 10 µm thick sections, using a Reichert-Jung semi-thin rotary microtome, and placed onto microscope slides before being de-waxed in a series of xylene concentrations. The histochemical staining of seed sections differed depending on the compound that was stained for; hence they are discussed individually below.

8.3.1.1 *Protein*

Intracellular protein was detected by staining seed sections in duplicate using two different protein stains namely, Light Green SF (Figure 8.1A) and Orange G (Figure 8.1B) according to James and Tas (1984). The same staining procedure was followed for both these stains. After de-waxing, sections were stained in either Orange G or Light Green SF for 30 min., followed by three rinses of 1% acetic acid and two rinses of distilled water. Sections were then briefly rinsed in three changes of *tert*-butanol, followed by two changes in xylene, before being mounted with synthetic resin. Protein stained bright green with the Light Green SF and bright orange with Orange G.

8.3.1.2 *Starch*

Starch reserves were detected using Periodic Acid-Schiff (PAS) (Figure 8.1C) reagent according to Merck (2011). De-waxed sections were rinsed in distilled water and placed into Sodium periodate solution for 5 min. each. After rinsing sections in distilled water, they were stained with PAS reagent for 15 min. and rinsed with sulfite water (6 min.), followed by distilled water (10 min.). Sulfite water was prepared by adding a mixture of 10 ml sodium disulfite solution (10 %) and 10 ml of hydrochloric acid (1 mol/l) to 200 ml of distilled water. Finally, sections were stained with hemtoxylin solution (2 min.) and rinsed with distilled water (3 min.) before being mounted. Starch (polysaccharides) stained purple-magenta with PAS.

8.3.1.3 *Oil*

Seed used for the lipid staining could not be embedded using the conventional procedure used for light microscopy as discussed above, given that seed lipids are xylene soluble. Alternatively, under such circumstances fresh seed specimens would merely be sectioned by hand. With this method, section thickness would have become inconsistent and this would in turn have affected staining, making different samples incomparable. Moringa seed posed an additional challenge in that they are relatively hard once mature and difficult to section uniformly thin. For this reason, the following customized method was developed for *Moringa oleifera* seed in order to perform lipid staining. Seed samples were fixed in FAA (80% ethanol: 37% formaldehyde: 100% acetic acid, in proportions 8:1:1 v/v/v) for 7-14 days, followed by 24-48 hours in distilled water to remove excess fixative prior to sectioning. Samples were then thinly sectioned using a freeze-microtome and transferred onto microscopy slides prior to staining. Preliminary trials found Sudan III (Sudan red) (Figure 8.1D) to be the best lipid stain for *Moringa oleifera* seed, when compared to Sudan IV and Sudan black B. Hence Sudan III was used for all subsequent

intracellular lipid staining according to Culling (1974). Sections were submerged in propylene glycol (2 min.) and then transferred into Sudan III solution (10 min.) Then, excess Sudan III was removed by rinsing sections in two changes of 85% and 50% propylene glycol. Lipids stained red/orange with Sudan III.

Randomly collected seed (n=10) of nine growth stages at each irrigation treatment were imbedded for sectioning and prepared for staining.

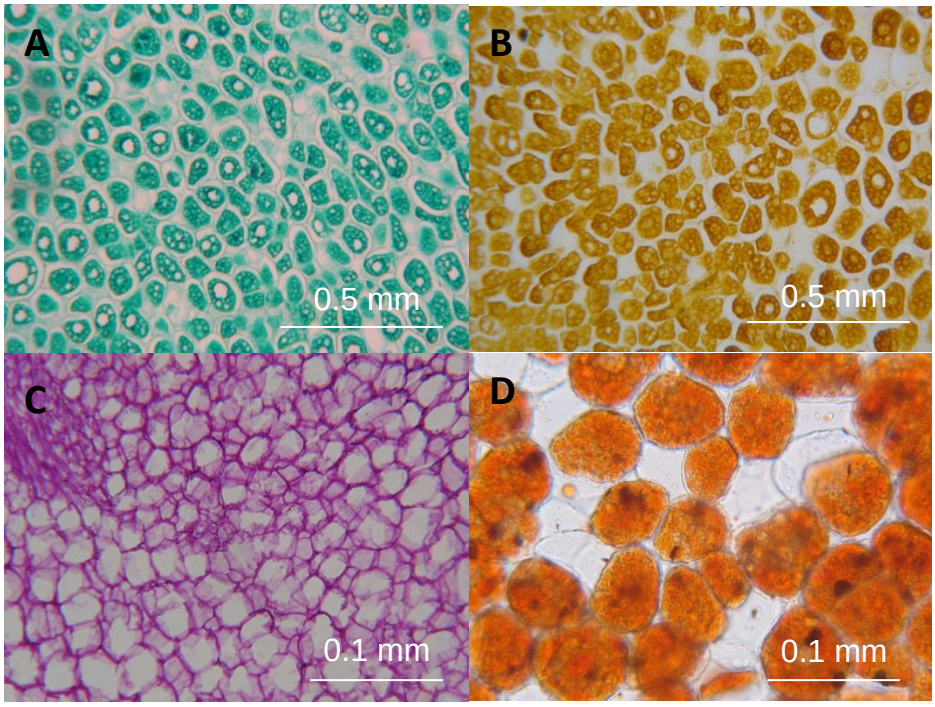

Figure 8.1 *Moringa oleifera* cotyledons stained with different compound detecting stains. A - Light Green SF (Protein), B - Orange G (Protein), C - Periodic Acid-Schiff (Starch), D - Sudan III (Oil).

8.3.2 *Image Analysis*

Once stained, seed sections were viewed using a Leitz Biomed light microscope, while photographs were taken using a Canon PowerShot A630 digital camera. Digital images of the sectioned seed stained with Orange G, Light Green SF, Sudan III and PAS were analysed using Adobe® Photoshop® CS6, Version 13.0 x32. Digital images were individually uploaded into Adobe® Photoshop® and the colour (RGB ratio) set to the specific stain in question (Figure 8.2). The RGB ratios were determined by visual comparison to match the specific stain colour and are given in Table 8.1.

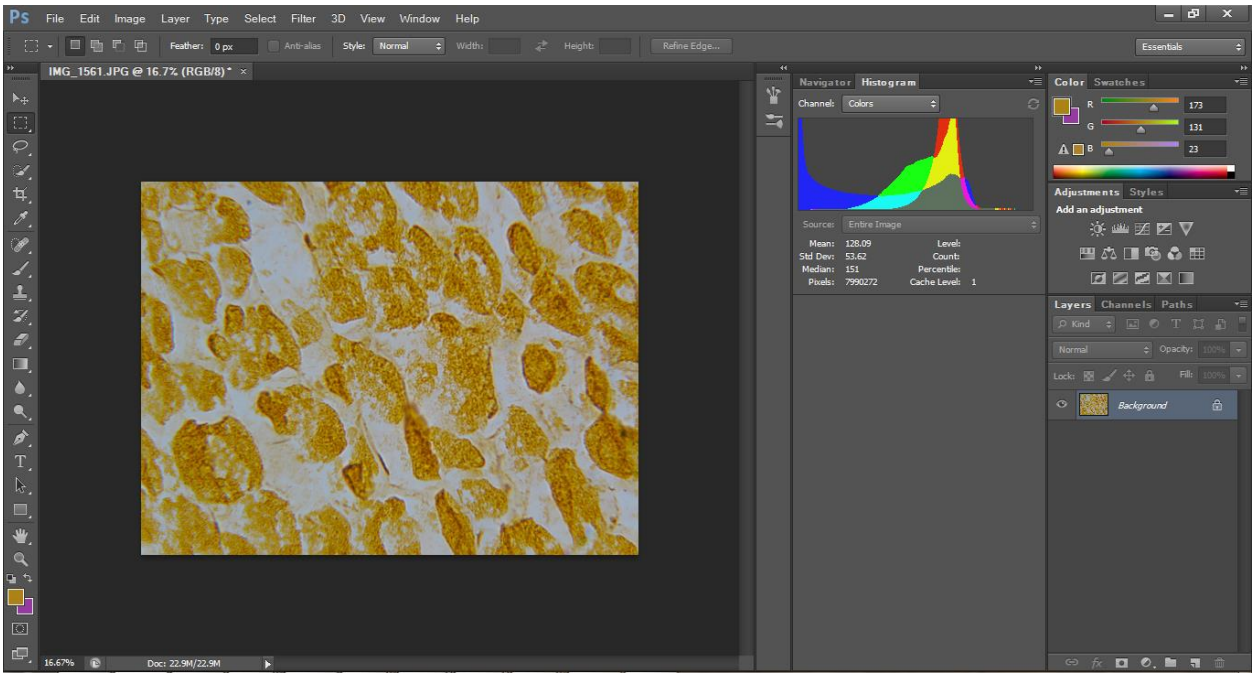

Figure 8.2 Screenshot of Adobe® Photoshop® CS6, with uploaded seed cross-section stained with the protein stain Orange G.

Table 8.1 RGB ratios used for the “color range” selection for various histochemical stains.

	Green SF	Orange G	Periodic Acid-Schiff	Sudan III
R	10	173	151	211
G	165	131	61	87
B	157	23	157	1

Subsequently, the “Color Range” command was selected under the “Select” drop-down menu. To ensure the selection was not limited to the set colour but included additional shades around the set colour, the “fuzziness” was set to 100. This meant that all pixels within 100 brightness levels (both lighter and darker) from the set colour (RGB ratio) were included in the selection. The selection area remained visible in the set colour range, while the unselected areas appeared black in the main window (Figure 8.3). Once all pixels within the set colour range were selected, the pixel count was displayed under the histogram section (Figure 8.4). Calculating the percentage coverage of a certain colour within an image was calculated using the equation below.

$$\%\ colour\ coverage = \left(\frac{number\ of\ selected\ pixels\ (x)}{total\ pixel\ count\ (7990272)}\right) \times 100$$

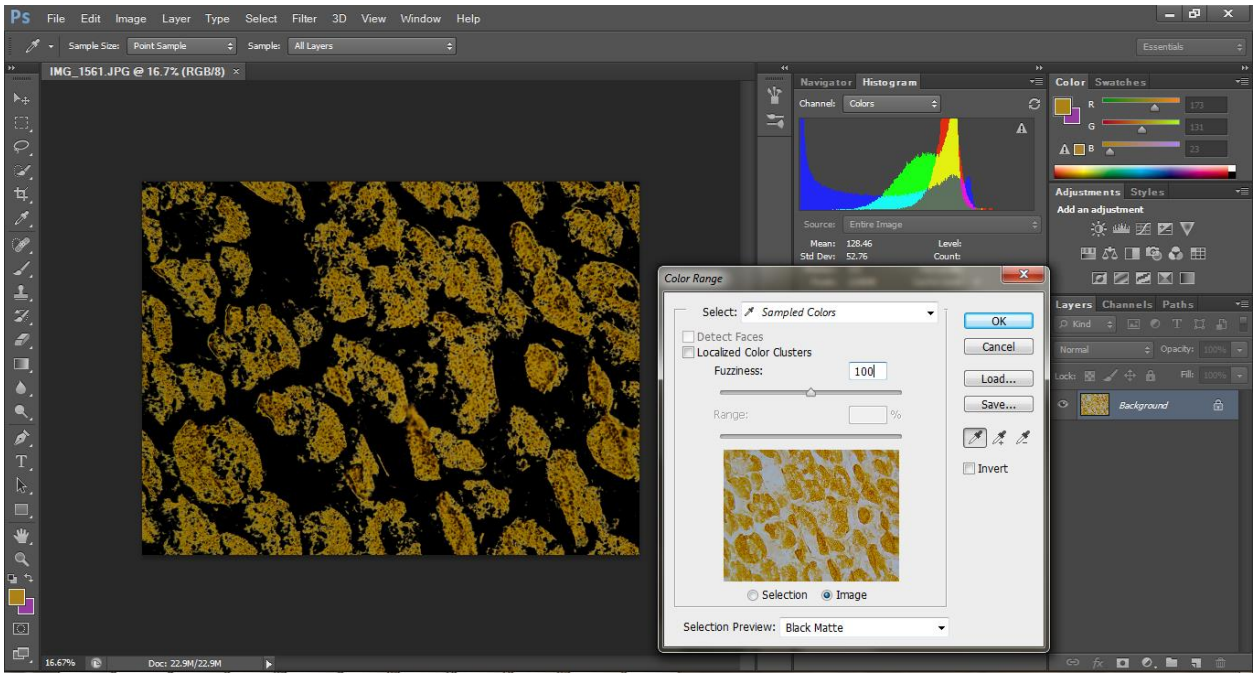

Figure 8.3 Screenshot of Adobe® Photoshop® CS6, illustrating the colour specific selection process using the colour range function.

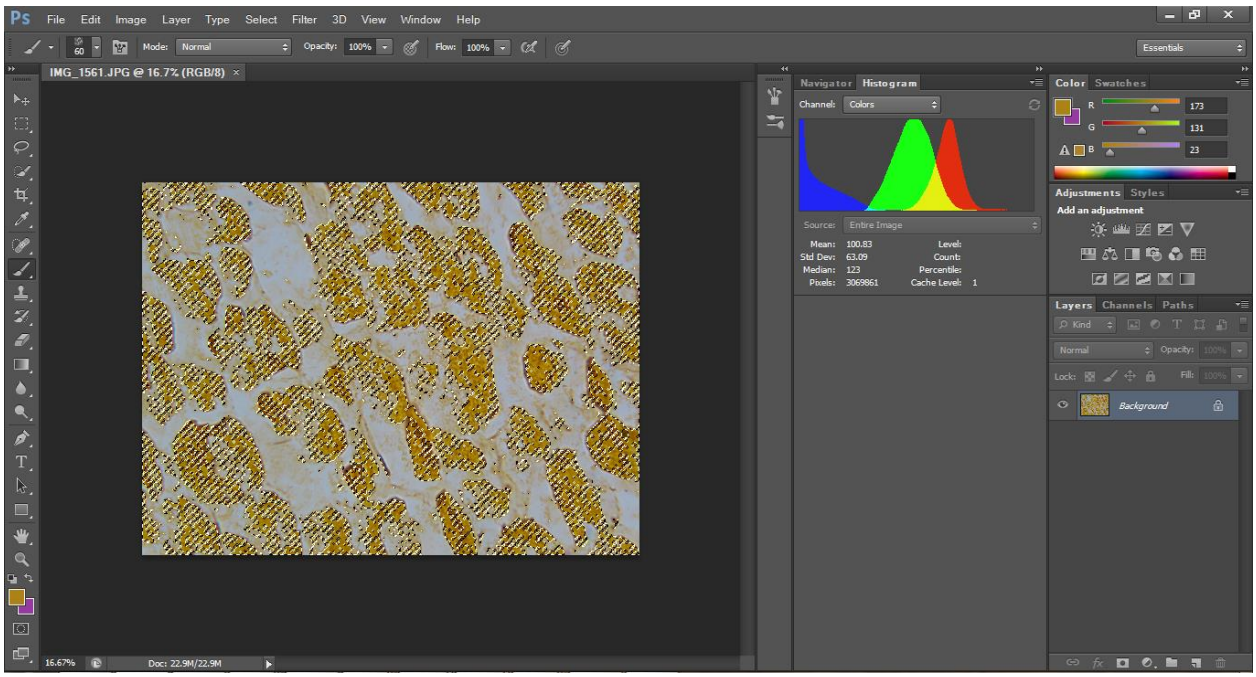

Figure 8.4 Screenshot of Adobe® Photoshop® CS6, displaying the total selection area within the set colour range as well as the histogram section revealing the number of pixels that have been selected.

The difference between a sample section stained with Orange G before and after selective extraction using Adobe® Photoshop® CS6 is illustrated in Figure 8.5.

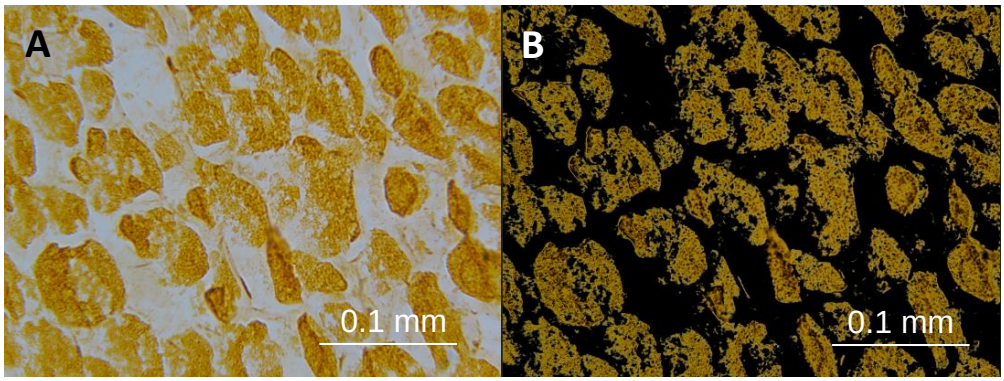

Figure 8.5 *Moringa oleifera* seed sections stained with Orange G before (A) and after (B) selective extraction using Adobe® Photoshop® CS6.

The analytical seed storage compound (oil, starch and protein) content percentages (g/100 g) across the various developmental stages were determined and discussed in Chapter 7. Results (seed storage compound content) from Chapter 7, were used in this study to perform comparisons as well as to quantify stain coverage of the seed sections.

Data were statistically analysed using the Statistical Analysis Software (SAS Version 9.2) program for Microsoft Windows, by the Statistics Department at the University of Pretoria. Means and standard errors were calculated using SAS PROC MEANS. Correlations between fruit diameter (mm) and various other measured parameters (seed count, seed/fruit mass) were determined using regression functions with the highest correlation coefficient.

8.4 Results and Discussion

8.4.1 *Storage compound content estimation from percentage stain coverage*

In order to compare the percentage coverage of the oil, protein and starch specific stains determined using digital image analysis with that of the oil, protein and starch content determined analytically, results from both methods were plotted against each other in Figure 8.6. Each storage compound is represented by a different colour, the lighter shade being the storage compound content percentage (%) determined analytically, while the darker shade is the percentage coverage (%) determined through digital image analysis. From this raw data, it is evident that there was no clear correlation between the percentage stain coverage and the percentage content between any of the storage compounds throughout the entire seed development phase.

Analytical protein, starch and oil content results were expressed as g/100 g, which essentially can also be referred to as the percentage content of a specific compound. This unit of measure takes the changes in seed size throughout their development into account.

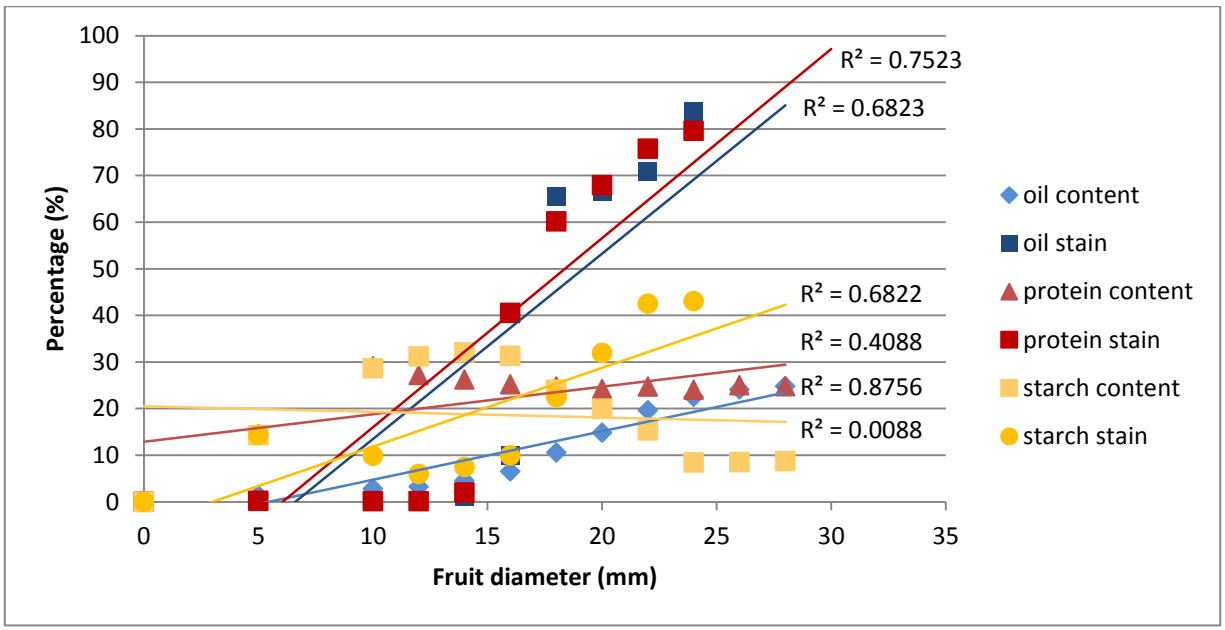

Figure 8.6 Comparison between the oil, protein and starch content percentage (%) and respective stain coverage (%) in developing *Moringa oleifera* seed.

In contrast, microscopical seed sections were photographed at the same magnification across all developmental stages. Due to cell expansion with seed growth, the seed portion examined decreased with the increase in seed size, ultimately affecting their comparability as illustrated in Figure 8.7. The number of cells under investigation at a fruit diameter of 10 mm (Figure 8.7A) were much greater when compared to the number of cells at a fruit diameter of 24 mm (Figure 8.7B), although the study area (mm^2) remained exactly the same.

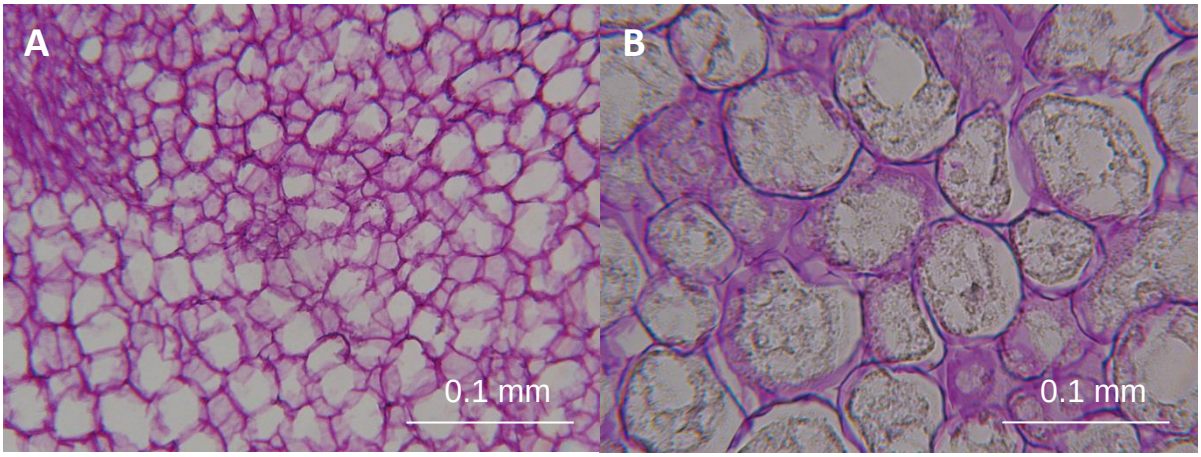

Figure 8.7 *Moringa oleifera* endosperm sections stained with Periodic Acid Schiff (PAS) reagent at two different developmental stages based on fruit diameter (mm). A - Ø10 mm, B – Ø24 mm.

In order to compensate for the increase in cell/seed size, staining results (percentage stain coverage) were expressed as mass (g)/seed by multiplying the percentage colour coverage at a particular developmental stage by the average seed mass (g) of that developmental stage. This conversion yielded much better correlation between the percentage stain coverage and the percentage content for both protein and oil stains, but for starch however, there was still no clear correlation as illustrated in Figure 8.8. This could possibly be attributed to the fact that PAS not only stained the storage polysaccharides (starch), but also structural polysaccharides (cellulose) inside the cell walls. As a result, the stain coverage area increased, while the actual starch content (g/100 g) decreased with seed growth. For this reason, seed sections stained with PAS offered very little correlation to starch content (g/100 g).

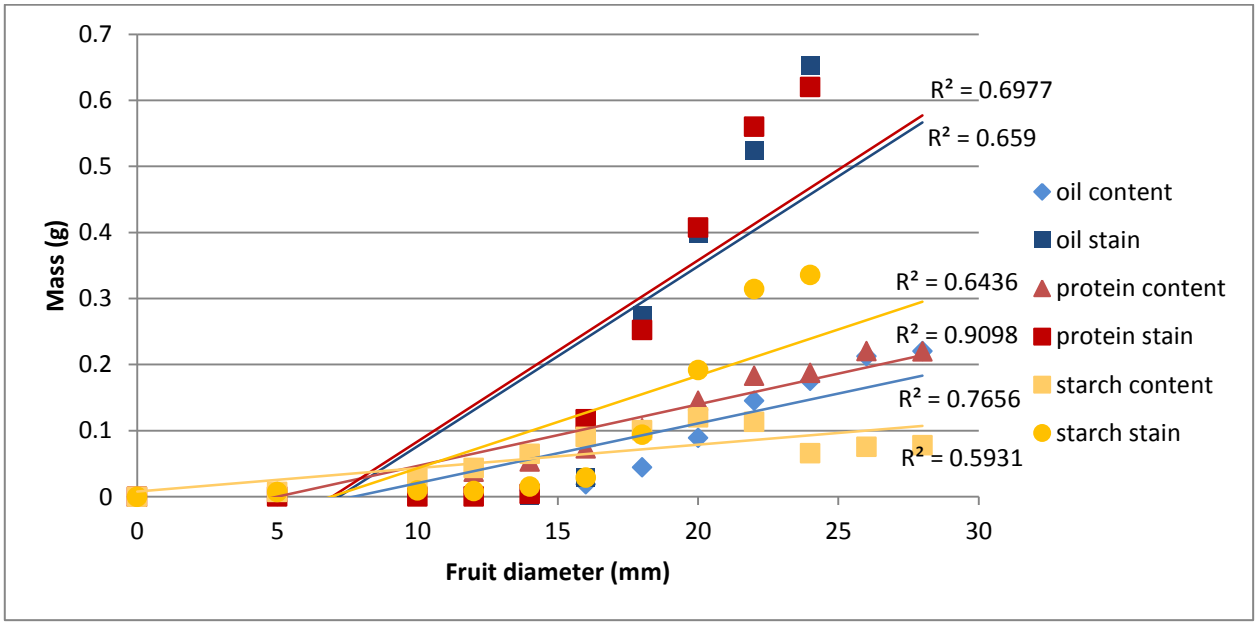

Figure 8.8 Comparison between the oil, protein and starch content (g) and respective stain coverage expressed as mass (g) in developing *Moringa oleifera* seed.

As a result, stain coverage (%) is best used to estimate the storage compound mass (g/seed) rather than percentage content (Figure 8.9).

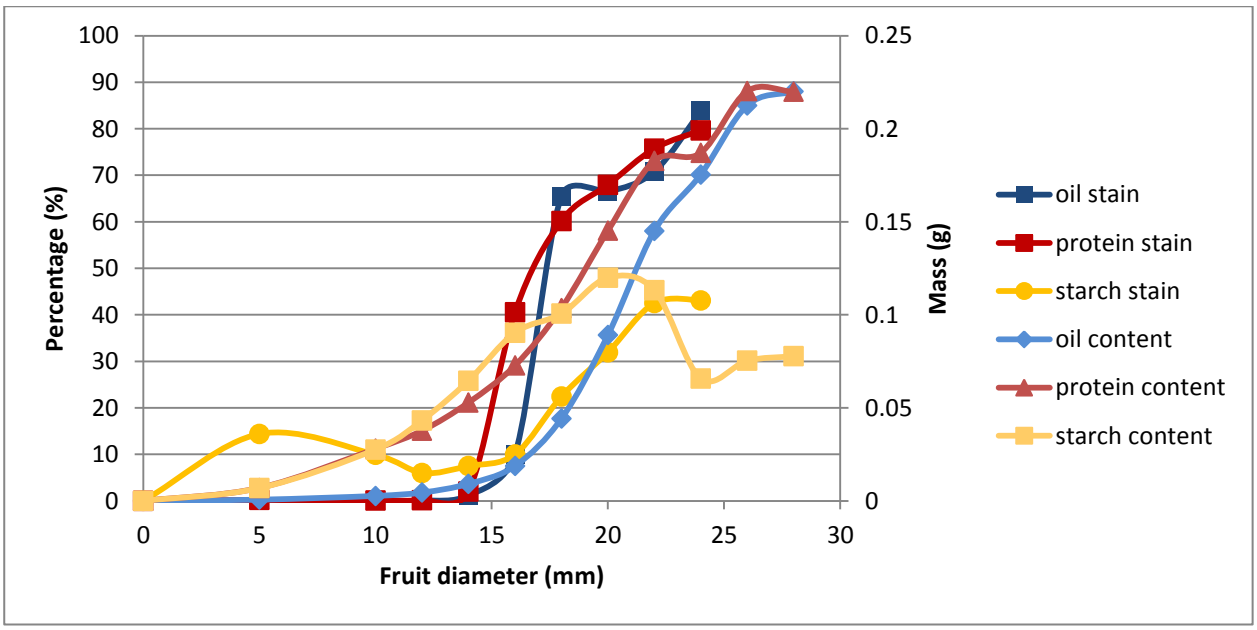

Figure 8.9 Comparison between the oil, protein and starch content (g) and respective percentage stain coverage in developing *Moringa oleifera* seed.

To calculate storage (oil, protein or starch) compound mass (g/seed) from stain coverage (%), a conversion factor (y) is required. Given the variability of the storage compound content (g) throughout seed development, such a conversion factor is both fruit diameter (mm) and stain dependant. To calculate the conversion factor (y) for a specific storage compound at any given fruit diameter (mm) (x), polynomial regression functions were fitted for each storage compound/stain as illustrated in Figure 8.10. The polynomial equation for each storage compound/stain is given in Table 8.2.

Table 8.2 Equation for each storage compound/stain to calculate the conversion factor (y) based on fruit diameter (mm) (x).

Storage compound/*Stain*	**Equation**	**R^2 value**
Oil/*Sudan III*	$y = 0.0003x^2 - 0.0113x + 0.118$	0.9577
Protein/*Light Green SF, Orange G*	$y = -0.0001x^3 + 0.0096x^2 - 0.2189x + 1.6228$	0.9063
Starch/*Periodic Acid Schiff*	$y = 2E\text{-}06x^4 - 0.0001x^3 + 0.002x^2 - 0.0152x + 0.0372$	0.9075

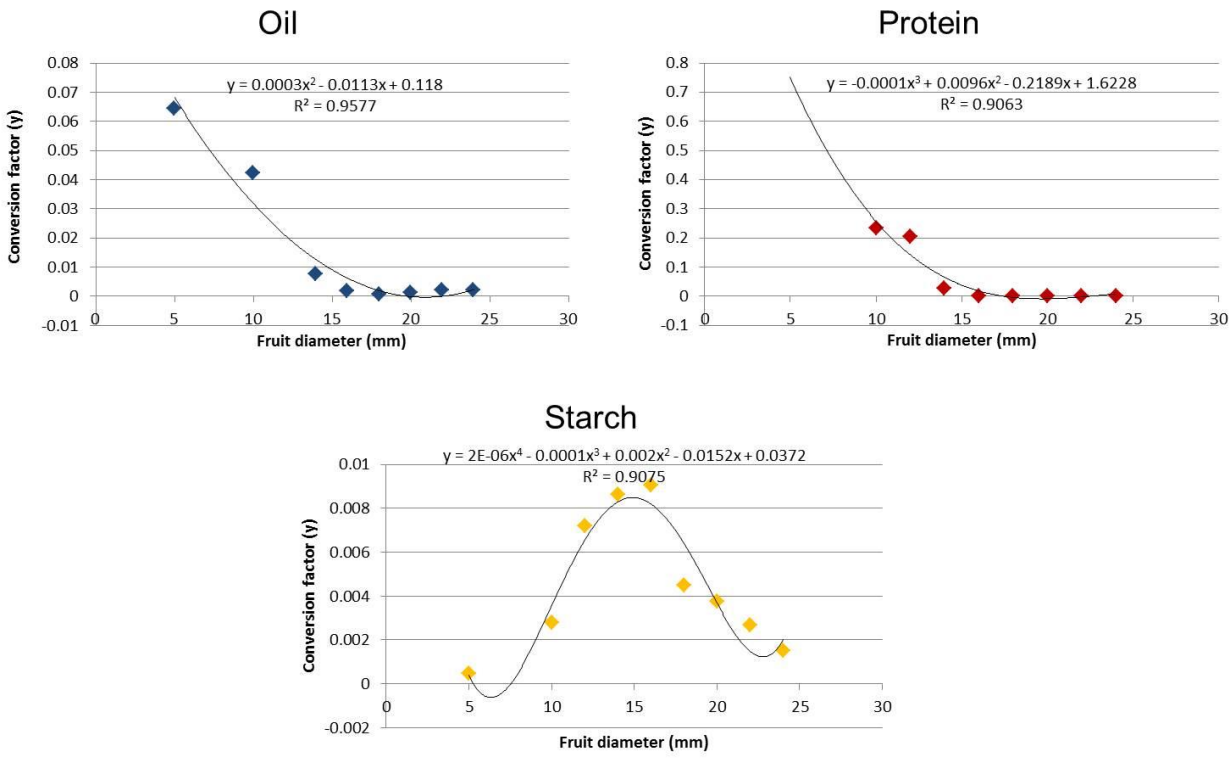

Figure 8.10 Polynomial regression functions for each storage compound (oil, protein and starch), to calculate the stain coverage (%) to storage content (g) conversion factor for any given fruit diameter (mm).

Once the conversion factor (y) for a specific storage compound and fruit diameter (mm) (x) has been calculated, the storage compound mass (g/seed) can be estimated by simply multiplying the conversion factor (y) with the stain coverage (%) as follows:

$$compound\ mass\ (g/seed) = stain\ coverage\ (\%) \times conversion\ factor\ (y)$$

8.4.2 *Stain quantification for measurable comparisons*

Stained seed sections offer very little tangible data as they can essentially only be used for visual comparisons. Quantification of such sections would enable differences between treatments and developmental stages to be statistically assessed. The percentage coverage of the oil, protein and starch detecting stains in digital images of sectioned seed

was determined using Adobe® Photoshop®. Average values for all three irrigation treatments at every developmental stage are illustrated in Figure 8.11.

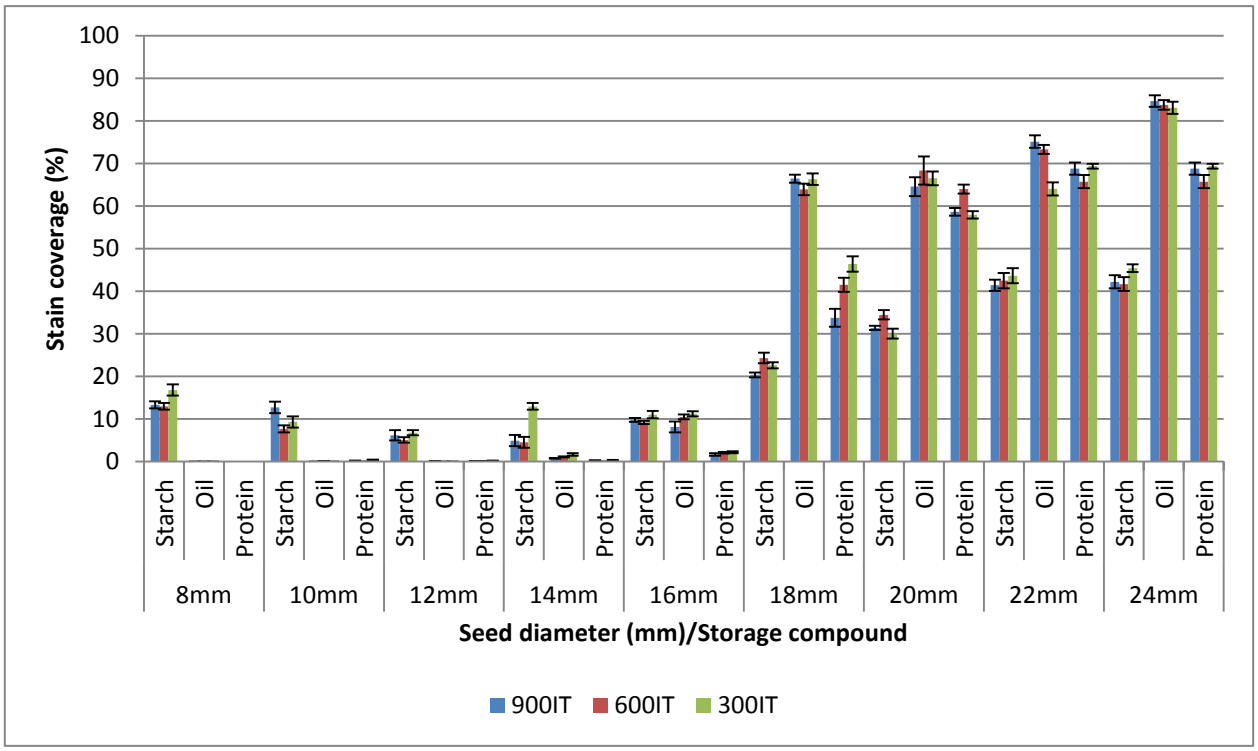

Figure 8.11 Stain coverage (%) of seed sections stained with starch, oil and protein detecting stains at various developmental stages (based on fruit diameter) across three irrigation treatments. (900IT – 900 mm/annum, 600IT – 600 mm/annum, 300IT – 300 mm/annum). Vertical bars (±) indicate standard errors of the mean (SEM).

By merely assessing the stain quantification results, compound (oil, protein and starch) synthesis remained seemingly unaffected by the different irrigation rates. Very slight, to no significant differences could be identified between the percentage stain coverage of the three irrigation treatments at most developmental stages. This could mainly be attributed to minor differences between treatment means and in some instances also a large standard error of the means (SEM). Individual images of a stained seed section merely offered a small representative sample, this accompanied by inter-sample variability made small differences between treatments less distinguishable. Seed staining might therefore not be the most appropriate method to detect slight differences between treatments. Across different developmental stages however, this method proved far more applicable,

as the variation between the average stain coverage at different developmental stages was much more discernible (Figure 8.12).

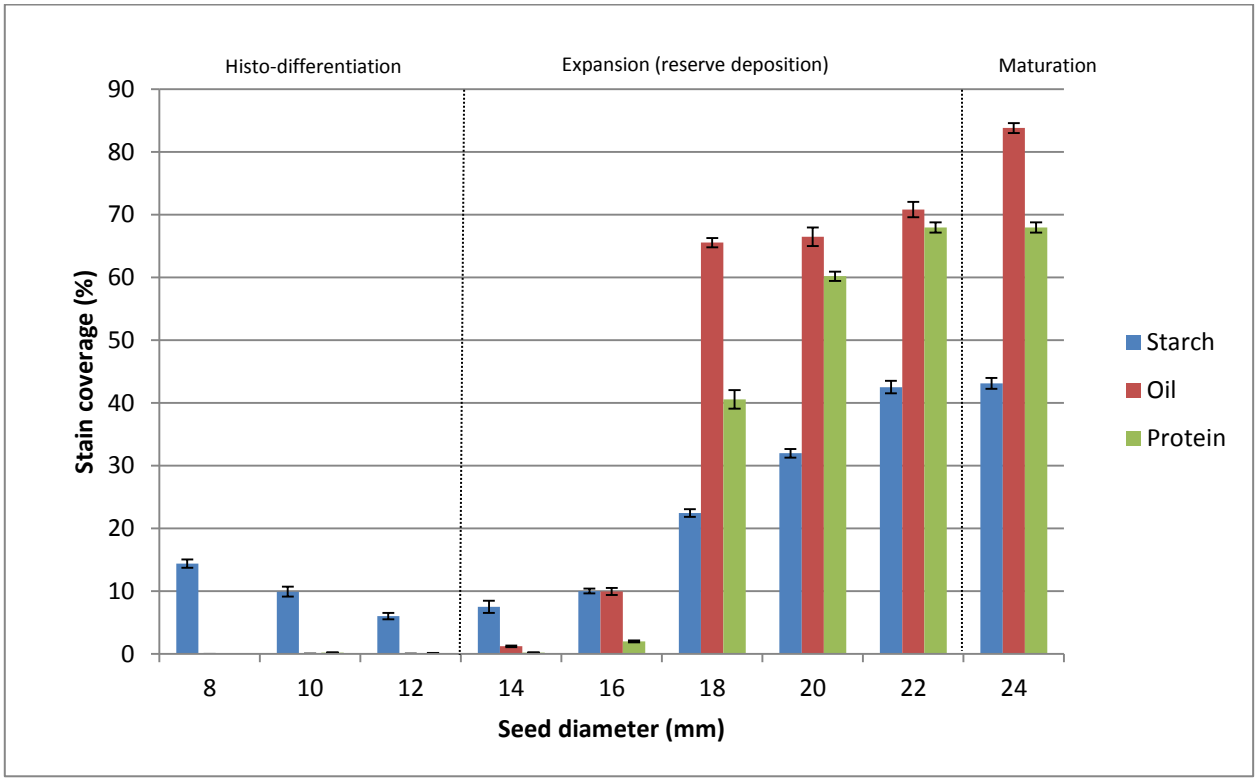

Figure 8.12 Average stain coverage (%) of seed sections from various developmental stages, across all three irrigation treatments, stained with starch, oil and protein detecting stains. Vertical bars (±) indicate standard errors of the mean (SEM).

Despite only slight differences between storage compounds (oil, protein and starch) during the initial growth stages, significant increases across all compounds were measured during the early reserve deposition phase, between the fruit diameters of 16 mm to 18 mm. While both starch and protein continued to increase significantly beyond these growth stages, differences in oil levels however, levelled off after seed reached a diameter of 18 mm, with only slight increases until maturity.

8.5 Conclusion

Since stained seed sections viewed under the microscope offer no tangible data for comparison, the quantification of these sections using digital imaging software enabled

seed samples from different treatments to be compared. The estimation of a storage compound content (g) based on the relative stain intensity was not equally effective for all stains and required the use of a factorial conversion. Orange G (protein stain) and Sudan III (oil stain) demonstrated a better correlation between the percentage stain coverage and the actual compound content (g) when compared to Periodic Acids Schiff reagent (starch stain). Nonetheless, stain quantification was used successfully to determine the onset of storage compound biosynthesis and the differentiation between storage compound amounts at different developmental stages. Although the success of digital image analysis might be stain dependant, it certainly is a cost effective technique for compound content estimation.

8.6 References

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CHAPTER 9

GENERAL DISCUSSION AND CONCLUSIONS

Besides the global challenges of food insecurity, rising levels of greenhouse gasses and the continued search for sustainable fuel/energy sources, the continent of Africa faces additional challenges such as malnutrition, poverty, diseases, water scarcity and large areas of sub-optimal agricultural land (Tirado *et al.*, 2010). Although, these challenges might seem multifarious, which indeed they are, there is a potential solution that could possibly address these challenges. This solution comes in the form of a tree, called *Moringa oleifera*.

This multipurpose tree, is considered to be one of the world's most useful tropical trees, as it is used as nutritious food, biofuel, medicine, water purifier and fodder (Foidl *et al.*, 2001, Palada and Chang, 2003, Rashid *et al.*, 2011). Also, *Moringa oleifera* trees have been found suitable for cultivation in areas that are less hospitable (Palada and Chang, 2003), and in so doing improve the environment through CO_2 sequestration and erosion aversion.

However, according to Padulosi *et al.* (2011), *Moringa oleifera* is still considered to be one of the worlds "underutilized species". Thus despite all its documented beneficial uses, this tree's full potential has not yet been recognized. According to Emongor (2009), one way of addressing this, is through innovative agronomic research that highlights this tree's benefits, thereby promoting Moringa related commodities both locally as well as internationally.

In an attempt to further the existing knowledgebase of Moringa research, the main aim of this study was to evaluate the effect of three different irrigation rates on flowering, fruiting and intracellular storage compound biosynthesis. In so doing, gaining a better understanding of how commodities such as oil yields for instance, might be affected should trees be cultivated in marginal/low rainfall production areas. In a water-scarce country like South Africa (Molobela and Sinha, 2012), a potential biofuel crop such as *Moringa oleifera* should ideally be cultivated under dryland conditions on marginal land. From an agronomic point of view, the identification of drought sensitive stages in the growth cycle is not only important from a water-use perspective, but also because water is often in short supply.

Flowering is not merely one of several important stages that will ultimately affect yield, but it is also a stage that is greatly affected by water stress (Mwanamwenge *et al.*, 1999, Fang *et al.*, 2010), and therefore a critical phase during the crop production cycle. As a result, a closer look had to be taken at the effect of three different irrigation treatments on flower initiation and flowering (Chapter 2). Surprisingly, this study has revealed that the highest number of flowers were initiated at the lowest (300IT) irrigation treatment. However, fewer flowers were fertilized compared to both the higher irrigation treatments (600IT and 900IT), resulting in low subsequent fruit/seed production. This was likely due to flower abortion caused by a combination of lower pollen viability and water stress in the styles, inhibiting pollen tube growth and fertilization. The highest irrigation rate (900IT) however, produced significantly less flowers, yet, a greater proportion of these were successfully fertilized to produce fruit/seed. Although *Moringa oleifera* has been found to tolerate low soil water levels (Morton, 1991, Palada and Chang, 2003, Stalin *et al.*, 2011), stress during the flowering phase can however have severe implications on the eventual yield. Moderate

water stress prior to floral initiation can be beneficial by stimulating flower initiation, while sufficient water supply thereafter would ensure better fruit set and greater yield.

Seed growth and storage compound biosynthesis are fundamental processes affecting both final yield and reproductive potential. By studying the post-fertilization seed anatomy, greater insight could be gained into how physiological and biochemical processes throughout seed growth and development might be affected, once trees are subjected to drought stress (Bewley and Black, 1994). From microscopical studies of developing seed, the endosperm was found to be nuclear, becoming cellular from the chalazal end towards the micropylar end at a fruit diameter of ±6 mm (Chapter 3). Once at a fruit diameter of ±8 mm the embryo had reached the globular stage, while the entire inner integument had also been covered by cellular endosperm. Visible cotyledon development commenced at a fruit diameter of ±12 mm and continued up until ±24 mm. During this seed developmental stage, the majority of storage compounds were synthesized and stored. As a result, the growth stages between ±12 mm up until ±24 mm were the most crucial and consequently also the most sensitive to environmental stresses. Water stress during these seed developmental stages can negatively affect seed mass and storage compound biosynthesis. At the end of this phase (fruit diameters >24 mm) the cotyledons had filled the entire space covered by the seed coat, while the unicellular epidermal layer of the inner integument remained distinctly visible between the cotyledons and the testa.

Since cell expansion is one of the first growth processes affected by water stress (Hsiao, 1973, Farooq *et al.*, 2009), the effect different irrigation rates had on fruit size, yield and growth rate was explored in Chapter 4. In addition, measurements collected from all fruit at various growth stages would enable the estimation of average seed count and seed mass at maturity level, based on the fruit diameter/length. The reduction in irrigation did not

simultaneously result in a significant decrease in fruit numbers, but rather a decrease in both the growth rate and final fruit size. Fruit from the higher irrigation rates however, reached maturity sooner with a higher average seed count per fruit. In contrast to the observations made during the flowering phase (Chapter 2), the fruit growth phase was seemingly less affected by the different irrigation treatments. This was however only in terms of the physical characteristics such as fruit size, yield and growth rate and not seed quality, results of which were discussed in Chapter 7. As a result, it appears as if the sensitivity of *Moringa oleifera* to water stress varies throughout the growing season.

Since photosynthesis is one the fundamental processes of primary plant metabolism, it is central to overall plant growth and development. Given that photosynthesis generally decreases with reduced water availability (Flexas and Medrano, 2002, Grassi and Magnani, 2005, Flexas *et al.*, 2009), any reduction in water availability will lower the photosynthetic rate, which sequentially will affect photo-assimilate partitioning throughout the plant (Chaves *et al.*, 2002, Chaves *et al.*, 2003, Flexas *et al.*, 2004, Lawlor and Tezara, 2009). As a result, Chapter 5 aimed to quantify the extent to which reduced plant water availability affect photosynthesis and ultimately yield. Results revealed a decrease in photosynthetic rate, both with a decrease in irrigation rate as well as with progression of the growing season. This reduction could not be attributed solely to either stomatal (conductance and SI) or non-stomatal (possible RuBP regeneration, ATP synthesis and mesophyll conductance) limitations as the primary limiting factor. Instead, photosynthesis was affected by a combination of both of the above mentioned limitations as well as a decrease in chlorophyll content throughout the growing season.

Studying the effect of water stress on seed storage component biosynthesis requires comprehensive understanding of the seed ultrastructure. For this reason and as a result of

the limited existing knowledge, microscopical analysis (light microscopy and TEM) of developing *Moringa oleifera* seed was performed. Various different histochemical staining techniques were used to identify storage compound biosynthesis initiation as well as final storage location, while TEM was used to determine their origin. As mentioned and illustrated in Chapter 6, both oil and protein were synthesized in association with the rough endoplasmic reticulum whereas starch granules are synthesized and transiently stored inside the plastids. Based on the staining results, storage compounds were only deposited in significant amounts from the fruit diameter of ±14 mm onwards.

The three main storage compounds found in mature *Moringa oleifera* seed are oil, protein and starch (Oliveira *et al.*, 1999, Abdulkarim *et al.*, 2005, Ferreira *et al.*, 2008). The aim of Chapter 7 therefore, was not only to determine when these storage compounds are synthesized during seed development, but also how their synthesis was affected by different irrigation rates. Results confirmed that the developmental stage most sensitive to soil water stress is the expansion phase (fruit diameters of 12 mm - 24 mm), since it was during this phase, with the exception of starch, when the majority of storage reserves were synthesized. Increased irrigation resulted in a shorter oil biosynthesis initiation time and higher accumulation rate. However, despite significant oil content percentage increases from the 300IT to the 600IT, final oil content percentage decreased again between the 600IT and 900IT. An increase in irrigation would almost certainly expedite oil production with higher protein and lower transient starch reserves, while excessive irrigation could result in lower final oil content percentages. Reduced irrigation (<600 mm/annum for the current trial site) however, will most likely result in lower seed oil content percentages and yield.

Histochemically stained seed sections prepared for the analytical studies in Chapter 6, unfortunately offered no quantitative data to accurately compare the starch, protein and oil

levels at different developmental stages from the three irrigation treatments. In an attempt to quantify these stained seed sections, digital imaging software (Adobe® Photoshop® CS6) was used to calculate the stain (colour) coverage, thereby enabling sections from different developmental stages/treatments to be compared. The estimation of a storage compound content based on the relative stain intensity was not equally as effective for all stains and required the use of a conversion factor, to convert percentage stain coverage (%) to storage compound content (g). The results showed that although image analysis might not be the most appropriate measurement tool for compound content (g) estimation, it proved to be very useful for comparison purposes, especially for oil and protein.

Once the findings of all the sub-studies are taken into account, it becomes evident that the effect of irrigation on seed growth and development is multifaceted and that their vulnerability to drought stress varies not only throughout the growing season, but also at different physiological growth stages.

This study contributes to existing knowledge by broadening our current understanding of Moringa flowering, seed growth and storage compound development and how they are affected by water supply. Simultaneously, it provides current and prospective Moringa growers with valuable insight into how the limitations of their production site could possibly affect yield. For this reason, and based on the knowledge attained from the aforementioned studies, Figure 9.1 can be used to determine the suitability of a prospective Moringa production site within South Africa. Based on this map (Figure 9.1), the irrigation amounts (300, 600 and 900 mm/annum) administered during this study represent the average annual rainfall of substantial portions across South Africa. Areas marked as 201-400 mm could be considered representing the 300IT, 401-600 mm as well as 601-800 mm the 600IT and 801-1000 mm the 900IT. According to the findings of the

study, production areas within low (±300 mm/year) annual rainfall would be considered sub-optimal for commercial Moringa production, without supplementary irrigation. Whilst flower initiation might be favoured by slight drought stress, subsequent fruit set, yield and storage compound synthesis would be negatively affected.

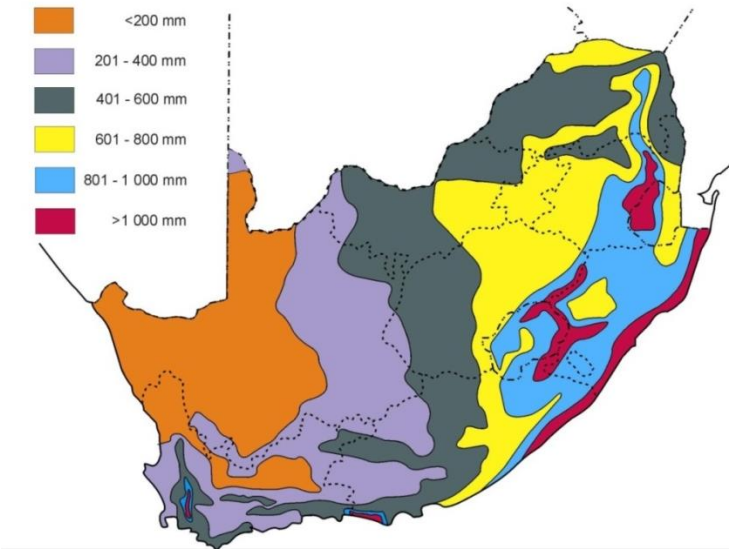

Figure 9.1 Mean annual precipitation (mm/year) of South Africa obtained from Siyavula Uploaders (2009).

Throughout this entire study, the 600IT consistently demonstrated the most favourable results across several measured parameters. Starting with flowering, although the 300IT evidently offered the necessary signalling to promote flower initiation, unlike the 600IT it was unable to retain these flowers. The 600IT, not only demonstrated satisfactory flower initiation, but also the ability to support satisfactory post-fertilisation fruit growth. Despite slightly reduced fruit growth and storage compound biosynthesis rates, when compared to the higher 900IT, the highest oil and protein content percentage (%) at seed maturity were measured at the 600IT. In addition, the 600IT also had the highest average seed mass amongst all three irrigation treatments. Although fruit growth and storage compound biosynthesis rates were highest at the 900IT, this irrigation treatment might have been slightly excessive, at least for the soil texture of the trial site (Chapter 2). After having subjected trees for two years in a row to the high 900IT, flower initiation was significantly less during the second year compared to the first. This could be attributed to the absence

of the flower promoting drought stress signal, as soil water levels remained consistently high according to soil water content measurement results (Chapter 2). Thus although soil water levels might be sufficiently high to ensure satisfactory fruit growth and development, reduced flowering will ultimately limit yield potential.

Based on all these observations, production areas with a medium (±600 mm/year) annual rainfall would therefore be recommended as most suitable for *Moringa oleifera.* Even though annual rainfall/irrigation amount is an important factor to consider during site selection, equally important climatic factors such as temperature (Muhl *et al.*, 2011), should not be overlooked. According to the findings of Muhl (2009), the mean annual temperature best suited for Moringa cultivation is 18-19°C or higher. As a result, the production areas best suited for *Moringa oleifera* production in South Africa have been identified based on both its temperature (Muhl, 2009) and rainfall requirements (Figure 9.2). Suggested production areas illustrated in Figure 9.2 were identified based upon weather data (mean annual temperature and rainfall) obtained from the Department of Environmental Affairs and Tourism (1999).

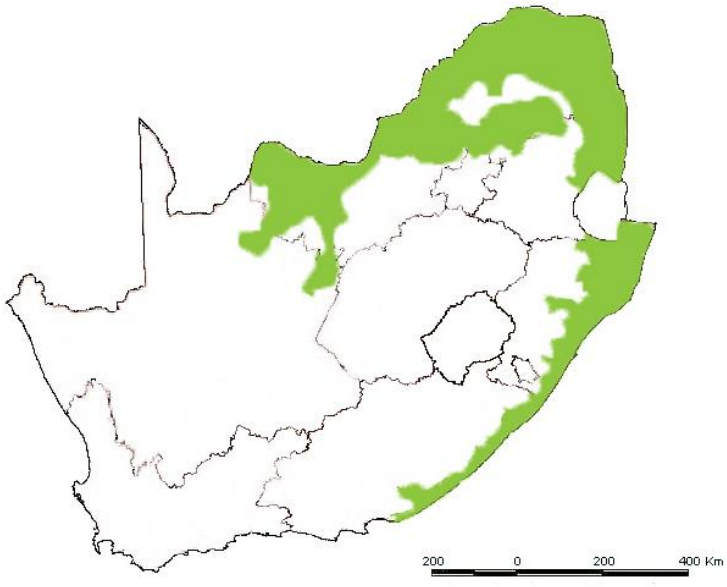

Figure 9.2 Suggested areas best suited for *Moringa oleifera* production in South Africa based on both average annual rainfall and temperature.

The suggested production areas were selected according to annual rainfall (mm/annum) and do not take into account the possibility of supplementary irrigation, which could make Moringa cultivation feasible in lower rainfall (<600 mm/annum) areas, beyond the suggested areas. Site specific limitations such as soil type and rainfall frequency, which could possibly affect Moringa production, should also be taken into consideration before plantation establishment, even if the proposed site might fall within an area identified as suitable for Moringa cultivation with regards to average annual temperature and rainfall according to Figure 9.2.

If the aim is however, to maximize yield in an existing orchard with limited resources (water), the irrigation management should be such, that trees are not stressed during sensitive stages. Reduced irrigation (<600 mm/year), would most likely result in slower biosynthesis rates of oil, protein and starch along with lower final contents of these storage compounds within seed. Moderate water stress prior to floral initiation could even be beneficial in stimulating flower initiation. This should however be followed by sufficient irrigation to ensure decent flower development, pollination, fruit set and yield. *Moringa oleifera* trees are least sensitive to water stress during the vegetative stage, more sensitive during flowering and fruit set, and most sensitive during reserve deposition (fruit diameters of ±12 – 24 mm).

Figure 9.3 depicts *Moringa oleifera* fruit and seed at various developmental stages (based on fruit diameter). From this figure the estimated storage compound initiation point and percentage content**s** (%) can also be estimated.

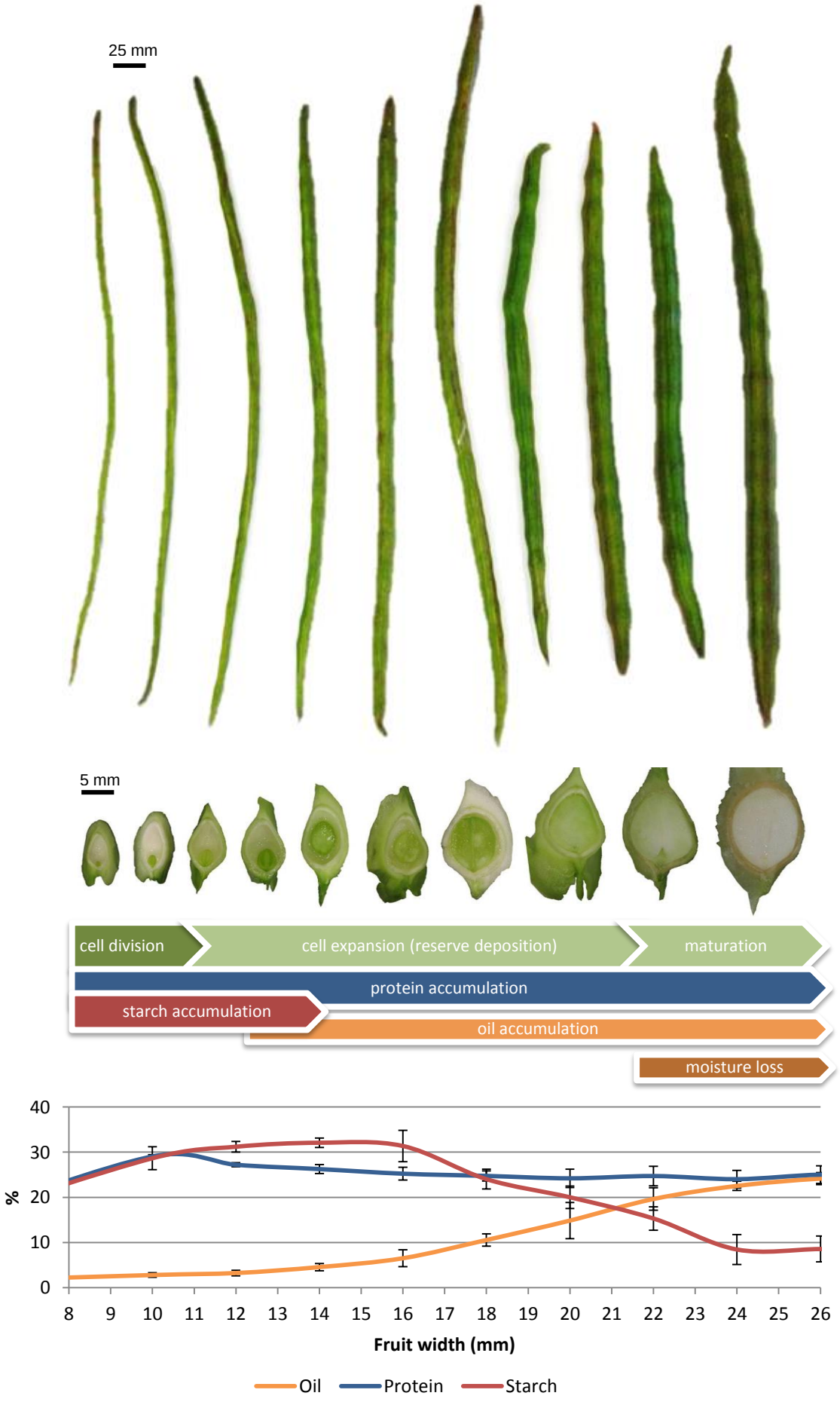

Figure 9.3 Oil, protein and starch biosynthesis initiation and percentage content (%) in developing *Moringa oleifera* seed.

In an attempt to enable Moringa growers to non-destructively estimate the protein, oil and starch content percentages (%) of their fruit/seed at any given developmental stage, Table 9.1 was drawn up. The percentage content of any of these major storage compounds (protein, oil and starch) can be estimated based on the annual rainfall/irrigation amount (mm/annum) as well as fruit diameter (mm). Fruit diameters are simply measured using a calliper while the fruit remain attached to the tree. With an average annual seed yield of approximately 503 g/tree at the 300IT, 566 g/tree at the 600IT and 563 g/tree at the 900IT, the seed and/or storage compound yield per hectare (kg/ha) can be estimated based on the planting density of the orchard (trees/ha).

Even though this study has provided considerable insight into the effect of different irrigation rates on fruit development throughout various stages, it has also revealed some aspects that may require further research to better understand their exact mode of action.

One such facet is the effect of drought stress on photosynthesis in *Moringa oleifera* trees. The decrease in photosynthetic rate with the reduction in irrigation, was found to be as a result of both stomatal (conductance and SI) and non-stomatal (possible RuBP regeneration, ATP synthesis and mesophyll conductance) limitations. The sensitivity of each of these limiting factors to drought stress and their subsequent effect on overall photosynthesis is still unknown, and a study area that future research could possibly explore in more detail.

Table 9.1 Guidelines for the estimation of *Moringa oleifera* seed protein, starch and oil content percentage (%) from fruit diameter (mm) measurements, at three annual rainfall/irrigation amounts (mm/annum).

PROTEIN %				
	Rainfall/irrigation (mm/annum)			
Fruit diameter (mm)	**900**	**600**	**300**	**Average**
5	16.0	14.7	13.5	14.7
10	32.1	29.4	27.0	29.5
12	29.4	26.3	26.1	27.3
14	28.8	25.8	23.2	25.9
16	27.8	23.0	21.8	24.2
18	27.0	23.0	20.8	23.6
20	26.4	23.7	21.8	24.0
22	25.6	24.2	21.4	23.7
24	24.8	23.4	21.5	23.2
26	24.8	25.2	23.1	24.2
28	24.8	25.3	23.1	24.2

STARCH %				
	Rainfall/irrigation (mm/annum)			
Fruit diameter (mm)	**900**	**600**	**300**	**Average**
5	13.1	14.7	15.9	14.3
10	26.1	29.4	31.7	28.7
12	29.5	34.9	33.2	31.2
14	29.3	34.9	37.7	32.1
16	29.6	37.2	38.2	31.4
18	21.5	27.6	37.0	24.0
20	13.7	27.1	32.6	20.0
22	10.1	22.1	23.9	15.3
24	5.4	8.0	14.5	8.5
26	5.4	6.6	10.3	7.8
28	5.4	5.8	16.1	7.9

OIL %				
	Rainfall/irrigation (mm/annum)			
Fruit diameter (mm)	**900**	**600**	**300**	**Average**
5	1.3	1.5	1.0	1.4
10	2.6	3.0	2.0	2.8
12	2.9	3.3	2.2	3.2
14	3.7	3.9	2.4	4.6
16	7.3	5.0	3.6	6.5
18	9.4	7.8	6.8	14.9
20	16.5	11.4	8.7	14.9
22	20.9	17.5	16.1	19.6
24	22.6	21.1	20.6	22.5
26	22.6	25.1	22.9	24.2
28	22.6	27.4	23.2	24.8

Further research that explores the effect of different irrigation/rainfall intensities on tree growth/biomass accumulation, water use and water use efficiency (WUE) would greatly enhance the accuracy to predict commercial feasibility within certain production areas.

Estimating storage compound content (g) based on the stain coverage area for comparative purposes by using digital imaging software, proved to be remarkably successful during this study. This appreciatively cost effective technique could potentially be used in various applications across numerous disciplines. Using colour selection, the percentage coverage (area) of any particular colour within a digital image can be determined by using this technique. Digital image analysis as a measurement tool could even be used to determine the coverage area (e.g. mm^2) of any material/substance given that it is similar in colour throughout and a reference to scale is available.

Not only has this study provided valuable insight into *Moringa oleifera* seed and storage compound development from flowering up until maturity, but also how all these growth phases are affected by different irrigation rates. In addition, this study enabled the identification of sensitive stages throughout fruit/seed development, by providing detailed insight into oil, starch and protein biosynthesis. Ultimately these findings provide current and prospective Moringa growers with recommendations to ensure suitable site selection as well as irrigation management guidelines. Furthermore, techniques such as the quantification of stained sections using Adobe® Photoshop®, developed for the purpose of this study, have potential applications beyond the scope of this study.

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FLOWERING, FRUIT GROWTH AND INTRACELLULAR STORAGE COMPONENT FORMATION IN DEVELOPING *MORINGA OLEIFERA* LAM. SEED AS INFLUENCED BY IRRIGATION

by

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SUMMARY

In recent times, *Moringa oleifera* has emerged as a new industrial crop due to its multitude of uses such as water purifier, nutritional supplement and biofuel feedstock. *Moringa oleifera* has also demonstrated high tolerance to sub-optimal growing conditions, particularly towards drought. As a result, the current and anticipated cultivation areas of this tree are in medium to low rainfall areas. This study therefore aimed to assess to what extent this drought tolerance is at the expense of flowering, fruit growth and storage compound biosynthesis.

Reduced irrigation initially affected flower bud initiation, where the reduction in irrigation increased the average number of flower buds. Fruit set however, declined with the decrease in irrigation amount, this was found to be as a result of floral abortion, reduced pollen viability as well as moisture stress in the style. Moderate water stress prior to floral initiation could possibly stimulate flower initiation, this should however be followed by sufficient irrigation/rainfall to ensure good pollination, fruit set and yield.

Anatomical studies of embryogenesis and the developing cotyledons, enabled greater insight into storage compound biosynthesis and accumulation. These observations revealed that the endosperm is nuclear, becoming cellular at a fruit diameter of ±6 mm from the micropylar side towards the emerging embryo covering the entire inner integument once the fruit had reached a diameter of ±8 mm. Cotyledon development and storage compounds biosynthesis commenced at a fruit diameter of ±12 mm and continued up until ±24 mm, as a result this stage of fruit development was found most susceptible to environmental stresses. At the end of this phase the cotyledons had filled the entire seed coat, while the unicellular epidermal layer of the inner integument remained distinctly visible between the perisperm and the seed coat.

Fruit growth measurements during the initial 60 days after flowering (DAF), revealed a significant increase in fruit growth rate, final fruit size, number of mature fruit, average seed count and time to maturity with the increase in irrigation amount.

Photosynthesis measurements during the vegetative, flowering and fruit development stages revealed not only a decrease in photosynthetic activity throughout the growing season (from flowering to fruiting), but also with the decrease in irrigation rate. Lower photosynthetic rates were primarily as a result of stomatal (conductance and SI) and non-stomatal (possible RuBP regeneration, ATP synthesis and mesophyll conductance) limitations, while decreases observed throughout the growing season were due to diminishing leaf chlorophyll concentrations.

Seed compositional studies found starch to be the primary compound synthesized during initial histo-differentiation phase (Ø0 mm-12 mm), while both oil and protein levels remained comparatively low. During the subsequent expansion phase (Ø12 mm-24 mm) however, stored starch reserves were remobilized and used in oil biosynthesis. The different irrigation treatments had less of an effect of final storage compound content percentage than on the time and rate of their synthesis throughout seed development. A decrease in irrigation amount, delayed the onset of oil biosynthesis, and as a result starch levels continued to increase, reaching much higher levels prior to their remobilization during oil biosynthesis. At maturity the average oil content percentage was 24.8%, while the protein content percentage was 24.7% and the starch content percentage was 8.8%.

Using light and electron microscopy in conjunction with histochemical staining techniques, the intracellular compound initiation and storage locality were determined. Starch was found to be synthesized by the plastids and transiently stored in the cell periphery. Protein and oil bodies instead were synthesized in association with the rough endoplasmic reticulum and finally stored in the cell centre.

Protein and oil body formation only commenced in significant amounts at fruit diameters of ≥14 mm.

The efficacy with which intracellular compounds could be identified using histochemical staining, prompted further investigation into the possibility of quantifying storage compound content using digital imaging software. The percentage stain coverage was calculated using Adobe® Photoshop® and then compared to the physical content percentage of the same compound at the corresponding developmental stage. The best correlation between measured seed storage compound and stain coverage was observed for the protein (Orange G) and oil (Sudan III) detecting stains. A stage specific factor based on seed mass was however necessary in order to estimate storage compound content (g) from image analysis results.

All things considered, this study found that an annual rainfall of ±600 mm/year should be sufficient to ensure satisfactory fruit growth and yields of *Moringa oleifera* even in marginal production areas. Similarly, this will enable prospective growers to identify suitable production sites based on the rainfall/irrigation requirements of *Moringa oleifera*. The identification of sensitive stages during seed development will enable Moringa growers to implement irrigation management strategies that optimise both storage compound synthesis and yield. Furthermore, as a result of this study, the storage compound (oil, protein and starch) content percentage (%) and fruit maturity can now be estimated non-destructively, by simply measuring the fruit diameter (mm).