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Understanding the genetic and physiological basis of drought resistance in Bambara groundnut (*Vigna subterranea* (L.) Verdc).

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Abstract

Bambara groundnut (*Vigna subterranea* (L) Verdc) is an underutilised African legume commonly known for its resistance to harsh environmental conditions such as drought, and equally known for its high protein content, and thus, having the potential to aid in the pursuit of food security as the climate is constantly changing. As an underutilised crop, Bambara groundnut has been relatively poorly researched in comparison to other more conventional crops such as maize, wheat, soybean, or rice.

To help Bambara groundnut farmers and breeding programs achieve the development of varieties with better agronomical potential, a better understanding of the physiological and genetic mechanisms, in relationship to drought resistance, is needed.

A series of experiments under controlled glasshouse conditions were conducted in order to gain a better understanding of the responses of Bambara groundnut to drought. Drought is defined as when the level of water content in the soil is below a healthy threshold for plants to continue normal physiological and biochemical processes. A selection of genotypes was assessed over a period of three years, in 10 L pots, and an Association Genetics Panel was assessed over one season in 5 L pots. Additionally, the transcriptome of a sub-set of 4 genotypes was studied through RNA-sequence, and a genome wide association study was conducted over the association genetics panel.

A combination of these approaches allowed to have a better understanding on a series of physiological mechanisms where two main approaches were detected, such as, **drought tolerance** and **drought avoidance**.

For **drought tolerance**, several genotypes, such as DodR, S19-3, and TN, showed a higher conservation of their canopy, their efficiency of the photosystem II, relative water content in leaves, and a warmer canopy under drought conditions. Additionally, genes related to several osmo-protectant compounds, and cuticular waxes were differently expressed in response to drought.

For **drought avoidance**, several genotypes, such as UnisR, Kano2, Kano3, and Gresik, showed a faster and higher rate of leaf senescence, lower efficiency of

the photosystem II, lower percentage of relative water content in leaves, and a cooler canopy temperature. However, the recovery rate when irrigation was re-introduced, was significantly higher than the **drought tolerant** genotypes. Additionally, in the case of Gresik, genes related to stomatal conductance were differently expressed in response to drought.

These findings will help stepping forward in the Bambara groundnut research by narrowing research objectives in future efforts through a selection of specific physiological traits and genes, and subsequent adaptation into breeding programs by marker assistant selection, or gene editing.

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ABA	Abscisic acid
AFLP	Amplified Fragment Length Polymorphism
AFLP	Amplified Fragment Length Polymorphism
AGP	Association Genetics Panel
ANOVA	Analysis of variance
bHLH	Basic helix–loop–helix
CO_2	Carbon dioxide
DAP	Days after planting
DArT	Diversity Array Technology
DAS	Days after seeding
DEG	Differentially Expressed Genes
DNA	Deoxyribonucleic acid
ECA	Estimate of canopy area
ECC	Estimate whole-canopy chlorophyll content
*F*m	Maximal possible value for fluorescence
*F*o	minimal fluorescence level
*F*v	Variable fluorescence
GbS	Genotyping-by-sequencing
GC-FID	Gas Chromatography Flame Ionization Detector
GEM	Gene expression markers
GWAS	Genome Wide Association Study
GxE	Genotype x environment
H_2O_2	Hydrogen peroxide
Hrs or h	Hours
HTC	Hard-to-cook phenomenon
IITA	International Institute of Tropical Agriculture
KEGG	Kyoto Encyclopedia of Genes and Genomes
Kg	Kilogram
LGs	Linkage groups
LRWC	Leaf relative water content
LTR	Long term repeats
LWUE	Leaf water use efficiency
MAS	Marker assisted selection
mg	Milligram
ml	Millilitre
mm	Millimetre
mRNA	Messenger RNA
NPQ	Non-photochemical chlorophyll fluorescence quenching

PhyB	Phytochrome B
PSI	Photo system I
PSII	Photo system II
PVC	Polyvinyl chloride
QTL	Quantitative Trait Loci
RAPD	Random Amplified Polyphormic DNA
RGB	Red green blue
RNA	Ribonucleic acid
RNA-seq	RNA sequencing
ROS	Reactive oxygen species
RSA	Root system architecture
RWC	Relative water content
SA	Salicylic acid
SC	Stomatal conductance
SNP	Single Nucleotide Polymorphism
SSD	Single seed decent
SSR	Single sequence repeat markers
TRL	Tap root length
uL	Microliter
WUE	Water use efficiency

Chapter 1: Introduction

Food security

It is a well-known fact, that due to the increase of the human population and the effects of climate change, a quest for food security must be undertaken. In the last 12 years the population has been increased by 1 billion to 7.9 billion people (https://www.worldometers.info/world-population/) and with an estimated population of around 9.7 billion people in 2050, and possibly reaching 11 billion by 2100 (James, 2015). An estimate given in 2019 by Mbow *et al*., suggested that 821 million people were undernourished, 151 million children under the age of five years old had difficulties developing properly, and 613 million women between the ages of 15 and 49 suffered from iron deficiency. Alongside current nutritional issues one of the current climate situations present in several regions in the world is the increased duration or variability of drought periods, which reduces the availability to predictably farm, as not many major crops or staple varieties are drought tolerant (Mbow *et al*., 2019). At the moment, farmers rely only on a very limited number of crop species, if these crops start to fail due to biotic and abiotic stresses (climate change), new alternative species will be needed to deal with food security in the future (Sean Mayes *et al*., 2015). The best replacement candidates should be those that have valuable traits such as drought tolerance, high nutritional content, possible nutritional or agricultural complementarity with other crops, and increased genetic diversity (S. Mayes *et al*., 2012; Varshney *et al*., 2010, 2013).

Climate change and its effects on crops through drought

Water deficit, extreme temperatures, and low atmospheric humidity are leading to the occurrence of periods of increased drought stress, which has become a worldwide issue that affects the geographical distribution of plants, limits their productivity in agriculture, and threatens food and nutritional security. The average global surface temperature increases by 1.5 °C by the

2019 according to the Intergovernmental Panel on Climate Change in their sixth assessment report (2020). At the same time, it is predicted that there will be an increase in evapotranspiration, therefore, increasing the frequency and intensity of drought. Drought affects several processes involved in photosynthesis, respiration, and/or nutrient metabolism, leading to a significant decrease of yield. For example, up to 60% decrease of bean production in developing countries (Abenavoli *et al*., 2016; Hamayun *et al*., 2010; Saglam *et al*., 2014; J.-K. Zhu, 2016), causing economic losses. Some of these processes are crop phenology, phasic development, growth, phytohormone levels, and assimilate partitioning. Examples of some representative field crops are highlighted by Farooq *et al*. (2009) and are given in Table 1 under drought in glasshouse conditions. For example, in maize, drought stress reduces yield by delaying silking, consequently increasing the anthesis-to-silking interval. In wheat, the kernel filling period was shortened (time from fertilization to maturity), and dry weight was reduced at maturity. In soybean, drought reduced total seed and branch yield. Drought tolerant pearl millets showed a greater partitioning of dry matter from stover to grains in comparison to irrigated counterparts.

Table 1 Yield reduction due to drought stress in some representative crops. Adapted from Farooq *et al*. (2009).

Crop	Growth stage	Yield reduction
Barley	Seed filling	49–57%
Maize	Grain filling	79–81%
Maize	Reproductive	63–87%
Maize	Reproductive	70–47%
Maize	Vegetative	25–60%
Maize	Reproductive	32–92%
Rice	Reproductive (mild stress)	53–92%
Rice	Reproductive (severe stress)	48–94%
Rice	Grain filling (mild stress)	30–55%
Rice	Grain filling (severe stress)	60%
Rice	Reproductive	24–84%
Chickpea	Reproductive	45–69%
Pigeonpea	Reproductive	40–55%
Common beans	Reproductive	58–87%
Soybean	Reproductive	46–71%
Cowpea	Reproductive	60–11%
Sunflower	Reproductive	60%
Canola	Reproductive	30%
Potato	Flowering	13%

The first and foremost effect of water deficit is impaired germination and poor establishment. There are two main periods in the life cycle of plants in

which harmful effects have the greatest impact, young seedlings, and before anthesis (Farooq *et al*., 2009; Filek *et al*., 2015). As mentioned before, drought affects several events such as cell division, cell enlargement and differentiation, leads to photoinhibition, among others, thus making drought resistance a highly complex trait (Farooq *et al*., 2009; Filek *et al*., 2015), therefore, an introduction to the different aspects of drought in plants will be described next.

An effect of water deficit in crops is stomatal, partial or complete, closure, which is associated with changes in both leaf water status and soil moisture content (Park *et al*., 2011; Talebi, 2011). This is mediated predominantly through signalling molecules produced by dehydration in the roots (in particular abscisic acid (ABA)) which plays a key role in adaptive responses to water deficit conditions and in the regulation of the expression of numerous stress-responsive genes that are involved in protective responses (Park *et al*., 2011; Talebi, 2011).

This stomatal partial or complete closure causes a decrease in transpiration (stomatal conductance) and photosynthetic rate. Gas exchange between leaf and atmosphere is reduced, causing low intercellular CO_2 concentration, therefore, the diffusion of CO_2 to the chloroplast is diminished and so is the net CO_2 assimilation rate, affecting photochemical efficiency (Souza *et al*., 2010). This happens in early stages of drought, while in more severe drought, these processes are often dramatically reduced, while increasing the generation of reactive oxygen species (ROS).

Photosynthesis is an essential process to maintain crop growth and development, and its sensitiveness to drought is well known, as decreasing activity of photosystems and of components of the electron transfer chain in chloroplasts due to drought have been observed (Filek *et al*., 2015). This leads to the exposure of chloroplasts to a surplus of excitation energy and increased production of ROS, such as superoxide O^{-}_{2}, singlet oxygen O^{2-}, hydrogen peroxide (H_2O_2) and a highly toxic hydroxyl radical (OH^{-}). These ROS are generated by the incomplete reduction of molecular oxygen, which can lead to lipid peroxidation and consequently, chlorophyll destruction, thus decreasing chlorophyll content (quantity of chlorophyll per unit area as an indicator for photosynthetic capacity in plants) and changing of leaf colour to yellow (chlorosis) (Filek *et al*., 2015; Khayatnezhad & Gholamin, 2012; Shivakrishna *et al*., 2016). This diminishes the amount of photosynthetic

assimilates available for sucrose and starch synthesis, and the action of sucrose phosphate.

Carbohydrates such as sucrose, glucose and fructose, are important components in the drought signalling pathways, however, their status in drought-stressed plants not only depends on the efficiency of the photosynthetic carbon reduction cycle and the sucrose/starch synthesis, it is also linked to the processes of osmotic adjustment (Khayatnezhad *et al*., 2011; Khayatnezhad & Gholamin, 2012; Talebi, 2011). Different classes of osmolytes such as, proline, glycine betaine, putrescine, among others, accumulate in plant cells (cytosol) when exposed to water deficiency. These osmolytes are non-toxic, even at high concentrations (Anjum *et al*., 2012).

Drought resistance

According to several authors (Blum, 2011; Fang & Xiong, 2015; Farooq *et al*., 2009; Xu *et al*., 2010), plants may respond to drought by several mechanisms:

1. Escape: plants may shorten their life cycle to allow them to generate the next generation before the environment becomes harmfully dry. This is commonly the case in annual crops, where an important trait in this mechanism is an early flowering time, in particular when growing seasons are restricted by terminal drought or high temperatures.

2. Avoidance: is the ability of plants to maintain fundamental normal physiological processes under mild and moderate drought conditions. Plants enhance their capacity of obtaining and/or control losing water by an extensive and prolific root system (biomass, length, density and depth as main characteristics), an efficient stomatal control of transpiration, a reduction of stomata, and leaf area/canopy cover, leaf rolling, and increasing wax accumulation on the leaf surface.

3. Tolerance: is the ability of plants to sustain a certain level of physiological activities under severe drought stress by regulating thousands of genes and a series of metabolic pathways to minimise or repair the damage caused by drought. Plants improve their osmotic adjustment ability and increase the cell wall elasticity to maintain tissue turgidity.

4. Drought recovery: is the ability to resume growth and produce yield after exposure to severe drought with subsequent access to water

Several of these responses have been studied to improve drought resistance in numerous research articles:

Chlorophyll related traits

One trait studied is chlorophyll content, where drought tolerant genotypes have shown higher values that correlate with lower canopy temperature, higher biomass and yield (Khayatnezhad *et al*., 2011; Khayatnezhad & Gholamin, 2012; Talebi, 2011). It has also been shown that *PhyB* (phytochrome B) mutants in rice developed a significant reduction in leaf area, which improved drought resistance through avoidance (J. Liu *et al*., 2012).

Chlorophyll exists as a green pigment-protein complex within plant leaves in Photo System II (PSII), Photo System I (PSI), and within the light-harvesting complexes, each associated with each other's reaction centres (Murchie & Lawson, 2013). These have the vital role of allowing plants to absorb energy from light. The amount of chlorophyll per area unit is an indicator of photosynthetic capacity of the plant and can be influenced by nutrient availability and environmental stresses such as drought (Palta, 1990).

When light energy is absorbed by the chlorophyll molecules, it can a) drive photosynthesis (photochemistry); b) be bounced back in the form of heat; or c) be reflected back as light (fluorescence) (Murchie & Lawson, 2013). These processes are in competition with each other; thus, the measure of

the chlorophyll fluorescence can give us valuable information about the quantum efficiency of the photochemistry and heat dissipation. For healthy leaves, a normal value of *F*v/*F*m ratio is around 0.83 and quite consistent; this correlates to the maximum quantum yield of photosynthesis and achieved by the application of a saturating pulse to a dark-adapted leaf (Murchie & Lawson, 2013). A healthy non-stressed plant would not show any Non-photochemical chlorophyll fluorescence quenching (NPQ) at this point, thus showing the maximal possible value for fluorescence, *F*m (Murchie & Lawson, 2013). On the other hand, the variable fluorescence (*F*v) is calculated as the difference between *F*o and *F*m, where *F*o is the minimal fluorescence level (Murchie & Lawson, 2013). After an appropriate period of dark adaptation, the measurement of *F*v/*F*m has been used as one of the most common techniques for measuring 'stress' in leaves (Murchie & Lawson, 2013).

It has been reported that both of these traits are positively correlated with drought tolerance in different crops such as Barley (LI *et al*., 2006), Sugarcane (de Almeida Silva *et al*., 2011), bread wheat (Geravandi *et al*., 2011). It has also been reported in tomato (Mishra *et al*., 2012), potato (Van der Mescht *et al*., 1999), pigeonpea and mungbean (Narina *et al*.,2013), among others.

Water use efficiency and stomatal conductance related traits

Another factor that has been looked at is water use efficiency (WUE), which can be divided into two different scales: plant and leaf. (Viger *et al*., 2013) describes WUE at a plant scale as the biomass production/water consumption over a given period. Meanwhile, leaf scale is described as the instantaneous ratio between the net CO_2 assimilation rate and the transpiration loss. This can be improved by lowering stomatal conductance, and/or increasing photosynthetic rates. It has been shown that WUE can be improved with stomatal closure at midday or opening stomata early in the morning (Benešová *et al*., 2012). Stomata are formed by two small symmetric guard cells on the epidermis of higher plants that play a central

role in the regulation of gas exchange between the inner air space of the leaf and the outer atmosphere (X. Zhu *et al*., 2018). Stomata enable CO_2 entry into the leaf for photosynthesis while limiting water loss. Stomatal conductance (g_s ; mmol m^{-2} s^{-1}) is regulated primarily by the aperture of the stomatal pore and stomatal density, as well as the water transport capacity of the guard cells on the leaf surface (X. Zhu *et al*., 2018). The aperture and closure of the stomata is induced by many factors including abscisic acid (ABA), salicylic acid (SA), CO_2, among others. Plants can improve drought resistance by closing their stomata to decrease water loss from leaves under drought stress (X. Zhu *et al*., 2018). The reduction or increase of stomatal conductance could lead to an increase or decrease of leaf temperature respectively (Hepworth *et al*., 2015). With improved and more sensitive infrared imaging technology there is the possibility of high-resolution studies of variation in stomatal conductance over leaf surfaces and their dynamics using leaf temperature as an indicator for transpiration cooling leaves (Bai & Purcell, 2018; Benavente *et al*., 2013; Liu *et al*., 2011). As mentioned before, another indicator of overall water status in plants is the difference between the canopy temperature in comparison to the ambient or air temperature, which could be a practical assessment of plant response to drought (Jokar *et al*., 2018). Additionally, leaf relative water content (RWC) is another measure of water status in plants, as it may reflect more closely the balance between water supply to the leaf tissue and transpiration rate than other water potential parameters under drought stress conditions (Lugojan & Ciulca, 2011).

As mentioned earlier, osmolytes have been described to play a role in drought tolerance. Among the different classes that accumulate in plant cells, proline is a class that has been studied in response to drought stress. Proline is an α-amino acid that has been associated with several osmoprotective roles including, osmotic adjustment, membrane stabilization, and signalling to activate anti-oxidizing enzymes that scavenge ROS (Mafakheri *et al*., 2010; Mwadzingeni *et al*., 2016). It has been reported that under drought–stress conditions, the accumulation of proline in drought tolerant wheat genotypes has been faster and in higher proportions than in sensitive counterparts (Mwadzingeni *et al*., 2016).

In addition, root architecture and growth are important traits for improving drought tolerance in crops suffering severe drought as an avoidance

mechanism (Abenavoli *et al*., 2016). It has been shown that increased root length, surface area and dry weight can be correlated with enhanced drought tolerance in common bean and chickpea, where it has been suggested that the canopy temperature is slightly cooler due to a more prolific and deeper root system leading to better water extraction (Kumar *et al*., 2012).

In summary, plants have a set of tools or morpho-physiological mechanisms to develop a combined response depending on the abiotic nature and severity of the stress. For example, in short term droughts, several possible changes may occur including, rapid stomatal closure, increased energy dissipation and down-regulation of photochemistry; whereas in long term droughts, plants could manifest changes including leaf area reduction, stomatal density decrease, or enhanced leaf thickness to increase water retention (Tapia *et al*., 2016).

Molecular tools

In addition to the morpho-physiological tools, and with the increase of sequencing technology development and cost reduction, molecular tools have been developed to aid in the biological research, such as plant responses to drought.

Genome Wide Association Study (GWAS) is an alternative and complementary approach to the Quantitative Trait Loci (QTL) analysis performed controlled crosses, especially when there is difficulty generating genetic crosses with a difficult flower location and/or size, or the life cycle of perennial crops. This approach consists of the sequencing of the DNA of a population of the same species (the larger the population, the more specific and powerful the analysis can be), by next generation sequencing, to identify natural genetic variants related to a given trait through the use of molecular markers.

GWAS has been carried out to better understand the genetic mechanisms in response to drought in different crops such as maize (Guo *et al*., 2020) and wheat (Mathew *et al*., 2019), locating several genes putatively associated with drought tolerant traits such as seminal root length (Guo *et al*., 2020) and root biomass (Mathew *et al*., 2019). However, and to the author's knowledge, no GWAS has been publish in Bambara groundnut for any trait.

Additionally to GWAS, RNA sequencing (RNA-seq) has become a very useful technique for detecting genome-wide gene expression patterns (Guo *et al*., 2020). Where the transcriptome of an individual is sequenced, revealing the presence and quantity of RNA transcripts at a given moment. This is a useful tool to perform comparisons between contrasting groups such as 'drought' and 'irrigated control', however, it is currently still costly and very sensitive, where an experiment with 2 lines with 4 replicates 1 treatment can cost at least £2k. Additionally, the data generated from RNAseq could aid in the assembly of a new genome, such as the case of the Bambara groundnut genome. It could be a great complementary tool to the other genetic techniques described above, due to the specificity of analysis.

Underutilised crops

Underutilised crops are plant species which are not as researched, invested in, consumed, or distributed as in comparison to major crops such as wheat, rice, soya, and maize.

Underutilised crops can also be defined as "Those species of minor importance that have been poorly documented/researched; either cultivated or wild, that have a great potential for agricultural development and production diversification, as well as preservation of cultural/ traditional uses; generating benefit to people living in marginal environments.

Due to the low human input into the agricultural systems associated with these crops, natural selection has evolved these species to adapt to different environments over millennia (such as drought), and thus developing resilient traits that could be of high significance in our quest for food security in response to climate change.

Bambara groundnut, a drought tolerant underutilised crop

Legumes are a good option for food security for the future, since they contribute nitrogen to the soil (thus being a good option for crop rotation

and intercropping) and produce high levels of non-animal protein for human diets (Mayes *et al*., 2012). An example is the Bambara groundnut ((*Vigna subterranea* (L.)Verdc) synonym [Voandzeia subterranea (L.) Thouars]) which is a bunchy/spreading Leguminosae, with trifoliate leaves, that self-pollinates, with a largely indeterminate growth habit (Descriptors for Vigna Subterranea Bambara Groundnut, n.d.; Molosiwa *et al*., 2015) (Figure 1-1). It produces 1 to 2 seeded pods, that grow at the surface or directly underneath, protecting them from flying insects that attack crops like cowpeas and beans. The subterranea species are divided furthermore into two groups: var. spontanea (wild forms from northern Cameroon and Nigeria) and var. subterranea (cultivated forms largely from sub-Saharan Africa) (Basu *et al*., 2007). The chromosome number in both wild and cultivated plants is 2n = 2x = 22 (www.nda.agric.za, 2011).

Figure 1-1 Bambara groundnut under field conditions in the United Kingdom in 2016 (52°50’08”N, 1°14’59”W).

The nutritional composition of this crop’s seeds is approximately 63% carbohydrates, 6.5% oil and 19% protein with a well-balanced combination of essential amino acids with relatively high lysine (6.8%) and methionine (1.3%), and a high mineral content per 100 g of seed for iron (59 mg), potassium (1240 mg), sodium (3.7 mg) and calcium (78 mg), among others

(Olaleke *et al*., 2006; Steve Ijarotimi & Ruth Esho, 2009; Yao *et al*., 2015); making Bambara groundnut a complete and low-cost food. A more complete analysis is shared in Chapter 2. Additionally, Bambara groundnut has been reported to have drought tolerance and the ability to adapt to marginal soils, while still producing reasonable yields compared to other legumes (Ahmad *et al*., 2016; Collinson, S. T., Azam-Ali, S. N., Chavula, K. M., & Hodson, 1996; Mwale *et al*., 2007; Yao *et al*., 2015). Therefore, Bambara groundnut is a good candidate to research in this matter.

Drought avoidance and tolerance could be the two main mechanisms for drought resistance, which involve a complex set of responses, however, shifting phenology to escape drought is also a very complex response. These mechanisms have been widely researched for at least 30 years in Bambara groundnut, resulting in the determination and agreement of the strategies that this species uses to cope with drought (Al Shareef *et al*., 2014; Chibarabada *et al*., 2015; Collinson, S. T., Azam-Ali, S. N., Chavula, K. M., & Hodson, 1996; Collinson *et al*., 1999; Jørgensen *et al*., 2010; Laary *et al*., 2012; Mabhaudhi & Modi, 2013; Nautiyal *et al*., 2017; Vurayai, R., Emongor, V., & Moseki, 2011). A few major strategies to have a better control of available water, are divided into a) leaf area; b) gas exchange; c) biochemical protection; d) root related traits such as root depth and density. The level of action would be different between landraces and their place of origin; the severity and velocity of the drought; and phenological stage where it takes place (Al Shareef *et al*., 2014; Chibarabada *et al*., 2015; Collinson, S. T., Azam-Ali, S. N., Chavula, K. M., & Hodson, 1996; Collinson *et al*., 1999; Jørgensen *et al*., 2010; Laary *et al*., 2012; Mabhaudhi & Modi, 2013; Nautiyal *et al*., 2017; Vurayai, R., Emongor, V., & Moseki, 2011).

According to several authors, below certain soil moisture levels (drought), Bambara groundnut reduces its leaf area by reducing leaf initiation and/or expansion, or increases senescence (Collinson *et al*., 1999; Jørgensen *et al*., 2010; Mabhaudhi & Modi, 2013; Saglam *et al*., 2014; Vurayai, R., Emongor, V., & Moseki, 2011); gas exchange (transpiration/ stomatal conductance) is also reduced by the closure of stomata and/or lower stomatal density (Chai *et al*., 2016; Mabhaudhi & Modi, 2013; Nautiyal *et al*., 2017); and additionally, levels of osmoprotectants, such as soluble sugars and proline, are increased (Chai *et al*., 2016; Khan *et al*., 2017; Mwale *et al*., 2007;

Nautiyal *et al*., 2017). Other more specific actions involve leaf temperature-transpiration (leaf orientation/ paraheliotropism), epicuticular wax and shorter phenological stages (Collinson *et al*., 1999; Nautiyal *et al*., 2017).

Several established landraces have shown the use of these strategies in different ways, or the lack of their presence (in more sensitive cases): DodR (from Tanzania, 600 mm/year average annual rainfall), in the presence of drought, has shown higher accumulation of proline and chlorophyll content, an active paraheliotropism and little reduction in leaf expansion, resulting in a greater biomass in comparison to other landraces such as UniswaR, Tz, and SB4-2 (Collinson *et al*., 1999; Nautiyal *et al*., 2017). A similar reported case is S19-3 from Namibia, where additionally it has shown a higher level of ABA (which is related to size of stomatal aperture and closure) in comparison to other landraces such as Uniswa and Uniswa Red (Nautiyal *et al*., 2017). A completely opposite case is LunT, (which comes from a high rainfall environment [3000 mm/ year] in Sierra Leone) where leaf initiation and expansion was reduced by almost 70% and a significant lower level of paraheliotropism was observed (Collinson *et al*., 1999).

Genetic populations have been used for the study of drought tolerance, generating linkage maps for QTL analysis. In the case of Bambara groundnut, research reported by Chai *et al*. (2016) using a cross between DipC and TN (both expected to be drought resistant landraces due to their origins), reported a higher stomatal density, smaller leaf area, and a rapid reduction of stomatal conductance observed in the presence of mild drought among the assessed F5 population (66 F5 lines plus both parents). The stomatal conductance declined gradually from 540 to 220 mmol $m^{-2}s^{-1}$, however, some of the F5 assessed lines showed a higher conductance in presence of mild drought, such as L101 (274.1 mmol $m^{-2}s^{-1}$), L89 (269.3 mmol $m^{-2}s^{-1}$), and L94 (261.8 mmol $m^{-2}s^{-1}$). In the case of stomatal density, the lines L37, L94, and L7 showed a significantly higher density in the presence of mild drought at values of 14, 13, and 11 pores cm^{-2} respectively. Additionally, QTLs for leaf carbon delta C^{13} isotope analysis and stomatal density were detected under irrigation treatment, meanwhile, for drought-related traits, QTL for stomatal conductance, carbon isotope discrimination, and stomatal density were located (Chai *et al*., 2017). A transcriptomic comparison assay was performed in the two parental landraces, by using cross-species hybridisation to a soybean microarray chip, which has shown

to be informative for Bambara groundnut using the XSpecies transcriptomics approach. Under irrigated conditions, a few ABA/ osmoprotectant-related genes were detected in both genotypes, such as ABI1 (ABA Insensitive 1), ABF1 (ABRE binding factor 1), ERD4 (Early responsive to dehydration 4), and RD19 (Response to dehydration 19); suggesting that, in comparison to other species, Bambara groundnut could be in a primed state for abiotic stress (Khan *et al*., 2017) depending on the different type of response to drought as assessed in this thesis.

Other approaches have been used in the wide range of drought research related to Bambara groundnut. Modelling for Bambara groundnut's response to the climate change has been published by Karunaratne (2009) and Mabhaudhi *et al*. (2018), where in the first case, a crop simulation model (BAMGRO) was developed for Bambara groundnut. This model was calibrated and validated against glasshouse data in 2002 and 2006 (Nottingham, UK) and field sites in Botswana and Swaziland during the study. The model successfully described effects on leaf area index and soil moisture for two landraces (Uniswa Red and S19-3) as reported before; equally for yield, which was compared with the field sites, thus BAMGRO successfully simulated the correlation with limited soil moisture conditions. In the second case by Mabhaudhi *et al*. (2018), the climate change impact model developed for Bambara groundnut, shows an increase in biomass and yield by 42.5% and 37.5% respectively. This was observed in simulations for the present, mid-century, and late-century periods, which could be contributed to by an increase of CO_2 emissions, therefore, increasing photosynthesis of C3 plants by 30-50%. Regardless of the different global circulation models, a trend towards a decrease of soil evaporation and crop transpiration was predicted with decreased rainfall, contributing to the reduction in total evapotranspiration over simulated periods [past (784 mm) > present (771 mm) > mid-century (752 mm) > late century (718 mm)]. The combination of these predictions (CO_2 and rainfall) could still meet the crop water requirements of Bambara groundnut, strengthening the assumption that Bambara groundnut is better adapted to the predicted future climate in South Africa than most common crops (such as wheat, maize, and soya) and should be better promoted/looked at, particularly in areas under increasing water scarcity (low and/or variable rainfall), with increased frequency and intensity of drought.

In Bambara groundnut, several species-specific genomic resources have been reported, including more than 200 single sequence repeats (SSR) markers and 201 Diversity Array Technology (DArT) markers (Ahmad *et al*., 2016; Molosiwa *et al*., 2015; Siise & Massawe, 2013; Varshney *et al*., 2010). Molecular markers are highly useful tools to aid and speed up plant breeding, alongside recent advances in next generation sequencing platforms which help in understanding the genetics behind several important traits (Sean Mayes *et al*., 2015). This has increased the efficiency and accuracy with which QTL and underlying candidate genes can be localised using high density markers, with the majority of agronomically important traits in crop plants controlled by multiple genes, each with relatively minor effects (Aliyu *et al*., 2016; Takagi *et al*., 2013). Many QTLs for several traits related to drought resistance in roots and leaves have been mapped, and some have been validated under drought conditions in several crops such as rice and maize (Fang & Xiong, 2015). In addition, QTLs have been reported for stomatal conductance and leaf carbon isotope composition in Populus (Viger *et al*., 2013), and in Maize for stomatal conductance with the aid of thermography systems (Liu *et al*., 2011). Ahmad *et al*. (2016) have reported for the first time a QTL analysis for phenotypic traits in Bambara groundnut. A total of 36 significant QTLs were revealed to be associated with 19 out of 29 assessed traits.

However, by putting into context the above, Bambara groundnut, as an underutilised crop, has been studied at a very limited level when compared with major crops. The lack of research, understanding of the physiology and genetic responses of this crop to drought, are highly scarce and the majority of the research papers published are from the latest 10 years. Additionally, the tools and information, as a well-established seed supply, genetic crosses, and the whole genome sequence are also lacking. This crop (as with other underutilised crops) could have a major support role in the future food security; however, we may not know yet to what extent. Therefore, in this project we attempt to aid in the above, which should help alleviate the un-underutilisation of this crop by generating different resources that could be implemented in breeding programs.

Conclusion

Due to the increase in food demand and climate change, new alternatives should be researched to aid in the pursuit of food security. Underutilised crops, such as Bambara groundnut, may play a role in this quest as a possible alternative crop due to its natural resilience to drought, regardless of the limited research performed in this crop.

Hypotheses

- Tolerant genotypes of Bambara groundnut will exhibit physiological traits related to tolerance

- Different Bambara groundnut genotypes will exhibit traits reflective of the agroecology and climate of their origin.

- Different Bambara groundnut genotypes will display different biological pathways in response to which could be genetically controlled.

Thesis overview

This thesis includes five chapters as described below:

Chapter 2 – "The Bambara groundnut genome".

This is a book chapter describing the latest advances in the Bambara groundnut genome and genetic related areas.

Chapter 3 – "Evaluation of drought stress response mechanisms in different Bambara groundnut genotypes.".

This chapter describes the effects of drought in different Bambara groundnut genotypes from a physiological perspective, as well as several possible physiological mechanisms used by Bambara groundnut to cope with drought.

Chapter 4 – "GWAS analysis reveals drought resistance related genes in Bambara groundnut (*Vigna subterranea* (L.) Verdc.)."

This chapter describes the results of the combination of the physiology knowledge acquired in the previous chapter applied to an association genetics panel, to unravel possible candidate genes through a genome wide association study, and have a closer understanding of the genetics behind the drought responses of Bambara groundnut.

Chapter 5 – "RNA-seq in Bambara groundnut leaf tissue reveals drought tolerant related genes".

This chapter describes the results of a RNAseq experiment in Bambara groundnut in response to drought stress, and few candidate genes based on the difference of expression levels under drought stress conditions.

Chapter 6 – General discussion of all the work conducted during the PhD.

References

Abenavoli, M. R., Leone, M., Sunseri, F., Bacchi, M., & Sorgonà, A. (2016). Root phenotyping for drought tolerance in bean landraces from Calabria (Italy). *Journal of Agronomy and Crop Science*, *202*(1), 1–12. https://doi.org/10.1111/jac.12124

Ahmad, N. S. N. S., Redjeki, E. S. E. S., Ho, W. K. W. K., Aliyu, S., Mayes, K., Massawe, F., Kilian, A., Mayes, S., & Belzile, F. (2016). Construction of a genetic linkage map and QTL analysis in Bambara groundnut. *Genome*, *59*(April), 1–14. https://doi.org/10.1139/gen-2015-0153

Al Shareef, I., Sparkes, D., & Azam-Ali, S. (2014). Temperature and drought stress effects on growth and development of Bambara groundnut (*Vigna subterranea* L.). *Experimental Agriculture*, *50*(1), 72–89. https://doi.org/10.1017/S0014479713000379

Aliyu, S., Massawe, F., & Mayes, S. (2016). Genetic diversity and population structure of Bambara groundnut (*Vigna subterranea* (L.) Verdc.): Synopsis of the past two decades of analysis and implications for crop improvement programmes. *Genetic Resources and Crop Evolution*, *63*(6), 925–943. https://doi.org/10.1007/s10722-016-0406-z

Anjum, S. A., Farooq, M., Xie, X. yu, Liu, X. jian, & Ijaz, M. F. (2012). Antioxidant defense system and proline accumulation enables hot pepper to perform better under drought. *Scientia Horticulturae*, *140*, 66–73. https://doi.org/10.1016/j.scienta.2012.03.028

Bai, H., & Purcell, L. C. (2018). Aerial canopy temperature differences between fast- and slow-wilting soya bean genotypes. *Journal of Agronomy and Crop Science*, *204*(3), 243–251. https://doi.org/10.1111/jac.12259

Basu, S., Mayes, S., Davey, M., Roberts, J. A., Azam-Ali, S. N., Mithen, R., & Pasquet, R. S. (2007). Inheritance of "domestication" traits in Bambara groundnut (*Vigna subterranea* (L.) Verdc.). *Euphytica*, *157*(1–2), 59–68. https://doi.org/10.1007/s10681-007-9396-4

Benavente, E., García-Toledano, L., Carrillo, J. M., & Quemada, M. (2013). Thermographic Imaging: Assessment of drought and heat tolerance in spanish germplasm of Brachypodium Distachyon. *Procedia Environmental Sciences*, *19*, 262–266. https://doi.org/10.1016/j.proenv.2013.06.030

Benešová, M., Holá, D., Fischer, L., Jedelský, P. L., Hnilička, F., Wilhelmová, N., Rothová, O., Kočová, M., Procházková, D., Honnerová, J., Fridrichová, L., & Hniličková, H. (2012). The physiology and proteomics of drought tolerance in Maize: Early stomatal closure as a cause of lower tolerance to short-term dehydration? *PLoS ONE*, *7*(6). https://doi.org/10.1371/journal.pone.0038017

Blum, A. (2011). Plant Breeding for Water-Limited Environments. *Springer New York*. https://doi.org/10.1007/978-1-4419-7491-4

Chai, H. H., Ho, W. K., Graham, N., May, S., Massawe, F., & Mayes, S. (2017). A cross-species gene expression marker-based genetic map and QTL analysis in Bambara groundnut. *Genes*, *8*(2).

https://doi.org/10.3390/genes8020084

Chai, H. H., Massawe, F., & Mayes, S. (2016). Effects of mild drought stress on the morpho-physiological characteristics of a Bambara groundnut segregating population. *Euphytica*, *208*(2), 225–236. https://doi.org/10.1007/s10681-015-1581-2

Chibarabada, T. P., Modi, A. T., & Mabhaudhi, T. (2015). Water use characteristics of a Bambara groundnut (*Vigna subterranea* L. Verdc) landrace during seedling establishment. *Water SA*, *41*(4), 472–482. https://doi.org/10.4314/wsa.v41i4.06

Collinson, S. T., Azam-Ali, S. N., Chavula, K. M., & Hodson, D. A. (1996). Growth, development and yield of Bambara groundnut (*Vigna subterranea*) in response to soil moisture. *The Journal of Agricultural Science*, *126*(1996), 307–318.

Collinson, S., Berchie, J., & Azam-Ali, S. (1999). The effect of soil moisture on light interception and the conversion coefficient for three landraces of Bambara groundnut (*Vigna subterranea*). *The Journal of Agricultural Science*, *133*(2), 151–157. https://doi.org/10.1017/S0021859699006875

de Almeida Silva, M., Jifon, J. L., Sharma, V., da Silva, J. A. G., Caputo, M. M., Damaj, M. B., Guimarães, E. R., & Ferro, M. I. T. (2011). Use of physiological parameters in screening drought tolerance in sugarcane genotypes. *Sugar Tech*, *13*(3), 191–197. https://doi.org/10.1007/s12355-011-0087-z, *Descriptors for Vigna subterranea Bambara groundnut*. (n.d.).

Fang, Y., & Xiong, L. (2015). General mechanisms of drought response and their application in drought resistance improvement in plants. *Cellular and Molecular Life Sciences*, *72*(4), 673–689. https://doi.org/10.1007/s00018-014-1767-0

Farooq, M., Wahid, A., Basra, N. K. D. F. S. M. a, Fujita, N. K. D., & Plant, S. M. a B. (2009). Plant drought stress : effects , mechanisms and management *Agronomy for Sustainable Development, Springer Verlag (Germany)*, *29*(1), 185–212. https://doi.org/10.1051/agro:2008021

Filek, M., Labanowska, M., Kościelniak, J., Biesaga-Kościelniak, J., Kurdziel, M., Szarejko, I., & Hartikainen, H. (2015). Characterization of barley leaf tolerance to drought stress by chlorophyll fluorescence and electron paramagnetic resonance studies. *Journal of Agronomy and Crop Science*, *201*(3), 228–240. https://doi.org/10.1111/jac.12063

Geravandi, M., Farshadfar, E., & Kahrizi, D. (2011). Evaluation of some physiological traits as indicators of drought tolerance in bread wheat genotypes. *Russian Journal of Plant Physiology*, *58*(1), 69–75. https://doi.org/10.1134/S1021443711010067

Guo, J., Li, C., Zhang, X., Li, Y., Zhang, D., Shi, Y., Song, Y., Li, Y., Yang, D., & Wang, T. (2020). Transcriptome and GWAS analyses reveal candidate gene for seminal root length of maize seedlings under drought stress. *Plant Science*,*292*(December2019).https://doi.org/10.1016/j.plantsci.2019.110380

Hamayun, M., Khan, S. A., Shinwari, Z. K., Khan, A. L., Ahmad, N., & Lee, I. J. (2010). Effect of polyethylene glycol induced drought stress on physio-

hormonal attributes of soybean. *Pakistan Journal of Botany*, *42*(2), 977–986.

Hepworth, C., Doheny-Adams, T., Hunt, L., Cameron, D. D., & Gray, J. E. (2015). Manipulating stomatal density enhances drought tolerance without deleterious effect on nutrient uptake. *New Phytologist*, *208*(2), 336–341. https://doi.org/10.1111/nph.13598

James, C. (2015). Anniversary of the Global Commercialization of Biotech Crops (1996 to 2015) and Biotech Crop Highlights in 2015 (Executive summary). *International Service for the Acquisition of Agri-Biotech Applications*, *51*(51), 1–17.

Jokar, F., Karimizadeh, R., Masoumiasl, A., & Amiri Fahliani, R. (2018). Canopy temperature and chlorophyll content are effective measures of drought stress tolerance in durum wheat. *Notulae Scientia Biologicae*, *10*(4), 575–583. https://doi.org/10.15835/nsb10410288

Jørgensen, S. T., Liu, F., Ouédraogo, M., Ntundu, W. H., Sarrazin, J., & Christiansen, J. L. (2010). Drought responses of two Bambara groundnut (*Vigna subterranea* L. Verdc.) landraces collected from a dry and a humid area of Africa. *Journal of Agronomy and Crop Science*, *196*(6), 412–422. https://doi.org/10.1111/j.1439-037X.2010.00435.x

Karunaratne, A. S. (2009). *Modelling the response of Bambara groundnut (Vigna subterranea (L.) Verdc) for abiotic stress*. http://etheses.nottingham.ac.uk/840/

Khan, F., Chai, H. H., Ajmera, I., Hodgman, C., Mayes, S., & Lu, C. (2017). A transcriptomic comparison of two Bambara groundnut landraces under dehydration stress. *Genes*, *8*(4), 1–19. https://doi.org/10.3390/genes8040121

Khayatnezhad, M., & Gholamin, R. (2012). The effect of drought stress on leaf chlorophyll content and stress resistance in maize cultivars (Zea mays). *African Journal of Microbiology Research*, *6*(12), 2844–2848. https://doi.org/10.5897/AJMR11.964

Khayatnezhad, M., Gholamin, R., Jamaati-e-Somarin, S., & Zabihi-e-Mahmoodabad, R. (2011). The leaf chlorophyll content and stress resistance relationship considering in corn cultivars (*Zea Mays*). *Advances in Environmental Biology*, *5*(1), 118–122.

KUMAR, N., NANDWAL, A. S., WALDIA, R. S., SINGH, S., DEVI, S., SHARMA, K. D., & KUMAR, A. (2012). Drought tolerance in chickpea as evaluated by root characteristics, plant water status, membrane integrity and chlorophyll fluorescence techniques. *Experimental Agriculture*, *48*(03), 378–387. https://doi.org/10.1017/S0014479712000063

Laary, J. K., Ofori, K., & Kumaga, F. K. (2012). The influence of soil moisture status on reproductive growth and development of bambara groundnut (*Vigna subterranea* (L .) Verdc) landraces in Ghana. *APRN Journal of Agricultural and Biological Science*, *7*(10), 845–851.

LI, R. Hua, GUO, P. Guo, Michael, B., Stefania, G., & Salvatore, C. (2006). Evaluation of chlorophyll content and fluorescence parameters as indicators of drought tolerance in barley. *Agricultural Sciences in China*,

5(10), 751–757. https://doi.org/10.1016/S1671-2927(06)60120-X

Liu, Y., Subhash, C., Yan, J., Song, C., Zhao, J., & Li, J. (2011). Maize leaf temperature responses to drought: Thermal imaging and quantitative trait loci (QTL) mapping. *Environmental and Experimental Botany*, *71*(2), 158–165. https://doi.org/10.1016/j.envexpbot.2010.11.010

Lugojan, C., & Ciulca, S. (2011). Evaluation of relative water content in winter wheat. *Forestry and Biotechnology*, *15*(2), 173–177.

Mabhaudhi, T., Chibarabada, T. P., Chimonyo, V. G. P., & Modi, A. T. (2018). Modelling climate change impact: A case of Bambara groundnut (*Vigna subterranea*). *Physics and Chemistry of the Earth*, *105*(December 2017), 25–31. https://doi.org/10.1016/j.pce.2018.01.003

Mabhaudhi, T., & Modi, A. T. (2013). Growth, phenological and yield responses of a Bambara groundnut (*Vigna subterranea* (L.) Verdc.) landrace to imposed water stress under field conditions. *South African Journal of Plant and Soil*, *30*(2), 69–79. https://doi.org/10.1080/02571862.2013.790492

Mafakheri, A., Siosemardeh, A., Bahramnejad, B., Struik, P. C., & Sohrabi, E. (2010). Effect of drought stress on yield, proline and chlorophyll contents in three chickpea cultivars. *Australian Journal of Crop Science*, *4*(8), 580–585.

Mathew, I., Shimelis, H., Shayanowako, A. I. T., Laing, M., & Chaplot, V. (2019). Genome-wide association study of drought tolerance and biomass allocation in wheat. *PLoS ONE*, *14*(12), 1–21. https://doi.org/10.1371/journal.pone.0225383

Mayes, S., Massawe, F. J., Alderson, P. G., Roberts, J. A., Azam-Ali, S. N., & Hermann, M. (2012). The potential for underutilized crops to improve security of food production. *Journal of Experimental Botany*, *63*(3), 1075–1079. https://doi.org/10.1093/jxb/err396

Mayes, S., Ho, W. K., Kendabie, P., Chai, H. H., Aliyu, S., Feldman, A., Halimi, R. A., Massawe, F., & Azam-Ali, S. (2015). Applying molecular genetics to underutilised species – problems and opportunities. *Malaysian Applied Biology*, *44*(4), 1–9.

Mbow, C., Rosenzweig, C., Barioni, L. G., G., B. T., Herrero, M., Krishnapillai, M., Liwenga, E., Pradhan, P., Rivera-Ferre, M. G., Sapkota, T., Tubiello, F. N., & Xu, Y. (2019). Chapter 5: Food Security. In *Climate change and land: an IPCC special report on climate change, desertification, land degradation, sustainable land management, food security, and greenhouse gas fluxes in terrestrial ecosystems*.

van der Mescht, A., de Ronde, J. A., Rossouw, F.T. (1999) Chlorophyll fluorescence and cholorphyll contesnt as a measure of drought tolerance in potato. *South African Journal of Science*, 95, 407-412.

Mishra, K. B., Iannacone, R., Petrozza, A., Mishra, A., Armentano, N., La Vecchia, G., Trílek, M., Cellini, F., Nedbal, L. (2012) Engineered drought tolerance in tomato plants is reflected in chlorophyll fluorescence emission. *Plant Science*, 182, 79-86.

Molosiwa, O. O., Aliyu, S., Stadler, F., Mayes, K., Massawe, F., Kilian, A., &

Mayes, S. (2015). SSR marker development, genetic diversity and population structure analysis of Bambara groundnut [*Vigna subterranea* (L.) Verdc.] landraces. *Genetic Resources and Crop Evolution*, *62*(8), 1225–1243. https://doi.org/10.1007/s10722-015-0226-6

Murchie, E. H., & Lawson, T. (2013). Chlorophyll fluorescence analysis: A guide to good practice and understanding some new applications. *Journal of Experimental Botany*, *64*(13), 3983–3998. https://doi.org/10.1093/jxb/ert208

Mwadzingeni, L., Shimelis, H., Tesfay, S., & Tsilo, T. J. (2016). Screening of bread wheat genotypes for drought tolerance using phenotypic and proline analyses. *Frontiers in Plant Science*, *7*(August). https://doi.org/10.3389/fpls.2016.01276

Mwale, S. S., Azam-Ali, S. N., & Massawe, F. J. (2007). Growth and development of Bambara groundnut (*Vigna subterranea*) in response to soil moisture. *European Journal of Agronomy*, *26*(4), 345–353. https://doi.org/10.1016/j.eja.2006.09.007

Narina, S. S., Phatak S. C., Bhardwaj H. L. (2014) Chlorophyll Fluorescence to Evaluate Pigeonpea Breeding Lines and Mungbean for Drought Tolerance. *Journal of Agricultural Science*, 6 (11), 238-246.

Nautiyal, P. C., Kulkarni, G., Singh, A. L., & Basu, M. S. (2017). Evaluation of water-deficit stress tolerance in Bambara groundnut landraces for cultivation in sub-tropical environments in India. *Indian Journal of Plant Physiology*, *22*(2), 190–196. https://doi.org/10.1007/s40502-017-0296-x

Olaleke, A. M., Olorunfemi, O., & Emmanuel, A. T. (2006). A comparative study on the chemical and amino acid composition of some Nigerian under-utilized legume flours. In *Pakistan Journal of Nutrition* (Vol. 5, Issue 1, pp. 34–38). https://doi.org/10.3923/pjn.2006.34.38

Palta, J. P. (1990). Leaf chlorophyll content. *Remote Sensing Reviews*, *5*(1), 207–213. https://doi.org/10.1080/02757259009532129

Park, M. Y., Kang, J. Y., & Kim, S. Y. (2011). Overexpression of AtMYB52 confers ABA hypersensitivity and drought tolerance. *Molecules and Cells*, *31*(5), 447–454. https://doi.org/10.1007/s10059-011-0300-7

Saglam, A., Terzi, R., & Demiralay, M. (2014). Effect of polyethylene glycol induced drought stress on photosynthesis in two chickpea genotypes with different drought tolerance. *Acta Biologica Hungarica*, *65*(2), 178–188. https://doi.org/10.1556/ABiol.65.2014.2.6

Shivakrishna, P., Ashok Reddy, K., & Manohar Rao, D. (2016). Effect of PEG-6000 imposed drought stress on RNA content, relative water content (RWC), and chlorophyll content in peanut leaves and roots. *Saudi Journal of Biological Sciences*, 8–12. https://doi.org/10.1016/j.sjbs.2017.04.008

Siise, A., & Massawe, F. J. (2013). Microsatellites based marker molecular analysis of Ghanaian Bambara groundnut (*Vigna subterranea* (L.) Verdc.) landraces alongside morphological characterization. *Genetic Resources and Crop Evolution*, *60*(2), 777–787. https://doi.org/10.1007/s10722-012-9874-y

Souza, B. D., Meiado, M. V., Rodrigues, B. M., & Santos, M. G. (2010). Water relations and chlorophyll fluorescence responses of two leguminous trees from the Caatinga to different watering regimes. *Acta Physiologiae Plantarum*, *32*(2), 235–244. https://doi.org/10.1007/s11738-009-0394-0

Steve Ijarotimi, O., & Ruth Esho, T. (2009). Comparison of nutritional composition and anti-nutrient status of fermented, germinated and roasted Bambara groundnut seeds (*Vigna subterranea*). *British Food Journal*, *111*(4), 376–386. https://doi.org/10.1108/00070700910951515

Takagi, H., Abe, A., Yoshida, K., Kosugi, S., Natsume, S., Mitsuoka, C., Uemura, A., Utsushi, H., Tamiru, M., Takuno, S., Innan, H., Cano, L. M., Kamoun, S., & Terauchi, R. (2013). QTL-seq: Rapid mapping of quantitative trait loci in rice by whole genome resequencing of DNA from two bulked populations. *Plant Journal*, *74*(1), 174–183. https://doi.org/10.1111/tpj.12105

Talebi, R. (2011). Evaluation of chlorophyll content and canopy temperature as indicators for drought tolerance in durum wheat (*Triticum durum* Desf.). *Australian Journal of Basic and Applied Sciences*, *5*(11), 1457–1462. http://www.ajbasweb.com/ajbas/2011/November-2011/1457-1462.pdf

Tapia, G., Méndez, J., & Inostroza, L. (2016). Different combinations of morpho-physiological traits are responsible for tolerance to drought in wild tomatoes *Solanum chilense* and *Solanum peruvianum*. *Plant Biology*, *18*(3), 406–416. https://doi.org/10.1111/plb.12409

Varshney, R. K., Glaszmann, J. C., Leung, H., & Ribaut, J. M. (2010). More genomic resources for less-studied crops. *Trends in Biotechnology*, *28*(9), 452–460. https://doi.org/10.1016/j.tibtech.2010.06.007

Varshney, R. K., Mohan, S. M., Gaur, P. M., Gangarao, N. V. P. R., Pandey, M. K., Bohra, A., Sawargaonkar, S. L., Chitikineni, A., Kimurto, P. K., Janila, P., Saxena, K. B., Fikre, A., Sharma, M., Rathore, A., Pratap, A., Tripathi, S., Datta, S., Chaturvedi, S. K., Mallikarjuna, N., Gowda, C. L. L. (2013). Achievements and prospects of genomics-assisted breeding in three legume crops of the semi-arid tropics. *Biotechnology Advances*, *31*(8), 1120–1134. https://doi.org/10.1016/j.biotechadv.2013.01.001

Viger, M., Rodriguez-Acosta, M., Rae, A. M., Morison, J. I. L., & Taylor, G. (2013). Toward improved drought tolerance in bioenergy crops: QTL for carbon isotope composition and stomatal conductance in Populus. *Food and Energy Security*, *2*(3), 220–236. https://doi.org/10.1002/fes3.39

Vurayai, R., Emongor, V., & Moseki, B. (2011). Effect of water stress imposed at different growth and developmental stages on morphological traits and yield of Bambara groundnut (*Vigna subterranea* L. Verdc). In *American journal of plant physiology* (Vol. 6, pp. 17–27).

Xu, Z., Zhou, G., & Shimizu, H. (2010). Plant responses to drought and rewatering. *Plant Signaling & Behavior*, *5*(6), 649–654. https://doi.org/10.4161/psb.5.6.11398

Yao, D. N., Kouassi, K. N., Erba, D., Scazzina, F., Pellegrini, N., & Casiraghi, M. C. (2015). Nutritive evaluation of the Bambara groundnut Ci12 landrace [*Vigna subterranea* (L.) Verdc. (Fabaceae)] produced in Côte d'Ivoire. *International Journal of Molecular Sciences*, *16*(9), 21428–21441.

https://doi.org/10.3390/ijms160921428

Zhu, J.-K. (2016). Abiotic stress signaling and responses in plants. *Cell*, *167*(2), 313–324. https://doi.org/10.1016/j.cell.2016.08.029

Zhu, X., Cao, Q., Sun, L., Yang, X., Yang, W., & Zhang, H. (2018). Stomatal conductance and morphology of arbuscular mycorrhizal wheat plants response to elevated CO2 and NaCl Stress. *Frontiers in Plant Science*, *9*(September), 1–10. https://doi.org/10.3389/fpls.2018.01363

Chapter 2: The Bambara Groundnut Genome

From the crop to the genome – the progress and constraints of genome-related studies in Bambara groundnut.

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Abstract

The combined effects of climate change, increase in world population and dependence on a relatively small selection of crops, are threating the global food security. Despite their limited promotion among farmers, seed companies, and researchers, underutilised crops could provide alternative sources of nutritionally dense foods and aid in the quest for food production due to their resilience and natural adaptation to marginal environments that could be too harsh for staple crops. Bambara groundnut (*Vigna subterranea* (L.) Verdc.) is a protein rich underutilised legume which has also long been recognised to be drought resistant, capable of fixing atmospheric nitrogen and producing yield in marginal soils. As a consequence of the rapid development of genomic technologies and their current accessibility, in this chapter we share the current progress in genomics using molecular tools, an overview of the genome sequence of Bambara groundnut, future work incorporating next generation sequencing technologies and bioinformatics, as well as an example that showcases the importance of linking trait data to the genome to benefit future breeding programs.

Keywords: Bambara groundnut, underutilised, genome, molecular markers, breeding, food security.

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Introduction

The quest for food security must be pursued around the world because of the increase in the human population worldwide combined with the effects of climate change. The global population has been increasing; in the last 12 years it increased by 1 billion to the current count of 7.9 billion (https://www.worldometers.info/world-population/). It is estimated that by 2050, the global population will reach 9.7 billion people, and possibly 11 billion by 2100 (James, 2015). Globally, 820 million people are currently suffering from chronic hunger and 2 billion are categorised as malnourishment by FAO, IFAD, UNICEF, WFP and WHO (2019). Sub-Saharan Africa was registered as having the world's highest proportion of undernourished people in 2016 according to FAO, IFAD, UNICEF, WFP and WHO (2017). Climate change events have also been predicted to have negative impacts on water resources, and hence, crop production (Piao *et al.*, 2010; Kole *et al.*, 2015). At present, there is an over reliance on a limited number of crop species for food. Less than twenty crops of the possible 50,000 documented edible plants provide over 90% of the plant based global food energy (Esquinas-Alcazar, 2005), with three staple crops (maize, wheat and rice) providing more than two thirds of this (IPES-Food 2016). If these staple crops start to fail due to biotic and abiotic stresses new alternative species will be needed to fulfil the global demand for food and nutrition (Mayes *et al.*, 2019). The best candidates should be those that have valuable traits such as drought tolerance, high nutritional density, possible nutritional or agricultural complementarity with other crops, and increased genetic diversity (Varshney *et al.*, 2010; Mayes *et al.*, 2019). Legumes, such as Bambara groundnut (*Vigna subterranea* (L.) *Verdc*.) (2n = 2x = 22), are a good alternative since they add nitrogen to the soil and provide good amounts of protein for human diets compared to cereals (Ahmad, 2016; Mayes *et al.*, 2019).

In this chapter we describe some of the advantages and disadvantages of Bambara groundnut, as well as the current and potential importance of this underutilised crop. Additionally, we present current developments in molecular research, from the genome to molecular breeding approaches. These developing resources will help researchers acquire knowledge and the molecular tools necessary to equip potential Bambara groundnut breeders to overcome the current restraints on wider adoption of this promising pulse.

Botanical description and general ecology

Bambara groundnut is an indigenous African protein-rich legume that is widely cultivated by subsistence and small-scale farmers in sub-Saharan Africa and South East Asia (mainly in West Java, Thailand and parts of Malaysia). Bambara groundnut is cultivated in the tropics at altitudes up to 2000 m above sea level. The crop is recognised to be tolerant to drought and is grown successfully

in areas with an average annual rainfall below 500 mm, although optimum yields are obtained when rainfall is higher (900–1000 mm year^{-1}) (Ocran *et al.*, 1998). Bambara groundnut can also be grown in humid conditions, such as in northern Sierra Leone, where the annual rainfall exceeds 2000 mm, although is seasonal. The crop grows on any well-drained soil, but light sandy loams with a pH of 5.0–6.5 rich in phosphorus and potassium are most suitable. Peduncle penetration and subsequent peduncle expansion to form pods can be aided by light sandy loam soils.

Bambara groundnut has a life cycle that ranges from 110 to 150 days, although some early maturing genotypes of Bambara groundnut have also been identified in Ghana, including 'Zebra coloured' with a maturation period of 90 days, and 'Mottled cream', which matures in 98 to 100 days (Berchie *et al.*, 2010a; Berchie *et al.*, 2010b). Bambara groundnut germinates between 7 to 15 days in temperature of between 28.5 °C and 32.5 °C (Makanda *et al.*, 2009), anthesis starts from 30 to 35 days after emergence and may continue until the end of the crop life cycle. The formation of pods takes 30 to 40 days after fertilisation and most genotypes require a photoperiod of 12 hours for optimal pod and seed development, although variation for this trait has been identified (Kendabie *et al.*, 2020). In many genotypes, flowering is not affected by photoperiod, however, long photoperiod can delay or inhibit pod set and/or seed development, such as in the genotypes 'Ankpa4' and 'Tiga Nicuru' (Linnemann 1993; Kendabie *et al.*, 2020), while other genotypes may produce increased yields under long photoperiods, with a delay in maturity date.

The morphology of Bambara groundnut is similar to that of groundnut (*Arachis hypogaea*). Bambara groundnut is an annual, herbaceous, intermediate legume with trifoliate leaves and erect petiole grown from short, creeping and multibranched lateral stems just above ground level (Figure 2-1) (Heller *et al.*, 1997). The cultivated forms of Bambara groundnut have stems with a limited creeping growth habit, which gives rise to either bunch or intermediate types (Linnemann and Azam-Ali, 1993). The petioles are long, stiff and grooved, they are grown from the nodes with a base of a range of colours including green, brown, and purple (Swanevelder, 1998). Bambara groundnut can grow up to 30 to 35cm in height with a well-developed tap root and lateral root branching under the soil (Mateva *et al.*, 2020), which are capable of forming root nodules in association with Rhizobia for nitrogen fixation (Foyer *et al.*, 2016; Considine *et al.*, 2017). Wild forms of Bambara groundnut (*Vigna subterranea* var. spontanea) demonstrate a slightly different morphology, such as a fully spreading growth habit, limited number of elongated lateral stems with pentafoliate leaves, and no distinct tap root (Swanevelder, 1998).

The flowers are generally described to be papilionaceous, and they are produced on long and hairy peduncles that elongate from the nodes of lateral stems (Swanevelder, 1998). The flower colour changes from yellow whitish in the morning, to pale yellow or light brown in the evening, and flowers generally open over 24 hours (Heller *et al.*, 1997). Upon pollination and fertilisation, the peduncles usually elongate and penetrate the soil surface, in some cases they would stay above

ground, and proceed to form pods with pod sizes ranging from 1.5 to 2.5 cm in diameter (Swanevelder, 1998). Approaching the maturity stage, various pod colours in Bambara groundnut are observed, ranging from cream yellow, pale or dark green, or red, depending on the genotypes (Massawe *et al.*, 2003a). Each of the pods can produce one seed, however, some genotypes are reported to have double-seeded seeds (Pasquet and Fotso, 1997; Gao *et al.*, 2020). Depending on genotypes, the seed colour in Bambara groundnut can be different, including cream, yellow, brown, red and black with or without hilum colouration and speckling (Swanevelder, 1998).

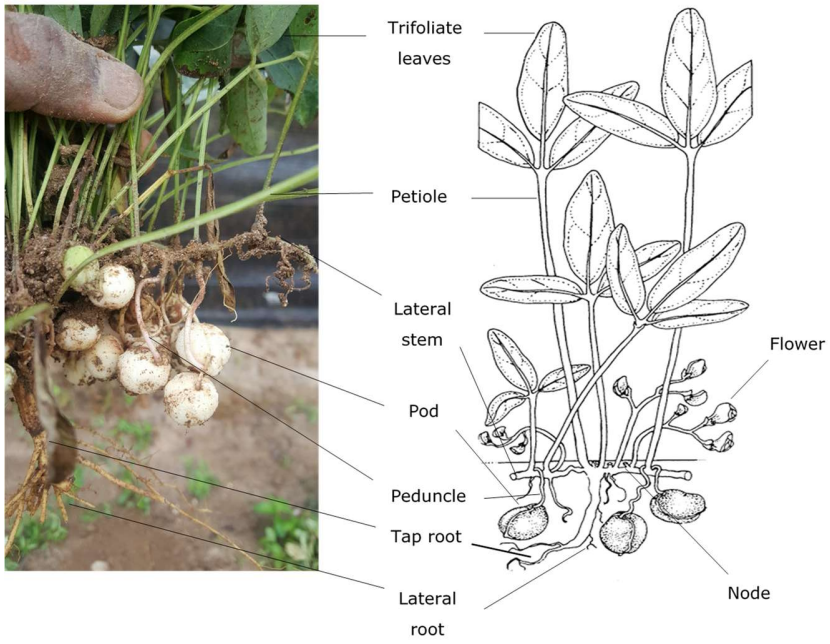

Figure 2-1 The morphology of Bambara groundnut (modified from National Research Council, 2006).

Geographical distribution

The name 'Bambara groundnut' is reputed to be derived from a tribe named Bambara, who mainly live in Mali today. However, no wild form of the crop has been found in Mali and the exact origin centre of Bambara groundnut has been unclear. Studies about the centre of origin of Bambara groundnut suggested that the crop originated from the African continent (Hepper 1970). As cited in Heller *et al.,* (1997), Guillemin *et al.* (1832) reported the discovery of wild forms near Senegal, it has also been suggested by Dalziel (1937) to have originated from the region between north eastern Nigeria (Yola province), and northern Cameroon (Garoua city).

The centre of domestication of Bambara groundnut is believed to extend from the Jos plateau and Yola region in Nigeria, to Garoua in Cameroon, and probably even to Central African Republic (Hepper, 1963; Begemann, 1988). Begemann (1988) carried out detailed analysis of seed diversity

for a large collection of Bambara groundnut from the International Institute of Tropical Agriculture (IITA). The results showed greater seed diversity in the samples collected within 200 km of the original putative centre, between Yola and Garoua (Hepper, 1963). In addition to seed traits, diversity was also observed in other traits, including number of days to maturity, pod length, and number of shoots per plant (Hepper, 1963). Interestingly, Somta *et al.* (2011) reported higher genetic diversity in accessions from Burkina Faso in contrast to those from Cameroon / Nigeria, hypothesising that the regions around Burkina Faso could be the more accurate place of domestication of Bambara groundnut. Though, recent work of Olukolu *et al.* (2012) using Diversity Array Technology (DArT) markers on 124 accessions from 25 African countries, revealed greater genetic diversity for the Cameroon / Nigeria region compared to other regions. Based on the analysis of 363 local varieties, Rungnoi *et al.* (2012) also concluded that West Africa is the centre of diversity/domestication of Bambara groundnut. Bambara groundnut has been reported to be grown in tropical regions since the 17th century, including Nigeria, Ghana, Haute Volta, Eastern Africa, and Madagascar. It is also grown in Central and South America, Oceania, Asia, including the Philippines, India, Sri Lanka, Indonesia, and Malaysia, and the South Pacific, as well as areas of northern Australia and Papua New Guinea. (Linnemann and Azam-Ali, 1993; Duke 1981; Baudoin and Mergeai, 2001).

Genetic resources, accessibility to/from seed banks

There are around 6,000 accessions of Bambara groundnut, mainly collected from African countries, and these collections are held by international or regional seed banks (Table 2). The largest Bambara groundnut germplasm collection is held by the International Institute of Tropical Agriculture (IITA) (Goli, 1997). The collection was gathered from 25 African countries, and has been characterised, evaluated, and documented (Goli, 1997). The crop is still largely grown as landraces and the variation harboured by these landraces is a great asset for breeding programmes (Olukolu *et al.* 2012; Kendabie *et al.* 2015; Mayes *et al.* 2015; Massawe *et al.* 2016). Phenotypic descriptors (IPRGI, 2000), biochemical markers (Pasquet *et al.* 1999), molecular markers including Amplified Fragment Length Polymorphism (AFLP) markers (Massawe *et al.* 2002), Random Amplified Polyphormic DNA (RAPD) (Massawe *et al.* 2003b), Single-Sequence Repeat (SSR) markers (Molosiwa *et al.* 2013; Aliyu and Massawe 2013; Redjeki *et al.* 2020), DArT markers (Olukolu *et al.* 2012) and Single Nucleotide Polymorphism (SNP) markers (Redjeki *et al.* 2020) have been used to assess genetic diversity within the available germplasm of Bambara groundnut.

Table 2 Bambara groundnut accessions held by countries or institutions (Begemann and Engels 1997; Muhammad *et al.* 2020).

Country/Institution	No. of accessions
Benin	3
Botswana	26
Botswana, Department of Agricultural Research (DAR)	338
Burkina Faso	143
France, Office de la Recherche Scientifique et Technique d'Outre-Mer	1416
Ghana, Plant Genetic Resources Unit (PGRC)	166
Ghana, Plant Genetic Resources Research Institute (PGRRI)	296
Ghana, Savanna Agricultural Research Institute (SARI)	90
Ghana, University of Ghana	80
Guinea	43
Kenya, Kakamega Regional Research Centre (KARI)	2
Kenya, National Genebank	6
Kenya, National Museums	2
Mali	70
Mozambique	12
Namibia	23
Niger	79
Nigeria, International Institute of Tropical Agriculture (IITA)	2035
South Africa, Department of Agriculture	20
South Africa, Grain Crops Institute	198
South Africa, Institute for Veld and Forage Utilization	117
Tanzania, National Plant Genetic Resources Committee (NPGRC)	222
Zambia, National Plant Genetic Resources Committee (NPGRC)	232
Zambia, University of Zambia	463
Zimbabwe	129
Total	6211

Bambara groundnut – an important but underutilised crop

Traits of importance – drought resistance

Bambara groundnut uses a combination of drought resistance mechanisms to produce yield under drought conditions. The adaptive characteristics that enable Bambara groundnut to survive under drought conditions have been studied (Collinson *et al.*, 1997; Collinson, Berchie and Azam-Ali, 1999; Jørgensen *et al.*, 2010; Sesay *et al.*, 2010; Vurayai, Emongor and Moseki, 2011; Tafadzwanashe Mabhaudhi and Modi, 2013; Chibarabada, Modi and Mabhaudhi, 2015; Chai, Massawe and Mayes, 2016; Muhammad, Mayes and Massawe, 2016) and could be explored further to develop Bambara groundnut varieties for drought prone areas.

Physiological changes – above ground

As reviewed in Mayes *et al.* (2019a), drought-resistance mechanisms in Bambara groundnut have been studied and evaluated over a period of 30 years (Collinson *et al.* 1997, 1999; Jorgensen *et al.* 2010; Vurayai *et al.* 2011; Laary *et al.* 2012; Mabhaudhi and Modi 2013; Al Shareef *et al.* 2014; Chibarabada *et al.* 2015; Berchie *et al.* 2016; Nautiyal *et al.* 2017). Given that Bambara groundnut is tolerant to drought, cultivation of Bambara groundnut may be one of the few options in drylands with minimal rainfall. Various reports with clear evidence have identified the potential of Bambara groundnut in response to drought stress through stomatal regulation and osmotic adjustment (Collinson *et al.*, 1997; Jorgensen *et al.*, 2010; Mabhaudhi *et al.*, 2013; Chai *et al.*, 2016ab). For example, the genotype S19-3, originating from Namibia, was reported to have late stomatal closure during drought stress (Jørgensen *et al.*, 2010). The authors further defined S19-3 as a "water-spender" exhibiting a slow decline in transpiration rate enabling the genotype to maximise use of available water. Accordingly, this is in line with a root system study by Mateva *et al.* (2020), identifying S19-3 as a genotype with a quick and high root length density in the deeper soil layers compared to the topsoil layer. The value in this would be increased root and soil contact enabling plants to access more water in lower soil depths. This value was also reported by Lynch (2007) and Blum (2011).

Bambara groundnut is also found to be able to escape from drought and this is related to phenological plasticity. Bambara groundnut was observed to have a reduced vegetative period, a reduced reproductive stage and earlier final maturity date in response to drought stress (Mabhaudhi *et al.*, 2013). For example, landraces 'Red' and 'Brown' from Jozini, South Africa, demonstrated a significantly earlier maturity date (mean: 122.8 days after planting (DAP)) when subjected to stress at 30% of crop water use (ET_a) compared to 100% ET_a (mean: 128 DAP; Mabhaudhi *et al.*, 2013). Although drought stress generally decreases the yield of most of the crops, Bambara groundnut is still able to produce a reasonable yields of up to 1.65 t ha^{-1} of seeds with a range of 1.3 to 2.1 t ha^{-1} (Mwale *et al.*, 2007a). These yields are reported to be similar to drought tolerant cultivars of groundnut and are higher than chickpea cultivars (0.3 to 0.5 t ha^{-1}) under comparable drought stress condition (Leport *et al.*, 1999; Collino *et al.*, 2000).

High efficiency of resource capture and conversion are believed to contribute to crop productivity under drought. Although Bambara groundnut was observed to have reduced radiation conversion coefficient (ε_s) from 1.51 to 1.02 g MJ^{-1} due to drought stress, the ε_s of Bambara groundnut reported in Mwale *et al.* (2007b) is higher than those of reported in soybean, ranging from 0.52 to 0.92 g MJ^{-1} (Board *et al.*, 1994; De Costa and Shanmugathasan, 2002), and cowpea (*Vigna unguiculata*; Craufurd and Wheeler, 1999) under minimal soil moisture conditions. In addition, the efficiency of plants to convert water into dry matter (ε_w) is essential for yield production. The ε_w of Bambara groundnut (1.65 g kg^{-1}) under drought stress (Mwale *et al.*, 2007b) was reported to be higher than most of the grain legumes grown in low rainfall Mediterranean environments, such as lentil (*Lens*

culinaris; 1.37 g kg^{-1}; Zhang *et al.*, 2000) and chickpea (*Cicer arietinum*; 1.11 g kg^{-1}; Siddique *et al.*, 2001).

Root trait system variation and its contribution to drought stress resistance

Roots are one of the most important organs for transporting various materials from the soil and thereby controlling productivity (Lynch, 1995). Plants can modify their root system architecture (RSA) to respond to a variety of conditions (Jovanovich *et al.*, 2008).

As an underutilised grain legume, Bambara groundnut has not been intensively studied for RSA. A better adapted RSA has been linked to alleviation of drought stress by increasing exploration for water in Bambara groundnut genotypes (Mateva *et al.*, unpublished). The root system of Bambara groundnut, as with many dicotyledons, is characterised by a well-defined taproot system, with numerous first-order lateral branches. These lateral roots further branch into second- and third-order laterals. The depth of rooting and distribution of lateral roots are determining factors for RSA in Bambara groundnut (Mateva *et al.*, 2020).

In a comparative analysis of RSA of eight Bambara groundnut genotypes derived from landraces, sourced from several countries, natural genetic variations in RSA has been reported, and could be utilised for improvement of drought resistance (Mateva *et al.*, unpublished). Using a lightweight polyvinyl chloride (PVC) columns evaluation, a known drought-resistant genotype (S19-3, from Namibia) showed a deeper tap root and more branching in the lower soil depths (Mateva *et al.*, 2020). Recently, the genotype DodR (sourced from Tanzania) was identified as showing promising RSA for extensive root length density in the 60-90cm of the soil and this was associated with grain yield. Mateva *et al.* (2020) suggested an adaptive response of Bambara groundnut for soil resource capture through an improved foraging capacity of the root system in the hot-dry region derived single genotypes. Furthermore, genotypes that evolved in drier areas could have adapted by increasing tap root length (TRL) and reducing their branching distribution to capture deep water more efficiently.

In addition, by screening of a bi-parental populations obtained from crossing two distinct single genotypes (i.e. S19-3 × DodR) (~22 lines), TRL and root length density in the 60-90cm region (RLD 60-90cm) of the soil were found to be useful traits for selecting Bambara groundnut lines for drought resistance (Mateva *et al.*, unpublished). In this study, lines with promising TRL and RLD 60-90cm were identified for further evaluation to breed more drought-resistant Bambara groundnut varieties. Quantitative Trait Loci (QTL) mapping could be deployed to identify chromosomal regions that have a substantial impact on root system variation particularly TRL and RLD 60 to 90cm in Bambara groundnut populations to further accelerate breeding outcomes.

Nutritional composition

As with most underutilised crops, there are limitations in terms of access to reliable datasets for analysis of the variation for traits within and between lines. For example, determining intra- and interspecies variation for nutritional components would enable direct comparison with commodity crops (Halimi *et al.*, 2019a, see example in Figure 2-). Such data could be useful at the policy making level to recognise the role underutilised crops may play alongside staple crops for food security (Mabhaudhi *et al.*, 2018, Pingali, 2015). Comparative analysis of the available literature on nutritional composition of Bambara groundnut and four taxonomically related legume species (Halimi *et al.,* 2019b) indicated that there is potential to develop the crop into a high protein or high oil species. The literature indicated a seed protein range of 9.6-30.7%, with larger variation than those reported for major legumes such as chickpea and cowpea.

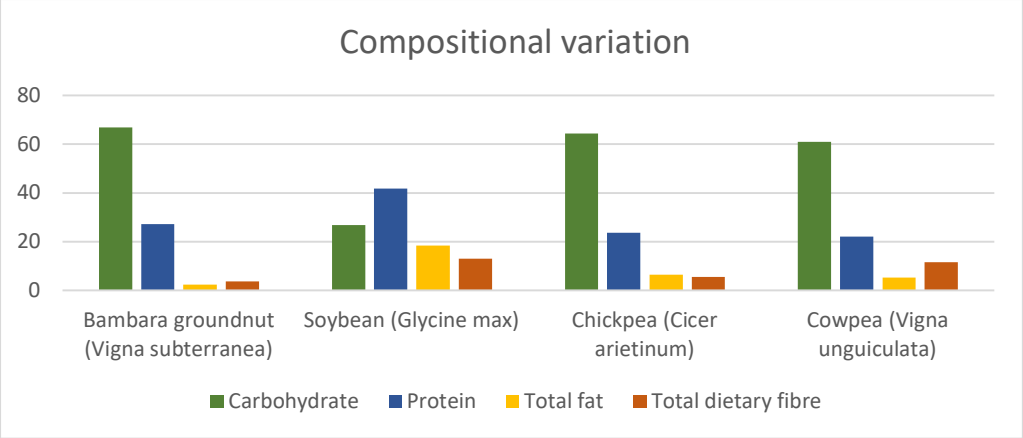

Figure 2-2 Compositional variation in the four proximate components for raw Bambara groundnut (Vigna subterranea) seeds, and selected crop comparators: soybean (Glycine max), chickpea (Cicer arietinum), and cowpea (Vigna unguiculata). Green - carbohydrate, blue - protein, yellow - total fat, and orange - total dietary fibre. Data are presented as calculated mean values expressed as % edible portion. Dataset for each compound for each crop was constructed from at least three data sources; dataset averages were calculated and normalized to 100%. Adapted from Halimi *et al*. (2019b).

The seed lipid of the 100 lines were used to determine the fatty acid composition on Gas Chromatography Flame Ionization Detector (GC-FID) using the Association of Official Analytical Chemist method 996.06 (Halimi *et al.,* 2019b). Twenty-one fatty acids were detected in Bambara groundnut seed lipid (Figure 2-and Table 3); a marked increase compared with the limited number of fatty acids reported for this species previously - oleic, linoleic, palmitic, myristic, stearic, behenic, and linolenic acids (Minka and Bruneteau, 2000, Mune *et al.*, 2007, Adeleke *et al.*, 2018). A study of a Bambara groundnut landrace found in Ivory Coast detected 13 fatty acids (Yao *et al.*, 2015) and this study has increased the knowledge base further. The predominant components observed were

linoleic acid (18:2 n-6) which accounted for 33 to 45% of the fatty acid, oleic acid (18:1, n-9) (15 to 27%), and palmitic acid (16:0) (16 to 23%). The concentration of oleic acid was similar to that present in soybean lines prior to modern selection for this trait.

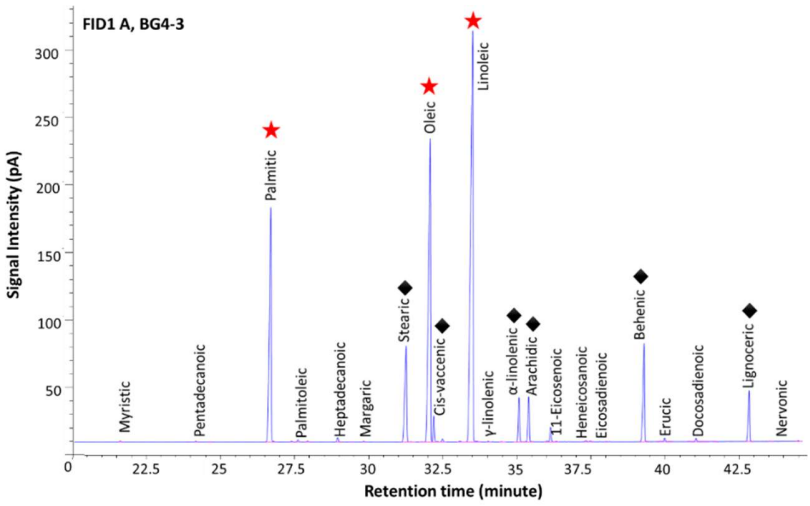

Figure 2-3 Typical GC-FID chromatogram showing separation (retention times, minutes) of fatty acid methyl esters from Bambara groundnut (V. subterranea) seed lipid. Red stars (★) indicate peaks of major fatty acids and black diamonds (♦) indicate minor fatty acids (*Halimi A*, unpublished).

Table 3 Typical GC-FID area percent report tabulating retention times (min) and composition of each fatty acid (area %) measured for Bambara groundnut (*V. subterranea*) seed lipid. Peak area for each fatty acvid is calculated by multiplying peak height (pA) by sample concentration (s).

Peak #	Retention	Width	Area [pA*s]	Area %	Fatty acid	Fatty acid
1	21.720	0.052	3.005	0.0608	Myristic	14:0
2	24.332	0.049	1.881	0.0381	Pentadecanoic	15:0
3	26.958	0.066	809.976	16.401	Palmitic ★	16:0
4	27.859	0.049	5.312	0.108	Palmitoleic	16:1(n-7)
5	29.230	0.053	11.497	0.233	Heptadecenoic	17:1
6	30.236	0.056	1.818	0.037	Magaric	17:0
7	31.626	0.069	352.930	7.147	Stearic ◆	18:0
8	32.469	0.068	1181.555	23.925	Oleic ★	18:1(n-9)
9	32.586	0.046	57.321	1.161	cis-vaccenic ◆	18:1 (n-11)
10	33.920	0.083	1768.274	35.806	Linoleic ★	18:2 (n-6)
11	34.707	0.046	0.341	6.928e-3	γ-Linolenic	18:3(n-3)
12	35.552	0.049	106.194	2.1503	α-Linolenic ◆	18:3(n-3)
13	35.820	0.052	112.602	2.2801	Arachidic ◆	20:0
14	36.591	0.049	34.557	0.6997	11-eicosenoic	20:1(n-11)
15	37.384	0.060	3.229	0.0654	Heneicosanoic	21:0
16	37.971	0.050	2.405	0.0487	Eicosadienoic	20:2
17	39.887	0.060	295.687	5.9874	Behenic ◆	22:0
18	40.537	0.049	9.205	0.1864	Erucic	22:1(n-9)
19	41.636	0.051	8.291	0.1679	Docosadienoic	22:2 (n-6)
20	43.441	0.050	136.987	2.7739	Lignoceric ◆	24:0
21	44.194	0.048	0.779	0.0158	Nervonic	24:1(n-9)
Totals			**4903.848**	**99.298**		

Underutilisation of Bambara groundnut

Bambara groundnut has been reported to have unpredictable yield (with planting material consisting of landraces), lack of commercial varieties, sensitivity to long photoperiods, long cooking time, and have few value-added product opportunities (Mayes *et al.,* 2019b). Additionally, climate change is happening too rapidly for crops, including Bambara groundnut, to passively adapt and may lead to erratic yields in currently used Bambara groundnut landraces, hence deliberate breeding in Bambara groundnut is required.

Bambara groundnut yield in Africa is estimated to be approximately 0.3 million tonnes annually, with Nigeria being the largest producer (0.1 million tonnes; Hillocks *et al.* 2012). The average annual production of legumes in Africa has been reported by Stanton *et al.,* 1966; Hillocks *et al.,* 2012; Nedumaran *et al.,* 2015; and reviewed by Mayes *et al.* 2019a (see Table 4). It is important to take note that the yield of Bambara groundnut in Africa varies between landraces and locations (0.5 to 3 t ha^{-1}), but the crop has yield potential of over 3 t ha^{-1} (Begemann 1988) and the average yield of 0.85 t ha^{-1} was reported to be comparable to other legumes (Stanton *et al.* 1966). Additionally, an estimated macronutrient comparison based on the findings of Halimi *et al.* (2019a) is presented in Table 4. In comparison to a major legume such as chickpea and cowpea, Bambara groundnut has higher fat content (55.3kg/ha for Bambara groundnut, 49.4kg.ha for chickpea and 11.9kg/ha for cowpea). Bambara groundnut also provides more protein per ha than cowpea. (Table 4). Compiled literature values represent the current situation for unimproved material as there are no commercial varieties released with improved nutritional composition (Halimi *et al*., 2019a). With Bambara groundnut showing approximately half the nutritional potential as soybean, there exists opportunity to develop varieties to meet the global demand for energy and protein.

Table 4 The yield and production of a subsection of legumes in Africa with estimated nutritional values (adopted from Mayes *et al.* 2019a as reported in Stanton *et al.*, 1966; Hillocks *et al.* 2012; Nedumaran *et al.* 2015, Halimi *et al.*, 2019a).

	Annual production (million tonnes)	**Yield (t ha^{-1})**	**Estimated macronutrient values in kg/ha**			
			Carbohydrate	**Protein**	**Total Fat**	**Dietary fibre**
Cowpea	4.9	0.49	327.12	133.13	11.86	17.93
Soybean	1.4	1.22	327.45	509.47	223.99	159.09
Bambara groundnut	0.3	0.85	547.66	200.52	55.34	46.75
Chickpea	0.3	0.94	573.21	207.55	49.35	109.79

Berchie *et al.* (2016) reported that the time of sowing affected the yield of Bambara groundnut, in which higher yields were observed in the dry minor rainfall season compared with the major rainy season. Pod yields of up to 4 t ha^{-1} were obtained in some landraces in the transition agro-ecological zone in Ghana, where temperatures are higher and rainfall is lower compared to forest agro-ecological zone (Berchie *et al.*, 2016). However, if the cultivation of Bambara groundnut occurs at the appropriate time, relatively high yields could be attained in the forest agro-ecological zone in Ghana (Berchie *et al.*, 2016).

As mentioned before, photoperiod was reported to influence the onset of flowering and podding of Bambara groundnut, depending on the genotypes (Linnemann and Craufurd, 1994; Linnemann *et al.,* 1995; Kendabie *et al.*, 2020). For instance, long photoperiods of 14 h and 16 h delayed flowering in genotype 'Ankpa4' which produced no pods, while genotype 'Tiga Nicuru' had delayed podding and decreased number of pods when photoperiod was increased from 12 to 14 h (Kendabie *et al.*, 2020). Nevertheless, significant differences amongst genotypes in response to long photoperiod have been identified (Kendabie *et al*., 2020), and crosses between 'quantitative long day' (IITA-686) and 'qualitative short day' genotype (Ankpa 4) in Bambara groundnut allows the generation of individual lines to be selected for future breeding programmes.

Similar to many pulses, long cooking time due to the 'hard-to-cook' (HTC) phenomenon, which was defined to reflect the amount of energy required for the legume to have desirable texture and edible, is recognised as a major limitation for the usage of Bambara groundnut. Bambara groundnut

generally needs 3–4 h of boiling, which is almost identical to that of soybean (3.6 h), but it is significantly longer than common bean (1.5 h), cowpea (2.4 h), and mung bean (0.5 h) (Mubaiwa *et al.* 2017), leading to greater fuel and water requirement, thus increasing the cost to cook in many developing countries (Adzawla *et al.*, 2016). Additionally, HTC also negatively impacts the eating and nutritional qualities on Bambara groundnut. Aging of seeds, as a result of long-term storage under increased temperature and humidity, was found to be associated with development of HTC traits, and aging also can reduce *in vitro* bioavailability of calcium and magnesium (Gwala *et al.*, 2020). The levels of minerals including magnesium, iron and zinc (Gwala *et al.*, 2020), and protein quality (Tuan and Philips, 1992) were also observed to be affected by prolonged cooking time on aged seeds. Research and investment in appropriate processing methods and machinery – particularly micro-manufacture - would be necessary to minimise the limitation and increase the uptake of Bambara groundnut in the market.

Although Bambara groundnut has been cultivated for centuries in Africa, it remains as one of the underutilised crops that has not been long associated with large-scale research programmes as has the case been for many other crops. Bambara groundnut has been largely ignored by the research and breeding community and received limited support from governmental or international agencies, as compared to major crop like soybean, which has received significant attention and considerable scientific and financial support since its introduction (Heller *et al.*, 1997; Oyeyinka *et al.*, 2015). Bambara groundnut also faces competition from groundnut (which was introduced into West Africa from Brazil), due to significant amounts of oil in the seeds, and hence groundnut can be cultivated as an oilseed crop. Bambara groundnut (along with other seed legumes) is commonly referred to as a 'poor man's crop'. The perception that underutilised crops, including Bambara groundnut, have lower economic potential and export value compared to major crops, thus influences the exploitation of the crop (Azam-Ali *et al.,* 2001). Although Bambara groundnut can have higher market prices than other legumes, including groundnut, due to seasonal crop supply there are only a limited number of value-added products which have been developed for Bambara groundnut. In addition, proper seed systems and best agronomic practices are yet to be established and shared with the Bambara groundnut production community, causing the crop to remain underutilised (Hillocks *et al.* 2012; Feldman *et al.* 2019). However, the results of specific research programmes indicate that Bambara groundnut is a crop with considerable potential that could contribute to food and nutritional security, especially as a food crop in dry areas with marginal soils. As a drought-tolerant legume, Bambara groundnut deserves to receive greater attention for further research and development.

Nevertheless, research attention is required to develop improved varieties and crop management practices to increase yield production as well as harvest index, especially under drought conditions. For many crop species it is often challenging to select for grain yield under drought conditions due to the interaction of genotype x environment (GxE), and thus, reliable and accurate phenotyping

tools are important to incorporate targeted traits into molecular breeding programme and dissect genes controlling traits of interest (Salekdeh *et al.*, 2009). In addition to yield traits, breeding work targeting traits such as tolerance to heat, disease resistance, seed nutrient quality, and palatability of the foliage would be of value, therefore, Bambara groundnut can be used as pasture crop as well as seed consumption and as a cash crop for use by resource-poor farmers (Mayes *et al.*, 2019a).

Molecular tools and their application in Bambara groundnut

Scientific work in plant genetics has used different molecular markers, but the reduction of costs and the development of technologies such as next generation sequencing has allowed a significant increase in molecular work. In the following sections we present a few examples of how this research has been implemented in Bambara groundnut until present day.

Molecular markers – development and applications

As an important component of both fundamental research and practical application in many studies of plants, animals, and microorganisms, a genetic linkage map represents the relative order of genetic markers along a chromosome and the relative distance between them, determined by recombination frequency (Yeboah *et al.* 2007; Liu *et al.* 1998). Understanding the genetic basis and identification of molecular markers for target traits are prerequisites for deploying molecular breeding for developing superior genotypes (Kullan *et al.* 2012).

The first genetic linkage map reported in Bambara groundnut was constructed using 67 AFLP and one SSR markers, consisting of 20 linkage groups and 516 cM in length using an F_2 segregating population derived from a cross between a wild accession, VSSP11, and a cultivated accession, DipC (Basu *et al.* 2007). QTL analysis in the F_2 population identified a range of QTLs associated with agronomic traits including *internode length, leaf water use efficiency* (LWUE), *carbon isotope discrimination* (Δ13C), *seed weight* and *testa colour* (Basu *et al.* 2007). The first intraspecific genetic linkage map between two cultivated accessions was constructed using 269 polymorphic markers, which included 236 DArT and 33 SSR markers, from a F_3 segregating population of Bambara groundnut derived from a narrow cross between cultivated accessions, DipC and Tiga nicuru (Ahmad *et al.* 2016). The genetic map consists of 21 linkage groups (LGs) with a total genetic distance of 608.3 cM, a total of 36 significant QTLs associated with various important phenotypic traits in Bambara groundnut were detected (Ahmad *et al.* 2016). In addition to linkage map construction, two significant QTLs were mapped for the *internode length* (LG4, 3.0 cM) and growth

habit (LG4, 0.0 cM) explaining more than 40% of phenotypic variation in the F_3 populations under controlled environment glasshouse and field conditions (Ahmad *et al.* 2016).

The first gene expression marker-based genetic map (GEM map) in a F_5 population of Bambara groundnut was developed for QTL analysis using 527 markers and covered 982.7 cM and 13 linkage groups (Chai *et al.* 2017). QTLs associated with *stomatal conductance, carbon isotope discrimination, and stomatal density*, were largely mapped on LG2 (Chai *et al.* 2017). QTLs for (Δ N15) isotope analysis (NID) mapped on LG1 and were associated with *internode length, pod number per plant, pod weight per plant* and *seed number per plant*, showing a positive relationship between nitrogen assimilation and biomass in plants (Chai *et al.* 2017). A combination of population-specific and pre-selected common markers were used to construct two individual intraspecific genetic maps in Bambara groundnut from the two crosses: a genetic map of IITA686 × Ankpa4, which was derived from 263 F_2 segregating population, gave 11 linkage groups comprising of 223 DArTSeq markers, and covered 1,395.2 cM; while a genetic map of Tiga Nicuru × DipC, derived from 71 F_3 segregating population, showed 11 linkage groups consisting of 293 DArTSeq markers, and covered 1,376.7 cM (Ho *et al.* 2017). A significant QTL for *internode length* was mapped on LG2 (50.6 cM; flanking markers between 47.6 – 54.4 cM), explaining 33.4% phenotypic variation observed in this cross. This was syntenic to *Pv03* (38.4 – 39.1 Mbp; common bean), *Va11* (12.5 – 17.4 Mbp; azuki bean) and *Vr07* (39.4 – 43.5 Mbp; mung bean) (Ho *et al.* 2017).

Microarrays

Microarrays have been widely adopted in past few years to generate expression-based markers for the development of expression-based genetic map, expression quantitative trait loci (eQTL) as well as conventional QTL studies (Winzeler *et al.*, 1998; Ronald *et al.*, 2005; West *et al.*, 2006; Potokina *et al.*, 2007; Hammond *et al.*, 2011; Chai *et al.*, 2017). Expression-based markers, such as gene expression markers (GEMs), can be developed for map construction on the basis of significant differences in hybridisation signal strength observed between individuals when mRNA or cRNA is hybridised to microarrays, as a result of either sequence polymorphisms effecting the hybridisation efficiencies, or genuine differences in the transcript abundance (Chai *et al.*, 2017). The potential of using microarrays developed for a major and/or model plant species to analyse less intensively studied species, such as Bambara groundnut, is known as XSpecies (cross-species) microarray approach. Some examples of proof-of-concept studies reported on XSpecies microarray approaches, including eggplant and pepper on tomato microarray (Moore *et al.*, 2005), potato on tomato microarray (Bagnaresi *et al.*, 2008), cowpea on soybean microarray (Das *et al.*, 2008), banana on rice microarray (Davey *et al.*, 2009), sweet sorghum on sugarcane microarray (Calvino *et al.*, 2009), and *Brassica oleracea* on *Arabidopsis* microarray (Hammond *et al.*, 2005; Hammond *et al.*, 2011).

Chai *et al.* (2017) also reported the generation of GEMs at the unmasked probe-pair level after Bambara groundnut leaf RNA was cross-hybridised onto Affymetrix Soybean Genome GeneChip, followed by construction of the first spaced GEM map consisting of 13 linkage groups containing 218 GEMs, covering 982.7 cM of the Bambara groundnut genome. Comprehensive QTL analysis with good genome coverage using the GEM map also demonstrated the use of XSpecies microarray pipeline in mapping both intrinsic and drought-related QTLs in Bambara groundnut, allowing targeted QTL to be identified and used for marker-assisted selection (MAS) breeding in the future (Chai *et al.*, 2017). Transcriptomic changes in two Bambara groundnut genotype, DipC and Tiga Nicuru, in response to drought stress were also studied by cross-hybridising cDNA onto the Soybean Affymetrix GeneChip (Khan *et al.*, 2017). According to Khan *et al.* (2017), this revealed different sets of transcription factors and dehydration-response genes in the two genotypes. For example, DipC displayed differential expression of transcription factors WRKY40, while Tiga Nicuru showed differential expression of CONSTANS-LIKE 1 and MYB60. The XSpecies microarray approach has been demonstrated to have the potential of investigating molecular mechanisms underlying traits of interest related to drought in Bambara groundnut.

Nevertheless, the hybridisation efficiency of transcripts onto the probes could be affected by sequence divergence, leading to inaccurate abundance signals that might be an obstacle in data analysis. It could even cause the loss of signal, especially when Affymetrix technology is utilised, as the cross-hybridisation is dependent upon a set of 11 oligonucleotides, which constitute a probe-set and each probe is only 25 nucleotides in length. Even with other microarray technology, such as Agilent, where the probe is a 60-mer, evolutionary distance between reference species and targeted species could still be a confounding factor. Divergence time between Bambara groundnut and soybean is reported to be 20 My (Cannon *et al.*, 2009). However, another complication of using the soybean microarray to study Bambara groundnut, is the duplication of soybean genome ($2n = 2x = 40$) since evolutionary divergence of the two species.

The XSpecies microarray approach offers an alternative feasible route to translate information from major, or model plant species, to underutilised and less researched crops, especially in some cases where there is limited public access to sequence resources. It is also important to take note that the XSpecies microarray approach was a cheaper alternative to next generation sequencing, although applications of both methods could be appropriate in different situations (Lai *et al.*, 2014). As sequencing technologies are evolving at a rapid pace, and the cost of sequencing is declining, RNA sequencing (RNAseq) technology offers benefits in studying transcriptomes for any species, including detection of novel transcripts as they do not require species- or transcript-specific probes like microarrays, have a greater dynamic range, higher specificity and sensitivity and allow detection of rare and low-abundance transcripts (Han *et al.*, 2015).

RNAseq in Bambara groundnut

Scientific efforts have been recently made to analyse the Bambara transcriptome of leaf tissue (unpublish data). A drought experiment involving four contrasting genotypes, under severe drought and well-watered conditions was performed, and leaf samples were collected for RNA isolation. The RNA was sequenced using Illumina NovaSeq platform. Figure 2- shows, in a Venn diagram, the significantly differentially expressed genes within each genotype in response to drought and their overlap between genotypes. Additionally, Figure 2-5 shows the number and statistical significance of up- and down-regulated genes in "Tiga nicuru" in response to drought (unpublished data).

The data resulting from the RNA sequencing will also serve in the future for transcriptome and gene expression analyses. Additionally, it is being used for future genome annotation.

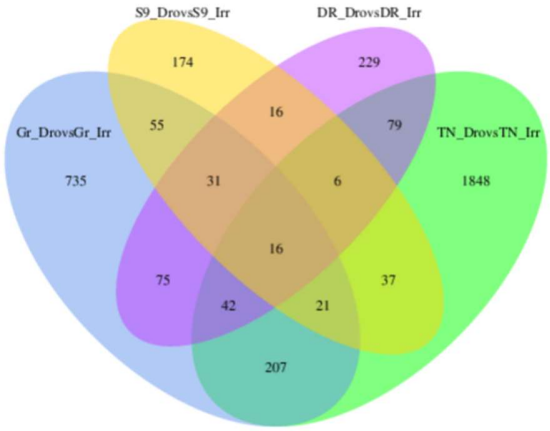

Figure 2-4 Venn diagram of four paired transcriptomes (Irrigated vs drought) of 4 genotypes (Gresik, S19-3, DodR, and Tiga nicuru), showing the number of common and unique genes expressed in response to drought (unpublished data).

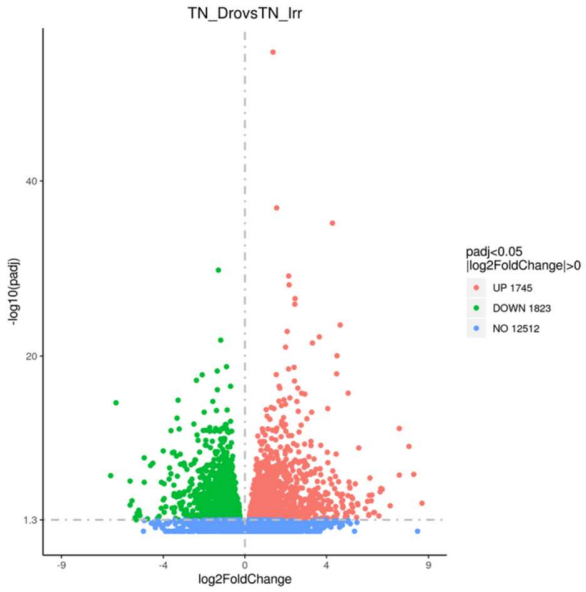

Figure 2-5 Volcano plot showing the up and down regulated genes of genotype 'Tiga nicuru' by level of significance in response to drought (unpublished data).

Bambara groundnut genome – current achievements

First glance at the genome

The genomes of a few *Vigna* species such as mung bean (*Vigna radiata*) and adzuki bean (*Vigna angularis*) have been sequenced and published (Kang *et al.*, 2014; Yang *et al.*, 2015). However, the first attempt in generating a genome sequence for Bambara groundnut was by the African Orphan Crops Consortium (AOCC), as this crop is among the 101 selected nutritious African orphan food crop genomes to be sequenced to act as a starting point for genetic improvement (AOCC, 2020; Chang *et al.*, 2019). Chang *et al.* (2019) published the first draft genome of Bambara groundnut along with four other species (*Lablab purpureus*, *Faidherbia albida*, *Sclerocarya birrea*, and *Moringa oleifera)* based on shotgun sequencing using the Illumina platform. For Bambara groundnut, this produced an assembly of 535Mb with N50 at 640,666 bp (N90 = 75,271 bp). With the genome size predicted to be 550Mb from k-mer analysis, this genome assembly is expected to cover 97.3% of the genome, despite being fragmented (65,586 scaffolds in total). From Benchmarking Universal Single-Copy Orthologs (BUSCO) analysis, the genome assembly completeness has been estimated to be at 92.1%.

With an average GC content of 33.2%, the GC content of Bambara groundnut is similar to other legume species, particularly soybean (*Glycine max*), and common bean (*Phaseolous vulgaris*) (Chang *et al.*, 2019). Long terminal repeat (LTR) mobile elements were predicted to be the most in

abundant class (38.4%) of the transposable elements (TEs) identified in Bambara groundnut. In both cultivated and wild soybean, a further characterisation found LTR/gypsy family to be predominant (Schmutz *et al.*, 2010; Xie *et al.*, 2019). On the other hand, short interspersed nuclear elements (SINEs), long interspersed nuclear elements (LINEs), satellite and simple repeats accounted for less than 1% of the Bambara groundnut genome (Chang *et al.*, 2019).

Coupled with the transcriptomic resources from different stages of leaf tissue and stem, 31,707 protein coding genes were predicted with 84.2% belonging to 16,307 gene families (Chang *et al.*, 2019). Among these, 7,541 were transcription factors predominantly basic helix–loop–helix (bHLH) type, occupying 10.5% of them (Chang *et al.*, 2019). A total of, 83.6% and 79.4% of these genes were found to have high similarity ($< e^{-30}$) with common bean and cowpea (*Vigna unguiculata*) genes, respectively. Within *Vigna* family, this has suggested a greater number of genes in comparison to mung bean (22,427), however, fewer than adzuki bean (34,183) (Chang *et al.*, 2019). The comparison done by Chang *et al.* (2019) of all-versus-all BLASTP alignment of the protein and nucleotide sequences from *Lablab purpureus*, *Faidherbia albida*, *Sclerocarya birrea*, *Moringa oleifera*, *Arabidopsis thaliana*, *Carica papaya*, *Citrus sinensis*, *G. max, Medicago truncatula, Oryza sativa, Phaseolus vulgaris, Sorghum bicolor, and Theobroma cacao*, showed a total of 609 gene families specific to Bambara groundnut. Presence of shared gene families between *L. purpureus*, *F. albida*, *S. birrea*, *M. oleifera*, and *V. subterranea* is presented in Figure 2-, where 789 gene families containing 3,118 genes specific to *V. subterranea*. It is not surprising that the expansion (1,322 *vs* 1,106) and contraction (2,098 *vs* 1,850) gene families within Bambara groundnut and common bean are similar. From the analysis of 141 single-copy genes, it has been estimated that Bambara groundnut diverged from common bean and soybean approximately 10.2 Mya and 25.1 Mya, respectively (Chang *et al.*, 2019). Additionally, Chang *et al.* (2019) reported that in Bambara groundnut, additional paralogs mainly related to carbon fixation, zeatin biosynthesis, and glyoxylate and dicarboxylate metabolism. Furthermore, the expanded gene families are enriched in the glucosinolate (ko00966) and secondary metabolites (ko01110) biosynthesis pathways (Chang *et al.*, 2019).

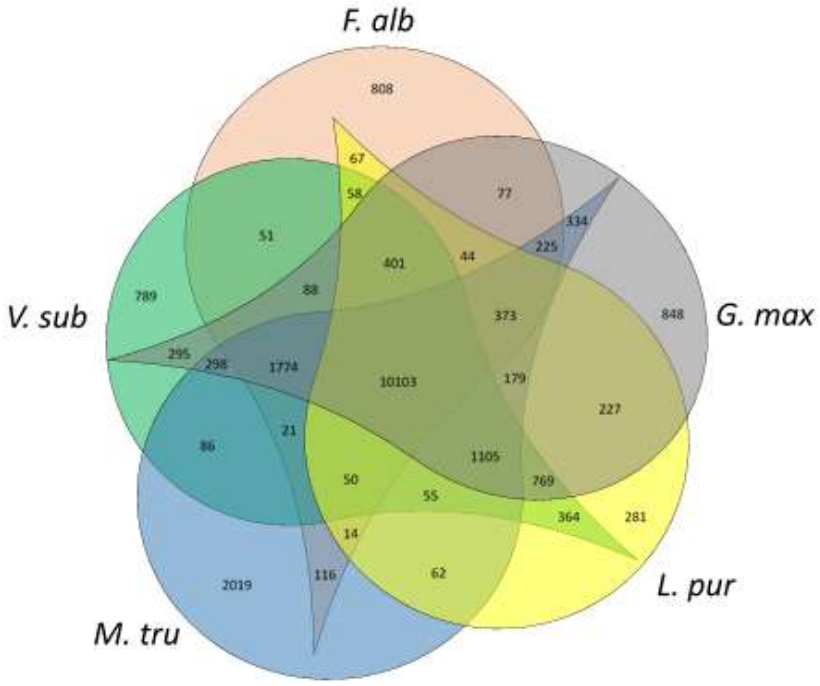

Figure 2-6 Orthologue groups shared between Lablab purpureus (L. pur), Faidherbia albida (F. alb), Glycine max (G. max), Medicago truncatula (M. tru), and Vigna subterranea (V. sub) adapted from Chang *et al.*, (2019).

Structure and nomenclature of traits in linkage to genome

Although Bambara groundnut has been proposed/adopted as an exemplar underutilised crop, the potential of genomic platforms contributing to crop improvement has yet to be realised. As for most underutilised crops, available phenotypic data for Bambara groundnut are fragmentary and dispersed, even when nominally associated with common genetic materials (Azam-Ali, 2007; Olukolu *et al.*, 2012; Mayes *et al.*, 2019a). The dissection of genetic and environmental contributions to trait variation would therefore benefit from clarity and standardisation of trait definitions and methodologies. There is also a pressing need for reliable trait data, especially for nutritional composition, to be compared directly with major or other competing crops. Moreover, statistical analysis that allows valid comparison of datasets and allocation of variance components is only possible when appropriate meta-data are available.

Data management in navigating between trait and genome

Reuse of trait (and genotype) datasets for GWAS of plant genetic resources or QTL analysis is typically limited by incomplete or explicit descriptions of germplasm status, provenance, pedigree and population relationships, and a lack of standardisation in trait names. Although these remain generic issues for both major and minor crops (Andrés-Hernández *et al.*, 2020), there is increased awareness of the need to develop appropriate standards and access to data that are 'Findable, Accessible, Interoperable, and Reusable' (FAIR; Wilkinson, 2016). Amongst efforts aimed at ensuring that data are both, human and machine-readable, has been the development of meta-data standards such as MIAPPE for plant phenotyping experiments (Papoutsoglou *et al.*, 2020), along with increased formal knowledge representation. Ontologies are now widely used in bioscience and elsewhere as formal controlled vocabularies that define sets of concepts, categories and relationships for particular domains (Bard and Rhee, 2004; Leonelli, 2008; Courtot *et al.*, 2011; Robinson and Bauer, 2011; Pan *et al.*, 2019).

Reuse and cross-referencing of existing ontology terms is important for data interoperability and reduces human effort and error by reusing concepts that have already been tested (Lonsdale *et al.*, 2010; Bandrowski *et al.*, 2016). This has led to establishment of principles outlined by the Open Biological and Biomedical Ontologies (OBO) Foundry, to ensure establishment of logically well-formed, scientifically accurate and interoperable controlled vocabularies (Smith *et al.*, 2007).

Data standards for Bambara groundnut and comparative traits

Recent efforts have aimed to help the curation and comparison of Bambara groundnut trait and genomic data. The topics covered and lessons learned are applicable to many underutilised crops.

In addition to a set of 54 trait descriptors previously collated by the International Plant Genetic Resources Institute (IPGRI, 2000), 80 independently defined, or additional descriptors, have since been defined by various research groups. Although the latter had been used within a small number of published studies, and as a basis for International Institute of Tropical Agriculture (IITA) (http://my.iita.org/accession2/collection.jspx?id=8) online datasets, there appeared to be no direct commonality or cross-reference of these trait descriptors with similar sets developed for other crops. However, based on the available information, a set of 134 defined trait terms for Bambara groundnut were registered within the Crop Ontology (CO) (Shrestha *et al.*, 2010) system in 2020 (Andrés-Hernández *et al., 2021*).

To date, CO has been the only major ontology initiative aimed at providing a consistent framework for describing crop traits in a form typically used by crop breeders, along with an indication of

measurement methods and scales. The trait terms within CO_366 for Bambara groundnut are classified within the major classes '*Abiotic stress trait*', '*Agronomical trait*', '*Biochemical trait*', '*Biotic stress trait*', '*Morphological trait*', '*Phenological trait*', '*Physiological trait*' and '*Quality trait*'. However, there are recognised limitations to the CO system, which does not fully meet criteria proposed by the OBO Foundry (Laporte *et al.*, 2016). These include a flat ontological structure for classifying traits, limited reuse of existing terms from other ontologies, and an absence of such terms being used within CO term definitions (Andrés-Hernández *et al.*, *2021*). In general, the 28 CO 'ontologies' lack consistency in the terms defined for each crop (Andrés-Hernández *et al.*, 2020). This limits reuse of associated trait data for comparative analysis of major and minor crops, and is particularly problematic for nutritional traits, which are variously categorised within the CO 'biochemical' or 'quality' classes (Andrés-Hernández *et al.*, 2020). However, for CO_366, an effort was made to ensure that 76 terms were in common with other CO sets, particularly legume crops.

It is hoped that in the future, a more robust and flexible ontology system will be developed to address the problem space for comparative crop studies. Specifications would require adherence to OBO principles and a wider integration of trait classes that reflect the different domain specialities associated with plant genetic resource management, agronomic and end-use traits.

A new approach for nutritional traits

Nutritional composition has been proposed as a key attribute justifying future development of Bambara groundnut (Onimawo, Momoh and Usman, 1998; Azam-Ali, 2007; Halimi A *et al.*, 2019; Mayes *et al.*, 2019a). Various studies have reported or collated nutritional composition, although comparison is often limited by lack of consistency in method or units used in analysis (Halimi A *et al.*, 2019). Moreover, few if any formal links exist between experimental data generated for underutilised crops**,** and nutritional data managed within national or international food composition databases (Pennington *et al.*, 2007) such as INFOODs (Charrondière *et al.*, 2013) or USDA's National Food and Nutrient Analysis Program (Haytowitz and Pehrsson, 2018).

The Crop Dietary Nutrition Ontology (CDNO) is a recently established OBO ontology that provides a formal human- and machine-readable controlled vocabulary to help find and navigate crop related nutritional information (Andrés-Hernández *et al.*, 2020). Datasets associated with Bambara groundnut were the first to be annotated using CDNO, with a wider range of other use-cases being collated. For data curation, terms defined within CDNO for 519 dietary nutritional components are used in conjunction with other formal ontology terms and definitions, including those from the Chemical Entities for Biological Interest (CHEBI) (de Matos *et al.*, 2010). This provides data curators with considerable flexibility in describing datasets from different crops and crop-derived products. It is expected that the use of the standardised, and well-defined nutritional terms, will

greatly enhance the ability to compare trait data between crops and resolve genetic contributions to such traits.

Experimental datasets for Bambara nutritional components have been curated within the CropStoreDB database (Eckes *et al.*, 2017; Leibovici *et al.*, 2017). Development of CDNO involved collaboration and co-development of terms with the Food Ontology (FoodOn) (Dooley *et al.*, 2018). During data curation, CDNO terms associated with nutritional component concentration were combined with terms reused from FOODON, Plant Ontology (PO) (Ilic *et al.*, 2006) and NCBI organismal classification ontology (NCBITaxon) (Schoch *et al.*, 2020) to describe typical crop nutritional datasets. For example, this enabled representation of organismal material terms (e.g. *Vigna subterranea* seed). This flexible approach facilitates data curation and downstream comparative analysis, by allowing different crops and production stages to be associated with commonly defined nutritional components.

Future goals and prospects

Current work on the Bambara groundnut genome

In addition to the African Orphan Crop Consortium (AOCC) sequence data, efforts are under way to sequence a single genotype S19-3, a bunchy, early maturing type originally from Namibia. The S19-3 sequencing effort combined long reads generated by Oxford Nanopore Technologies with accuracy corrected by the Illumina reads together with Bionano optical mapping. Initial results are promising with 23 hybrid scaffolds and a total of 552,045,261 bp (N50 = 38,635,177; N90 = 16,759,050), covering 100.3% of the genome size (550 Mb) estimated by Chang *et al.* (2019). From two genetic maps, our preliminary results suggests that five scaffolds are at chromosome level. In addition, with the preliminary insight into the genetic diversity of single seed decent (SSD) materials with DArTSeq markers (GBS), together with the phenotypic data from a number of traits, close to 100 SSD accessions are currently being selected for resequencing at the minimum of 10x depth, aiming to generate higher density of SNP and presence-absence variation (PAV) information for the identification of marker loci associated with traits of interest.

Genome assembly evolution – a practical case using Association Genetics Panel and Genome Wide Association Studies

Genome Wide Association Study (GWAS) is an alternative and complementary approach to QTL analysis using controlled crosses, especially when there is difficulty generating genetic crosses with flower location and/or size difficult to reach (which is the case of Bambara groundnut). However, GWAS could be a difficult approach in the case of underutilised crops, as it requires significant genomic resources.

A joint effort by IITA, Crops for the Future Research Centre, and University of Nottingham has generated an initial Association Genetics Panel (AGP) for Bambara groundnut after a round of single plant descent purification from a subset of genebank accessions (n=229) from different places of origin, thus suggesting a good genetic representation of the species (Mayes *et al.*, unpublished data).

To be able to use this AGP in an initial GWAS analysis, the population was genotyped using the DArTseq platform, which is based on Illumina next generation sequencing, generating dominant silicoDArT markers, and co-dominant SNP markers (SNP variation within the sequence tags of the markers; Alam *et al.* 2018; Barilli *et al.* 2018; Kilian *et al.* 2012). According to Mayes *et al.* (2019a), the level of heterozygosity using 7894 markers ranged from 0.8 to 5.0% between all 229 genotypes belonging to the AGP. A principal component analysis was carried out using the molecular markers generated using the DArTseq platform, allowing us to generate a genetic clustering x place of origin in Figure 2-.

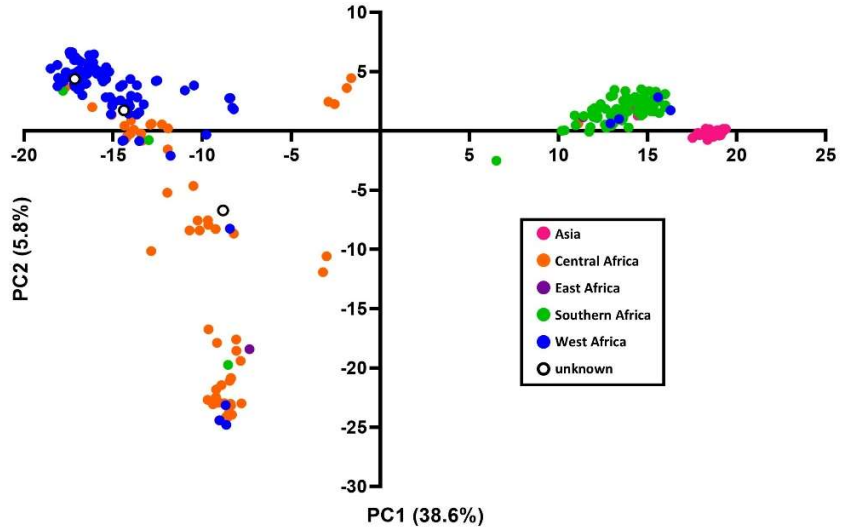

Figure 2-7 Genetic clustering of 229 genotypes from different regions of the world. The principal component analysis was derived from 11,964 SNPs.

No GWAS analysis has been published in Bambara groundnut to date. In this chapter we share provisional test results on *days-to-anthesis* (Figure 2-; Salazar-Licea *et al.* unpublished) and illustrate how the ongoing genome sequence and assembly of Bambara genome has allowed for improved genetic analysis (Figure 2-), where a comparison between Figure 2- and Figure 2-, shows how the new assembly has reduced the number of scaffolds from 64K, closer to the 11 pairs of chromosomes (14 scaffolds).

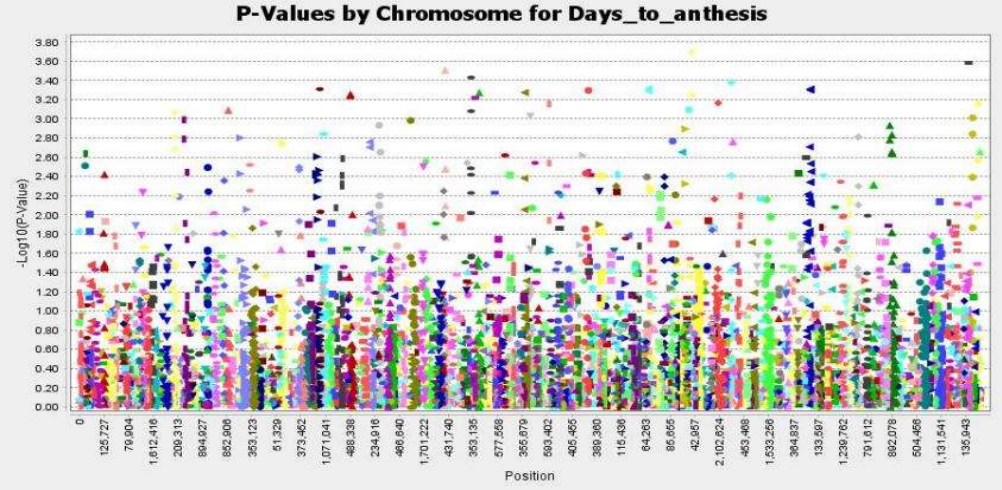

Figure 2-8 First Manhattan plot. The location in different scaffolds are represented by different colours – reference TASSEL version. The Manhattan plot shows the trait for days to anthesis (Salazar-Licea *et al.*, unpublished).

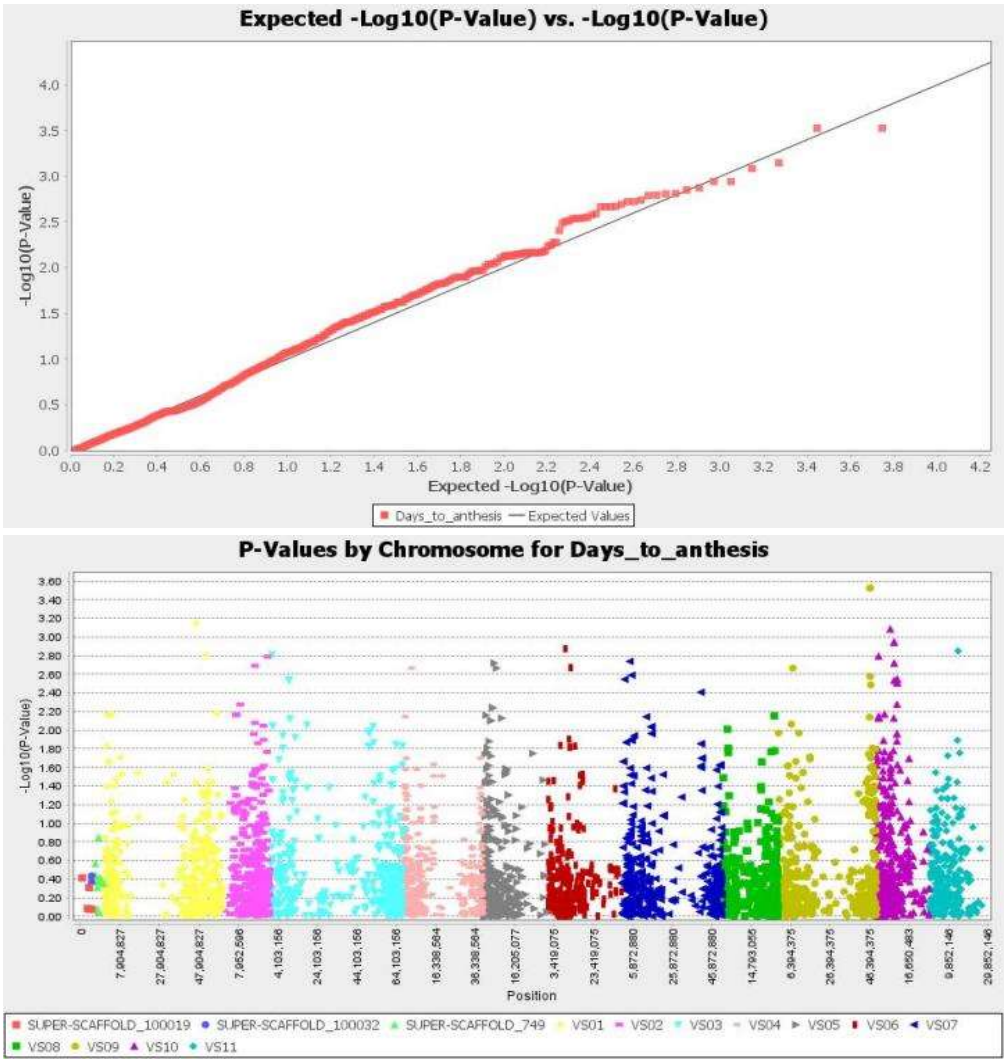

Figure 2-9 Refined GWAS analysis on new genome assembly. Top: Quantile-Quantile plots, bottom: Manhattan plots. There are 11 linkage groups identified (Vs01-11), and 3 scaffolds yet to be allocated.

Conclusion

It's important to have alternative crops to aid with the future food security by having crops which can yield in harsh environments, and by producing a nutritious product for human consumption. Diverse agricultural systems may also be more resilient than extensive monoculture. As sequencing technologies become more advanced and affordable, new doors open to the less studied crops that could aid food and nutritional security, such as Bambara groundnut, allowing the development of genomic resources to facilitate genetic improvement and breeding.

Phenotyping studies in Bambara groundnut have been established under different locations, climates and treatments, the generation of reliable trait data is important to contribute to the detection of linkage between genetic and phenotypic resources, and hence, to breeding programs as well. Alongside the generation of genetic and phenotypic data, the constant improvement of data management platforms are also required to maintain clarity and standardisation of trait definitions and methodologies so that datasets and trait variation recorded as a result of genetic and environmental interactions can be compared directly and statistically across different locations of studies, but also that it becomes possible to translate methods and information from other related species.

With the objective to fully understand, target, and efficiently select the traits of interest with farmers, in particular in cultivated areas with harsh weather conditions, the combination of genomic technologies and resources, including genetic mapping, QTL, GWAS and RNAseq analyses, should be able to aid breeding programs by generating: a) a better understanding of the crop; b) molecular markers for MAS; and c) gene identification for gene editing.

References

Adeleke OR, *et al* (2018) Nutritional, physicochemical, and functional properties of protein concentrate and isolate of newly-developed Bambara groundnut (*Vigna subterranea* L.) cultivars. *Food Science & Nutrition,* 6, 229-242.

Adzawla W, Donkoh SA, Nyarko G, O'Reilly P, Mayes S. Use patterns and perceptions about the attributes of Bambara groundnut (*Vigna subterranea* (L.) Verdc.) in Northern Ghana. Ghana J Sci Technol Dev. (2016) 4:1–10. doi: 10.47881/2016.749x.88

AOCC (African Orphan Crops Consortium), Healthy Africa through nutritious, diverse and local food crops. http://africanorphancrops.org/

Ahmad NS, Redjeki ES, Ho WK, Aliyu S, Mayes K, Massawe F, Kilian A, Mayes S (2016) "Construction of a Genetic Linkage Map and QTL Analysis in Bambara Groundnut" 14 (April): 1–14.

Alake CO (2018) Genetic variability, gains from selection and genetic correlations for pod yield and nutritional traits in African landraces of Bambara groundnut. *Biological Agriculture & Horticulture,* 34, 71-87.

Alake CO, Alake OO (2016) Genetic Diversity for Agro-Nutritional Traits in African Landraces of *Vigna subterranea*n Germplasm. *J. Crop Improv.,* 30, 378-398.

Aliyu S, Massawe FJ (2013) Microsatellites based marker molecular analysis of Ghanaian Bambara groundnut (*Vigna subterranea* (L.) Verdc.) landraces alongside morphological characterization. Genet Resour Crop Evol 60:777–787. doi:10.1007/s10722-012-9874-y

Alam M, Neal J, O'Connor K, Kilian A, Topp B (2018) Ultra-highthroughput DArTseq-based silicoDArT and SNP markers for genomic studies in macadamia. PLoS One. https ://doi.org/10.1371/journal.pone.02034 65

Al Shareef I, Sparkes D, Azam-Ali S (2014) Temperature and drought stress effects on growth and development of Bambara groundnut (*Vigna subterranea* L.). Exp Agric 50(1):72–89. https ://doi.org/10.1017/S0014 47971 30003 79

Andrés-Hernández L *et al.* (2020) 'Knowledge representation and data sharing to unlock crop variation for nutritional food security', *Crop Science*, pp. 516–529. doi: 10.1002/csc2.20092.

Andrés-Hernández L, Halimi RA, Mauleon R, Mayes S, Baten A, & King GJ (2021) 'Challenges for FAIR-compliant description and comparison of crop phenotype data with standardized controlled vocabularies'. *Database*, 2021, baab028.Azam-Ali SN, Sesay A, Karikari SK, Massawe F, Aguilar-Manjarrez J, Bannayan M, Hampson KJ (2001) Assessing the potential of an underutilized crop—a case study using Bambara groundnut. Exp Agric 37:433–472. https ://doi.org/10.1017/S0014 4797010004 12

Azam-Ali, S. (2007) 'Agricultural diversification: The potential for underutilised crops in Africa's changing climates', *Rivista di Biologia - Biology Forum*, 100(1), pp. 27–37. doi: 3581 [pii].

Bandrowski A *et al* (2016) 'The Ontology for Biomedical Investigations', *PLoS ONE*, 11(4), pp. 1–19. doi: 10.1371/journal.pone.0154556.

Bard JBL and Rhee SY (2004) 'Ontologies in biology: Design, applications and future challenges', *Nature Reviews Genetics*, 5(3), pp. 213–222. doi: 10.1038/nrg1295.

Barilli E, Cobos MJ, Carrillo E, Kilian A, Carling J, Rubiales D (2018) A high-density integrated DArTseq SNP-based genetic map of *Pisum fulvum* and identification of QTLs controlling rust resistance. Front Plant Sci. https://doi.org/10.3389/fpls.2018.00167

Basu S, Roberts JA, Azam-Ali SN, Mayes S (2007) "Development of Microsatellite Markers for Bambara Groundnut (*Vigna subterranea* L. Verdc.) - An Underutilized African Legume Crop Species." *Molecular Ecology Notes* 7 (6): 1326–28. https://doi.org/10.1111/j.1471-8286.2007.01870.x.

Baudoin JP and Mergeai G 2001 Grain Legumes: Bambara Groundnut [*Vigna subterranean* (L.) Verd.]. In: Crop Production in Tropical Africa, Romain, H. and Raemaekers (Ed.). A DGIC Publication, CIP Royal Library Albert I, Brusels, pp: 313-317.

Begemann F (1988) Ecogeographic differentiation of Bambara groundnut (*Vigna subterranea*) in the collection of the international institute of tropical agriculture (IITA). PhD Thesis, Technical University of Munich, Germany

Begemann HJF and Mushonga J (1997) Bambara groundnut (*Vigna subterranea* (L.) Verdc.). Promoting the conservation and use of underutilized and neglected crops. 9. Proceedings of the workshop on Conservation and Improvement of Bambara Groundnut (*Vigna subterranea* (L.) Verdc.), 14–16 November 1995

Berchie JN, Adu-Dapaah H, Sarkodie-Addo J, Asare E, Agyemang A, Addy S, Donkor J (2010b) Effect of seed priming on seedling emergence and establishment of four Bambara groundnut landraces. J Agron 9(4):180–183

Berchie JN, Sarkodie-Addo J, Adu-Dapaah H, Agyemang A, Addy S, Asare E, Donkor J (2010a) Yield evaluation of three early maturing Bambara groundnut (*Vigna subterranea*n (L) Verdc.) landraces at the CSIR-Crops Research Institute, Fumesua-Kumasi. Ghana. J Agron 9(4):175–179

Blum A (2011) "Plant Water Relations, Plant Stress and Plant Production BT - Plant Breeding for Water-Limited Environments." In ed. Abraham Blum. New York, NY: Springer New York, 11–52. https://doi.org/10.1007/978-1-4419-7491-4_2.

Board JE, Harville BG, Kamal M (1994) Radiation-use efficiency in relation to row spacing for late-planted soybean. Field Crops Research. 36: 13–19.

Calvino M, Miclaus M, Bruggmann R, Messing J (2009) Molecular markers for sweet sorghum based on microarray expression data. Rice 2: 129-142

Cannon SB, Gregory DM, Jackson SA (2009) Three sequenced legume genomes and many crop species: rich opportunities for translational genomics. Plant Physiology 151: 970-977

Chai HH, Massawe F, Mayes S (2016a) "Assessment of a Segregating Population for the Improvement of Drought Tolerance in Bambara Groundnut." Acta Horticulturae 1127: 339–45.

Chai HH, Massawe F, Mayes S (2016b) Effects of mild drought stress on the morpho-physiological characteristics of a Bambara groundnut segregating population. Euphytica 208, 225–236 (2016). https://doi.org/10.1007/s10681-015-1581-2

Chai HH, Ho WK, Graham N, May S, Massawe F, Mayes S (2017) "A Cross-Species Gene Expression Marker-Based Genetic Map and QTL Analysis in Bambara Groundnut." *Genes* . https://doi.org/10.3390/genes8020084.

Chang Y, Liu H, Liu M, Liao X, Sahu SK, Fu Y, Song B, Cheng S, Kariba R, Muthemba S, Hendre PS, Mayes S, Ho WK, Yssel AEJ, Kendabie P, Wang S, Li L, Muchugi A, Jamnadass R, Lu H, Peng S, Van Deynze A, Simons A, Yana-Shapiro H, Van de Peer Y, Xu X, Yang H, Wang J, Liu X (2019) The draft genomes of five agriculturally important African orphan crops. Gigascience 1;8(3):giy152. doi: 10.1093/gigascience/giy152. PMID: 30535374; PMCID: PMC6405277.

Chibarabada TP, Modi AT, Mabhaudhi T (2015) Water use characteristics of a Bambara groundnut (*Vigna subterranea* L. Verdc) landrace during seedling establishment. Water SA 41(4):472–482. https ://doi.org/10.4314/wsa.v41i4 .06

Collino DJ, Dardanelli JL, Sereno R, Racca RW (2000) Physiological responses of argentine peanut varieties to water stress. Water uptake and water use efficiency. Field Crops Research. 68, 133– 142.

Collinson S, Berchie J, Azam-Ali S (1999) The effect of soil moisture on light interception and the conversion coefficient for three landraces of Bambara groundnut (*Vigna subterranea*). J Agric Sci 133(2):151–157. https ://doi.org/10.1017/S0021 85969 90068 75

Collinson ST, Clawson EJ, Azam-Ali SN, Black CR (1997) Effects of soil moisture deficits on the water relations of Bambara groundnut *(Vigna subterranea L.* Verdc.). J Exp Bot 48:877–884

Considine, M. J., *et al.* (2017). "Nature's pulse power: legumes, food security and climate change." Journal of Experimental Botany **68**(8): 1815 - 1818.

Courtot, M. *et al.* (2011) 'Controlled vocabularies and semantics in systems biology', *Molecular Systems Biology*, 7(543). doi: 10.1038/msb.2011.77.

Craufurd PQ, Wheeler TR (1999) Effect of drought and plant density on radiation interception, radiation-use efficiency and partitioning of dry matter to seeds in cowpea. Experimental Agriculture 35: 309–325.

Das S, Bhat PR, Sudhakar C, Ehlers JD, Wanamaker S, Roberts PQ, Cui XP, Close TJ (2008) Detection and validation of single feature polymorphisms in cowpea (*Vigna unguiculata* L. Walp) using a soybean genome array. *BMC Genomics* 9: 1-12

Dalziel JM (1937) Voandzeia Thou. In: The useful plants of west tropical Africa: Crown Agents, London. pp 269–271

Davey MW, Graham NS, Vanholme B, Swennen R, May ST, Keulemans J (2009). Heterologous oligonucleotide microarrays for transcriptomics in a non-model species; a proof-of-concept study of drought stress in *Musa*. *BMC Genomics* 10: 1-19

De Costa WAJM, Shanmugathasan KN (2002) Physiology of yield determination of soybean (*Glycine max* (L.) Merr.) under different irrigation regimes in the sub-humid zone of Sri Lanka. Field Crops Research. 75: 23–35.

de Matos P *et al.* (2010) 'ChEBI: a chemistry ontology and database', *Journal of Cheminformatics*, 2(Suppl 1), p. P6. doi: 10.1186/1758-2946-2-S1-P6.

Dooley DM *et al.* (2018) 'FoodOn : a harmonized food ontology to increase global food traceability , quality control and data integration', (September). doi: 10.1038/s41538-018-0032-6.

Duke JA (1981) Voandzeia subterranea (L). Thouars, In: Handbook of legumes of world economic importance. Plenum Press, New York, pp: 307-310.

Eckes AH *et al.* (2017) 'Introducing the Brassica Information Portal: Towards integrating genotypic and phenotypic Brassica crop data', F1000Research, 6(0), p. 465. doi: 10.12688/f1000research.11301.1.

Esquinas-Alcazar, J. (2005). "Science and society: protecting crop genetic diversity for food security: political, ethical and technical challenges." Nat Rev Genet **6**(12): 946-953.

FAO, IFAD, UNICEF, WFP, WHO. 2017. The State of Food Security and Nutrition in the World 2017. Building resilience for peace and food security. Rome, FAO. http://www.fao.org/3/a-I7695e.pdf

FAO, IFAD, UNICEF, WFP and WHO. 2019. The State of Food Security and Nutrition in the World 2019. Safeguarding against economic slowdowns and downturns. Rome, FAO. Licence: CC BY-NC-SA 3.0 IGO.

Feldman A, Ho WK, Massawe F, Mayes S (2019) Bambara groundnut is a Climate-resilient crop: how could a drought-tolerant and nutritious legume improve community resilience in the face of climate change? In: Sarkar A *et al* (eds) Sustainable solutions for food security. Springer, Berlin

Foyer, C. H., *et al.* (2016). "Neglecting legumes has compromised human health and sustainable food production." Nature Plants **2**.

Gao, X, Bamba ASA, Kundy AC, Mateva KI, Chai HH, Ho WK, Musa M. Mayes S, Massawe F, (2020) Variation of Phenotypic Traits in Twelve Bambara Groundnut (*Vigna subterranea* (L.) Verdc.) Genotypes and Two F_2 Bi-Parental Segregating Populations. *Agronomy* 2020, *10*, 1451. https://doi.org/10.3390/agronomy10101451

Goli AE (1997) Bibliographical review of Bambara groundnut, p. 4-10. In: J. Heller, *et al.* (Eds.), Proceedings of the Workshop on Conservation and Improvement of Bambara Groundnut (*Vigna subterranea* [L.] Verdc.) 14-16 November, 1995, Harare, Zimbabwe, International Plant Genetic Resources Institute, Rome, Italy, 162 pp

Guillemin JA, Perrottet GS, Richard A (1832) Florae Senegambiae Tentamen. Paris

Gwala S, Kyomugasho C, Wainaina I, Rousseau S, Hendrickx M, Grauwet T (2020) Ageing, dehulling and cooking of Bambara groundnuts: consequences for mineral retention and: in vitro bioaccessibility. Food Funct. (2020) 11:2509–21. doi: 10.1039/C9FO01731C

Halimi AR, Barkla BJ, Mayes S, King GJ (2019a) Characteristics of the Underutilised Pulse Bambara Groundnut (*Vigna subterranea* (L.) *Verdc.*) Relevant to Food & Nutritional Security. *Proceedings,* 36, 199.

Halimi AR, *et al.* (2019b) 'The potential of the underutilized pulse Bambara groundnut (*Vigna subterranea* (L.) Verdc.) for nutritional food security', *Journal of Food Composition and Analysis*. Elsevier, 77(December 2018), pp. 47–59. doi: 10.1016/j.jfca.2018.12.008.

Hammond JP, Broadley MR, Craigon DJ, Higgins J, Emmerson ZF, Townsend HJ, White PJ, May ST (2005) Using genomic DNA-based probe-selection to improve the sensitivity of high-density oligonucleotide arrays when applied to heterologous species. *Plant Methods* 1: 1-10

Hammond JP, Mayes S, Bowen HC, Graham NS, Hayden RM, Love CG, Spracklen WP, Wang J, Welham SJ, White PJ, King GJ, Broadley MR (2011) Regulatory hotspots are associated with plant gene expression under varying soil phosphorus supply in *Brassica rapa. Plant Physiology* 156: 1230-1241

Han Y, Gao S, Muegge K, Zhang W, Zhou B (2015) Advanced Applications of RNA Sequencing and Challenges. *Bioinformatics and biology insights*, *9*(Suppl 1), 29–46. https://doi.org/10.4137/BBI.S28991

Haytowitz D B, Pehrsson PR (2018) 'USDA's National Food and Nutrient Analysis Program (NFNAP) produces high-quality data for USDA food composition databases: Two decades of collaboration', *Food Chemistry*, 238, pp. 134–138. doi: 10.1016/j.foodchem.2016.11.082.

Heller J, Begemann F, Mushonga J (1997) Bambara groundnut (*Vigna subterranea* (L.) *Verdc.*). In: Promoting the conservation and use of underutilized and neglected crops. 9. Proceedings of the workshop on Conservation and Improvement of Bambara Groundnut (*Vigna subterranea* (L.) *Verdc.*), 14–16 November 1995, Harare, Zimbabwe. International Plant Genetic Resources Institute (IPGRI), Rome, Italy.

Hepper FN (1963) The Bambara groundnut (*Voandzeia subterranea*) in West Africa. *Kew Bull*, **16**, 398–407

Hepper FN (1970) "Bambara Groundnut (Voandzeia Subterranea)." Field Crop 23(1): 1–6.

Hillocks RJ, Bennett C, Mponda OM (2012) Bambara nut: a review of utilisation, market potential and crop improvement. Afr Crop Sci J 20(1):1–16

Ho WK, Chai HH, Kendabie P, Ahmad NS, Jani J, Massawe F, Kilian A, Mayes S (2017) "Integrating Genetic Maps in Bambara Groundnut [*Vigna subterranea* (L) Verdc.] and Their Syntenic Relationships among Closely Related Legumes." *BMC Genomics* 18 (1). https://doi.org/10.1186/s12864-016-3393-8.

IPES-Food (2016). From uniformity to diversity: a paradigm shift from industrial agriculture to diversified agroecological systems. International Panel of Experts on Sustainable Food systems. http://www.ipes-food.org/

IPGRI, IITA, BAMNET (2000) Descriptors for Bambara groundnut (*Vigna subterranea*). International Plant Genetic Resources Institute, Rome, Italy; International Institute of Tropical Agriculture, Ibadan, Nigeria; The International Bambara Groundnut Network, Germany. ISBN 92-9043-461-9

James C (2015) Global Status of Commercialized Biotech/GM Crops: 2015. *ISAAA Brief No. 51*: 1-17.

Jorgensen ST, Liu F, Ouédraogo M, Ntundu WH, Sarrazin J, Christiansen JL (2010) Drought responses of two Bambara groundnut (*Vigna subterranea* L. Verdc.) landraces collected from a dry and a humid area of Africa. J Agron Crop Sci 196(6):412–422. https ://doi.org/10.1111/j.1439-037X.2010.00435 .x

Jovanovic M *et al* (2007) "How the Environment Regulates Root Architecture in Dicots." In Academic Press, 35–74. https://www.sciencedirect.com/science/article/pii/S0065229607460025.

Kang YJ, Kim SK, Kim MY, Lestari P, Kim KH, Ha BK, Jun TH, Hwang WJ, Lee T, Lee J, Shim S, Yoon MY, Jang YE, Han KS, Taeprayoon P, Yoon N, Somta P, Tanya P, Kim KS, Gwag JG, Moon JK, Park BS, Bombarely A, Doyle JJ, Jackson SA, Schafleitner R, Srinives P, Varshney RV, Lee SH (2014) Genome sequence of mungbean and insights into evolution within Vigna species. *Nature communications*, *5*, 5443. https://doi.org/10.1038/ncomms6443

Kilian A, Wenzl P, Huttner E, Carling J, Xia L, Blois H *et al* (2012) Diversity arrays technology: a generic genome profiling technology on open platforms. In: Bonin FPAA (ed) Data production and analysis in population genomics: methods and protocol. Springer, New York, pp 67–89

Kole C, Muthamilarasan M, Henry R, Edwards D, Sharma R, Abberton M, Batley J, Bentley A, Blakeney M, Bryant J, Cai H, Cakir M, Cseke LJ, Cockram J, Oliveira AC, Pace CD, Dempewolf H, Ellison S, Gepts P, Greenland A, Hall A, Hori K, Hughes S, Humphreys MW, Iorizzo M, Ismail AM, Marshall A, Mayes S, Nguyen HT, Ogbonnaya FC, Ortiz R, Paterson AH, Simon PW, Tohme J, Tuberosa R, Valliyodan B, Vershney RK, Wullschleger SD, Yano M, Prasad M (2015) Application of genomics-assisted breeding for generation of climate resilient crops: progress and prospects. Frontiers in Plant Science 6: 1-16.

Kendabie P, Massawe F, and Mayes S (2015) "Developing Genetic Mapping Resources from Landrace-Derived Genotypes That Differ for Photoperiod Sensitivity in Bambara Groundnut (*Vigna subterranea* L .)." *Aspects of Applied Biology* 124: 1–8.

Kendabie P, Jørgensen S, Massawe F, Fernández J, Azam-Ali S, Mayes S (2020) Photoperiod control of yield and sink capacity in Bambara groundnut (*Vigna subterranea*) genotypes. *Food and Energy Security, 9*.

Khan F, Chai HH, Ajmera I, Hodgman C, Mayes S, Lu C (2017) A Transcriptomic Comparison of Two Bambara Groundnut Landraces under Dehydration Stress. Genes (Basel). 2017 Apr 18;8(4):121. doi: 10.3390/genes8040121.

Kullan ARK, van Dyk MM, Jones N, Kanzler A, Bayley A, Myburg AA (2012) "High-Density Genetic Linkage Maps with over 2,400 Sequence-Anchored DArT Markers for Genetic Dissection in an F2 Pseudo-Backcross of Eucalyptus Grandis × E. Urophylla." *Tree Genetics and Genomes* 8 (1): 163–75. https://doi.org/10.1007/s11295-011-0430-2.

Lai HM, May ST, Mayes S (2014) Pigeons: A novel GUI software for analysing and parsing high density heterologous oligonucleotide microarray probe level data Microarrays. 3(1), 1-23

Laporte MA *et al* (2016) 'Comparison of ontology mapping techniques to map plant trait ontologies', *CEUR Workshop Proceedings*, 1747, pp. 1–2. doi: 10.5281/zenodo.47337.

Leibovici DG, Anand S, Santos R, Mayes S, Ray R, Al-Azri, M, Baten A, King G, Karunaratne AS, Azam-Ali A, Jackson M (2017) Geospatial binding for transdisciplinary research in crop science: the GRASP gfs initiative. Open Geospatial Data, Software and Standards doi:10.1186/s40965-017-0034-3

Leonelli S (2008) 'Bio-ontologies as tools for integration in biology', *Biological Theory*, 3(1), pp. 7–11. doi: 10.1162/biot.2008.3.1.7.

Leport L, Turner NC, French RJ, Barr MD, Duda R, Daves SL, Tennant D, Siddique KHM (1999) Physiological responses of chickpea genotypes to terminal drought in a Mediterranean-type environment. European Journal of Agronomy 11: 279–291

Linnemann AR (1993) Phenological development in Bambara groundnut (*Vigna subterranea*) at constant exposure to photoperiods of 10 to 16 h. *Annals of Botany*, *71*, 445–452. https://doi.org/10.1006/anbo.1993.1058

Linnemann AR, Azam–Ali SN (1993) Bambara groundnut (*Vigna subterranea*) literature review: A revised and updated bibliography. Tropical Crops Communication No. 7. Wageningen Agricultural University

Linnemann AR, Craufurd PQ (1994) Effects of Temperature and Photoperiod on Phenological Development in Three Genotypes of Bambara Groundnut (*Vigna subterranea*). *Annals of Botany*, *74*, 675–681. https://doi.org/10.1006/anbo.1994.1170

Linnemann AR, Westphal E, Wessel M (1995) Photoperiod regulation of development and growth in Bambara groundnut (*Vigna subterranea*). *Field Crops Research*, *40*, 39–47. https://doi.org/10.1016/0378-4290(94)00080-V

Liu Q, Kasuga M, Sakuma Y, Abe H, Miura S, Yamaguchi-Shinozaki K, Shinozaki K (1998) "Two Transcription Factors, DREB1 and DREB2, with an EREBP/AP2 DNA Binding Domain Separate Two Cellular Signal Transduction Pathways in Drought- and Low-Temperature-Responsive Gene Expression, Respectively, in Arabidopsis." *The Plant Cell* 10 (8): 1391. https://doi.org/10.2307/3870648.

Lonsdale D *et al.* (2010) 'Reusing ontologies and language components for ontology generation', *Data and Knowledge Engineering*. Elsevier B.V., 69(4), pp. 318–330. doi: 10.1016/j.datak.2009.08.003.

Lynch J (1995) "Root Architecture and Plant Productivity." Plant physiology 109(1): 7–13.

Lynch JP (2007) "Roots of the Second Green Revolution." Australian Journal of Botany 55(5): 493–512.

Mabhaudhi T, Modi AT (2013) Growth, phenological and yield responses of a Bambara groundnut (*Vigna subterranea* (L.) Verdc.) landrace to imposed water stress under field conditions. S Afr J Plant Soil 30(2):69–79. https ://doi.org/10.1080/02571862.2013.79049 2

Mabhaudhi T, Modi AT, Beletse YG (2013) Growth, phenological and yield responses of a Bambara groundnut (*Vigna subterranea* L. Verdc) landrace to imposed water stress: II. Rain shelter conditions. Water SA 39:191-198.

Mabhaudhi T, Chibarabada TP, Chimonyo VGP, Murugani VG, Pereira LM, Sobratee N, Govender L, Slotow R, Modi AT (2018) Mainstreaming Underutilized Indigenous and Traditional Crops into Food Systems: A South African Perspective. Sustainability, 11, 172.

Makanda I *et al* (2008) "Evaluation of Bambara Groundnut Varieties for Off-Season Production in Zimbabwe." AFRICAN JOURNALS ONLINE (AJOL) 16. 10.4314/acsj.v16i3.54370.

Massawe FJ, Dickinson M, Roberts JA, Azam-Ali SN (2002) "Genetic Diversity in Bambara Groundnut (Vigna subterranea (L.) Verdc) Landraces Revealed by AFLP Markers." Genome 45 (6): 1175–80. https://doi.org/10.1139/g02-093.

Massawe F, Schenkel W, Basu S, Temba EM (2003a) Artificial hybridization in Bambara groundnut (Vigna subterranea (L.) Verdc.). In: Proceedings of the international Bambara groundnut symposium, Botswana, vol 1, pp 193–209

Massawe F, Mayes S, Cheng A (2016) "Crop Diversity: An Unexploited Treasure Trove for Food Security." Trends in Plant Science 21 (5): 365–68. https://doi.org/http://doi.org/10.1016/j.tplants.2016.02.006.

Mateva KI, Chai HH, Mayes S, Massawe F (2020) Morphological characterisation of root trait variability in diverse Bambara groundnut (Vigna subterranea (L.) Verdc.) genotypes – implications for water capture; MDPI Plants 2020, 9, 645; doi:10.3390/plants9050645

Mayes S, Ho WK, Chai HH, Gao X, Kundy AC, Mateva KI, Zahrulakmal M, Hahiree MKIM, Kendabie P, Licea LCS, Massawe F, Mabhaudhi T, Modi AT, Berchie JN, Amoah S, Faloye B, Abberton M, Olaniyi O, Azam-Ali SN. Bambara groundnut: an exemplar underutilised legume for resilience under climate change. Planta. 2019 Sep;250(3):803-820. doi: 10.1007/s00425-019-03191-6.

Mayes S, Ho WK, Chai HH, Song B, *et al* (2019b) "Bambara Groundnut (Vigna subterranea (L) Verdc)—A Climate Smart Crop for Food and Nutrition Security." http://doi.org/10.1007/978-3-319-96932-9_8.

Mayes S, Ho WK, Kendabie P, Chai HH, Aliyu S, Feldman A, Halimi AR, Massawe F, Azam-Ali S (2015) "Applying Molecular Genetics to Underutilised Species – Problems and Opportunities." *Malaysian Applied Biology* 44 (4): 1–9.

Minka SR, Bruneteau M (2000) Partial chemical composition of Bambara pea [*Vigna subterranea* (L.) Verde]. *Food Chem.,* 68, 273-276.

Molosiwa O, Basu SM, Stadler F, Azam-Ali S, Mayes S (2013) "Assessment of Genetic Variability of Bambara Groundnut (*Vigna subterranea* (L.) Verde.) Accessions Using Morphological Traits and Molecular Markers." *Acta Horticulturae* 979 (May 2019): 779–90. https://doi.org/10.17660/ActaHortic.2013.979.87.

Moore S, Payton P, Wright M, Tanksley S, Giovannoni J (2005) Utilisation of tomato microarrays for comparative gene expression analysis in the Solanaceae. *Journal of Experimental Botany* 56: 2885-2895

Mubaiwa J, Fogliano V, Chidewe C, Linnemann AR (2017) Hard-to-cook phenomenon in Bambara groundnut (*Vigna subterranea* (L.) Verdc.) processing: options to improve its role in

providing food security. Food Rev Int. (2017) 33:167–94. doi: 10.1080/87559129.2016.1149864

Muhammad YY, Mayes S and Massawe F (2015) Effects of short-term water deficit stress on physiological characteristics of Bambara groundnut (*Vigna subterranea* (L.) Verdc.) South African Journal of Plant and Soil. 33 (1), 51-58

Muhammad I, Rafii MY *et al* (2020) Exploration of Bambara Groundnut (*Vigna subterranea* (L.) Verdc.), an Underutilized Crop, to Aid Global Food Security: Varietal Improvement, Genetic Diversity and Processing. *Agronomy* 2020, *10*, 766. https://doi.org/10.3390/agronomy10060766

Mune MAM, Mbome IL, Minka SR (2007) Improving the Nutritional Quality of Cowpea and Bambara bean flours for Use in Infant Feeding. *Pak. J. Nutr.,* 6, 660 - 664.

Mwale SS, Azam-Ali SN, Massawe FJ (2007a) Growth and development of Bambara groundnut (*Vigna subterranea*) in response to soil moisture 1. Dry matter and yield. European Journal of Agronomy 26: 345-353.

Mwale SS, Azam-Ali SN, Massawe FJ (2007b) Growth and development of Bambara groundnut (*Vigna subterranea*) in response to soil moisture 1. Resource capture and conversion. European Journal of Agronomy 26: 354-362.

National Research Council (2006) *Lost Crops of Africa: Volume II: Vegetables*. Washington, DC: The National Academies Press. https://doi.org/10.17226/11763.

Nautiyal PC, Kulkarni G, Singh AL, Basu MS (2017) Evaluation of water-deficit stress tolerance in Bambara groundnut landraces for cultivation in sub-tropical environments in India. Indian J Plant Physiol 22(2):190–196. https ://doi.org/10.1007/s40502-017-0296-x

Nedumaran S, Abinaya P, Jyosthnaa P, Shraavya B, Rao P, Bantilan C (2015) Grain legumes production, consumption and trade trends in developing countries. Working paper series no.

Ocran VK, Delimini LL, Asuboah RA, Asiedu EA (1998) Seed Management Manual for Ghana. Publication of Ministry of Food and Agriculture, Accra Ghana

Piao S, Ciais P, Huang Y, Shen Z, Peng S, Li J, Zhou L, Liu H, Ma Y, Ding Y, Friedlingstein P, Liu C, Tan K, Yu Y, Zhang T, Fang J (2010) The impacts of climate change on water resources and agriculture in China. *Nature* 467: 43-51.

Olukolu BA, Mayes S, Stadler F *et al* (2012) Genetic diversity in Bambara groundnut (*Vigna subterranea* (L.) Verdc.) As revealed by phenotypic descriptors and DArT marker analysis. Genet Resour Crop Evol 59:347–358. doi:10.1007/s10722-011-9686-5

Onimawo IA, Momoh AH, Usman A (1998) 'Proximate composition and functional properties of four cultivars of Bambara groundnut (Voandezeia subterranea)', Plant Foods for Human Nutrition, 53(2), pp. 153–158. doi: 10.1023/A:1008027030095.

Oyeyinka SA *et al* (2015) "Physicochemical Properties of Starches with Variable Amylose Contents Extracted from Bambara Groundnut Genotypes." Carbohydrate polymers 133: 171–78.

Pan H *et al* (2019) 'Biomedical ontologies and their development, management, and applications in and beyond China', Journal of Bio-X Research, 2(4), pp. 178–184. doi: 10.1097/jbr.0000000000000051.

Papoutsoglou EA *et al* (2020) 'Enabling reusability of plant phenomic datasets with MIAPPE 1.1', *New Phytologist*, 227(1), pp. 260–273. doi: 10.1111/nph.16544.

Pasquet RS, Fotso M (1997) The ORSTOM Bambara groundnut collection. In: Heller J, Begemann F, Mushonga J (eds) Bambara groundnut, *Vigna subterranea* (L.) Verdc. IPGRI, Rome, pp 119–123

Pasquet RS, Schwedes S, Gepts P (1999) "Isozyme Diversity in Bambara Groundnut." https://doi.org/10.2135/cropsci1999.0011183X003900040045x.

Pennington JAT *et al* (2007) 'Food Composition Data: The Foundation of Dietetic Practice and Research', *Journal of the American Dietetic Association*, 107(12), pp. 2105–2113. doi: 10.1016/j.jada.2007.09.004.

Peter NR, Bauer S (2011) *Introduction to Bio-Ontologies*.

Pingali P (2015) Agricultural policy and nutrition outcomes – getting beyond the preoccupation with staple grains. *Food Security,* 7, 583-591.

Potokina E, Druka A, Luo ZW, Wise R, Waugh R, Kearsey MJ (2007) eQTL analysis of 16,000 barley genes reveals a complex pattern of genome wide transcriptional regulation. *The Plant Journal* 53: 90–101.

Redjeki ES, Ho WK, Shah N, Molosiwa, N. R. Ardiarini, Kuswanto, and Sean Mayes. 2020. "Understanding the Genetic Relationships between Indonesian Bambara Groundnut Landraces and Investigating Their Origins." *Genome* 63 (6): 319–27. https://doi.org/10.1139/gen-2019-0137.

Ronald J, Akey JM, Whittle J, Smith EN, Yvert G, Kruglyak L (2005) Simultaneous genotyping, gene-expression measurement, and detection of allele-specific expression with oligonucleotide arrays. *Genome Reserach.* 15: 284–291.

Rungnoi O, Suwanprasert J, Somta P, Srinives P (2012) Molecular genetic diversity of Bambara groundnut (*Vigna subterranea* L. Verdc.) revealed by RAPD and ISSR marker analysis. SABRAO J Breed Genet 44:87–101

Ruth CU *et al.* (2013) 'FAO/INFOODS food composition database for biodiversity', *Food Chemistry*, 140(3), pp. 408–412. doi: 10.1016/j.foodchem.2012.08.049.

Salekdeh GH, Reynolds M, Bennett J, Boyer J (2009) Conceptual framework for drought phenotyping during molecular breeding.Trends Plant Sci 14:488–496

Schmutz J, Cannon S, Schlueter J *et al* (2010) Genome sequence of the palaeopolyploid soybean. Nature 463, 178–183 (2010). https://doi.org/10.1038/nature08670

Schoch CL *et al* (2020) 'NCBI Taxonomy: a comprehensive update on curation, resources and tools', Database : the journal of biological databases and curation, 2020(2), pp. 1–21. doi: 10.1093/database/baaa062.

Sesay, A *et al*. (2010) "Preliminary Assessment of Bambara Groundnut (Vigna subterranea l.) Landraces for Temperature and Water Stress Tolerance under Field Conditions in Botswana." South African Journal of Plant and Soil 27(4): 312–21.

Shrestha R *et al* (2010) 'Multifunctional crop trait ontology for breeders' data: field book, annotation, data discovery and semantic enrichment of tSShe literature', AoB Plants, 2010(0), pp. plq008–plq008. doi: 10.1093/aobpla/plq008.

Siddique KHM, Regan KL, Tennant D, Thomson BD (2001) Water use and water use efficiency of cool season grain legumes in low rainfall Mediterranean-type environments. European Journal of Agronomy 15: 267– 280.

Smith B *et al* (2007) 'The OBO Foundry: coordinated evolution of ontologies to support biomedical data integration', Nature Biotechnology, 25(11), pp. 1251–1255. doi: 10.1038/nbt1346.

Somta P, Chankaew S, Rungnoi O, Srinives P (2011) Genetic diversity of the Bambara groundnut (Vigna subterranea (L.) Verdc.) as assessed by SSR markers. Genome 54:898–910. doi:10.1139/G11-056

Stanton WR, Doughty J, Orraca-Tetteh R, Steele W (1966) Voandzeia subterranea Thouars. In: Stanton WR (ed) Grain legumes in Africa. Food and Agriculture Organization, Rome, pp 128–133

Swanevelder CJ (1998) Bambara—food for Africa (Vigna subterranea – Bambara groundnut). Pretoria: National Department of Agriculture.

Tuan YH, Phillips RD (1992) Nutritional quality of hard-to-cook and processed cowpea. J Food Sci. (1992) 57:1371– 4. doi: 10.1111/j.1365-2621.1992.tb06861.x

Varshney RK, Glaszmann JC, Leung H, Ribaut JM (2010) More genomic resources for less-studied crops. Trends in Biotechnology 28: 452-460.

Vurayai R, Emongor V, Moseki B (2011) Effect of water stress imposed at different growth and developmental stages on morphological traits and yield of Bambara groundnut (Vigna subterranea L. Verdc). Am J Plant Physiol 6(1):17–27. https ://doi.org/10.3923/ajpp.2011.17.27

Wilkinson MD (2016) 'Comment : The FAIR Guiding Principles for scientific data management and stewardship', Nature, 3(160018 |), pp. 1–9.

West MAL, van Leeuwen H, Kozik A, Kliebenstein DJ, Doerge RW, St Clair DA, Michelmore RW (2006) High-density haplotyping with microarray-based expression and single feature polymorphism markers in Arabidopsis. Genome Research 16: 787–795.

Winzeler, EA, Richards DR, Conway AR, Goldstein AL, Kalman S, McCullough MJ, McCusker JH, Stevens DA, Wodicka L, Lockhart DJ, Davis RW (1998) Direct allelic variation scanning of the yeast genome. Science 281: 1194–1197

Xie M, Chung CYL, Li MW *et al* (2019) A reference-grade wild soybean genome. Nat Commun 10, 1216 (2019). https://doi.org/10.1038/s41467-019-09142-9

Yeboah MA, Chen X, Rong FC, Guohua L, Minghong G (2007) "A Genetic Linkage Map of Cucumber (Cucumis Sativus L) Combining SRAP and ISSR Markers." African Journal of Biotechnology 6 (24): 2784–91. https://doi.org/10.5897/ajb2007.000-2445.

Yang K, Tian Z, Chen C, *et al* (2015). Genome sequencing of adzuki bean (Vigna angularis) provides insight into high starch and low fat accumulation and domestication. Proceedings of the National Academy of Sciences of the United States of America, 112(43), 13213–13218. https://doi.org/10.1073/pnas.1420949112

Yao DN, Kouassi KN, Erba D, Scazzina F, Pellegrini N, Casiraghi MC (2015). Nutritive Evaluation of the Bambara Groundnut Ci12 Landrace [*Vigna subterranea* (L.) Verdc. (Fabaceae)] Produced in Cote d'Ivoire. *Int J Mol Sci,* 16, 21428-41.

Zhang H, Pala M, Oweis T, Harri H (2000) Water use and water-use efficiency of chickpea and lentil in a Mediterranean environment. Australian Journal of Agricultural Research 51: 295–304.

Chapter 3: Evaluation of drought stress response mechanisms in different Bambara groundnut genotypes.

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Abstract

Bambara groundnut (*Vigna subterranea* (L) Verdc) is a drought resistant underutilised legume crop with place of origin in the African continent. The present study aimed to better understand the physiological mechanisms and strategies for which Bambara groundnut employs in response to severe drought conditions. An initial set of twelve genotypes was assessed under drought and irrigated conditions in a controlled-environment tropical glasshouse. Furthermore, a sub-set of 8 genotypes was subsequently researched further in two additional seasons. Drought stress significantly reduced the *stomatal conductance* and increased canopy temperature in year one. Additionally, in all three years of experiments, *leaf relative water content* was significantly reduced, as well as the *photochemistry efficiency of the photosystem II under light and dark conditions* and the *estimated canopy area*. *Estimated chlorophyll content* was assessed using imaging software in years two and three, where a significant reduction was observed in response to drought in both years.

The response behaviour of the different genotypes (interaction genotype x treatment) to drought conditions was significantly different over three years in *estimated canopy area* and *efficiency of the photosystem II under light and dark conditions*.

Our results suggest two main strategies for coping with drought in the genotypes studied, **drought tolerance** and **drought avoidance**, where UnisR, DipC, Gresik, Kano2, and Kano3 could be considered more as drought avoidant genotypes based on their greater reduction of canopy size, high relative water content in leaves, overall higher reduction of the efficiency of

the PSII, and their higher rate of recovery after water was supplied. Meanwhile, DodR, TN, and S19-3, could be considered more as drought tolerant, as they cope better with the severe drought without reducing their canopy size as much, keeping a higher LRWC and a slightly reduced overall efficiency of the PSII. Additionally, their recovery rate was not significantly changed after water was supplied at the end of the drought period, which their places of origin have a mean rainfall of 350-750 mm per year. Our findings should help selecting traits and genotypes of interest for furthermore specific research, such as genetic crosses or transcriptome studies.

Introduction

With the current increase in human population, the effects of climate change, and our dependency on a low number of crops to fulfil the current and future needs for food and nutrition, further research is needed in the pursuit of food security. An estimate given in 2019 by Mbow *et al*., suggested that 821 million people were undernourished in 2019, 151 million children under the age of five years old had difficulties developing properly, and 613 million women between the ages of 15 and 49 suffered from iron deficiency. Alongside current nutritional issues, one of the current climate situations present in several regions in the world is the increased duration or variability of drought periods, which reduces the availability to predictably farm, as not many major crops or staple varieties are drought tolerant (Mbow *et al*., 2019). According to several authors (Blum, 2011; Farooq *et al*., 2009; Xu *et al*., 2010), there are three main responses to drought by crops in pursuit of survival to produce seed for the next generation. One of these mechanisms is **drought escape**, whereby plants may shorten their life cycle, trying to make the best of the water resources available in a short period (such as ground water remaining from a defined rain season), to allow them to produce the next generation before the environment becomes harmfully dry. **Drought-avoidance** is when plants attempt to adapt to changing water availability, such as deeper root growth to seek further water, to avoid reaching a point of damagingly low moisture levels in the crop before the next water becomes available (rain, or irrigation). This can be assisted by, for example, reducing stomatal conductance, restricting leaf area expansion, increasing root depth and density, among other traits. The last mechanism is **drought-tolerance**, which is considered as the ability to regulate thousands of genes and a series of metabolic pathways to minimise or repair damage caused by drought, and thus sustain physiological activities under severe drought conditions (Fang & Xiong, 2015).

Underutilised crops are plants that have been not widely cultivated, improved, and/or researched, which have a range of limitations, such as lack of producer and market interest, of developed cultivars and germplasm availability and a lack of research and technical information due to the low interest shown by researchers, agronomists, and extension workers. However, some of these plants have naturally adapted to harsh conditions over decades (Mabhaudhi *et al*., 2018), such as drought environments,

making them potential candidates for research and further development into possibly future crop varieties which could be cultivated in places where the more conventional and commercial crops cannot grow, or perform poorly.

Bambara groundnut (*Vigna subterranea* (L) Verdc) is an underutilised African pulse known for its drought resistance and high protein content. There are relatively few published studies about the drought resistance mechanisms of Bambara groundnut in comparison to some major crops. Previous studies have shown that drought may reduce leaf and canopy size, stomatal conductance, leaf relative water content, yield, and increase abscisic acid and osmoprotectant compounds in Bambara groundnut (Chai *et al*., 2016; Jørgensen *et al*., 2010; Mabhaudhi & Modi, 2013; Mwale *et al*., 2007; Nautiyal *et al*., 2017), yet this has only been tested in a few landraces.

In this paper, we present data on how a series of Bambara groundnut genotypes respond to drought through several drought-related physiological traits, and how different genotypes may have different mechanisms to cope with drought.

Materials and methods

Plant material, experimental design, drought treatment

Three years of drought experiments were carried out in the FutureCrop glasshouses (52°50’02”N, 1°15’00”W) of the Sutton Bonington Campus of the University of Nottingham in the summers of 2018 to 2020. Growing conditions were 23°C at night and 28°C day temperatures, respectively, in 12 h photoperiods controlled by automatic blackouts, with individual plants grown in 10 L pots. Irrigation was supplied by a dripper system to individual pots with two irrigation periods per day at 0600 and 1800 h to maintain a uniform field capacity of 75%. Irrigation was manually controlled to raise the pots to 75% field capacity at the beginning of the experiment and prior to beginning the drought treatment for 24 days in year 1 and 15 days in years 2 and 3, by removing the irrigation drippers for the drought treatment samples. This was performed at first flower per biological replicate, while in years 2 and 3, it was as soon as 50% of the biological replicates had reached first flower. After 15 days with no irrigation in the treatment plants, the field

capacity was raised manually to 75% and the irrigation drippers were re-introduced to the pots in years 2 and 3. In year 1, irrigation was restored at 24 days after the drought treatment began.

A set of 12 genotypes from different places of origin were initially assessed in a 50/50 soil mix of sand and John Innes No 2 compost, 4 replicates per treatment per genotype. The drought treatment was imposed at the production of the first flower for a genotype. The genotypes assessed were DipC, DodR, Getso, Gresik, IITA, Kano2, Kano3, LunT, S19-3, TN, UniswaG, and UniswaR, with places of origin described in Table 5 (Mateva *et al*. 2020). The experimental work was carried out in a randomised block design, with each block having one replicate of each treatment and each genotype, with a total of 4 blocks across the glasshouse. The main physiological traits measured were leaf relative water content, chlorophyll fluorescence, stomata conductance, relative canopy size, and canopy temperature.

Table 5 Reported countries of origin of the different genotypes used.

Genotype	Country of origin	Climate	Rainfall Mean (mm year^{-1})
TigaNecuru (TN)	Mali	Subtropical	450
S19-3	Namibia	Subtropical desert	350
DipC	Botswana	Semi-arid	500
DodR	Tanzania	Tropical dry	>570
IITA-686	Tanzania	Tropical dry	>750
LunT	Sierra Leone	Tropical wet	>2000
Uniswa Red (UnisR)	Eswatini		
Uniswa Green (UnisG)	Eswatini		
Kano2	Nigeria		600
Kano3	Nigeria		600
Getso	Nigeria		630
Gresik	Indonesia	Tropical wet	>2000

In the second- and third-year drought experiments, a smaller set of 8 of the most contrasting genotypes (DipC, DodR, S19-3, and TN; Kano2, Kano3, Gresik, and UniswaR) were taken forward and evaluated at higher replication, with 6 replicates per treatment per genotype, sown in topsoil with a sandy-loam texture. Drought treatment was introduced once 50% of the replicates per genotype had achieved their first flower. The main physiological traits measured were leaf relative water content, chlorophyll fluorescence, stomata conductance, estimate canopy size, and estimate chlorophyll content.

Field capacity is the maximum amount of water retained in a given volume of soil. This value changes depending on the structure and composition of the soil. All pots were filled to the same weight during the experiment set up, including an additional set of 4 pots. To determine the field capacity of the soil used, four random pots were irrigated until over-saturation was achieved. The pots were left until there were no signs of water drainage by gravity (12 hours) and subsequently weighed. To determine the soil mass in the absence of water, the soil was thereafter dried for 72 h at 80°C and a weight measurement was taken. The field capacity was calculated as follow:

$$\theta m = (\frac{\theta w - \theta d}{\theta d}) \times 100\%$$

where: θm = gravimetric field water capacity,

θw = soil mass at 100% field capacity, and

θd = soil mass in absence of water.

Physiological traits

Stomatal Conductance and canopy temperature

In the first year of experiments, stomatal conductance (*SC*) was measured on 3 random fully expanded leaflets per biological replicate at 24 days into the drought, these measurements were done at constant intervals until reaching a stable value, which was recorded per leaflet. Measurements were made between 0800 and 1200 h using a AP4 Porometer (Delta-T devices) according to the manufacturer's instructions, where different calibration points would take place based on the changes in relative humidity and temperature pointed out by the equipment. Canopy temperature was taken at 15 days in drought in the first year by taking a thermal image using a C2 thermal camera (FLIR) at 1600 h and at a 1.5 m distance from the plant, then temperature values were obtained using the FLIR Tools software, selecting the average temperatures of all the leaves of the canopy (T_{can}). Air temperature (T_{air}) was defined by averaging the air temperatures between a Sensirion electric thermometer, and the glasshouse internal control sensor thermometer (Cambridge HOK, UK) for the time when the thermal images were taken.

Mineral content of leaves

For the ionome determination, 3 leaflets were collected per replicate using a ceramic knife at 15 days into the drought treatment, dried at 80°C and transferred to 2 ml Eppendorf tubes. The dried Bambara samples were processed and analysed by the Ionomics lab of the University of Nottingham, where the samples were ground and transferred into Pyrex test tubes (16 x 100 mm) and weighted. The trace metal grade nitric acid Primar Plus (Fisher Chemicals) spiked with an indium internal standard was added to the tubes (2 mL per tube). The samples were pre-digested overnight in the fume hood at room temperature. Then, the samples were digested in a dry block heater (DigiPREP MS, SCP Science; QMX Laboratories, Essex, UK) at 115°C for 4 hours. Once the samples cooled down, 1 mL of hydrogen peroxide (Primar, for trace metal analysis, Fisher Chemicals) was added to the tubes and samples were digested in the dry block heater for an additional 2h at 115°C. The digested samples were diluted to 20 mL with 18.2 MΩcm Milli-Q Direct water (Merck Millipore) and mixed with an array mixer. Elemental analysis was performed with an inductively coupled plasma-mass spectrometry (ICP-MS) PerkinElmer NexION 2000 equipped with Elemental Scientific Inc. autosampler, in collision mode (He). Twenty-three elements (Li, B, Na, Mg, P, S, K, Ca, Ti, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Rb, Sr, Mo, Cd and Pb) were reported. Liquid reference material composed of mixture of samples was prepared before the sample run and was used throughout the runs. The control was run after every ninth sample to correct for variation within the ICP-MS analysis run (Danku *et al*., 2013). The calibration standards (with indium internal standard and blanks) were prepared from single element standards (Inorganic Ventures; Essex Scientific Laboratory Supplies Ltd, Essex, UK) solutions. Sample concentrations were calculated using an external calibration method within the instrument software.

Fluorpen measurements of photosynthesis

In all three years, the Maximum quantum efficiency of the Photosystem II (PSII, *QYDA*) was measured after 15 days of drought treatment by measuring the quantum yield after 30 min of dark adaptation in 3 leaflets per biological replicate using a Fluorpen (PSI), one measurement was made

per leaflet. Meanwhile, the maximum efficiency (*QYLA*) was measured in the same way under normal light conditions in the afternoon and in presence of supplemental light generated by 600w high pressure sodium lights after 25 days drought for year 1, and 15 days in drought for year 2 and 3. Measurements were done on the irrigated controls as well, to give a better understanding of any intrinsic genotypic differences between the genotypes.

Leaf Relative water content (LRWC)

Meanwhile, for relative water content in leaves, a total of three leaf discs of 0.78 cm² were punched out using a biopsy punch from a single leaflet and from 3 random fully expanded leaflets, adding to a total of 3 discs per biological replicate, at 15 days in drought in all three years. These were weighted to determine fresh weight (Fw), and soon after, the leaf discs were incubated under deionised water and in presence of light for 24 h and weighted again for a turgid weight (Tw). The discs were immediately transferred to an oven at 80°C for 48 h and then weighed for dry weight (Dw). The formula below was used to determine the *LRWC*:

$$RWC = \frac{Fw - Dw}{Tw - Dw} \; x\,100\%$$

Estimating Canopy Area

An estimate of canopy area (*ECA*) was determined by analysing photographs taken from the top of the canopy which were taken using a Canon D7000 camera fixed to a metal frame with a white background and white LED, with a reference ruler in the photograph. The photographs were taken after 25 days drought treatment in year 1, and after 15 days drought treatment in year 2 and 3 and were processed using ImageJ software to measure the canopy area. An image analysis code was specifically written for this experiment and is shared in appendix 1.

Estimating Chlorophyll Content

For an estimate whole-canopy chlorophyll content (*ECC*), photographs in the second and third years were processed using ImageJ software by calculating the red, green, and blue (RGB) pixel values of all the leaves present in the pictures. These RGB values were then calculated using the formula

$$RGBtoCh = G - \frac{R}{2} - \frac{B}{2}$$

reported by Ali *et al*. (2013). To transform these values into a chlorophyll indicator, individual leaves were photographed and simultaneously measured using SPAD, and the reading was plotted against the RGB calculated value. This gave the following best-fit equation to apply to these values:

$$ECC = (-21.28) * (LN(RGBtoCh)) + 123.28$$

Where: ECC = Estimated Chlorophyll content

RGBtoCh = value calculated based on the RGB values of the leaf pixels as described above

To allow a better understanding of early sensing of drought by Bambara groundnut and to assess changes in recovery period, 3 time points were taken in the third year for the measurements of (*QYDA*), (*QYLA*), *ECA*, *ECC* at day 7, 11, and 15 during the drought treatment, and two time points at recovery, at 1 day after irrigation was re-introduced, and at day 3.

Statistical analysis

Statistical analysis was performed using Genstat 20th edition (VSN International) and GraphPad Prism 9. Depending on the year and data set, two-way or three-way Analyses Of Variance (ANOVA) were carried out.

Results and Discussion

Year one results

Flowering time

In relationship to flowering time, there were significantly difference between the genotypes in each year ($P<0.05$), with TN consistently being the genotype to reach anthesis first (39, 45, and 40 days after sowing (DAS) in 2018, 2019, and 2020 respectively) while UnisR and Gresik were the later genotypes with averages of 48, 45 and 46 DAS for UnisR, and 47, 54, and 49 DAS for Gresik. These results are in agreement with the results published

by Kendabie *et al*. (2020). It has been reported by Blair *et al.* (2012) that the most serious effects of drought take place when drought occurs during the anthesis phase.

Since the drought treatment was linked to anthesis, the differences between the earliest genotype (TN) and the latest (Gresik) was 9 days, which is linked to a slightly (yet possibly not significant) older plant, however, the canopy structures and size differences were already expressing significant values prior to anthesis.

Stomatal conductance

During the first year, after 15 days in the drought treatment, *stomatal conductance* had decreased significantly in the treatment compared to the control ($F_{(1,66)}$=314.6, P < 0.001) However, there was no significant differences among genotypes (Figure 3-1). Drought stressed plants had averages for *stomatal conductance* between 3 and 30 mol s^{-1} m^{-2}, meanwhile irrigated controls had averages between 100 and 180 s^{-1} m^{-2}. This is consistent with Jørgensen *et al*. (2010), Mabhaudhi *et al*. (2013), and Chai *et al*. (2015), where similar values were presented, however, conductance was at values between 200 and 905 mol s^{-1} m^{-2}, measured before flowering, where Gresik had the highest conductance at an average of 905±209 mol s^{-1} m^{-2}, followed by IITA at 868±189 mol s^{-1} m^{-2}, meanwhile the lowest genotype was TN at 626±41 mol s^{-1} m^{-2}.

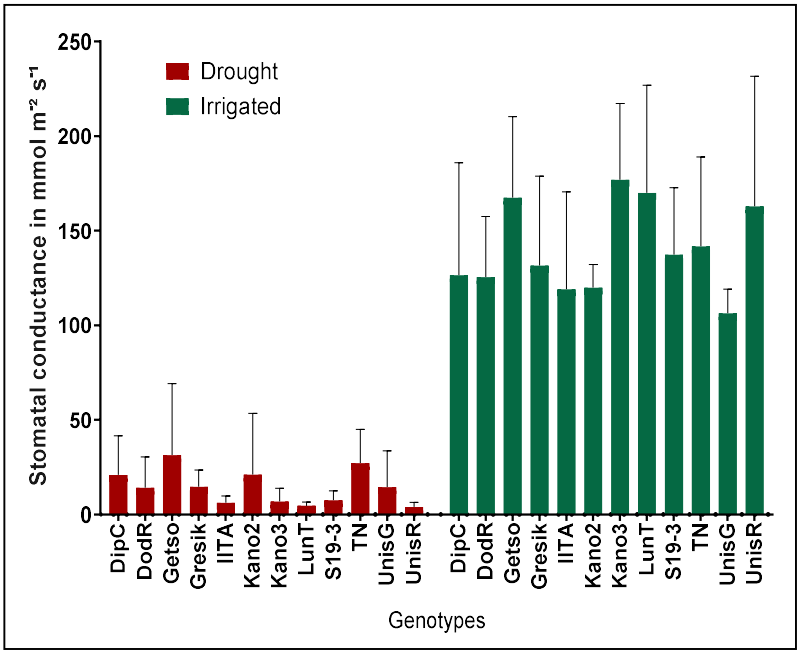

Figure 3-1 Stomatal conductance in year 1. In red is drought treatment and in green is irrigated control. All genotypes were significantly affected by the drought treatment ($F_{(1,61)}$=60.2, P < 0.001).

Canopy temperature

Meanwhile, the canopy temperature was significantly increased in the drought treatment plants ($F_{(1,61)}$=60.2, P < 0.001), with significant differences among the genotypes ($F_{(11,61)}$=7.93, P < 0.001), and an overall significantly different behaviour in response to drought ($F_{(11,61)}$=2.34, p<0.018).

Since different genotypes and replicates started the drought treatment at different dates, while comparing the leaf temperature against the air temperature, all genotypes showed a significant increase in temperature in response to the drought treatment in comparison to the irrigated controls ($F_{(1,62)}$=79.2, P < 0.001). The average response to drought was 0.2±1.9 °C warmer, compared to -4.1±1.0 °C cooler in the irrigated controls, and even though there were significant differences between genotypes ($F_{(11,62)}$=2.36, P=0.0168), there was no significant interaction between both factors. Among both drought and irrigated plants, Gresik had the lowest leaf to air temperature difference at -3.2±0.6 °C in the drought treatment and -5.5±0.8 °C in the irrigated control, meanwhile S19-3 had the warmest temperature difference during drought conditions being +2.9±2.1 °C warmer than the air temperature, while in the irrigated controls, Kano2 and Kano3 had the warmest temperature differences at -2.9±0.6 °C and -2.4±0.7 °C respectively. To date, no canopy temperature data has previously been published in Bambara groundnut, and our results are very relevant, as all the material were in pots (thus root depth/length might not reflect a significant difference between genotypes due to same volume

restriction, same in the case of shadowing due to plant/canopy density).

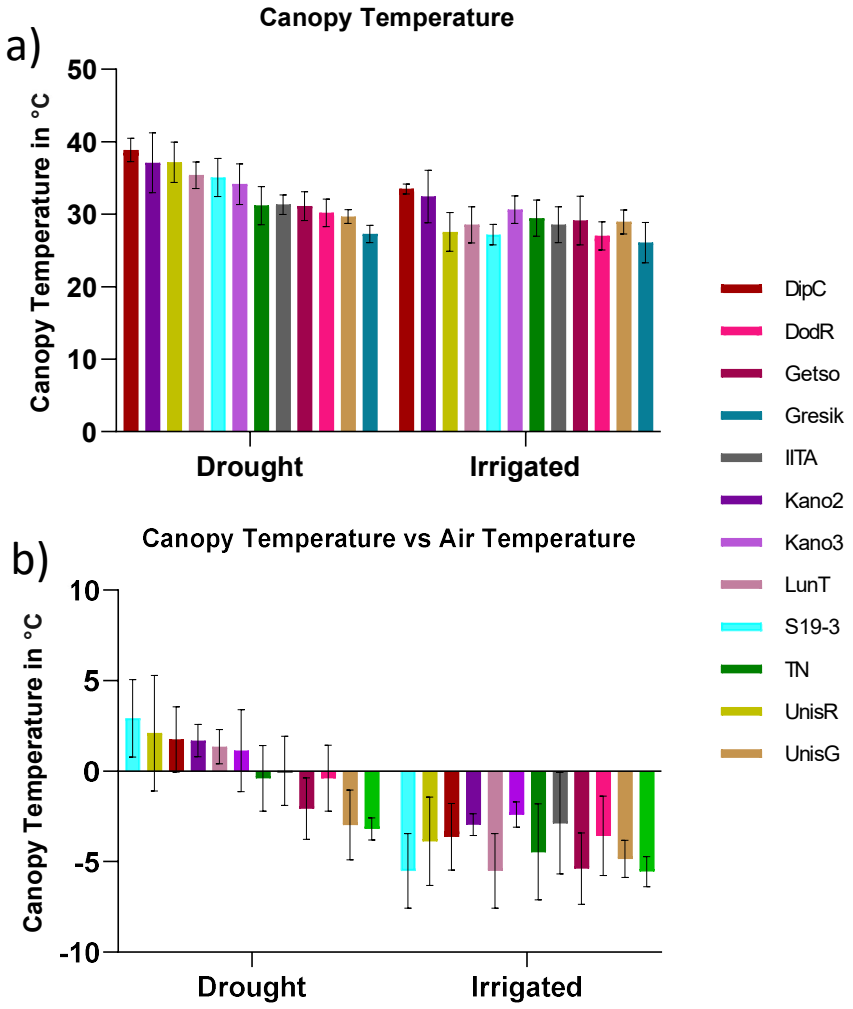

Figure 3-2 Effect of drought in canopy temperature. a) canopy temperature, showing a significant increase in temperature under drought conditions ($F_{(1,61)}$=60.2, P < 0.001); b) temperature difference between canopy and air, expressing a significant increase in temperature when comparing between drought and irrigated conditions ($F_{(1,62)}$=79.2, P < 0.001).

In the first year, Gresik did not survive after 15 days of drought treatment, thus a series of measurements did not include this genotype in this particular year.

Leaf ionome

The ionome composition results showed significant differences in certain elements, however, overall, there was no significant interaction between

treatment and the genotypes. Among the differences, drought significantly increased the content of Li, Na, S, K, Fe, Zn, As, Rb, and Mo in leaves (p<0.05). The most significant amounts and increases of ion content in response to drought in the different genotypes where in UnisR (Li), Getso (Li, Mo, S, K), Kano2 (S, Fe), Kano3 (Li, S), DodR (Rb), IITA (Rb, Fe, K) and S19 (increase of Rb and decrease of As). Further research will be needed to understand better the role of the ionome in drought, for example the roles of K and Ca, where the first is related to stomatal responses, and the second one is related to enzymatic activity. A few of these elements have more important biological roles than others, thus, further research is required in this area, however, all the minerals that were significantly different under drought conditions (in comparison to the irrigated counter parts) are presented in annex 2.

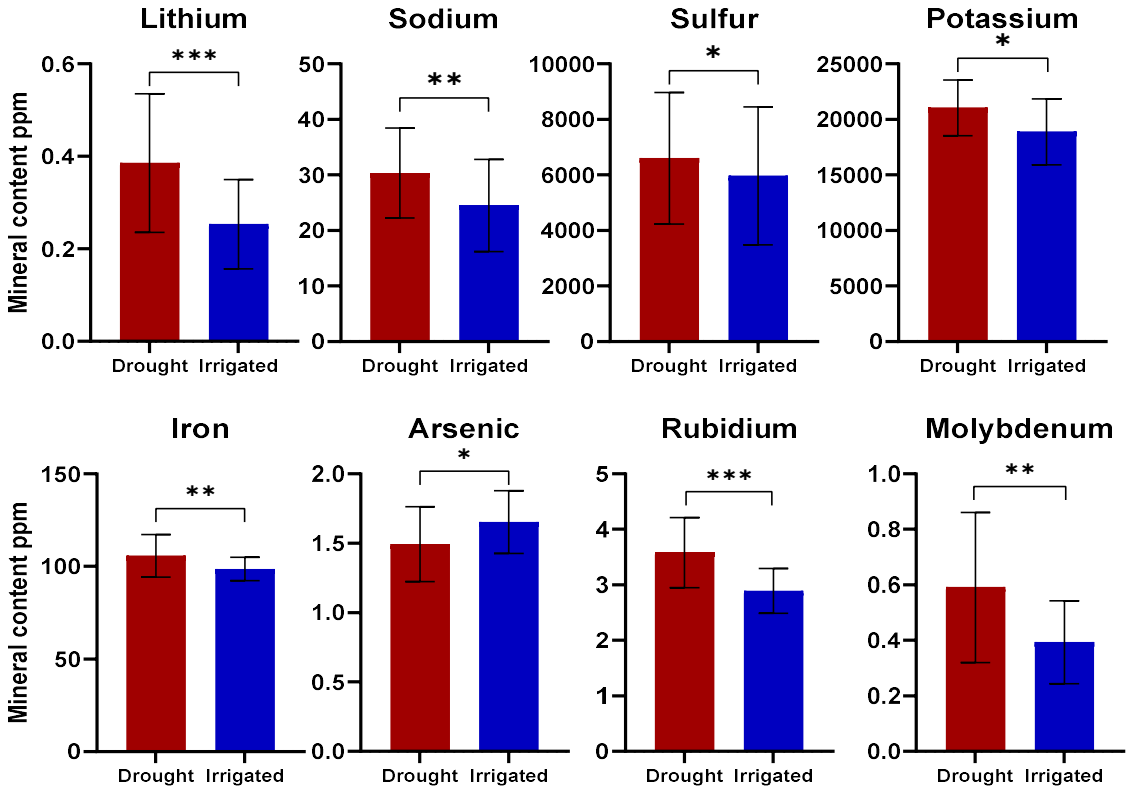

Figure 3-3 Drought effect in diverse mineral compositions in leaves.

Year one, two, and three results

Leaf relative water content

For the *LRWC* (Figure 3-), in all three years there was a significant decrease in response to drought treatment ($F_{(1,51)}$=56.5, P < 0.001; $F_{(1,70)}$=354.1 P < 0.001; $F_{(1,70)}$=563.7, P < 0.001), with the genotypes showing clearly significantly differences in years 2 and 3. However, there was a slightly less clear difference in year one due to higher variance ($F_{(10,51)}$=2.8, P=0.008; $F_{(7,70)}$=21.32, P < 0.001; $F_{(7,70)}$=10.39, P < 0.001). The overall behaviour was more variable across years, where in year 1, LunT, IITA, Gresik, and UnisR had the lowest *LRWC* under drought conditions (56.58±4.1; 39.81±3; 21.92±23.9; 44.75±2.14 respectively); in year 2, DodR and TN (37.16±2.9; 38.91±7.02); and in year 3, Kano3 and DipC had the lowest values (44.15±6.33; 41.19±5.3). Additionally, when analysing for the reduction of leaf water content in response to drought treatment by comparing the irrigated control values against its drought counterpart, DodR had the least reduction in year 1 (1.29±6.38). Interestingly, in year 2 DodR, alongside UnisR, showed the highest reduction (27.33±3.03; 27.05±3.52); while in year 3, DodR, UnisR, and Kano2 showed lowest reduction (22.85±4.26; 22.17±6.07; 23.94±5.74). The overall reduction of *LRWC* in response to drought has been reported previously by Jørgensen *et al*., (2010), Muhammad *et al*., (2016), Nautiyal *et al*., (2017), however, the variable behaviour of the different genotypes across the different years could be result of the possible heterozygosity in the genotypes. The *LRWC* values on the drought treated plants were also lower in comparison to the results presented by Jørgensen *et al*., (2010), Muhammad *et al*., (2016), Nautiyal *et al*., (2017). Nautiyal *et al*., (2017) reported a higher *LRWC* in UnisR compared to DodR and S19-3, which is consistent with our results only in year 2.

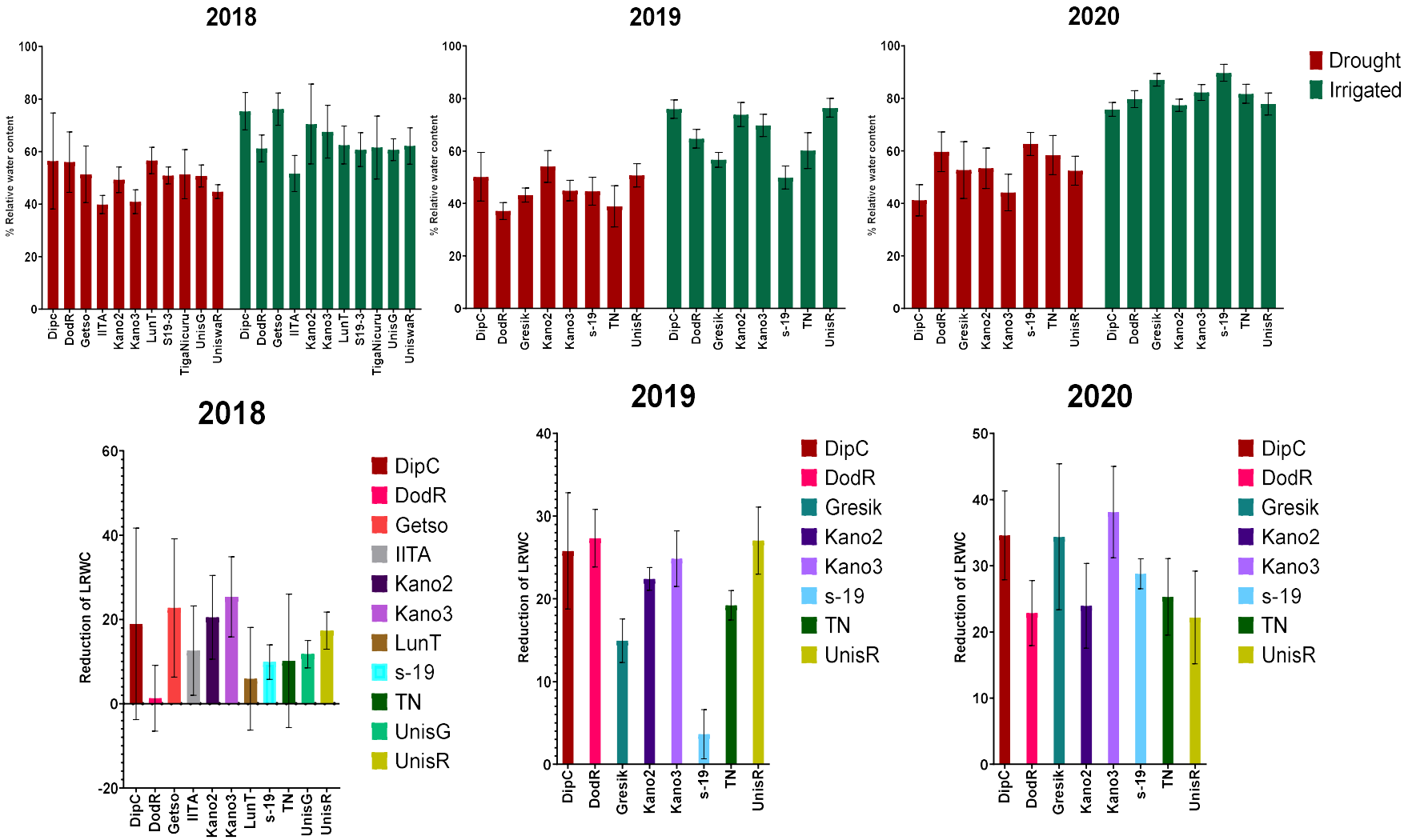

Figure 3-4 Leaf relative water content over three years of experiments. Top set shows the leaf relative water content, whereas the bottom row shows the difference between the irrigation control and the drought treatment. There is an overall descrease of *LRWC* in all three years in the drought treatment ($F_{(1,51)}$=56.5, P < 0.001; $F_{(1,70)}$=354.1 P < 0.001; $F_{(1,70)}$=563.7, P < 0.001).

Chlorophyll fluorescence

In all three years, *QYDA*, and the *QYLA* were significantly decreased in response to the drought treatment ($P < 0.001$; Figure 3-), the genotypes behaved significantly differently in response to drought stress ($P < 0.001$), where S19-3 (0.60±0.19), DodR (0.69±0.08), and TN (0.70±0.07) showed over the three years the overall highest values of the *QYDA under drought stress*, while Gresik (0.57±0.09), Kano2 (0.56±0.20), Kano3 (0.45±0.13), and UnisR (0.47±0.16) showed the lowest values under drought stress. Meanwhile, for *QYLA*, TN (0.55±0.08) and S19-3 (0.41±0.11) had the highest values in drought overall during all 3 years, meanwhile Kano3 (0.45±0.13), DipC (0.26±0.07) and Gresik (0.29±0.11) had the lowest.

Similar results were reported by Muhhamad *et al*. (2015), where Bambara dark adapted leaves showed a highly significant decrease in the efficiency of the PSII in comparison to their irrigated counterpart. However, our results show a higher decrease rate in the case of Gresik, where major decrease is appreciated from 11 days in drought, compared to 21 days reported by Muhhamad *et al*. (2015). This could be attributed to several factors, such as higher relative humidity in the air (Malaysia) and younger plants (seedling stage).

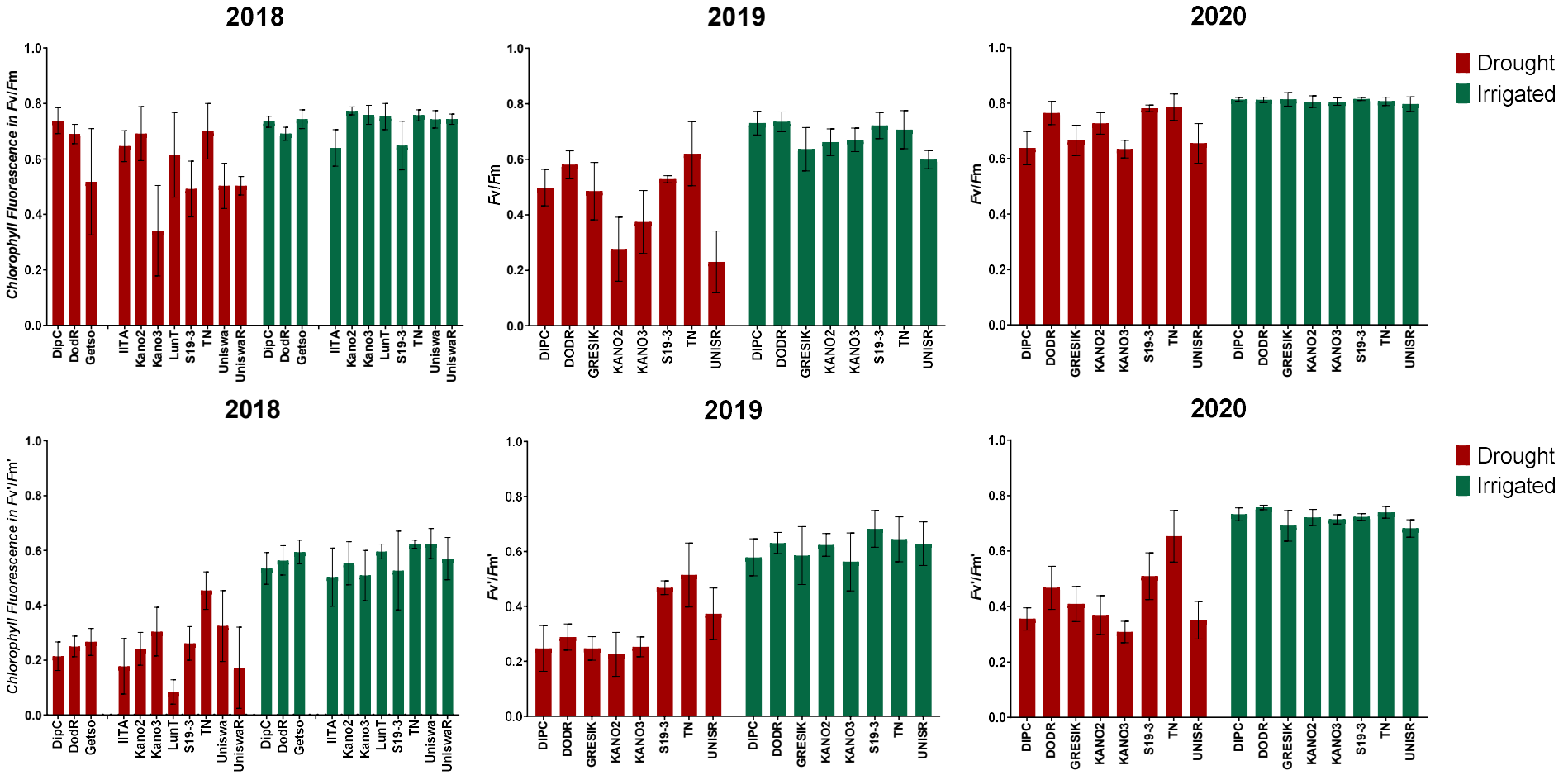

Figure 3-5 Chlorophyll fluorescence in Bambara groundnut. On top row set Maximum quantum efficiency of the PSII (QYDA), where there was an overall reduction in response to drought in all three years ($F_{(1,59)}$=58.07, $P < 0.001$; $F_{(1,72)}$=222.6, $P < 0.001$; $F_{(1,78)}$=185.58, $P < 0.001$). Bottom row shows the Maximum efficiency of the PSII in prescence of light (LA) where a reduction of efficiency is observed in response to drought in all three years ($F_{(1,52)}$=280.7, $P < 0.001$; $F_{(1,70)}$=319.2, $P < 0.001$; $F_{(1,78)}$=713.58, $P < 0.001$).

Estimated canopy area

In all three years, the estimated canopy area was significantly reduced in all genotypes due to the drought stress ($F_{(1,64)}$=513.2, P < 0.001; $F_{(1,69)}$=92.79, P < 0.001; $F_{(1,68)}$=5.14, P < 0.001; Figure 3-), which is consistent with several authors who reported a significant reduction in leaf number, leaf expansion index, and finally canopy area in Bambara groundnut (Jørgensen *et al*., 2010; Mabhaudhi & Modi, 2013; Mwale *et al*., 2007; Nautiyal *et al*., 2017), as well as in other related crops such as groundnut (*Arachis hypogaea*) (Collino *et al*., 2001), cowpea (*Vigna unguiculata*) (Anyia & Herzog, 2004) and chickpea (*Cicer arietinum*) (Singh, 1991). Due to the natural genetic differences of the genotypes in terms of canopy size, all irrigated control plants were also significantly different (P < 0.001; P < 0.001; P < 0.001), thus, a direct comparison of the difference between the irrigated control and drought stressed plants was performed by comparing both individuals per block, allowing us to better represent how the canopy changed in response to drought stress (Figure 3-). In all three years, there was a significant reduction of the estimated canopy size in response to drought ($F_{(11,28)}$=9.026, P < 0.001; $F_{(7,25)}$=12.55, P < 0.001; $F_{(7,23)}$=8.65, P < 0.001), where overall Gresik (815±6; 1350±128; 1401±149), Kano3 (694±77; 1070±294; 1583±63), and UnisR (1009±15; 154±198; 1560±97) were the genotypes which had a greater change/reduction of their canopy size, meanwhile TN (250±92; 441±393; 891±368), S19-3 (276±89; -39.97±228; 1112±186) , and DodR (455±127; 409±283; 1039±194) were the genotypes with the least canopy reduction. Contrary to the observations reported by Mwale *et al*. (2007), DipC and S19-3 did not behave similarly in any of the three years, however, as mentioned before, there were significantly different behaviours among all the genotypes assessed. These differences could be due to the different environments, length and level of drought, and pot vs land. On the other hand, our results are in agreement with those reported by Nautiyal *et al*., (2017), where UnisR had a higher reduction of the canopy size in comparison to DodR and S19-3.

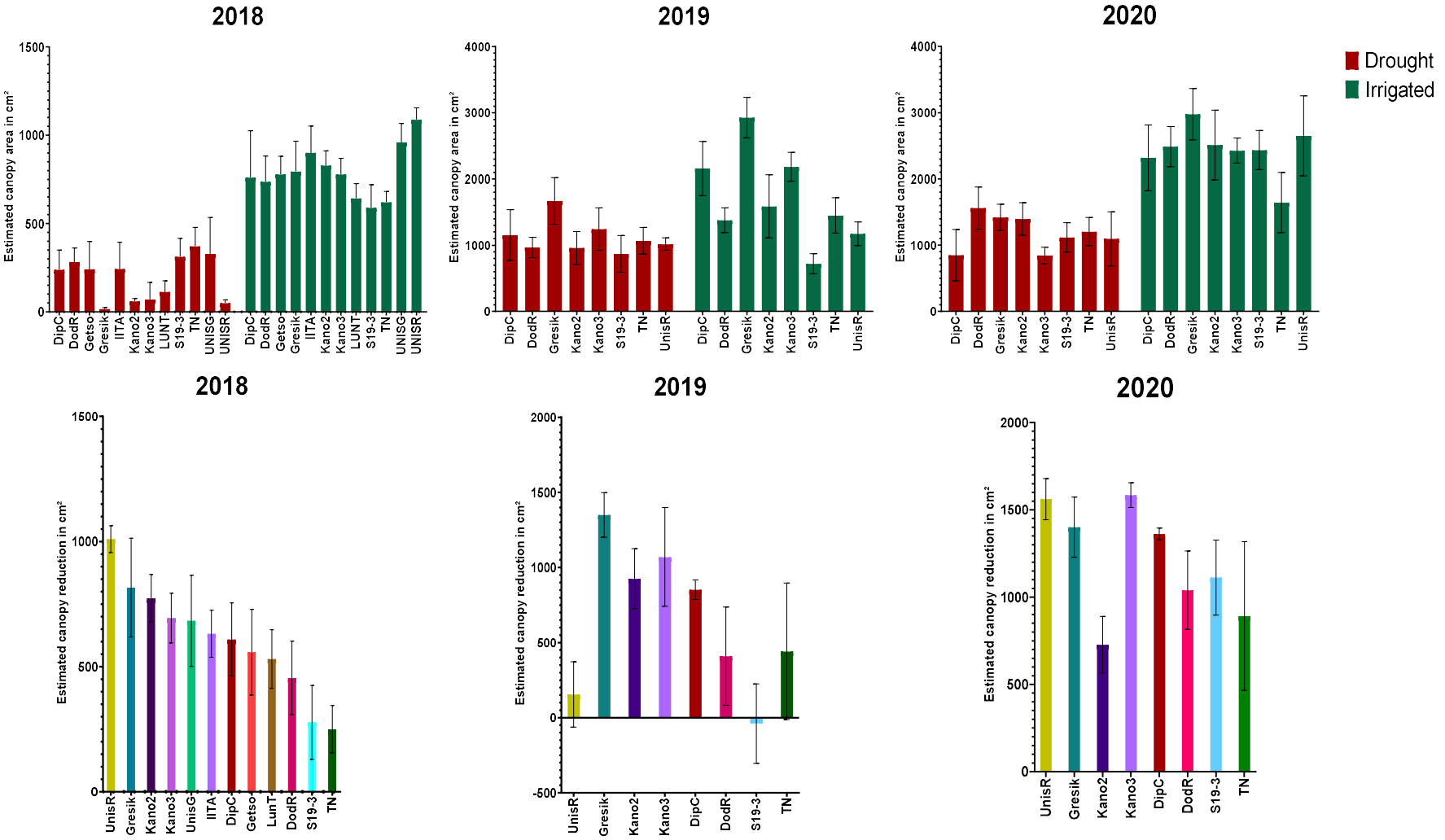

Figure 3-6 Estimated canopy size over three years of experiments. Top row set shows the estimated canopy area, where an overall decrease of canopy size is observe in all three years ($F_{(1,64)}$=513.2, P < 0.001; $F_{(1,69)}$=92.79, P < 0.001; $F_{(1,68)}$=5.14, P < 0.001). The bottom row set shows the different reduction of the estimated canopy area between genotypes in response to drought, where a significantly different behaviour is observed ($F_{(11,28)}$=9.026, P < 0.001; $F_{(7,25)}$=12.55, P < 0.001; $F_{(7,23)}$=8.65, P < 0.001).

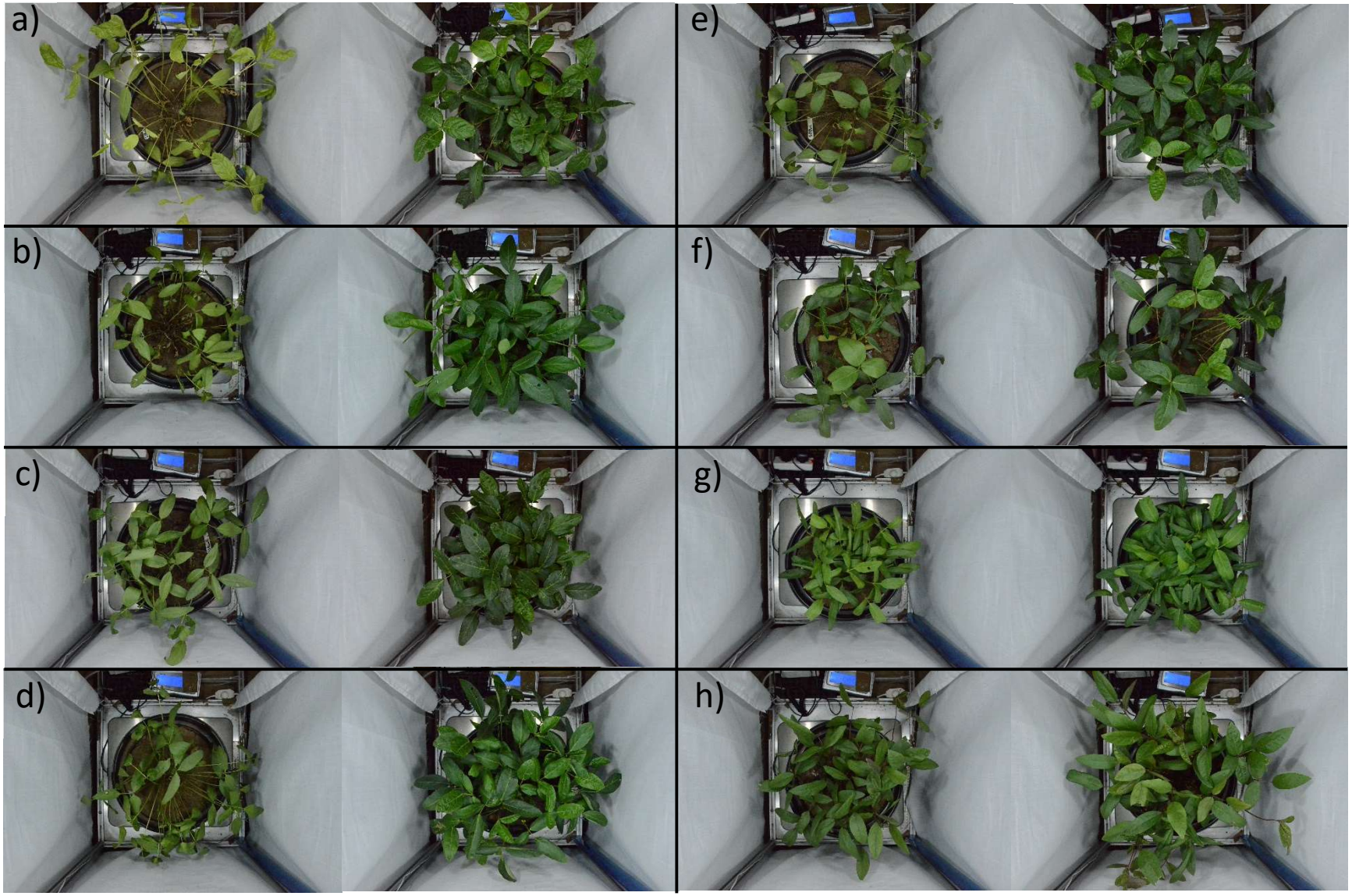

Figure 3-7 Effects of drought in Bambara groundnut in year 3 (2020). Left picture represents drought treatment, right side represents irrigation control. a) Gresik; b) Kano3; c) Kano2; d) UnisR; e) DipC; f) S19-3; g) TN; h) DodR. There was a significant reduction in canopy size in response to drought ($F_{(1,68)}$=5.14, P < 0.001)

Estimated chlorophyll content

From the different individual leaf assessment and best fitted equation, RGB values were plotted against SPAD values, Figure 3- shows the SPAD values manually obtained, versus the values obtained by running the conversion of RGB using the equation "(-21.28)*(LN(RGBtoCh))+123.28" mentioned in the material and methods section. A paired t-test showed no significant difference between both values ($t_{(d.f.\ 47)}$=1.36, *P*=0.186).

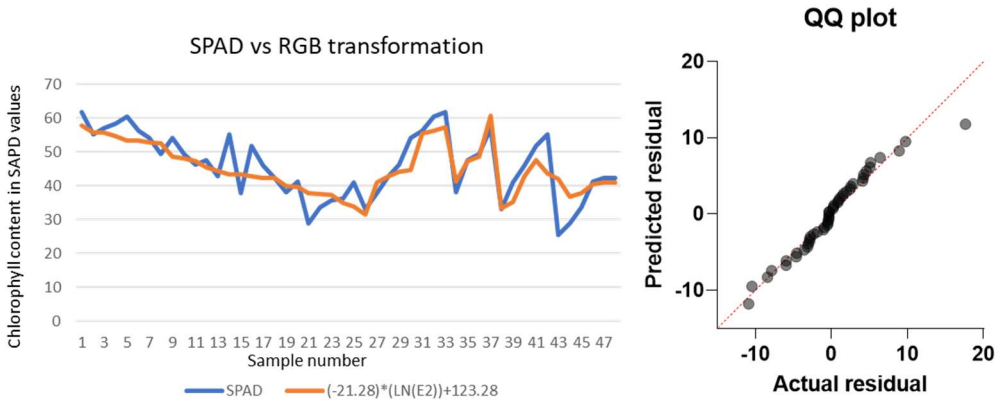

Figure 3-8 Chlorophyll content values obtained by SPAD and plotted against *Estimated Chlorophyll Content* equation values, where E2= RGB pixel values calculated as "G – R/2 – B/2". Paired t-test showed no significant difference (*P*=0.186) and QQ plot showing a general normal distribution.

In years 2 and 3, the estimated chlorophyll content was significantly lower in drought stressed plants ($F_{(1,79)}$=57.81, $P < 0.001$; $F_{(1,68)}$=4.37, $P = 0.04$), where a clear genotype effect was seen ($F_{(7,79)}$=21.52, $P < 0.001$; $F_{(7,68)}$=5.26, $P < 0.001$), with the interaction between genotype and treatment significant in year 2, yet not significant in year 3 ($F_{(7,79)}$=21.52, $P < 0.001$; $F_{(7,68)}$=0.60, $P = 0.75$). A summary of findings is shown in Appendix Tables 5 to 7.

Even though there are different methods in the literature, our approach shows an initial assessment of high throughput phenotyping for full canopy chlorophyll content, which further validation is required for more precision (chlorophyll extraction and quantification and more disperse lights to avoid possible shadows).

In year three, repeated measures ANOVA showed a significant difference between genotypes in response to drought over time for *ECA* ($F_{(14,249)}$=5.1, $P < 0.001$), *QYDA* ($F_{(14,280)}$=4.84, $P < 0.001$), *QYLA* ($F_{(14,280)}$=2.47, P=0.005), and *ECC* ($F_{(14,253)}$=4.43, $P < 0.001$). Compilation of results and data is presented in the Appendix Table 7 and 8.

Correlation analysis showed different results in different years. A summary of the different years are given in Tables 6, 7 and 8. Two correlation sets per year were evaluated, with the first taking place between measurements in the drought treatment, showing the effect during drought; meanwhile, the second set shows correlations in response to drought, which is how the plant responded when comparing the irrigated control with the drought treatment. In year one, there was a positive correlation in the drought treatment between several traits such as between *ECA* and *QYDA*, *ECA* and *QYLA*, *ECA* and stomatal conductance, and among some mineral concentrations (Table 6). Meanwhile, when the drought treatment was compared to the irrigated controls, significant positive correlations were found in the case of *ECA* and *QYDA*, and a few others involving different mineral concentrations in leaves. In year 2, the only significant correlations were, in the drought treatment, *ECC* and *QYLA* (-0.34, P=0.036), and *QYDA* with *LRWC* (-0.46, P=0.004), while in direct comparison between the irrigated control and drought treatment identified a significant correlation between *ECA* with *ECC* (-0.7, P<0.001). In year 3, more significant positive correlations were identified between the measurements in the drought treatment, with *ECA*, *QYDA*, AND *LRWC* were positively correlated (Table 10). When comparing against the irrigated controls, *ECA*, *QYDA*, *QYLA*, and *LRWC*, were positively correlated (Table 11).

Table 6 Correlation results from year 1 (2018).

2018 Response to drought			2018 Effect in Drought		
Traits	Correlation	*P*	Traits	Correlation	*P*
ECA.QYDA	0.44	0.012	ECA.QYDA	0.42	0.014
Tcan.Tdiff	0.60	<0.001	ECA.QYLA	0.55	0.002
QYLA.Pb	0.37	0.031	ECA.SC	0.45	0.005
ECA.P	-0.40	0.014	Tcan.Tdiff	0.75	0.000
Tdif.Zn	-0.44	0.006	Tcan.Ca	0.36	0.033
QYDA.P	-0.35	0.031	QYDA.Mg	0.37	0.020
QYDA.Ca	-0.41	0.013	QYDA.SC	0.32	0.047
QY.Mn	-0.43	0.007	Ca.LRWC	0.31	0.054
QYDA.Mo	-0.37	0.025	Tcan.Rb	-0.50	0.002
QYLA.Ca	-0.36	0.035	QYDA.Na	-0.41	0.010
QYLA.Sr	-0.34	0.049	QYDA.P	-0.46	0.003
Mo.LRWC	-0.47	0.005	QYLA.Mg	-0.38	0.030
Li.LRWC	-0.39	0.022	QYLA.Ca	-0.38	0.028
P.LRWC	-0.45	0.008	QYLA.Co	-0.44	0.011
			Fe.LRWC	-0.39	0.014
			Mo.LRWC	-0.35	0.029

Table 7 Correlation results in year 3 in drought (2020).

	Area	QYDA	QYLA	RWC
Area	**1**			
QYDA	0.38 *P*=0.02	**1**		
QYLA	0.13 *P*=0.41	0.73 *P*<0.001	**1**	
LRWC	0.42 *P*=0.01	0.71 *P*<0.001	0.49 *P*<0.001	**1**

Table 8 Correlation results in year 3 Irr-Dro(2020).

	Area	QYDA	QYLA	RWC
Area	**1**			
QYDA	0.65 *P*<0.001	**1**		
QYLA	0.40 *P*=0.03	0.69 *P*<0.001	**1**	
LRWC	0.40 *P*=0.04	0.60 *P*<0.001	0.35 *P*=0.02	**1**

In terms of the recovery phase (Figure 3- to Figure 3-), *ECA, QYDA,* and *QYLA*, showed an overall significant increase at 24 h after the soil capacity was raised to 75% ($F_{(2,105)}$=18.26, $P < 0.001$; $F_{(2,116)}$=39.22, $P < 0.001$; $F_{(2,116)}$=212.5, $P < 0.001$), whereas only *QYLA* had a further significant increase between 24 to 72h (from 0.63±.01 to 0.68±0.05, P=0.0005). UnisR, Gresik, and Kano3 showed the greatest recovery of *ECA*, whereas DipC and Kano2 had a lower recovery increase. S19-3, DodR, and TN did not show a significant increase. Similarly, for *QYDA*, whereas Kano3, UnisR, Gresik, and Kano2 had a more significant increase at 24 h (0.078±0.053; 0.112±0.053; 0.089±0.071; 0.071±0.035), DipC was slower (0.055±0.11). DodR, S19-3, and TN did not show a significant increase (0.042±0.053; 0.005±0.0178; 0.033±0.043). Meanwhile, for *QYLA*, DodR, Gresik, Kano2, Kano3, S19-3, and UnisR, had a significant recovery (0.20±0.063; 0.23±0.093; 0.322±0.064; 0.262±0.095; 0.160±0.078; 0.254±0.059), while DipC once more had a slower recovery (0.092±0.117). TN show a borderline significant increase (P=0.028) from drought to 24h in recovery. DipC and UnisR where the only genotypes to have a significant increase from 24 to 72 h.

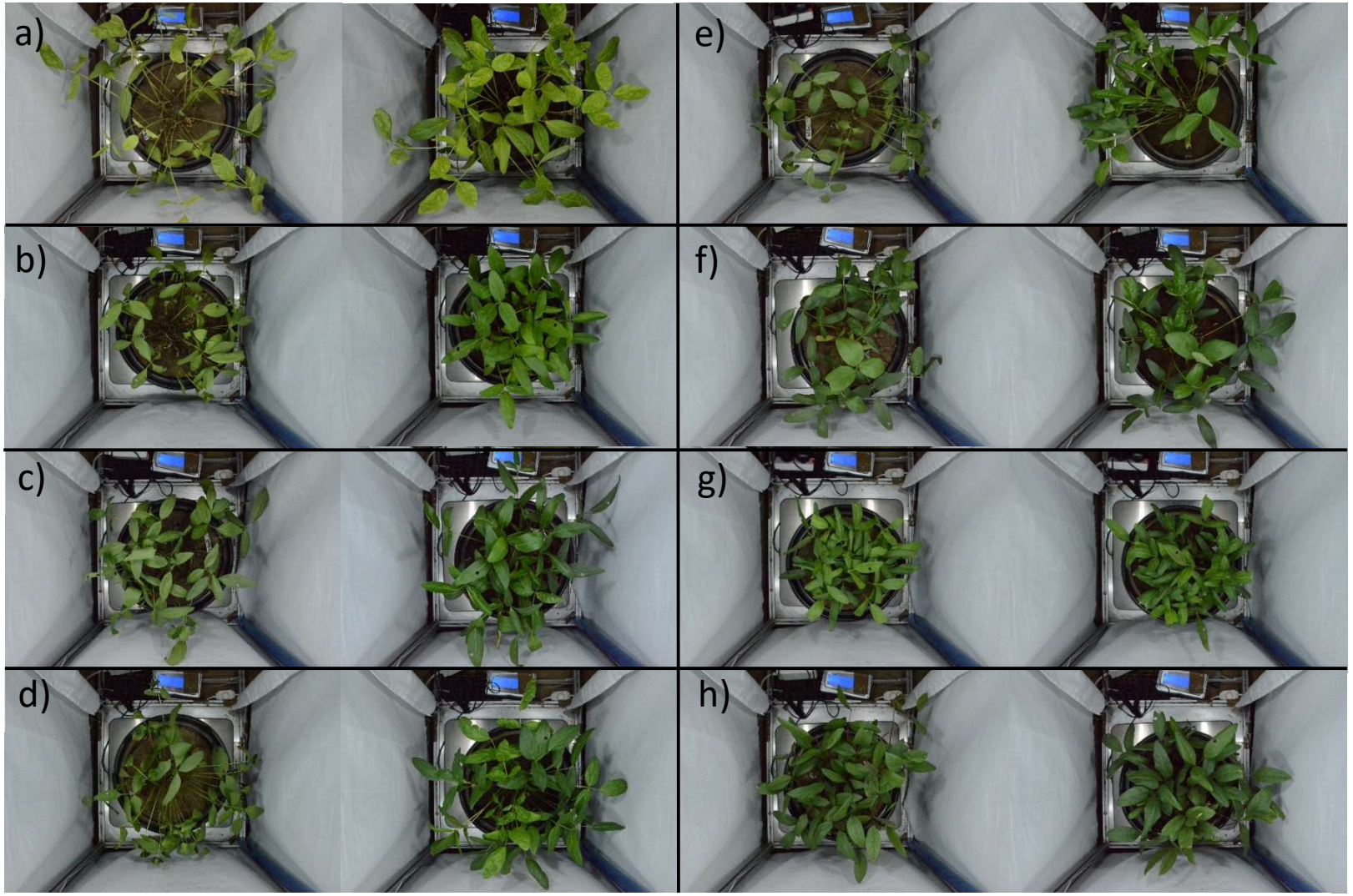

Figure 3-10 Effects of drought and recovery in Bambara groundnut year 3 (2020). Left panel represents drought treatment, right panel shows same plant 24 h after water was re-introduced to 75% field capacity. a) Gresik; b) Kano3; c) Kano2; d) UnisR; e) DipC; f) S19-3; g) TN; h) DodR

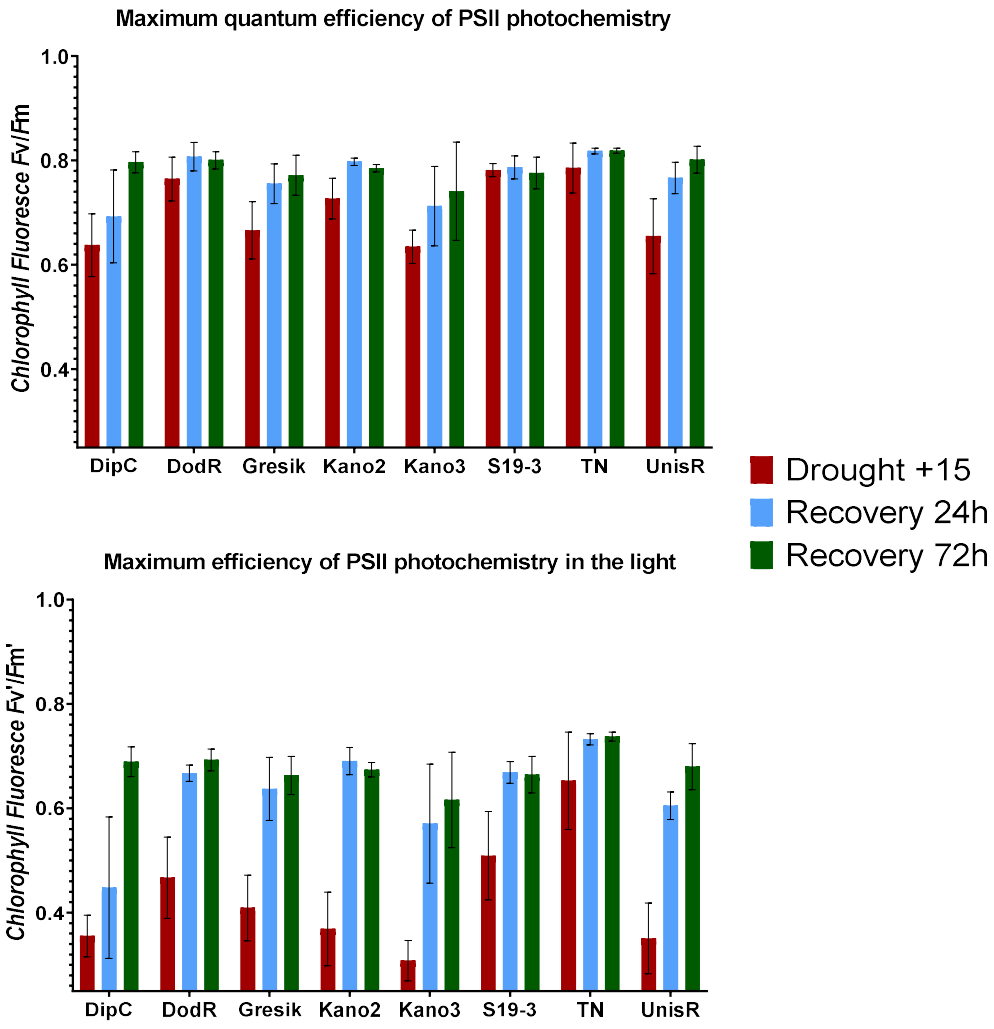

Figure 3-11 Recovery effect in PSII efficiency in Bambara groundnut drought treated plants. Top graph is QYDA, and bottom is QYLA. There were significant increases at 24 h after irrigation field capacity was re-stablished at 75% in both QYDA ($F_{(2,116)}$=39.22, P < 0.001) and QYLA ($F_{(2,116)}$=212.5, P < 0.001). QYDA did not show significant difference between 24 and 72 h, however, QYLA did show a further significant increase in the cases of DipC and TN (P<0.01).

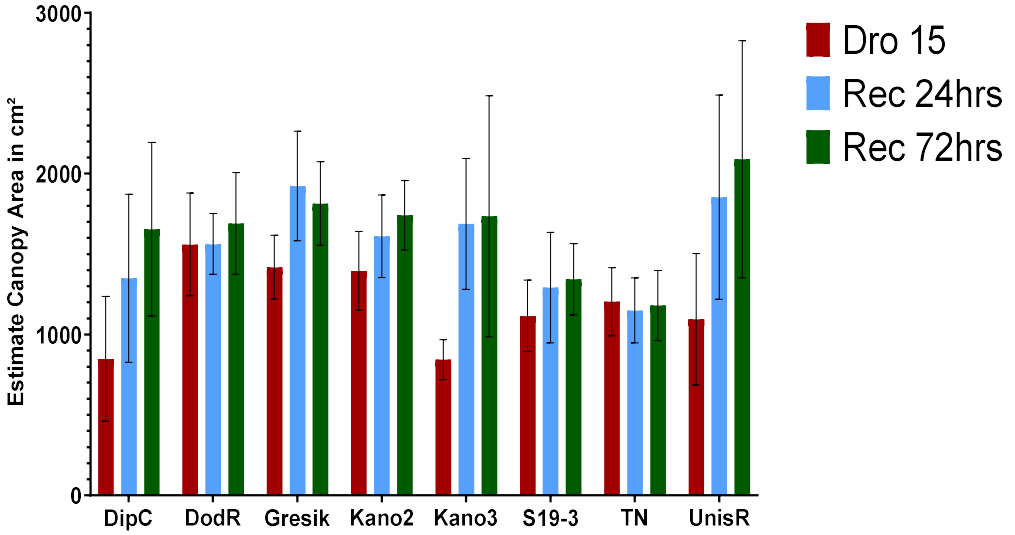

Figure 3-12 Effect of recovery in *estimate canopy area* in drought treated Bambara groundnut, where there was a significant increase at 24 h after field capacity was re-stablished at 75% ($F_{(2,105)}$=18.26, P < 0.001).

Overall possible meaning

The physiological response observed over the different years, might suggest the division and/or combination of drought tolerant and drought avoidant strategies. Clear examples were S19-3, TN, and DodR, where their physiology was affected by drought, yet not at such a significant rate in comparison to the other genotypes. Looking at the places of origin from table 5, these genotypes come from climates with less than 570 mm of rainfall per year, in dry environments. Their recovery rate was also minimal, possibly meaning that in a more in-depth way, they would be in a slow state, making the best out of the remaining resources until achieving the next generation.

Meanwhile, in the case of UnisR, Kano2 and Kano3, and Gresik, in particular Gresik, are genotypes with big canopies which had a very significant rate of senescence. This could correspond to the avoidant strategy of not reducing the organs that are using water until the next rain falls. This could be in a way confirmed due to their high recovery rate at 24 h after water was re-applied.

There could be other strategies involved that yet need further understanding, such as the different mineral composition in the leaves, and how these may (or may not) have an effect in drought resistance, as little is known about these interactions.

Conclusion

Based on the results reported in this chapter, it appears UnisR, DipC, Gresik, Kano2, and Kano3 could be considered more as drought avoidant genotypes based on their greater reduction of canopy size, high relative water content in leaves, overall higher reduction of the efficiency of the PSII, and their higher rate of recovery after water was supplied. Meanwhile, DodR, TN, and S19-3, could be considered more as drought tolerant, as they cope better with the severe drought without reducing their canopy size as much, keeping a higher LRWC and a slightly reduced overall efficiency of the PSII. Additionally, their recovery rate was not significantly changed after water was supplied at the end of the drought period.

Acknowledgments

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References

Anyia, A., & Herzog, H. (2004). Water-use efficiency, leaf area and leaf gas exchange of cowpeas under mid-season drought. *European Journal of Agronomy - EUR J AGRON*, *20*, 327–339. https://doi.org/10.1016/S1161-0301(03)00038-8

Blum, A. (2011). *Plant Breeding for Water-Limited Environments*. Springer New York. https://doi.org/10.1007/978-1-4419-7491-4

Chai, H. H., Massawe, F., & Mayes, S. (2016). Effects of mild drought stress on the morpho-physiological characteristics of a Bambara groundnut segregating population. *Euphytica*, *208*(2), 225–236. https://doi.org/10.1007/s10681-015-1581-2

Collino, D., Dardanelli, J., Sereno, R., & Racca, R. (2001). Physiological responses of argentine peanut varieties to water stress. *Field Crops Research - FIELD CROP RES*, *70*, 177–184. https://doi.org/10.1016/S0378-4290(01)00137-X

Danku, J. M. C., Lahner, B., Yakubova, E., & Salt, D. E. (2013). Chapter 17: Large-scale plant ionomics. In *Plant Mineral Nutrients: Methods and Protocols* (Vol. 953, Issue July, pp. 255–277). https://doi.org/10.1007/978-1-62703-152-3

Fang, Y., & Xiong, L. (2015). General mechanisms of drought response and their application in drought resistance improvement in plants. *Cellular and Molecular Life Sciences*, *72*(4), 673–689. https://doi.org/10.1007/s00018-014-1767-0

Farooq, M., Wahid, A., Basra, N. K. D. F. S. M. a, Fujita, N. K. D., & Plant, S. M. a B. (2009). Plant drought stress : Effects , mechanisms and management, *Agronomy for Sustainable Development, Springer Verlag (Germany)*, *29*(1), 185–212. https://doi.org/10.1051/agro:2008021

Jørgensen, S. T., Liu, F., Ouédraogo, M., Ntundu, W. H., Sarrazin, J., & Christiansen, J. L. (2010). Drought responses of two Bambara groundnut (Vigna subterranea L. Verdc.) landraces collected from a dry and a humid area of Africa. *Journal of Agronomy and Crop Science*, *196*(6), 412–422. https://doi.org/10.1111/j.1439-037X.2010.00435.x

Mabhaudhi, T., Chibarabada, T. P., Chimonyo, V. G. P., & Modi, A. T. (2018). Modelling climate change impact: A case of Bambara groundnut (Vigna subterranea). *Physics and Chemistry of the Earth*, *105*(December 2017), 25–31. https://doi.org/10.1016/j.pce.2018.01.003

Mabhaudhi, T., & Modi, A. T. (2013). Growth, phenological and yield responses of a Bambara groundnut (Vigna subterranea (L.) Verdc.) landrace to imposed

water stress under field conditions. *South African Journal of Plant and Soil*, *30*(2), 69–79. https://doi.org/10.1080/02571862.2013.790492

Mbow, C., Rosenzweig, C., Barioni, L. G., G., B. T., Herrero, M., Krishnapillai, M., Liwenga, E., Pradhan, P., Rivera-Ferre, M. G., Sapkota, T., Tubiello, F. N., & Xu, Y. (2019). Chapter 5: Food Security. In *Climate Change and Land: an IPCC special report on climate change, desertification, land degradation, sustainable land management, food security, and greenhouse gas fluxes in terrestrial ecosystems*.

Mateva, K. I., Chai, H. H., Mayes, S., Massawe F (2020) Root Foraging Capacity in Bambara Groundnut (*Vigna Subterranea* (L.) Verdc.) Core Parental Lines Depends on the Root System Architecture during the Pre-Flowering Stage. *Plants*, 9(5), 645. https://doi.org/10.3390/plants9050645

Muhammad, Y. Y., Mayes, S., & Massawe, F. (2016). Effects of short-term water deficit stress on physiological characteristics of Bambara groundnut (Vigna subterranea (L.) Verdc.). *South African Journal of Plant and Soil*, *33*(1), 51–58. https://doi.org/10.1080/02571862.2015.1056847

Mwale, S. S., Azam-Ali, S. N., & Massawe, F. J. (2007). Growth and development of Bambara groundnut (Vigna subterranea) in response to soil moisture. 2. Resource capture and conversion. *European Journal of Agronomy*, *26*(4), 354–362. https://doi.org/10.1016/j.eja.2006.12.008

Nautiyal, P. C., Kulkarni, G., Singh, A. L., & Basu, M. S. (2017). Evaluation of water-deficit stress tolerance in Bambara groundnut landraces for cultivation in sub-tropical environments in India. *Indian Journal of Plant Physiology*, *22*(2), 190–196. https://doi.org/10.1007/s40502-017-0296-x

Singh, P. (1991). Influence of water-deficits on phenology, growth and dry-matter allocation in chickpea (Cicer arietinum). *Field Crops Research*, *28*(1–2), 1–15. https://doi.org/10.1016/0378-4290(91)90070-C

Xu, Z., Zhou, G., & Shimizu, H. (2010). Plant responses to drought and rewatering. *Plant Signaling & Behavior*, *5*(6), 649–654. https://doi.org/10.4161/psb.5.6.11398

Chapter 4: GWAS analysis reveals drought resistance related genes in Bambara groundnut (*Vigna subterranea* (L.) Verdc.).

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Abstract

Bambara groundnut (*Vigna subterranea* (L) Verdc) is an underutilised African legume with a known drought resistance. As an underutilised crop, not much genetic research has been reported in the literature in comparison to several major crops such as wheat, maize, rice, and soybean. When the generation of controlled crosses is challenging, Genome Wide Association Studies (GWAS) could be an alternative to QTL analysis, allowing molecular mechanisms regulating traits of interest to be unravelled. To the best knowledge of the author, this is the first GWAS published in Bambara groundnut.

In the present study, an association genetic panel comprising 229 single plant purified accessions was genotyped using 9804 single nucleotide polymorphism markers and the level of heterozygosity was found to be ranging from 0.8 to 5.0%, in consistent with caliginous nature of Bambara groundnut.

A sub-set of 137 genotypes were assessed for water deficit response using controlled-environment tropical glasshouse. Drought stress has reduced significantly *leaf relative water content* ($P < 0.001$), *estimated canopy area* ($P<0.001$), and *estimated chlorophyll content* ($P<0.001$). Genotypic difference has also contributed to the variations in *leaf relative water content* ($P < 0.001$), *estimated canopy area* ($P<0.001$), and *estimated chlorophyll content* ($P<0.001$) in response to terminal drought.

GWAS analysis has identified eleven significant marker-trait associations with these three traits, as a result of limited water availability. These markers are linked with genes involved in osmo-protection, stomatal

conductance, cuticular wax formation, and drought memory priming which have been previously reported to be associated with drought stress.

This, thus, has demonstrated that developing an association genetics panel has formed a valuable source for marker-trait association studies in order to dissect genes affecting traits of interest which could be applied in Bambara groundnut breeding improvement programmes.

Introduction

Bambara groundnut (*Vigna subterranea* (L) Verdc) is an underutilised African pulse known for its drought resistance and relatively high protein content. As an underutilised crop, there are not many molecular genetic resources available in comparison to some major crops such as wheat, maize, rice, and soybean. However, its natural adaptation to harsh conditions, such as drought, has been receiving more attention over the decades, making this species a potential candidate to be researched and developed further into future varieties which could be cultivated in places where more conventional staples cannot, or where they perform very poorly (Mayes *et al*., 2019).

According to a number of authors (Blum, 2011; Farooq *et al*., 2009; Xu *et al*., 2010), there are three main responses to drought in pursuit of survival to the next generation. One of these mechanisms is **drought escape**, where plants may shorten their life cycle, trying to make the best use of the water resources available in short periods (such as a defined rain seasons), to allow them to produce the next generation before the environment becomes harmfully dry. **Drought avoidance** is when plants perform changes which results in reducing use of the immediate available water while seeking for more available water, to avoid reaching a point of damaging low moisture levels in the soil before the next water becomes available (rain, or irrigation). This could be achieved for example, by reducing stomatal conductance, restricting leaf area expansion, increasing root depth and density, among other traits. The last mechanism is **drought tolerance**, which the ability of the plant to regulate thousands of genes and a series of metabolic pathways to reduce or repair damage cause by drought, and thus sustaining a certain level of physiological activity under severe drought conditions (Fang and Xiong, 2015).

In order to improve landraces into varieties with agriculturally desirable traits, more genetic research is necessary aiming to decrease the time required for conventional breeding programs through the application of genetic markers. Genome Wide Association Study (GWAS) is an alternative and complementary approach to QTL analysis using controlled crosses, especially when there is difficulty generating genetic crosses. However, GWAS could be a difficult approach in the case of underutilised crops, as it requires significant genomic resources. DArTseq can be an efficient, cheap, easy, and reliable platform for genotyping-by-sequencing (GbS), which

allows genome-wide marker discovery, generating two types of data: codominant Single Nucleotide Polymorphism (SNP) markers and dominant SilicoDArT markers (Edet *et al*., 2018). SilicoDarT markers are the presence/absence variation of tag sequences, while SNP is the nucleotide polymorphism found in tag sequences (Edet *et al*., 2018).

In this study we combined phenotypic trait data, under drought and irrigated conditions, with DArTseq data to perform a GWAS to pursue further understanding of some of the mechanisms that might be involved in the drought resistance behaviour of Bambara groundnut.

Materials and methods

Plant material, experimental design, drought treatment

A joint effort by IITA, Crops for the Future Research Centre, and University of Nottingham has generated an initial Association Genetics Panel (AGP) for Bambara groundnut after a round of single plant descent purification from a subset of genebank accessions (n=229) from different places of origin, with GBS data suggesting a good genetic representation of the species (Mayes *et al.*, unpublished data). The association genetics panel was generated from single seed decent and was test assessed in the FutureCrop glasshouses (52°50’02”N, 1°15’00”W) of the Sutton Bonington Campus of the University of Nottingham in the summer of 2019. Growing conditions were 23°C at night and 28°C during the day, respectively, in 12 h photoperiods controlled by automatic blackouts, with individual plants grown in 5 L tall pots in a fully randomised block design. Irrigation was supplied by a dripper system to individual pots with two irrigation periods per day at 0600 and 1800 h to maintain a uniform field capacity of 75%. Irrigation was manually controlled to raise the pots to 75% field capacity at the beginning of the experiment and prior to beginning the drought treatment for 15 days by removing the irrigation drippers for the drought treatment samples. This was performed as soon as 50% of the biological replicates had reached first flower. After 15 days with no irrigation in the treatment plants, the field capacity was raised manually to 75% and the irrigation drippers were re-introduced to the pots.

From the 229 genotypes of the AGP, an initial set of 165 genotypes from different places of origin were selected, from which 137 were assessed in

topsoil with a sandy-loam texture, with four replicates per treatment per genotype. The experimental work was carried out in a randomised block design, with each block having one replicate of each treatment and each genotype, with a total of 12 blocks across 2 glasshouses. The main physiological traits measured were leaf relative water content, estimated canopy size, and estimated chlorophyll content.

Field capacity is the maximum amount of water that can be retained in a given volume of soil. This value changes depending on the structure and composition of the soil. All pots were filled to the same weight during the experiment set up, including an additional set of 4 pots. To determine the field capacity of the soil used, four random pots were irrigated until over-saturation was achieved. The pots were left until there were no signs of water drainage by gravity (12 hours) and subsequently weighed. To determine the soil mass in the absence of water, the soil was thereafter dried for 72 h at 80°C and a weight measurement was taken. The field capacity was calculated as follow:

$$\theta m = \frac{\theta w - \theta d}{\theta d} \times 100\%$$

where: θm = gravimetric field water capacity,

θw = soil mass at 100% field capacity, and

θd = soil mass in absence of water.

Leaf relative water content (LRWC)

Meanwhile, for relative water content in leaves, a total of three leaf discs of 0.78 cm² were punched out using a biopsy punch from a single leaflet from three random selected and fully expanded leaflets, giving a total of three discs per biological replicate, at 15 days into drought. These were weighed to determine fresh weight (Fw), and soon after, the leaf discs were incubated floating on deionised water and in presence of light for 24 h and weighed again for a turgid weight (Tw). The discs were immediately transferred to an oven at 80°C for 48 h and then weighed for dry weight (Dw). The formula below was used to determine the *LRWC*:

$$RWC = \frac{Fw - Dw}{Tw - Dw} x\ 100\%$$

Table 9 Countries of origin of the different genotypes researched. BEN=Benin, BFO=Burkina Faso, BWA=Botswana, CAR=Central African Republic, CdI=Côte d'Ivoire, CMN=Cameroon, EWI=Eswatini, IND=Indonesia, MLW=Malawi, NGA=Nigeria, SLE=Sierra Leone, TZN=Tanzania, ZBE=Zimbabwe, ZMA=Zambia, U=Unknown.

Exp ID.	Genotype	Country	Exp ID.	Genotype	Country	Exp ID.	Genotype	Country	Exp ID.	Genotype	Country
1	TVSu-7	NGA	42	TVSu-491	CMN	84	TVSu-759	ZMA	125	TVSu-1177	BFO
2	TVSu-9	NGA	43	TVSu-492	CMN	85	TVSu-760	ZMA	126	TVSu-1188	BFO
3	TVSu-11	U	44	TVSu-506	CMN	86	TVSu-764	ZMA	127	TVSu-1190	U
4	TVSu-22	NGA	45	TVSu-508	CMN	87	TVSu-765	ZMA	128	TVSu-1191	BFO
5	TVSu-115	CdI	46	TVSu-509	CMN	88	TVSu-770	ZMA	129	TVSu-1221	NGA
6	TVSu-116	CdI	47	TVSu-510	CMN	89	TVSu-774	ZMA	130	TVSu-1231	NGA
7	TVSu-182	NGA	48	TVSu-521	CMN	90	TVSu-782	U	131	TVSu-1243	U
8	TVSu-188	BEN	49	TVSu-534	CMN	91	Getso	NGA	132	TVSu-1244	NGA
9	TVSu-194	BEN	50	TVSu-535	CMN	92	TVSu-873	ZMA	133	TVSu-1251	NGA
10	Kano 3	NGA	51	TVSu-547	CMN	93	TVSu-892	ZMA	134	TVSu-1258	NGA
11	TVSu-266	NGA	52	TVSu-573	U	94	TVSu-896	U	135	TVSu-1260	NGA
12	TVSu-283	NGA	53	TVSu-593	NGA	95	TVSu-915	U	136	TVSu-1276	U
13	TVSu-286	NGA	54	TVSu-595	NGA	96	TVSu-920	ZMA	137	TVSu-1277	U
14	TVSu-288	BEN	55	TVSu-600	NGA	97	TVSu-921	ZMA	138	TVSu-1280	CAR
15	TVSu-308	BFO	56	TVSu-648	NGA	98	TVSu-922	ZMA	139	TVSu-1285	CAR
16	TVSu-312	BFO	57	TVSu-658	NGA	99	TVSu-928	U	140	TVSu-1289	CAR
17	TVSu-315	BFO	58	TVSu-673	NGA	100	TVSu-932	UU	141	TVSu-1290	CAR
18	TVSu-326	NGA	59	TVSu-677	ZMA	101	DodR	TZN	142	TVSu-1296	U
19	TVSu-328	NGA	60	TVSu-681	ZMA	102	TVSu-941	ZMA	143	TVSu-1309	CAR
20	TVSu-338	NGA	61	TVSu-682	ZMA	103	UniswaG	EWI	144	TVSu-1373	CAR

21	TVSu-352	NGA	62	TVSu-683	ZMA	104	TVSu-978	U	145	TVSu-1727	ZMA
22	TVSu-353	NGA	63	TVSu-686	ZMA	105	TVSu-1014	ZBE	146	TVSu-1822	CMN
23	IITA	TZN	64	TVSu-687	ZMA	106	TVSu-1015	ZBE	147	TVSu-1824	CMN
24	TVSu-362	NGA	65	TVSu-688	ZMA	107	TVSu-1018	ZBE	148	TVSu-1827	U
25	TVSu-397	CMN	66	TVSu-689	ZMA	108	TVSu-1022	ZBE	149	TVSu-1860	U
26	TVSu-447	CMN	67	TVSu-690	ZMA	109	TVSu-1023	ZBE	150	TVSu-1870	U
27	TVSu-460	CMN	68	TVSu-691	ZMA	110	TVSu-864	ZMA	151	TVSu-1872	ZBE
28	TVSu-461	CMN	69	TVSu-692	ZMA	111	TVSu-1051	ZBE	152	TVSu-1881	ZBE
29	TVSu-462	CMN	70	TVSu-699	ZMA	112	TVSu-1056	U	153	TVSu-1991	ZBE
30	TVSu-464	CMN	71	TVSu-709	ZMA	113	TVSu-1066	U	154	TVSu-2017	U
31	TVSu-466	CMN	72	TVSu-716	ZMA	114	TVSu-1078	ZBE	155	TVSu-2018	U
32	TVSu-467	CMN	73	LunT	SLE	115	TVSu-1081	ZBE	156	TVSu-4631	U
33	TVSu-470	U	75	TVSu-731	ZMA	116	TVSu-1085	ZBE	157	TVSu-434	CMN
34	TVSu-474	CMN	76	TVSu-736	ZMA	117	TVSu-1092	BEN	158	TVSu-1027	U
35	TVSu-479	CMN	77	TVSu-742	U	118	TVSu-1099	ZBE	159	DIPC	BWA
36	TVSu-481	U	78	TVSu-750	ZMA	119	TVSu-1102	U	160	GETSO	NGA
37	TVSu-482	CMN	79	TVSu-751	ZMA	120	TVSu-1110	ZBE	161	Gresik	IND
38	TVSu-484	CMN	80	TVSu-754	ZMA	121	TVSu-1126	ZBE	162	IITA	TZN
39	TVSu-486	CMN	81	TVSu-755	ZMA	122	TVSu-1130	ZBE	163	LUNT	SLE
40	TVSu-487	CMN	82	TVSu-757	ZMA	123	TVSu-1162	BFO	164	TN	MLW
41	TVSu-488	CMN	83	TVSu-758	ZMA	124	TVSu-1175	U	165	UNISR	EWI

Estimating Canopy Area

An estimate of canopy area (*ECA*) was determined by analysing photographs taken from the top of the canopy using a Canon D7000 camera fixed to a metal frame, with a white background and white LED. A reference ruler was included in each photograph. The photographs were taken after 15 days drought treatment and were processed using Fiji software to measure the canopy area. An image analysis code was specifically written for this experiment and is shared in appendix 1.

For an estimate of whole-canopy chlorophyll content (*ECC*), photographs in the second and third years were processed using Fiji software by calculating the red, green, and blue (RGB) pixel values of all the leaves present in the pictures. These RGB values were then analysed using the formula:

$$RGBtoCh = G - \frac{R}{2} - \frac{B}{2}$$

reported by Ali *et al*. (2013). To transform these values into a chlorophyll indicator, individual leaves were photographed and simultaneously measured using SPAD, and the reading was plotted against the RGB calculated value. This gave the following best-fit equation to apply to these values:

$$ECC = (-21.28) * (LN(RGBtoCh)) + 123.28$$

Where: ECC = Estimated Chlorophyll content

RGBtoCh = value calculated based on the RGB values of the leaf pixels as described above

To allow a better understanding of early sensing of drought by Bambara groundnut and to assess changes in recovery period, three time points were taken in the third year for the measurements of (*QYDA*), (*QYLA*), *ECA*, *ECC*

at day 7, 11, and 15 during the drought treatment, and two time points at recovery, at 1 day after irrigation was re-introduced, and at day 3.

Isolation of genomic DNA

One leaflet per genotype was sampled in aluminium foil and frozen immediately in the presence of liquid nitrogen. Subsequently, frozen leaves were transported to the laboratory. Leaf tissues were ground to a fine powder using mortar and pestle in the presence of liquid nitrogen and up to 100mg were collected in 1.5 ml Eppendorf tubes and place in liquid nitrogen until the DNA isolation protocol took place. DNA isolation was achieved using the Qiagen DNeasy Plant mini kit (Germany) and according to the manufacturer's instruction. A total of 400 µl of buffer AP1 was added to the tubes followed by 4 µl of RNAse A and the sample vortexed rigorously until the tissue was dispersed with no clumps. Samples were incubated at 65°C for 10 minutes in a hot plate (TECHNE, U.K.), during this period tubes were inverted 2-3 times. Next, 130 µl of AP3 Buffer was added, mixed, and transferred to ice for 5 minutes. The lysate was centrifuged (Thermo Fisher Scientific, USA) at 14,000 rpm for 5 minutes then pipetted into a QIAshredder spin column placed in a 2 ml collection tube and centrifuged once more for 2 more minutes.

The flow-through was transferred to a new 1.5 ml tube and 1 volume of Buffer AW1 added and mixed by pipetting. 650 µl of this mixture was then transferred into a DNeasy Mini spin column placed in a 2 ml collection tube, centrifuged at 8,000 rpm for 1 minute and the flow-through was discarded. This process was repeated with the rest of the mixture. The spin column was placed in a new 2 ml collection tube, then 500 µl of AW2 Buffer was added to the spin column and centrifuged at 8,000 rpm for 1 minute. The flow-through was discard and another 500 µl of AW2 Buffer was added and centrifuged at 14,000 rpm for 2 minutes.

The spin column was carefully removed from the collection tube, making sure that the flow-through didn't contact the column, and placed in a 1.5 ml labelled tube for final DNA storage. 50 µl of Buffer AE was added to the spin column for elution, incubated at room temperature for 5 minutes and then centrifuged at 8,000 rpm. The same process was repeated in a new 1.5 ml tube, in this case substituting the Buffer AE for 30 µl of pre-warmed (65°C) sterile distilled water (SDW) for the second elution.

Quantity and quality control, DArT sequence

To check quantity and quality of all DNA samples, 5 uL were ran for 2 hours at 80 volts in a 1% agarose gels in presence of ethidium bromide alongside a reference made form a known concentration of lambda DNA (Thermo Fisher) for visual assessment of the quality and quantity. Additionally, the purity of extracted DNA was evaluated by taking 1ul of gDNA for in an enzymatic restriction assessment using 10 U (unit) of *Eco*RI (New England Biolab) for 2 hours 37 °C, and subsequently ran on a 1% agarose gels in presence of ethidium bromide for visual assessment. Lastly, samples were also analysed using the Nanodrop ND-1000. Samples with good quality and quantity gDNA were diluted to a final concentration of 50 to 100 ng/ul in a final volume of 15 ul and sent to Diversity Arrays Technology Pty Ltd (http://www.diversityarrays.com/) for sequencing and marker identification by genotyping-by-sequencing (HiSeq 2500, Illumina Inc.) (Alam *et al*., 2018). SNPs were mapped to the in-house unannotated S19 reference genome having 23 super-scaffolds. Significant associations between SNP markers and the phenotypic trait data were identified using the Tassel 5 software v5.x.y with the Generalised Linear Model (GLM). The genetic data were filtered with a minimum allele frequency of 0.01, numeralized, imputed, analysed by a relatedness principal component under default settings, and the GLM association analysis. From the list of SNPs, only those with a $-Log^{10}$(P-Value)>=3.7 were retained. From the marker positions, the ORCAE platform was use (https://bioinformatics.psb.ugent.be/orcae/aocc/overview/Vigsu) to find which Bambara groundnut genes were closer/targeted by the marker of importance. From the list of Bambara genes tagged by these markers of interest (by referring to BG genome assembly, Chang *et al*., 2018), were checked for their orthologues in *Arabidopsis thaliana*, *Vigna unguiculata*,

Glycine max, and *Phaseolus vulgaris.* Only the genes that had been reported to have a role related to drought stress in Arabidopsis were retained. Due to lack of full-size genome data and marker density, and time, only the preliminary results are presented below.

Statistical analysis was performed using Genstat 20th edition (VSN International) and GraphPad Prism 9. Depending on the year and data set, one-way or two-way Analyses Of Variance (ANOVA) were carried out.

Results and Discussion

Physiological traits

Plant establishment and flowering time

There was a significant difference in flowering time between genotypes ($p<0.001$), where 134 and 2 were the earliest genotypes to reach anthesis at 54±1.5 and 55±1.1 days after sowing, meanwhile 142 was the latest at 65±0.6 days after sowing.

Leaf relative water content

For the *LRWC* (Figure 4-1 to Figure 4-3), there was a significant decrease in response to the drought treatment ($F_{(1,685)}$=2085, $P < 0.001$), with genotypes significantly different ($F_{(137,685)}$=7.124, $P < 0.001$), and interaction between treatment and genotype ($F_{(137,685)}$=7.499, $P < 0.001$). TVSu-326, TVSu-758, TVSu-352, and TVSu-573 genotypes showed the lowest values in the drought treatment samples at 21.4±4.2%, 26.6±2.3%, 26.9±4.6%, and 27.5±7.8% respectively. Meanwhile TVSu-487, TVSu-648, TVSu-892, TVSu-116, TVSu-482, and TVSu-462 where the highest values in the drought treatment with values of 81.3±5.0%, 82.1±4.0%, 82.7±1.7%, 82.9±2.8%, 83.3±2.6%, and 86.4±1.5%, respectively. In the case of the irrigated controls, the majority of values were between 68.4% and 94%, with a few genotypes had values outside this range. Similarly, on the low side were TVSu-692, TVSu-688, TVSu-687, Getso, LunT, TVSu-686, TN, TVSu-690, with values of 58.9±7.5, 60.5±7.5, 64.4±3.9, 64.8±2.2, 65.8±3.5, 66.9±3.2, 67.6±3.8, 67.9±6.3 respectively; and the high side with TVSu-1373 (90.7±1.7), TVSu-920 (91.9±4.3), and TVSu-352 (95.9±1.9).

An overall reduction of *LRWC* in response to drought has been reported in Bambara groundnut previously by Jørgensen *et al*., (2010), Muhammad *et al*., (2016), Nautiyal *et al*., (2017) and Chapter 3. Interestingly, a few of the genotypes that showed the highest values of *LRWC* during the drought treatment, showed no significant difference to their irrigated counterparts, with the top three genotypes being TVSu-116, TVSu-482, and TVSu-462

with values average values of 82.4±0.5, 84.2±0.8, and 85.4±0.9 respectively.

Trait correlations

There was a significantly positive correlation between *LRWC* and *Estimated Chlorophyll Content* under drought conditions ($r^2_{(672)}$= 0.51, *P*<0.001), and a significantly negative correlation between *LRWC* and *Estimated Canopy Area* under irrigated conditions ($r^2_{(672)}$= -0.16, *P*=0.004).

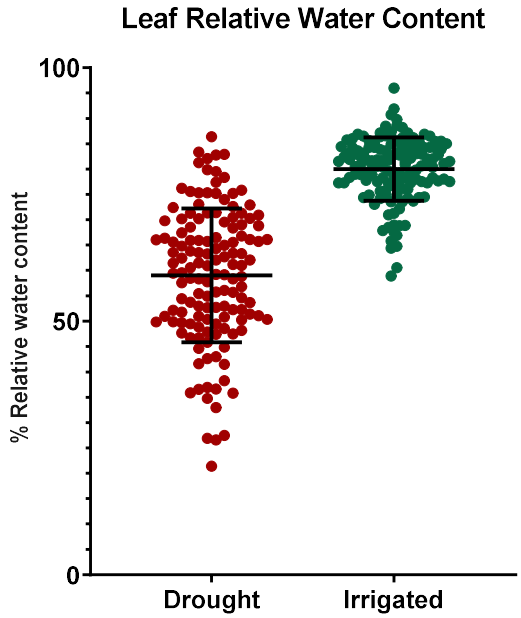

Figure 4-1 Effect of drought in *LRWC*. Drought treated plants showed a significant decrease ($F_{(1,685)}$=2085, *P* < 0.001).

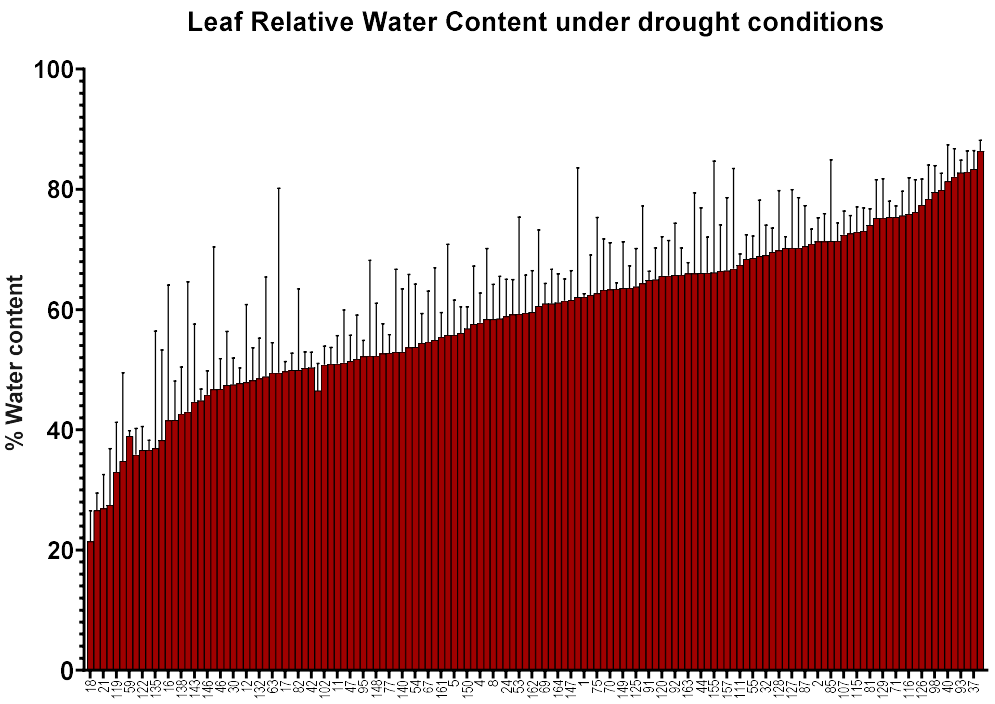

Figure 4-2 *LRWC* under drought conditions. Genotypes responded significantly different ($F_{(137,685)}$=7.499, P < 0.001).

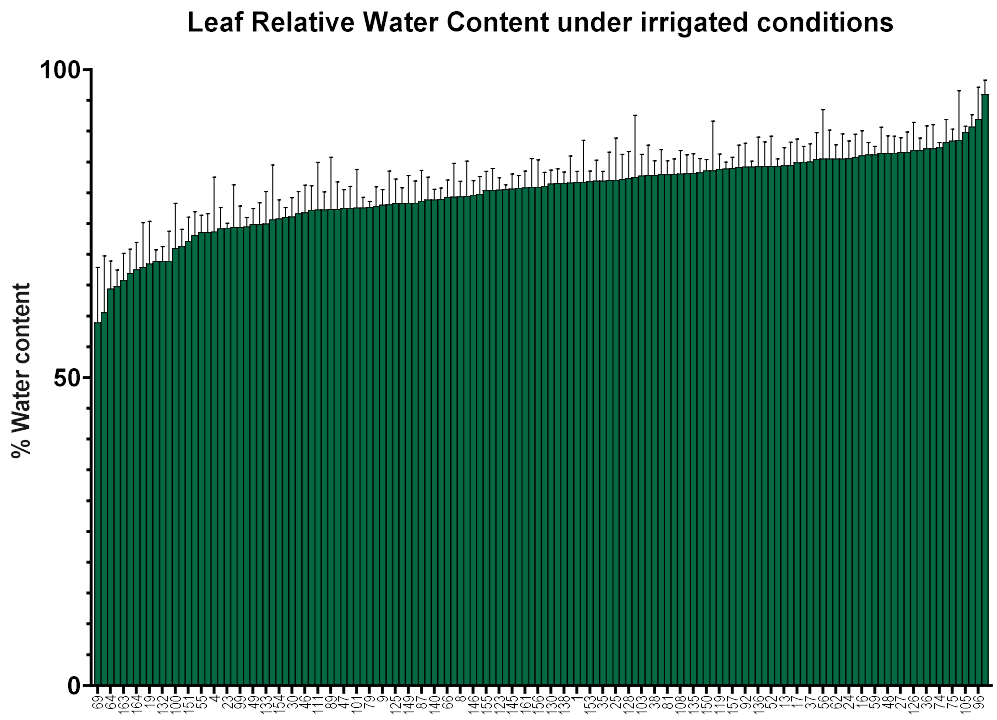

Figure 4-3 *LRWC* under irrigated conditions. The irrigated controls behaved differently under irrigation (P<0.05).

Estimated canopy area

For the *estimated canopy area* (Figure 4-4 to Figure 4-7), there was a significant decrease in response to the drought treatment ($F_{(1,478)}$=347.2, P<0.001), with the genotypes showing clearly significantly differences in ($F_{(96,478)}$=12.8, P<0.001), and interaction between treatment and genotype ($F_{(96,478)}$=4.5, P<0.001). Under drought conditions the majority of genotypes had an average estimated canopy size between 551 cm² and 1523 cm², with TVSu-1260, TVSu-1014, TVSu-2017, and TVSu-467 having the lowest values at 145±48 cm², 284±67 cm², 369±259 cm², 450±132 cm², respectively. Additionally, TVSu-1296, TVSu-1015, TVSu-328, TVSu-699, TVSu-22, and TVSu-686 had the greatest estimated canopy at 1578±97 cm², 1600±147 cm², 1612±127 cm², 1712±209 cm², 1725±141 cm², and 1836±364 cm², respectively. Meanwhile, the irrigated controls averaged between 715 cm² and 2326 cm², having TVSu-1188, TN, Getso, TVSu-1014, TVSu-487, and TVSu-1190 with the smallest canopy at 366±147 cm²,

456±175 cm², 458±175 cm², 458±81 cm², 506±28 cm², 523±107 cm², and 591±82 cm² respectively, while TVSu-686 and TVSu-1102 had the greatest estimated canopy at 2454±116 cm² and 2512±243 cm² respectively.

To have a better understanding of the intrinsic effects of canopy size in response to drought stress, the normalisation of the irrigated canopy vs the drought effect canopy showed a significant difference between the genotype behaviour in terms of *estimated canopy reduction*, or increase, due to drought stress ($F_{(86,194)}$=6, P<0.001; Figure 4-7). TVSu-864 and TVSu-1188 showed an estimated canopy size increase (645 cm² and 497 cm² respectively) in response to drought, while TVSu-326 and TVSu-352 had the highest reduction in estimated canopy size (1566 cm² and 1595 cm² respectively) due to the drought stress.

There was a significantly negative correlation between *estimated canopy area* and *estimated chlorophyll content* in drought conditions (-0.23, P<0.001), irrigated conditions (-0.43, P<0.001), and when comparing both treatments (irrigated vs drought; -0.24, P<0.001).

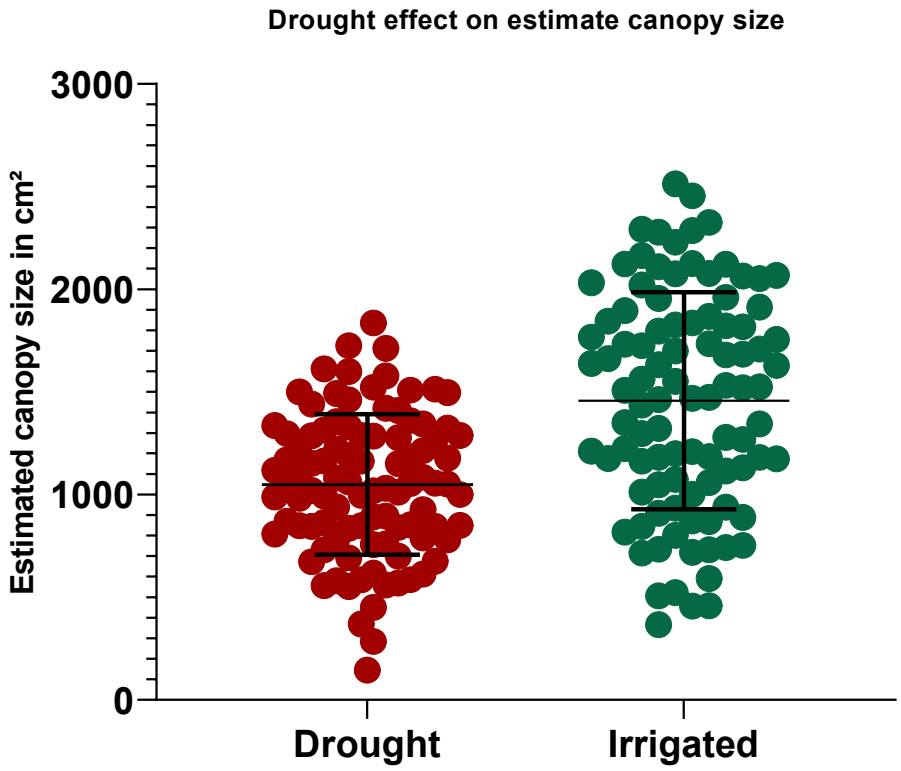

Figure 4-4 Effect of drought in *estimated canopy area*. Drought treated plants showed an overall significant decrease in response to drought ($F_{(1,478)}$=347.2, P<0.001).

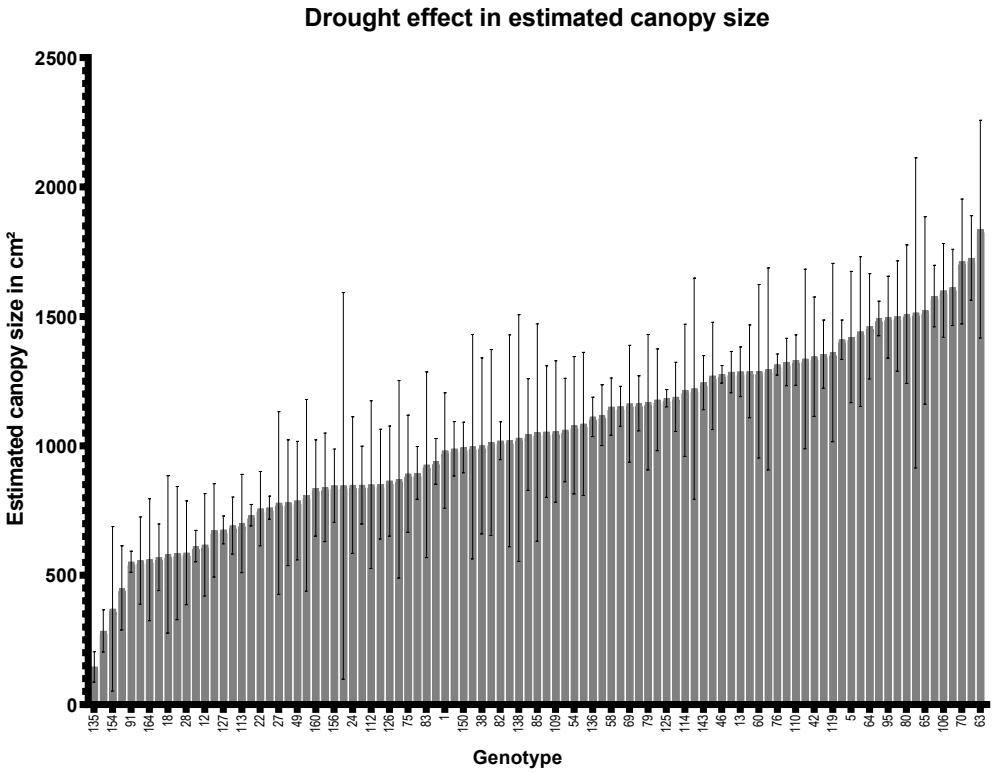

Figure 4-5 *Estimated canopy area* of Bambara groundnut under drought conditions.

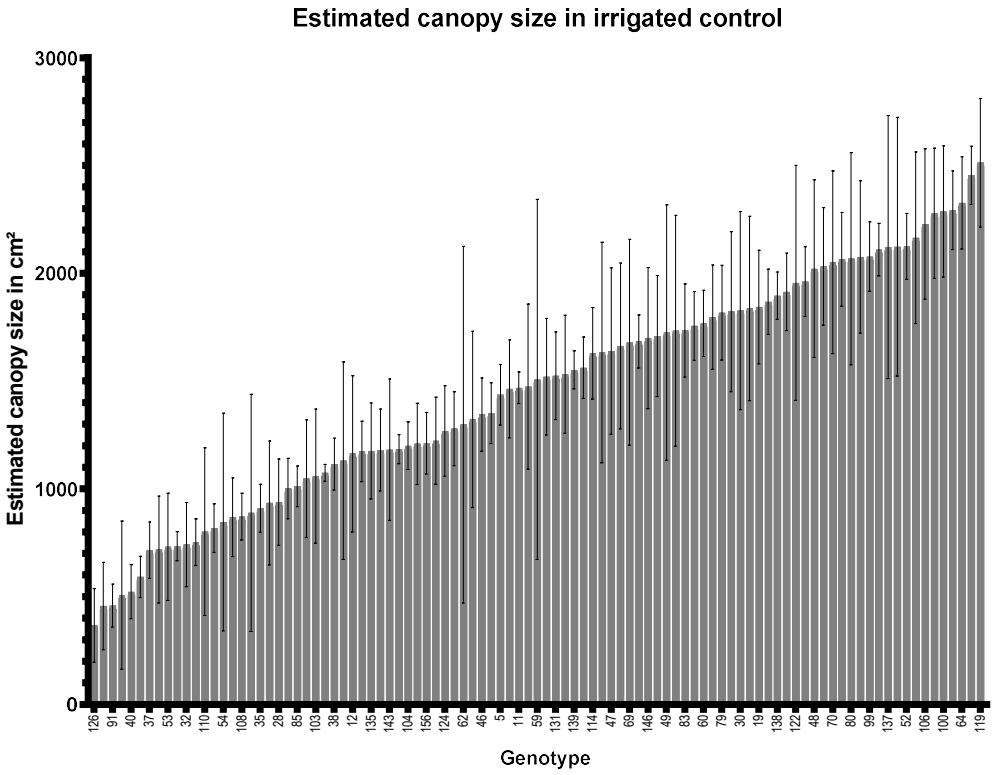

Figure 4-6 *Estimated canopy area* of Bambara groundnut under irrigated conditions.

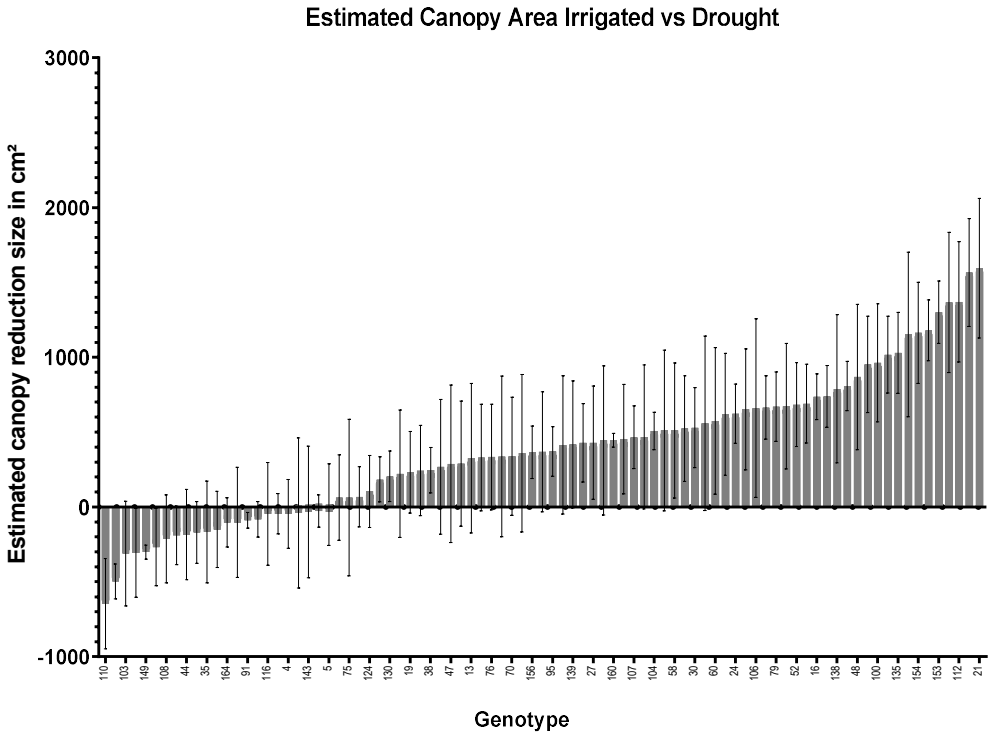

Figure 4-7 *Estimated canopy area* reduction in different Bambara groundnut genotypes.

Estimated Chlorophyll Content

For the *estimated chlorophyll conten*t (Figure 4-8 to

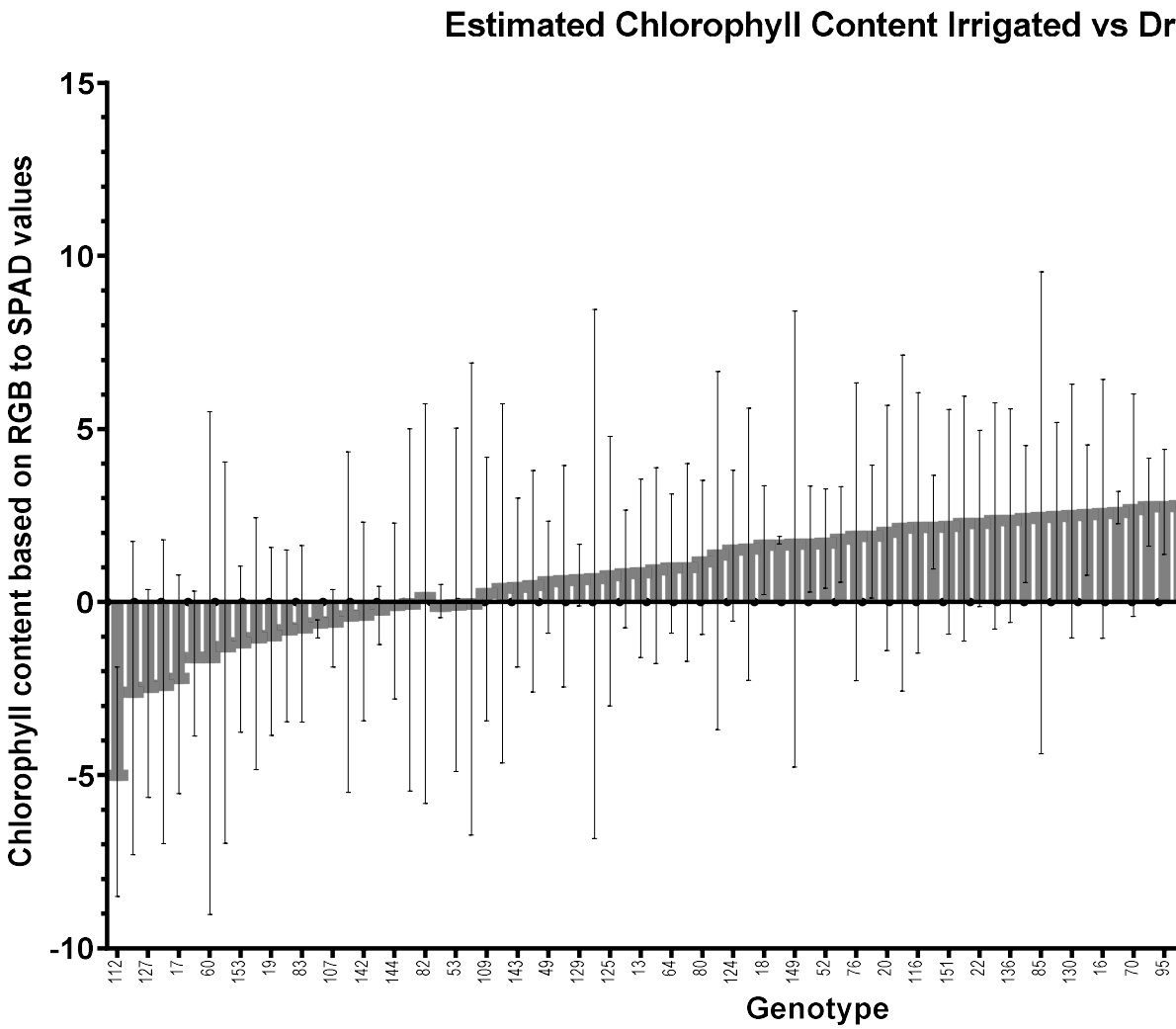

Figure 4-11), there was a significant decrease in response to drought treatment ($F_{(1,466)}$=94.4, P<0.001), with the genotypes showing clearly significantly differences in ($F_{(96,466)}$=7.25, P<0.001), and interaction between treatment and genotype ($F_{(96,466)}$=1.63, P<0.001). Under drought conditions the majority of genotypes had an average estimated chlorophyll content value between 49 and 60, with TVSu-1280, TVSu-266, TVSu-286, TVSu-1177, and TVSu-461 with the lowest values at 45.7±1.5, 47.8±1.4, 48.0±2.2, 48.2±2.1 respectively. Additionally, Getso and TVSu-1014 showed the greatest *estimated chlorophyll content* at 60.2±2.4 and 60.3±0.3 respectively. Meanwhile, the irrigated controls averaged between 50.5 and 61, having TVSu-315, TVSu-1177, and TVSu-286, with the smallest canopy at 48.1±1.8, 49.1±1.1, and 50.1±1.5 respectively, while TVSu-1860, TVSu-760, TVSu-506, TVSu-978, and TVSu-600 had the

greatest estimated canopy at 61.3±3.2, 61.5±1.5, 61.7±3.2, 62.1±1.3 and 62.3±1.6 respectively. Same comparative between the irrigated controls vs the drought treatment was performed in the *estimated chlorophyll content* showing a significantly difference in behaviour between genotypes in response to drought ($F_{(96,218)}$=1.75, P<0.001). TVSu-1056 showed the highest increase by 5.19±2.7 in response to drought, while TVSu-460 and TVSu-461 showed the highest reduction of estimated chlorophyll content by 9.39±2.3 and 9.5±2.3 respectively.

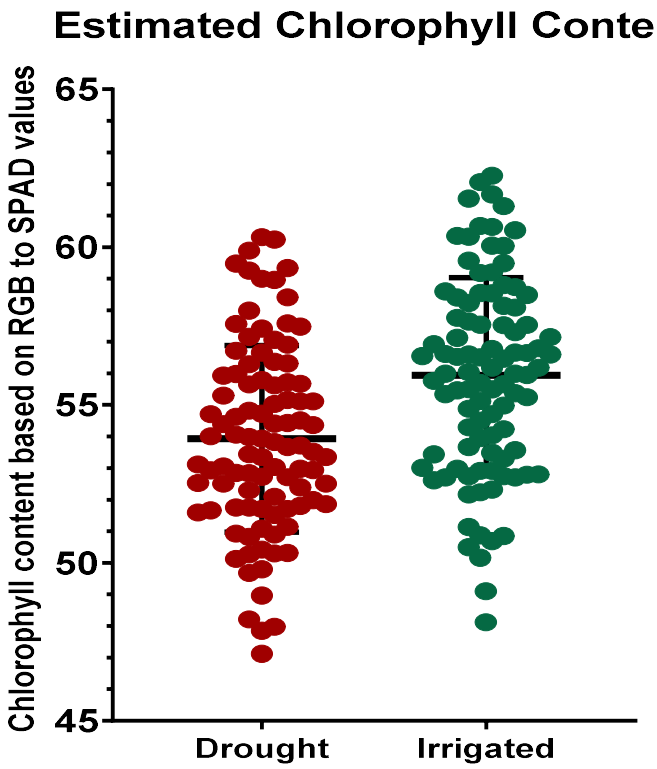

Figure 4-8 Effect of drought in *estimated chlorophyll content*. Drought treated plants showed an overall significant decrease in response to drought ($F_{(1,466)}$=94.4, P<0.001).

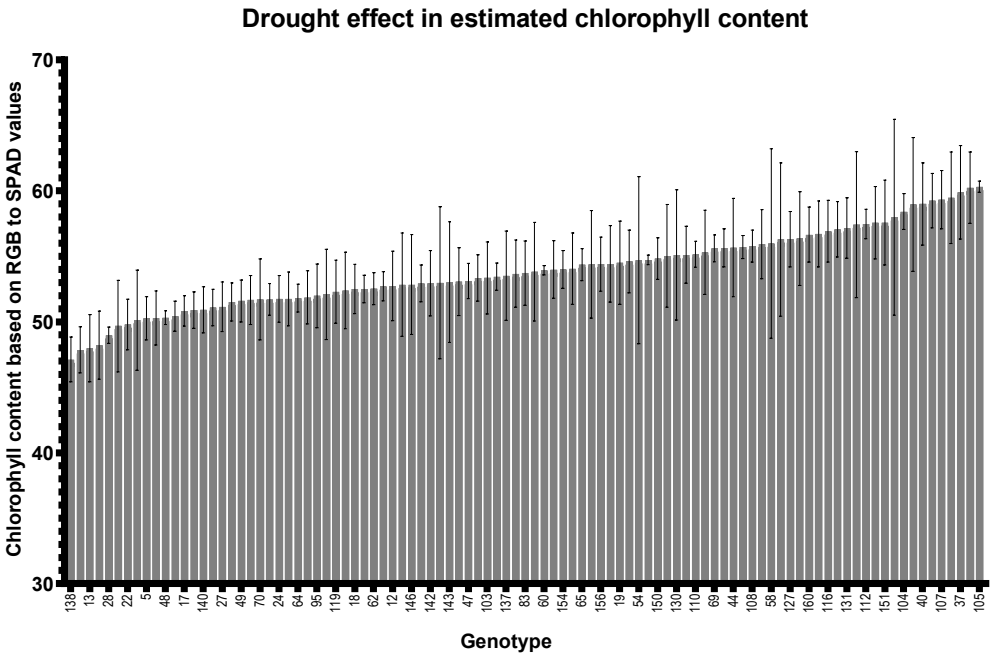

Figure 4-9 *Estimated chlorophyll content* of Bambara groundnut under drought conditions.

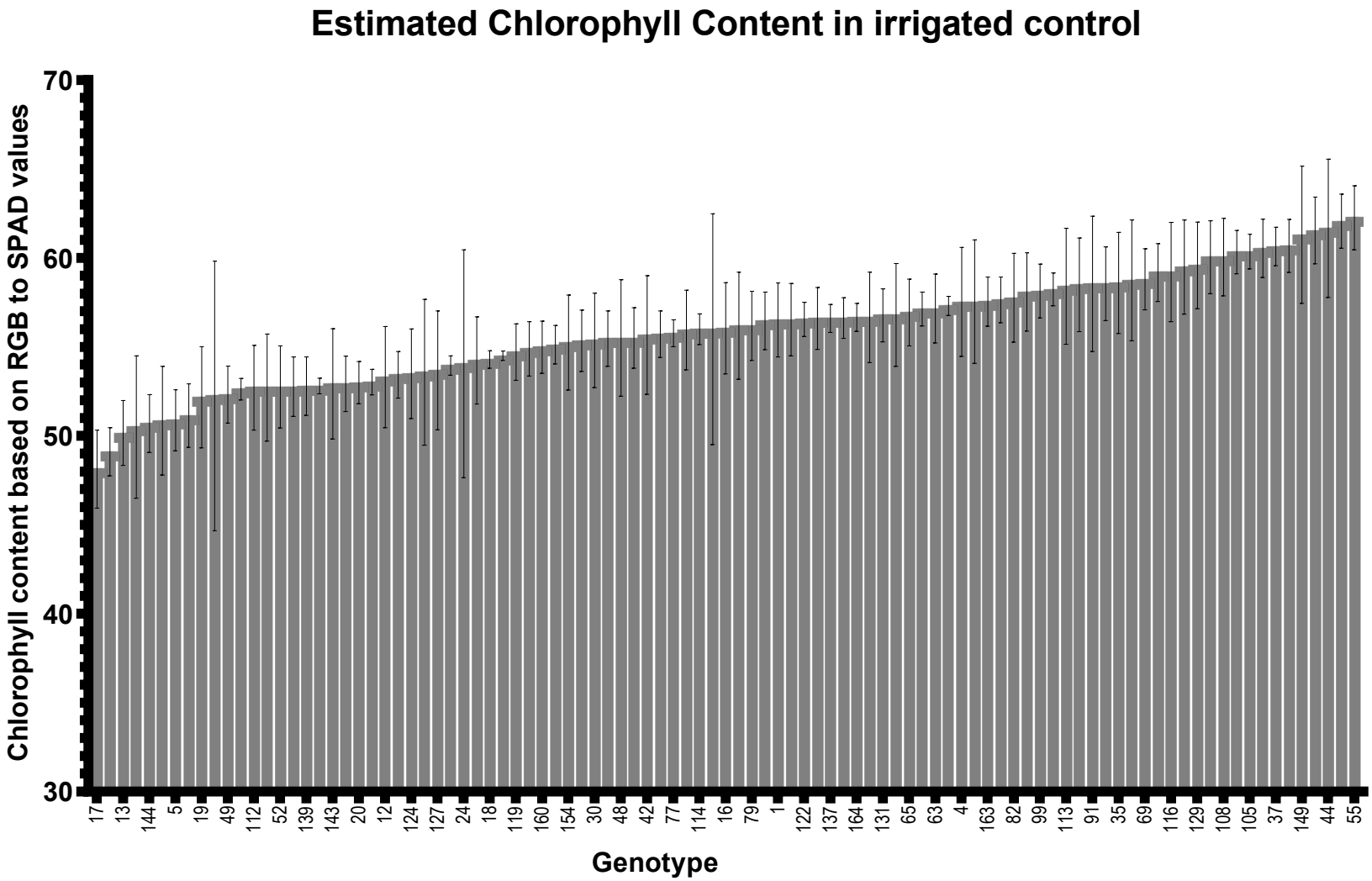

Figure 4-10 *Estimated chlorophyll content* of Bambara groundnut under irrigated conditions.

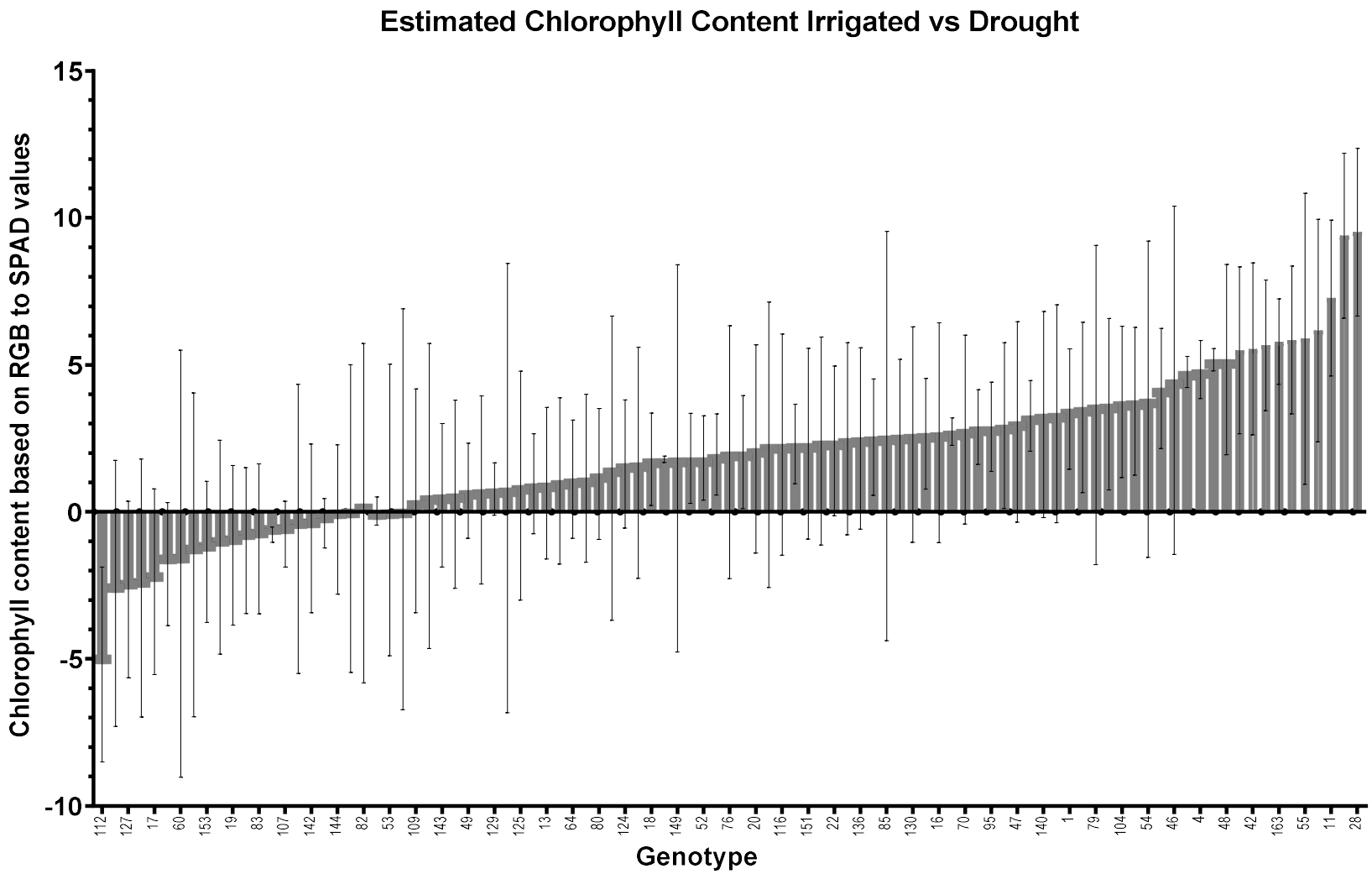

Figure 4-11 *Estimated chlorophyll content* reduction in different Bambara groundnut genotypes. The irrigated canopy vs the drought effect chlorophyll content showed a significant difference between the genotype behaviour in terms of estimated chlorophyll content reduction, or increase, due to drought stress ($F_{(96,218)}$=1.75, P<0.001).

Overall physiology

This Association Genetics Panel showed a diverse response to drought, and a general genetic diversity in the irrigated controls. Unfortunately, we do not count with the climate data for these genotypes and their places of origin, however, we can see some drought avoidant traits, and some drought tolerant traits. Within the initial 155 genotypes, DodR, S19-3, Gresik, and TN from chapter 3 and 5 were used as well, showing the same responses, which could be an indication of a possible evolutionary adaptative response to certain climates, yet climate data and replication of this experiment would be required to be closer to answer that.

In a short comparative analysis by ranking, a small set of lines behaved relative poorly in response to drought in *ECC, RWC,* and *Area diff*, some of these being lines 18, 21, and 119; meanwhile, on the opposite case were the lines 37, 40, 91, and 149.

DArTseq and GWAS

A total of 9804 SNP markers were obtained with a level of heterozygosity ranging from 0.8 to 5.0% between all 229 genotypes belonging to the AGP. This level of genetic markers would be considered low, and further re-sequencing is taking place as part of this project, and thus do a more in depth GWAS. As the current published draft genome of Bambara groundnut is highly fragmented (65,586 scaffolds, with N50 scaffold length at 640,666 bp), the marker density and LD have not been added into the analysis. However, this data would be included when the genome assembly at pseudo chromosomal scale is available. A principal component analysis was carried out using the molecular markers generated using the DArTseq platform, allowing us to generate a genetic clustering x place of origin in Figure 4-1 and the phylogenetic tree is presented in Figure 4-. The first principal component explained 38.6% of the data, separating Southern African materials from the Western African, and a small separation between the Western African materials and the Asian. The second principal component explained 5.8% of the data, having the Central African materials closer to the Western African on the X axis, yet showing a wider spread distribution

along the Y axis, which is one of the first of its kind on the literature and gives a better understanding on the genetic resources that our AGP provides.

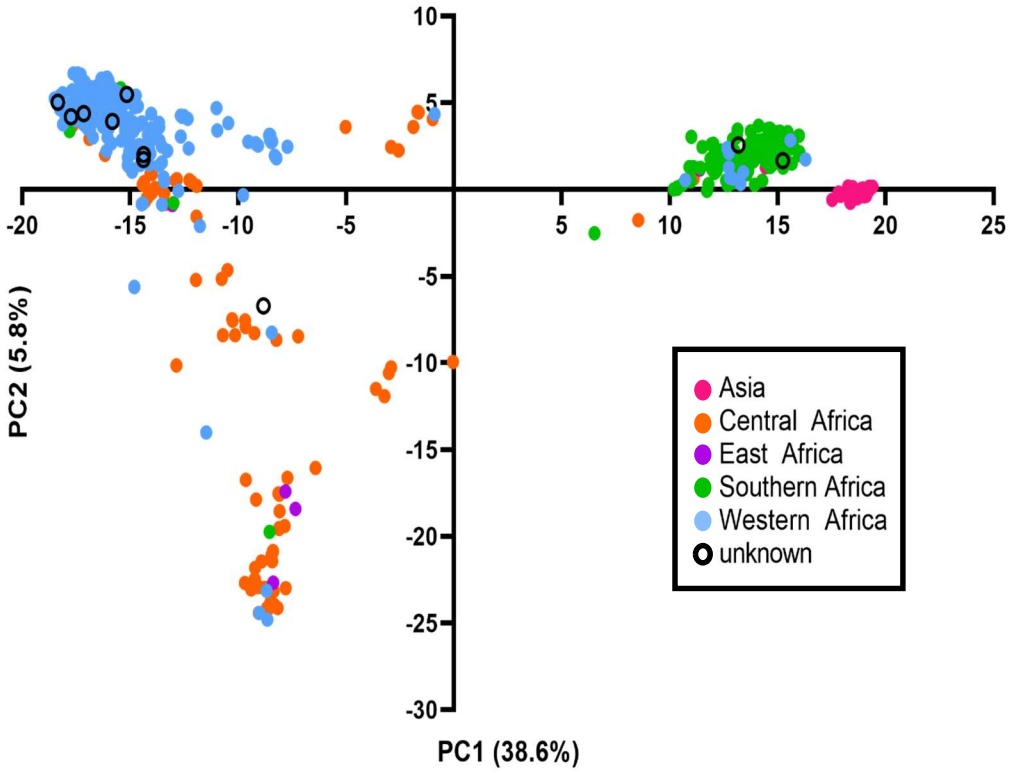

Figure 4-12 Genetic clustering of 229 genotypes from different regions of the world. The principal component analysis was derived from 11,964 SNPs.

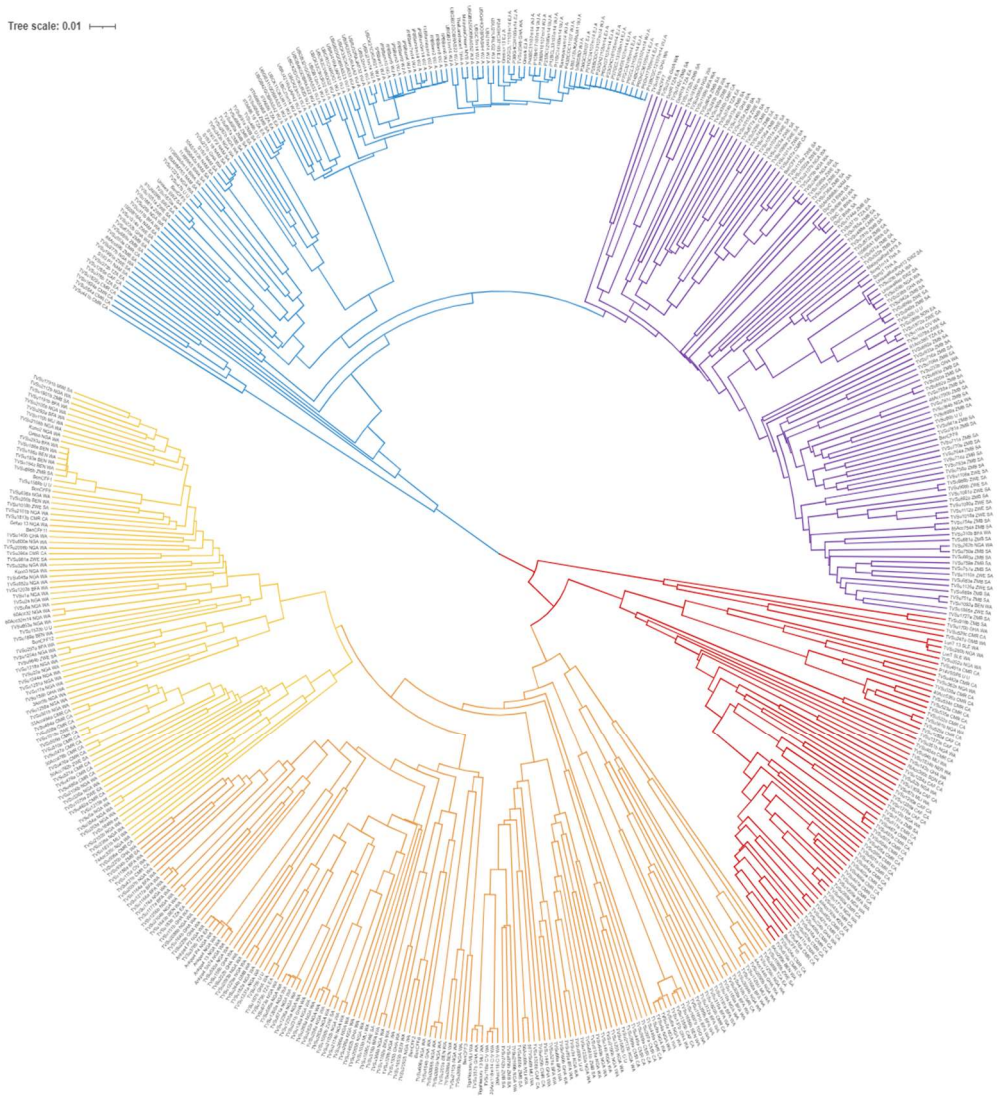

Figure 4-13 Phylogenetic tree, showing the genetic relatedness between the 229 DArT sequenced genotypes. Principal branches are coloured for easier visual representation.

From the GWAS analysis of the traits described previously, a QQ plot graph is presented in Figure 4-, where *LRWC* of the irrigated vs drought (LRWC_Irr-Dro), *Estimated Canopy Area* of the drought treated plants (Area_Drought) had the closest to expected distribution, followed by *Estimated Canopy Area* of the irrigated controls (Area_Irrigated) and *LRWC* of the drought treated plants (LRWC_Dro). The distribution was less uniform in the case of the *Estimated Canopy Area* and *Estimated Chlorophyll Content* on the comparatives between irrigated against drought (Area_Irr-Dro and CC_Irr-Dro, respectively), *Estimated Chlorophyll Content* of the drought treated plants and the irrigated controls (CC_Dro and CC_Irr, respectively), *LRWC*

(LRWC_Irr) of the irrigated controls, and days to anthesis (Days_to_anthesis).

Due to the ongoing assembly of the Bambara groundnut genome, the Manhattan plots are showed in "Supper-Scaffolds" which are close to chromosomes, however, new developments in the genome have allowed us to allocate the different significant genes onto their respective chromosome in Table 13. The Manhattan plots showing the genes at higher -log^{10}(P-value) of 4.0 were Area_Dro, Area_Irr, CC_Dro, CC_Irr, LRWC_Dro, LRWC_Irr, CC_Irr-Dro, and Days_to_anthesis.

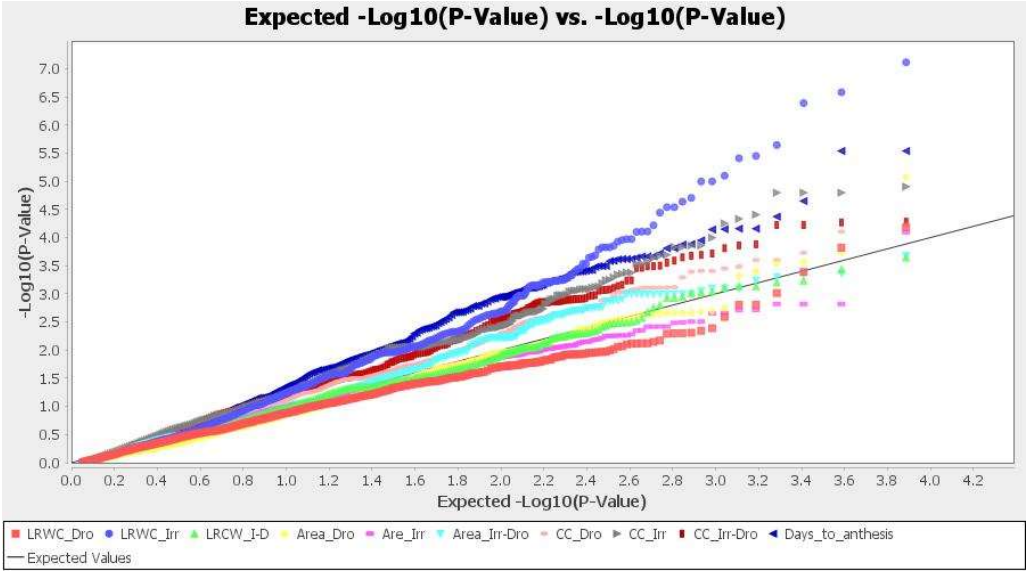

Figure 4-14 QQ plot graph. LRWC_Irr-Dro showed in green triangles; Area_Dro showed in yellow circles; Area_Irr, showed in pink squares; LRWC_Dro showed in orange squares; Area_Irr-Dro showed in teal blue triangles; CC_Irr-Dro showed in dark read squares; CC_Dro showed in beige squares; CC_Irr showed in grey triangles; LRWC_Irr showed in light green triangles; and Days_to_anthesis showed in dark blue triangles.

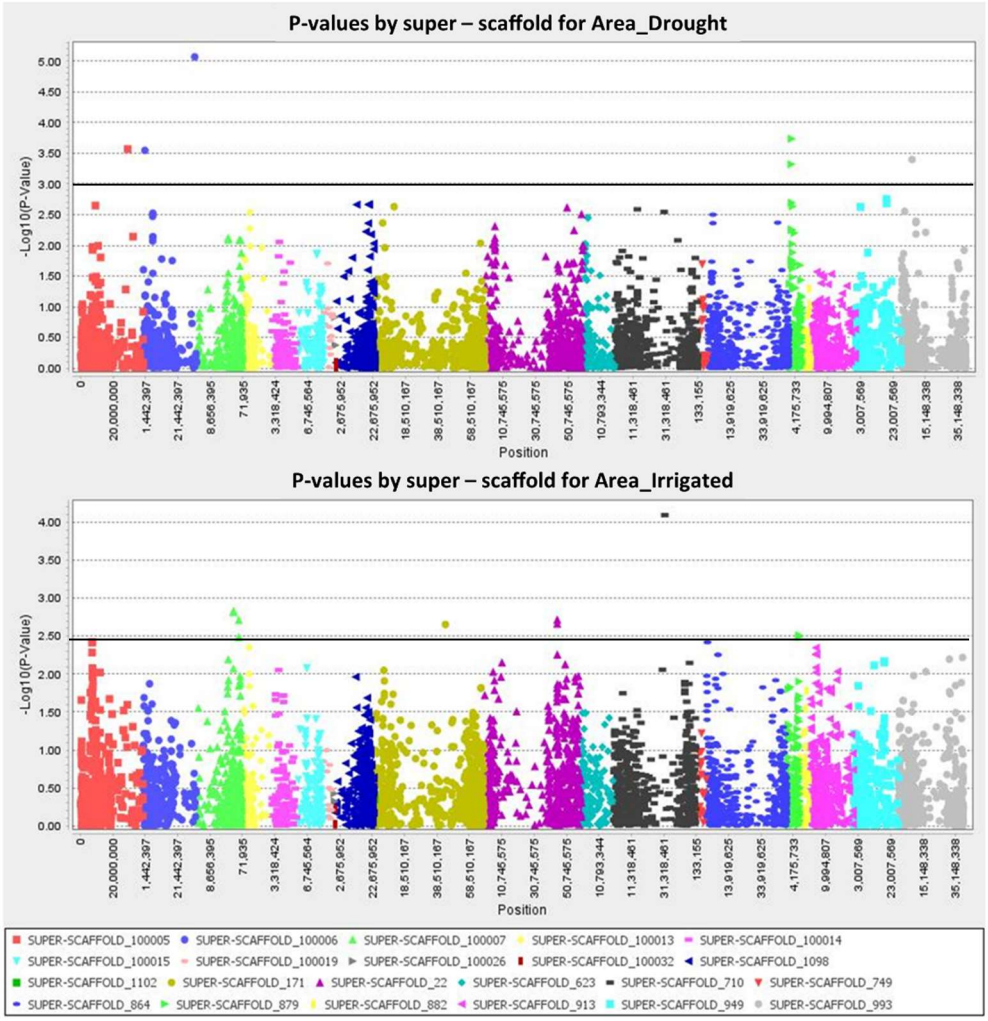

Figure 4-15 Manhattan plots for *estimated canopy area*. Top section shows the results under drought conditions; bottom section showed results under irrigated condition. Black line depicts the threshold above which markers selection were chosen for further analysis.

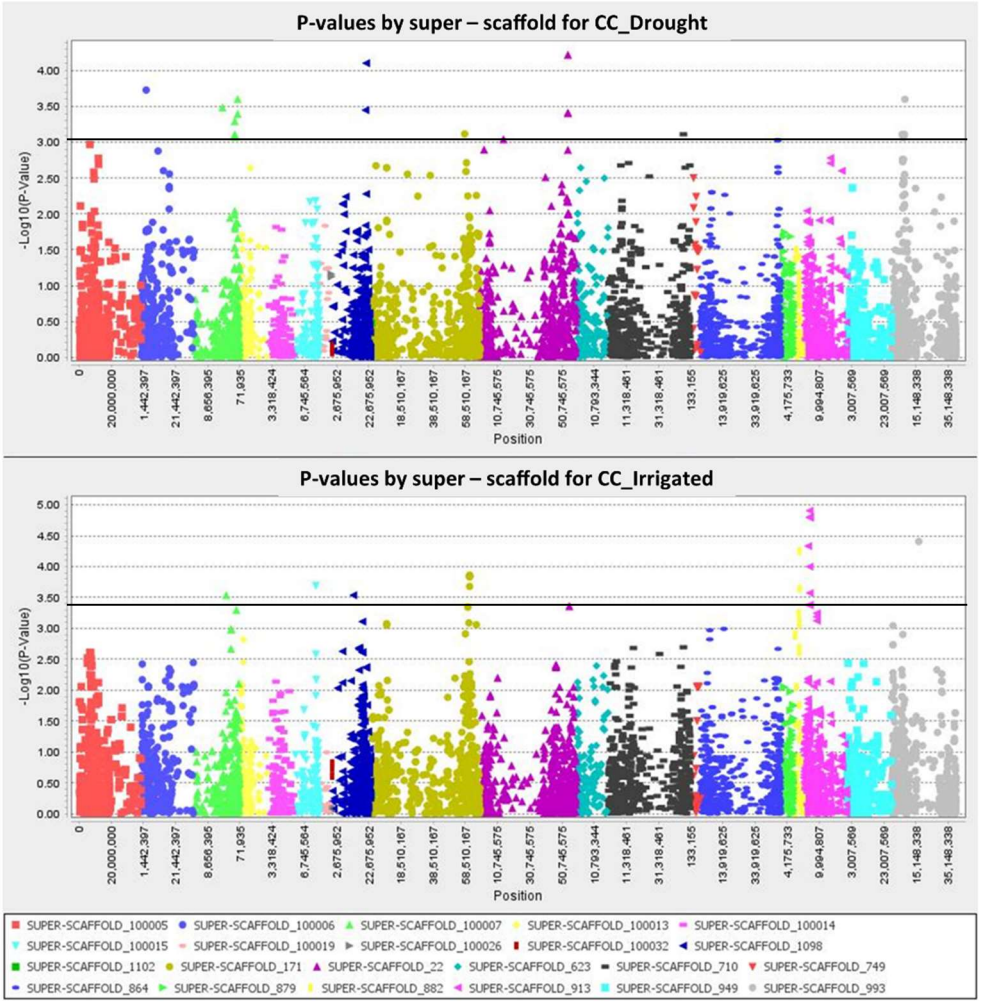

Figure 4-16 Manhattan plots for *estimated chlorophyll content*. Top section shows the results under drought conditions; bottom section showed results under irrigated condition. Black line depicts the threshold above which markers selection were chosen for further analysis.

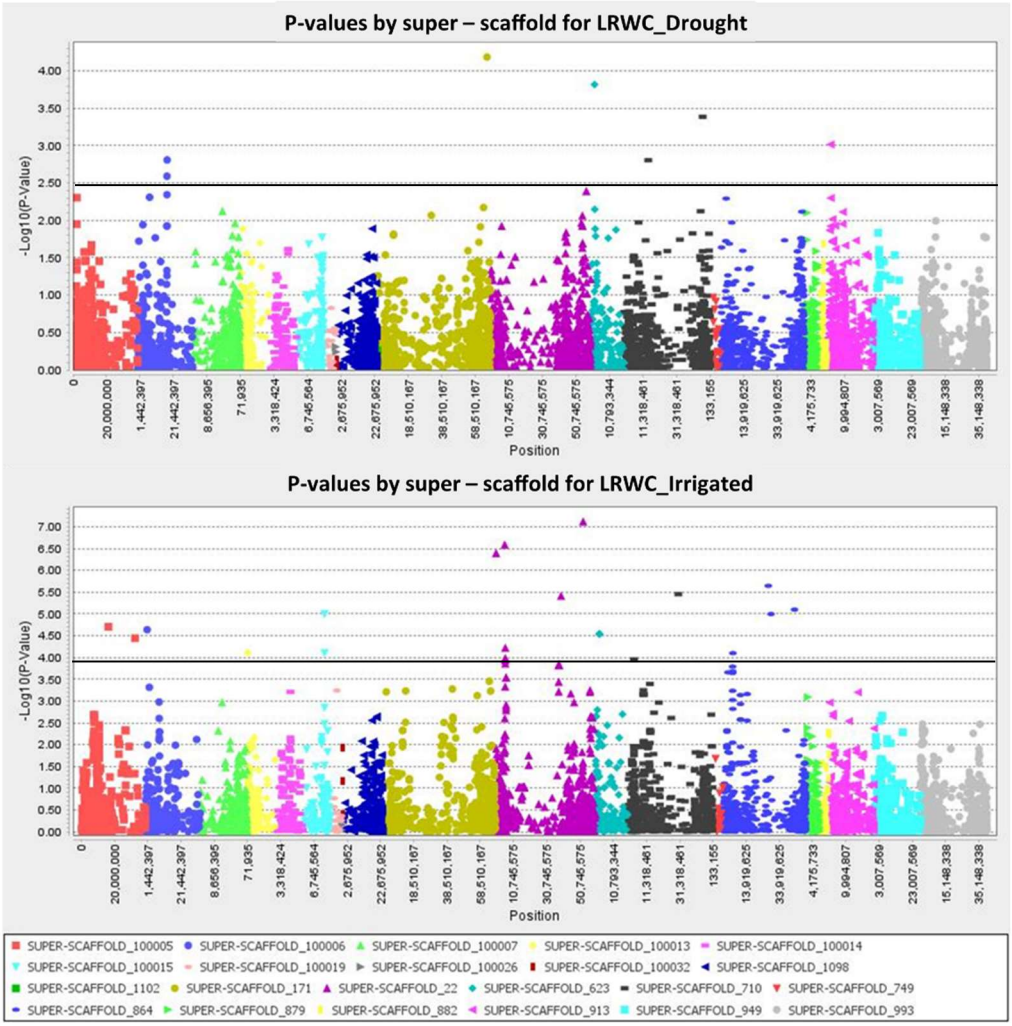

Figure 4-17 Manhattan plots for *estimated LRWC*. Top section shows the results under drought conditions; bottom section showed results under irrigated condition. Black line depicts the threshold above which markers selection were chosen for further analysis.

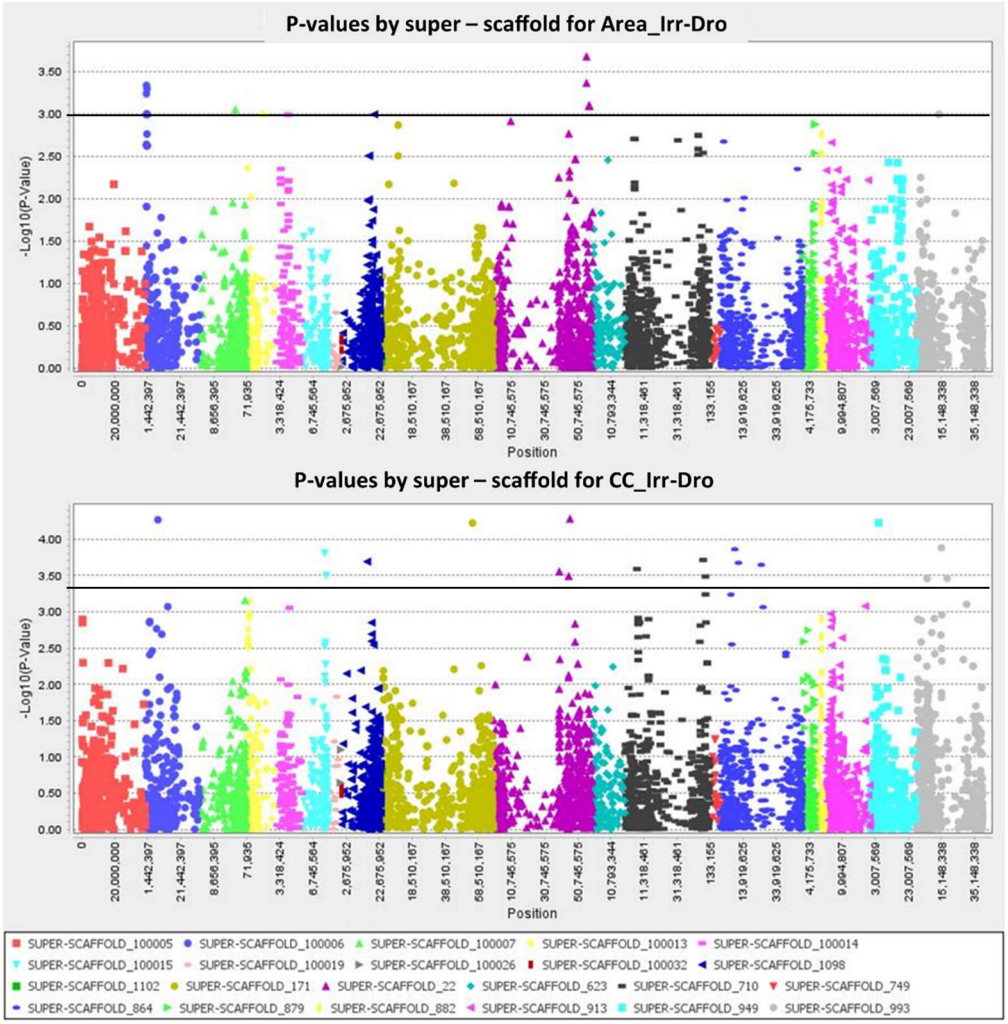

Figure 4-18 Manhattan plots for *estimated canopy area* difference (top section), and *estimated chlorophyll content* difference (bottom section). Black line depicts the threshold above which markers selection were chosen for further analysis.

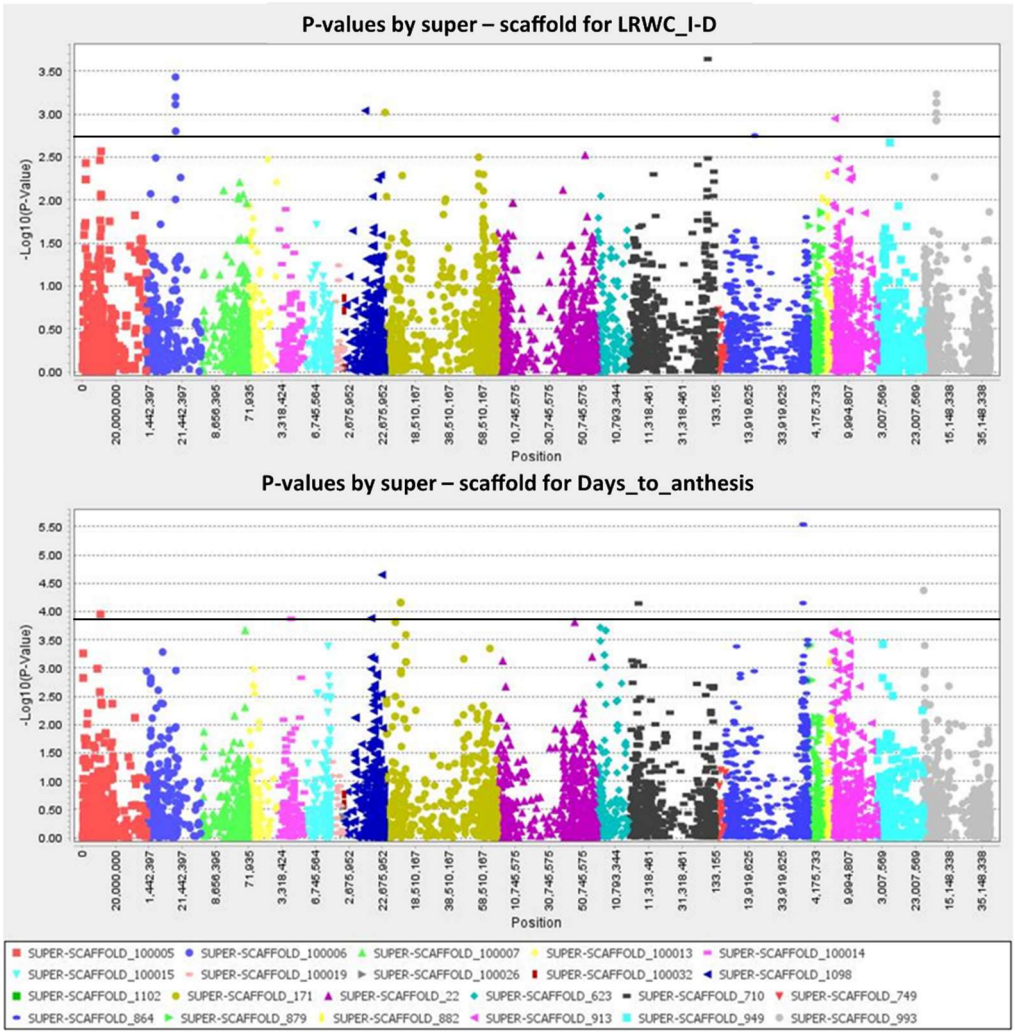

Figure 0-19 Manhattan plots for *LRWC* difference (top section), and *days to anthesis* (bottom section). Black line depicts the threshold above which markers selection were chosen for further analysis.

From the markers with the smaller *P* values, different genes were matched from the draft genome on the location provided by Tassel 5. From which *Table* 13 summarises the putative candidate genes affecting reduction in leaf relative water content, leaf area and chlorophyll content in response of drought stress. As mentioned previously, this analysis was based on the smallest *P* values, due to the use of a draft genome during the analysis, which hinders the depth of the analysis, in particular the surrounding to those peak markers. Two were found in chromosome Vs01, two in chromosome Vs02, one in chromosome Vs03, two in chromosome Vs05, one in chromosome Vs07, one in chromosome Vs08, one in chromosome Vs09, and one in chromosome Vs10. CC_Dro and CC_Irr shared one same gene (*Vs108784g0010*), which is reported as *AUTOINHIBITED CA2+ -ATPASE, ISOFORM 8* (*ACA8*) in *Arabidopsis thaliana* expressed mainly in the stomata guard cells, controlling opening or closing (Schiøtt & Palmgren, 2005), and thus, stomatal conductance, which is a trait commonly related to drought resistance through the drought avoidance mechanism. The remaining 10 genes had varied functions such as priming and memory of stress (*Vs108202g0027, FORGETTER 1,* Leuendorf *et al*., 2020); encoding of essential proteins for cell growth, development, and response to hormones, environmental stresses, and detoxification (*Vs108248g0002, Ankyrin repeat family protein; Vs107919g0023*, *ATNUDX19*; *Vs107698g0006, SPL13*; Corpas *et al*., 2016; Feyissa *et al*., 2019; Zhao *et al*., 2020)*;* synthesis of malate as part of the pathway for gluconeogenesis (*Vs107725g0079*, *MALATE SYNTHASE*, Ebeed *et al*., 2018) among others (refer to Table 13). In the case of *Vs107725g0079* it was reported by Maruyama *et al*. (2020) that the upregulation of *Glyma.17G128000* soybean gene had a positive effect in response to drought, by possibly having a role in monosaccharide accumulation in leaves and stems under drought conditions. Other genes were not reported in the literature as tested under drought conditions un *Vigna unguiculata, Glycine max,* and *Phaseolus vulgaris*, however, there was research published in *Arabidopsis thaliana*.

Table 10 Summary of candidate gene results in GWAS

Trait	Chr	*P*	Log-10	Gene	Homologous gene	Description	Reference
Area_Dro	Vs07	8.54E-06	5.07	Vs108248g0002	AT3G01750.2	Ankyrin repeat family protein, encodes for essential proteins for cell growth, development, and response to hormones and abiotic stresses.	Zhao *et al*., 2020
Area_Irr-Dro	Vs02	2.10E-04	3.68	Vs107894g0090	AT3G17600.1 Glyma.13g117100	*IAA31*, auxin signalling repressor which could result in an inhibition of some shoot responses regulated by auxin under water deficit.	Vargas *et al*., 2014
CC_Dro	Vs10	7.84E-05	4.11	Vs108091g0063	AT5G47120.1 Phvul.002G001400.1 Vigun02g161300.1 GlymaLee.01G168600.1	*BAX INHIBITOR 1*, implicated in suppression of H2O2- and endoplasmic reticulum-stress-induced plant cell death. Reported to confer drought tolerance.	Ishikawa *et al*., 2015; Nagano *et al*., 2019; Ramiro *et al*., 2016
CC_Dro	Vs02	6.03E-05	4.22	Vs108784g0010	AT5G57110.2	ACA8, Ca+2-ATPase expressed in stomata guard cells and in vascular tissues.	Schiøtt & Palmgren, 2005
CC_Irr	Vs05	1.59E-05	4.80	Vs083224g0001	AT3G07100.1	*ERMO2/*SEC24a, involve in the maintenance of the endoplasmic reticulum, which supports diverse cellular functions, such as protein and lipid synthesis, maintenance of calcium homeostasis, and quality control of proteins.	Nakano *et al*., 2009
CC_Irr-Dro	Vs03	5.96E-05	4.22	Vs108030g0001	AT5G64400.1	*At12cys-1,* lost of function led to enhanced tolerance to drought and light stress and increased anti-oxidant capacity.	Wang *et al*., 2016
CC_Irr-Dro	Vs02	5.22E-05	4.28	Vs107725g0079	AT5G03860.2 Glyma.17G128000	*MALATE SYNTHASE,* part of the pathway for gluconeogenesis. Reported to have drought tolerance effect in Soy bean and Arabidopsis.	Ebeed *et al*., 2018 Maruyama *et al*., 2020
CC_Irr-Dro	Vs08	1.31E-04	3.88	Vs107987g0013	AT2G33260.2	Tryptophan/tyrosine permease, involved in osmo-protectant action.	Bowne *et al*., 2012
LRWC_Irr	Vs01	7.96E-06	5.10	Vs107919g0023	AT5G20070.1	*NUDX19,* involved in hydrolyzation of NADPH, an important cofactor in cell growth, proliferation, and detoxification (oxidative stress).	Corpas *et al*., 2016
LRWC_Irr	Vs09	1.01E-05	5.00	Vs108202g0027	AT1G79350.1	*FGT1, FORGETTER 1* plays a role in abiotic stress memory.	Leuendorf *et al*., 2020
LRWC_Irr	Vs01	1.01E-05	5.00	Vs107698g0006	AT5G50570.1	*SPL13*, involve in regulation of a network of downstream genes affecting plant development and physiology by binding to gene promoters. Silencing of SPL13 has shown an enhance in drought resistance in *Medicago sativa L.* (alfalfa).	Feyissa *et al*., 2019

To the best of the author's knowledge, these are the results of the first GWAS published in Bambara groundnut, where interestingly, the set of genes and functions are completely different from the genes published chapter 5 and Khan *et al*., (2017). From our results, the *BAX INHIBITOR 1 (BI1)* gene has been reported to confer drought tolerance in *Arabidopsis thaliana*, *Saccharum officinarum* (sugarcane), *Nicotiana tabacum* (tobacco), and *Oryza sativa* (rice) (Ishikawa *et al*., 2015; Nagano *et al*., 2019; Ramiro *et al*., 2016). This gene has been reported to encode a cytoprotective protein in the endoplasmic reticulum membrane, which is induced during leaf senescence, as well as during abiotic stresses, modulating programmed cell death by inducing the release of cytochrome C (Ramiro *et al*., 2016). This has suggested that when water availability becoming a limitation, *BI1* might affect the amount of chlorophyll as a means to reduce the photosynthesis rate. Nevertheless, the reduction of chlorophyll content in the stressed plants in comparison to their well-watered counterpart might have suggested *At12cys-1* orthologue and osmotic adjustment through malic acid play a role in the extent of chlorophyll reduction. Wang *et al*. (2016) have demonstrated that the mitochondrial and chloroplast functions are affected from the single deletion of either paralogue (At12cys-1 or At12cys-2). It has been reported that Malate could be converted to starch to prevent stomata closure by reducing osmotic potential and turgor (Arve *et al*., 2013). The *Vs107894g0090* (*IIA31)* has recently been reported in Chapter 5, where this gene was downregulated in irrigated controls, which is involved in drought stress response and leaf senescence (Gadallah, 2000; Youzhi Zhang *et al*., 2020).

Similarly, *ERMO2/*SEC24a gene has been reported to be involved in the maintenance of the endoplasmic reticulum, supporting protein and lipid synthesis, as well as maintenance of calcium homeostasis, and controlling the quality of proteins (Nakano *et al*., 2009). These mechanisms could be more attributed to drought tolerance, by expressing several genes in pursuit to maintain biological processes and functions as normal as possible under water deficit. Similar is the case of *AtCys-1* and *AtCys-2*, which together are negatively involved in the increase of antioxidant capacity, where the deletion of both genes in *Arabidopsis thaliana* enhance tolerance to drought and light stresses (Wang *et al*., 2016). This gene was detected from the control plants, which requires further studies to understand better the

pathways that this gene might be involve, and how this affects under drought stress. Interestingly, GWAS revealed two significant SNP markers (4182906 and 24382669) in CC_Irr, located within two exons of *Vs083224g0001* gene or others close to it, suggesting that this gene might play a major role in maintaining chlorophyll content during optimum growing conditions.

This is the first GWAS performed in Bambara groundnut, which with a future higher marker density would aid on having a better genetic resolution, to allow a better detection of genes of interest, where resequencing could be a sensible approach on this AGP by generating more genotyping resources. As well as having a full genome sequenced could aid on finding more genes or regions of interest on this GWAS, or this AGP. The validation of the genes of interest is also required, to confirm their function in drought resistance, and possible application in molecular breeding programs.

References

Alam, M., Neal, J., O'Connor, K., Kilian, A., & Topp, B. (2018). Ultra-high-throughput DArTseq-based silicoDArT and SNP markers for genomic studies in macadamia. *PLoS ONE*, *13*(8), 1–20. https://doi.org/10.1371/journal.pone.0203465

Arve, L. E., Terfa, M. T., Gislerød, H. R., Olsen, J. E., & Torre, S. (2013). High relative air humidity and continuous light reduce stomata functionality by affecting the ABA regulation in rose leaves. *Plant, Cell and Environment*, *36*(2), 382–392. https://doi.org/10.1111/j.1365-3040.2012.02580.x

Bowne, J. B., Erwin, T. A., Juttner, J., Schnurbusch, T., Langridge, P., Bacic, A., & Roessner, U. (2012). Drought responses of leaf tissues from wheat cultivars of differing drought tolerance at the metabolite level. *Molecular Plant*, *5*(2), 418–429. https://doi.org/10.1093/mp/ssr114

Chang, Y., Liu, H., Liu, M., Liao, X., Sahu, S. K., Fu, Y., Song, B., Cheng, S., Kariba, R., Muthemba, S., Hendre, P. S., Mayes, S., Ho, W. K., Yssel, A. E. J., Kendabie, P., Wang, S., Li, L., Muchugi, A., Jamnadass, R., … Liu, X. (2018). The draft genomes of five agriculturally important african orphan crops. *GigaScience*, *8*(3), 1–16. https://doi.org/10.1093/gigascience/giy152

Corpas, F. J., Aguayo-Trinidad, S., Ogawa, T., Yoshimura, K., & Shigeoka, S. (2016). Activation of NADPH-recycling systems in leaves and roots of Arabidopsis thaliana under arsenic-induced stress conditions is accelerated by knock-out of Nudix hydrolase 19 (AtNUDX19) gene. *Journal of Plant Physiology*, *192*, 81–89. https://doi.org/10.1016/j.jplph.2016.01.010

Ebeed, H. T., Stevenson, S. R., Cuming, A. C., & Baker, A. (2018). Conserved and differential transcriptional responses of peroxisome associated pathways to drought, dehydration and ABA. *Journal of Experimental Botany*, *69*(20), 4971–4985. https://doi.org/10.1093/jxb/ery266

Edet, O. U., Gorafi, Y. S. A., Nasuda, S., & Tsujimoto, H. (2018). DArTseq-based analysis of genomic relationships among species of tribe Triticeae. *Scientific Reports*, *8*(1), 1–11. https://doi.org/10.1038/s41598-018-34811-y

Feyissa, B. A., Arshad, M., Gruber, M. Y., Kohalmi, S. E., & Hannoufa, A. (2019). The interplay between miR156/SPL13 and DFR/WD40-1 regulate drought tolerance in alfalfa. *BMC Plant Biology*, *19*(1), 1–19. https://doi.org/10.1186/s12870-019-2059-5

Gadallah, M. A. A. (2000). Effects of indole-3-acetic acid and zinc on the growth, osmotic potential and soluble carbon and nitrogen components of soybean plants growing under water deficit. *Journal of Arid Environments*, *44*(4), 451–467. https://doi.org/10.1006/jare.1999.0610

Ishikawa, T., Aki, T., Yanagisawa, S., Uchimiya, H., & Kawai-Yamada, M. (2015). Overexpression of BAX INHIBITOR-1 links plasma membrane

microdomain proteins to stress. *Plant Physiology*, *169*(2), 1333–1343. https://doi.org/10.1104/pp.15.00445

Jørgensen, S. T., Liu, F., Ouédraogo, M., Ntundu, W. H., Sarrazin, J., & Christiansen, J. L. (2010). Drought responses of two Bambara Groundnut (Vigna subterranea L. Verdc.) landraces collected from a dry and a humid area of Africa. *Journal of Agronomy and Crop Science*, *196*(6), 412–422. https://doi.org/10.1111/j.1439-037X.2010.00435.x

Khan, F., Chai, H. H., Ajmera, I., Hodgman, C., Mayes, S., & Lu, C. (2017). A transcriptomic comparison of two Bambara groundnut landraces under dehydration stress. *Genes*, *8*(4), 1–19. https://doi.org/10.3390/genes8040121

Leuendorf, J. E., Frank, M., & Schmülling, T. (2020). Acclimation, priming and memory in the response of Arabidopsis thaliana seedlings to cold stress. *Scientific Reports*, *10*(1), 1–11. https://doi.org/10.1038/s41598-019-56797-x

Mayes, S., Ho, W. K., Chai, H. H., Gao, X., Kundy, A. C., Mateva, K. I., Zahrulakmal, M., Hahiree, M. K. I. M., Kendabie, P., Licea, L. C. S., Massawe, F., Mabhaudhi, T., Modi, A. T., Berchie, J. N., Amoah, S., Faloye, B., Abberton, M., Olaniyi, O., & Azam-Ali, S. N. (2019). Bambara groundnut: an exemplar underutilised legume for resilience under climate change. *Planta*, *250*(3), 803–820. https://doi.org/10.1007/s00425-019-03191-6

Maruyama, K., Urano, K., Kusano, M., Sakurai, T., Takasaki, H., Kishimoto, M., Yoshiwara, K., Kobayashi, M., Kojima, M., Sakakibara, H., Saito, K., Shinozaki, K. (2020) Metabolite/phytohormone-gene regulatory networks in soybean organs under dehydration conditions revealed by integration analysis. *The Plant Journal,* 103, 197-211

Muhammad, Y. Y., Mayes, S., & Massawe, F. (2016). Effects of short-term water deficit stress on physiological characteristics of Bambara groundnut (Vigna subterranea (L.) Verdc.). *South African Journal of Plant and Soil*, *33*(1), 51–58. https://doi.org/10.1080/02571862.2015.1056847

Nagano, M., Kakuta, C., Fukao, Y., Fujiwara, M., Uchimiya, H., & Kawai-Yamada, M. (2019). Arabidopsis Bax inhibitor-1 interacts with enzymes related to very-long-chain fatty acid synthesis. *Journal of Plant Research*, *132*(1), 131–143. https://doi.org/10.1007/s10265-018-01081-8

Nakano, R. T., Matsushima, R., Ueda, H., Tamura, K., Shimada, T., Li, L., Hayashi, Y., Kondo, M., Nishimura, M., & Hara-Nishimura, I. (2009). Gnom-like1/ermo1 and sec24a/ermo2 are required for maintenance of endoplasmic reticulum morphology inarabidopsis thaliana. *Plant Cell*, *21*(11), 3672–3685. https://doi.org/10.1105/tpc.109.068270

Nautiyal, P. C., Kulkarni, G., Singh, A. L., & Basu, M. S. (2017). Evaluation of water-deficit stress tolerance in Bambara groundnut landraces for cultivation in sub-tropical environments in India. *Indian Journal of Plant Physiology*, *22*(2), 190–196. https://doi.org/10.1007/s40502-017-0296-x

Ramiro, D. A., Melotto-Passarin, D. M., Barbosa, M. de A., Santos, F. Dos, Gomez, S. G. P., Massola Júnior, N. S., Lam, E., & Carrer, H. (2016). Expression of Arabidopsis Bax Inhibitor-1 in transgenic sugarcane confers drought tolerance. *Plant Biotechnology Journal*, *14*(9), 1826–1837. https://doi.org/10.1111/pbi.12540

Schiøtt, M., & Palmgren, M. G. (2005). Two plant Ca2+ pumps expressed in stomatal guard cells show opposite expression patterns during cold stress. *Physiologia Plantarum*, *124*(2), 278–283. https://doi.org/10.1111/j.1399-3054.2005.00512.x

Vargas, L., Brígida, A. B. S., Mota Filho, J. P., De Carvalho, T. G., Rojas, C. A., Vaneechoutte, D., Van Bel, M., Farrinelli, L., Ferreira, P. C. G., Vandepoele, K., & Hemerly, A. S. (2014). Drought tolerance conferred to sugarcane by association with gluconacetobacter diazotrophicus: A transcriptomic view of hormone pathways. *PLoS ONE*, *9*(12), 1–37. https://doi.org/10.1371/journal.pone.0114744

Wang, Y., Lyu, W., Berkowitz, O., Radomiljac, J. D., Law, S. R., Murcha, M. W., Carrie, C., Teixeira, P. F., Kmiec, B., Duncan, O., Van Aken, O., Narsai, R., Glaser, E., Huang, S., Roessner, U., Millar, A. H., & Whelan, J. (2016). Inactivation of Mitochondrial Complex i Induces the Expression of a Twin Cysteine Protein that Targets and Affects Cytosolic, Chloroplastidic and Mitochondrial Function. *Molecular Plant*, *9*(5), 696–710. https://doi.org/10.1016/j.molp.2016.01.009

Zhang, Y., Li, Y., Hassan, M. J., Li, Z., & Peng, Y. (2020). Indole-3-acetic acid improves drought tolerance of white clover via activating auxin, abscisic acid and jasmonic acid related genes and inhibiting senescence genes. *BMC Plant Biology*, *20*(1), 1–12. https://doi.org/10.1186/s12870-020-02354-y

Zhao, J. Y., Lu, Z. W., Sun, Y., Fang, Z. W., Chen, J., Zhou, Y. Bin, Chen, M., Ma, Y. Z., Xu, Z. S., & Min, D. H. (2020). The Ankyrin-Repeat Gene GmANK114 Confers Drought and Salt Tolerance in Arabidopsis and Soybean. *Frontiers in Plant Science*, *11*(October), 1–20. https://doi.org/10.3389/fpls.2020.584167

Chapter 5: RNA-seq in Bambara groundnut leaf tissue reveals drought tolerant related genes.

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Abstract

Bambara groundnut (*Vigna subterranea* (L) Verdc) is an underutilised African legume known for its resistance to drought conditions. However, little is known on how this crop species cope with terminal drought stress.

Here we evaluate transcriptomic changes in four Bambara groundnut genotypes after 15 days without irrigation to have a better understanding on the mechanisms involved when water becomes a limitation. These four genotypes varied in chlorophyl fluorescence. Corresponding to that, a total of 2,986 differentially expressed genes in response to drought were found uniquely stated among the different genotypes, meanwhile, only 585 genes were commonly expressed between genotypes. Genotype TN, had a larger number of DEG (n=1848) uniquely expressed in response to the drought treatment while Genotype S19-3 had the lowest number of differentially expressed genes that are unique to that genotype (174 genes).

Our preliminary analysis has suggested that genotypic variations might have contributed to differences related to phytohormones such as ABA (related in stomatal conductance), IAA and jasmonic acid (which could be related to leaf senescence), which could be attributed as **drought avoidance** mechanisms. In addition, increase in the production or synthesis of soluble sugars as a means for osmotic protection could have contributed to **drought tolerance** mechanism, as observed in some genotypes.

The RNAseq resources developed in this study will also be used in gene annotation of an in-house developed reference genome. Further validations of potential DEGs could aid in developing more drought tolerance Bambara groundnut varieties.

Introduction

Bambara groundnut (*Vigna subterranea* (L.) Verdc.; 2x = 2n = 22) is a drought resistant underutilised pulse from sub-Saharan Africa which is also grown in some regions of southeast Asia (Mayes *et al*., 2019). It may have potential to aid in future food security; as a legume it can fix atmospheric nitrogen into the soil, produce nutritionally protein rich beans, and provide reasonable yields under drought conditions. These characteristics could position Bambara groundnut as an alternative crop for places with soils or environments where normal crops might not develop or produce well.

The present effects of climate change such as water deficit and extreme temperatures, have become a worldwide problem, with increasing periods of drought affecting crop yields (Raza *et al*., 2019). According to several authors (Blum, 2011; Farooq *et al*., 2009; Xu *et al*., 2010), there are three main responses to drought in pursuit of survival to produce seed for the next generation. One of these mechanisms is drought escape, whereby plants may shorten their life cycle, trying to make the best of the water resources available in a short period (such ground water remaining from a defined rain season), to allow them to produce the next generation before the environment becomes harmfully dry. Drought avoidance is when plants attempt to adapt to changing water availability, through strategies such as deeper root growth to seek further water and try to avoid reaching a point of damagingly low moisture levels in the soil before the next water becomes available (rain, or irrigation). This could be achieved for example, by reducing stomatal conductance, restricting leaf area expansion, increasing root depth and density, among other traits. The last mechanism is drought tolerance, which is considered to be the ability to regulate thousands of genes and a series of metabolic pathways to minimise (e.g. osmotic adjustment) or repair damage cause by drought, and thus sustain physiological activities under severe drought conditions (Fang & Xiong, 2015). A couple of examples are, in Groundnut (*Arachis hypogaea*), where it has been reported that a fatty acid elongase gene (*ß-Ketoacyl Co-A Synthase1, KCS1*) affected the cuticular wax of the aerial plant parts, and thus, minimizing water loss and conferring drought tolerance (Lokesh *et al*., 2019). Another case has been reported in rice (*Oryza sativa*), where *Myo-inositol oxygenase* (*MIOX*) is suggested to decrease oxidative damage by increasing the activity of Reactive Oxygen Species scavenging enzymes, as well as increasing proline content.

There are a range of desirable traits for breeding programs that are linked to drought resistance, however, conventional breeding is time consuming (Luo *et al*., 2019). Molecular tools could aid in speeding up the process of selection of desirable breeding lines (Luo *et al*., 2019). Measuring chlorophyll fluorescence is one of the common techniques for measuring 'stress' in leaves, as it's a fast, efficient, and consistent (Murchie & Lawson, 2013). Healthy leaves would show a dark-adapted fluorescence value of around 0.83, as healthy leaves should not express any non-photochemical quenching due to the absence of stress (Murchie & Lawson, 2013).

Next generation sequencing has in recent years become more accessible, less expensive, more productive, and faster. From different alternatives for small sequencing reads, such as 454 sequencing, SOLiD, and Illumina, the latter has been reported to be the most accurate and cheapest, with the ability to handle thousands of samples simultaneously (Liu *et al*., 2012). One of the major uses for the Illumina platform technology is for RNA sequencing (RNA-seq), which is a powerful technique for the effective study of the transcriptome, and thus, gene expression under different treatment conditions, such as drought. Bambara groundnut, as an underutilised crop, has not been studied intensely and more research is needed to fully understand this crop's hidden potential and uses in comparison to wheat, maize, soya, and rice. The resistance to drought in Bambara groundnut has been reported from a morpho-physiological point of view, however, little is known about its associated transcriptome, and how it is involved in the mechanisms induced to cope with drought. A first report of transcriptome assessment of two Bambara groundnut genotypes was published by Khan *et al*. (2017), where a full experiment on different stages of water deficit and recovery were sampled for a cross-species hybridisation to soybean microarray chip. This experiment revealed a number of dehydration-associated transcription factors such as *CONSTANS-LIKE 1* and *MYB60.* While highly informative, there are limitations to the Affymetrix microarray sequencing, where in the case of underutilised crops chips of the closest major crop species must be used. Conversely, RNA-seq is a true sequence representation of the transcriptome, where any species could be sequenced without the need of a particular chip or genome data, generating a more comprehensive analysis (Rao *et al*., 2019).

In this study, we report the first RNA-seq experiment conducted in Bambara groundnut using the Illumina sequencing platform, to begin to understand

some of the genetic mechanisms behind the physiological responses to drought in this species, and how these fit with the different drought resistance mechanisms. We evaluate whether S19-3, DodR, and TN express gene regulation in metabolic pathways to induce drought tolerance, while Gresik regulates ABA and/or leaf senescence related traits as drought avoidance mechanism.

Materials and Methods

Plant material, stress treatment and experimental design, tissue collection.

The experiment was conducted in the FutureCrops Glasshouse 1 in the Sutton Bonington Campus of the University of Nottingham (52°50′02″N, 1°15′00″W) in the months of July to September of 2020. The growing environmental conditions were set at 12 h daylength and 28°C/23°C day/night temperatures. Four different genotypes (TN, Gresik, DodR, and S19-3) with contrasting responses to drought were grown in six replicates per treatment according to Salazar-Licea *et al.* (2021, chapter 3), each in a 10L pot in a fully randomised block design. The drought stress was imposed at 50% flowering (i.e., three out of six replicates had the first flower open) and this varied across genotypes. Watering was halted for 15 days from 50% flowering, before the irrigation regime was resumed.

Soil field capacity

To determine the field capacity of the soil-type used, four pots were irrigated until fully saturated. The pots were left until there were no signs of water drainage by gravity (more than 12 hours) and subsequently weighted. To determine the soil mass, the soil was dried for 72 h at 80°C and the weight was retaken. The field capacity was calculated as follows:

$$\theta m = \left(\frac{\theta w - \theta d}{\theta d}\right) \times 100\%$$

where: θm = gravimetric field water capacity,

θw = soil mass at 100% field capacity, and

θd = soil mass in absence of water.

Chlorophyll fluorescence

Maximum quantum efficiency of the photosystem II (PSII) was assessed by measuring the quantum yield under 30 min of dark adaptation in three leaflets per replicate using a Fluorpen (Photon Systems Instruments, Czech Republic). These measurements were taken at 6 pm at 7, 11, and 15 days after imposing drought stress as well as during recovery stage (1 and 3 days after rewatering).

Statistical analysis was carried out using Genstat 20th edition (VSN International) and GraphPad Prism 9 for Analyses Of Variance (ANOVA).

Leaf tissues were collected at 1600 h after 11 days of drought, at which the plants had shown initial symptoms of drought stress (lower PSII efficiency, leaf wilting, and lower chlorophyll content). Leaves of the same age were selected from which one individual leaflet per replicate was collected.

RNA isolation and quality assessment

Total RNA was isolated from the leaf samples by using the RNeasy PlantMini Kit (Qiagen) according to the manufacturer's instructions and DNA was removed by DNase I digestion during the washing step. The quality, quantity, and integrity of the RNA was checked using Nanodrop ND-1000, 1% agarose gel electrophoresis, and Agilent Bioanalyzer 2100. Sample requirements were, concentration above 20ng/µL, total yield of RNA above 0.4µg, Agilent RNA integrity number above 6.3 and both Nanodrop ratios of ODO260/280 and 260/230 of above 2.0. Only the samples that met the quality requirements of Novogene were considered further. Four out of six RNA samples per treatment, from each genotype were selected, were packed in dry ice, and shipped to Novogene, Cambridge, UK.

Whole-transcriptome sequencing (RNAseq) and bioinformatics

Quality control, sequencing, and bioinformatics was additionally performed by Novogene (UK) Company Limited (https://en.novogene.com/), as shown in the pipeline in Figure 5-1 and Figure 5-2. Quality control involved a degradation and contamination assessment by running 1 µl on 1% agarose gel; the purity was checked using the NanoPhotometer spectrophotometer; and integrity and quantitation were assessed using the RNA Nano 6000 Assay kit of Bioanalyzer 2100 systems. According to Novogene, to ensure good data quality, quality control was performed at each step from the RNA sample to the final data, as shown in green in Figure 5-1.

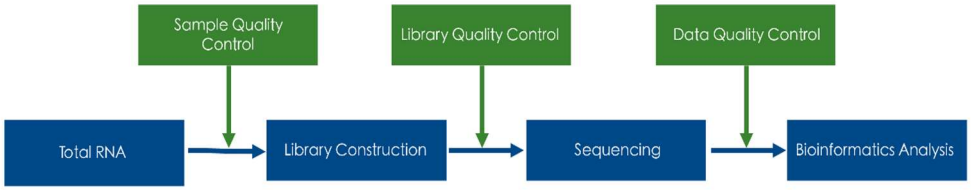

Figure 5-1 Overall pipeline for RNA-seq of RNA samples upon reception by Novogene.

Sequencing libraries were generated using NEBNext® UltraTM RNA Library Prep Kit for Illumina® (NEB, USA) following the manufacturer's recommendations and index codes were added to attribute sequences to each sample. Briefly, mRNA was purified from 1 µg of total RNA using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations under elevated temperature in NEBNext First Strand Synthesis Reaction Buffer (5X). First strand cDNA was synthesized using random hexamer primer and M-MuLV Reverse Transcriptase (RNase H-). Second strand cDNA synthesis was subsequently performed using DNA Polymerase I and RNase H. Remaining overhangs were converted into blunt ends via exonuclease/polymerase activities. After adenylation of 3' ends of DNA fragments, NEBNext Adaptor with hairpin loop structure were ligated to

prepare for hybridization. In order to select cDNA fragments of preferentially 150~200 bp in length, the library fragments were purified with AMPure XP system (Beckman Coulter, Beverly, USA). Then 3 μL USER Enzyme (NEB, USA) was used with size-selected, adaptor-ligated cDNA at 37 °C for 15 min followed by 5 min at 95 °C before PCR. Then PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index (X) Primer. Finally, PCR products were purified (AMPure XP system) and library quality was assessed on the Agilent Bioanalyzer 2100 system. The clustering of the index-coded samples was performed on a cBot Cluster Generation System using PE Cluster Kit cBot-HS (Illumina) according to the manufacturer's instructions before sequencing.

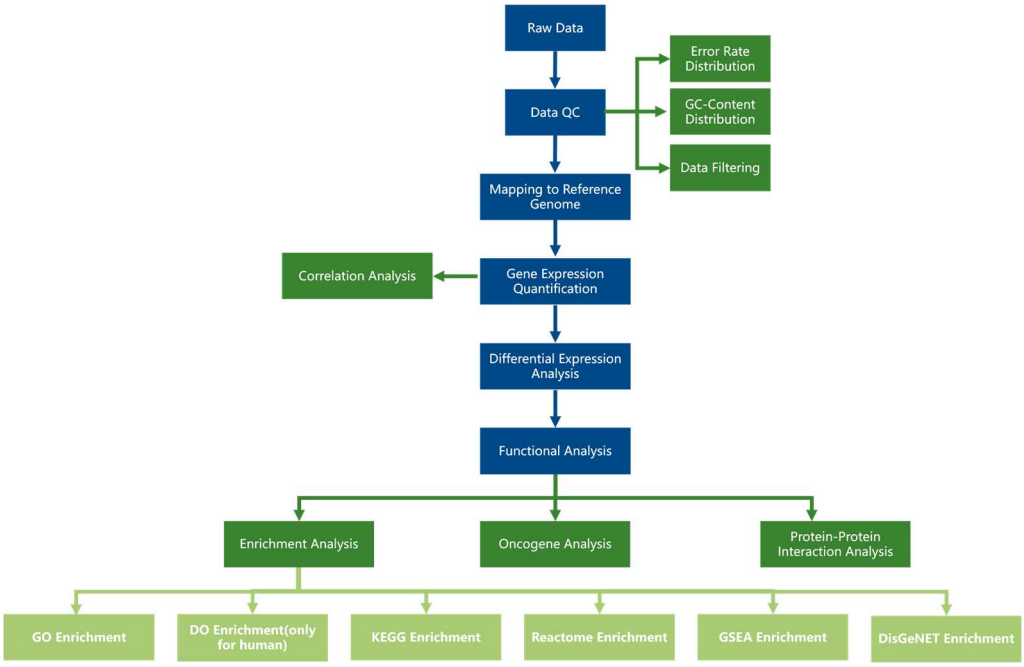

Figure 5-2 Pipeline of the bioinformatic analysis of RNA-seq.

For the data processing, raw data (raw reads) of FASTQ format were firstly processed through fastp. In this step, clean data (clean reads) were obtained by removing reads containing adapter and poly-N sequences and reads with low quality from raw data. At the same time, Q20, Q30 and GC content of the clean data were calculated. All the downstream analyses were based on the clean data with high quality. Thereafter, reference genome and gene model annotation files were downloaded from genome website browsers (NCBI/UCSC/Ensembl) directly. Paired-end clean reads were mapped to the

reference genome published by Chang *et al.* (2018) using HISAT2 software (Kim *et al*., 2019). HISAT2 uses a large set of small GFM indexes that collectively cover the whole genome. These small indexes (called local indexes), combined with several alignment strategies, enable rapid and accurate alignment of sequencing reads. Because transcriptome annotations are still incomplete for this species, most RNA-seq studies will reveal novel genes and transcripts. Stringtie (Kovaka *et al*., 2019) was used to assemble the set of transcript isoforms of each bam file obtained in the mapping step. Gffcompare (Pertea and Pertea, 2020) can compare Strintie assemblies to reference annotation files and help sort out new genes from known ones. Featurecounts (Liao *et al*., 2014) was used to count the read numbers mapped of each gene, including known and novel genes. Thereafter, RPKM of each gene was calculated based on the length of the gene and reads count mapped to this gene. RPKM, Reads Per Kilobase of exon model per Million mapped reads, considers the effect of sequencing depth and gene length for the reads count at the same time, and is currently the most commonly used method for estimating gene expression levels.

Prior to differential gene expression analysis for each sequenced library, the read counts were adjusted by Trimmed Mean of Mvalues (TMM) through one scaling normalized factor. Differential expression analysis of two conditions was performed using the edgeR R package (Robinson *et al*., 2010). The P values were adjusted using the Benjamini and Hochberg methods (Benjamini & Hochberg, 1995). Corrected p-value of 0.005 and |log2 (Fold Change) | of 1 were set as the threshold for significantly differential expression.

For the differential expression, enrichment, and alternative splicing analyses, the following methods were carried out. Differential expression analysis between two conditions/groups (four biological replicates per condition) was performed using DESeq2 R package (http://bioconductor.org/packages/devel/bioc/vignettes/DESeq2/inst/doc/DESeq2.html). DESeq2 provides statistical routines for determining differential expression in digital gene expression data using a model based on the negative binomial distribution. The resulting P values were adjusted using the Benjamini and Hochberg's approach for controlling the False Discovery Rate (FDR). Genes with an adjusted P value < 0.05 found by DESeq2 were assigned as differentially expressed.

A common way for searching shared functions among genes, is to incorporate the biological knowledge provided by biological ontologies. Gene Ontology (GO) annotates genes to biological processes, molecular functions, and cellular components in a directed acyclic graph structure, and Kyoto Encyclopedia of Genes and Genomes (KEGG) annotates genes to pathway. GO enrichment analysis of differentially expressed genes was implemented by the clusterProfiler R package (Yu *et al*., 2012), in which gene length bias was corrected. GO terms with corrected *P* value less than 0.05 were considered significantly enriched by differential expressed genes. For the KEGG pathway enrichment analysis, clusterProfiler R package was implemented to test the statistical enrichment of differential expression genes in KEGG pathways.

Alternative splicing analysis was performed by the software rMATS, a statistical method for robust and flexible detection of differential AS from replicate RNA-Seq data. It identifies alternative splicing events corresponding to all major types of alternative splicing patterns and calculates the P value and FDR for differential splicing. These types include exon skipping (SE), alternative 5′ splice sites (A5SS), alternative 3′ splice sites (A3SS), mutually exclusive exons (MXE), and retained introns (RI).

Two Microsoft Excel files with all the significant results ($p<0.05$) results from the differential expression analysis, GO, and KEGG enrichment was compiled (GSEA in Appendix 3). This list was manually filtered by excluding genes where gene expression values were missing in any replicate, or the standard deviations were higher than 900 per treatment, or the log-fold change was lower than 0.7 in up-regulated genes, or higher than -0.7 in down-regulated genes. This filtering still left more than 200 genes differentially expressed in some genotypes. Genes were sorted based on the log-fold change and the top 120 genes (where applicable) were used to identify the most important differentially expressed genes in response to drought in both DEG and KEGG results. BLAST searches were carried out against the *Arabidopsis thaliana*, *Vigna unguiculata*, *Glycine max*, and *Phaseolus vulgaris* genomes, in the case of Arabidopsis. The majority of genes were found in the Arabidopsis genome, thus the TAIR website was used (https://www-arabidopsis-

org.ezproxy.nottingham.ac.uk/Blast/) and only drought related genes reported in the literature were highlighted.

Statistical analysis of the physiological traits was performed using Genstat 20th edition (VSN International) and GraphPad Prism 9.

Results and discussion

Plant material and stress assessment

Although there were variations in the soil moisture loss among pots, as illustrated in Figure 5-, there was 10 - 20 % reduction in the soil moisture

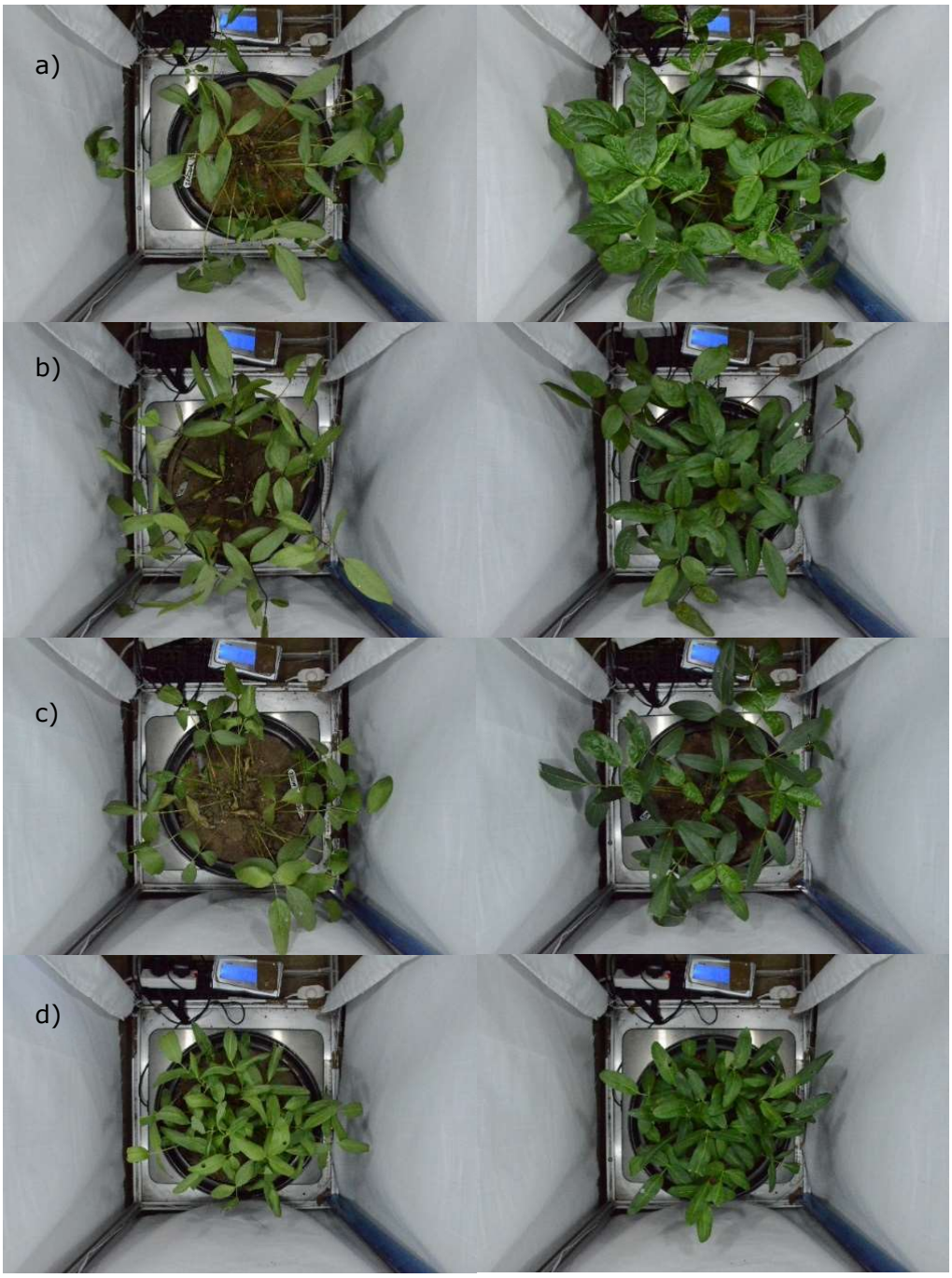

Figure 5-3 Visual effects of drought in Bambara groundnut at 15 days without irrigation. Left side is the drought treatment, while right side is the irrigated control. Genotypes are a) Gresik, b) DodR, c) S19-3, and d) TN.

in the treatments as compared to their control counterparts, after 11 days

of water being withheld in the treatment samples. The water content in the soil between both treatments was significantly different ($p<0.001$) and the effects on the plants are illustrated in Figure 5-. The maximum efficiency of PSII equally showed the initial impacts of drought stress as shown in Figure 5- and Figure 5-.

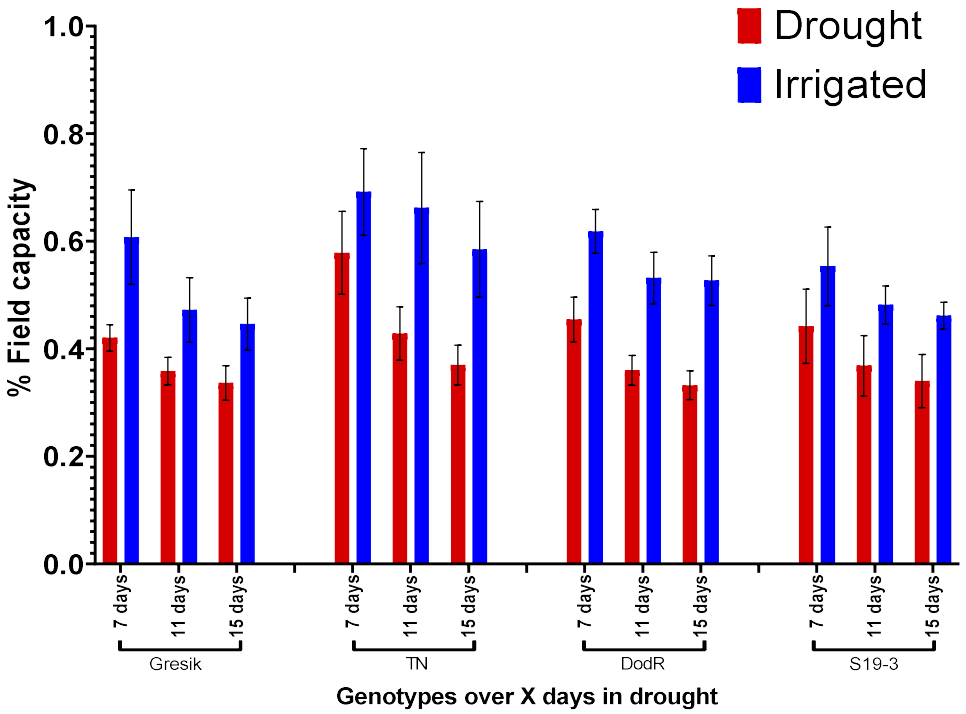

Figure 5-4 Percentage of field capacity on 4 different genotypes under irrigation and non-irrigated conditions. Measurements on day 7, 11 and 15.

After 11 days of water deficit stress, all four genotypes showed a significant decrease of leaf area greenness in comparison with their control sets ($P<0.001$), illustrated in Figure 5-. These observations were further confirmed by the maximum efficiency of PSII, equally demonstrated the initial effects of drought stress as shown in Figure 5- and Figure 5-. Although the soil moisture was less than 50% field capacity in the irrigated Gresik and S19-3 genotypes, the maximum efficiency of the PSII (Figure 5-) indicated the absence of stress.

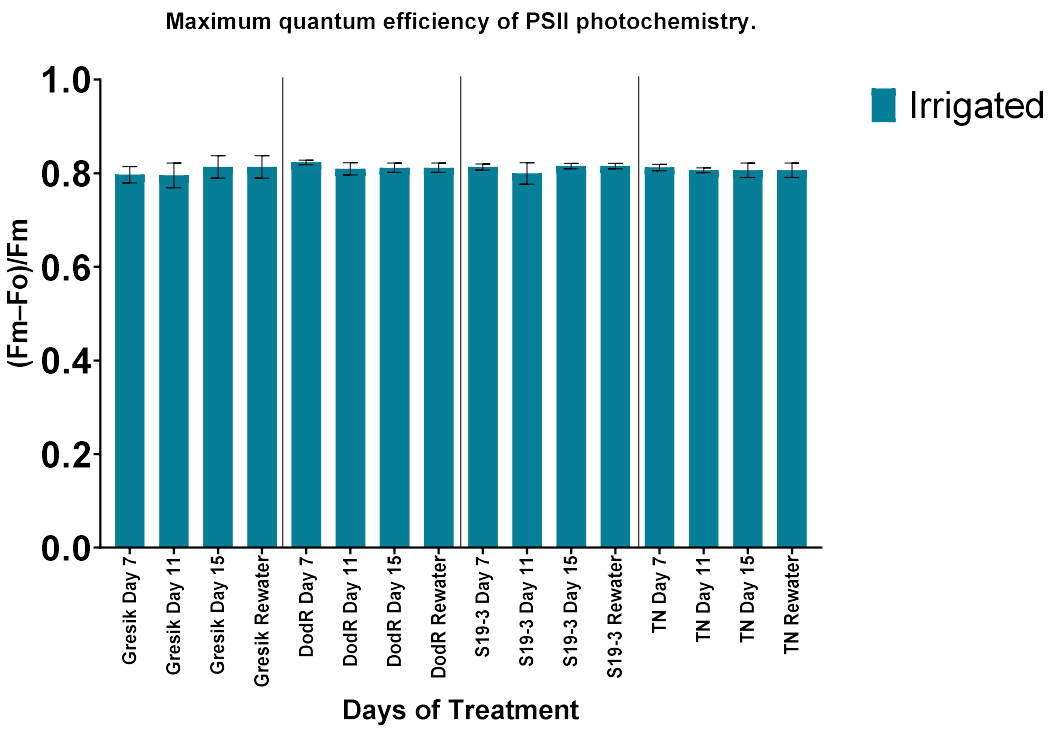

Figure 5-5 Maximum efficiency of the PSII photochemistry over time in four different genotypes after 30 min in dark adaptation. There is not a significant difference during the drought between irrigated genotypes (0.78 ± 0.05, $P<0.58$).

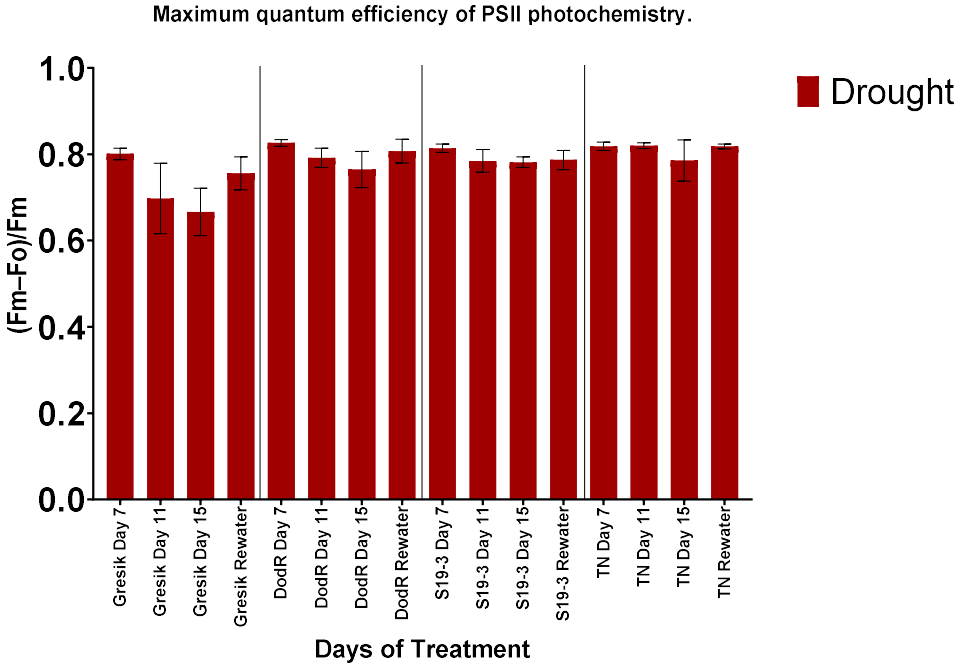

Figure 5-6 Maximum efficiency of the PSII photochemistry over time in four different genotypes after 30 min in dark adaptation. There is a significant difference during drought between Gresik and the other genotypes ($P<0.01$).

The plants behaved as expected, showing the same behaviour as described in Salazar *et al.* (2021, Chapter 4). It is important to determine the best

time point to harvest leaf tissue for RNA isolation. Our results suggest that 11 days of drought could be an adequate time point for leaf harvesting, where the drought effect is significantly detected (Treatment $P<0.01$; Genotype $P<0.01$, and interaction $P<0.01$), however, not reaching to the point of reducing the quality of the tissue in those less resistant genotypes, where in previous studies (Chapter 3) these less resistant genotypes have reached terminal damage at 15 days in drought. Additionally, this time point may allow enough drought effect to be expressed in the more resistant genotypes. Even though there were initial problems with 3 samples, these were substituted with new isolated RNA, having all 32 samples passing the quality control test by Novogene.

RNA isolation and quality check

All the samples selected for RNAseq are presented in Figure 5-. Samples 29 to 31 were re-extracted, due to quality issues. Final assessment and concentrations are presented in Table 14.

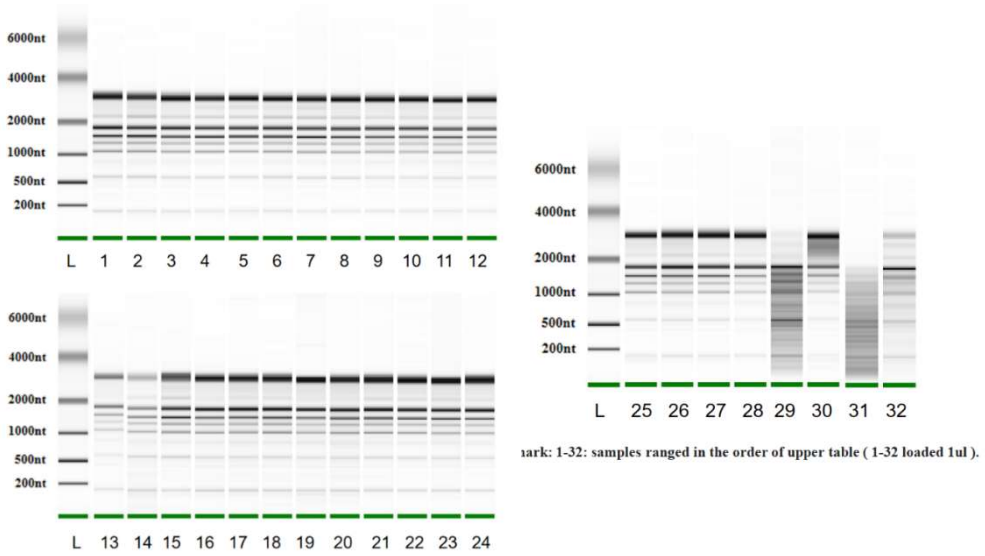

Figure 5-7 Quality control using Bioanalyzer. Sample names are according to Table 14.

Table 11 Details of the different biological samples after quality check upon reception.

N	Sample Name	Concentration (ng/ul)	Volume(ul)	Total amount (ug)	Integrity value	Sample QC Results	Sample QC Memo
1	1DTN	190	13	2.47	7.2	P	/
2	3ITN	196	13	2.548	7.1	P	/
3	4DTN	140	13	1.82	7.6	P	/
4	4ITN	110	14	1.54	7.4	P	/
5	5ITN	133	14	1.862	7.5	P	/
6	5DTN	127	14	1.778	7.3	P	/
7	6ITN	129	14	1.806	7.5	P	/
8	6DTN	132	14	1.848	7.6	P	/
9	1IDodR	132	14	1.848	7.6	P	/
10	5IDodR	118	12	1.416	7.7	P	/
11	6IDodR	124	13	1.612	7.5	P	/
12	5DS19-3	113	12	1.356	7.9	P	/
13	6DS19-3	43	12	0.516	7.9	P	/
14	1IS19-3	42	12	0.504	7.3	P	/
15	5IS19-3	59	7	0.413	7.5	P	/
16	2DGresik	152	14	2.128	8	P	/
17	4DGresik	133	11	1.463	7.8	P	/
18	2IGresik	128	12	1.536	7.2	P	/
19	5DGresik	88	8	0.704	7.6	P	/
20	3DGresik	169	14	2.366	7.1	P	/
21	1DDodR	117	11	1.287	7.4	P	/
22	3DDodR	121	10	1.21	7.7	P	/
23	4DDodR	98	10	0.98	7.6	P	/
24	5DDodR	96	9	0.864	8	P	/
25	1IGresik	83	9	0.747	7.4	P	/
26	2DS19-3	200	17	3.4	7.2	P	/
27	4DS19-3	147	16	2.352	7.6	P	/
28	4IS19-3	138	16	2.208	8.2	P	/
29	5IGresik	191	9	1.719	2.8	F	Unqualified RIN
30	6IGresik	102	9	0.918	7.3	P	/
31	4IDodR	125	3	0.375	2.2	F	Unqualified RIN,
32	3IS19-3	103	14	1.442	5.9	P	/
1	3IS19-3y	171	17	2.907	8.5	P	/
2	5IGresiky	112	16	1.792	8.7	P	/
3	4IDodRy	127	16	2.032	7.9	P	/

Bioinformatic analysis

This is the first Bambara groundnut RNAseq experiment reported in the literature to the author's best knowledge and the results shared by Novogene generated a total of 298 Gb of data, which additionally has been shared and used for the assembly and annotation of the Bambara groundnut genome. A Venn diagram in Figure 5- shows the number of genes commonly or uniquely expressed by each individual genotype in response to drought. Specifically, TN, has a larger number of DEG uniquely expressed in response to the drought treatment (irrigated vs drought, 1848 genes; with a minimum fold-change of 0.20/-0.20, a maximum of 8.68/-8.68, and an average of 1.29/-1.29; and an adjusted *P* value lower than 0.05), while S19-3 has the lowest number of differentially expressed genes that are unique to that genotype (174 genes). From a molecular to physiological perspective, Figure 5- may provide some support that each genotype may have different or a combination of different mechanisms to cope with drought, as only 16 genes were commonly differentially expressed in all four genotypes in response to drought (minimum fold-change of 0.20/-0.20, a maximum of 8.68/-8.68, and an average of 1.29/-1.29; and an adjusted *P* value lower than 0.05). In response to drought, a total of 506 genes commonly differentially expressed

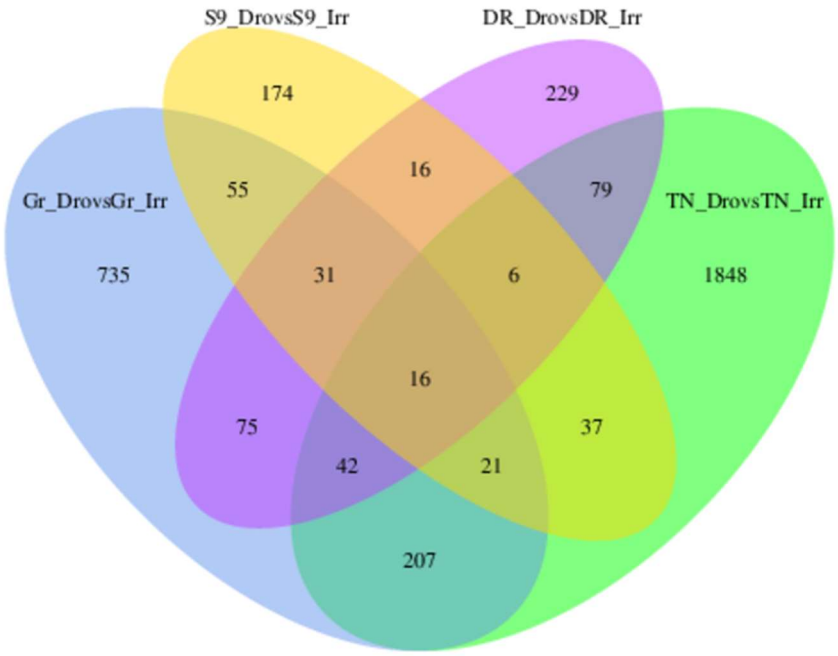

Figure 5-8 Venn diagram showing the common and differentially expressed genes in response to drought per genotype. Genotypes are shown as Gr (Gresik), S9 (S19-3), DR (DodR), and TN.

among the different genotypes, compared to the total of 2,989 differently expressed genes exclusively within individual genotypes in response to drought ($P < 0.05$; FC > 0.2, and FC < -0.2).

Overall, this leads to a higher number of genes found in comparison to the Affymetrix results reported by Khan *et al.* 2017.

In Figure 5, the volcano plots give a clear representation of the significance in the gene expression in response to drought in each genotype; meanwhile, Figure 5-10 shows the number of gene counts significantly differently expressed (adjusted $p < 0.05$) in response to drought in the different genotypes, showing TN with the highest number of DEG while DodR, Gresik, and S19-3 had a comparatively similar count. A larger proportion of genes are downregulated compared to upregulated genes. Interestingly, TN shows almost a balance between down and upregulated genes. Khan *et al*. (2017) also reported more down regulated than up regulated genes in response to drought.

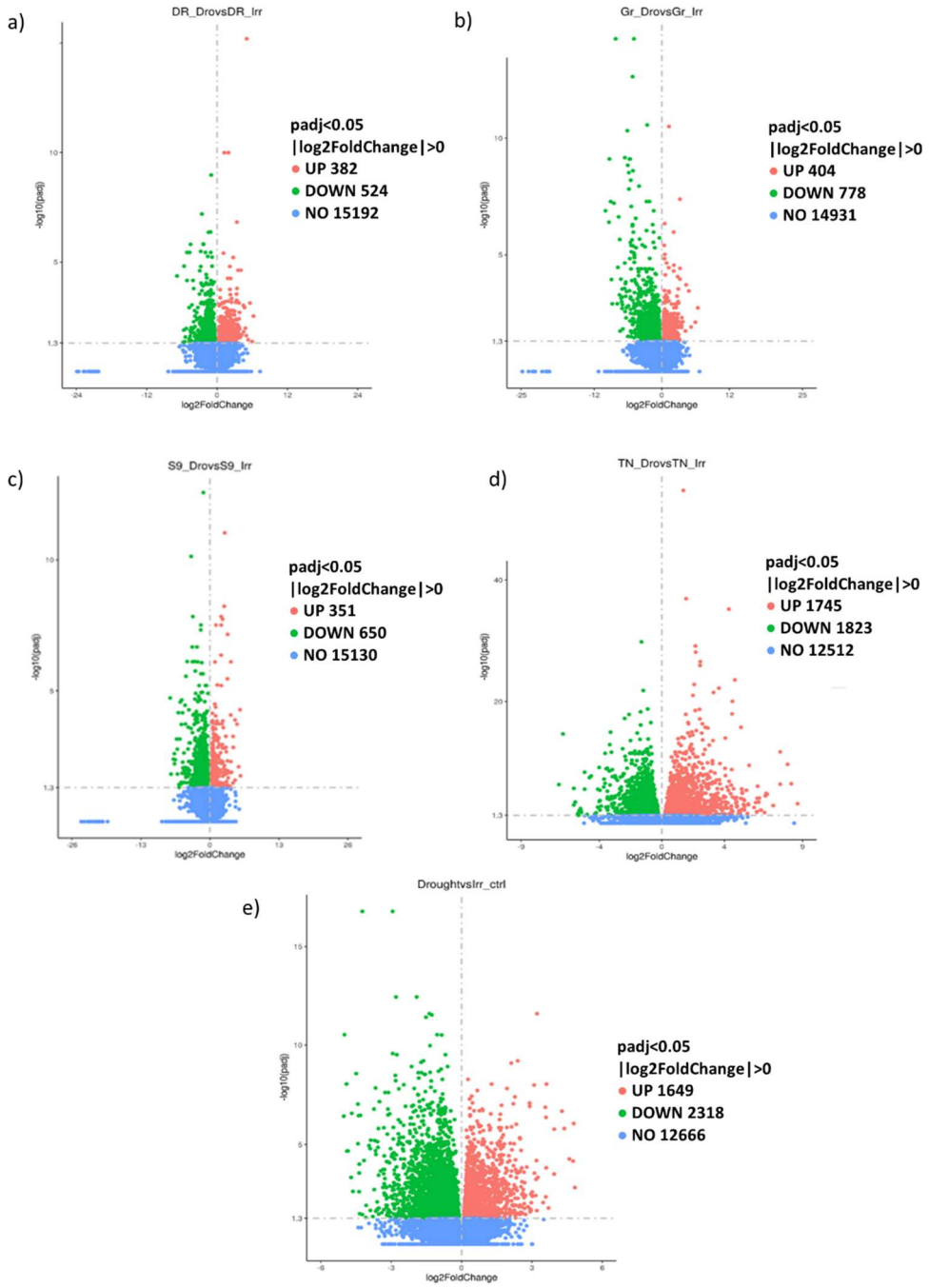

Figure 5-8 Volcano plots of DEG in response to drought in all genotypes. a) DodR; b) Gresik; c) S19-3; d) TN; e) All drought vs All Irrigated

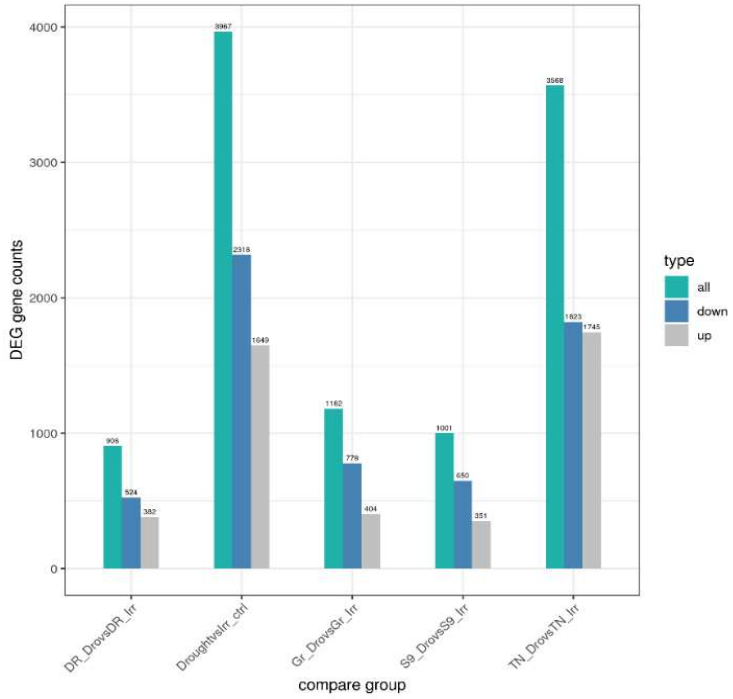

Figure 5-9 Number of DEG counts in response to drought. Sets of columns from left to right: DodR, All Drought vs All Irrigated, Gresik, S19-3, and TN.

The combination of Differentially Expressed Genes (DEG) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) revealed a list divided between Tables 15 to 21, of genes of interest that have been reported in other species as drought related. This list shows genes involved in sugar production, cuticular waxes, different phytohormonal pathways such as cytokinins, strigolactones, ABA, brassinosteroids, ethylene, and auxin. An interesting case was *BRI1*, which is a gene related to the signalling of brassinosteroids, which has been suggested to have an important role in drought tolerance by regulating expression of key drought-responsive genes (Feng *et al*., 2015), and in our results it was up regulated by S19-3 but down regulated by TN. There were additionally a series of drought-resistant related genes differently expressed in genotypes from the more drought resistant group (DodR, S19-3, and TN), but not expressed in Gresik (less resistant), and shown in Table 15. Raffinose synthase 5 was up regulated in DodR and TN, meanwhile Raffinose synthase 4 was upregulated in Gresik, TN, and all irrigated genotypes. Cytokinin related genes were only shown in the more resistant group, while genes shared with Gresik were more ABA related. Additionally, Ethylene response factor 1 and 1B were shown to be down regulated in Gresik, although upregulated in S19-3. These response factors have been reported to help in drought stress enhancement due to its

involvement in both ethylene and jasmonic acid signalling pathways (Cheng *et al*., 2013; Lestari *et al*., 2018; Sengupta *et al*., 2020).

Table 12 DEG of Bambara groundnut in response to drought. Genotype abbreviation goes as follows: DodR(D), Gresik (G), S19-3 (S), TN (TN), and all irrigated (AI).

Vs Gene	Homologous Gene	Chromosome	Fold Change	Expression regulation				Gene name	Summary	Reference
				D	G	S	T			
Vs107692g0034	AT1G77920.1	N/F	-0.80/-0.73/-0.83/-0.65	D	D	D		TGACG SEQUENCE-SPECIFIC BINDING PROTEIN 7	In Arabidopsis thaliana, TGA7 may respond to plant drought stress by negatively regulating the expression of downstream gene AtBGI.	Chen *et al.*, 2021
Vs108114g0057	AT1G03055.1	Vs01	-3.84/-1.74		D		D	DWARF27	The Arabidopsis ortholog of rice DWARF27 acts upstream of MAX1 in control of plant development by strigolactones acting as positive regulator of plant responses to drought and salt stress, which was associated with shoot-related traits.	Ha *et al.*, 2014; Waters *et al.*, 2012
Vs106038g0014	AT1G15080.1	Vs02	-3.79/-1.37		D		D	LIPID PHOSPHATE PHOSPHATASE 2, PHOSPHATIDIC ACID PHOSPHATASE 2	AtLPP2 is a part of ABA signalling and participate to the regulation of stomatal movements.	Katagiri *et al.*, 2005; Paradis *et al.*, 2011
Vs107617g0131	AT1G23800.1	Vs04	-3.18/-1.84/-1.68		D	D		ALDEHYDE DEHYDROGENASE 2B, ALDEHYDE DEHYDROGENASE 2B7	The Arabidopsis histone deacetylase 6 (HDA6) mutant exhibits increased tolerance to drought stress by negatively regulating the expression of ALDH2B7 and PDC1.	Rasheed *et al.*, 2018; Tola *et al.*, 2021
Vs107734g0027	AT2G31230.1	Vs07	-1.69		D			ETHYLENE-RESPONSIVE ELEMENT BINDING FACTOR 15	Transgenic lines were hypersensitive to high salinity and high osmolarity at the seedling establishment stage, and the transgenic seedlings were drought-tolerant. Collectively, data suggest that AtERF15 is a positive regulator of ABA response.	Kang *et al.*, 2014
Vs103800g0098	AT3G56850.1	Vs07	-2.36/-1.24/-1.64		D		D	ABA-RESPONSIVE ELEMENT BINDING PROTEIN 3, DPBF3	Indication that ADF5 participates in drought stress by regulating stomatal closure and may also serve as a potential downstream target of the drought stress/ABA signaling pathway via members of the ABF/AREB transcription factors family. DPBF3 expressed mainly in leaves.	Fujita *et al.*, 2005; Qian *et al.*, 2019
Vs000363g0054	AT4G01970.2 Phvul.001G214300 Glyma.19G217700	Vs08	2.23/3.48/1.25		U		U	ATSTS, RAFFINOSE SYNTHASE 4, STACHYOSE SYNTHASE	Raffinose appeared in drought stressed *AtRS4,5* plants, but not under other abiotic stress conditions. Drought stress leads to novel transcripts of raffinose synthase 6 suggesting that this isoform is a further stress inducible raffinose synthase in *Arabidopsis*.	Gangl & Tenhaken, 2016

Table 13 DEG significantly expressed in DodR in response to drought.

Vs Gene	At Gene	Chromosome	Fold Change	Expression	Gene name	Summary	Reference
Vs000353g0050	AT4G20780.1	Vs05	2.75	Up	CALMODULIN LIKE 42	CML42 negatively regulates ABA levels upon drought stress. It was shown that both CML37 and CML42 are involved in drought stress response but show antagonistic effects.	Scholz *et al.*, 2015; Vadassery *et al.*, 2012
Vs001305g0327	AT5G13630.2	Vs06	-2.2	Down	CYTOKININ OXIDASE/DEHYDROGENASE 1	The overexpression of AtCKX1 and AtCKX3 resulted in the enhancement of root elongation and lateral root development, and leaf mineral enrichment, as well as drought tolerance in the transgenic Arabidopsis and tobacco (Nicotiana tabacum)	Hai *et al.*, 2020; Macková *et al.*, 2013
Vs108544g0010	AT3G12120.2	Vs07	2.03	Up	FATTY ACID DESATURASE 2	The activity of FAD2 leads to an increase in the content of dienoic fatty acids, and hence increases the resistance toward cold and salt stress.	Dar *et al.*, 2017

Table 14 DEG in Gresik in response to drought.

Vs Gene	Homologous Gene	Chromosome	Fold Change	Expression	Gene name	Summary	Reference
Vs108470g0009	AT4G25700.1 Glyma.16G179100	sf108470	-0.55	Down	BETA CAROTENOID HYDROXYLASE 1, BETA-HYDROXYLASE 1, CHY1	Production of zeaxanthin, the first oxygenated carotenoid, is catalyzed by β-carotene hydroxylases encoded by two homologous genes (BCH1 and SCH2) in Arabidopsis and many other species. Overexpression of ZEP in transgenic plants conferred greater tolerance to salt and drought stress, indicating that this enzyme may be limiting for some stress responses.	Du *et al.*, 2010; Finkelstein, 2013
Vs107794g0148	AT4G29080.1	Vs01	-0.56	Down	INDOLE-3-ACETIC ACID INDUCIBLE 27, PHYTOCHROME-ASSOCIATED PROTEIN 2	In white clover, exogenous IAA improved drought tolerance possibly due to endogenous plant hormone concentration changes (such as IAA27) and modulation of genes involving in drought stress response and leaf senescence.	Gadallah, 2000; Zhang *et al.*, 2020
Vs001305g0135	AT3G23240.1	Vs06	-2.63	Down	ETHYLENE RESPONSE FACTORS 1,1B;	Overexpression of a number of ERFs enhances salt, drought, light stress, and cold and heat tolerance, as well as pathogen resistance in Arabidopsis plants. ERF1 is involved in both ethylene and jasmonic acid signaling pathways. Moreover, plants that overexpress ERF1 enhance ABA levels under drought stress, indicating that ERF1 may regulate ABA biosynthesis.	Cheng *et al.*, 2013; Lestari *et al.*, 2018; Sengupta *et al.*, 2020
Vs107784g0069	AT3G52990.1	Vs07	-0.77	Down	Pyruvate kinase family protein	Pyruvate kinase participates in glycolysis and is very important for the regulation of pyruvate metabolic pathway under stress conditions.	Li *et al.*, 2016

Table 15 DEG in S19-3 in response to dought.

Vs Gene	At Gene	Chromosome	Fold Change	Expression	Gene name	Summary	Reference
Vs107907g0180	AT3G23240.1	Vs07	1.52	Up	ETHYLENE RESPONSE FACTORS 1,1B;	Overexpression of a number of ERFs enhances salt, drought, light stress, and cold and heat tolerance, as well as pathogen resistance in Arabidopsis plants. ERF1 is involved in both ethylene and jasmonic acid signaling pathways. Moreover, plants that overexpress ERF1 enhance ABA levels under drought stress, indicating that ERF1 may regulate ABA biosynthesis.	Cheng *et al*., 2013; Lestari *et al*., 2018; Sengupta *et al*., 2020
Vs105183g0062	AT2G28630.2	Vs11	-4.11	Down	3-KETOACYL-COA SYNTHASE 12	KCS12 and KCS3, which showed higher expression in stem epidermis than in stem, might be involved in cuticular wax biosynthesis. Other KCS are more related, than KCS12	Kim *et al*., 2013; Yang *et al*., 2020

Table 16 DEG in TN in response to drought

Vs Gene	Homologous Gene	Chromosome	Fold Change	Expression	Gene name	Summary	Reference
Vs005417g01 31	AT5G6514 0.1	Vs01	2.5	Up	TREHALOSE-6-PHOSPHATE PHOSPHATASE J	Trehalose-6-phosphate phosphatase (TPP) deficient Arabidopsis plants showed hypersensitiviness to salinity stress, while overexpressions showed higher tolerance in correlation with high starch levels and increased accumulation of trehalose, sucrose, and total soluble sugars under drought conditions; these compounds may play a role in scavenging reactive oxygen species.	Eastmond *et al*., 2003; Krasensky *et al*., 2014; Lin *et al*., 2019
Vs107719g01 18	AT1G14520.2	Vs01	2.5	Up	MYO-INOSITOL OXYGENASE 1	Transgenic rice lines overexpressing OsMIOX showed a specific function in drought stress tolerance by decreasing oxidative damage.	Duan *et al*., 2012
Vs107787g00 70	AT3G30180.1 Phvul.004G04 1700 Glyma.13G052 900	Vs03	2.74	Up	BRASSINOSTEROID-6-OXIDASE 2, CYP85A2	In A. thaliana, reduced BR accumulation, due to either loss of CYP85A2 activity or CYP85A farnesylation, increased drought tolerance. Lack of active BR molecules in maize causes a pleiotropic effect on plant development and improves seedling tolerance of drought.	Castorina *et al*., 2018
Vs105693g00 45	AT5G35750.1	Vs08	-0.45	Down	HISTIDINE KINASE 2	The loss of function ahk2, ahk3 single mutants and ahk2-ahk3 double mutants displayed strong tolerance toward drought and salinity indicating that AHK2 and AHK3 function as negative regulators of salt and osmotic stress.10	Nongpiur *et al*., 2012
Vs108592g00 20	AT1G01120.1	Vs09	1.89	Up	3-ketoacyl-CoA synthase 1	Overexpression of AhKCS1 in transgenic groundnut plants exhibited an increase in the cuticular wax content, reduction of water loss, lower membrane damage, decreased MDA content, and high proline content compared to that of non-transgenic groundnut plants.	L. Chen *et al*., 2020; Lokesh *et al*., 2019

Table 17 DEG in all genotypes in irrigated vs drought.

Vs Gene	Homologous gene	Chromosome	Fold Change	Expression	Gene name	Summary	Reference
Vs107894g0090	AT3G17600.1 Glyma.13g117100	Vs01	-1.5	Down	INDOLE-3-ACETIC ACID INDUCIBLE 31	In white clover, exogenous IAA improved drought tolerance possibly due to endogenous plant hormone concentration changes (such as IAA27) and modulation of genes involving in drought stress response and leaf senescence.	Gadallah, 2000; Youzhi Zhang *et al*., 2020
Vs000715g0013	AT2G33310.1	Vs03	-1.5	Down	AUXIN-INDUCED PROTEIN 13	In this study, gene enrichment analysis showed the number of genes contributing to the growth under drought stress related to auxin hormones including auxin-induced protein (IAA13, IAA16, IAA33), auxin response factor (ARF-1,9,11,19)	Sarwar *et al*., 2019

Table 18 DEG of Bambara groundnut in response to drought. Genotype abbreviation goes as follows: DodR(D), S19-3 (S), TN (TN), and all irrigated (AI).

Vs Gene	Homologous Gene	Chromosome	Fold Change	Expression regulation				Gene name	Summary	Reference
				D	S	T	AI			
Vs006039g0001	AT2G41510.1	sf6039	-4.05/-2.16	D	D			CYTOKININ OXIDASE/DEHYDROGENASE 1	The overexpression of *AtCKX1* and *AtCKX3* resulted in the enhancement of root elongation and lateral root development, and leaf mineral enrichment, as well as drought tolerance in the transgenic Arabidopsis and tobacco (*Nicotiana tabacum*).	Hai *et al*., 2020; Macková *et al*., 2013
Vs107959g0025	AT4G17870	Vs02	-1.90/-1.79			D	D	PYRABACTIN RESISTANCE 1, REGULATORY COMPONENT OF ABA RECEPTOR 11	The overexpression of *PtPYRL1* and *PtPYRL5* substantially improved ABA sensitivity and drought stress tolerance in transgenic plants.	Takahashi *et al*., 2016
Vs000114g0036	AT1G10370.1	Vs02	2.19/2.14	U		U		CYTOKININ OXIDASE/DEHYDROGENASE 1	The overexpression of *AtCKX1* and *AtCKX3* resulted in the enhancement of root elongation and lateral root development,	Hai *et al*., 2020; Macková *et al*., 2013

Vs000719g0218	AT1G11600.1	Vs03	0.90/1.68	U	U	CYTOCHROME P450, FAMILY 77 SUBFAMILY B POLYPEPTIDE 1	and leaf mineral enrichment, as well as drought tolerance in the transgenic Arabidopsis and tobacco (*Nicotiana tabacum*). Cytochromes P450 are involved in biosynthesis or catabolism of all hormone and signalling molecules, of pigments, odorants, flavours, antioxidants, allelochemicals and defence compounds, and in the metabolism of xenobiotics.	Ma *et al*., 2010; Magwanga *et al*., 2019
Vs108004g0016	AT5G40390.1 Glyma.05G003900 Phvul.004G007100	Vs04	1.95/2.55	U	U	RAFFINOSE SYNTHASE 5	RAFS in maize and its product raffinose contributes to plant drought tolerance. *ZmRAFS* overexpression in Arabidopsis enhanced drought stress tolerance by increasing myo-inositol levels via *ZmRAFS*-mediated galactinol hydrolysis in the leaves due to sucrose insufficiency in leaf cells.	T. Li *et al*., 2020

Vs000353g0064	AT2G45400.1 Glyma.18G220600	Vs05	2.82/-1.35		U	D		BRI1-5 ENHANCED 1	In *Brachypodium*, *BRI1* RNAi mutants exhibited enhanced drought tolerance, accompanied by highly elevated expression of drought-responsive genes, *BdP5CS*, *BdCOR47*/*BdRD17*, suggesting that BR signalling plays an important role in drought tolerance by directly regulating expression of key drought-responsive genes.	Feng *et al*., 2015
Vs001305g0042	AT3G21510.1	Vs06	-4.17/-1.55		D		D	HISTIDINE-CONTAINING PHOSPHOTRANSMITTER 1	Plants carrying the *AHK5* loss-of-function alleles of the AHP genes have defects in stomatal closure in response to ethylene and H2O2.	Mira-Rodado *et al*., 2012
Vs107736g0002	AT3G06120.1	Vs08	-3.2/-3.1	D		D		MUTE	Basic Helix-Loop-Helix Transcription Factor, involve in stomata development and ABA signalling	Castilhos *et al*., 2014
Vs000363g0125	AT1G09530.5	Vs08	2.31/1.34	U		U		PHOTOCURRENT 1, PHYTOCHROME INTERACTING FACTOR 3, PHYTOCHROME-ASSOCIATED PROTEIN 3	Phytochrome-interacting factor 3 (*PIF3*) expressed in maize leaves increased relative water content,	Gao *et al*., 2015

Vs105183g0062	AT2G28630.2	Vs11	-4.11/-1.10	D	D	CYTOKININ OXIDASE/DEHYDROGENASE 1	chlorophyll content, and chlorophyll fluorescence, as well as significantly enhanced cell membrane stability under stress conditions. The over-expression of *ZmPIF3* increased the expression of some stress-responsive genes. The overexpression of *AtCKX1* and *AtCKX3* resulted in the enhancement of root elongation and lateral root development, and leaf mineral enrichment, as well as drought tolerance in the transgenic Arabidopsis and tobacco (Nicotiana tabacum)	Hai *et al*., 2020; Macková *et al*., 2013

As pointed out previously, Bambara groundnut, as an underutilised crop, lacks research in comparison to most mainstream crops, reflecting the scare genetic information available, making it difficult to understand how DEGs might interact in certain pathways. To overcome this, it has been reported by Ho *et al*. (2017) the conserved synteny between *Phaseolus vulgaris* (a more researched crop, with more genetic information available) and Bambara groundnut, thus allowing us to use pathways reported in *Phaseolus vulgaris* to further understand the relationship of several of our DEG (Figure 5- and Appendix 3 Figure 0- to Figure 0-. The main pathways were: plant hormonal signal transduction (Appendix 3 Figure 0-); ascorbate and aldarate metabolism (Appendix 3 Figure 0-); glycolysis/gluconeogenesis (Appendix 3 Figure 0-); inositol phosphate metabolism (Appendix 3 Figure 0-); galactose metabolism (Appendix 3 Figure 0-); and carotenoid biosynthesis (Appendix 3 Figure 0-). In these figures, the downstream effects of down or up regulated genes is shown.

Several genes were found in these pathways as differentially expressed between drought and control in the different genotypes, however, only the circle depicted in red passed multiple filters explained previously for drought related expression genes. As described previously, indole acetic acid (IIA), and Ethylene response factors 1 and 1b could be related to leaf senescence and stress responses to drought (Cheng *et al*., 2013; Lestari *et al*., 2018; Sengupta *et al*., 2020), which is consider a drought avoidant response; in the case of *IIA31*, this gene was found also in common bean and soybean as response to stress, which correlates with our findings. Similar is the case of the ABF transcription factor participates in the closure of stomata (Fujita *et al*., 2005; Qian *et al*., 2019) as part of drought avoidance, while Raffinose synthases 4 and 5 are part of the galactinol hydrolysis, and thus, increasing myo-inositol (similar case with *MYO-INOSITOL OXYGENASE 1*), which are reported to enhance drought tolerance by reducing oxidative damage (Duan *et al*., 2012; Gangl & Tenhaken, 2016; T. Li *et al*., 2020) and our blast results showed, in both Raffinose synthase 4 and 5, a stress protection in common bean, soybean, maize, and arabidopsis.

The results showed a mixture of drought resistant related mechanisms in all four genotypes, suggesting the possibility that Bambara groundnut may implement different actions which could fit as drought tolerant, or drought avoidant mechanisms. However, in the particular case of TN and DodR, they expressed up-regulation of Raffinose synthase 5 (fold change 1.95 and 2.55

respectively), corroborating the findings of Salazar-Licea *et al.* (2021, Chapter 3) where these genotypes have been classified more as drought tolerant.

Interestingly, TN showed down regulation of all the ABA related genes and shared 3 out of 5 with Gresik. In the case of TN, we speculate that this could be due to the amount of water conserved in the soil by the plant as shown in Figure 5- (probably the result of a lower overall loss of water due to transpiration), however, in the case of Gresik (which had a significantly lower water availability in the soil) this could be related to an inability to fully close its stomata, due to its place of origin (Indonesia), where the vapor pressure deficit would be lower due to high humidity, and thus inefficiently controlling the amount of water transpired

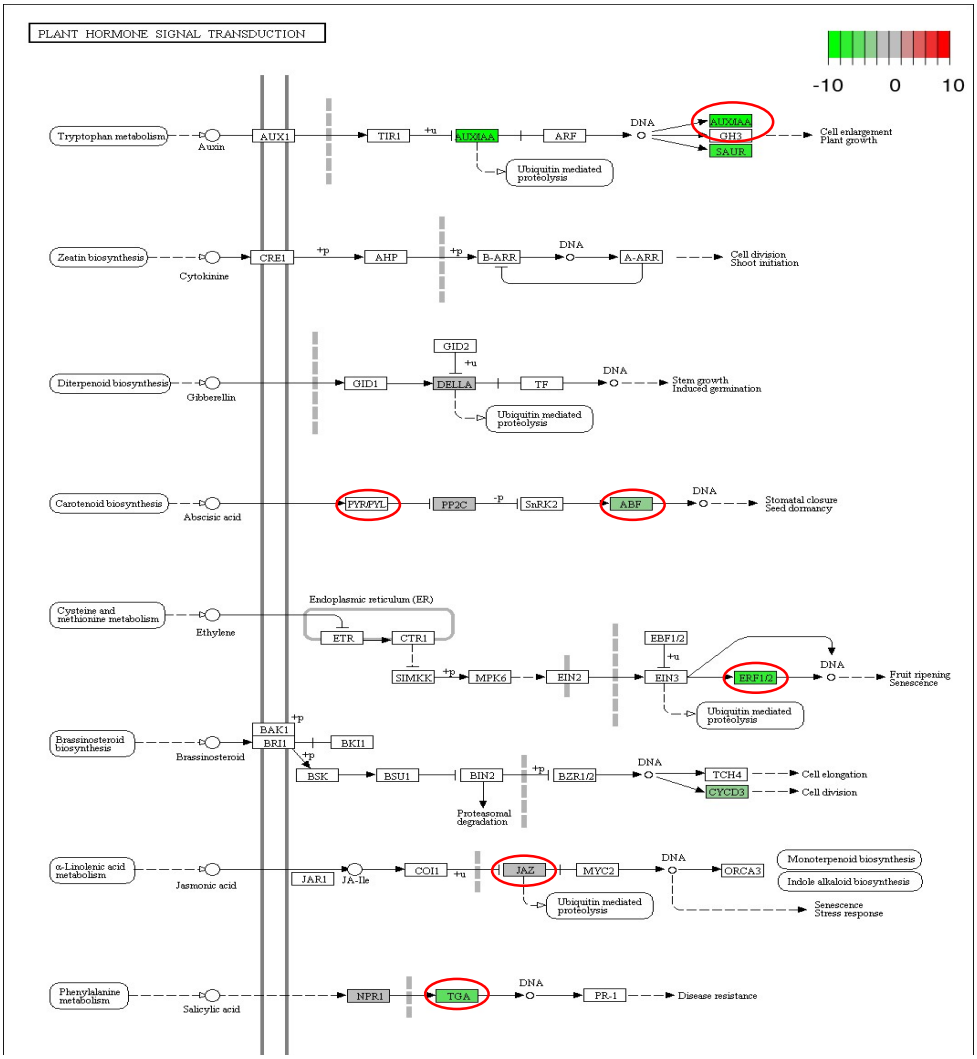

Figure 5-10 Plant hormone signal transduction pathway in common bean. Green colour represents down regulation, and red up regulation. Red circles represent genes of more importance in response to drought according to the filter of DEG and KEGG.

Future work

In order to validate findings from this study and gain more insight how the different genotypes cope with drought further work is required. This could include more in-depth transcriptomic analysis, validation by qPCR, hormonal phenotyping, an increase of less tolerant genotypes for better contrast of gene networks in response to drought, and the addition of more time points to detect early responses to drought at 7 days in drought, and at 1 day after irrigation has been re-introduced, as this could be linked to drought tolerance mechanisms where plants have a better recovery mechanism to survive in the presence of very sporadic rainfalls. These would aid in gaining an even more comprehensive understanding of the responses expressed by Bambara groundnut to drought.

Further detail work should be taken in the case of Raffinose synthases 4 and 5, as we found in the literature a strong correlation in response to drought or other stresses in arabidopsis, maize, common bean, and soybean, thus making it an interesting gene work with.

Additionally, further investigation of the down regulated genes in Gresik would be ideal, as well as to research whether the up regulation of these genes would confer a higher drought resistance, or whether the silencing of these genes would develop a more sensitive plant.

Conclusion

RNAseq has revealed differently expressed genes in response to drought which have been reported in other crop species. Preliminary results suggest that Gresik employs more "drought avoidant" mechanisms to cope with drought, due to the physiological and genetic responses expressed under drought conditions, which can be related to its place of origin in Indonesia. Meanwhile TN, S19-3, and DodR have a more "drought tolerant" behaviour due to different genes expressed related to osmo-protection and security of biological functions under drought stress conditions. If confirmed in

subsequent molecular work, this could aid in breeding programs to target different genes, in pursuit of better adaptation to different drought environmental conditions.

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References

Benjamini, Y., & Hochberg, Y. (1995). Controlling the False Discovery Rate : A Practical and Powerful Approach to Multiple Testing Author (s): Yoav Benjamini and Yosef Hochberg Source : Journal of the Royal Statistical Society . Series B (Methodological), Vol . 57 , No . 1 (1995), Publi. *Journal of the Royal Statistical Society*, *57*(1), 289–300.

Blum, A. (2011). *Plant Breeding for Water-Limited Environments*. Springer New York. https://doi.org/10.1007/978-1-4419-7491-4

Castilhos, G., Lazzarotto, F., Spagnolo-Fonini, L., Bodanese-Zanettini, M. H., & Margis-Pinheiro, M. (2014). Possible roles of basic helix-loop-helix transcription factors in adaptation to drought. *Plant Science*, *223*, 1–7. https://doi.org/10.1016/j.plantsci.2014.02.010

Castorina, G., Persico, M., Zilio, M., Sangiorgio, S., Carabelli, L., & Consonni, G. (2018). The maize lilliputian1 (lil1) gene, encoding a brassinosteroid cytochrome P450 C-6 oxidase, is involved in plant growth and drought response. *Annals of Botany*, *122*(2), 227–238. https://doi.org/10.1093/aob/mcy047

Chang, Y., Liu, H., Liu, M., Liao, X., Sahu, S. K., Fu, Y., Song, B., Cheng, S., Kariba, R., Muthemba, S., Hendre, P. S., Mayes, S., Ho, W. K., Yssel, A. E. J., Kendabie, P., Wang, S., Li, L., Muchugi, A., Jamnadass, R., … Liu, X. (2018). The draft genomes of five agriculturally important African orphan crops. *GigaScience*, *8*(3), 1–16. https://doi.org/10.1093/gigascience/giy152

Chen, L., Hu, W., Mishra, N., Wei, J., Lu, H., Hou, Y., Qiu, X., Yu, S., Wang, C., Zhang, H., Cai, Y., Sun, C., & Shen, G. (2020). AKR2A interacts with KCS1 to improve VLCFAs contents and chilling tolerance of Arabidopsis thaliana. *Plant Journal*, *103*(4), 1575–1589. https://doi.org/10.1111/tpj.14848

Chen, Z., Fang, X., Yuan, X., Zhang, Y., Li, H., Zhou, Y., & Cui, X. (2021). Overexpression of transcription factor gmtga15 enhances drought tolerance in transgenic soybean hairy roots and arabidopsis plants. *Agronomy*, *11*(1). https://doi.org/10.3390/agronomy11010170

Cheng, M. C., Liao, P. M., Kuo, W. W., & Lin, T. P. (2013). The arabidopsis ETHYLENE RESPONSE FACTOR1 regulates abiotic stress-responsive gene expression by binding to different cis-acting elements in response to different stress signals. *Plant Physiology*, *162*(3), 1566–1582. https://doi.org/10.1104/pp.113.221911

Dar, A. A., Choudhury, A. R., Kancharla, P. K., & Arumugam, N. (2017). The FAD2 gene in plants: Occurrence, regulation, and role. *Frontiers in Plant Science*, *8*(October), 1–16. https://doi.org/10.3389/fpls.2017.01789

Du, H., Wang, N., Cui, F., Li, X., Xiao, J., & Xiong, L. (2010). Characterization of the β-carotene hydroxylase gene DSM2 conferring drought and oxidative stress resistance by increasing xanthophylls and abscisic acid synthesis in rice. *Plant Physiology*, *154*(3), 1304–1318. https://doi.org/10.1104/pp.110.163741

Duan, J., Zhang, M., Zhang, H., Xiong, H., Liu, P., Ali, J., Li, J., & Li, Z. (2012). OsMIOX, a myo-inositol oxygenase gene, improves drought tolerance through scavenging of reactive oxygen species in rice (Oryza

sativa L.). *Plant Science*, *196*, 143–151. https://doi.org/10.1016/j.plantsci.2012.08.003

Eastmond, P. J., Li, Y., & Graham, I. A. (2003). Is trehalose-6-phosphate a regulator of sugar metabolism in plants? *Journal of Experimental Botany*, *54*(382), 533–537. https://doi.org/10.1093/jxb/erg039

Fang, Y., & Xiong, L. (2015). General mechanisms of drought response and their application in drought resistance improvement in plants. *Cellular and Molecular Life Sciences*, *72*(4), 673–689. https://doi.org/10.1007/s00018-014-1767-0

Farooq, M., Wahid, A., Basra, N. K. D. F. S. M. a, Fujita, N. K. D., & Plant, S. M. a B. (2009). Plant drought stress : effects , mechanisms and management To cite this version : Review article. *Agronomy for Sustainable Development, Springer Verlag (Germany)*, *29*(1), 185–212. https://doi.org/10.1051/agro:2008021

Feng, Y., Yin, Y., & Fei, S. (2015). Down-regulation of BdBRI1, a putative brassinosteroid receptor gene produces a dwarf phenotype with enhanced drought tolerance in Brachypodium distachyon. *Plant Science*, *234*, 163–173. https://doi.org/10.1016/j.plantsci.2015.02.015

Finkelstein, R. (2013). Abscisic Acid Synthesis and Response. *The Arabidopsis Book*, *11*, e0166. https://doi.org/10.1199/tab.0166

Fujita, Y., Fujita, M., Satoh, R., Maruyama, K., Parvez, M. M., Seki, M., Hiratsu, K., Ohme-Takagi, M., Shinozaki, K., & Yamaguchi-Shinozaki, K. (2005). AREB1 is a transcription activator of novel ABRE-dependent ABA signaling that enhances drought stress tolerance in Arabidopsis. *Plant Cell*, *17*(12), 3470–3488. https://doi.org/10.1105/tpc.105.035659

Gadallah, M. A. A. (2000). Effects of indole-3-acetic acid and zinc on the growth, osmotic potential and soluble carbon and nitrogen components of soybean plants growing under water deficit. *Journal of Arid Environments*, *44*(4), 451–467. https://doi.org/10.1006/jare.1999.0610

Gangl, R., & Tenhaken, R. (2016). Raffinose family oligosaccharides act as galactose stores in seeds and are required for rapid germination of Arabidopsis in the dark. *Frontiers in Plant Science*, *7*(JULY2016), 1–15. https://doi.org/10.3389/fpls.2016.01115

Gao, Y., Jiang, W., Dai, Y., Xiao, N., Zhang, C., Li, H., Lu, Y., Wu, M., Tao, X., Deng, D., & Chen, J. (2015). A maize phytochrome-interacting factor 3 improves drought and salt stress tolerance in rice. *Plant Molecular Biology*, *87*(4–5), 413–428. https://doi.org/10.1007/s11103-015-0288-z

Ha, C. Van, Leyva-Gonzalez, M. A., Osakabe, Y., Tran, U. T., Nishiyama, R., Watanabe, Y., Tanaka, M., Seki, M., Yamaguchi, S., Dong, N. Van, Yamaguchi-Shinozaki, K., Shinozaki, K., Herrera-Estrella, L., & Tran, L. S. P. (2014). Positive regulatory role of strigolactone in plant responses to drought and salt stress. *Proceedings of the National Academy of Sciences of the United States of America*, *111*(2), 851–856. https://doi.org/10.1073/pnas.1322135111

Hai, N. N., Chuong, N. N., Tu, N. H. C., Kisiala, A., Hoang, X. L. T., & Thao, N. P. (2020). Role and regulation of cytokinins in plant response to drought stress. *Plants*, *9*(4), 10–12.

https://doi.org/10.3390/plants9040422

Ho, W. K., Chai, H. H., Kendabie, P., Ahmad, N. S., Jani, J., Massawe, F., Kilian, A., & Mayes, S. (2017). Integrating genetic maps in Bambara groundnut [Vigna subterranea (L) Verdc.] and their syntenic relationships among closely related legumes. *BMC Genomics*, *18*(1). https://doi.org/10.1186/s12864-016-3393-8

Kang, Y. J., Kim, S. K., Kim, M. Y., Lestari, P., Kim, K. H., Ha, B.-K., Jun, T. H., Hwang, W. J., Lee, T., Lee, J., Shim, S., Yoon, M. Y., Jang, Y. E., Han, K. S., Taeprayoon, P., Yoon, N., Somta, P., Tanya, P., Kim, K. S., … Lee, S.-H. (2014). Genome sequence of mungbean and insights into evolution within Vigna species. *Nature Communications*, *5*, 5443. https://doi.org/10.1038/ncomms6443

Katagiri, T., Ishiyama, K., Kato, T., Tabata, S., Kobayashi, M., & Shinozaki, K. (2005). An important role of phosphatidic acid in ABA signaling during germination in Arabidopsis thaliana. *Plant Journal*, *43*(1), 107–117. https://doi.org/10.1111/j.1365-313X.2005.02431.x

Khan, F., Chai, H. H., Ajmera, I., Hodgman, C., Mayes, S., & Lu, C. (2017). A transcriptomic comparison of two Bambara groundnut landraces under dehydration stress. *Genes*, *8*(4), 1–19. https://doi.org/10.3390/genes8040121

Kim, J., Jung, J. H., Lee, S. B., Go, Y. S., Kim, H. J., Cahoon, R., Markham, J. E., Cahoon, E. B., & Suh, M. C. (2013). Arabidopsis 3-ketoacyl-coenzyme a synthase9 is involved in the synthesis of tetracosanoic acids as precursors of cuticular waxes, suberins, sphingolipids, and phospholipids. *Plant Physiology*, *162*(2), 567–580. https://doi.org/10.1104/pp.112.210450

Kim, D., Paggi, J.M., Park, C., *et al.* (2019) Graph-based genome alignment and genotyping with HISAT2 and HISAT-genotype. *Nature Biotechnology,* 37, 907-915.

Kovaka, S., Zimin, A.V., Pertea, G.M., Razaghi, R., Salazberg, S.L., Pertea, M. (2019) Transcriptome assembly from long-read RNA-seq alignments with StringTie2. *Genome Biology*, 20, 278, doi:10.1186/s13059-019-1910-1

Krasensky, J., Broyart, C., Rabanal, F. A., & Jonak, C. (2014). The Redox-Sensitive chloroplast Trehalose-6-Phosphate Phosphatase AtTPPD regulates salt stress tolerance. *Antioxidants \& Redox Signaling*, *21*(9), 1289–1304. https://doi.org/10.1089/ars.2013.5693

Lestari, R., Rio, M., Martin, F., Leclercq, J., Woraathasin, N., Roques, S., Dessailly, F., Clément-Vidal, A., Sanier, C., Fabre, D., Melliti, S., Suharsono, S., & Montoro, P. (2018). Overexpression of Hevea brasiliensis ethylene response factor HbERF-IXc5 enhances growth and tolerance to abiotic stress and affects laticifer differentiation. *Plant Biotechnology Journal*, *16*(1), 322–336. https://doi.org/10.1111/pbi.12774

Li, C., Nong, Q., Solanki, M. K., Liang, Q., Xie, J., Liu, X., Li, Y., Wang, W., Yang, L., & Li, Y. (2016). Differential expression profiles and pathways of genes in sugarcane leaf at elongation stage in response to drought stress. *Scientific Reports*, *6*(December 2015), 1–11. https://doi.org/10.1038/srep25698

Li, T., Zhang, Y., Liu, Y., Li, X., Hao, G., Han, Q., Dirk, L. M. A., Downie, A.

B., Ruan, Y. L., Wang, J., Wang, G., & Zhao, T. (2020). Raffinose synthase enhances drought tolerance through raffinose synthesis or galactinol hydrolysis in maize and Arabidopsis plants. *Journal of Biological Chemistry*, *295*(23), 8064–8077. https://doi.org/10.1074/JBC.RA120.013948

Liao, Y., Gordon, K.S., Shi, W. (2014) featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics*, 30 (7), 923-930, https://doi-org.nottingham.idm.oclc.org/10.1093/bioinformatics/btt656

Lin, Q., Yang, J., Wang, Q., Zhu, H., Chen, Z., Dao, Y., & Wang, K. (2019). Overexpression of the trehalose-6-phosphate phosphatase family gene AtTPPF improves the drought tolerance of Arabidopsis thaliana. *BMC Plant Biology*, *19*(1), 1–15. https://doi.org/10.1186/s12870-019-1986-5

Liu, L., Li, Y., Li, S., Hu, N., He, Y., Pong, R., Lin, D., Lu, L., & Law, M. (2012). Comparison of next-generation sequencing systems. *Journal of Biomedicine and Biotechnology*, *2012*. https://doi.org/10.1155/2012/251364

Lokesh, U., Venkatesh, B., Kiranmai, K., Nareshkumar, A., Amarnathareddy, V., Rao, G. L., Johnson, A. M. A., Pandurangaiah, M., & Sudhakar, C. (2019). Overexpression of ß-Ketoacyl co-a synthase1 gene improves tolerance of drought susceptible groundnut (Arachis hypogaea L.) cultivar K-6 by increased leaf epicuticular wax accumulation. *Frontiers in Plant Science*, *9*(January), 1–13. https://doi.org/10.3389/fpls.2018.01869

Luo, L., Xia, H., & Lu, B. R. (2019). Editorial: Crop breeding for drought resistance. *Frontiers in Plant Science*, *10*(March), 1–2. https://doi.org/10.3389/fpls.2019.00314

Ma, L.-J., van der Does, H. C., Borkovich, K. A., Coleman, J. J., Daboussi, M.-J., Di Pietro, A., Dufresne, M., Freitag, M., Grabherr, M., Henrissat, B., Houterman, P. M., Kang, S., Shim, W.-B., Woloshuk, C., Xie, X., Xu, J.-R., Antoniw, J., Baker, S. E., Bluhm, B. H., Rep, M. (2010). Comparative genomics reveals mobile pathogenicity chromosomes in Fusarium. *Nature*, *464*(7287), 367–373. https://doi.org/10.1038/nature08850

Macková, H., Hronková, M., Dobrá, J., Turečková, V., Novák, O., Lubovská, Z., Motyka, V., Haisel, D., Hájek, T., Prášil, I. T., Gaudinová, A., Štorchová, H., Ge, E., Werner, T., Schmülling, T., & Vanková, R. (2013). Enhanced drought and heat stress tolerance of tobacco plants with ectopically enhanced cytokinin oxidase/dehydrogenase gene expression. *Journal of Experimental Botany*, *64*(10), 2805–2815. https://doi.org/10.1093/jxb/ert131

Magwanga, R. O., Lu, P., Kirungu, J. N., Dong, Q., Cai, X., Zhou, Z., Wang, X., Hou, Y., Xu, Y., Peng, R., Agong, S. G., Wang, K., & Fang, L. (2019). Knockdown of cytochrome P450 genes Gh_D07G1197 and Gh_A13G2057 on chromosomes D07 and A13 reveals their putative role in enhancing drought and salt stress tolerance in Gossypium hirsutum. *Genes*, *10*(3). https://doi.org/10.3390/genes10030226

Mayes, S., Ho, W. K., Chai, H. H., Gao, X., Kundy, A. C., Mateva, K. I., Zahrulakmal, M., Hahiree, M. K. I. M., Kendabie, P., Licea, L. C. S., Massawe, F., Mabhaudhi, T., Modi, A. T., Berchie, J. N., Amoah, S.,

Faloye, B., Abberton, M., Olaniyi, O., & Azam-Ali, S. N. (2019). Bambara groundnut: an exemplar underutilised legume for resilience under climate change. *Planta*, *250*(3), 803–820. https://doi.org/10.1007/s00425-019-03191-6

Mira-Rodado, V., Veerabagu, M., Witthöft, J., Teply, J., Harter, K., & Desikan, R. (2012). Identification of two-component system elements downstream of AHK5 in the stomatal closure response of Arabidopsis thaliana. *Plant Signaling & Behavior*, *7*(11), 1467–1476. https://doi.org/10.4161/psb.21898

Murchie, E. H., & Lawson, T. (2013). Chlorophyll fluorescence analysis: A guide to good practice and understanding some new applications. *Journal of Experimental Botany*, *64*(13), 3983–3998. https://doi.org/10.1093/jxb/ert208

Nongpiur, R., Soni, P., Karan, R., Singla-Pareek, S. L., & Pareek, A. (2012). Histidine kinases in plants: Cross talk between hormone and stress responses. *Plant Signaling and Behavior*, *7*(10), 1230–1237. https://doi.org/10.4161/psb.21516

Paradis, S., Villasuso, A. L., Aguayo, S. S., Maldiney, R., Habricot, Y., Zalejski, C., Machado, E., Sotta, B., Miginiac, E., & Jeannette, E. (2011). Arabidopsis thaliana lipid phosphate phosphatase 2 is involved in abscisic acid signalling in leaves. *Plant Physiology and Biochemistry*, *49*(3), 357–362. https://doi.org/10.1016/j.plaphy.2011.01.010

Pertea G and Pertea M (2020). GFF Utilities: GffRead and GffCompare [version 1; peer review: 3 approved]. *F1000Research*, 9:304. https://doi.org/10.12688/f1000research.23297.1

Qian, D., Zhang, Z., He, J., Zhang, P., Ou, X., Li, T., Niu, L., Nan, Q., Niu, Y., He, W., An, L., Jiang, K., & Xiang, Y. (2019). Arabidopsis ADF5 promotes stomatal closure by regulating actin cytoskeleton remodeling in response to ABA and drought stress. *Journal of Experimental Botany*, *70*(2), 435–446. https://doi.org/10.1093/jxb/ery385

Rao, M. S., Van Vleet, T. R., Ciurlionis, R., Buck, W. R., Mittelstadt, S. W., Blomme, E. A. G., & Liguori, M. J. (2019). Comparison of RNA-Seq and microarray gene expression platforms for the toxicogenomic evaluation of liver from short-term rat toxicity studies. *Frontiers in Genetics*, *10*(JAN), 1–16. https://doi.org/10.3389/fgene.2018.00636

Rasheed, S., Bashir, K., Kim, J. M., Ando, M., Tanaka, M., & Seki, M. (2018). The modulation of acetic acid pathway genes in Arabidopsis improves survival under drought stress. *Scientific Reports*, *8*(1), 1–15. https://doi.org/10.1038/s41598-018-26103-2

Raza, A., Razzaq, A., Mehmood, S. S., Zou, X., Zhang, X., Lv, Y., & Xu, J. (2019). Impact of climate change on crops adaptation and strategies to tackle its outcome: A review. *Plants*, *8*(2). https://doi.org/10.3390/plants8020034

Robinson, M.D., McCarthy, D.J., Smyth, G.K. (2010) edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics*, 26(1), 139-140, doi: 10.1093/bioinformatics/btp616

Sarwar, M. B., Ahmad, Z., Rashid, B., Hassan, S., Gregersen, P. L., Leyva, M. D. la O., Nagy, I., Asp, T., & Husnain, T. (2019). De novo assembly of Agave sisalana transcriptome in response to drought stress provides

insight into the tolerance mechanisms. *Scientific Reports*, *9*(1), 1–14. https://doi.org/10.1038/s41598-018-35891-6

Scholz, S. S., Reichelt, M., Vadassery, J., & Mithöfer, A. (2015). Calmodulin-like protein CML37 is a positive regulator of ABA during drought stress in Arabidopsis. *Plant Signaling and Behavior*, *10*(6), 5–7. https://doi.org/10.1080/15592324.2015.1011951

Sengupta, S., Ray, A., Mandal, D., & Nag Chaudhuri, R. (2020). ABI3 mediated repression of RAV1 gene expression promotes efficient dehydration stress response in Arabidopsis thaliana. *Biochimica et Biophysica Acta - Gene Regulatory Mechanisms*, *1863*(9), 194582. https://doi.org/10.1016/j.bbagrm.2020.194582

Takahashi, Y., Somta, P., Muto, C., Iseki, K., Naito, K., Pandiyan, M., Natesan, S., & Tomooka, N. (2016). Novel genetic resources in the genus vigna unveiled from gene bank accessions. *PLoS ONE*, *11*(1), 1–18. https://doi.org/10.1371/journal.pone.0147568

Tola, A. J., Jaballi, A., Germain, H., & Missihoun, T. D. (2021). Recent development on plant aldehyde dehydrogenase enzymes and their functions in plant development and stress signaling. *Genes*, *12*(1), 1–18. https://doi.org/10.3390/genes12010051

Vadassery, J., Reichelt, M., Hause, B., Gershenzon, J., Boland, W., & Mithöfer, A. (2012). CML42-mediated calcium signaling coordinates responses to Spodoptera herbivory and abiotic stresses in Arabidopsis. *Plant Physiology*, *159*(3), 1159–1175. https://doi.org/10.1104/pp.112.198150

Waters, M. T., Brewer, P. B., Bussell, J. D., Smith, S. M., & Beveridge, C. A. (2012). The Arabidopsis ortholog of rice DWARF27 acts upstream of MAX1 in the control of plant development by Strigolactones. *Plant Physiology*, *159*(3), 1073–1085. https://doi.org/10.1104/pp.112.196253

Xu, Z., Zhou, G., & Shimizu, H. (2010). Plant responses to drought and rewatering. *Plant Signaling & Behavior*, *5*(6), 649–654. https://doi.org/10.4161/psb.5.6.11398

Yang, Z., Yang, X., Dong, S., Ge, Y., Zhang, X., Zhao, X., & Han, N. (2020). Overexpression of β-Ketoacyl-CoA Synthase from Vitis vinifera L. improves salt tolerance in Arabidopsis thaliana. *Frontiers in Plant Science*, *11*(November), 1–11. https://doi.org/10.3389/fpls.2020.564385

Zhang, Youzhi, Li, Y., Hassan, M. J., Li, Z., & Peng, Y. (2020). Indole-3-acetic acid improves drought tolerance of white clover via activating auxin, abscisic acid and jasmonic acid related genes and inhibiting senescence genes. *BMC Plant Biology*, *20*(1), 1–12. https://doi.org/10.1186/s12870-020-02354-y

Zhang, Yue, Zhou, Y., Zhang, D., Tang, X., Li, Z., Shen, C., Han, X., Deng, W., Yin, W., & Xia, X. (2020). PtrWRKY75 overexpression reduces stomatal aperture and improves drought tolerance by salicylic acid-induced reactive oxygen species accumulation in poplar. *Environmental and Experimental Botany*, *176*(May), 104117. https://doi.org/10.1016/j.envexpbot.2020.104117

Chapter 6 – Summary and conclusions

In this chapter, a summary of the knowledge gained throughout the PhD will be described and conclusion drawn. I will also outline other areas where I have been involved which are outside of the scope of the main chapters.

The research carried in this thesis project had the aim to better understand the Bambara groundnut's physiology and genetics in response to drought stress. My personal objectives were to develop better techniques for phenotyping, genotyping, and to develop critical thinking when addressing biological questions in a scientific manner.

Conclusions on chapter one

With the climate changing, and the population increasing, our dependence on a limited number of crops it's not the best strategy for the future of food security. The wonderful crops that are out there, not being utilised or researched, have lots of potential to aid in the quest for more and better food. Bambara groundnut has a natural potential that has not been assess in great detail compared to other crops, which is where I hope to take part in making a positive change with my work presented in this thesis, and hopefully future work.

In terms of background knowledge, the places of origin of Bambara do lead to think of a natural adaptation to harsh conditions, which makes this species very interesting, where the efforts to underutilise it has been growing, slowly, but growing.

Conclusions on chapter two

Following the literature review in previous chapter, this chapter shows a positive step forward in terms of the genetic studies of Bambara groundnut in particular. The ability to have a draft genome, or even full genome sequence, can lead to incredible genetic studies, gene editing techniques, and enhancement of breeding programs. With the reduction of sequencing costs, and the development of new technologies, I do not see how Bambara groundnut won't have a chance in the near future to increase the genetic resources and knowledge to accelerate the improvement of this crop.

I strongly believe we are heading in the correct direction in this topic.

Conclusions on chapter three

In this first experiment chapter, several ideas have materialised due to the experiences observed during the experiments:

There was a clear differentiation between genotypes in response to drought where different genotypes showed different mechanisms to cope with drought. Less tolerant genotypes showed a higher recovery rate once water was re-introduced after 15 days of drought. This could be due to a place of origin with rainfall dispersed over a whole season.

More tolerant genotypes had a lower rate of senescence with a higher water content, this could be due to an early stomata closure. This could be due to a place of origin with rainfall only at the first stages of the biological cycle of Bambara groundnut.

As a general effect, more drought tolerant genotypes had better efficiency in the PSII, lower senescence rate, a higher leaf relative water content, and chlorophyll content. In our 10 L confined set up, this could have promoted a 'above ground' only effect, as the roots wouldn't be able to reach far for extra water, and thus, giving us a specific understanding of what happens above ground, allowing us to understand how leaf related traits behaved during absence of water in the soil. The drought tolerant effects observed in this chapter make sense on a physiology perspective, with the very particular case of S19-3, which in my experience, it doesn't care what happens in the outside, this genotype still lives and produces like nothing changes in the environment. To be able to pinpoint what series of mechanisms take place in a physiological and genetic level take place in s19-3 and the other drought resistant genotypes would be ideal, as this could help other minor crops by translating the outcomes into them (more discussed in chapter five). The results also show interesting differences between the resistant genotypes, in terms of some keeping a smaller canopy, others moving their leaves, and other having possible background processes implemented to reduce oxidative damages, which a genetic cross between these genotypes might be able to help generate a more overall drought resistant genotype, rather than a specific type of drought climate resistant genotype.

The variety of responses by different genotypes encourages to try to think outside of the box and find whether something not reported in other crops/legumes might be happening here as well, which is part of the positive points of working with underutilised species. An idea would be based on the observations of certain mineral content in leaves, which might have a correlation with some biochemical processes (such as potassium).

Further physiological aspects need to be observed in Bambara groundnut that might lead to interesting traits that might help to resist drought, or even reduce the amount of water required, possibly making the use of every drop more efficiently.

Conclusions on chapter four and five

The physiology part of this GWAS chapter showed the potential of our genetic panel to be used for further studies, as there was a highly significant difference between genotypes. It also helped to confirm the general observations found in chapter three as these were conserved. However, it would be very interesting (although very laborious and expensive) to research other traits related to drought resistance and see the different strategies implemented by the different genotypes, to make the best of this diverse panel. If these trait differences could be then mapped against places/climates of origin, if would also give us a better picture of the overall evolution of this crop in response to the different harsh environments.

When comparing to their places of origin and their climates, a possible correlation could be inferred as whether the genotypes with low rainfall might be more tolerant, and thus, try to maintain their biological function throughout the drought period as mentioned above. Where different set of genes expressed might have helped with the protection against stress in a biochemical manner, alluding to the maintenance like if the drought was milder than it really was. These genotypes had a less impact from the drought, but as well from the recovery.

On the other hand, those genotypes, which are more drought avoidant, come from climates with a higher rainfall, spread in a longer period, which could justify the strategy leaf senescence (in presence of drought) and leaf production (in recovery) in a way of conserving water until the next rainfall, which they would expect to come due to the history of their places of origin.

These genotypes suffered a high impact from drought, however, also had the highest recovery rate.

In terms of the genetics, as also discussed in the conclusions of chapter 2, there is a lack of genetic resources, which are been addressed and worked on, however, even though with the reduced genetic resource available, significant markers were found in relationship to traits of interest in drought resistance through our GWAS. Additionally, given the relative short about of genetic data available at present, and the results given by this, when the full genome sequence gets released, this should reveal other genes that might have been skipped or ignored due to the power of the analysis/resources.

As result of our GWAS, homologous genes were found in other species such as common bean, soybean, and cowpea, however, for the exception of 1 gene, the rest of the genes haven't been reported in the literature as researched in response to drought. This could be an opportunity to do some qPCR to test these non-drought-reported genes found in Bambara groundnut for confirmation and then try to translate to other legume crops. In the case of the malate synthase gene found in our GWAS and in soybean, is an interested example of osmo-protectant relate genes upregulated in both species, which could be a similar case with our other possible drought resistant genes found in our analysis.

From our RNAseq experiments, the Raffinose synthase 4 and 5 seem the next step forward, where it would be very interesting to confirm this gene using qPCR, and then see how we could introduce or breed these genes into the less tolerant genotypes, as it seems to be reported in other legumes, in arabidopsis, and maize. This could possible help increase production under drought conditions, as the larger leaf areas of the more drought avoidant genotypes could possibly be conserved due to the osmoprotection of the Raffinose synthase, and thus increasing yield in comparison with smaller canopies of the drought tolerant types.

This could also be the case of leaf senescent related genes, where in the case of Raffinose we are trying to protect from damage, maybe by reducing the expression of *IIA37* we could avoid senescence in the drought avoidant genotypes, although this would have to be paired with ABA related genes to avoid the possible overuse of transpiration as well.

Additionally, once the complete genome is released, a new analysis might reveal even more important genes involved in the drought resistance mechanisms of Bambara groundnut.

Overall, from the knowledge gathered from this thesis, confirmation on the different candidate genes is needed, as well with the oncoming resequencing data and genome, a deeper and more comprehensible analysis can take place to understand more the genetics behind the different responses by Bambara groundnut to drought. From the physiology perspective, replication in field or higher volume container will be ideal, to confirm the genetic behaviour expressed in our glasshouses. However, the results presented here are definitely a step forward in the right direction, giving important firsts steps towards breeding and understanding Bambara groundnut under drought conditions.

Research collaborations

As part of this research degree, the author also took part in a series of collaborations in different crops and areas of research.

Proso millet

- A small population of 60 different individuals were received from collaborators in India for future work. This population was grown by the author, stored and labelled for future experiments.

Winged bean

- The author grew the first winged bean in the University of Nottingham (UoN), additionally taking part in the generation of leaf tissue for the genome sequencing using Oxford Nanopore and Bionano technologies in collaboration with Deepseq (UoN).

- Co-author on a book chapter titled "The Winged Bean Genome"

Bambara groundnut

- Took part as co-author on a review for *Planta* in 2019 (Mayes *et al*., 2019)

- Took a small part in growing the Bambara genotype used by Deepseq for the genome sequence and assembly using bio-nano and Oxford nanopore technologies. Additionally, the RNA data generated in chapter 5 has been used for the annotation and assembly of the Bambara groundnut genome (manuscript in preparation).

- In 2020, the author won the UNICAS graduate funding call as principal applicant on a collaboration with Dr Dong-Hyun in Life Science, Dr Yang in Nutritional Science, and Dr Flis in Plant Science. This would have allowed a detailed analysis of Bambara groundnut metabolome, aroma profile and mineral composition in seeds. Due to the COVID-19 pandemic, the funding for this project was re-directed by the University of Nottingham to the response action plan, thus the research did not not taken place. This funding was for £5,000.00.

- However, initial assessments of the seed's ionome and aroma compounds of Bambara groundnut was achieved, showing the following:

- Three seeds from each biological replicate, three technical replicates, are enough for a seed ionome experiment to develop reliable data.

- There is a general difference between genotypes in terms of their ionome composition in the seeds.

- Different elements are more present in the testa than in the rest of the seed (Ca, Mn, Cu, Zn, and Mo) and vice versa (P, S, K), however, this is dependant on the genotype in some cases (Mg, Fe).

- Thirty-three aroma compounds were identified in twelve different varieties of BG. Odour Activity Value suggested pyrazines, phenolics and sulfurs were the main aroma functional group contributing to nutty, earthy and beany profile of BG samples. PCA and AHC results were able to categorise twelve varieties of BG samples into three main groups. Group 1(Getso, TN, DipC, and Kano2, which have a predominant cream colour) being the most aromatic and nutty, followed by Group 3 (DodR, UniswaG, Gresik, and LunT) and finally Group 2 (UniswaR and Kano3) with the least aromatic profile.

-A functional prototype software for automatic stomata counting was developed under the joint efforts of the Mayes, Murchie, and Gilles groups.

Foxtail millet

- A collaboration between the Mayes group (UoN), the Hunt research group in archaeogenetics (University of Cambridge), Bennett group (UoN), and Han group (Shanxi Agricultural University, China) generated an Association Genetics Panel grown, harvested, documented, and stored by the author for future work.

- With the aid of 3 undergraduate students, the whole population was sent to genotype by sequencing to DArTseq for future GWAS.

- Took part in the mentoring of a total of 4 B.Sc. research projects, and 1 M.Sc.

- Took part on the physiological experiments in response to drought, alongside a post-doc visitor from China, generating data for GWAS, as well as root phenotyping in 2D.

- Took a small part in the DNA isolation for re-sequencing of 150 genotypes.

This collaboration has now expanded to other research groups within the UoN looking at transformation, single cell RNA, and phenotyping automation.

Appendix 1

ImageJ macro for estimated canopy area and RGB values for estimated chlorophyll content

```
run("Set Measurements...", "area mean limit display redirect=None decimal=2");

path= getDirectory("Choose a directory containing leaf images");

list= getFileList(path);

//For loop to apply processing on all images found in folder

for(i=0;i<list.length;i++){

open(path+list[i]);


orig = getTitle();

setTool("line");

waitForUser("Please Draw your scale for setting");

run("Set Scale...");

run( "Duplicate...", "title=result" );

run( "Duplicate...", "title=temp" );

run("Color Threshold...");

setTool("wand");

waitForUser("Please correctly set Colour Histogram adjustment and ROI");

run("Analyze Particles...", "add");

roiManager("Combine");

selectImage(orig);

run("Restore Selection");

roiManager("Delete");

selectImage("temp");

close();

selectImage(orig);

roiManager("Add");

run("Make Composite");

roiManager("multi-measure measure_all one append");
```

```
selectImage("result");
run("Restore Selection");
run("From ROI Manager");
selectImage(orig);
close();
//selectWindow("ROI Manager");
//run("Close");

// Color Thresholder 1.51s
function hsbThres( bmax ) {
min=newArray(3);
max=newArray(3);
filter=newArray(3);
a=getTitle();
run("HSB Stack");
run("Convert Stack to Images");
selectWindow("Hue");
rename("0");
selectWindow("Saturation");
rename("1");
selectWindow("Brightness");
rename("2");
min[0]=0;
max[0]=255;
filter[0]="pass";
min[1]=0;
max[1]=255;
filter[1]="pass";
min[2]=0;
max[2]=bmax;
filter[2]="pass";
for (i=0;i<3;i++){
  selectWindow(""+i);
```

```
  setThreshold(min[i], max[i]);
  run("Convert to Mask");
  if (filter[i]=="stop")  run("Invert");
}
imageCalculator("AND create", "0","1");
imageCalculator("AND create", "Result of 0","2");
for (i=0;i<3;i++){
  selectWindow(""+i);
  close();
}
selectWindow("Result of 0");
close();
selectWindow("Result of Result of 0");
rename(a);

};
roiManager("delete");
close("*");
}
setBatchMode( false );
waitForUser("Process complete");
exit();

///set correct scale
//waitForUser("Please correctly set scale");

///Run Color Thresholding
//run("Color Threshold...");
//waitForUser("Please correctly set Brightness adjustment");



///Choose Region of interest (ROI)
//run("ROI Manager...");
```

```
//waitForUser("Please correctly place ROI");



///Multi-measure all leaves
//roiManager("multi-measure measure_all");

//close();
//}
```

Table 19 Summary of findings in year 1 (2018). T=treatment; SC = stomata conductance; T_{can} = canopy temperature; $T_{air\text{-}can}$ = temperature of canopy vs air; ECA = estimate canopy area.

		2018						
Genotype	T	SC	T_{can}	$T_{air\text{-}can}$	ECA	QYDA	QYLA	LRWC
DipC	Drought	21 ±17.7	38.87 ±1.6	1.76 ±1.8	238 ±90.6	0.72 ±0.11	0.21 ±0.05	56.4 ±14.9
DipC	Irrigated	127 ±51.4	33.49 ±0.7	-3.62 ±1.8	761 ±228.9	0.73 ±0.02	0.53 ±0.05	75.4 ±5.8
DodR	Drought	14 ±14.0	30.20 ±1.9	-0.38 ±1.8	283 ±67.9	0.65 ±0.05	0.29 ±0.04	56.0 ±9.9
DodR	Irrigated	126 ±27.6	27.01 ±1.9	-3.57 ±2.2	738 ±125.4	0.69 ±0.02	0.56 ±0.05	61.2 ±4.2
Getso	Drought	31 ±32.6	31.12 ±2.0	-2.06 ±1.7	241 ±128.6	0.52 ±0.16	0.27 ±0.04	51.4 ±9.3
Getso	Irrigated	168 ±37.1	29.13 ±3.4	-5.38 ±1.9	779 ±88.89	0.74 ±0.03	0.59 ±0.04	76.2 ±5.0
Gresik	Drought	15 ±7.6	27.26 ±1.2	-3.18 ±0.6	15 ±6.9	0.67 ±0	0.18 ±0.18	NA
Gresik	Irrigated	132 ±40.8	26.10 ±2.8	-5.54 ±0.8	796 ±148.2	0.65 ±0.0	0.56 ±0.08	50.0 ±10.5
IITA	Drought	6 ±3.0	31.32 ±1.3	0.03 ±1.9	243 ±123.1	0.65 ±0.05	0.18 ±0.08	39.8 ±4.0
IITA	Irrigated	119 ±44.6	28.54 ±2.5	-2.88 ±2.8	901 ±131.2	0.64 ±0.06	0.50 ±0.09	51.6 ±5.6
Kano2	Drought	28 ±29.1	37.09 ±4.1	1.69 ±0.9	60 ±11.9	0.69 ±0.15	0.24 ±0.05	49.3 ±4.2
Kano2	Irrigated	120 ±10.5	32.46 ±3.6	-2.95 ±0.6	828 ±72.4	0.77 ±0.01	0.55 ±0.07	70.5 ±12.4
Kano3	Drought	7 ±6.0	34.15 ±2.8	1.14 ±2.3	71 ±77.5	0.34 ±0.21	0.30 ±0.07	40.9 ±3.9
Kano3	Irrigated	177 ±34.9	30.62 ±1.9	-2.39 ±0.7	778 ±79.0	0.69 ±0.12	0.51 ±0.08	67.5 ±8.2
LunT	Drought	5 ±1.7	35.40 ±1.8	1.36 ±0.9	112 ±54.2	0.62 ±0.12	0.08 ±0.04	56.6 ±4.1
LunT	Irrigated	170 ±49.3	28.54 ±2.5	-5.50 ±0.8	643 ±72.2	0.75 ±0.04	0.60 ±0.02	62.5 ±5.9
S19-3	Drought	8 ±4.2	35.07 ±2.6	2.92 ±2.1	312 ±89.9	0.49 ±0.08	0.26 ±0.05	50.9 ±2.6
S19-3	Irrigated	137 ±30.6	27.19 ±1.4	-4.45 ±2.7	589 ±112.9	0.54 ±0.20	0.53 ±0.12	60.7 ±5.2
TN	Drought	27 ±15.4	31.20 ±2.6	-0.39 ±1.8	371 ±250	0.70 ±0.09	0.45 ±0.06	51.4 ±7.6
TN	Irrigated	142 ±41.0	29.45 ±2.5	-3.87 ±2.4	621 ±52.7	0.76 ±0.02	0.62 ±0.1	61.5 ±9.7
Uniswa	Drought	14 ±16.6	29.67 ±0.9	-2.96 ±1.9	328 ±167.7	0.50 ±0.07	0.32 ±0.10	50.7 ±3.6
Uniswa	Irrigated	107 ±10.9	28.95 ±1.6	-4.31 ±1.1	959 ±92.9	0.74 ±0.03	0.63 ±0.01	60.7 ±3.6
UniswaR	Drought	4 ±2.1	37.18 ±2.8	2.10 ±3.2	50 ±15.1	0.50 ±0.12	0.17 ±0.12	44.7 ±2.1
UniswaR	Irrigated	163 ±59.4	27.55 ±2.7	-4.84 ±1.0	1089 ±57.5	0.74 ±0.02	0.57 ±0.06	62.1 ±5.7
Treatment		<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Genotype		0.53	<0.01	0.02	<0.01	<0.01	<0.01	0.01
T x G		0.22	0.02	0.19	<0.01	<0.01	0.05	0.23

Table 20 Summary of findings in year 2.

		2019				
Genotype	Treatment	ECA	*QYDA*	QYLA	LRWC	ECC
DipC	Drought	1151.74 ±332.48	0.46 ±0.09	0.25 ±0.08	53.41 ±10.51	53.43 ±1.29
DipC	Irrigated	2160.07 ±365.92	0.73 ±0.04	0.58 ±0.06	75.96 ±3.22	53.56 ±2.47
DodR	Drought	966.25 ±137.37	0.60 ±0.06	0.29 ±0.04	39.10 ±5.08	52.84 ±0.99
DodR	Irrigated	1376.31 ±166.86	0.73 ±0.04	0.63 ±0.03	63.08 ±4.55	64.65 ±3.48
Gresik	Drought	1667.74 ±322.12	0.48 ±0.09	0.24 ±0.04	43.25 ±2.45	56.11 ±2.52
Gresik	Irrigated	2928.40 ±273.51	0.64 ±0.07	0.58 ±0.10	54.52 ±5.22	52.68 ±2.90
Kano2	Drought	958.94 ±226.53	0.24 ±0.12	0.22 ±0.07	56.75 ±7.63	53.33 ±1.92
Kano2	Irrigated	1587.55 ±411.34	0.66 ±0.05	0.61 ±0.04	72.53 ±4.86	60.18 ±6.52
Kano3	Drought	1242.47 ±292.31	0.41 ±0.12	0.23 ±0.10	44.91 ±3.55	50.36 ±1.58
Kano3	Irrigated	2183.66 ±200.86	0.67 ±0.04	0.54 ±0.09	69.76 ±3.87	48.37 ±1.97
S19-3	Drought	870.76 ±253.71	0.45 ±0.16	0.39 ±0.11	44.68 ±4.85	53.57 ±2.90
S19-3	Irrigated	720.73 ±134.92	0.72 ±0.04	0.68 ±0.06	49.89 ±4.01	67.12 ±4.60
TN	Drought	1068.09 ±184.22	0.64 ±0.10	0.51 ±0.10	44.64 ±14.33	49.84 ±3.37
TN	Irrigated	1450.36 ±233.37	0.71 ±0.06	0.61 ±0.10	62.74 ±7.97	49.09 ±3.20
UniswaR	Drought	1017.05 ±85.29	0.26 ±0.09	0.36 ±0.11	52.48 ±5.34	52.47 ±1.37
UniswaR	Irrigated	1171.98 ±153.22	0.60 ±0.03	0.56 ±0.17	78.80 ±6.02	66.57 ±1.61
Treatment		<0.001	<0.001	<0.001	<0.001	<0.001
Genotype		<0.001	<0.001	<0.001	<0.001	<0.001
T x G		<0.001	<0.001	0.0027	<0.001	<0.001

Table 21 Summary of findings in year 3.

2020						
Genotype	Treatment	ECA	*QYDA*	QYLA	LRWC	ECC
DipC	Drought	849.00 ±347.08	0.64 ±0.05	0.35 ±0.03	41.19 ±5.30	51.83 ±1.05
DipC	Irrigated	2319.30 ±443.69	0.81 ±0.01	0.73 ±0.02	75.79 ±2.33	52.68 ±2.49
DodR	Drought	1559.96 ±291.67	0.76 ±0.04	0.68 ±0.07	59.65 ±6.76	51.67 ±3.71
DodR	Irrigated	2488.18 ±271.75	0.81 ±0.01	0.75 ±0.01	79.73 ±2.95	51.07 ±3.43
Gresik	Drought	1418.94 ±181.42	0.66 ±0.05	0.36 ±0.05	52.70 ±9.70	46.90 ±2.86
Gresik	Irrigated	2976.05 ±347.57	0.81 ±0.02	0.69 ±0.05	87.07 ±2.11	44.02 ±3.16
Kano2	Drought	1396.60 ±219.55	0.73 ±0.04	0.37 ±0.6	53.42 ±6.90	49.50 ±1.93
Kano2	Irrigated	2511.61 ±482.17	0.81 ±0.02	0.72 ±0.03	77.36 ±2.07	48.61 ±4.14
Kano3	Drought	844.80 ±111.16	0.63 ±0.03	0.31 ±0.4	44.15 ±6.33	47.85 ±2.04
Kano3	Irrigated	2428.42 ±168.11	0.81 ±0.01	0.71 ±0.02	82.25 ±2.76	43.93 ±3.99
S19-3	Drought	1116.70 ±198.99	0.78 ±0.01	0.51 ±0.07	62.65 ±4.03	49.96 ±3.95
S19-3	Irrigated	2436.00 ±271.14	0.82 ±0.01	0.72 ±0.01	89.70 ±2.94	49.29 ±4.48
TN	Drought	1126.53 ±117.69	0.79 ±0.04	0.65 ±0.08	58.40 ±6.68	46.35 ±2.62
TN	Irrigated	1645.17 ±405.75	0.81 ±0.01	0.74 ±0.02	81.74 ±3.18	47.46 ±5.96
UniswaR	Drought	1094.49 ±366.20	0.66 ±0.07	0.35 ±0.06	52.44 ±5.03	48.94 ±3.43
UniswaR	Irrigated	2650.10 ±541.30	0.80 ±0.02	0.68 ±0.03	77.87 ±3.73	44.16 ±3.93
Treatment		P<0.0001	< 0.001	< 0.001	P<0.0001	P=0.0403
Genotype		P<0.0001	< 0.001	< 0.001	P<0.0001	P<0.0001
T x G		P=0.0062	< 0.001	< 0.001	P=0.0017	P=0.7544
T x G x Time		0.05	<.001	0.01		<.001

Appendix 2

Mineral content between genotypes.

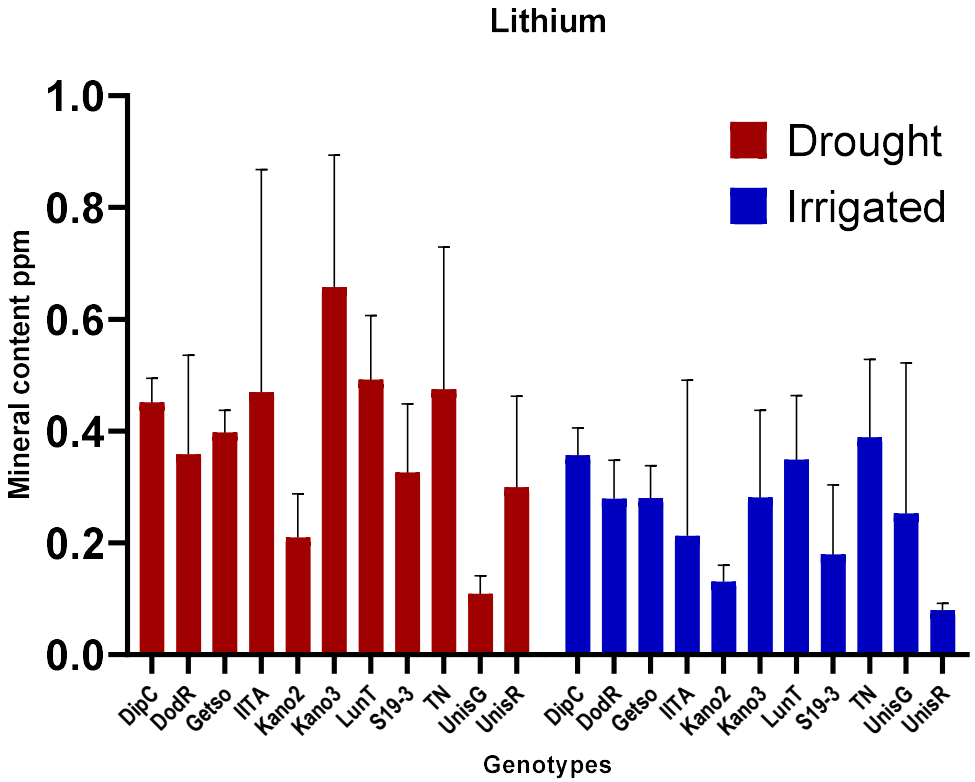

Figure 0-1 Lithium content in leaves. Significant increase in the drought treated genotypes (p<0.001), and a significant genotype effect (p<0.002).

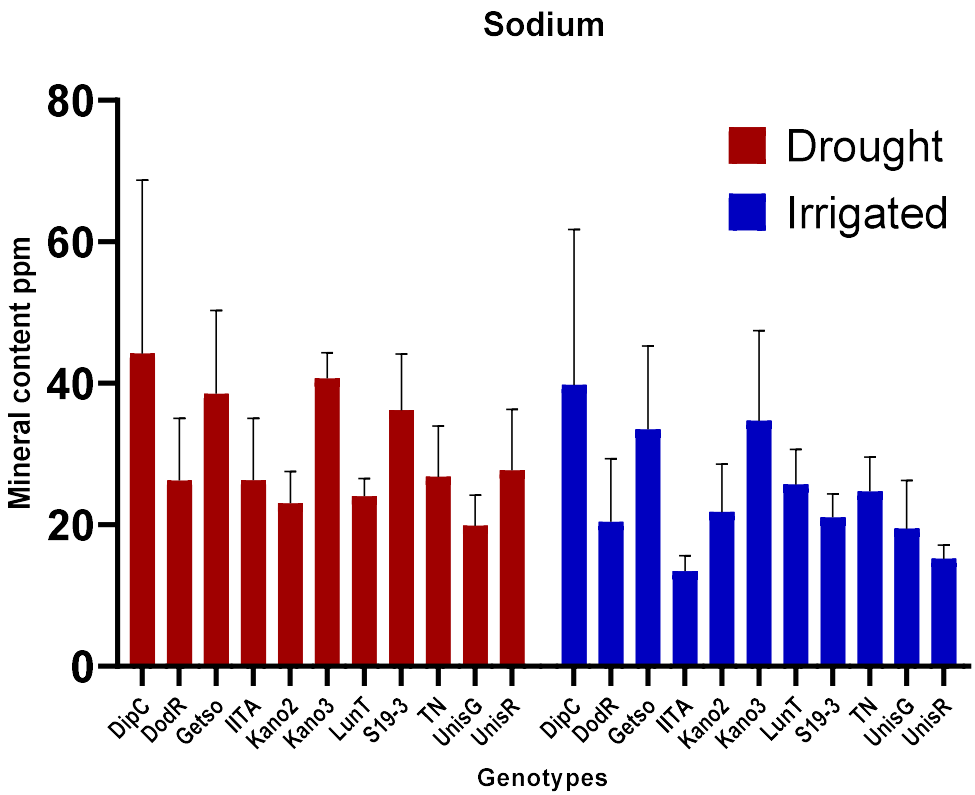

Figure 0-2 Sodium content in leaves. Significant increase in the drought treated genotypes (p<0.01), and a significant genotype effect (p<0.001).

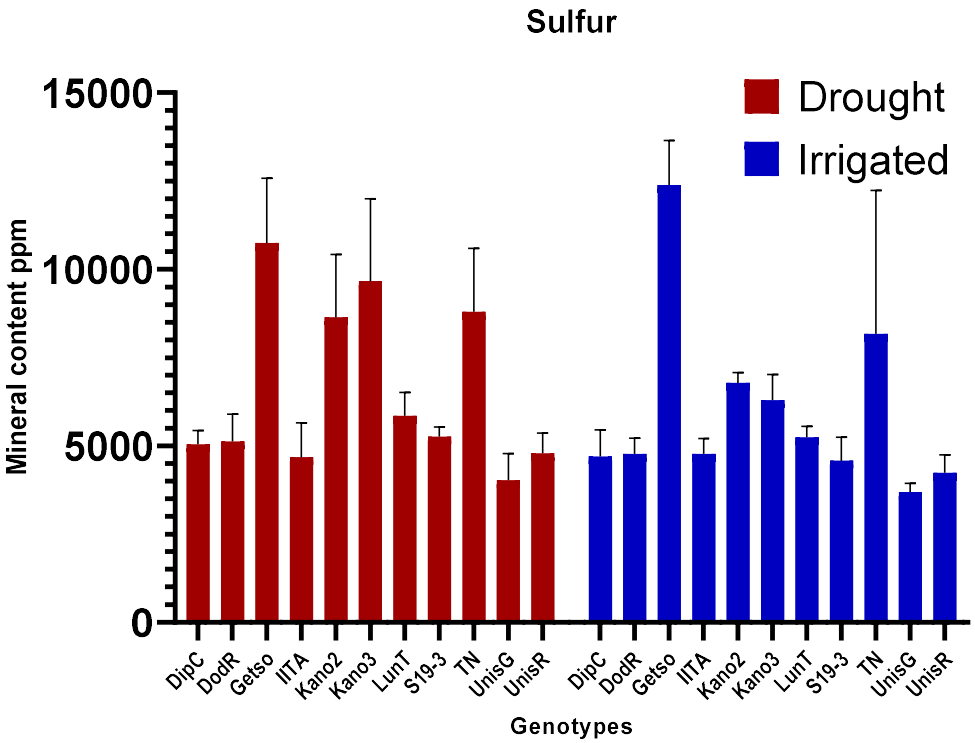

Figure 0-3 Sulfur content in leaves. Significant increase in the drought treated genotypes (p=0.03), and a significant genotype effect (p<0.001).

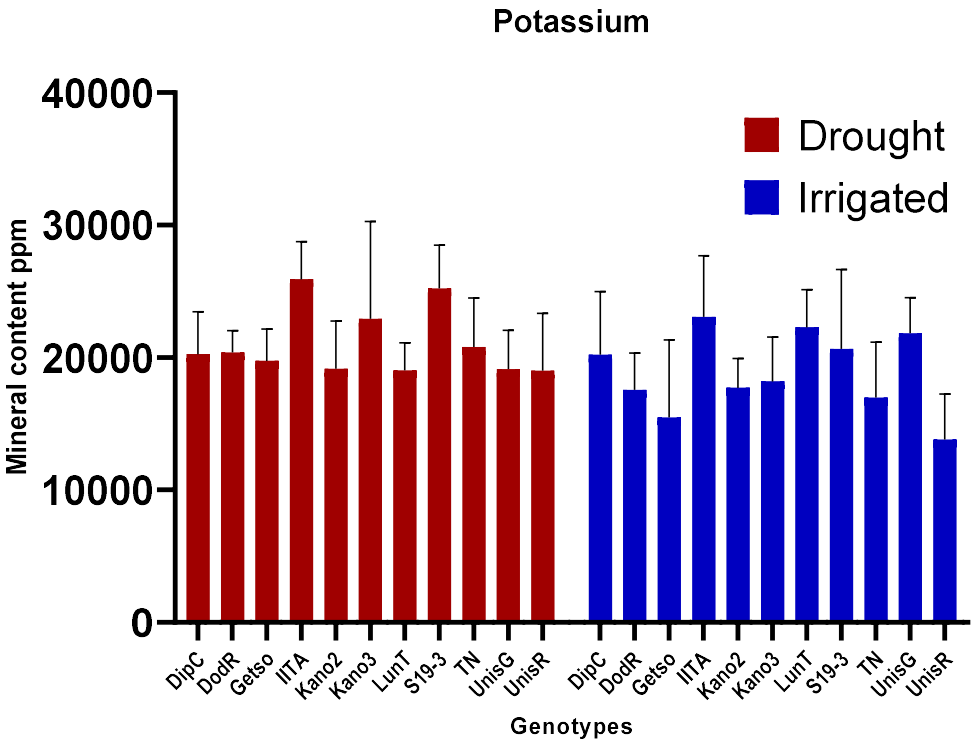

Figure 0-4 Potassium content in leaves. Significant increase in the drought treated genotypes (p=0.011), and a significant genotype effect (p<0.006).

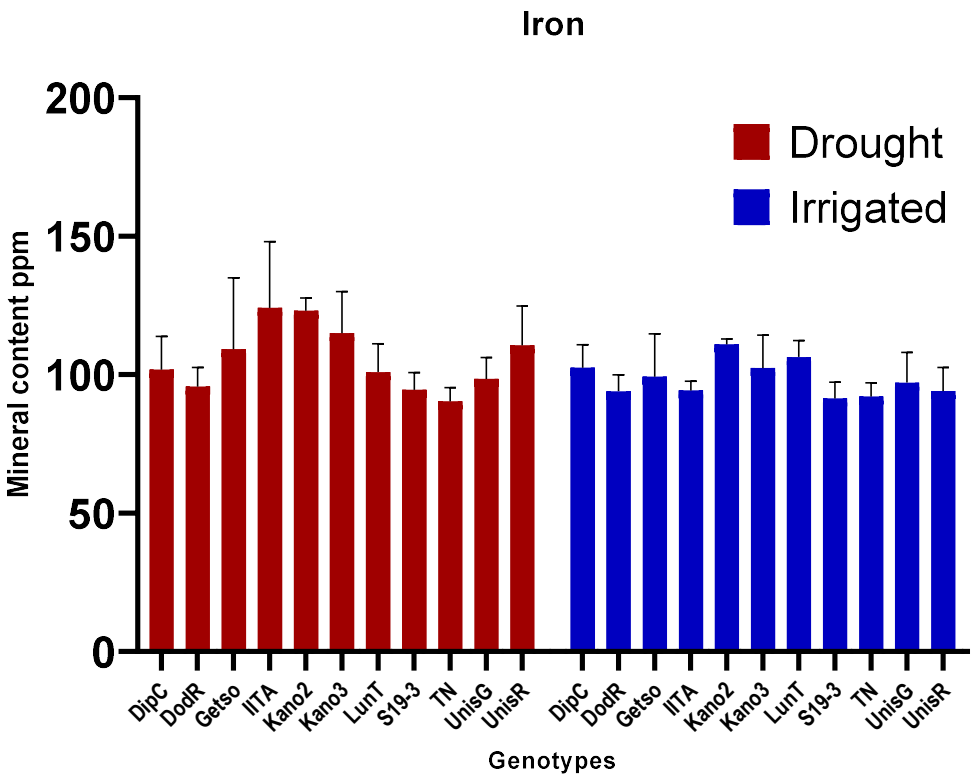

Figure 0-5 Iron content in leaves. Significant increase in the drought treated genotypes (p<0.005), and a significant genotype effect (p<0.001).

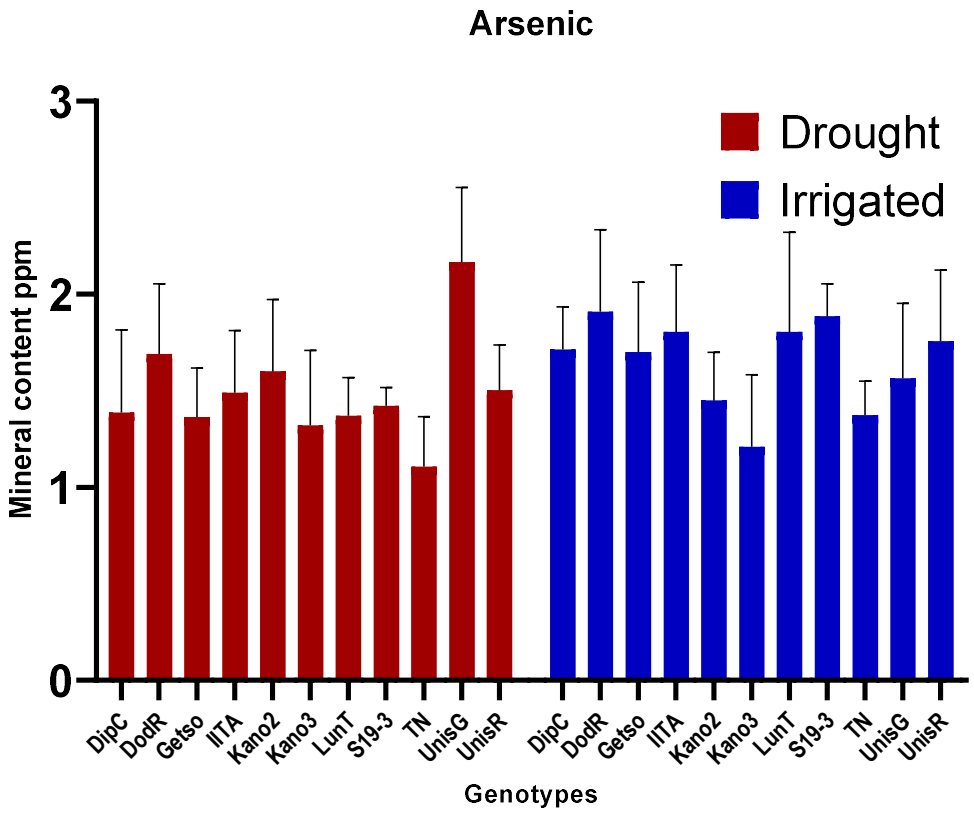

Figure 0-6 Arsenic content in leaves. Significant decrease in the drought treated genotypes (p=0.026), and a significant genotype effect (p<0.01).

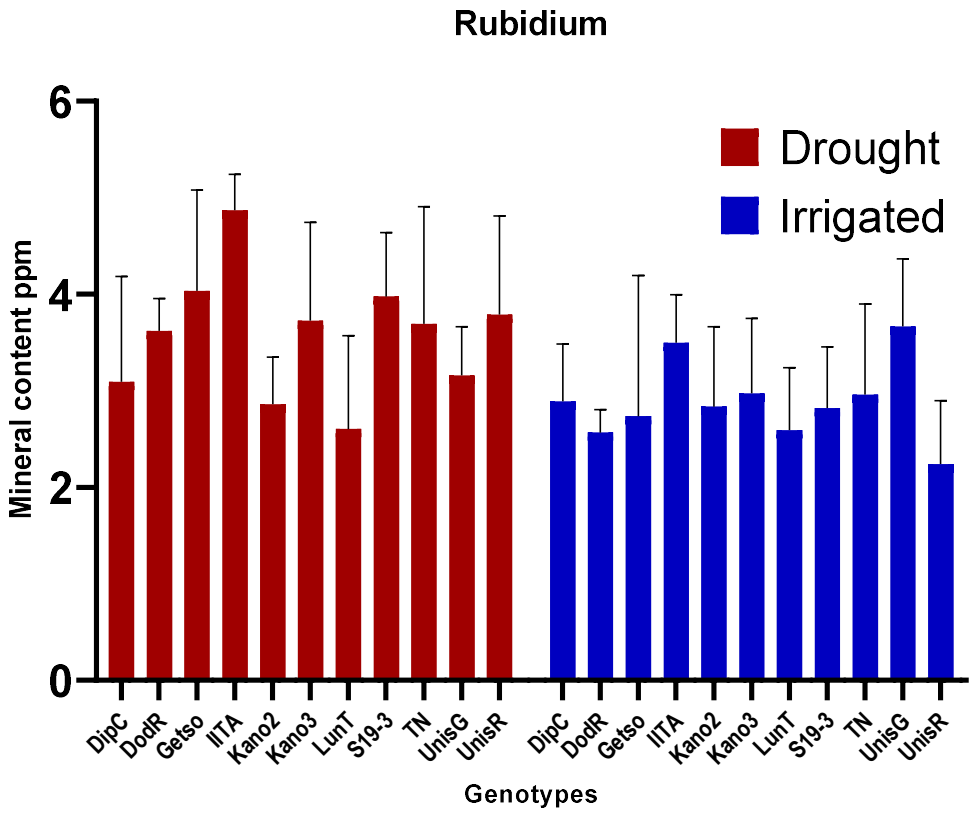

Figure 0-7 Rubidium content in leaves. Significant increase in the drought treated genotypes (p<0.01), and a significant genotype effect (p<0.05).

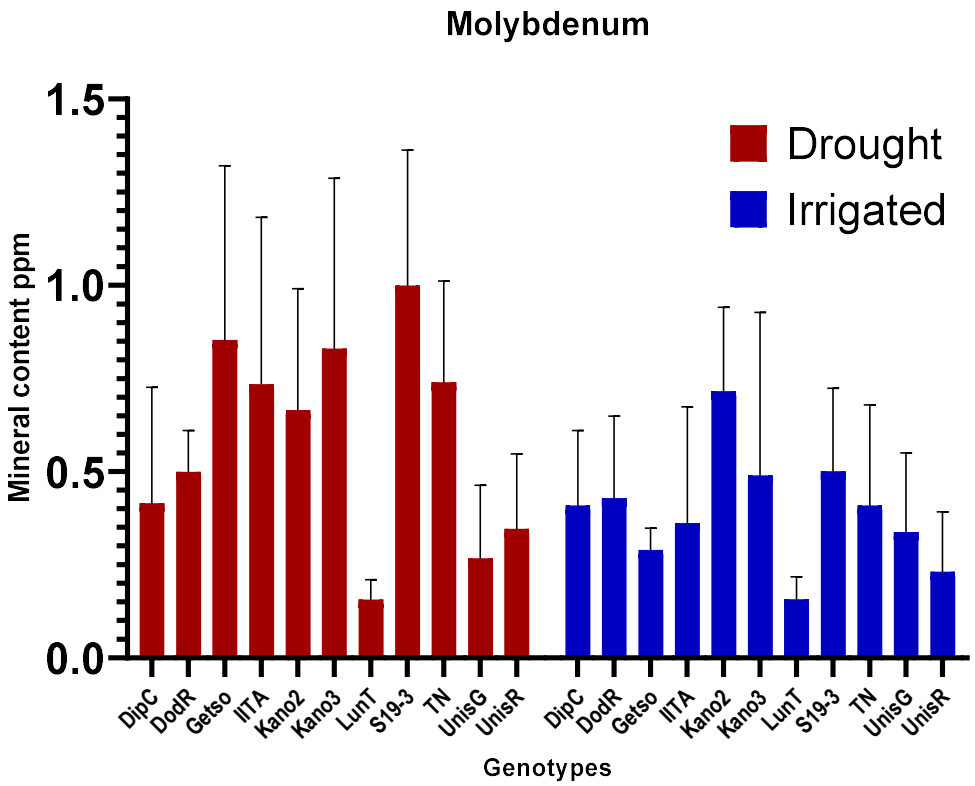

Figure 0-8 Molybdenum content in leaves. Significant increase in the drought treated genotypes (p<0.002), and a significant genotype effect (p<0.002).

Appendix 3

DEG in Bambara groundnut showed in *Phaseolus vulgaris* pathways.

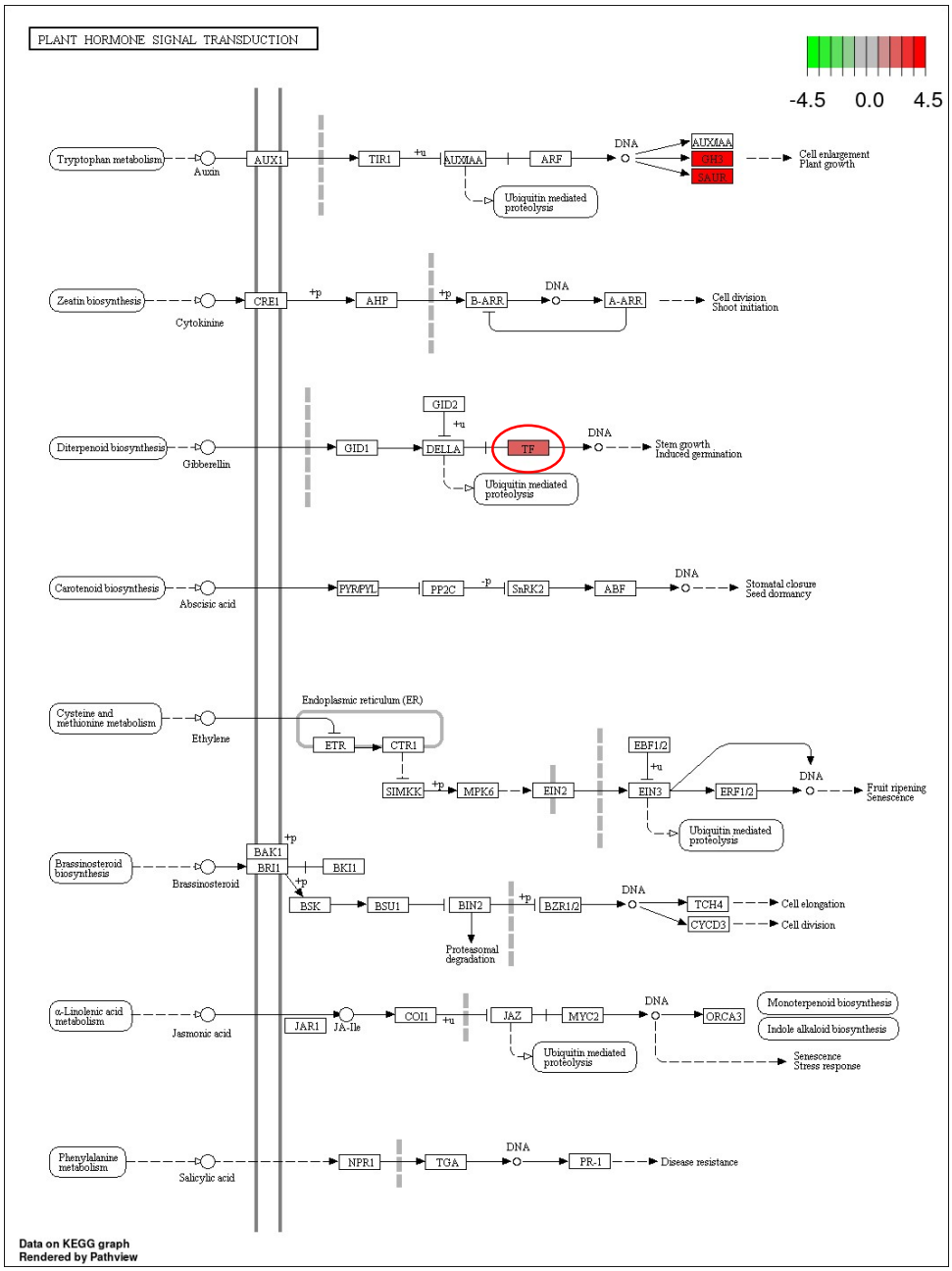

Figure 0-9 Plant hormone signal transduction pathway in common bean. Green colour represents down regulation, and red up regulation. Red circles represent genes of more importance in response to drought according to the filter of DEG and KEGG.

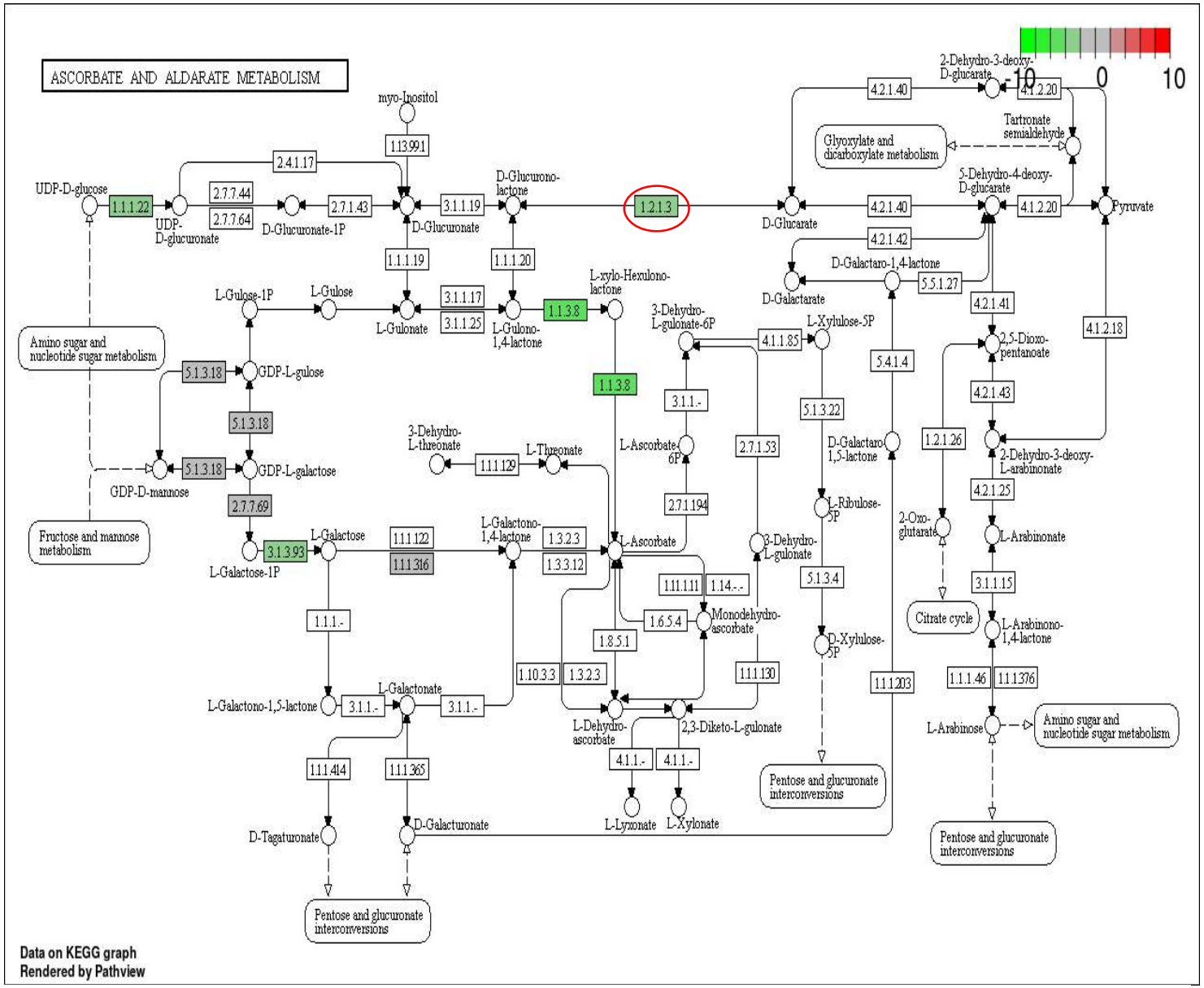

Figure 0-10 Ascorbate and alderate metabolism pathway in common bean. Green colour represents down regulation, and red up regulation. Red circles represent genes of more importance in response to drought according to the filter of DEG and KEGG.

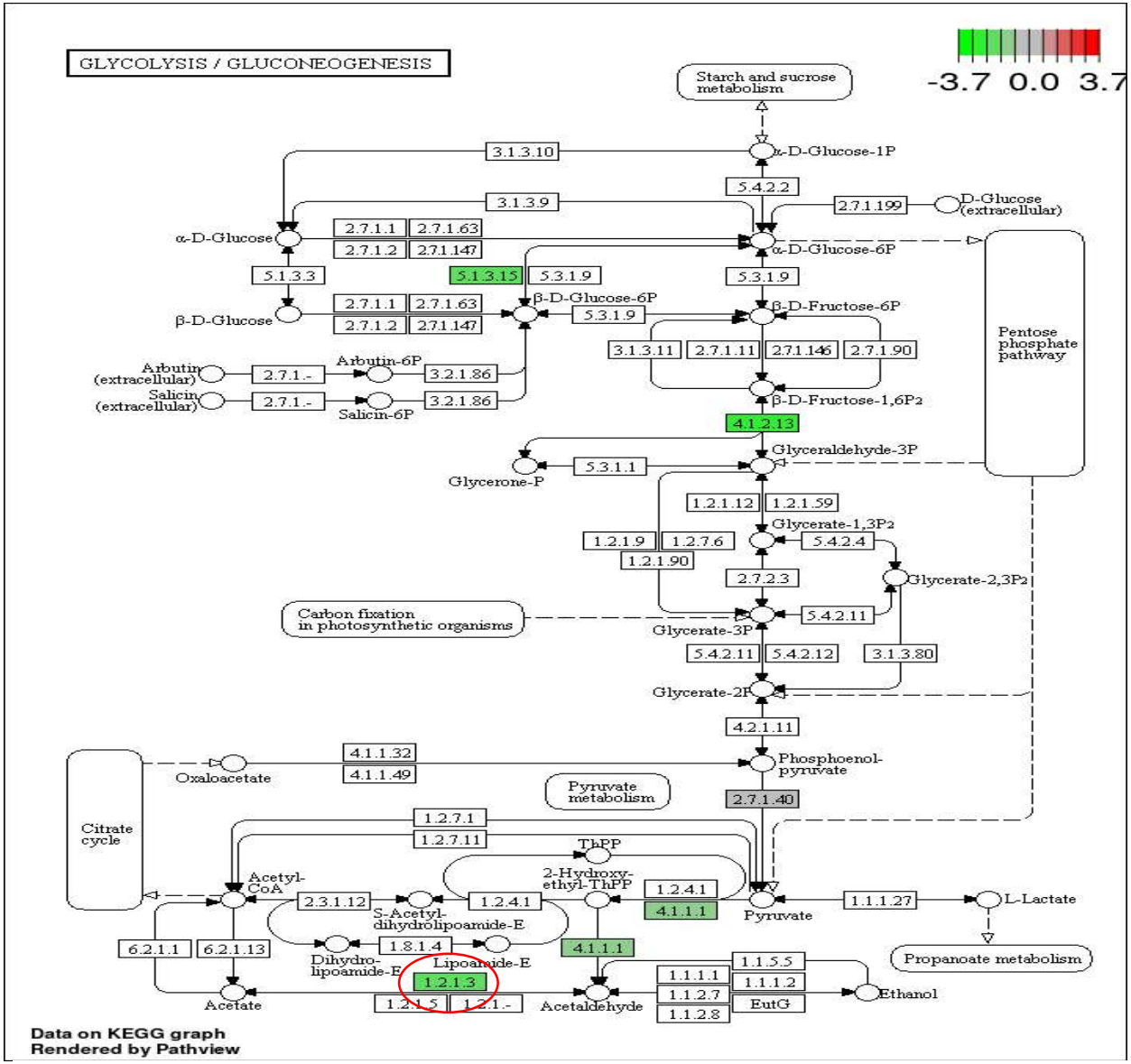

Figure 0-11 Glycolysis pathway in common bean. Green colour represents down regulation, and red up regulation. Red circles represent genes of more importance in response to drought according to the filter of DEG and KEGG.

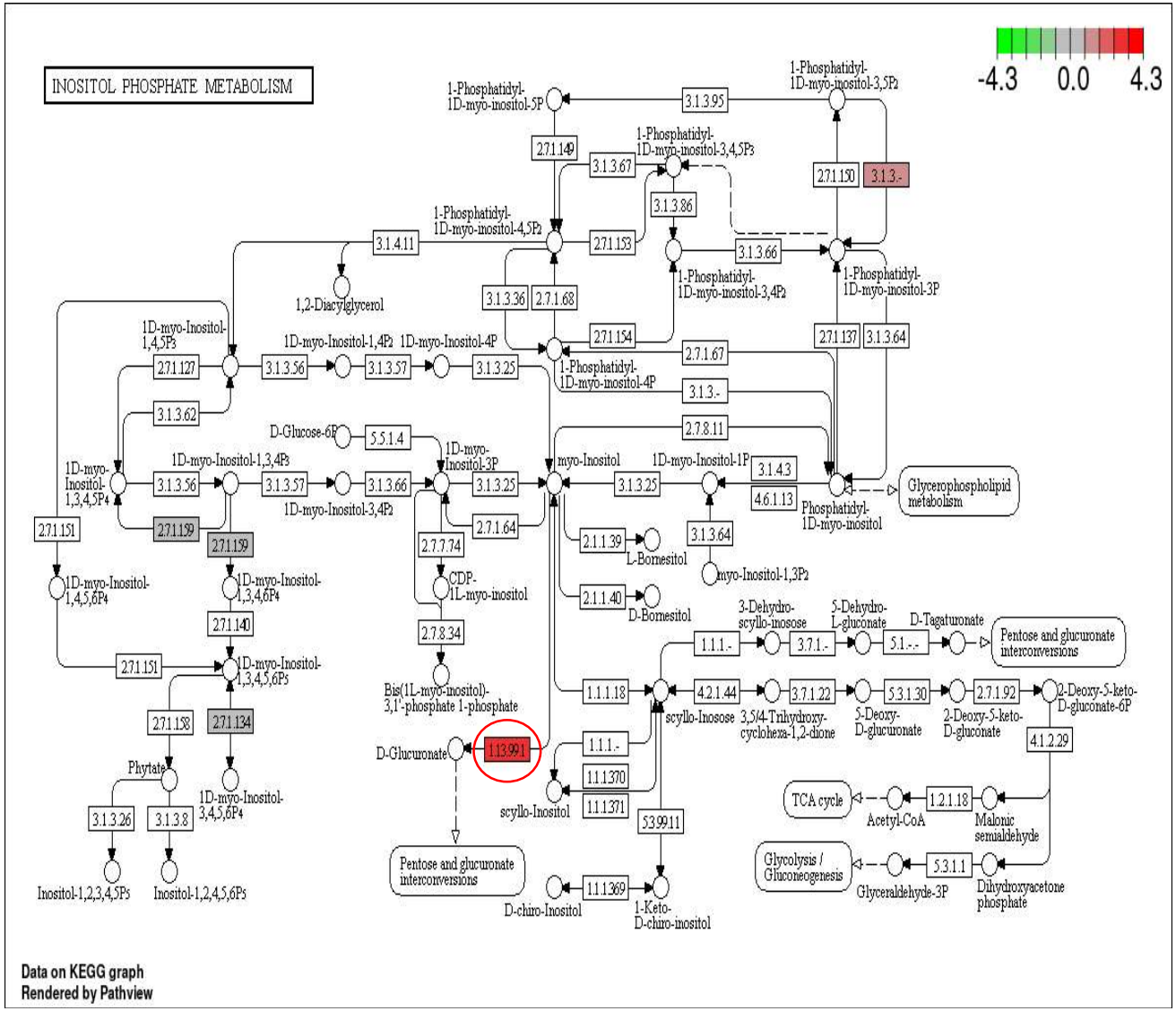

Figure 0-12 Inositol phosphate metabolism pathway in common bean. Green colour represents down regulation, and red up regulation. Red circles represent genes of more importance in response to drought according to the filter of DEG and KEGG.

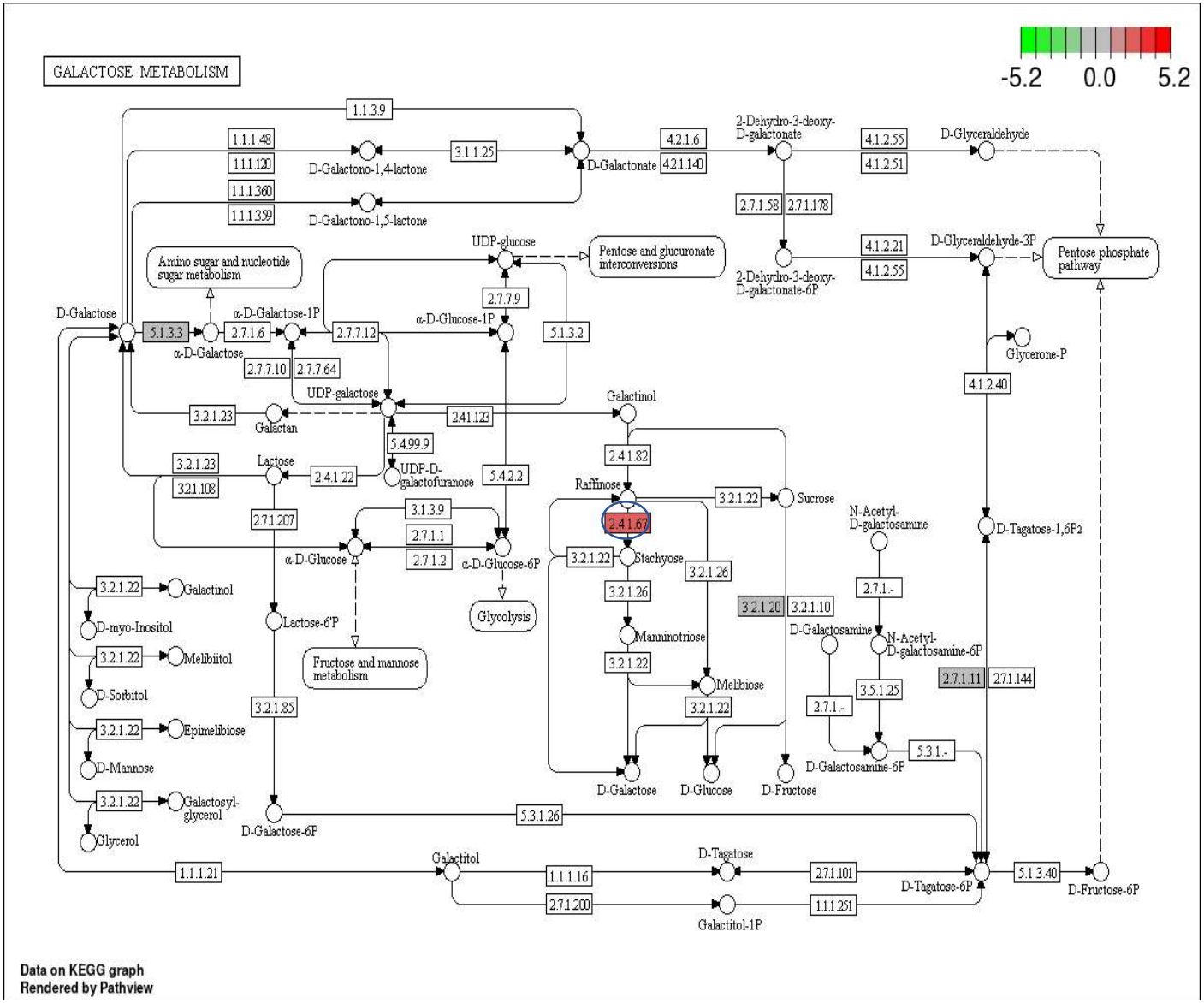

Figure 0-13 Galactose metabolism pathway in common bean. Green colour represents down regulation, and red up regulation. Red circles represent genes of more importance in response to drought according to the filter of DEG and KEGG.

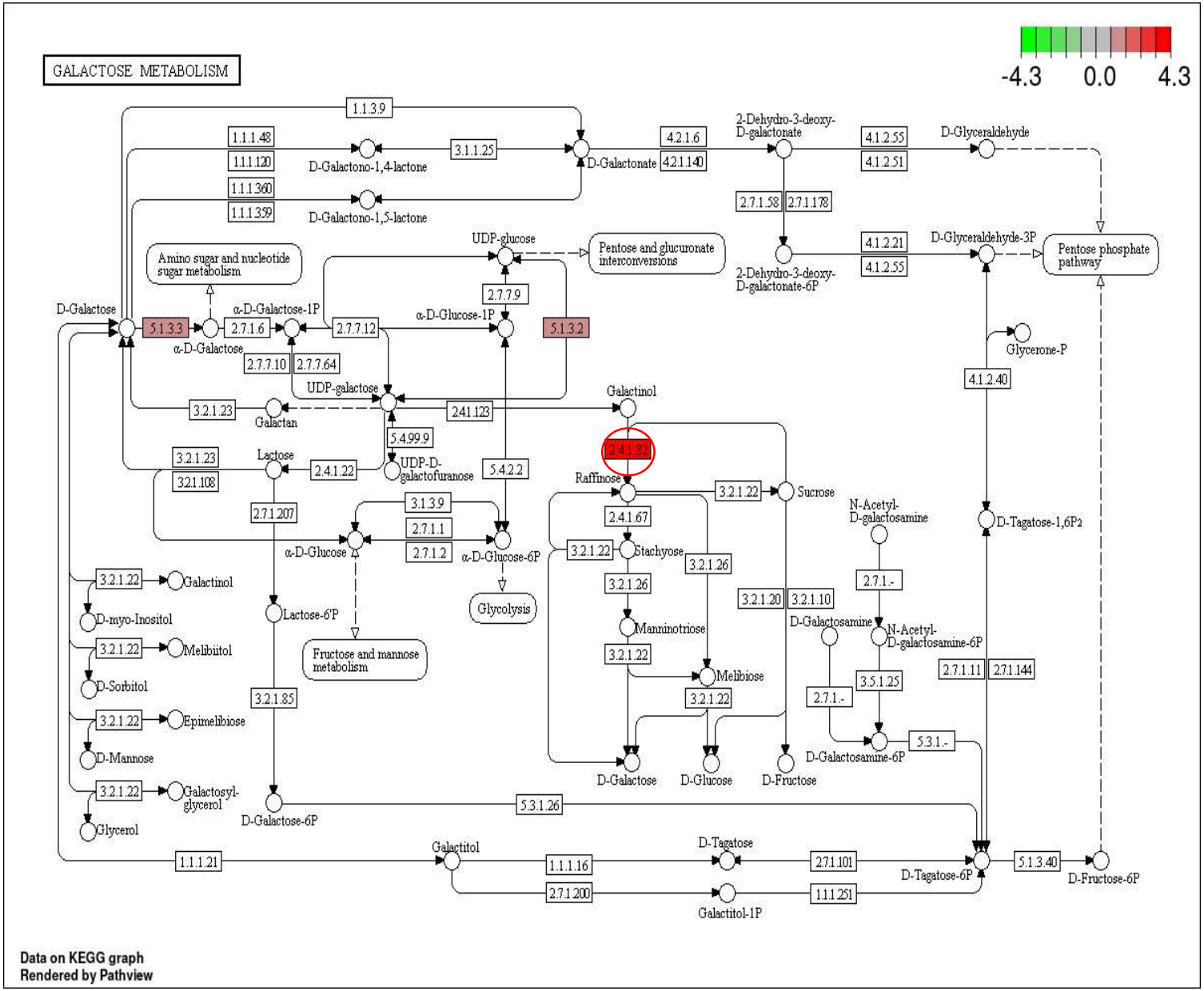

Figure 0-14 Galactose metabolism pathway in common bean. Green colour represents down regulation, and red up regulation. Red circles represent genes of more importance in response to drought according to the filter of DEG and KEGG.

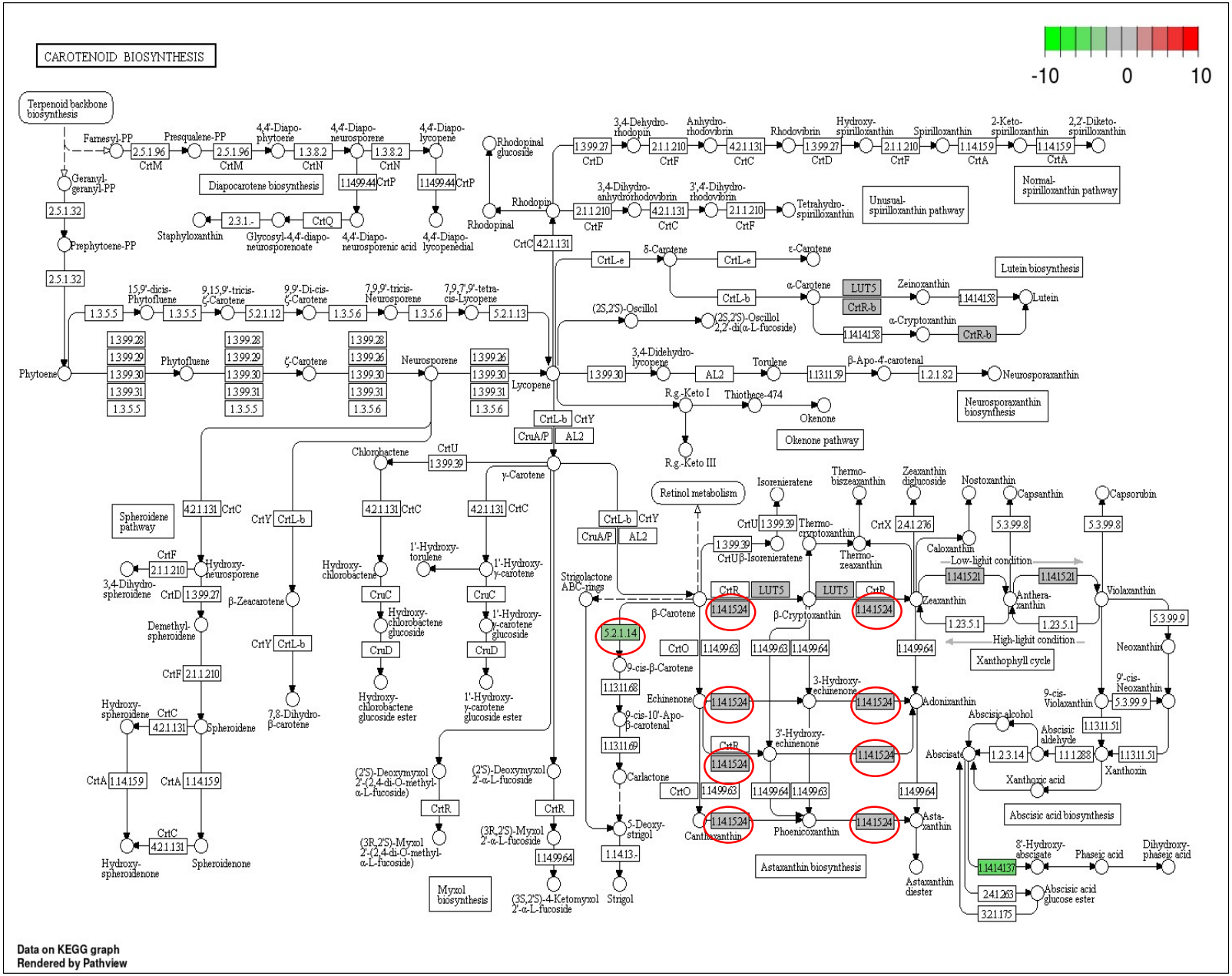

Figure 0-15 Carotenoid Biosynthesis pathway in common bean. Green colour represents down regulation, and red up regulation. Red circles represent genes of more importance in response to drought according to the filter of DEG and KEGG.