Analysis of starch metabolism in South African pigeon pea (*Cajanus cajan*) varieties

by

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Declaration

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Abstract

Starch is a major storage polyglucan in plants that is composed of two fractions, amylose and amylopectin. The biosynthesis and degradation pathways of starch are well documented, with phosphoglucomutase (PGM) and ADP-glucose pyrophosphorylase (AGPase) catalysing the first two steps in its biosynthesis. This project examined starch in five pigeon pea (*Cajanus cajan*) varieties: uDhali, SEFA, Nondolo, Lari and India by measuring both total and resistant starches in the seeds and leaves, activities of PGM and AGPase as well as expression of the genes encoding these enzymes. The findings demonstrated that the seeds from these South African pigeon pea varieties are rich in starch, containing an average of 47% starch on a dry weight basis; however, one variety (SEFA) contained only 0.3% starch. The starch in the high-starch varieties contained a minimum of 50% resistant starch, with the India variety reaching 70%. Assessment of soluble sugars in seeds revealed sucrose to be the only sugar present in abundance in all varieties while amounts of galacto-oligosaccharides were low in all seeds. Starch in leaves was observed to be 10 fold less than that found in seeds and the amount of resistant starch in leaves was less than 2 mg/g fresh weight (7.6% of the total). The *AGPase* gDNA nucleotide sequence from one variety was identical to an already sequenced pigeon pea variety, whereas amplification *PGM* gDNA was unsuccessful. Amplification of coding sequences (CDSs) for both *AGPase* and *PGM* were also identified to be the same as the already sequenced *AGPase* and *PGM* genes from the pigeon pea genome resource database. Gene expression for both genes varied throughout a 24 h period and was at its peak during the day (light period). Activities of both AGPase and PGM were determined in seeds from all varieties whereas the AGPase enzyme activity was the same in leaves throughout the day while PGM activity varied between the day (light) and night (dark).

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List of Abbreviations

AGPase	Glucose-1-phosphate adenylyltransferase
AMG	Amyloglucosidase
BRICS	Brazil, Russia, India, China and South Africa
CAF	Central Analytical Facilities
cDNA	Complementary DNA
CDS	Coding DNA sequence
cm	Centimeter
DI	Deionized
DNA	Deoxyribonucleic acid
Dr	Doctor
fwd	Forward
G-6-P	Glucose-6-phosphate
g	Gram
GI	Glycemic index
GOPOD	Glucose oxidase/peroxidase
h	Hour(s)
HK/G-6-PDH	Hexokinase/glucose-6-phosphate dehydrogenase
HSP90	Heat shock protein 90
IPB	Institute for Plant Biotechnology
KOH	Potassium hydroxide
L	Litre
LB medium	Luria-Bertani medium
LC-MS	Liquid chromatography-mass spectrometry
M	Moles
mg/L	Milligram per litre
min	Minute(s)
ml	Millilitre

mM	Millimolar
n.d.	No data
NADP+	Nicotinamide adenine dinucleotide phosphate
NCBI	National Center of Biotechnology Information
NCD	Non-communicable disease
NRF	National Research Foundation
PCR	Polymerase chain reaction
PGM	Phosphoglucomutase
ppm	Parts per million
rev	Reverse
RNA	Ribonucleic acid
SA	South Africa
sec	Seconds
T7	T7 promoter region
TUB6	Tubulin beta chain
UDP-glucose	Glucose-1-phosphate adenylyltransferase
UPLC	Ultra Performance Liquid Chromatography
U/ml	Enzyme units per millilitre
WHO	World Health Organization
WSC	Water soluble carbohydrate(s)
w/v	Weight per volume
w/w	Weight per weight
℃	Degrees Celsius
µg	Microgram
µl	Microlitre(s)
µmol	Micromoles
18s rRNA	18s Ribosomal RNA
%	Percent

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Chapter 1: Background

The current rise in the global population is putting increased pressure on the agricultural sector to expand food grain production to meet nutritional and food security demands (Pawlak and Kołodziejczak, 2020; Davis *et al*., 2016). Cereals such as wheat (*Triticum aestivum*), maize (*Zea mays*) and rice (*Oryza sativa*) are known as grass crops and are cultivated for their edible grains. They are considered the main staple foods worldwide and because of this much previous research has concentrated on them. There is a current shift in research from examining those crops, to studying others. These are ones that can be beneficial for human and animal health, which can sequester nitrogen and carbon in soil and which display climate-resilience in the context of global warming and climate variability (Stagnari *et al*., 2017). Many of these proposed alternate crop models are legumes and in developing countries subsistence farmers are the major producers of legumes. They are grown in such countries mainly for their dry seeds (pulses) for human and animal consumption, as well as to help in soil conservation as part of crop rotation strategies (IITA, 2009).

Legumes are ancient crops belonging to the Fabaceae family, forming one of the world's largest and most important food types after grains (Popoola *et al*. 2019; Maphosa and Jideani, 2017). Domestic cultivation of legumes can be dated back to 7000 B.C. during the Neolithic age in Turkey (Gajzago, 2004). Large-scale cultivated legumes include alfalfa (*Medicago sativa*), mung bean (*Vigna radiata*), lentil (*Lens culinaris*), clover (*Trifolium spp*.), soybean (*Glycine max*), cowpea (*Vigna unguiculata*), peanut (*Arachis hypogaea*), pea (*Pisum sativum*) and kidney bean (*Phaseolus vulgaris*); but there are other legumes grown at small-scale that are classified as orphan crops of which little research has been performed and which lack established markets. Examples of these include marama bean (*Tylosema esculentum*) and pigeon pea (*Cajanus cajan*) (Ahmed and Hasan, 2014). There is a growing paradigm shift toward the use of alternate crops to bolster future food production. This was highlighted recently by the food and agricultural organization (FAO) of the United Nations (UN), which declared 2016 to be the "year of the pulse" to raise awareness about the nutritional value of pulses (FAO, 2017).

Production of legumes has been increasing throughout Africa due to the need for affordable nutritious plants that are easy to cultivate, which have short production cycles and allow a consistent supply of nutrient rich food (Popoola *et al*., 2019). Legumes form symbiotic relationships with *Rhizobium* species allowing them to fix atmospheric nitrogen into the soil,

making them particularly suitable for inclusion in low-input cropping systems (Stagnari *et al*., 2017). The value of pulses goes beyond their status as nutritional and nitrogen fixing-crops as they can also provide aerial protection for companion plants and forage for animals (Frohberg *et al*., 2003).

Pulses are highly nutritious and are considered to be a major source of proteins, minerals and carbohydrates. In addition, they contain all essential microelements, B vitamins, bioactive phytochemicals and antioxidants, as well as being low in fat and are generally cholesterol-free (Figure 1; Polak *et al*., 2015). Because of this in some countries, and especially in developing countries, legumes are regarded as a meat substitute, often being referred to as the "poor man's meat" (Maphosa and Jideani, 2017; Devindra *et al.*, 2006).

As they mature legume seeds accumulate both proteins and carbohydrates. The main proteins present are albumin and globulin while carbohydrates include both structural and storage polymers (Gajzago, 2004). One storage carbohydrate - starch - is present in legume seeds at varying concentrations dependent on species (Maphosa and Jideani, 2017; Ahmed and Hasan, 2014; Gajzago, 2004). Other major storage carbohydrates are the raffinose family oligosaccharides (RFOs), which represent water-soluble galactosyl extensions of sucrose (Obendorf and Gorecki, 2012; Martin-Cabrejas *et al.*, 2008; Sprenger and Keller, 2000).

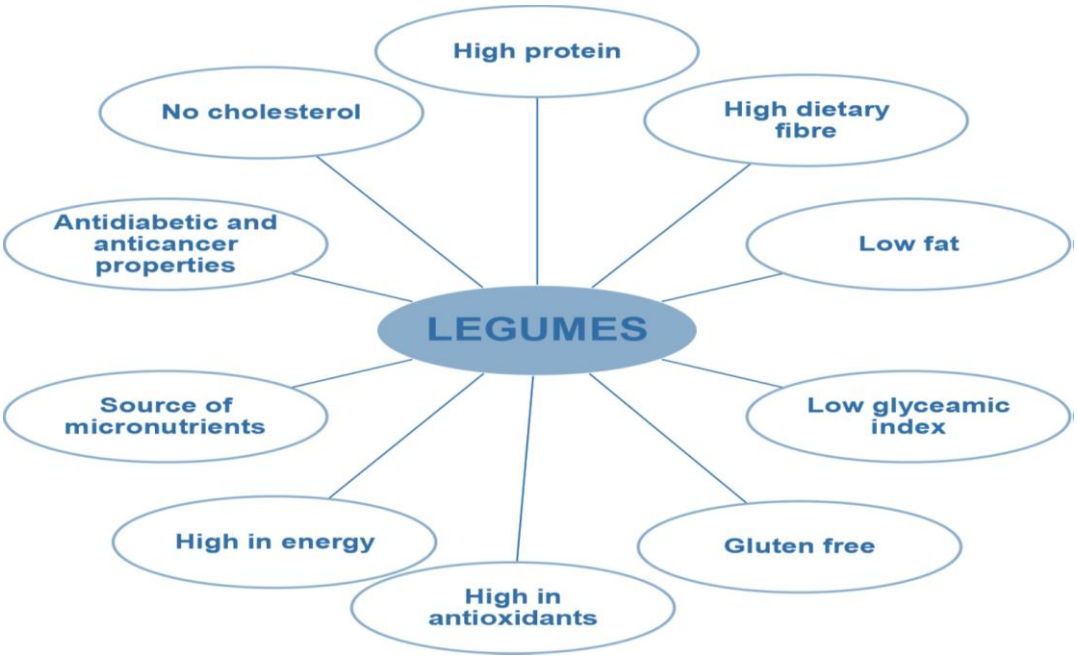

Figure 1: Schematic representation of the benefits of legumes to humans (Maphosa and Jideani, 2017).

While staple crops are considered to be of global importance, orphan crops are defined as minor crops that have only regional importance and which lack an established global market, meaning that they receive little research attention in terms of yield improvement (Cullis and Kunert, 2016). It is thought that increasing knowledge of orphan crops will lead to them becoming integrated in global food production. This is of particular importance in developing countries where pulse crops are mostly cultivated by subsistence farmers. Although orphan crops are often staples for some resource deprived farmers, barriers around crop developments still pose a huge challenge. For such minor legumes these include low seed set and a lack of efficient harvesting techniques (Cullis and Kunert, 2016), often due to a lack of genetic resources and knowledge of efficient farming practices.

One orphan crop, pigeon pea, is mostly grown in tropical and subtropical regions and has become of interest in developing countries because it is both drought-tolerant and contains highly nutritional components (Popoola *et al*., 2019). India is currently both the largest producer and consumer of pigeon pea which is ranked second only to chickpea (*Cicer arietinum)* in terms of legume production (Sarkar *et al*., 2018: FAO, 2017). It is mainly cultivated for its edible seeds, as well as for animal feed and forage. Due to its ability to produce appreciable amounts of grain during drought, and its high content of available nutrients, pigeon pea has the potential to become a marketable climate-resilient crop in agriculture of the future. Indeed, it holds the distinction of being the first orphan crop to have a fully and publicly available genome resource – an initiative led by the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT; Varshney *et al*., 2011).

The presence of slowly digestible nutritious compounds in legumes has increased their demand by health-conscious individuals, diabetics and people managing their weight (Maphosa and Jideani, 2017). Literature suggests that insoluble fibre in legumes could attenuate the rate of digestion and absorption of carbohydrates reducing post-prandial glycemia, and have the potential of lowering production of intestinal short-chain fatty acids through fermentation of resistant starch (Bahadoran and Mirmiran, 2015). While cereal crops are rich in digestible starch, legume seeds contain significant amounts of resistant starch that is digested slowly in the gut, leading to their low glycemic index (Table 1). They also contain appreciable amounts of readily available sugars that support plant metabolism (Rosa *et al*., 2009). Such sugars include sucrose, raffinose and stachyose which are involved in seed desiccation tolerance and longevity but have also recently become regarded as effective prebiotics which, stimulate the

growth of health beneficial microbes in the digestive tract (Elango *et al*., 2022; Davani-Davari *et al*., 2019).

Table 1: Different glycemic indices of legumes and the main cereals (adapted and modified from Atkinson *et al*., 2008 and Rizkalla *et al*., 2003).

Glycemic Index (GI)	**Glycemic index rating**	**Source of food**
High	70 and above	Oatmeal, rye, maize (corn).
Medium	56 to 69	Brown rice, grain sorghum, sweet corn, couscous.
Low	55 and less	Soybeans, beans, pigeon pea, peanuts, lentils, split peas, chickpeas.

During the accumulation of soluble sugars, specific carbohydrate-active genes and enzymes become upregulated, which help to convert them into long term storable nutrients such as starch or raffinose family oligosaccharides. These enzymes include plastidial phosphoglucomutase (PGM, EC 5.4.2.2), ADP-glucose pyrophosphorylase (AGPase, EC 2.7.7.27) and galactinol (EC 2.4.1.123), raffinose (EC 2.4.1.82) and stachyose synthases (EC 2.4.1.67) (Lee *et al*., 2016; Oiestad *et al*., 2016; Hendriks *et al*., 2003). Both PGM and AGPase are enzymes that are essential for carbon-flux into the starch biosynthetic pathway and are found in the plastidial stroma (Baris *et al*., 2009). The plastidial isoform of PGM forms glucose-1-phosphate, a substrate for AGPase, which synthesises ADP-glucose and pyrophosphate. Both enzymes are essential for starch biosynthesis as mutants lacking them often contain almost no starch (Malinova *et al*., 2014). Plant AGPase's are heterotetrametric, being composed of two small subunits which play a catalytic role and two large subunits which help regulate activity (Cejudo *et al*., 2019; Orzechowski, 2008). Tissue specific isoforms are present with different kinetic properties reflecting the differing roles of these tissues (Lee *et al*., 2016; Akihiro *et al*., 2005; Tetlow *et al*., 2004).

Overexpression of AGPase and PGM encoding genes increases yield output and decreases ovule abortion in grain crops during fertilization (MacNeill *et al*., 2017; Saripalli and Gupta, 2015; Stitt and Zeeman, 2012). However, mutation of these genes results in adverse production with reduced starch, increased ovule abortion and poor yields (Lee *et al*., 2016; Oiestad *et al*.,

2016; Egli *et al*., 2010). Therefore, optimal expression of PGM and AGPase can help increase crop yield leading to reductions food shortages and food prices.

1.1 The role of legumes in food security

Food security is the ability of an individual to access enough satisfactory food that is nutritionally safe for human consumption and which is obtained in a socially acceptable way (Parnell and Smith, 2008). Measurements for food security differ from household to national and supra-national scales; for example, South Africa is food secure at a national level but food insecure at a household level (Stats SA, 2019). Within that context physical quantities of food produced as well as economic and social resources directly influence the severity of food security.

Four pillars help define food security: availability, access, utilisation, and stability. Supplying sufficient food is an ongoing problem first predicted in 1798 by Thomas Robert Malthus (Rosegrant and Cline, 2004; Napoli *et al*., 2011). By 1970 public awareness of food security began to rise, influencing households as well as politicians at national and global levels to help reduce food insecurity (Napoli *et al*., 2011). Global food security is heavily dependent on major cereal crops such as maize, wheat and rice which provide approximately 60% of the energy consumed by the world population (Cullis *et al*., 2019).

In low-income countries, more than one third of children display stunted growth because of long-term nutritional deprivation (WHO, 2019). Low availability of nutritious food containing sufficient vitamins and minerals contributes to this. Increased amounts of food crops in low-income countries are needed to deliver the basic nutrients necessary to combat food insecurity and malnutrition (Sharma *et al*., 2011). To accomplish this South Africa adopted the Integrated Food Security Strategy in 2002 which aimed to eradicate hunger and nutritional deficits among low-income households by the year 2030 (D'Haese *et al*., 2011). In 2017 South Africa was not on track to meeting this deadline as 10.5% of the population were vulnerable to hunger, despite 78.7% households having adequate access to food (Stats SA, 2019).

Income plays a crucial role in food acquisition. Low income is often associated with poor food nutritional quality and security, resulting in an increase of hunger related cases (FRAC, 2017) and global production of legumes has the potential to provide a sustainable solution to overcome this. Legume production has been rapidly increasing in Africa due to the need for

nutritious crops due to their affordability, ease of cultivation and short production cycles which enables consistent cultivation and supply.

Legumes are used in balanced diets to supply protein, calories and fibre. For people consuming plant-based diets, legumes serve as a meat substitute (Considine, 2017; Varshney *et al*., 2011) due to their high protein content. Increased vegetarianism and veganism are increasing legume consumption, putting pressure on agriculture to meet the increased demand, while trying to maintain sustainable agricultural practices.

Due to their symbiotic relationship with rhizobia, legumes can fix atmospheric nitrogen without being genetically modified, making them suitable for inclusion in low-input cropping systems (Stagnari *et al*., 2017). This helps replenish nitrogen in the soil in inter-and rotational cropping-systems where legumes grown in one season then supply the crops in the next growing season with fixed soil nitrogen. They can also act as a cover crop during the early stages of growth for the main crop. The value of pulses, therefore, goes beyond their consumption due to their importance in cropping systems and soil nitrogen fixing.

1.2 Drought as a physical and mechanical stressor in plants

Drought is one of the most detrimental consequences of climate change. This abiotic stress is predicted to increase as climate change leads to rises in average temperatures and this will limit growth performance in plants, adversely affecting food security and increasing poverty (Pape and Wollburg, 2019; Hallegatte *et al*., 2014). The consequences of this are not limited to plants but also affect socio-economic development, natural environments and agriculture (Figure 2; Schreiner *et al*., 2018; Silva *et al*., 2013, Ding *et al*., 2010) as drought leads to an increase in water scarcity for agriculture, which acts as a catalyst of famine (Farooq *et al*., 2009).

Agriculture is responsible for the largest consumption of water worldwide (Figure 3) and amounts used in agricultural production rise with increasing temperatures (Malhi *et al*., 2021; Brauman *et al*., 2016). With low water availability most agricultural practices suffer through drought which increases vegetation mortality leading to negative effects on the agriculture sector as well as industries and sectors that rely on it (Silva *et al*., 2013; Ding *et al*., 2010). The stage, severity and duration of water stress often determines the harvestable yield (Farooq *et al*., 2009), but some plants can tolerate water deficit better than others.

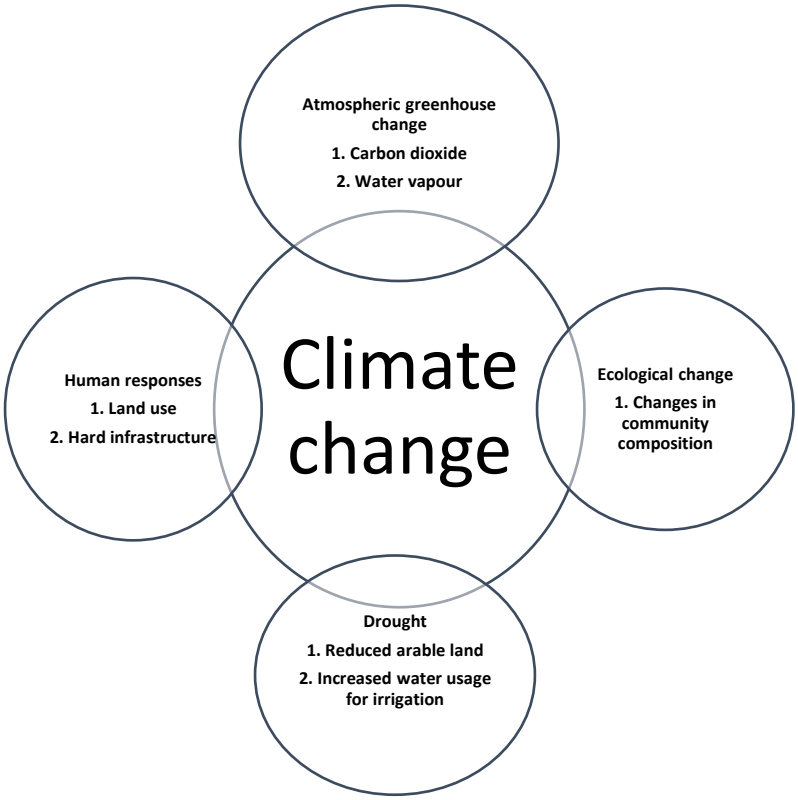

Figure 2: Schematic diagram of the effects of climate change on humans and environment. Modified from studies by Hallegatte *et al*., (2014) and Brinkman and Hendrix (2011).

Plants respond to drought using a variety of morphological, physiological, biochemical and molecular mechanisms (Abobatta, 2019; Time *et al*., 2018; Weidje *et al*., 2017). These are achieved through root and leaf modifications with activation of stress proteins that signal the plant to close leaf stomata and reduce transpiration. Some plants also modify their root system and produce needle leaves to minimise transpiration (Zhang *et al*., 2018; Maseda and Fernandez, 2006). Even with these natural coping mechanisms, prolonged drought can have a catastrophic impact on crop production and so production of novel crop varieties better able to cope with drought stress is needed (Wang *et al*., 2020; Pape and Wollburg, 2019). Conventional plant breeding can accomplish this by utilising molecular variation to bring about permanent changes to plants which help increase yield (Ahmar *et al*., 2020). Such variation can be introduced either as naturally induced mutations that enable plants to become ecologically fitter, through mutagenesis programs utilising either chemical or radioactive mutagens or, more recently, through genome editing technologies such as those using CRISPR/Cas9.

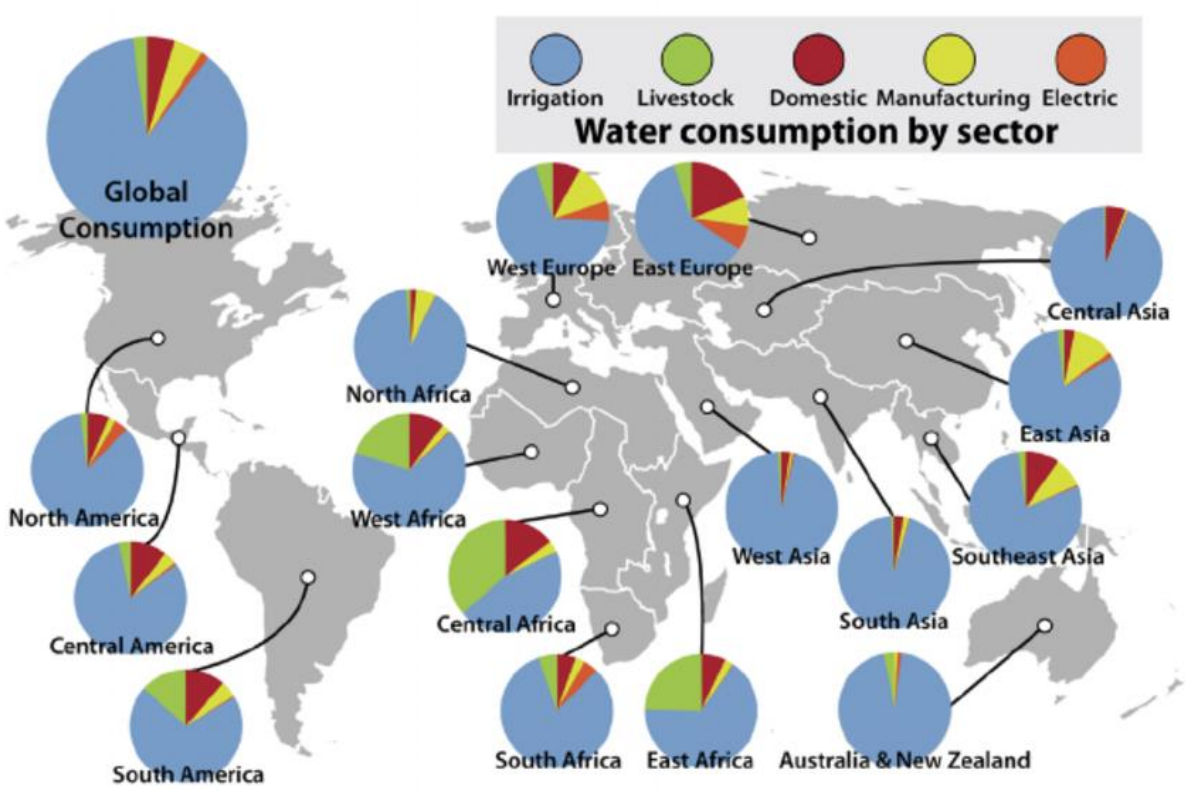

Figure 3: Global water consumption by different sectors: irrigation, livestock, domestic, manufacturing and electric. The agriculture sectors (irrigation and livestock) are responsible for consumption of more water than the other sectors, irrigation alone is responsible for the highest water consumption (Brauman *et al*., 2016).

1.3 Mutation in plants

Mutations are sudden, heritable genetic changes in a living cell which can occur spontaneously or be induced, and they have the potential to permanently change the functioning of the affected individual (Pathirana, 2011; Sigurbjornsson, 1971). Spontaneous mutations are random in nature and through this process crops can potentially adapt better to different environments, increasing their chances of survival. Although mutations can be lethal, they can also improve traits and this has led to scientists inducing mutations to create genetic variation for selection of beneficial traits (Raina *et al*., 2018; Oliadosu *et al*., 2015; Parry *et al*., 2009).

Mutation breeding in plants is a process of exposing plant material to mutagens to introduce new genetic variation. Plants are then selected based on their traits to produce improved crops that help to reduce food shortages (Raina *et al*., 2016). Rice, barley, wheat, and maize have been the main crops subjected to mutation breeding due to their role as the major calorie source

of much of the world's population (Pathirana, 2011; Parry *et al*., 2009). Induced mutations in maize and wheat have been used to improve output yields and have led to improved resistance to environmental stress and pathogens (Table 2). This has been evident since the green revolution in the 1960s where yields of staple crops were increased using induced mutation, and later the introductory use of breeding and molecular techniques to increase resistance to herbicides and insect pests (Bailey-Serres *et al*., 2019).

Table 2: Important quantitative traits in maize used to improve crop traits through breeding and mutation (Liu *et al*., 2019).

Gene	**Phenotype**	**Functional annotation**
ZmRap2.7	Flowering time	AP2-like transcription factor
ZmCCT10	Flowering time and disease resistance	CCT transcription factor
ZmCCT9	Flowering time	CCT transcription factor
ZmGA3ox2	Plant height	GA3 β-hydroxylase
qph1	Plant height	ABC transporter
UB3	Kernel and row number	SBP-box transcription factor
ids1/Ts6	Kernel and row number	AP2 transcription factor
ZmBAM1d	Kernel size and weight	CLV1/BAM-related receptor kinase-like protein
DGAT1-2	Oil content and composition	Diacylglycerol acyltransferase
ZmAuxRP1	Disease resistance	Domain of unknown function 966

More recently a shift towards breeding legumes has been observed. Because of its agricultural importance, soybean has been the main crop of interest, having more mutation related studies than other legumes (Oliadosu *et al*., 2015). The use of mutations in most legumes has been to improve crop yields and such breeding efforts resulted in substantial improvements during the green revolution (Parry et al., 2009). Breeders often induce mutation to generate new traits such as disease resistance, early maturity, strengthened stems, tolerance to abiotic stresses and increased yields (Oliadosu et al., 2015).

The use of mutations in plant breeding has been an on-going process to increase production while meeting food demands from growing populations and minimising environmental disruption. Other benefits that have been developed through their use in plants include

improving taste in fruits, colour in flowers and increased phytochemicals linked to health (Mostafa, 2015; Predieri, 2001).

1.4 Health benefits of legumes in the regulation of insulin

High intake of vegetables has been correlated with increased health benefits (Varadaraju, 2019), such as decreasing the risk of stroke, type-2 diabetes and hypertension (Maphosa and Jideani, 2017; Uchegbu and Ishiwu, 2015; Bouchenak and Lamri-Senhadgi, 2013). Studies indicate that various legume nutritional properties can help promote a healthy inexpensive lifestyle (Maphosa and Jideani, 2017).

Non-communicable diseases (NCDs), which include obstructive pulmonary disease, diabetes, dementia and stroke, were the leading chronic diseases that reduced life expectancy worldwide in 2018 (Coetzee *et al*., 2020; Ritchie and Roser, 2020; WHO, 2019). The prevalence of some of these diseases are rising; for example, diabetes - considered a silent killer - has increased by 3.8% worldwide in the past two decades (Coetzee *et al*., 2020; Mutyambizi *et al*., 2019). In South Africa, NCDs were the major cause of death in the past four years (Coetzee *et al*., 2020; Mutyambizi *et al*., 2019) with diabetes mellitus (also known as type-2 diabetes) being a major driving force behind this.

Type-2 diabetes is a multi-clustered metabolic disorder accompanied by abnormal glucose homeostasis, developing insulin resistance, impaired lipid and lipoprotein metabolism as well as increased oxidative stress at a cellular level (Krauss, 2004). Its development has many causes, including the rapid transport of sugar into the bloodstream after eating. Legume rich-diets have been reported to protect against the development of this NCD (Steinle and Chandrasekran, 2011) as they contain high amounts of resistant starch. High intake of vegetables has been correlated with increased health benefits (Varadaraju, 2019), such as decreasing the risk of stroke, type-2 diabetes and hypertension (Maphosa and Jideani, 2017; Uchegbu and Ishiwu, 2015; Bouchenak and Lamri-Senhadgi, 2013). Studies indicate that various legume nutritional properties can help promote a healthy inexpensive lifestyle (Maphosa and Jideani, 2017).

There are now well-known mechanisms that help slow the release of insulin, some at the molecular level. For example, the α-amylase inhibitory peptides 7S globulin α chain and conglutin γ are some of the bioactive compounds in legumes that reduce digestion and absorption of dietary carbohydrates, modulate post-prandial glycemia response, regulate lipid

metabolism and normalise lipid and lipoprotein levels (Steinle and Chandrasekran, 2011; Bahadoran and Mirmiran, 2015).

1.5 Water soluble sugars, their roles in ameliorating abiotic stress and as important storage carbohydrates

A small number of monosaccharides and disaccharides are the main sugar solutes readily available in plants. These are easily absorbed by humans but also act as osmo-protectants in plants for the stabilisation of biomolecules and membranes (Yasseen *et al*., 2018; Turhan and Ergin, 2012; Coue´e *et al*., 2006). During plant development, soluble sugars play a major role in developing cellular structure, acting as nutrients for the synthesis of structural polymers and as metabolic signalling molecules, regulating processes associated with plant growth (Coue´e *et al*., 2006).

During seed development an increase in water soluble sugar accumulation has been observed (Obendorf *et al*., 2012). Sucrose and raffinose family oligosaccharides (RFOs) are some of the sugars observed to accumulate at this stage and have been correlated with the acquisition of desiccation tolerance and the ability to germinate upon rehydration (Obendorf *et al*., 2012; Samarah *et al*., 2009; Steadman *et al*., 1995). Decreases in concentrations of soluble sugars in seeds have been reported to lead to low germination percentages. If raffinose and stachyose are the only sugars lowered, however, seeds exhibit normal field germination if galactosyl cyclitols (which act at cryo- and desiccation-protectants) are normal or elevated (Obendorf *et al*., 2012; Samarah *et al*., 2009). Because accumulation of specific soluble sugars can be sensitive to physiological and biochemical changes, plant metabolism often entails plasticity to allow different sugars to mitigate abiotic stress caused by environmental changes.

Plants respond to abiotic stresses using different mechanisms to minimise the loss of nutrients. Under drought and cold stresses one of these mechanisms is to accumulate sugars, especially sucrose (Turhan and Ergin, 2012). Soluble sugars also assume a dual role with respect to reactive oxygen species (ROS). The sugars are involved in ROS-producing metabolic pathways in high oxidising organelles such as chloroplasts where sugars can also fuel NADPH-producing metabolic pathways such as the oxidative pentose-phosphate pathway. Different stress situations causing ROS accumulation are associated with soluble sugar accumulation, and this is considered to be an adaptive response to help ameliorate accumulation of increased ROS (Keunen *et al*., 2013; Coue´e *et al*., 2006). Raffinose family oligosaccharides are known to

stabilise membranes during abiotic stress (Hincha *et al.*, 2003). They occur abundantly in seeds, and they have historically been considered as anti-nutritional due to their α-galactosidic linkages being non digestible by humans leading to flatulence. Studies have now shown RFO's to have prebiotic properties and can promote growth of beneficial gut bacteria (Davari *et al.*, 2019; Johnson et al., *2014*; Peterbauer and Richter, 2001).

1.6 Starch as a major storage carbohydrate in plants

Sugars are not only used to help plants cope with abiotic stress but can also be converted to polymers such as the storage polyglucan starch. This is a significant nutrient within crop plants for several reasons. Firstly, it is important for plant growth as its presence in leaves acts as a carbon buffer at night. Because of this, plants unable to synthesise starch or which degrade it slowly, grow at reduced rates (Paparelli *et al.*, 2013; Stitt and Zeeman, 2012). Secondly, it is a major substance isolated from plants that is used by several different industries. Understanding the types of starch present in a plant can help in identifying its potential industrial uses (Zeeman *et al.*, 2010). Finally, starch is often a major source of calories within plant material and is, therefore, important for both human and animal nutrition. Its digestibility can also be important for human health. Starches that are digested slowly are advantageous compared to those that are quickly digested as they lead to a smaller spike in post-prandial blood glucose, which is associated with a decreased risk of development of type-2 diabetes (see section 1.4; Miao *et al.*, 2015).

Starch is a polyglucan that is stored in plastids in the form of insoluble granules. It is composed of two fractions, amylopectin and amylose which are structurally distinct. The amylopectin molecule normally makes up about 70% of the granule and contains many short α-1,4 linked glucose chains, which are linked together by α-1,6 branch points. These links form clusters of chains that bind together to form double helices (Figure 4; Raguin and Ebenhöh, 2017). This ordered structure makes amylopectin semi-crystalline, a property it confers to the starch granule (Zeeman *et al.*, 2010).

Amylose makes up the remainder of the starch molecule and also contains α-1,4 linked glucose chains, but these are normally several hundred glucose units in length and contain few (if any) α-1,6 branch points. It is amorphous, does not form an ordered structure and is, therefore, non-crystalline (Zeeman *et al.*, 2010).

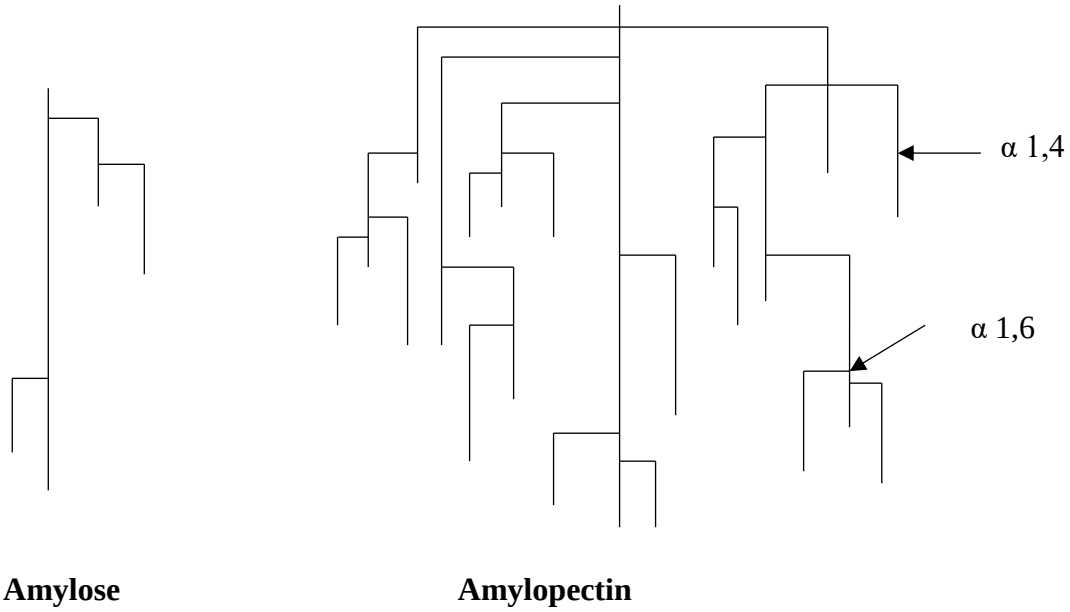

Figure 4: Structure and components of starch granules: amylopectin contains many α-1,4 linked chains linked by α-1,6 branching chains; amylose is composed of longer α-1,4 chains with few α1,6 links.

1.7 Starch synthesis and the roles of phosphoglucomutase and ADP-glucose pyrophosphorylase

The pathway of starch metabolism has been examined in detail over the past century, and the major catalytic enzymes involved in its biosynthesis and degradation have been identified. Two enzymes, PGM and AGPase, are key regulators of starch synthesis. In storage organs such as seeds and tubers, sucrose is imported from leaves into the cytosol where it is converted to glucose-6-phosphate (G6P) by the actions of several enzymes. The final step in this pathway is catalysed by an isoform of PGM that is targeted to the cytosol (Figure 5; Malinova *et al*., 2014; Egli *et al*., 2010). A specific protein transports G6P into the plastid, the site of starch synthesis and degradation, where it is used to synthesise glucose-1-phosphate (G1P) by a second, plastidial, PGM isoform (Yu *et al*., 2000; Harrison *et al*., 1998). ADP-glucose pyrophosphorylase then catalyses the formation of ADP-glucose and inorganic pyrophosphate (PPi) from G1P and ATP (Lee *et al*., 2016). ADP-glucose is the substrate used to synthesise

the starch polymer by two enzymes; starch synthase and starch branching enzyme, both of which exist as multiple isoforms (Zeeman *et al*., 2010).

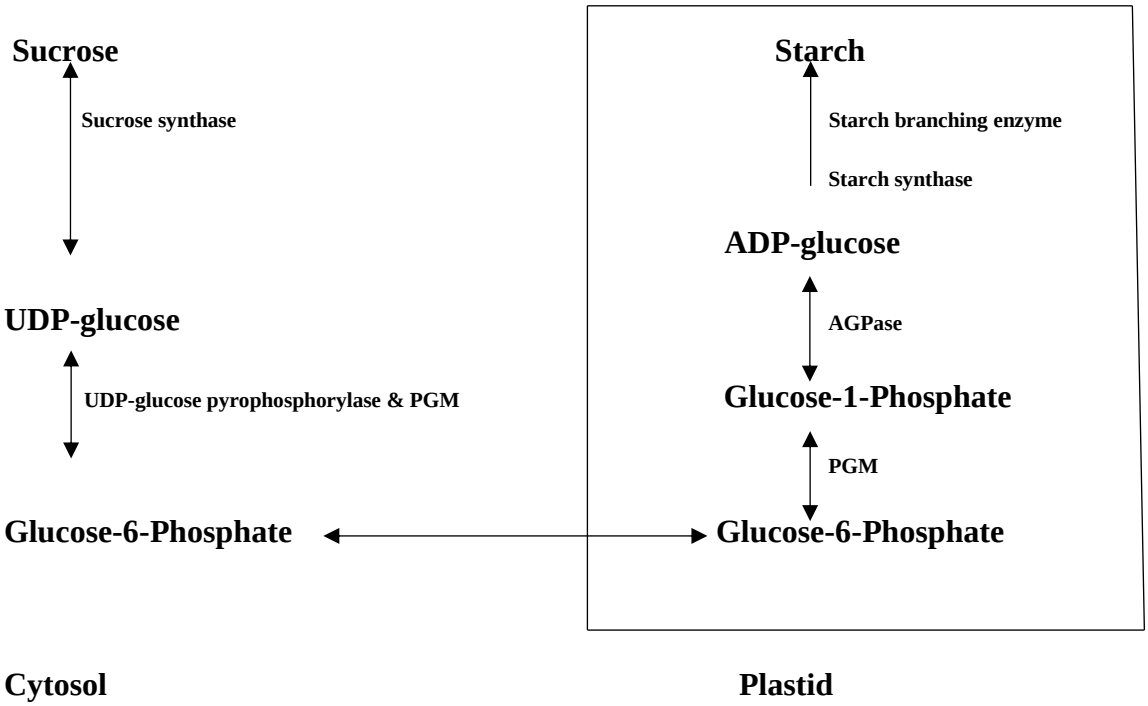

Figure 5: A putative pathway for sucrose breakdown and starch synthesis in legume seeds, Modified from Kleczkowski (1996).

One key regulatory step that controls the flux of carbon into starch is catalysed by AGPase and mutations in this enzyme can result in plants containing almost no starch (Rosti et al., 2020). The AGPase pathway for cereal endosperm and legume seeds differs, with cereals having both cytosolic and plastidial isoforms alongside a plastidial ADP-glucose transporter. Overexpression of cytosolic AGPase can enhance starch synthesis and seed weight in cereals, however, this is not the case for legume seeds (Nagai *et al*., 2009; Patron and Keeling, 2009). Angiosperm ADP-glucose pyrophosphorylase enzymes are heterotetrameric, composed of two small and two large subunits and plants lacking either type of AGPase subunits accumulate reduced starch (Szydlowski *et al*., 2009; Hofvander *et al*., 2004; Schwall *et al*., 2000).

Though research on starch metabolism has been extensively conducted for decades, more research on its role in underutilised crops needs to be performed. Understanding the molecular

basis behind starch synthesis in these plants will allow the alteration of pathways to influence their use in industries and may also provide alternative uses.

1.8 Pigeon pea as a potential alternate climate-resilient crop model

Cajanus cajan (L.) Millsp. (pigeon pea) is an ancient crop belonging to the Fabaceae family and is characterised by having bright coloured flowers at maturity (Figure 6 and Table 3). It is the 6th largest legume crop worldwide with India being both the largest consumer and producer (Ayenan *et al*., 2017; FAO 2017; Varshney *et al*., 2011). The origin of pigeon pea is thought to be between India and Northeast Africa (Hluyako *et al*., 2017; Eco-crop, 2016; Feedpedia, 2016; van der Maesen, 1989). In South Africa, the crop is believed to have been introduced by Indian indentured labour in Natal during the 19th century where it is still grown today, with local varieties now considered landraces (DAFF, 2017).

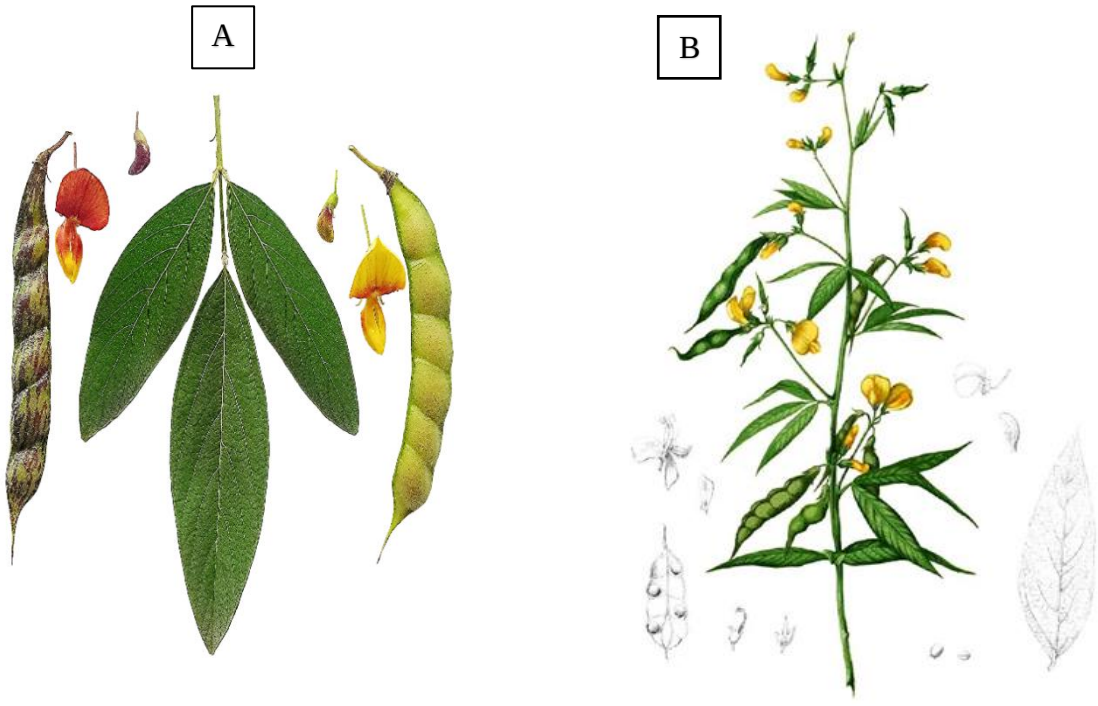

Figure 6: Morphological features of *Cajanus cajan* (pigeon pea). (A) trifoliate leaves from different plants with red flower and specked mature seed pod on the left and yellow flower and juvenile seed pod on the right. (B) Stem diagram at maturity with flowers and seedpods (Snapp, 2018).

Table 3: Classification of *Cajanus cajan* (L.) Millsp.

Domain	Eukaryota
Kingdom	Plantae
Phylum	Spermatophyta
Class	Dicotyledonae
Order	Fabales
Family	Fabaceae
Taxon Name	*Cajanus cajun* (L.) Millsp.
Common Names/s	Pigeon pea, Red gram, Dhal, Nondolo, Kacang

Since its introduction into South Africa, pigeon pea has been grown as a subsistence crop by farmers in rural areas of KwaZulu-Natal, Mpumalanga and Limpopo, mainly for poultry feed (Hluyako *et al*., 2017). Though an orphan crop, pigeon pea had its genome sequenced and published by ICRISAT making it the first non-industrial orphan crop where this information is available (Varshney *et al*., 2011).

This neglected and underutilised crop is able to grow on marginal land and significantly replenish soil with nitrogen (Popoola *et al*., 2019). Its root system allows it to source minerals from deep soils (Sharma *et al*., 2011). As well as being one of the most drought-tolerant legumes, pigeon pea is a highly nutritious crop, capable of boosting food nutritional security in the world, especially in developing and tropical regions where it is mostly grown (Popoola *et al*., 2019).

Its mature seeds contain more than 50% starch, 18.8% protein, 2.3% fat and the rest being crude fibre, vitamins and minerals such as potassium, iron, magnesium and calcium (Popoola *et al*., 2019; Ayenan *et al*., 2017). Research has found that pigeon pea seeds contain many essential amino acids in relatively high amounts but, like other legumes, it accumulates low levels of cysteine and methionine (Nwokolo, 2010; Saxena *et al*., 1987). The starch and protein compositions of pigeon pea are the main nutritional aspects that have led to the recent increase in studies concerning pigeon pea.

Lack of research on pigeon pea in South Africa has left a knowledge gap about local landraces, production, potential yields and commercialisation of the crop. The potential of pigeon pea to be an affordable staple crop relies on extensive research being performed on local landraces and increased cultivation of the crop. Though South Africa has no commercial pigeon pea industry, it recently signed a memorandum of understanding with India as part of a BRICS

(Brazil, Russia, India, China and South Africa) agreement that will provide a market to export 1000 tons of pigeon pea per annum to India (DAFF, 2016).

There is an urgent need for the intensification of basic and applied research into legumes to form a cornerstone for future food and nutritional security as well as a contribution to the understanding of the global legume distribution (Considine, 2017). Like other orphan crops, pigeon pea still requires intensive research for it to become a high yielding crop in terms of seed production. There is still a lack of knowledge surrounding its genetic diversity and how its growth is influenced by environmental factors (Saxena *et al*., 2010). In South Africa, the lack of knowledge about the crop has contributed to the small available market for pigeon pea and, consequently for its exploitation as a crop.

Aims and objectives

This project aimed to characterize starch and its metabolism in pigeon pea varieties. To enable this, the following objectives were attempted:

- **Biochemical analysis**

1. Survey of five South African genotypic entries of pigeon pea in terms of seed, leaf and starch and water-soluble carbohydrate profiles in seeds.
2. Determination of phosphoglucomutase and ADP-glucose pyrophosphorylase activities.

- **Genetic analysis**

1. Amplification of genes encoding either plastidial PGM or subunits of AGPase from genomic DNA (gDNA) and complementary DNA (cDNA) by PCR. Sequence analysis of PCR amplicons to examine the intron/exon structure of the genes by in silico analyses against the coding sequences isolated from cDNA.
2. Examination of transcript accumulation using quantitative RT-PCR of genes encoding phosphoglucomutase or the small subunit of *AGPase*.

Chapter 2: Examination of starch and soluble sugars in pigeon pea

2.1 Introduction

The prime nutritional advantages of slowly digested starch are its benefits to intestinal microflora and its relatively low glycemic index (Tayade *et al*., 2019; Birt *et al*., 2013). Analysis of starch metabolism in crop plants is therefore, of importance as knowledge about it can be used to manipulate starches to improve their properties for human consumption. Legume seeds are a good source of resistant starch which is thought to be caused by hydrogen bonding between the hydroxyl group of glucose moieties and water inter-spread bonding structures (Nissar *et al.*, 2017). Like all crops, pulses retain and can even increase this resistance to digestion after cooking leading to better insulin control. This is because resistant starch is not digested in the small intestine, delaying glucose release into the bloodstream (Tayade *et al*., 2019).

Mature pigeon pea seeds contain similar starch concentrations to those found in other legumes; however, they are reported to contain more resistant starch in their seeds (approximately 40%) than most pulses (between 20% - 30%; Tayade *et al*., 2019; Narina *et al*., 2014). In legumes, seeds are considered mature once the seed filling stage is complete and the seeds have become desiccated (Weber *et al*., 2005). Increased consumption of pigeon pea has been attributed to their low GI regulating blood glucose, making them suitable for consumption by diabetics and people with elevated risk of developing diabetes (Maphosa and Jideani, 2017). The fact that pigeon pea is mostly grown in low-input and risk-prone marginal environments means, however, that their potential yield is often not attained (Varshney *et al*., 2011).

Since its introduction in South Africa, pigeon pea has not been properly exploited to help elevate the standard of living in rural communities and strengthen national food security. This is despite its consistent growth under drought conditions where it has been recorded to produce appreciable yields and maintain a good level of germination (Kumar *et al*., 2011). Although South Africa is food secure as a country, at the household level it is still food insecure leading to malnutrition (Schönfeldt and Pretorius, 2011; DAFF, 2011; Ntombela, 2012). An introduction of such new, easy to cultivate nutritious crops, to communities can help reduce this. Legume orphan crops have the potential to be integrated in agricultural practices to increase yield and they also offer appreciable amounts of nutrients, mainly carbohydrates and

proteins. This chapter helps to increase knowledge about different pigeon pea varieties through examination of starch present in a number of varieties collected within South Africa.

2.2 Materials and methods

Seed collection and plant tissue preparation

Seeds of different pigeon pea varieties were gathered and classified according to the place they were obtained. These were: SEFA seeds bought from Seeds for Africa in 2017; Lari obtained in Durban from a local shop; Nondolo seeds originally from Malawi; India seeds originally from India and the uDhali seeds obtained from local garden in Cape Town. UDhali seeds were harvested from a plant in December 2018, but the ages of seeds from the Lari, Nondolo and India varieties are unknown. Seeds were germinated and grown in the greenhouse in February 2019, and were harvested in May for staining for the presence of starch.

Visualisation of starch

All seeds were examined for the presence of starch using Lugol's solution [5 g (w/v) I_2, 10 *g* (w/v) KI in 100 ml water]. To allow for successful visualisation of the presence of starch in mature seeds they were first imbibed in 65% (v/v) ethanol overnight at 4 ℃. Once the seeds were dissected along the embryo on the axis, they were immersed in Lugol's solution for two minutes and rinsed in cold water before imaging.

Leaves were cut from the main stems of the uDhali pigeon pea variety to create an excised leaf system similar to one previously reported for the common bugle (*Ajuga reptans*, Peters and Keller, 2009). Their petioles were recut under water and leaf sets were then incubated with petioles immersed in water for 2 days to allow for acclimation. Leaves in the excised system were then harvested every 12 h for 24 h and immersed twice in 80% (v/v) ethanol at 90 ℃ to remove chlorophyll, and then rinsed in cold water. Bleached leaves were stained for the presence of starch using Lugol's solution.

To assess the presence of starch, leaves were grown under different environments following an excised leaf system mentioned above. After the leaves had acclimatised, they were divided and kept in controlled growth chambers under constant light for 5 days, and the others darkness induced environments for 5 days. Leaves were harvested daily and immediately stained for starch using the same method described above.

Total starch determination assays

Seeds were freeze-dried and ground for 15 min using a Retsch M400 ball mill until the resultant powder was able to pass through a 5 µm screen. Leaves were frozen in liquid nitrogen and ground to fine powder in a pestle and mortar. Starch was determined in plant material using a Total Starch Assay Kit (Megazyme) using the protocol below where all centrifugation steps were performed using the Eppendorf 5420 centrifuge (Inqaba Biotech, South Africa):

To remove water soluble carbohydrates, duplicates of tissue samples (10 mg) were heated in 1 ml 80% (v/v) ethanol at 85 °C for 10 min. After 5 min centrifugation at 1 800 *g* the supernatants were discarded. The remaining pellets were re-suspended in 2 ml 80% (v/v) ethanol and centrifuged for 10 min at 1 800 *g* and the supernatants discarded. Twenty microliters of 80% (v/v) ethanol was then added to each sample to aid dispersion, followed by addition of 300 µl of thermostable α-amylase. The samples were then incubated for 12 min at 100 °C with vigorous shaking, followed by addition of amyloglucosidase (AMG) capable of liberating 33 µmol glucose min^{-1} and incubation at 50 °C with vigorous shaking for 30 min. The final volume was adjusted to 1 ml using distilled water, mixed well and centrifuged for 10 min at 1 800 *g*.

Two hundred microlitres of distilled water, 5 µl sample solutions, 10 µl buffer with sodium azide (0.02% w/v) and 10 µl $NADP^+$ and ATP solutions provided with the kit were combined and added to a microtiter plate well. The reaction was initiated by the addition of 2 µl hexokinase and glucose-6-phosphate dehydrogenase suspension and the increase in absorbance at 340 nm was measured. Changes in absorbance were determined using a VersaMax™ Tunable Microplate Reader (Molecular Devices). Total starch content was determined following calculation based on the increase in absorbance, using the extinction co-efficient for NADPH according to the manual provided with the Megazyme Total Starch Assay Kit.

Resistant starch determination assays

For determination of resistant starch, a Resistant Starch Kit (Megazyme) was used following the adjusted protocol and all centrifugation steps were performed using an Eppendorf 5420 centrifuge (Inqaba Biotech, South Africa):

Four hundred microlitres of pancreatic α-amylase (10 mg ml^{-1}) containing AMG (3 µmol glucose min^{-1}) was added to 10 mg of seed and leaf powder. Samples were mixed and incubated for 16 h at a 37 °C. Four hundred microlitres of 99% (v/v) ethanol was added, mixed vigorously

and centrifuged at 1 500 *g* for 10 min. The supernatants were decanted and pellets re-suspended in 200 µl of 50% (v/v) ethanol. After vortexing 600 µl of 50% (v/v) ethanol were added. Samples were mixed and centrifuged at 1500 *g* for 10 min before the supernatant was discarded. This process was repeated twice.

To measure resistant starch, 200 µl of 2 M KOH were added to each pellet before being mixed and incubated in a shaker for 20 min. Eight hundred microlitres of 1.2 M sodium acetate buffer (pH 3.8) were then added to each sample followed by AMG to a final concentration of 30 µmol glucose min^{-1}. Samples were mixed and incubated at 50 °C for 30 min with intermittent shaking. The contents were transferred to a tube with the volume adjusted to 10 ml using distilled water and mixed. A 100 µl aliquot of this solution was centrifuged at 1 500 *g* for 10 min and 10 µl aliquots of the supernatant were transferred into new 1.5 ml micro-centrifuge tubes. Three hundred microlitres of glucose oxidase/peroxidase (GOPOD) reagent was added and incubated at 50 °C for 20 min. The OD_{510} was determined for each sample and glucose determined using a standard curve (VersaMax™ Tunable Microplate Reader; Molecular Devices).

For the measurement of non-resistant starch, all supernatants from hydrolysis and solubilisation assays were carefully combined, adjusted to a volume of 10 ml with 100 mM sodium acetate buffer (pH 4.5) and mixed. Ten microlitre aliquots of the solution were incubated with 1 µmol glucose min^{-1} of AMG solution in 100 mM sodium malate buffer (pH 6) for 20 min at 50 °C. Three hundred microlitres of GOPOD reagent were added and samples incubated for 20 min at 50 °C. Absorbance were measured at 510 nm against the D-glucose standard (0.1 mg/ml and 300 µl of GOPOD) and reagent blank (10 µl of 100 Mm sodium acetate buffer and 300 µl of GOPOD). All measurements for this assay were observed using a VersaMax™ Tunable Microplate Reader, Molecular Devices instrument. Final determination of total starch content was obtained following calculation manual provided with the Megazyme Total Starch Assay Kit.

Sugar assay:

Wash solutions

One hundred milligrams of tissue were heated in 1 ml of 80% (v/v) ethanol at 85 °C for 10 min with shaking. The sample was centrifuged at 13 000 *g* (Eppendorf 5420 centrifuge; Inqaba Biotech, South Africa) for 10 min at room temperature and supernatant collected. This step

was repeated, and supernatants combined. The pellet was then incubated in 1 ml of 50% (v/v) ethanol at 85 ℃ for 10 min. The sample was centrifuged at 13 000 *g* (Eppendorf 5420 centrifuge; Inqaba Biotech, South Africa) and supernatant removed and combined with the previous supernatants. All subsequent centrifuge steps for washing and desalting used the same centrifuge. This step was repeated before the pellet was incubated in 1 ml distilled autoclaved water for 10 min at 85 ℃. The sample was then centrifuged at 13 000 *g* for 10 min and the supernatant collected and combined with the previously collected supernatants. The volume was then adjusted to 6 ml using distilled water (dH_2O).

Desalting of soluble sugar extracts

One millilitre aliquots of soluble sugar extracts were vacuum dried for approximately 5 h. These were re-suspended in 200 µl dH_2O before desalting. The following contents were packed into a spin column (MoBiTec GmbH, Goettingen, Germany): 150 µl AG 1-X8 resin (Bio-Rad Laboratories, Johannesburg, South Africa), 100 µl PVPP and 50 µl AG 50W-X8 (Bio-Rad Laboratories, Inc, Johannesburg, South Africa). Columns were placed in a 2 ml microcentrifuge tube and 600 µl of dH_2O was added directly to the resins and centrifuged at 3000 *g* for 2 min at 4 ℃. This was repeated and all flow-through discarded. Two hundred microliters of each sample were placed on a column and centrifuged at 3000 *g* for 2 min at 4 ℃. The flow-through from the spin was collected before 200 µl of dH_2O was added to the resins and centrifuged as before. The water-soluble sugar flow-through and the water flow-through steps were combined.

Tandem mass spectrometry analyses

Liquid chromatography-mass spectrometry (LC-MS) analysis was performed with a Waters Acquity Ultra Performance Liquid Chromatography (UPLC) linked to a Waters Synapt G2 quadrupole time-of-flight mass spectrometer (Waters Corporation, Milford, MA, USA). Desalted samples were separated on a Waters UPLC BEH Amide column (2.1 x 100 mm; 1.7 µm) at a flow rate of 0.17 ml/min at 35 °C. Solvent A consisted of acetonitrile/water (30:70) containing 0.1% (v/v) ammonium hydroxide and solvent B was acetonitrile/water (80:20) containing 0.1% (v/v) ammonium hydroxide. The mobile phase gradient was from 0% to 60% solvent A over 5 min, maintained for 2 min at 60% solvent A before the column was re-equilibrated to the initial conditions. Electrospray ionization was applied in the negative mode and the scan range was from m/z 150 to 1500. The capillary voltage was set at 2.5 kV, the cone

voltage was 15 V, the source temperature 120 °C and the desolvation temperature was 275 °C. The desolvation gas and cone gas flows were 650 L/h and 50 L/h, respectively. Water-soluble carbohydrates (WSCs) were monitored using their deprotonated quasi-molecular ions and base peak ion chromatograms were extracted (Waters MassLynx V4.1V software) to indicate potential accumulation. From the base peak chromatograms, the m/z spectra were isolated to predict certain carbohydrates. None of the WSCs were quantified and were predicted purely on mass similarities.

Statistical analysis

All statistical analyses were performed using GraphPad Prism software (GraphPad Prism version 7.04 for Mac OS X, GraphPad Software, La Jolla California USA, www.graphpad.com). Experimental values are expressed as the mean ± standard error of mean (SEM) of three independent experiments. Significant differences between groups were identified using one-way ANOVA, with Tukey *posthoc* tests. Differences were considered significant when $P < 0.05$.

2.3 Results

Visual appearance of the seeds

All seeds were spherical and demonstrated noticeable differences in seed coat appearance among the five varieties, which varied from white to red or speckled (Table 1). The lowest recorded seed weight was observed in SEFA seeds (145.2 mg) and the highest in Nondolo seeds at 229.2 mg.

Table 1: Seed weights and seed coat appearance of the pigeon pea varieties. Seed weights were determined using 50 seeds per variety and samples marked with different letters are statistically different ($P < 0.05$).

Variety	Mean seed weight (mg) ± SEM	Seed coat appearance
uDhali	170.9 ± 1.0 [a]	Red
SEFA	145.2 ± 1.2 [b]	White (speckled)
Lari	202.6 ± 1.9 [c]	Brown
Nondolo	229.2 ± 1.0 [d]	Multi-colored with speckles
India	181.0 ± 2.1 [e]	White

Staining seeds for the presence of starch

Four of the five varieties were stained for the presence of starch using Lugol's solution. The India variety was omitted due to a lack of seed material after the seeds allocated for staining were damaged during the imbibition step. Staining of the different seed varieties with Lugol's solution demonstrated the presence of starch in all varieties except SEFA for which no seed part stained. The intensity of staining indicated a clear distinction between the cotyledons, seed coats and embryonic axes, with only the cotyledons staining positive for starch and the other parts not staining (Figure 1). The intensity of the staining solution of all varieties that stained positive was the same in both whole and dissected seeds indicating the presence of starch throughout the cotyledons.

Figure 1: Seeds stained for the presence of starch using Lugol's solution on the four different seed varieties where enough seeds were available. Letters A-D2 indicate the various seed components before and after staining. The presence of starch is normally indicated by the development of a deep blue colouration.

Seed starch content

To quantitatively investigate the variation in starch in mature dry seeds, measurements of total (Figure 2) and resistant (Figure 3) starch were undertaken. The seeds could be divided into high- and low-starch varieties. The high starch varieties included uDhali (483.9 mg/g seed DW), Nondolo (489.0 mg/g seed DW), Lari (549.2 mg/g seed DW) and India (520.2 mg/g seed DW) (Figure 2). SEFA was the only variety observed to have low-starch, with seeds containing 3.0 mg/g seed DW.

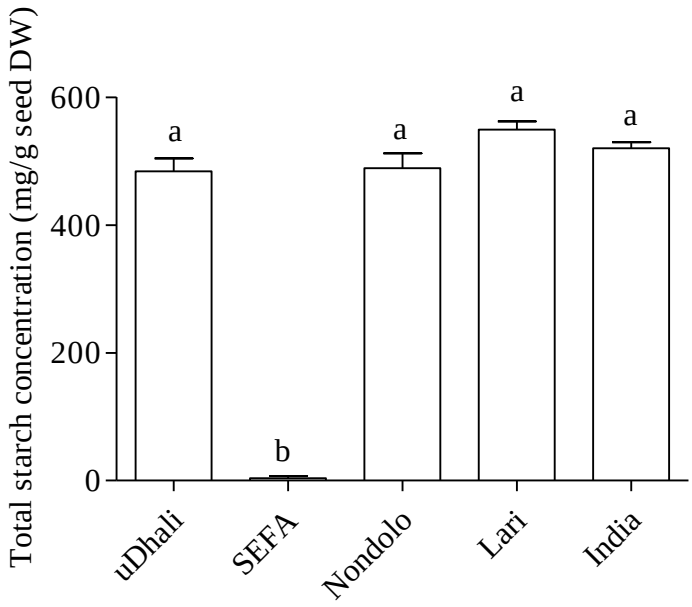

Figure 2: Seed total starch accumulation in different pigeon pea variety seeds at maturity. Data represent means ± SEM of three biological samples (n = 5). Statistical analysis was performed using one-way ANOVA and Tukey *posthoc* test. Letters represent groups with similar means ($P<0.05$).

Resistant starch assays indicated that starch from the high-starch varieties were composed of more than 40% resistant starch (Figure 3). Of the high-starch varieties, India demonstrated the highest resistant starch concentration of 395.0 mg/g seed dry weight, which amounted to 75.9% resistant starch. The Nondolo seeds contained resistant starch amounting to 50.2% (245.5 mg/g) of the total starch, while Lari and uDhali seeds contained 57.2% (314.1 mg/g) and 56.0% (270.9 mg/g) resistant starch respectively. Accumulation of resistant starch on SEFA seeds was 1.3 mg/g dry weight, amounting to 43.4%

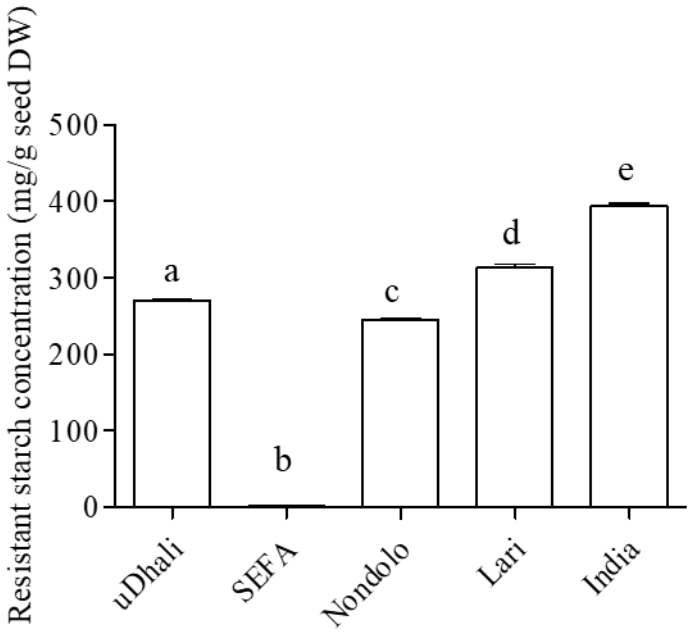

Figure 3: Resistant seed starch in mature pigeon pea seeds. Data represent means ± SEM of three biological samples (n = 5). Statistical analysis performed using one-way ANOVA and Tukey *posthoc* test. Letters represent groups with similar means ($P<0.05$).

Leaf starch content

For assessments of leaf starch, only the uDhali variety was used as it proved the easiest to germinate. Iodine staining indicated that all leaves contained starch with the intensity of staining being similar for all the leaves (Figure 4). Leaves harvested over a diurnal cycle in a controlled environment chamber under a 12h light/dark cycle showed no clear difference in starch amounts using Lugol's solution, with both day and night harvested leaves displaying the presence of starch. Observations of leaves harvested at the end of the dark period (Figure 4: A1, A2, C1, C2) and the end of the light (Figure 4: B1 and B2) indicated a lack of variation in the presence of starch in the leaves. This prompted us to a quantitatively measure total starch in leaves.

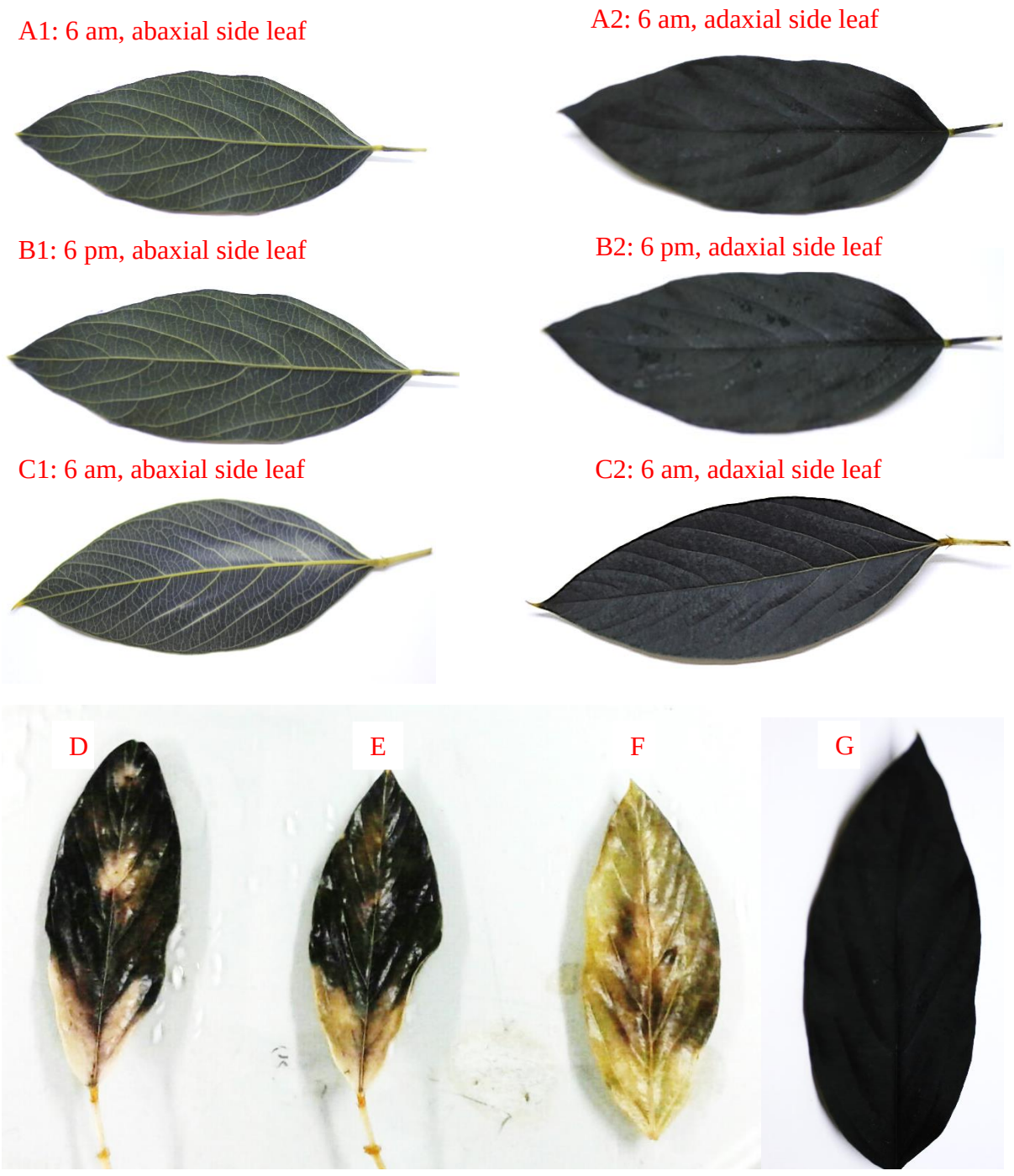

Figure 4: Examination of starch in uDhali leaves using Lugol's solution. Excised leaves were kept in controlled growth chambers under different conditions. Figures (A1 - C2) show leaves harvested at 06h00, 18h00 and 06h00 during a 24 h 12 h light/12h dark cycle where the light came on at 06h00 am. (D) a leaf grown in darkness for two days, (E) a leaf grown in continuous light for three days (F) a leaf grown in darkness for five days. (G) a leaf grown under a 12 h light /12 h dark diurnal cycle.

When grown in darkness for up to five days the starch was almost completely depleted and the leaves started to show signs of wilting (Figure 4F). Reduced starch was only visible after two days of darkness (Figure 4D), while after 5 days almost no starch remained (Figure 4F). Under continuous light, a decrease in starch became visible after three days (Figure 4E).

To further understand the amount of starch present in leaves, total and resistant starch assays were conducted on leaf material over a 24 h period when grown under a 12 h light/ 12 h dark photoperiod. Total starch was approximately 27 mg/g FW after 4 hours of light, after which it gradually decreased until it reached the lowest amount of 14 mg/g FW at the end of dark period (Figure 5).

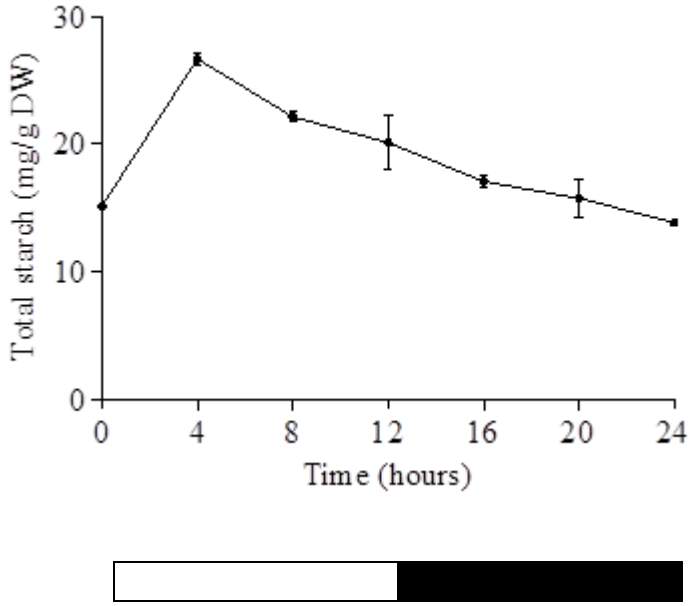

Figure 5: Total starch in pigeon pea leaves in a controlled environment (growth chamber) over a 24 h period with 12 h day/night with the day starting at 08h00 (T0). The black box represents darkness and the white box light periods. Data represent means ± SEM of three biological repeats.

Resistant starch was also measured in the same samples and was present in leaves, although the amounts were relatively low (less than 2 mg/g fresh weight; Figure 6). This means that resistant starch in leaves was less than 10% of the total starch present. The maximum resistant starch results observed in leaves were at its highest after 8 hours of light and lowest at the end

of the night. Though amounts of both total and resistant starches decreased, this began earlier in the total starch (after 4 hours) than the resistant starch (after 8 hours). Total starch breakdown was observed to be constant throughout the night whereas for resistant starch, starch break down was greater for the first four hours (T8-T12) than between T12 to T24.

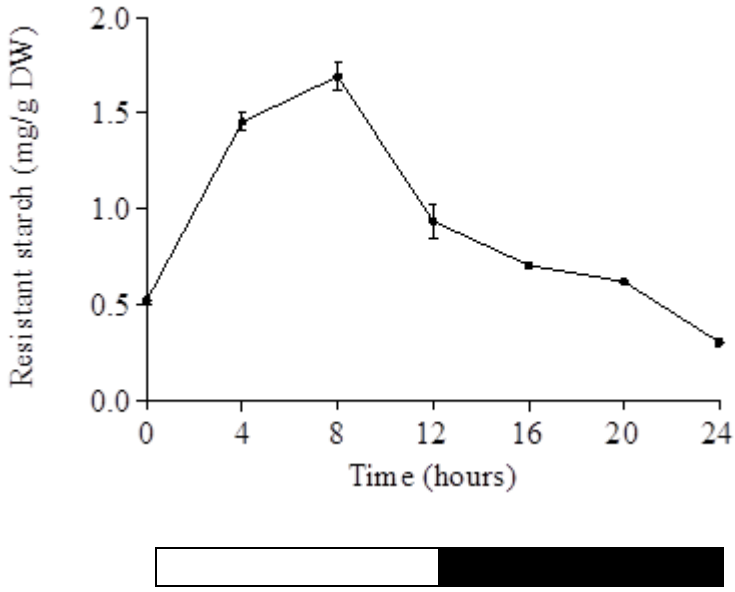

Figure 6: Resistant starch in pigeon pea leaves in a controlled environment over a 24 h period with 12 h night/day with 4 h intervals and day starting at 08h00 (T0). The black box represents darkness and the white box light periods. Data represent means ± SEM of three biological repeats.

Soluble sugars in pigeon pea seeds

An assessment of soluble sugars was conducted in seeds of each variety (Figure 7). The results demonstrated as mass spectra gave an indication of relative abundance (%) of sugars present in the seeds. Three main sugars – putatively identified as sucrose/galactinol, raffinose and stachyose - were observed to be present in low amounts in these mature dry seeds. Peaks for sucrose/galactinol, raffinose and stachyose were present in extracts of all varieties, except uDhali which lacked raffinose. The putitative sucrose/galactinol accounted for approximately 40% of all soluble sugars in Nondolo seeds, 35% in Lari seeds, 25% in India and 20% in uDhali and SEFA seeds.

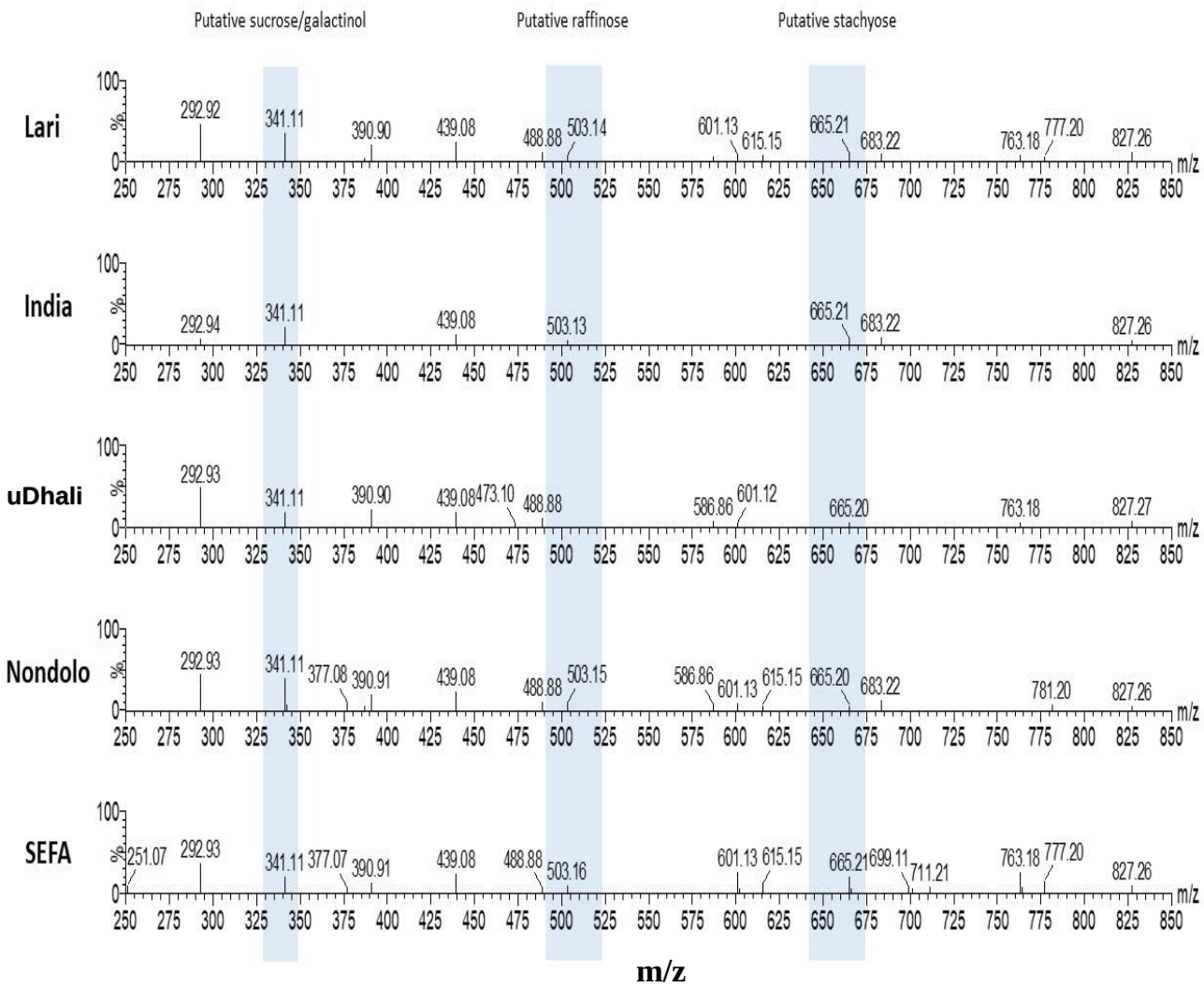

Figure 7: Water-soluble sugar composition of pigeon pea seeds at seed maturity presented in relative abundance (%) using Liquid Chromatography-Mass Spectrometry (LC-MS/MS) for analysis.

Raffinose family oligosaccharides constituted at most 10% of the soluble sugars in seeds from all the varieties except uDhali which contained none. Nondolo contained the highest proportion of raffinose (10% of total sugars), while uDhali, India and Lari all contained raffinose as approximately 5% of total sugars. The proportion of stachyose varied between seed varieties, with uDhali and Nondolo containing it at less than 5% of total soluble sugars and India and Lari at approximately 10% of total sugars. One variety (SEFA) contained a very high proportion (38% of total sugars) of stachyose in the soluble sugar profile.

2.4 Discussion

The lack of research on pigeon pea has placed it at a disadvantage when compared to other legumes in terms of developing it as crop. Increasing knowledge of this orphan crop will help integrate it in both local and global food production, as well as leading to a better understanding of its potential seed yield and nutritional value. One of the main nutritional interests in pigeon pea is its resistant starch. In plants, starch is normally the main carbohydrate reserve. In leaves it is synthesised during the day and degraded at night, while in seeds it is mobilised during germination to produce sprouts (Stitt and Zeeman, 2012; Wang *et al*., 1998).

The present study was conducted to assess starch in local pigeon pea varieties. There was considerable variation in seed size and appearance, with each variety differing from the other in weight, which ranged between 145-229 mg with the weights of seeds from all varieties being different from each other. The SEFA variety produced seeds with the lowest weights while Nondolo seeds were the heaviest (Table 1). Seed coat colours were also observed to be different in all seeds, ranging from red, white or speckled. It was only possible to source small numbers of seeds from local sources, however, and it should be acknowledged that future experiments using larger numbers of seeds will be needed to confirm these data.

Iodine staining demonstrated that seeds from all varieties except SEFA contained starch in cotyledons, but not the testa and embryo (Figure 1). Those parts contained little to no starch in all varieties, which is common for seed coats from mature seeds as most food reserves are stored in cotyledons for later use during germination (Aguirre *et al*., 2018: Gaikwad *et al*., 2013; Kabeya and Sakai, 2003). Cross sections of the cotyledons revealed that starch was present in cells throughout the seed of most varieties, but examination of cross sections from the SEFA variety indicated that they contained almost no starch throughout.

Measurements of total starch revealed most varieties contained high amounts or, in the case of SEFA, almost none. Mutants in other plant species have been isolated previously with similar low starch levels to SEFA, and these have been described as essentially starchless or starchless (Ventrigilia *et al*., 2008; Harrison *et al.* 2000; Lin *et al*., 1988). In the starch positive varieties, the total seed starch content varied between 54.4% to 54.9% mg/g dry weight (Figure 2). These results are consistent with previous reports in legume seeds where starch concentrations of more than 40% were found (Popoola *et al*., 2019; Tayade *et al*., 2019; Sarkar *et al*., 2018; Talari and Shakappa, 2018; Ayenan *et al*., 2017; Narina *et al*., 2014). Seed starch contents can be affected by environmental factors including agro-ecological production, soil fertility,

weather and drought all of which affect the amount of starch produced by each crop (Famera *et al*., 2015). As we obtained the seeds from various sources and did not control the growth of the mother plants, it is possible that the differences noted may not be present in seeds isolated from plants grown under identical conditions.

All seeds were spherical and not wrinkled, which indicates that there is a difference between the almost starchless variety (SEFA) and a similar low starch *Pisum sativum* mutant (*rug-3*), which is wrinkled (Rayner *et al*., 2017; Harrison *et al*., 2000). The rugosus (wrinkled) phenotype - after which *rug-3* is named - is common in peas that contain reduced starch and it is thought to be due to increased soluble sugars leading to greater water uptake by the developing embryo, which leads to a wrinkled phenotype in the dessicated mature seed (Wang and Hedley, 1991). This indicates that the physiological response of pigeon pea seeds to low starch differs from that of *P. sativum*.

The low starch content of the SEFA variety may indicate a genetic difference between it and the other varieties. It is interesting to speculate which enzymes may be affected to lead to this phenotype and there are a few possibilities. Similar low seed starch mutants have been isolated in other legumes, namely pea and *Lotus japonicus*, which are mutated in plastidial phosphoglucomutase (Vriet *et al*., 2010; Harrison *et al*., 2000), and so it is possible that this enzyme is affected in pigeon pea. A second possiblilty is that ADP-glucose pyrophosphorylase activity is reduced. Although mutations affecting that enzyme can lead to a starchless phenotype in leaves (Lin *et al*., 1988), in seeds similar mutations appear to reduce starch by about half, but not lead to near starchlessness (Vriet *et al*., 2010; Hylton and Smith, 1992). Finally, mutations in genes encoding a recently discovered protein (Protein Targeting to Starch) that affects starch metabolism through mitigating protein-protein interractions eliminates starch in barley endosperm (Zhong *et al*., 2018). It is possible that the low starch phenotype in the SEFA variety may be caused by a lesion in one of the genes encoding these proteins, but it cannot be ruled out that other genes may be affected.

Resistant starch amounts in legumes vary between species. The analysis of resistant starch in seeds in the current study revealed that the high-starch varieties contained a high percentage of resistant starch (ranging between 43% to 76% of the total starch; Figure 3). Other studies have found that pigeon pea seeds contain less resistant starch, generally between 22% to 36% (Sarkar *et al*., 2018; Narina *et al*., 2014; Sharma *et al*., 2011). This difference may be due to varietal differences or to differences in growth conditons used during growth of the pigeon pea

plants in this study. Variations in growth habitats, may have also influenced local diversity in seeds and the starch composition of the varieties assessed together with the different ages of the seeds since increase in age is known to affect the starch and amylose content (Wang *et al*., 2017; Mengarda *et al*., 2015).

The India variety contained the highest proportion of resistant starch at 76%. Such a high amount is unusual for legumes, with other studies reporting amounts ranging between 35%-52% for crops such as soybeans, lentils and chickpea (Tayade *et al*., 2019; Sarkar *et al*., 2018; Jideani and Diedricks, 2014; Narina *et al*., 2014). The SEFA variety contained less than 1% total starch and also contained relatively high amounts of resistant starch in its seeds (43%). The relatively high amounts of resistant starch in seeds from the uDhali, Nondolo and Lari varieties indicates that these varieties may be useful in the management of type-2 diabetes through regulation of insulin by the slower release of glucose into the bloodstream this would confer.

Developing countries such as Tanzania and Uganda that grow pigeon pea have used it to help manage type-2 diabetes (Sarkar *et al*., 2018; Uchegbu and Ishiwu, 2015). In South Africa, this disease is one of the four leading NCDs responsible for health problems (Stats SA, 2017; Wandai *et al.,* 2017; Sheik *et al.,* 2016; Bradshaw *et al.,* 2011). The current study is one of the first to identify seeds containing very high levels of resistant starch of up to 76% which may be useful in helping to control this disease. However, it needs to be pointed out that the data here showing the presence of resistant starch is based on an *in vitro* assay. Clearly the potential use of pigeon pea seeds as a food that will help control type-2 diabetes will need to be confirmed through clinical trials. An added advantage of pigeon pea is it being low-input cost crop and nourishing, which may help to both combat nutritional imbalance and lead to improved food security. Nevertheless the high amounts of resistant starch may help this orphan crop to establish and maintain a stable commercial market in the business sector by marketing it as a healthier legume.

Unlike seeds which contain starch as a long-term carbon store, leaves synthesise starch during the day and degrade it at night. Catabolised leaf starch becomes partitioned to different parts of the plant in the form of sugars which are used in various metabolic pathways. Within a diurnal cycle, leaves are known to contain little starch at the end of the night although this is influenced by the plant species, day length and light intensity (Fernandez *et al*., 2017; Orzechowski, 2008; Zeeman *et al*., 2004). The results of iodine staining in excised leaves

showed that pigeon pea leaves contained starch throughout a 24 h period undergoing a 12 h day/night cycle (Figure 4A-C). Even though this demonstrated that starch was broken down at night this was not observed using semi-quantitative iodine staining (Fig 4A-C).

To assess how much time it takes for pigeon pea leaves to start degrading starch, excised leaves were kept under continuous darkness or continuous light (Figure 4D-F). Leaves grown under these conditions started to eliminate starch from portions of the leaves. In prolonged darkness, breakdown of starch was observed after 2 days and by the time the leaves reached day 5, starch was almost completely eliminated, and the leaves had showed signs of wilting. Leaves kept in prolonged light started showing starch degradation on day 3 and by day 5 all leaves had almost degraded all starch and also showed signs of wilting. This indicated that they were undergoing physiological changes, most likely caused by the inability to synthesise new starch in extended darkness due to lack of light needed for photosynthesis and after reaching a peak for starch production when grown under continues light. The reason for the wilting may be due to the excised system leading to food reserves being mobilised throughout the day, decreasing energy reserves. Although such an excised system is useful in terms of allowing easy control of environmental conditions, its effects on leaf physiology need to be assessed in more detail.

To measure total and resistant starch in leaves, excised leaves were kept in 12 h light/dark. Unlike the visualisation of leaf starch by iodine staining (Figure 4), starch concentrations were shown to vary during the day when starch was determined quantitatively (Figure 5). Total and resistant starch amounts peaked at midday, with a decline during the rest of the day and night (Figures 5 & 6). These results aligned with past studies on leaf starch, indicating starch is synthesised during the day and then broken down and used to fuel metabolism at night (Fernandez *et al*., 2017; Zeeman *et al*., 2004).

This study also revealed that the pigeon pea leaves contained a maximum amount of starch of approximately 20 mg/g FW, which is twenty fold lower than the amounts found in most seeds. Such findings are common in plants as seeds are known to possess high amounts of starch. For example soybean is known to have ten times more starch in seeds than leaves (Tayade *et al*., 2019; Chatterton and Silvius, 1979). Resistant starch in pigeon pea leaves was also less than that in seeds showing that leaves have low resistant starch.

Further analysis examined soluble sugars in seeds from all five pigeon pea varieties. The seeds were observed to contain very low water-soluble sugars, with sucrose/galactinol being the only sugar presented in the mass spectra that gave an indication of being greater than the other sugars

of interest in this study (Figure 7). The results revealed that RFOs were present at about 10% of total soluble sugars in mature seeds of pigeon pea. Soluble sugars are known to be at their greatest concentrations early in seed development before starch deposition, however, stachyose and raffinose concentrations increase as the seeds mature to prevent dessication (Obendorf and Gorecki, 2012; Sharma *et al*., 2011; Samarah *et al*., 2009; Steadman, 1995).

The SEFA seeds contained a greater proportion of raffinose and stachyose than the other seeds while Lari and Nondolo seeds contained more sucrose than the others and uDhali and India the least raffinose. Although stachyose is the main RFO in many mature legume seeds and has been known to reach 50% of total soluble sugars in cowpea seeds (Martın-Cabrejas *et al*, 2007), this was not the case in pigeon pea. The two RFOs examined in the study were present in low percentages, and in some seeds like uDhali they were absent (Figure 7). Similar to other studies, sucrose was the dominant sugar in seeds from all varieties when compared to RFOs (Obendorf and Gorecki, 2012;Karner *et al*., 2004; Gill *et al*., 2002; Sanchez-Mata *et al*., 1998). It is known that the presence of raffinose and stachyose in seeds decreases with age whereas the other water-soluble sugars remain constant. Although the age for some of the seeds in this study is unknown, it has been shown previously that raffinose and stachyose remain in seeds that have been stored for 2 years (Sharma *et al*., 2011; Samarah *et al*., 2009; Steadman, 1995).

These findings contrast with reports of soluble sugars in other legumes, such as cowpea, which accumulate high amounts of RFOs in mature seeds and have also been reported to contain high amounts of sucrose and galactose (Obendorf and Gorecki, 2012; Martın-Cabrejas *et al*, 2007). The percentages of sugars in this study, especially raffinose and stachyose, may be attributed to several factors, which include the presence of phenolics in the samples that may bind with the sugar molecules and interefere with LC-MS. Pigeon pea is known to contain high phenolics (Rinthong and Maneechai, 2018; Al-Saeedi and Hossain, 2015; Rani *et al*., 2014) which may have interfered with the purification process. For better understanding, quantification of soluble sugars in all variaties is recommended, with improved purification steps.

Although pigeon pea is grown mainly for its seeds in other parts of the world, in Soutrh Africa it is mostly grown by subsistence farmers for fodder and as vegetable soup, mostly in Mpumalanga (DAFF, 2013). As a result South Africa lacks a market for this legume which has potential to become a conventional crop. A lack of research on it has disadvantaged it when compared with staple crops, as its nutritional benefits including high starch, protein and low fats have gone unnoticed. In the sub-Saharan region, pigeon pea may be used together with

cereal staples to provide a healthy mixture of different amino acids as cereals are rich in methionine and legumes in lysine.

To our knowledge, this study is the first in South Africa to assess starch composition of local pigeon pea varieties. Subsistance farmers have grown this crop for generations without knowledge of its nutritional composition. Extensive research on the available varieties is still required to increase knowledge of other nutritional aspects of the seeds such as protein and amino acid contents, especially in the starchless variety. Since low carbohydrates in seeds are associated with increased lipid and amino acid content (Meyer *et al.*, 2012; Mello *et al.*, 2010), research on these nutritional aspects is needed to further understand the benefits of this crop (Talari and Shakappa, 2018; Hlukayo, 2015; Sharma *et al*., 2011; Saxena *et al*., 2010).

Chapter 3: Analysis of expression of starch biosynthetic genes in *Cajanus cajan* seeds and leaves

3.1 Introduction

The pathway of starch synthesis in seeds starts with sucrose degradation in the cytosol leading to sugars entering the amyloplast as glucose 6-phosphate where a plastidial phosphoglucomutase uses it to form glucose 1-phosphate. This is the substrate of ADP-glucose pyrophosphorylase (AGPase) that forms ADP-glucose (Lee *et al*., 2016; Pfister and Zeeman, 2016), an activated sugar that is used to synthesise the starch polymer by a combination of starch synthases, starch branching enzymes and debranching enzymes (Tayade *et al*., 2019; Saripalli and Gupta, 2015). The hetero-tetrameric AGPase is composed of two large and two small subunits and catalyses the committed step of starch synthesis (Batra *et al*., 2017; Cheng *et al*., 2016; Lee *et al*., 2016; Saripalli and Gupta, 2015; Georgelis *et al*., 2007).

Impaired AGPase enzyme activity is linked to the presence of shrivelled seeds (Hylton and Smith, 1992), a trait thought to be caused by high levels of soluble sugars as a result of reduced amounts of starch in the seeds (Cheng *et al*., 2016; Lee *et al*., 2016). Absence of the plastidial PGM enzyme has resulted in plants being able to synthesise only small amounts of starch which are often referred to as starchless or essentially starchless (Ventrigilia *et al*., 2008; Harrison *et al.* 2000; Lin *et al*., 1988). Similar to AGPase deficiency, the lack of plastidial PGM can lead to the appearance of wrinkled seeds (Harrison *et al*., 2000; Periappuram *et al*., 2000).

The starch biosynthetic pathway is composed of multiple isoforms of the synthesizing enzymes encoded by independent genes. Legumes synthesise starch in their seeds that is more slowly digestible than starch found in cereal endosperm (Tayade *et al*., 2019). The presence of near-starchless varieties in legumes have been reported previously (Vriet et al. 2010; Harrison *et al*., 2000), and in chapter 2 I identified a pigeon pea variety that also demonstrates this phenotype. Low starch in legume seeds is associated with lesions in genes encoding the small subunit of AGPase or PGM (Tayade *et al*., 2019). This led to the first step to examine South African pigeon pea seeds for expression of genes encoding AGPase or PGM alongside examination of the activities of these enzymes in the seeds and leaves.

3.2 Materials and methods

Amplification of PGM and AGPase genes

It was not possible to germinate seeds from the SEFA variety and so total RNA was extracted only from leaves of the uDhali variety (Chapter 2). This was performed using the RNeasy® Plant Mini Kit (Qiagen, Whitehead Scientific, South Africa) following the manufacturer's protocol, all centrifugation steps for amplification of the two genes were performed using the Eppendorf 5420 centrifuge (Inqaba Biotech, South Africa). Complementary DNA was synthesised using a ThermoFisher Scientific RevertAid first strand cDNA synthesis kit following the manufacturer's instruction. Reactions included cDNA synthesised from 1 µg total RNA, 1 µl oligo dT18 primer, 4 µl 5X reaction buffer, 1 µl RiboLock RNase inhibitor, 10 mM dNTP mix, 1 µl reverse transcriptase and water in a final volume of 20 µl.

Genomic DNA was extracted from the same variety using a method adapted from Edwards *et al*. (1990) as follows: Ground tissue material (20 mg-100 mg) was placed in a 1.5 ml tube with 400 µl gDNA extraction buffer (200 mM Tris-Cl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% (w/v) SDS and water) and vortexed for 1 min. The mixture was centrifuged for 10 min at 13 000 *g* before 300 µl of supernatant was placed in a new 1.5 ml tube on ice. Three hundred microlitres of isopropanol was added and mixed by inversion before being incubated at -20 ℃ for 60 min. Samples were then centrifuged for 15 min at 13 000 *g* and the supernatant discarded. The remaining pellet was rinsed with 1 ml ethanol and air-dried until all residual ethanol had evaporated. The gDNA pellet was re-suspended in 100 µl TE buffer at pH 8.0.

Coding sequences for both phosphoglucomutase and the small AGPase subunit were amplified using primers designed using *C. cajan* gene sequences with the following accession numbers: *C. cajan*_07432 and *C. cajan*_06002 from the Noble Legumes database (Li *et al*., 2016; Li *et al*., 2012). All primers used were obtained from Inqaba Biotech (South Africa).

Q5 high fidelity thermostable polymerase (New England BioLabs Inc., Inqaba, South Africa) was used to amplify the two genes using gene specific primers (Table 1) with the following thermocycling conditions for *PGM* and *AGPase:* Initial denaturation temperature 98 ℃, 30 sec; followed by 25 cycles of denaturation temperature 98 ℃, 10 sec; annealing temperature 60 ℃, 30 sec (*PGM*) and 61 ℃, 50 sec (*AGPase*); initial extension temperature 72 ℃, 2 min final extension temperature 72 ℃, 2 min (*PGM*) and 5 min (*AGPase*).

Table 1: Primer sequences used for amplification of genes encoding plastidial phosphoglucomutase and glucose-1-phosphate adenylyltransferase as well as T7 vector primer used during molecular analysis and cloning of the two genes.

Name	Annotation	Sequence 5'- 3'	Reference
*PGM*_F *PGM*_R	phosphoglucomutase, chloroplastic	ATGGCTTTCTCTTCTACGCTTG TTATGTGATGACTGTAGGCTTGTC	Varshney *et al*., 2011
*AGPase*_F *AGPase*_R	*Cajanus cajan* glucose-1-phosphate adenylyltransferase small subunit 2, chloroplastic	ATGGCTTCCATGGCTGC TTAGATGACAGTTCCACTGGG	Varshney *et al*., 2011
T7		TAATACGACTCACTATAGGG	Yin and Steitz, 2004; Chamberlin and Ring, 1973

Amplicons of the two genes were purified using Wizard® Plus SV Gel and PCR Clean-Up system. They were A-tailed with the Go*Taq* DNA polymerase in the following mix: PCR fragment (1 µg); dATP (20 mM); *Taq* DNA polymerase (0.25 µl); 10x Buffer (5 µl) and adjusted with water to final volume of 50 µl before being incubated at 72 ℃ for 30 min. DNA from this reaction was purified using the Wizard® Plus SV Gel and PCR Clean-Up system, cloned into the pGEM-T-easy vector system and transformed into *Escherichia coli* (*E. coli;* DH5α strain) chemically competent cells.

Transformed cells were spread on Petri dishes containing sterile Luria-Bertani agar (1% (w/v) tryptone, 1% (w/v) NaCl, 0.5% (w/v) yeast extract, 1.5% (w/v) agar) with ampicillin (100 µg/ml) for selection. PCR was performed using bacterial colonies to determine the presence and orientation of inserts using Go*Taq* DNA polymerase (Promega) and a combination of gene specific and T7 promoter (Table 1) primers under the following thermocycling conditions: initial denaturation temperature 95 ℃, 2 min; denaturation 95 ℃, 50 sec; annealing 58 ℃, 50 sec (*PGM*) and 59 ℃, 50 sec (*AGPase*); extension 72 ℃ for 2 min (*PGM*) or 1:30 sec (*AGPase*); final extension 72 ℃ 10 min. All reagents used for PCR clean-up systems, Go*Taq* DNA polymerase and *pGEM-T* easy vector were obtained from Promega, Anatech.

Vectors containing inserts in the correct orientation were isolated from the bacterial cells using the Wizard® Plus SV Minipreps DNA Purification systems and sequenced at the Central

Analytical Facility (CAF, Stellenbosch University, South Africa) to validate the fidelity of the amplification process.

Molecular analysis of starch metabolism in pigeon pea:

I. Quantitative real-time PCR (RT-qPCR)

Gene expression analysis in pigeon pea leaves was performed using RT-qPCR (QuantStudio 3 applied biosystems, Thermo Fisher Scientific). The same excised leaf system that was used to visualise starch (Chapter 2) was established in a controlled environment. Leaves were acclimatised over a period of 48 h in controlled growth chambers. The leaves were placed in growth chambers set at 12 h light (26 ℃)/dark (19 ℃) for 24 h, half of the leaves were used to examine the expression of genes over a day night cycle. The other leaves were used for a second RT-qPCR experiment, which assessed the expression of *phosphoglucomutase* in a sucrose solution under environments with extended light and darkness. The leaves for the first experiment were harvested every 4 h for 24 h, frozen in liquid nitrogen and ground to a powder in a pestle and mortar. For the second experiment the leaves were divided into two groups with some kept in a 2% (w/v) sucrose (Sigma-Aldrich, South Africa) solution and the rest in water for an additional 24 h. Leaves grown in sucrose or water were then subdivided into being grown in darkness for 24 h or cycle of 16 h light/8 h darkness for 24 h before being harvested.

Reactions were performed in total volume of 10 µl each [1 µl cDNA (synthesised from 1 µg of RNA), 2 µL (1 µM) each primer (Table 2), 5 µl SYBR Green Master-mix]. The denaturation/annealing temperature (95 ℃/15 sec, 60 ℃/1 min) per cycle of 40 cycles. The threshold cycle values (ΔCT) were used to calculate the relative fold change ΔΔCT and T1 used as a calibrator sample. All qPCR experimentation was conducted in compliance with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE).

Table 2: Primer sequences used in RT-qPCR experiments.

Primer	Annotation	Primer sequence 5'- 3'	Amplicon Size (bp)	Reference
*PGM*_Q_F *PGM*_Q_R	phosphoglucomutase, chloroplastic	AGCTTCAGTGTGGAAGTCATAG AAATCCGGTCGTGAAAGAAGA	104	This study
*AGPase*_Q_F *AGPase*_Q_R	glucose-1-phosphate adenylyltransferase small subunit 2, chloroplastic	CTCCCAAGGCAGTTTCTGAT AGCACCACCTCCAAGTATAATG	95	This study

18SrRNA_Q_F 18SrRNA_Q_R	18Sr-RNA	CCACTTATCCTACACCTCTC ACTGTCCCTGTCTACTATCC	102	Sinha *et al*., 2015
TUB6_Q_F TUB6_Q_R	Tubulin beta-6.	GCCCTGACAACTTCGTCTTC GCAGTTTTCAGCCTCTTTGC	100	Sinha *et al*., 2015
HSP90_Q_F HSP90_Q_R	*Cajanus cajan* Heat shock protein 90	TGTCGAGCAAGAAGACGATG GGGCAGTTTCAAAGAGCAAG	103	Sinha *et al*., 2015

II. Phosphoglucomutase and ADP-glucose pyrophosphorylase assays

The activities of PGM and *A*GPase were assessed as follows:

AGPase

Protein was extracted from seeds using an extraction buffer composed of 100 mM HEPES-NaOH pH 7.5, 8 mM MgCl2, 1 mM DTT, 2 mM EDTA, 12.5 % (v/v) glycerol and 5% (w/v) polyvinylpyrrolidone. Aliquots of crude protein (50 µl) were then mixed with 373 µl reaction mixture consisting of HEPES-NaOH (pH 7.5), 6.0 mM MgCl2, 3 mM DTT, 5 mM sodium pyrophosphate 50 mM and dH2O to a volume of 423 µl. The mixture was incubated at 30 °C for 20 min followed by addition of ADP-glucose to a final concentration of 1.2 mM. This was incubated for 20 min at 30 °C. The reaction was terminated at 100 °C for 1 min and centrifuged at 10 000 *g* for 10 min. The supernatant was mixed with 0.1 ml of 6 mM NADP+, 0.3 ml of 50 mM HEPES-NaOH pH 7.5, 0.08 U of PGM and 0.07 U of G6PDH and the increase in OD340 was used to calculate activity (Kulichikhin et al., 2016).

Protein was extracted from leaves using an extraction buffer consisting of 50 mM HEPES-KOH (pH 7.4), 5 mM MgCl2, 1 mM EDTA, 5mM DTT, 0.1% (v/v) Triton X-100, 10% (v/v) glycerol (Geigenberger and Stitt, 1993). The assay consisted of 20 µl of desalted extract in 25 mM HEPES-KOH (pH 7.4), 2 mM MgCl2, 0.5 mM NAD+, 2 mM 3-phosphoglyceric acid (3PGA) and 1.5 mM ADP-glucose, 0.3 U G6PDH, 0.4 U PGM. The reaction was started by the addition of tetrasodium pyrophosphate to a final concentration of 2 mM.

PGM

Activity of PGM in seeds was measured using Sigma-Aldrich phosphoglucomutase colorimetric assay kit following manufacturer's protocol. Seed powder was homogenized with

200 µl ice cold assay buffer and centrifuged for 5 min at 13 000 *g* (Eppendorf 5420 centrifuge, Inqaba Biotech, South Africa) before supernatant was collected. An aliquot of the supernatant mixed with 4.1 M ammonium sulfate solution to a final concentration of 3.2 M and incubated on ice for 20 min. The sample was centrifuged at 13 000 *g* (Eppendorf 5420 centrifuge, Inqaba Biotech, South Africa) for 5 min and the supernatant discarded. The pellet was then resuspended using 200 µl of assay buffer.

An NADH standard curve with 0, 2.5, 5, 7.5, 10 and 12.5 nmol/well NADH was prepared to a final volume of 50 µl/well using the supplied buffer assay buffer to determine the linear range of sample to NADH. For the reaction mix, PGM assay buffer (44 µl), PGM enzyme mix (2 µl), PGM developer (2 µl) and PGM substrate (2 µl) were added in a microplate. The reaction was initiated by addition of 50 µl sample, and for the standard curve 50 µl NADH bringing the final volume to 100 µl. Absorbance was determined at 450 nm.

Protein was extracted from leaves in a buffer composed of 50 mM Tris-HCl (pH 7.5), 5 mM MgCl2, 2 mM EDTA, 0.1 mM PMSF, 0.1% (v/v) Triton X-100, 1 mM DTT. The assay consisted of desalted extract in 50 mM Tris-HCl (pH 7.5), 5 mM MgCl2, 1 mM DTT and 2 mM glucose-1-phosphate (G1P) in a volume of 450 µl. The reaction was initiated by the addition of 50 µl protein extract to the reaction mixture and incubated at 30 °C for 30 min. The reaction was terminated by boiling at 100 °C for 2 min. After centrifugation at 10 000 *g* for 5 min the supernatant was collected. In a microplate, a 200 µl assay buffer consisting of 50 mM Tris-HCl pH 7.5, 5 mM MgCl2, 1 mM NAD+ was added and combined with 50 µl of supernatant before being incubated at room temperature for 10 min. The reaction was initiated by the addition 0.2 U G6PDH and the increase in OD_{340} determined.

Statistical analysis

All statistical analyses were performed using GraphPad Prism software (GraphPad Prism version 7.04 for Mac OS X, GraphPad Software, La Jolla California USA, http://www.graphpad.com) and Geneious Prime for sequence analysis. Experimental values were expressed as the mean ± standard error of mean (SEM) of three independent experiments. Significant differences between two groups were identified using nonparametric one-way ANOVA with Tukey *posthoc* test. Mean differences were considered significant at $P < 0.05$.

3.3 Results

Molecular analysis: *AGPase* and *PGM* gene expression in uDhali leaves

Amplification of both genes from cDNA and gDNA was attempted using PCR. The *AGPase* cDNA amplicon was determined to be around 1500 bp and *PGM* to be 1900 bp (Figure 1). Amplicon sizes of *AGPase* were similar when either gDNA or cDNA was used as template, but amplification of *PGM* from gDNA was unsuccessful despite effort in optimizing the reaction.

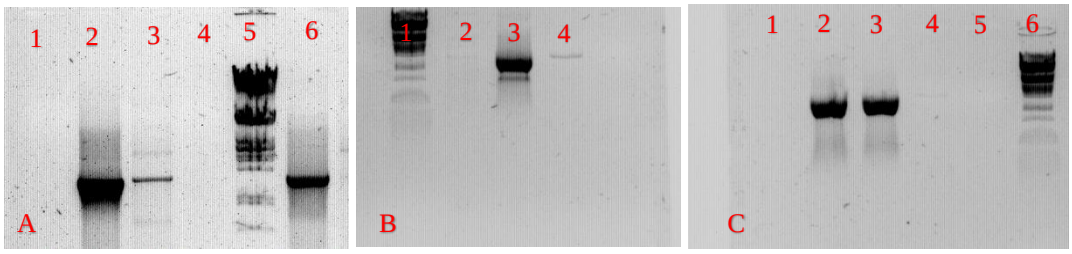

Figure 1: cDNA and gDNA amplification of *AGPase* and *PGM* in uDhali leaves. A shows PCR amplicons of *AGPase* from gDNA cloned into the pGEMT-easy vector, lane 1 is the blank, 2, 3, 4 and 6 are AGPase samples and 5 is the molecular weight marker (MW; λ DNA digested with *PstI*). B and C represent *AGPase* and *PGM* cDNA, respectively. In B lane 1 is the MW marker, 2 as the blank and 3 and 4 were *AGPase* samples. In C lane 1 was the blank, 2-5 were *PGM* samples and 6 the MW marker.

Gel electrophoresis and sequencing of amplicons demonstrated that the *AGPase* was the same size for both cDNA and gDNA. Sequence alignment of the cDNA and gDNA amplicons revealed both *AGPase* sequences were identical and a BLASTn search showed that both sequences were identical to a pigeon pea mRNA sequence thought to encode the small subunit of AGPase (XM_020353377) that is already sequenced from a different pigeon pea variety. A similar search using sequence obtained from the phosphoglucomutase (*PGM*) cDNA showed that, the sequenced aligned to a putative pigeon pea chloroplastic PGM isoform (XM_020378966).

RT-qPCR gene expression in uDhali leaves

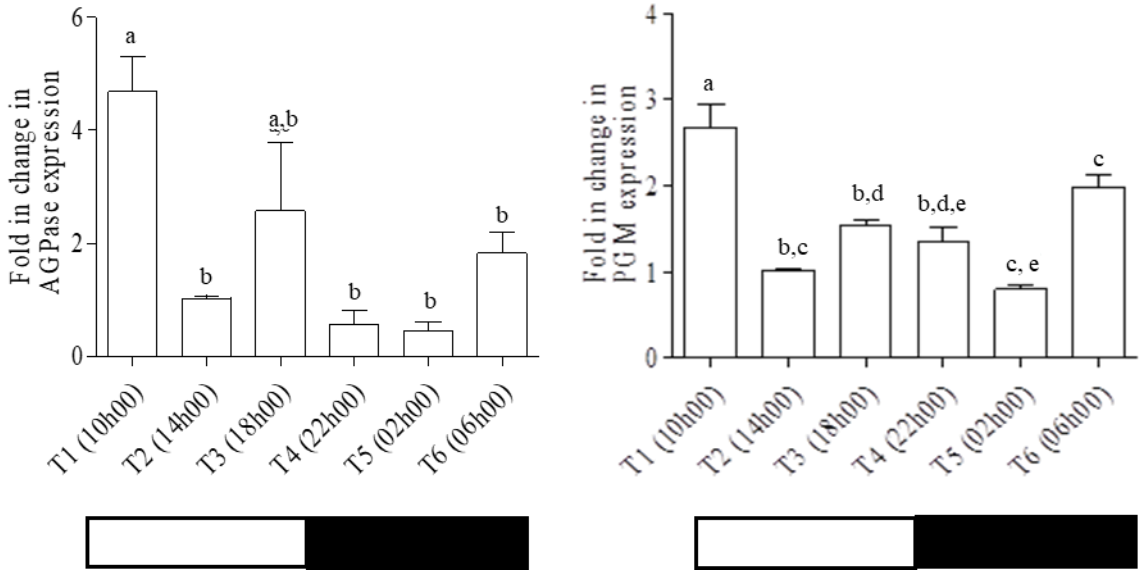

Figure 2: Quantitative expression analysis of pigeon pea uDhali variety leaf *ADP-glucose pyrophosphorylase* (*AGPase*) and *phosphoglucomutase* (*PGM*) genes.

Samples were taken every 4 h over a day/night cycle (12 h day/night with day starting at 06h10), and are calibrated against T2 (14h00). Block bars represent time of the day with being the day and black representing the night. Statistical analysis was performed using one-way ANOVA with Tukey *posthoc* tests., data was found significant at $P < 0.05$. The letters $^{a} - ^{e}$ indicate significance differences between data points.

Expression of *AGPase* and *PGM* in leaves during the day and night was assessed using RT-qPCR over a 24 h period. The results revealed that both *AGPase* and *PGM* genes were expressed at varying levels throughout the day. *AGPase* was most highly expressed early in the morning (T1) and had its lowest expression was at night [T4 (22h00) and T5 (02h00)]. For this gene, expression at T1 was observed to be significantly different to all time points except for T3. The time point T6 which fell under the night period, showed an increase in the expression *of AGPase* at 06h00 before dawn unlike T4 and T5 which also which was also at night but had reduced *AGPase* expression.

Expression of *PGM* (Figure 2) showed a similar pattern to that of *AGPase*. The T2 to T6 timepoints had lower expression that T1 with a rise in expression just before dawn at the T6

time point. These findings are consistent with the role of these genes being involved in daytime starch synthesis. For both the genes, expression at night varied as observed with T5 and T6 of both genes especially for *PGM* (Figure 2).

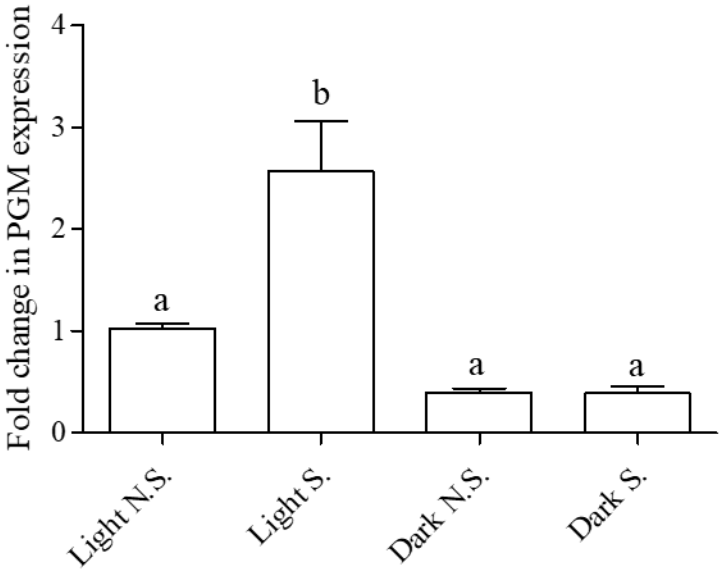

Figure 3: Quantitative gene expression analysis in excised leaves of pigeon pea uDhali variety *phosphoglucomutase* (*PGM*). Statistical analysis was performed using one-way ANOVA with a Tukey *posthoc* test, with $P < 0.05$. The figure legend is presented as: Light N.S. is light no sucrose, Light S. is light with sucrose, Dark N.S. is darkness no sucrose and Dark S. darkness with sucrose. The letters ^{a} – ^{b} indicate significance difference between data points.

An RT-qPCR experiment to evaluate expression levels of *PGM* in pigeon pea leaves in extended light/darkness with or without exogenous sucrose supply revealed the leaves to have significantly higher gene expression in conditions with extended light exposure and sucrose than all other conditions (Figure 3). Interestingly, even with exogenous sucrose supply, leaves kept in darkness did not show increased transcript accumulation, having the same expression as the leaves kept in darkness without any sucrose.

Enzyme activity

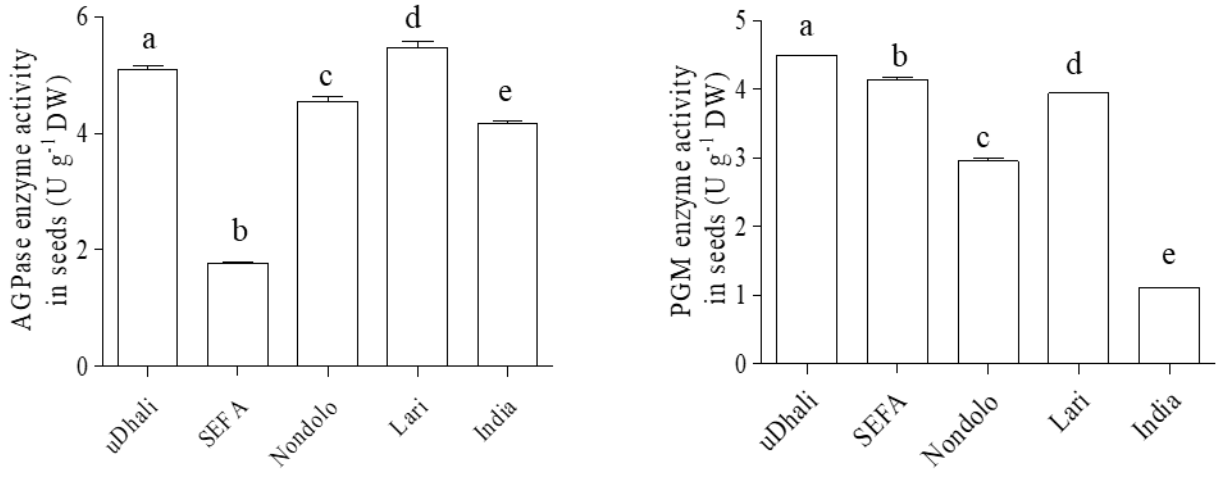

Figure 4: Enzyme activity of ADP-pyrophosphorylase (AGPase) and phosphoglucomutase (PGM). Mature seed varieties were subjected to the same enzyme assay and statistical analysis was performed using one-way ANOVA with Tukey *posthoc* tests. Different letters indicate significant differences ($P<0.05$). The letters ^{a} – ^{e} indicate significance difference between data points.

Enzyme activities of AGPase and PGM were measured in all five varieties in both mature seeds and leaves. In seeds, AGPase was more active than PGM. AGPase activity was detected in all seeds in varying amounts with significant variation present between varieties. At seed maturity, AGPase activity was determined to be about 2-3 times greater in the starch positive seeds than in SEFA (Figure 4).

Analysis of PGM activity showed that this varied in seeds of the different varieties. India demonstrated the lowest PGM activity and uDhali the greatest. Activities in seeds of all varieties were significantly different from each other for both AGPase and PGM.

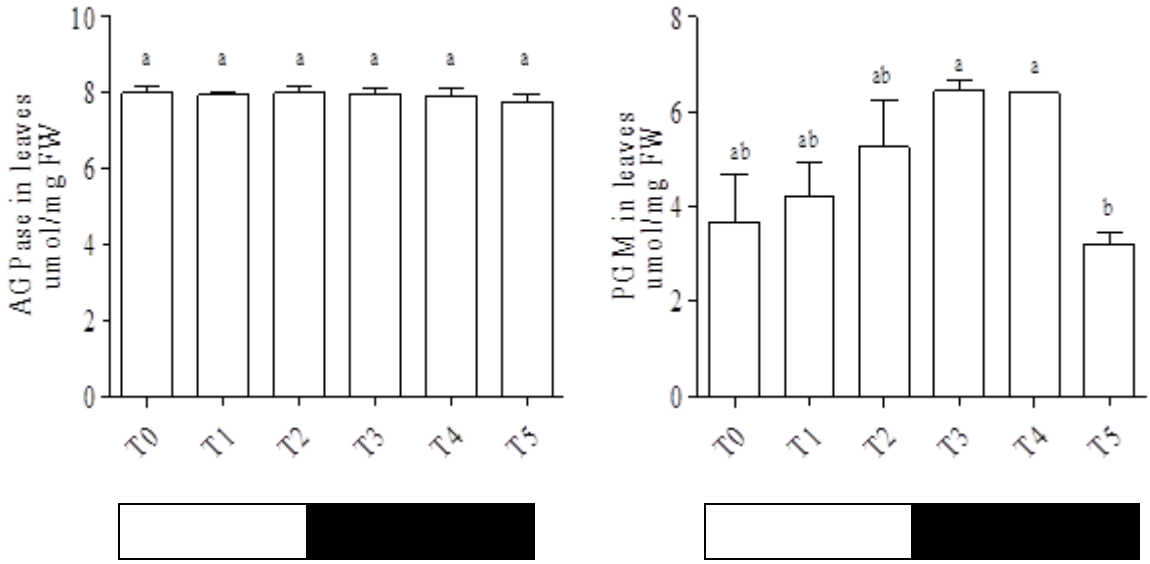

Figure 5: ADP-pyrophosphorylase (AGPase) and phosphoglucomutase (PGM) enzyme activity in uDhali leaves over 24 h (12 h day/night), at 4 h harvest intervals with the day starting at 06h10. The blocks represent the time of the day, white being day time and black representing the night period. Statistical analysis was performed using one-way ANOVA with Tukey *posthoc* test. Different letters indicate significance differences ($P<0.05$). The letters ^{a} – ^{b} indicate significance difference between data points.

Activities of both enzymes were determined in leaves of the uDhali variety over a day night cycle. All leaves demonstrated the presence of activity from both enzymes. AGPase was invariable throughout the day (Figure 5). Unlike the AGPase activity, PGM displayed varying activities, with T6 being significantly decreased compared with T4 and T5.

3.4 Discussion

As variation in starch content was observed in seeds from the different varieties (Chapter 2), molecular analysis examining genes involved in starch synthesis was undertaken in uDhali. This was used rather than the starchless SEFA variety as SEFA seeds failed to germinate when grown in soil, peat or tissue culture. It is unclear if this inability to germinate was caused by the lack of starch or poor seed storage prior to its acquisition. SEFA seeds lacked starch and would often die after the emergence of the radicle, and none germinated sufficiently to allow for the emergence of the plumule. It could be theorised that the low starch may have caused this as starch acts as a carbon reserve for geminating seeds, however, a similar low starch phenotype in the pea *rug3* mutant has never been reported to affect seed viability (Harrison *et al*., 2000).

Amplification of the two coding sequences for *AGPase* and *PGM* revealed the lengths of the amplicons to be 1500 bp and 1900 bp respectively. (Figure 1). Sequences of both amplicons demonstrated them to encode a small subunit of *AGPase* and plastidial *PGM* from pigeon pea. Amplicons from both cDNA and gDNA were the same length for *AGPase*. The most likely explanation for the similarity in size is that the gDNA amplicon is the result of contamination as the corresponding pigeon pea gene is reported to contain 9 exons and be 3572 bp in length (https://www.ncbi.nlm.nih.gov/gene/109793910), although varietal differences cannot be ruled out.

Amplification of *PGM* was only possible from cDNA and the amplicon was shown to be identical in sequence to a pigeon pea *PGM* cDNA. Due to the challenges encountered amplifying the gDNA and limited time, other methods such as amplifying and sequencing multiple short fragments of gDNA and, then assembling them together could not be done. In future studies it would be best to explore other method of obtaining genomic sequence from this variety. Difficulties in amplifying genomic DNA are associated with long fragments and hydrogen bonding which can be overcome through optimisation of PCR conditions (Hill *et al*., 2013; Strien *et al*., 2013; Mamedov *et al*., 2008). Although much effort was spent in optimising conditions for amplification of the 2683 bp *PGM* gene (https://www.ncbi.nlm.nih.gov/gene/?term=XM_020378966), all attempts were unsuccessful.

The present study demonstrated that expression of both *AGPase* and *PGM* in leaves changed over a day/night cycle (Figure 2). Expression of *AGPase* at T1 was higher than all the other time points except T3. *AGPase* was observed to have the lowest expression at night (T4 and

T5). This would be expected as leaves cannot synthesise starch at night and it is known that starch synthesising genes are often downregulated in the dark (Batra *et al*., 2017; Saripalli and Gupta, 2015; Huang *et al*., 2013).

PGM expression was observed to follow as similar pattern to that of *AGPase* (Figure 2). Two time points (T1 and T6) demonstrated the highest and second highest expression, respectively. These were just before (T6) and just after (T1) the lights were turned on which indicates that plants can sense and anticipate dawn as has been shown for the expression of several genes involved in starch metabolism (Arias *et al*., 2014). Interestingly, *PGM* expression at T3 and T4 were at similar levels, despite T3 being during the day and T4 at night. One explanation for this is the mRNA degradation was not occurring rapidly enough for us to see a decrease at T4, but it is also possible that expression of *PGM* in pigeon pea differs from other plants. .

PGM expression was examined in leaves supplied with external sucrose under extended light or dark see if the presence of sucrose enhances the expression. This revealed that *PGM* expression increased only in the combination of sucrose and extended light (Figure 3), an observation that differs from studies where sucrose was shown to induce expression of genes involved in starch synthesis (Duwenig et al 1997; Kossman *et al*., 1991; Müller-Röber *et al*., 1990; Baysdorfer and Robinson; 1985). Those experiments, however, used leaf discs placed on sugar solutions and differ, therefore, from the experimental setup that was used in the current study where entire leaves were used.

Analysis of enzyme activity in seeds and leaves revealed that both organs contained both AGPase and PGM activities. Variation in activity within seeds of the different varieties was observed with the low AGPase activity in SEFA potentially helping to explain the lack total starch present in this variety (Chapter 2). When compared to the starch positive varieties, AGPase enzyme activity was 2 times lower in SEFA than the other varieties (Figure 4, Chapter 2).

PGM enzyme activity in SEFA was greater than the other varieties, indicating the seeds have functional PGM activity even though they lacked starch. This does not rule out the possibility that a mutation in plastidial *PGM* is responsible to the essentially starchless phenotype observed in SEFA seeds as the activity observed may come from the cytosolic isoform. To examine this a zymogram where the two isoforms are separated could be performed. The India variety had the least PGM activity amongst all seeds, even though it was starch positive whereas the rest of the seed varieties displayed similar activities.

Most legume crops are known to have similar activities of these enzymes which are higher that our findings. Those other studies were performed on immature embryos (Atlthammer *et al*., 2020; Kaur *et al*., 2017) rather than mature ones used in this study. Activities should be measured, therefore, at various stages during embryo development to examine the enzymes at times of active starch synthesis.

Enzyme activity in leaves differed from that of seeds especially for AGPase, significant differences were observed in all enzyme activities in seeds where as in leaves it was only observed within the PGM activity. Measures of AGPase activity in leaves were the same at all time points. For PGM that was not the case as two time points at night (T4 and T5; Figure 5) were observed to exhibit higher enzyme activity than the three that fell during the day (T1, T2 and T3). The activity at the end of the night (T6) was also lower than at T4 and T5. The use of zymograms to examine the plastidial and cytosolic PGM isoforms would again be helpful in this instance to examine if one, or both isoforms are increasing in activity at T4 and T5.

Since this is the first study of its kind on South African pigeon pea seeds and leaves, further studies and repeats of the current ones still need to be undertaken with possible increased sample size to have more knowledge on the local varieties. This will allow better knowledge of starch metabolism in these plants and potentially help develop them as a crop.

Closing commentary

Studies on legumes have increased in the past few decades and neglected legume crops are now starting to become the new focus of scientific endeavour. The orphan crop, pigeon pea, which has good drought tolerance and nutritional content is gaining traction because of these attributes. From this study, the seeds contained high amounts of resistant starch of more than 50% which is unusual in crops. Seeds from most varieties contained high resistant starch indicating that pigeon pea seeds would be a good addition to daily meals for a healthy diet as the resistant starch would release carbohydrates into the bloodstream relatively slowly. This would improve the health of individuals who consume them, especially those with type-2 diabetes where they would help manage the disease reducing cardiovascular problems.

The relatively high amounts of resistant starch (up to 76% in the India variety) could give this variety a competitive edge as a food. Further studies are needed to determine the chemical properties of starch from the India variety to help understand why the starch is so resistant to digestion. A second key finding was the identification of the low starch (0.3%) variety (SEFA) The lack of wrinkled seeds in SEFA make them phenotypically distinct from the near starchless pea *rug-3* mutant, which demonstrates a wrinkled seeded phenotype (Harrison *et al*., 2000). The reduced starch in that mutant is known to be accompanied by high amounts of soluble sugars. Quantification of sugars in SEFA seeds was not performed, although LC-MS data indicates that they contain similar amounts of sugars at maturity as the other varieties.

Variations in expression of *AGPase* and *PGM* and determination of PGM and AGPase activities in seeds demonstrate differences between varieties. Low activities of AGPase and PGM were observed respectively in seeds from India and SEFA, whereas in leaves similar AGPase activity was observed throughout the day. The presence of starch in both seeds and leaves indicates that both genes studied encode enzymes that are capable of synthesising the precursors for starch polymer formation. I only measured activities in mature seeds and determining enzyme activity during different seed development stages would add more insight to how AGPase and PGM enzyme activities differs during seed development. In addition separation and analysis of the cytosolic and plastidial PGM activities could be performed using activity gels to provide insight into the activities of both isoforms. It is possible for example that the PGM activity measured in the SEFA variety comes from the cytosolic protein. A more indepth survey of other starch synthesising enzymes will need to be provide a full understanding of starch biosynthesis in pigeon pea.

Leaves of the uDhali variety contained starch and when enzyme activities were determined, AGPase was observed to be unchanged throughout the day whereas PGM activity differed between day and night. The expression of these genes was also observed to be similar to previous work examining starch metabolic genes, being more highly expressed during the day than at night (Batra *et al*., 2017; Janneke *et al*., 2003). All data from the leaves was produced using an excised leaf system which is useful in terms of allowing easy control of environmental conditions, but its effects on leaf physiology need to be assessed in more detail.

It can be concluded that pigeon pea contains more resistant starch than most legumes at seed maturity which might help it be exploited in the food industry, however, more research on the crop and different growth stages is still needed. Different seed developmental stages should be studied to examine nutritional values of starch and proteins as well as accumulation of secondary metabolites that may have medicinal properties. For analysis of soluble sugars, the use of better separation conditions to distinguish and quantify the various sugars is recommended to improve resolution of soluble sugars would be helpful.

References

- Abobatta WF. 2009. Drought adaptive mechanisms of plants – A review. *Advances in Agriculture and Environmental Science: Open Access*. Vol. 2(1), pp. 62−65. DOI: 10.30881/aaeoa.00022.
- Aguirre M, Kiegle E, Leo G, and Ezquer I. 2018. Carbohydrate reserves and seed development: An overview. *Plant Reproduction*. Vol. 31(3), pp. 263–290. DOI: org/10.1007/s00497-018-0336-3.
- Ahmar S, Gill RA, Jung KH, Faheem A, Qasim MU, Mubeen M and Zhou W. 2020. Conventional and molecular techniques from simple breeding to speed breeding in crop plants: recent advances and future outlook. Review. *International Journal of Molecular Sciences*. Vol. 21(2590), pp. 1-24. DOI:10.3390/ijms21072590.
- Ahmed S and Hasan M. 2014. Legumes: An overview. *Journal of Pharmacy and Pharmaceutical Sciences*. Vol. 2(1), pp 34-38. ISSN (online) 2321-8484.
- Akihiro T, Mizuno K and Fujimura T. 2005. Gene expression of ADP-glucose pyrophosphorylase and starch contents in rice cultured cells are cooperatively regulated by sucrose and ABA. *Plant and Cell Physiology*. Vol. 46(6), pp. 937–946. DOI: 10.1093/pcp/pci101.
- Alcázar-Alay SC and Meireles MAA. 2015. Physicochemical properties, modifications and applications of starches from different botanical sources. *Journal of Food Science and Technology*. Vol. 35(2), pp. 215–236. DOI: 10.1590/1678-457X.6749.
- Allen ON. and Allen EK. 1981. The Leguminosae: A source book of characteristics, uses and nodulation. University of Wisconsin Press. pp.812. ISBN 0-333-32221-5.
- Althammer M, Blöchl Constantin, Reischl Roland, Huber CG and Tenhaken R. 2020. Phosphoglucomutase is not the target for galactose toxicity in plants. *Frontiers in Plant Science*. Vol.11, pp. 1–9. DOI: 10.3389/fpls.2020.00167.
- Arias1 MC, Pelletier S, Hilliou F, Wattebled F, Renou JP and D'Hulst C. 2014. From dusk till dawn: The Arabidopsis thaliana sugar starving responsive network. *Frontiers in Plant Science*. Vol. 482(5), pp.1–9. DOI: 10.3389/fpls.2014.00482.
- Atkinson FS, Foster-Powell K and Brand Miller JC. 2008. International tables of glycemic index and glycemic load values: 2008. *Diabetes Care, Peer-reviewed Journal*. Vol. 31(12), pp. 18–20. DOI: 10.2337/dc08-1239.J.B.M.

- Ayenan MAT, Ofori K, Ahoton LE and Danquah A. 2017. Pigeon pea (*Cajanus cajan* (L.) Millsp.) production system, farmers' preferred traits and implications for variety development and introduction in Benin. *Journal of Agriculture and Food Security*. Vol. 6(48), pp. 1–11. DOI 10.1186/s40066-017-0129-1.
- Bahadoran Z and Mirmiran P. 2015. Potential properties of legumes as important functional foods for management of type 2 diabetes: a short review. *International Journal of Nutrition and Food Sciences*. Special Issue: Functional Foods and Nutraceuticals for Management of Type 2 Diabetes. Vol. 4(2), pp. 6-9. DOI: 10.11648/j.ijnfs. s.2015040201.12.
- Bahaji A, Li J, Ovecka M, Ezquer I, Mun FJ, Romero JM, Almagro G, Montero M, Hidalgo M, and Baroja-Ferna, E. 2011. *Arabidopsis thaliana* mutants lacking ADP-glucose pyrophosphorylase accumulate starch and wild-type ADP-glucose content: further evidence for the occurrence of important sources, other than ADP-glucose pyrophosphorylase, of ADP-glucose linked to leaf starch biosynthesis. *Plant and Cell Physiology*. Vol. 52, pp. 1162–1176. DOI: org/10.1093/pcp/pcr067.
- Bailey-Serres J, Parker JE, Ainsworth EA, Oldroyd GED and Schroeder JI. 2019. Genetic strategies for improving crop yields. *Nature*. Vol. 575(7781), pp. 109–118. DOI: org/10.1038/s41586-019-1679-0.
- Baris I, Tuncel A, Ozber N, Keskin O, Kavakli IH. 2009. Investigation of the interaction between the large and small subunits of potato ADP-glucose pyrophosphorylase. *PLOS Computational Biology*. Vol. 5(10), 1–14. DOI: 10.1371/journal.pcbi.1000546.
- Batra R, Saripalli G, Mohan A, Gupta S and Gill KS, Varadwaj PK, Baylan HS and Gupta PK. 2017. Comparative analysis of AGPase genes and encoded proteins in eight monocots and three dicots with emphasis on wheat. *Frontiers in Plant Science*. Vol. 8(19), pp. 1–18. DOI: 10.3389/fpls.2017.00019.
- Baysdorfer C and Robinson JM. 1985. Sucrose and starch synthesis in spinach plants grown under long and short photosynthetic periods. *Plant Physiology*. Vol. 79(3), pp. 838–842. DOI: 10.1104/pp.79.3.838.
- Birt DF, Boylston T, Hendrich S, Jane JL Hollis J, Li L *et al*. 2013. Resistant starch: Promise for improving human health. *Advances in Nutrition*. Vol. 4(6), pp. 587–601. DOI: 10.3945/an.113.004325.
- Blair EJ, Bonnot T, Hummel M, Hay E, Marzolino JM, Quijada IA and Nagel DH. 2019. Contribution of time of day and the circadian clock to the heat stress responsive

transcriptome in *Arabidopsis*. *Scientific Reports*. Vol. 9(1), pp. 36–40. DOI: 10.1038/s41598-019-41234-w.

- Boehlein SK, Shaw JR, Boehlein TJ, Boehlein EC, Hannah LC. 2018. Fundamental differences in starch synthesis in the maize leaf, embryo, ovary and endosperm. *The Plant Journal*. Vol. 96(3), pp. 595–606. DOI: 10.1111/tpj.14053.
- Bouchenak M and Lamri-senhadji M. 2013. Nutritional quality of legumes, and their role in cardiometabolic risk prevention: A Review. *Journal of Medicinal Food*. Vol. 16(3), pp. 185-98. DOI: 10.1089/jmf.2011.0238.
- Bradshaw D, Steyn K Levitt, N and Nojilana, B. 2011. Non-communicable diseases: A race against time. *South African Medical Research Council*. Chronic Disease Initiative for Africa, Department of Medicine, University of Cape Town.
- Brauman KA, Richter BD, Postel S, Malsy M and Flörke M. 2016. Water depletion: An improved metric for incorporating seasonal and dry-year water scarcity into water risk assessments. *Elementa: Science of the Anthropocene*. Vol. 4, pp. 1–13. DOI: 10.12952/journal.elementa.000083.
- Bravo L, Siddhuraju P and Saura-Calixto F. 1999. Composition of underexploited Indian pulses: comparison with common legumes. *Food Chemistry*. Vol. 64(2), 185–192. DOI: 10.1016/S0308-8146(98)00140-X.
- Brinkman HJ and Hendrix C. 2011. Food insecurity and violent conflict: Causes, consequences, and addressing challenges. *World Food Programme*. Occasional paper number 24, pp. 1–33. DOI: 10.13140/2.1.3379.2003.
- Brock RD. 1965. Induced mutations affecting quantitative characters. *Radiation Botany*. Vol. 5, pp. 451-464.
- Cejudo FJ, Ojeda V, Delgado-Requerey V, González M and Pérez-Ruiz JM. 2019. Chloroplast redox regulatory mechanisms in plant adaptation to light and darkness. *Frontiers in Plant Science*. Vol. 10(380), pp. 1–11. DOI: 10.3389/fpls.2019.00380.
- Chatterton NJ and Silvius J. 1979. Photosynthate partitioning into starch in soybean leaves: effects of photoperiod versus photosynthetic period duration. *Plant Physiology*. Vol. 64, pp. 749-753. DOI: 10.1104/pp.64.5.749
- Cheng L, Liu X, Yin J, Yang J, Li Y, Hui L, et al. 2016. Activity and expression of ADP-glucose pyrophosphorylase during rhizome formation in lotus (*Nelumbo nucifera* Gaertn.). *Botanical Studies*. Springer Berlin Heidelberg, 57(1), pp. 1-11. DOI: 10.1186/s40529-016-0140-z.

- Coetzee A, Taljaard JJ, Hugo SS, Conradie M, Conradie-Smit M and Dave J. A. 2020. Diabetes mellitus and COVID-19: a review and management guidance for South Africa. *South African Medical Journal*. Vol. 110(8), pp. 761–766. DOI: 10.7196/SAMJ.2020.v110i8.14881.
- Considine MJ, Siddique KHS, and Foyer CH. 2017. Nature's pulse power: Legumes, food security and climate change. *Journal of Experimental Botany*. Vol. 68(8), pp. 1815–1818. DOI: 10.1093/jxb/erx099
- Couée I, Sulmon C, Gouesbet G, and El Amrani A. 2006. Involvement of soluble sugars in reactive oxygen species balance and responses to oxidative stress in plants. *Journal of Experimental Botany*. Vol. 57(3), pp. 449–459. DOI: 10.1093/jxb/erj027.
- Cullis C and Kunert KJ. 2016. Unlocking the potential of orphan legumes. *Journal of Experimental Botany*. Vol. 68(8), pp. 1895–1903. DOI: 10.1093/jxb/erw437.
- Cullis C, Lawlor DW, Chimwamurombe P, Bbebe N, Kunert K and Vorster J. 2019. Development of marama bean, an orphan legume, as a crop. *Food and Energy Security*. Vol. 8(3), pp. 1–12. DOI: 10.1002/fes3.164.
- D'Haese L, Van Rooyen J, Vink N, Kirsten J, Staelens L, Vandamme E, Shonfeld H, Remaut-Dewinter AM, D'Haese M. 2011. Food security in Limpopo province. Limpopo project in partnership with University of Pretoria, Stellenbosch University, University of Antwerpen, University of Gent, Limpopo Provincial Government, National Agricultural Marketing Council and Red Meat Research and Development South Africa (RMDR SA). pp. 1-97.
- D'Hulst C, Wettebled F and Szydlowski N. 2015. Starch biosynthesis in leaves and its regulation. Book: Starch metabolism and structure. pp. 221-237. DOI:10.1007/978-4-431-55495-0_6.
- DAFF (Department of Agriculture, Forestry and Fisheries). 2011. Production guidelines for Cowpeas. Compiled by Directorate Plant Production in collaboration with the ARC. pp. 1-24.
- DAFF (Department of Agriculture, Forestry and Fisheries). 2016. Agriculture, Forestry and Fisheries on BRICS (Brazil, Russia, India, China, South Africa) meetings on Agriculture ministers. Annual report. pp. 1-3
- DAFF (Department of Agriculture, Forestry and Fisheries). 2017. From http://www.daff.gov.za/Daffweb3/Portal/0/Brochures%20and%20Production%20guidelines/Pigeonp%25peas%20-Brochure.pdf.

- Davani-Davari D, Negahdaripour I, M, Mohkam M, Masoumi SJ, Berenjian A and Ghasemi Y. 2019. Prebiotics: Definition, types, sources, mechanisms, and clinical applications. Foods: MPDI. Vol. 8(3), pp. 92. DOI: 10.3390/foods8030092.
- Davis KF, Gephart JA, Emery KA, Leach AM, Galloway JN and D'Odorico P. 2016. Meeting future food demand with current agricultural resources. *Global Environmental Change*. Vol. 39, pp. 125–132. DOI: 10.1016/J.GLOENVCHA.2016.05.004.
- Devindra S, Shankar SK and Mulimani, VH. 2006. Reduction of raffinose oligosaccharides in red gram flour by microbial α-galactosidase. *Indian Journal of Plant Physiology*. Vol. 11(2), pp. 123–129.
- Ding Y, Hayes MJ and Widhalm M. 2010. Measuring economic impacts of drought: A review and discussion. *Natural Resources*. Vol. 20(4), pp. 434–446. DOI: 10.1108/09653561111161752.
- Eashwarage IS, Herath HMT and Gunathilake KGT.2017. Dietary fibre, resistant starch and in-vitro starch digestibility of selected eleven commonly consumed legumes (Mung bean, Cowpea, Soybean and Horse dietary) in Sri Lanka. *Research Journal of Chemical Sciences*. Vol. 7(2), pp 1-7. ISSN 2231-606X. www.isca.in, www.isca.me.
- Ecocrop. 2016. Food and Agriculture Organization (FAO), Rome, Italy. https://ecocrop.fao.org/ecocrop/srv/en/home.
- Egli B, Kölling K, Köhler C, Zeeman SC and Streb S. 2010. Loss of cytosolic phosphoglucomutase compromises gametophyte development in *Arabidopsis*. *Plant Physiology*. Vol. 154, pp. 1659–1671. DOI: 10.1104/pp.110.165027.
- Elango D, Rajendran K, Van der Laan L, Sebastiar S, Raigne J, Thaiparambil NA, Haddad NE, Raja B, Wang W, Ferela A, Chiteri KO, Thudi M, Varshney RK, Chopra S, Singh A and Singh AK. 2022. Raffinose Family Oligosaccharides: Friend or Foe for Human and Plant Health? Frontiers in Plant Science. Vol. 13, pp. 1-16. DOI: 10.3389/fpls.2022.829118.
- Fabbri ADT, Schacht RW and Crosby GA. 2016. Evaluation of resistant starch content of cooked black beans, pinto beans, and chickpeas. *Journal of Nutrition and Food Science*. Vol. 3(2), pp. 8–12. ISSN (online): 2352-3646.
- Faměra1 O, Mayerová M, Burešová I, Kouřimská L and Prášilová M. 2015. Influence of selected factors on the content and properties of starch in the grain of non-food wheat. *Plant Soil and Environment*. Vol. 61(6), pp. 241–246. DOI: 10.17221/13/2015-PSE.

- FAO (Food and Agriculture Organization) of the United Nations. 2017. International year of pulses: nutritious seeds for a sustainable future. http://www.fao.org/3/a-bb029e.
- FAO (Food and Agriculture Organization). 2003. Focus on Food Insecurity and Vulnerability – A review of the UN system common country assessments and world bank poverty reduction strategy papers. FIVIMS Secretariat and Wageningen University and Research Centre: www.fao.org/DOCREP/006/Y5095E/Y5095E00.htm.
- FAO (Food and Agriculture Organization). 2017. Pigeon pea.
- FAOSTAT (Food and agriculture Organization of the United Nations, Statistics). 2015. Agricultural production: Pigeon pea.
- Farooq M, Wahid A, Kobayashi N, Fujita D and Basra SMA. 2009. Plant drought stress: effects, mechanisms and management. *Agronomy for Sustainable Development*. Vol. 23, pp. 185-212. DOI: 10.1051/agro:2008021.
- Feedpedia. 2016. Pigeon pea (*Cajanus cajan*) seed. Animal feed resources information system. From: Feedpedia.org/node/329.
- Fernandez O, Ishihara H, George GM, Mengin V, Flis A, Sumner D, Arrivault S, Feil R, Lunn JE, Zeeman SC, Smith AM and Stitt M. 2017. Leaf starch turnover occurs in long days and in falling light at the end of the day. *Plant Physiology*. Vol. 174(4), pp. 2199–2212. DOI: 10.1104/pp.17.00601.
- Foyer CH, Lam H-M, Nguyen HT, Kadambot SHM, Varshney R, Colmer TD, *et al*. 2016. Neglecting legumes has compromised human health and sustainable food production. *Nature Plants*. Vol. 2. 16112. ISSN 2055-026X. Nature Plants, In press. DOI: https://doi.org/10.1038/nplants.2016.112.
- FRAC (Food Resource and Action Center). 2017. The impact of poverty, food insecurity, and poor nutrition on health and well-being. *Hunger and Health*. Vol. 2, pp. 1-14. https://frac.org/wp-content/uploads/hunger-health-impact-poverty-food-insecurity-health-well-being.
- Frohberg C, Leube M and Rumpler I. 2003. Transgenic fodder plants with an increased leaf starch content. *U.S. Patent Application.* Vol. 10 (371), pp.077.
- Gaikwad S, Sharma K and Kanungo VK. 2013. Comparison of changes in sugar contents in the cotyledons of *Trigonella* and *Tamarindus*. *Recent Research in Science and Technology*. Vol. 5(4), pp. 29–31. ISSN: 2076-5061. http://recent-science.com.
- Gajzago I. S. 2004. Nutritional aspects of legumes. In: Cultivated plants, primarily as food sources. *Encyclopedia of Life Support Systems* (EOLSS). Vol. 1, pp. 101-114.

- Gangola MP, Jaiswal S, Kannan U, Gaur PM, Båga M and Chibbar RN. 2016. Galactinol synthase enzyme activity influences raffinose family oligosaccharides (RFO) accumulation in developing chickpea (*Cicer arietinum* L.) seeds. *Phytochemistry*. Vol. 125, pp. 88-98. DOI: 10.1016/j.phytochem.2016.02.009.
- Garcia IS, Souza A, Barbedo CJ, Dietrich SMC and Figueiredo-Ribeiro RCL. 2006. Changes in soluble carbohydrates during storage of *Caesalpinia echinata* LAM. (Brazilwood) seeds, an endangered leguminous tree from the Brazilian Atlantic forest. *Brazilian Journal of Biology*. Vol. 66(2), pp. 739–745. DOI: 10.1590/s1519-69842006000400018.
- Georgelis N, Braun EL, Shaw JR and Hannah LC. 2007. The two AGPase subunits evolve at different rates in angiosperms, yet they are equally sensitive to activity-altering amino acid changes when expressed in bacteria. *The Plant Cell*. Vol. 19(5), pp. 1458–1472. DOI: 10.1105/tpc.106.049676.
- Gill PK, Sharma AD, Singh P and Bhullar SS. 2002. Changes in germination, growth and soluble sugar contents of Sorghum bicolor (L.) Moench seeds under various abiotic stresses. *Plant Growth Regulations*. Vol. 40, pp. 157-162. DOI: 10.1023/A:1024252222376.
- Goto LS, Vessoni AA, Malvessi PC, Silva MC, D'Muniz PH, Brandão-Neto, Jé, *et al*. 2016. Structural and functional characterization of the phosphoglucomutase from *Xanthomonas citri* subsp. *citri*. *Biochimica et Biophysica Acta - Proteins and Proteomics*. Elsevier B.V., Vol. 1864(12), pp. 1658–1666. DOI: 10.1016/j.bbapap.2016.08.014.
- Guillon F and Champ MM. 2002. Carbohydrate fractions of legumes: uses in human nutrition and potential for health. *British Journal of Nutrition*. Vol. 88(3), pp. 293-306. DOI: 10.1079/BJN2002720.
- Hajduch M, Matusova R, Houston NL and Thelen JJ. 2011. Comparative proteomics of seed maturation in oilseeds reveals differences in intermediary metabolism. *Journal of Proteomics*. Vol. 11(9), pp. 1619–1629. DOI: 10.1002/pmic.201000644.
- Hallegatte S, Green C, Nicholls RJ and Corfee-Morlot J. 2014. “Future flood losses in major coastal cities”. *Nature Climate Change*. Vol. 3. pp. 802–806.
- Hamblin J. 2015. Quickening nature's pulse: Atomic agriculture at the International Atomic Energy Agency. *Dynamis*. DOI: 10.4321/S0211-95362015000200006.

- Harrison CJ, Hedley CL and Wang TL. 1998. Evidence that the *rug3* locus of pea (*Pisum sativum* L.) encodes plastidial phosphoglucomutase confirms that the imported substrate for starch synthesis in pea amyloplasts is glucose-6-phosphate. *The Plant Journal*. Vol. 13(6), pp. 753–762. DOI: 10.1046/j.1365-313X.1998.00077.x.
- Harrison CJ, Mould RM, Leech MJ, Johnson SA, Turner L, Schreck SL, Baird KM, Jack PL, Rawsthrone S, Hedley CL and Wang TL. 2000. The *rug3* locus of pea encodes plastidial phosphoglucomutase. *Plant Physiology*. Vol. 122(4), pp. 1187–1192. DOI: org/10.1104/pp.122.4.1187.
- Hartline-Grafton H and Dean O. 2017. Hunger and health: the impact of poverty, food insecurity, and poor nutrition on health and well-being. Washington, DC: *Food Research and Action Center*. Vol. 7, pp. 1–14.
- Hill PJ and Stewart GSAB. 1992. The polymerase chain reaction in molecular and micro-biology. *Biotechnology and Genetic Engineering Reviews*. Vol. 10:1, pp. 342-376. ISSN: 343-378, DOI: 10.1080/02648725.1992.10647892.
- Hincha DK, Zuther E and Heyer AG. 2003. The preservation of liposomes by raffinose family oligosaccharides during drying is mediated by effects on fusion and lipid phase transitions. Biochim. Biophys. Acta 1612, pp. 172–177. DOI: 10.1016/S0005-2736(03)00116-0.
- Hlukayo L. 2015. Characterization of pigeon pea (*Cajanus cajan*) landraces grown in two climatic zones in KwaZulu-Natal province, South Africa. www.researchgate.net/journal/02571862_South_African_Journal_of_Plant_and_Soil. DOI: 10.1080/02571862.2016.1266044.
- Hofman K. 2014. Non-communicable diseases in South Africa: A challenge to economic development. *South African Medical Journal*. Vol. 104(10), pp. 1-2. DOI: 10.7196/samj.8727.
- Hofvander P, Andersson M, Larsson C-T and Larsson H. 2004. Field performance and starch characteristics of high-amylose potatoes obtained by antisense gene targeting of two branching enzymes. *Plant Biotechnology Journal*. Vol. 2, pp. 311-320. DOI: 10.1111/j.1467-7652.2004.00073.x.
- Huang B, Hennen-Bierwagen TA, and Myers A.M. 2013. Functions of multiple genes encoding ADP-glucose pyrophosphorylase subunits in maize endosperm, embryo, and leaf. *Plant Physiology*. Vol. 164(2), pp. 596–611. DOI: 10.1104/pp.113.231605.

- Hwang SK, Nagai Y, Kim D and Okita TW. 2008. Direct appraisal of the potato tuber ADP-glucose pyrophosphorylase large subunit in enzyme function by study of a novel mutant form. *Journal of Biological Chemistry*. Vol. 283, pp. 6640-6647. DOI: 10.1074/jbc.M707447200.
- Hylton C and Smith AM. 1992. The *rb* mutation of peas causes structural and regulatory changes in ADP-glucose pyrophosphorylase from developing embryos. *Plant Physiology*. Vol. 99, pp. 1626–1634. DOI: 10.1104/pp.99.4.1626
- IITA (International Institute of Tropical Agriculture). 2009. Cowpea. Ibadan, Oyo State, Nigeria. In: Menyonga JM, Bezuneh JB, Yayock JY, Soumana I (editors). *Progress in food grains research and production in semiarid Africa*. pp. 77–85
- Jideani VA and Diedricks CF. 2014. Nutritional, therapeutic, and prophylactic properties of *Vigna subterranea* and *Moringa oleifera*. *Intech Open Science|Open Minds*. Chapter 8, pp. 189-207. DOI: 10.5772/57338.
- Johnson C, Thavarajah D, Thavarajah P and Kumar S. 2014. Probiotic oligosaccharides, resistant starch, and sugar alcohols in lentils: Implications for obesity. International Center for Agricultural Research in the Dry Areas (ICARDA). Pp. 49-83. ISBN: 978-1-62948-328-3.
- Kabeya D and Sakai S. 2003. The role of roots and cotyledons as storage organs in early stages of establishment in *Quercus crispula*: A quantitative analysis of the non-structural carbohydrate in cotyledons and roots. *Annals of Botany*. Vol. 92, pp. 637-545. DOI: org/10.1093/aob/mcg165.
- Karner U, Peterbauer T, Raboy V, Jones D A, Hedley CL and Richter A. 2004. myo - Inositol and sucrose concentrations affect the accumulation of raffinose family oligosaccharides in seeds. *Journal of Experimental Botany*. Vol. 55(405), pp. 1981–198. DOI: 10.1093/jxb/erh216.
- Keunen E, Peshev D, Vangronsveld J, Van Den Ende W and Cuypers A. 2013. Plant sugars are crucial players in the oxidative challenge during abiotic stress: Extending the traditional concept. *Plant, Cell and Environment*. Vol. 36(7), pp. 1242–1255. DOI: 10.1111/pce.12061.
- Kleczkowski LA. 1996. Back to the drawing board: Redefining starch synthesis in cereals. *Trends in Plant Science*. doi.org/10.1016/S1360-1385(96)80304-9.
- Krauss RM. 2004. Lipids and lipoproteins in patients with type 2 diabetes. *Diabetes Care*. Vol. 27(6), pp. 1496-1504. DOI: 10.2337/diacare.27.6.1496.

- Kumar N, Gopinath KA, Srivastva AK and Mahajan V. 2008. Performance of pigeon pea (*Cajanus cajan* L. Millsp.) at different sowing dates in the mid-hills of Indian Himalaya. *Archives of Agronomy and Soil Science*. Vol.54(5), pp. 507–514. DOI: 10.1080/03650340802287018.
- Lee S, Eom J, Hwang S, Shin D, An G and Okita, TW. 2016. Plastidic phosphoglucomutase and ADP-glucose pyrophosphorylase mutants impair starch synthesis in rice pollen grains and cause male sterility. *Journal of Experimental Botany*. Vol. 67(18), pp. 5557–5569. DOI: org/10.1093/jxb/erw324.
- Li C, Powell PO and Gilbert RG. 2017. Recent progress toward understanding the role of starch biosynthetic enzymes in the cereal endosperm: a review. *DE GRUYTER OPEN*. Vol. 1, pp. 59–74. DOI: 10.1515/amylase-2017-0006.
- Lin T, Caspar T, Somerville C and Preiss J. 1988. Isolation and characterization of a starchless mutant of *Arabidopsis thaliana* (L.) Heynh lacking ADP-glucose pyrophosphorylase activity. *Plant Physiology*. Vol. 86, pp. 1131–1135. DOI: 10.1104/pp.86.4.1131.
- Liu J, Fernie, AR and Yan J. 2019. The past, present, and future of maize improvement: Domestication, genomics, and functional genomic routes toward crop enhancement. *Plant Communications*. Vol. 1(1). DOI: 10.1016/j.xplc.2019.100010.
- Lloyd JR and Kötting, O. 2016. Starch biosynthesis and degradation in plants. In: eLS. John Wiley and Sons, Limited: Chichester. pp. 1–10. DOI: 10.1002/9780470015902.a0020124.pub2.
- MacNeill GJ, Mehrpouyan S, Minow MAA, Patterson JA, Tetlow IJ, and Emes MJ. 2017. Starch as a source, starch as a sink: The bifunctional role of starch in carbon allocation. *Journal of Experimental Botany*. Vol. 68(16), pp. 4433–4453. DOI: 10.1093/jxb/erx291.
- Malhi GS, Kaur M and Kaushik P. 2021.Impact of climate change on agriculture and its mitigation strategies: a review. *Sustainability*. Vol. 13(1318), pp. 1-21. DOI: 10.3390/su13031318.
- Malinova I, Kunz HH, Alseekh S, Herbst K, Fernie AR. Gierth M, *et al*. 2014. Reduction of the cytosolic phosphoglucomutase in *Arabidopsis* reveals impact on plant growth, seed and root development, and carbohydrate partitioning. *PLoS One*. Vol 9(11), pp. 1–11. DOI: 10.1371/journal.pone.0112468.

- Mamedov TG, Pienaar E, Whitney SE, TerMaat JR, Carvill G, Goliath R, Subramanian A and Viljoen HJ. 2008. A fundamental study of the PCR amplification of GC-rich DNA templates. *Computational Biology and Chemistry*. Vol. 32(6), pp. 452–457. DOI: 10.1016/j.compbiolchem.2008.07.021.
- Manjunath S, Lee CK, Vanwinkle P and Bailey-Serres, J. 1998. Molecular and biochemical characterization of cytosolic phosphoglucomutase in maize: Expression during development and in response to oxygen deprivation. *Plant Physiology*. Vol. 117, pp. 997-1006. DOI: 10.1104/PP.117.3.997.
- Maphosa Y. and Jideani VA. 2017. The role of legumes in human nutrition. Functional Food - Improve Health through Adequate Food. *Intech Open Science|Open Minds*. DOI: 10.5772/intechopen.69127.
- Martın-Cabrejas MA, Diaz FE, Aguilera Y, Benitez V, Molla E and Esteban RM. 2008. Influence of germination on the soluble carbohydrates and dietary fibre fractions in non-conventional legumes. Science Direct, *Food Chemistry*. Vol. 107, pp. 1045–1052. DOI: 10.1016/j.foodchem.2007.09.020.
- Maseda PH and Ferna´ndez RJ. 2006. Stay wet or else: three ways in which plants can adjust hydraulically to their environment. *Journal of Experimental* Botany. Vol. 57(15), pp. 1-15. DOI:10.1093/jxb/erl127.
- Mello J, Barbedo CJ, Salatino A, Figueiredo-Ribeiro, CL. 2010. Reserve carbohydrates and lipids from the seeds of four tropical tree species with different sensitivity to desiccation. *Brazilian Archives of Biology and Technology an International Journal*. Vol. 53(4), pp. 889–899. ISSN 1516-8913. DOI: 10.1590/S1516-89132010000400019.
- Meyer K, Stecca KL, Ewell-hicks K, Allen SM, and Everard JD. 2012. Oil and protein accumulation in developing seeds is influenced by the expression of a cytosolic pyrophosphatase in *Arabidopsis*. *Plant Physiology*. Vol. 159, pp. 1221–1234. DOI: 10.1104/pp.112.198309.
- Miao M, Jiang B, Cui SW, and Zhang T. 2013. Slowly digestible starch: a review. *Critical Reviews in Food Science and Nutrition*. Vol. 55(12), pp. 1642-1657. DOI: 10.1080/10408398.2012.704434.
- Mutyambizi C, Booysen F, Stokes A, Pavlova M and Groot W. 2019. Lifestyle and socio-economic inequalities in diabetes prevalence in South Africa: A decomposition analysis. *PLoS One*. Vol. 14(1), pp. 1–21. DOI: 10.1371/journal.pone.0211208.

- Nagai YS, Sakulsingharoj C, Edwards GE, Satoh H, Greene TW, Blakeslee and Okita TW. 2009. Control of starch synthesis in cereals: metabolite analysis of transgenic rice expressing an up-regulated cytoplasmic ADP-glucose pyrophosphorylase in developing seeds. *Plant and Cell Physiology*. Vol. 50(3), pp. 635–643. DOI: 10.1093/pcp/pcp021.
- Nakamichi N. 2020. The transcriptional network in the *Arabidopsis* circadian clock system. *Genes*. 11(11), 1–13. DOI: 10.3390/genes11111284.
- Napoli M. 2011. Towards a food insecurity multidimensional index (FIMI). Master's thesis in Human Development and Food Security. Roma Tre, Universita Degli Studi. pp. 1-72. https://www.developmentaid.org/api/frontend/cms/file/2021/03/FIMI.pdf.
- Narina SS, Bhardwaj HL, Hamama AA, Burke JJ, Pathak SC and Xu Y. 2014. Seed protein and starch qualities of drought tolerant pigeon pea and native tepary beans. *Journal of Agricultural Science*. Vol. 6(11), pp. 247-259. DOI: 10.5539/jas.v6n11p247.
- Nissar J, Ahad T, Naik HR and Hussain SZ. 2017. Resistant starch - chemistry and nutritional properties. *International Journal of Food Science and Nutrition*. Vol. 2(6), pp. 95–108.
- Ntombela Z. 2012. Growth and yield responses of cowpeas (*Vigna unguiculata* L.) to waterstress and defoliation. Master of Science thesis in Agriculture (Crop Science), University of KwaZulu-Natal.
- Nwokolo E. 1987. Nutritional evaluation of pigeon pea meal. *Plant Foods for Human Nutritionl*. Vol. 37:283-90. DOI: 10.1007/BF01092203.
- Obendorf RL and Gorecki RJ. 2012. Soluble carbohydrates in legume seeds. *Seed Science Research*. Vol. 22, pp. 219–242. DOI: 10.1017/S0960258512000104.
- Oiestad AJ, Martin JM and Giroux MJ. 2016. Overexpression of ADP-glucose pyrophosphorylase in both leaf and seed tissue synergistically increase biomass and seed number in rice (*Oryza sativa* ssp. japonica). *Functional Plant Biology*. Vol. 43(12), pp. 1194–1204. DOI: 10.1071/FP16218.
- Ojiewo C, Keatinge DJDH, Hughes J, Tenkouano A, Nair R, Varshney R, Siambi M, Monyo E, Ganga-Rao NVPR and Silim S. 2015. The role of vegetables and legumes in assuring food, nutrition, and income security for vulnerable groups in Sub-Saharan Africa. *World Medical and Health Policy*. Vol. 7(3), pp. 187–210. DOI: 10.1002/wmh3.148.

- Orzechowski, S. 2008. Starch metabolism in leaves. *Acta Biochimica Polinica*. Vol. 55(3), pp. 435–445. http://www.actabp.pl.
- Pan Q, Shikano I, Hoover K, Liu TX and Felton GW. 2019. Enterobacter ludwigii, isolated from the gut microbiota of *Helicoverpa zea*, promotes tomato plant growth and yield without compromising anti-herbivore defences. *Arthropod-Plant Interactions*. Vol. 13(2), pp. 271–278. DOI: 10.1007/s11829-018-9634-9.
- Paparelli E, Parlanti S, Gonzali S, Novi G, Mariotti L, Ceccarelli N, Dongen JT, Kolling K, Zeeman SC and Parata P. 2013. Nighttime sugar starvation orchestrates gibberellin biosynthesis and plant growth in *Arabidopsis. The Plant Cell*. Vol. 25(10), pp. 3760–3769. DOI: 10.1105/tpc.113.115519.
- Pape U and Wollburg P. 2019. Impact of drought on poverty in Somalia. *Policy Research Working Papers*. pp. 1-23. DOI: 10.1596/1813-9450-8698.
- Parnell WR and Smith C. 2008. Food security: current research initiatives, globally and in New Zealand. PowerPoint presentation, Nutrition Society of New Zealand Conference.
- Parry MAJ, Madgwick PJ, Bayon C, Tearall K, Hernandez-lopez A, Baudo M, Rakszegi M, et al. 2009. Mutation discovery for crop improvement. *Journal of Experimental Botany*. Vol. 60(10), pp. 2817–2825. DOI: 10.1093/jxb/erp189.
- Pathirana R. 2011. Plant mutation breeding in agriculture. *CAB Reviews: Perspectives in Agriculture Veterinary Science, Nutrition and Natural Resources*. Vol. 6(32), pp. 1-20. DOI: 10.1079/PAVSNNR20116032.
- Patron NJ and Keeling PJ. 2005. Common evolutionary origin of starch biosynthetic enzymes in green and red algae. *Journal of Phycology*. Vol. 41, pp. 1131–1141. DOI: 10.1111/j.1529-8817.2005.00135.x.
- Pawlak K and Kołodziejczak M. 2020. The role of agriculture in ensuring food security in developing countries: considerations in the context of the problem of sustainable food production. *Sustainability*. Vol. 12, pp. 1–20. DOI:10.3390/su12135488.
- Periappuram C, Steinhauer L, Barton DL, Taylor DC, Chatson B and Zhou J. 2000. The plastidic phosphoglucomutase from Arabidopsis. A reversible enzyme reaction with an important role in metabolic control. Plant Physiology. Vol. 122, pp. 1193–1200. DOI: 10.1104/pp.122.4.1193.

- Peterbauer T and Andreas Richter A. 2001. Biochemistry and physiology of raffinose family oligosaccharides and galactosyl cyclitols in seeds. Invited review. Seed Science Research. Vol. 11, pp. 185-197. DOI: 10.1079/SSR200175.
- Peters S and Keller F. 2009. Frost tolerance in excised leaves of the common bugle (Ajuga reptans L.) correlates positively with the concentrations of raffinose family oligosaccharides (RFOs). Plant, Cell and Environment. Vol. 32, pp. 1099–1107. DOI: 10.1111/j.1365-3040.2009.01991.x
- Petreikov M, Eisenstein M, Yeselson Y, Preiss J and Schaffer AA. 2010. Characterization of the AGPase large subunit isoforms from tomato indicates that the recombinant L3 subunit is active as a monomer. *Biochemical Journal*. Vol. 428(2), pp. 201-212. DOI: org/10.1042/BJ20091777.
- Pfister B and Zeeman SC. 2016. Formation of starch in plant cells. *Cellular and Molecular Life Sciences*. Springer International Publishing. Vol. 73(14), pp. 2781-2807. DOI: 10.1007/s00018-016-2250-x.
- Polak R, Phillips EM and Campbell A. 2015. Legumes: health benefits and culinary approaches to increase take. *American Diabetes Association*. Institute of Lifestyle Medicine, Joslin diabetes center, Department of Physical Medicine and Rehabilitation, Havard Medical School, Boston, MA. pp. 198-205. DOI: 10.2337/diaclin.33.4.198.
- Popoola J, Ojuederie O, Omonhinmin C and Adegbite A. 2019. Neglected and underutilized legume crops: improvement and future prospects. *Intech OpenBook series: Recent Advances in Grain Crops Research, Chapter 8. Science|Open Access.* pp. 1-22. DOI: 10.5772/intechopen.87069.
- Raguin A and Ebenhöh O. 2017. Design starch: Stochastic modelling of starch granule biogenesis. *Biochemical Society Transactions*. Vol. 45(4), pp. 885–893. DOI: 10.1042/BST20160407.
- Raina A, Laskar RA, Jahan R, Amin, R, Khursheed S, Wani R, Nisa NT and Khan S. 2018. Mutation breeding for crop improvement. Enriched Publications PTY. LTD. pp. 293-307.
- Raina A, Laskar RA, Khursheed S and Amin R. 2016. Role of mutation breeding in crop improvement - past, present and future. *Asian Research Journal of Agriculture*. Vol. 2(2), pp. 1-16. DOI: 10.9734/ARJA/2016/29334.
- Rayner T, Moreau C, Ambrose M, Isaac PG, Ellis N and Domoney C. 2017. Genetic variation controlling wrinkled seed phenotypes in *Pisum*: How lucky was Mendel?

International Journal of Molecular Sciences. Vol. 18, pp. 1-22. DOI: 10.3390/ijms18061205.

- Ritchie H and Roser M. 2020. Causes of death. *Published online at OurWorldInData.org*. Retrieved from: 'https://ourworldindata.org/causes-of-death'.
- Rizkalla SW, Bellisle F and Slama G. 2002. Health benefits of low glycaemic index foods, such as pulses, in diabetic patients and healthy individuals. *British Journal of Nutrition*. Vol. 88(3), pp. 255–262. DOI: 10.1079/bjn2002715.
- Rosa M, Prado C, Podazza G, Interdonato R, González JA, Hilal M and Prado FE. 2009. Soluble sugars-metabolism, sensing and abiotic stress a complex network in the life of plants. *Plant Signaling and Behavior*. Vol. 4, pp. 388–393. DOI: 10.4161/psb.4.5.8294.
- Rosegrant MW and Cline SA. 2003. Global food security: Challenges and policies. *Science*. Vol. 302(5652), Pp. 1917-1919. DOI: 10.1126/science.1092958.
- Sa´nchez-Mata MC, Pen˜ uela-Teruel MJ, Ca´mara-Hurtado M, Dı´ez-Marque´s C and Torija-Isasa ME. 1998. Determination of mono-, di-, and oligosaccharides in legumes by high-performance liquid chromatography using an amino-bonded silica column. Journal of Agriculture, food and Chemistry. Vol. 46(9), pp. 3648−3652.
- Samarah NH, Mullen RE and Anderson I. 2009. Soluble sugar contents, germination, and vigor of soybean seeds in response to drought stress. *Journal of New Seeds*. Vol. 10(2), pp. 63-73. DOI:%2010.1080/15228860902786525.
- Saripalli G and Gupta PK. 2015. AGPase: Its role in crop productivity with emphasis on heat tolerance in cereals. *Theoretical and Applied Genetics*. Vol. 128(10), pp. 1893–1916. DOI: 10.1007/s00122-015-2565-2.
- Sarkar S, Panda S, Yadav KK and Kandasamy P. 2018. Pigeon pea (*Cajanus cajan*) an important food legume in Indian scenario: A review. *Legume Research - An International Journal*. pp. 0–10. ISSN (online): 0976-0571DOI: 10.18805/lr-4021.
- Saxena KB, Kumar, RV, and Sultana R. 2010. Quality nutrition through pigeon pea: A review. *Scientific Research*. Vol. 2(11), pp.1335–1344. DOI: 10.4236/health.2010.211199.
- Schonfeldt HC and Pretorius B. 2011. The nutrient content of five traditional South African dark green leafy vegetables-a preliminary study. *Journal of Food Composition and Analysis*. Vol. 24(8), pp. 1141–1146. DOI: 10.1016/j.jfca.2011.04.004.

- Schreiner BG, Mungatana ED and Baleta H. 2018. Impacts of drought induced water shortages in South Africa: Sector policy briefs. Water Research and Commission, Issue number: 2604/1/18, pp. 1-85. ISBN 978-0-6392-0055-2.
- Shaik SS, Carciofi M, Martens HJ, Hebelstrup KH and Blennow A. 2014. Starch bioengineering affects cereal grain germination and seedling establishment. *Journal of Experimental Botany*. Vol. 65(9), pp. 2257–2270. DOI: 10.1093/jxb/eru107.
- Sharma S, Agarwal N and Verma P. 2011. Pigeon pea (*Cajanus cajan* L.): A hidden treasure of regime nutrition. *Journal of Functional and Environmental Botany*. Vol. 1(2), pp. 91–101. DOI: 10.5958/j.2231-1742.1.2.010.
- Sheik S, Evans J, Morden E and Coetzee, D. 2016. Non - communicable diseases in the Western Cape. Burden of Disease Update. Epidemiology and Surveillance sub-directorate, Health Impact Assessment Unit Western Cape Government: Department of Health. pp. 1-34.
- Sigurbjornsson B. 1971. Quickening the pulse of nature. Review paper. FAO/ IAEA Joint Division of Atomic Energy in Food and Agriculture. pp. 20-27.
- Silva EC, Albuquerque MB, Azevedo Neto AD and Silva Junior CD. 2013. Drought and its consequences to plants - from individual to ecosystem. *Responses of Organisms to Water Stress*. In book: Responses of Organisms to Water Stress, Chapter: Drought and Its Consequences to Plants – From Individual to Ecosystem. pp.17-47.DOI: 10.5772/53833.
- Sinha P, Saxena RK, Singh VK, Krishnamurthy L and Varshney RK. 2015. Selection and validation of housekeeping genes as reference for gene expression studies in pigeon pea (*Cajanus cajan*) under heat and salt stress conditions. *Frontiers in Plant Science*. Vol. 6, pp.1071. DOI: 10.3389/fpls.2015.01071.
- Snapp SS, Grabowski P, Chikowo R, Smith A, Anders E, Sirrine D, Chimonyo V and Bekunda M. 2018. Maize yield and profitability trade-offs with social, human and environmental performance: Is sustainable intensification feasible? *Agricultural Systems*. Vol. 162, pp. 77–88. DOI: 10.1016/j.agsy.2018.01.012.
- Sprenger N and Keller F. 2000. Allocation of raffinose family oligosaccharides to transport and storage pools in *Ajuga reptans*: the roles of two distinct galactinol synthases. *Plant Journal*. Vol. 21(3), pp. 249–258. DOI:10.1046/j.1365-313x.2000.00671.x.

- Stagnari F, Maggio A, Galieni A and Michele M. 2017. Multiple benefits of legumes for agriculture sustainability: An overview. *Chemical and Biological Technologies in Agriculture*. Vol. 4(2), pp. 1-13. DOI 10.1186/s40538-016-0085-1.
- Stats SA (Statistics South Africa). 2017. Census of commercial agriculture. 2017. Financial and production statistics.
- Stats SA (Statistics South Africa). 2019. Towards measuring the extent of food security in South Africa: an examination of hunger and food inadequacy. Report: 03-00-14.
- Steadman KJ, Pritchard HW and Dey PM. 1995. Tissue-specific soluble sugars in seeds as indicators of storage category. *Annals of Botany*. Vol. 77(6), pp. 667–674. DOI: 10.1006/anbo.1996.0083.
- Steinle NI and Chandrasekran. 2011. Dry beans in the diet may benefit people with diabetes. Dry bean quarterly: *Smart Choice for a Healthy Life*. Vol. 1(4), pp. 1-4. http://www.beaninstitute.com.
- Stitt M and Zeeman S. 2012. Starch turnover: Pathways, regulation and role in growth. *SciVerse ScienceDirect.* Current Opinion in Plant Biology. Vol. 15(3), 282-292. DOI: 10.1016/j.pbi.2012.03.016.
- Streb S, Egli B, Eicke S and Zeeman SC. 2009. The debate on the pathway of starch synthesis: A closer look at low-starch mutants lacking plastidial phosphoglucomutase supports the chloroplast-localized pathway. *Plant Physiology*. Vol. 151(4), pp. 1769–1772. DOI: 10.1104/pp.109.144931.
- Strien J, Sanft J, Mall and G. 2013. Enhancement of PCR amplification of moderate GC-containing and highly GC-rich DNA sequences. *Molecular Biotechnology*. Vol. 54(3), pp. 1048-54. DOI: org/10.1007/s12033-013-9660-x.
- Su C, Yuan J, Zhao H, Zhao Y, Ji H, Wang Y and Li X. 2017. *RUG3* is a negative regulator of plant responses to ABA in *Arabidopsis thaliana. Plant Signalling and Behavior*. Vol. 12(6), pp. 1-5. DOI: 10.1080/15592324.2017.1333217.
- Talari A, and Shakappa D. 2018. Role of pigeon pea (*Cajanus cajan* L.) in human nutrition and health: A review. *Asian Journal of Diary and Food Resources*. Vol. 37(3), pp. 212-220. ISSN:0976-0563. DOI: 10.18805/ajdfr.DR-1379.
- Tayade R, Kulkarni KP, Jo H, Song JT, Lee J, and Lee J. 2019. Insight into the prospects for the improvement of seed starch in legume: A review. *Frontiers in Plant Science*. Vol. 10, pp. 1–17. DOI: 10.3389/fpls.2019.01213.

- Tetlow IJ, Morell MK and Emes MJ. 2004. Recent developments in understanding the regulation of starch metabolism in higher plants. *Journal of Experimental Botany*. Vol. 55(406), pp. 2131–2145. DOI: 10.1093/jxb/erh248.
- Time A, Garrido M and Acevedo E. 2018. Water relations and growth response to drought stress of *Prosopis tamarugo* phil. A review. *Journal of Soil Science and Plant Nutrition*. Vol. 18(2), pp. 329–343. DOI: 10.4067/S0718-95162018005001103.
- Turhan E and Ergin S. 2012 Soluble Sugars and Sucrose-Metabolizing Enzymes Related to Cold Acclimation of Sweet Cherry Cultivars Grafted on Different Rootstocks. *The Scientific World Journal*. Vol. 2012, pp. 1-8. DOI:10.1100/2012/979682.
- Uchegbu NN and Ishiwu CN. 2015. Nutritional composition of crackers produced from blend of sprouted pigeon pea (*Cajanus cajan*), unripe plantain (*musa parasidiaca*) and brewers' spent grain flour and blood glucose level of diabetic rats fed the biscuit. *World Academy of Science, Engineering and Technology*. Vol. 10, pp. 1-5.
- Varadaraju,R and Patel, P. 2019. Health benefits of vegetables. *International Journal of Chemical Studies*. Vol. 7(2), pp. 82–87. ISSN (online): 2321-4902.
- Varshney RK, Chen W, Li Y, Bharti AK, Saxena RK, Schlueter JA, *et al*. 2012. Draft genome sequence of pigeon pea (*Cajanus cajan*), an orphan legume crop of resource-poor farmers. *Nature Cell Biology*. Vol. 30(1), pp. 83-92. DOI:10.1038/nbt.2022.
- Ventriglia T, Kuhn ML, Ruiz MT, Ribeiro-Pedro M, Valverde F, Ballicora MA, Preiss J and Romero JM. 2008. Two *Arabidopsis* ADP-glucose pyrophosphorylase large subunits (APL1 and APL2) are catalytic. *Plant Physiology*. Vol. 148, pp. 65–76. DOI: 10.1104/pp.108.122846.
- Vriet C, Welham T, Brachmann A, Marilyn PM, Pike J, Perry J, Parniske M, Sato S, Tabata S, Smith AM and Wang TL. 2010. A suite of *Lotus japonicus* starch mutants reveals both conserved and novel features of starch metabolism. *Plant Physiology*. Vol. 154(2), pp. 643–655. DOI: 10.1104/pp.110.161844.
- Wandai M, Aagaard-Hansen J, Day C, Sartorius B, and Hofman KJ. 2017. Available data sources for monitoring non-communicable diseases and their risk factors in South Africa. *South African Medical Journal*. Vol. 107(4), pp. 331–337. DOI: 10.7196/SAMJ.2017.v107i4.11438.
- Wang F, Qi J, Guo Y, Zheng X Gong L, Wang S, Chen A, Huang X, Li Z and Song R. 2017. Effect of aging on starch and amylase activity of barley seeds in early

germination. *Journal of the Chinese Cereals and Oil association*. Vol. 32(8), pp. 14-21.

- Wang SM, Chu B, Lue WL, You TS, Eimert K and Chen J. 1997. Adg2-1 represents a missense mutation in the ADP-glucose pyrophosphorylase large subunit gene of *Arabidopsis thaliana*. *The Plant Journal*. Vol. 11(5), pp. 1121-1126. DOI: 10.1046/j.1365-313x.1997.11051121.x.
- Wang TL and Hedley CL. 1991. Seed development in peas: Knowing your three 'r's' (or four, or five). *Seed Science Research*. Vol. 1, pp. 3–14. DOI: 10.1017/S096025850000057X.
- Wang TL, Bogracheva TY, Hedley CL, Centre JI and Nr N. 1998. Starch: as simple as A, B, C? *Journal of Experimental Botany*. Vol. 49(320), pp. 481–502. DOI: 10.1093/jxb/49.320.481.
- Weber H, Borisjuk L, and Wobus U. Molecular physiology of legume seed development.2005. *Annual Review of Plant Biology*. Vol. 56, pp. 253-279. DOI: 10.1146/annurev.arplant.56.032604.144201.
- WHO (World Health Organization). 2019. More than one in three low- and middle-income countries face both extremes of malnutrition. Twin presence of obesity and undernutrition reflects shifts in food systems. Geneva. https://www.who.int/news/item/16-12-2019-more-than-one-in-three-low--and-middle-income-countries-face-both-extremes-of-malnutrition.
- Yassen BT, A.-Thani RF, Alhadi FA and Abbas RAA. 2018. Soluble sugars in plants under stress at the Arabian Gulf Region: Possible roles of microorganisms. *Journal of Plant Biochemistry and Physiology*. Vol. 6(4), pp. 1-17. DOI: 10.4172/2329-9029.1000224.
- Yu TS, Lue WL, Wang SM and Chen J. 2000. Mutation of *Arabidopsis* plastid phosphoglucose isomerase affects leaf starch synthesis and floral initiation1. *Plant Physiology*. Vol. 123, 319-326. DOI: 10.1104/pp.123.1.319.
- Zabawinski C, Van Den Koornhuyse, D'Hulst C, Shlichtung R, Giersch C, Delrue B, Lacroix JM, Preiss J and Balll S. 2001. Starchless mutants of Chlamydomonas reinhardtii lack the small subunit of a heterotetrameric ADP-glucose pyrophosphorylase. *Journal of Bacteriology*. Vol. 183, pp. 1069–1077. DOI: 10.1128%2FJB.183.3.1069-1077.2001.

- Zeeman S, Kossmann J and Smith AM. 2010. Starch: its metabolism, evolution, and biotechnological modification in plants. *Annual Review in Plant Biology*. Vol. 61(15), pp. 1-15.26. DOI: 10.1146/annurev-arplant-042809-112301.
- Zeeman SC, Smith SM and Smith AM. 2004. The breakdown of starch in leaves. *New Phytologist*. Vol. 163(2), pp. 247–261. DOI: org/10.1111/j.1469-8137.2004.01101.x.
- Zhang J, Jiang H, Song X, Jin J and Zhang X. 2018. The responses of plant leaf CO2/H2O exchange and water wse efficiency to drought: a meta-analysis. *Sustainability*. Vol. 10(551), pp. 1-13. DOI: 10.3390/su10020551
- Zhong Y, Blennow A, Kofoed-enevoldsen O, Jiang D,and Hebelstrup KH. 2018. Protein targeting to starch 1 is essential for starchy endosperm development in barley. *Journal of Experimental Botany*. Vol. 70(2), pp. 485–496. DOI: 10.1093/jxb/ery398.