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Full Length Article

Irrigation Amounts Affect the Compositional Changes of *Moringa oleifera* Seeds throughout Different Developmental Stages

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Abstract

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. 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 reached 24.8%, while the protein contents were 24.8% and the starch contents were 8.7%. The different irrigation treatments had less of an effect on the final starch, oil and protein content than on the time and rate of their synthesis throughout seed development. Higher irrigation levels principally favoured oil biosynthesis. The highest final oil contents were measured at the intermediate irrigation treatment (600 mm/annum), suggesting that both lower and higher irrigation levels could possibly reduce final oil contents. The reduction in irrigation amount delayed the onset of oil biosynthesis and as a result the starch content reached higher levels prior to its remobilization during oil biosynthesis.

Keywords: Biosynthesis; Oil; Protein; Starch

Introduction

The entire *Moringa oleifera* tree can be utilized and offers asource of nutrition, medicine, water purification, cosmetics and even biofuel, making it one of the most useful trees currently found throughout the tropics of the world (Jahn, 1988; Fuglie, 2001; Anwar *et al*., 2007; Rashid *et al*., 2008). In addition, this fast growing, multipurpose tree is also known to tolerate sub-optimal growing conditions (Muhl *et al*., 2011; Nouman *et al*., 2012), making it suitable for the cultivation in marginal/degraded growing environments.

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 contents are 30.8% ± 2 (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; 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 of 38.3% ± 1.03 (Mean ± SD), while Makkar and Becker (1997) reported a protein content 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 contents 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 absorption and neutralization of charges (Santos *et al*., 2012).

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Similarly to oil and protein content, the reported starch content 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. First lyhisto-differentiation, 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.

Materials and Methods

Trials were conducted on six-year-old *Moringa oleifera* trees at the field trial section on the 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 and each of the three groups were subjected to different irrigation treatments. Irrigation water was applied through a surface drip irrigation system at three levels. The three administered irrigation levels were 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. According to Palada and Chang (2003) the minimum annual rainfall requirement for *Moringa oleifera* is 250 mm/year. The irrigation amounts were administered, simulating total annual rainfall (mm/year). Three dipper lines were installed at 900IT, two dripper lines at 600IT, while 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 L/h/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. The plastic sheets were then covered with organic mulch 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 Hydro-probe), to verify differences in soil water levels between treatments.Trees were subjected to the three irrigation treatments for twelve months prior to the initial seed sampling. Subsequent seed sampling continued overthe two consecutive growing seasons (24 months).

Six months after flowering, all fruit were harvested and grouped according to treatment and 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. After harvesting the fruit, seeds were freeze dried for at least 72 h 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 Fig. 1. All quantitative analysis results are expressed as percentages (g/100 g) of the dry seed mass.

Assessment of Oil Content

Oil content determination was performed using a soxhlet extractor fitted with a 500mL 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 h 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 sample mass.

Fig. 1: Cross-sections of *Moringa oleifera* seed at different developmental (8 mm – 28 mm) stages based on the fruit diameter (mm)

Assessment of Starch Content

Starch contents 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 h. 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 h. Subsequently, samples were transferred into 100 mL volumetric flasks, filled with distilled water, mixed and filtered. One millilitre of filtrate was then transferred into a 50mL volumetric flask and filled with distilled water, after which 0.5mL of that sample solution and 2.5mLof twice filtered colour reagent was 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. Specs were read using an Analytikjena Spekol 1300 machine at 540 nm and measured against a set of standards.

Assessment of Protein Content

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

Data were statistically analysed using the Statistical Analysis System (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 ProcGLM (SAS).

Results

Starch was the first storage compound to be synthesised, however, the amount fluctuated throughout most of the seed development phase. From the compositional content results illustrated in Fig. 2, starch was transiently stored at relatively high levels, peaking at 32.1% once fruit reached a diameter of 14 mm. Thereafter starch content 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.

Unlike starch, the oil content increased sigmoidally until reaching an average final oil content of 24.8% at maturity (Fig. 2).

Protein accumulation on the other hand remained fairly constant throughout seed development, decreasing slightly from its peak of 29.1% at a fruit diameter of 10 mm to 24.8% at maturity (Fig. 2). As the individual seed increased in size/mass, so did the protein content (g). Thus despite a slight decrease in the protein portion (%) (Fig. 2), the protein content (g) continued to increase with the increase in seed size as illustrated in Fig. 3.

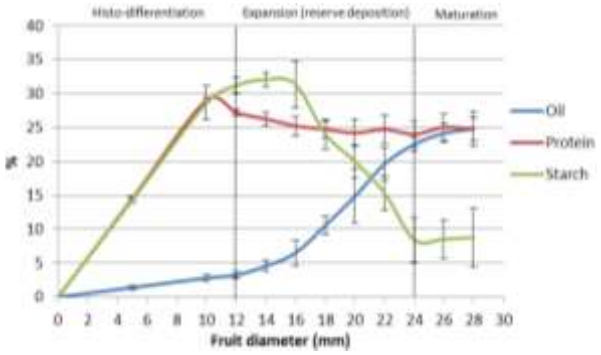

Fig. 2: Changes in the compositional content (%) of storage compounds in developing *Moringa oleifera* seed. Vertical bars (±) indicate standard errors

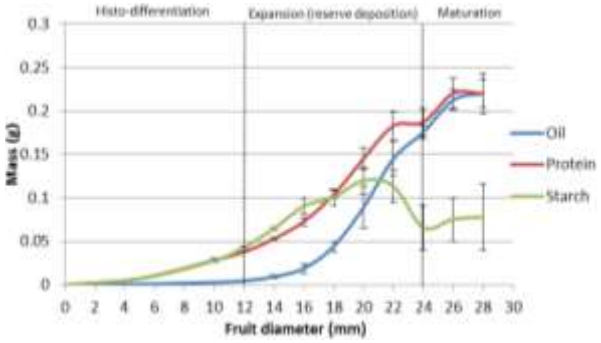

Fig. 3: Storage compound mass (g) in the average *Moringa oleifera* seed at different developmental stages. Vertical bars (±) indicate standard errors

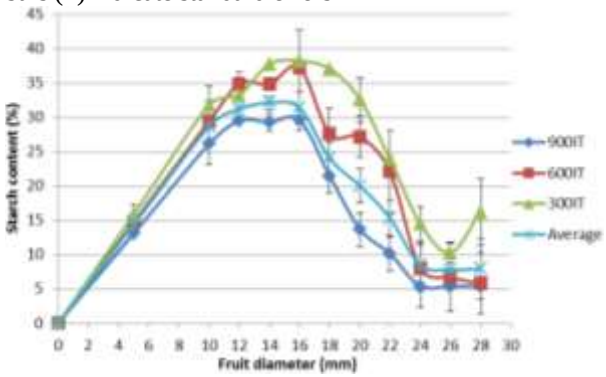

Fig. 4: Changes in starch content (%) of developing *Moringa oleifera* seed at various developmental stages as affected by three irrigation treatments (IT). 900IT – 900 mm/annum, 600IT – 600 mm/annum, 300IT – 300 mm/annum. Vertical bars (±) indicate standard errors

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 levels was then investigated.

Starch Biosynthesis

The measured starch contents (%) 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 reached, as well as the onset of starch remobilization to oil (Fig. 4).

Oil Biosynthesis

Results illustrated in Fig. 5 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 levels.

Protein Biosynthesis

Seed protein content (%) remained rather consistent, with only a slight decrease throughout seed development across all irrigation treatments (Fig. 6). Throughout the entire trial period, protein content (%) was lowest for the 300IT, slightly (not significantly) higher for the 600IT and highest for the 900IT.

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 levels on the storage compound biosynthesis can be discussed, the interaction of the various storage compounds throughout seed development needed to be studied.

Sucrose entering the developing seed was used for starch, protein and oil biosynthesis. Starch is 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). 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

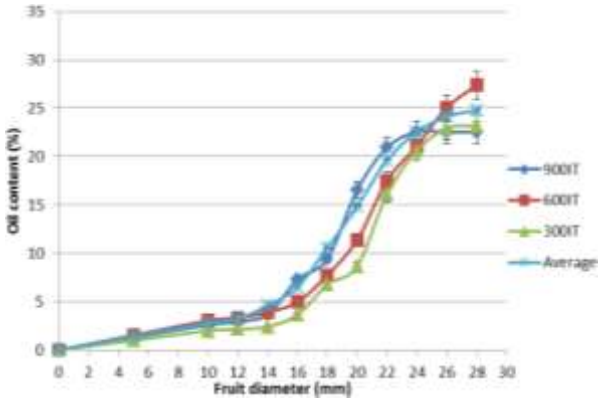

Fig. 5: Changes in oil content (%) of developing *Moringa oleifera* seed at various developmental stages as affected by three irrigation treatments (IT). 900IT – 900 mm/annum, 600IT – 600 mm/annum, 300IT – 300 mm/annum. Vertical bars (±) indicate standard errors

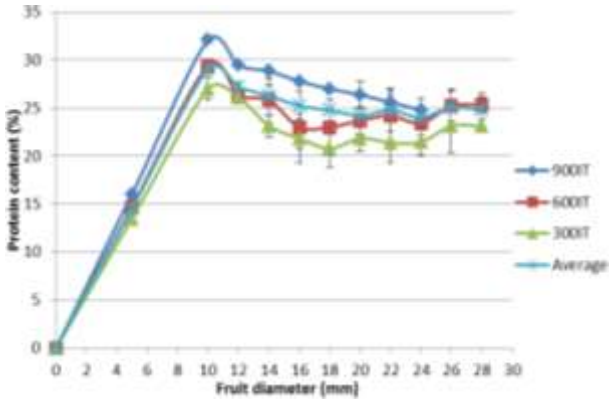

Fig. 6: Changes in protein content (%) of developing *Moringa oleifera* seed at various developmental stages as affected by three irrigation treatments (IT). 900IT – 900 mm/annum, 600IT – 600 mm/annum, 300IT – 300 mm/annum. Vertical bars (±) indicate standard errors

starch contents decreased after fruit reached a width of 14mm (Fig. 2), which corresponded with the onset of oil accumulation. As a result, the 72.7% reduction in starch content from when fruit reached 14 mm in diameter to maturity could possibly be attributed to the biosynthesis of oil (Angeles-Núñez and Tiessen, 2010; Luthra *et al.*, 1991).

The starch breakdown marked by a decrease in starch content coincided with the increase in oil content, 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, 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.

A decrease in irrigation amount consistently increased the maximum starch content level, 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 at the low 300IT decreased later compared to the 600IT and even later than the 900IT (Fig. 4). As a result, seed from drought stressed trees typically had a higher starch content 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 cannot be viewed in isolation as oil biosynthesis is at least partly dependent on the mobilization of starch reserves.

Since the decrease in irrigation amount delayed starch remobilization, the commencement of oil biosynthesis was also postponed (Fig. 5). 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 for most of the developmental stages amongst the treatments. Subsequently, oil biosynthesis initiation was delayed with the decrease in irrigation treatment.

When comparing oil content (%) at seed maturity, the 600IT yielded the highest average oil content at 27.4%, which was significantly higher compared to the 23.2% at the 300IT and 22.6% at the 900IT (Fig. 5). 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 level throughout the initial growth and development phase, it was the intermediate 600IT that had the highest oil content at maturity. This unexpected result, whereby the oil content 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 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.

In this study, protein accumulation was seemingly least affected by the reduction in irrigation amount (Fig. 6). 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.

In conclusion, increased irrigation resulted in a shorter oil biosynthesis initiation time and higher accumulation rate. However, despite significant oil content increases from the 300IT to the 600IT, final oil content decreased again between the 600IT and 900IT.An increase in irrigation would almost certainly expedite oil production with higher protein and transient starch reserves, while excessive irrigation could result in lower oil contents. Reduced irrigation (<600mm per annum for the current trial site) on the other hand will most likely result in lower seed oil content. 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.

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