DROUGHT TOLERANT TRAITS OF TRITICALE AND COWPEA GENOTYPES UNDER SEMI-ARID CONDITIONS

by

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THESIS

Submitted in fulfilment of the requirements for the degree of

DOCTOR OF PHILOSOPHY (PhD)

in

AGRICULTURE (SOIL SCIENCE)

in the

FACULTY OF SCIENCE AND AGRICULTURE

(School of Agricultural and Environmental Sciences)

at the

UNIVERSITY OF LIMPOPO

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2017

DECLARATION

I declare that the thesis hereby submitted to the University of Limpopo, for the degree of Doctor of Philosophy in Agriculture (Soil Science) has not previously been submitted by me for a degree at this or any other university; that it is my work in design and in execution, and that all material contained herein has been duly acknowledged.

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Acknowledgements

First and foremost, I give glory to God. It’s not by my own wisdom that I have reached this milestone in my life.

Secondly, I would like to sincerely thank my promoters: Prof. Dr. ir. Pascal Boeckx, Prof. Dr. ir Geert Haesaert at Ugent and Prof Kingsley K. Ayisi and Dr. Frank Vanassche at UL. I thank them for the guidance throughout the duration of this study. I feel blessed, because I had four of you on my team. Special thanks to Prof Boeckx, for giving me this opportunity. You were my promoter for my MSc thesis and you believed in me and showed confidence that I could do this…so I really thank you. Prof Boeckx you are indeed very efficient. You were the quickest in replying emails, the quickest in giving feedback and the first to ask for progress reports. Prof Boeckx I will always remember the email I received from you after sending you a draft of my very first paper; you said “*Dear Lawrence, Please find attached my comments to draft 1 of your first paper. No panic... the first time it is almost always like that.*” That was my introduction to article writing. Suffice to say you shredded the article apart. Prof. Ayisi, you were and you are more than my supervisor. You are the most kind and humble person I have met so far. You were the first person that I would reported any challenges to and I thank you for your open-door policy. Prof Geert, I thank you for all the support, the office space, and the discussions we had. As a breeder, you always had a different view on how things are reported and that brought in an interesting dynamic to my writing. Dr Vanassche I thank you for establishing the experiments and the technical support and the reviews to this thesis.

I also would like to express my profound gratitude to the laboratory staff at ISOFYS: Katja Van Nieuland, Dr. Samuel Bode, Eric Gillis. You helped me with the analyses of my samples which I really appreciate. Many thanks to Saskia Van der Looven for the logistics; you were the first port of call every time I came to Gent. I really thank you for making my stays less stressful. I also would like to thank other staff members at ISOFYS and fellow PhD students. Special mention to MSc Bram Vanderwalle for the assistance with the field experiment and data collection. You reduced the burden and made the research monitoring more enjoyable.

I wish to thank a lot of people at UL. I spent most of my four years of this study at UL. I felt at home away from home. I want to say thank you very much to Dimakatso Tlaka and Flora at VLIR house (UL). Words alone cannot express how grateful I am for everything that you have done for me. Dima, you were and you are like a mom to me. May you continue to treat others the way you treated me. I want to say thank you to all my friends and colleagues at RVSC: Nompumelelo Mobe, Kabisheng Mabitsela, Paulina Mabapa, Shila Dolo, Edwin Mudongo, Thabang Maphanga, Patricia Sebola, Dr Manyevere and Josephine Khosa. Kabisheng, *ke a leboga* for teaching some Sepedi. I also thank Elizabeth and our uncles that helped with the field work I say thank you to all the staff at the UL Experimental Farm. Special mention to Louis Eloff, Jimmy, and Lucas for your assistance.

I want to thank my friends in Gent. It’s not easy to be 800 km from home but its far much worse to be 9 000 km away. Without listing, all of you I want to say thanks for the social gatherings and social soccer games we had, thanks for the Friday night pool games at Overpoort. Thanks to Nicky for the chess games in the park (you know who the champion is). My fellow Zimbabweans in Gent, you are appreciated.

Lastly and most importantly, I would to acknowledge the tremendous support and love I received from my wife Shelter and our two daughters Tanatswa and Tafadzwa. To my wife, I know it’s not been easy for you to travel up and down between Polokwane and Harare…thank you. I also thank my father for the sacrifices he made for me to be where I am today.

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Abstract

Climate change and variability pose a significant challenge to future global food security due to changes in mean climatic conditions which are rendering many areas marginally suitable for crop production. Erratic rains, drought, heat stress and declining soil fertility are some of the factors limiting crop productivity in many arid and semi-arid areas. Furthermore, water resources that could be used to mitigate drought and erratic rains, are also threatened by both scarcity and overuse. To ensure food security in the future, adaptation strategies at crop and management levels should be explored. Adaptation to future drier and warmer climates calls, among others, for improvement in drought resistance of crops through measures such as screening traits for drought tolerance and water use efficiency.

This PhD study was carried out at University of Limpopo experimental farm, Limpopo Province, South Africa, a region with a semi-arid climate and mean annual rainfall of ca. 500 mm. The main aim of the study was to evaluate the use of plant $\delta^{13}C$ and $\delta^{18}O$ as screening traits for potential yield and water use efficiency of crop genotypes under drought conditions. The study also determined the agronomic performance of cowpea (*Vigna unguiculata* L. Walp.) and triticale (x. *Triticosecale* Wittmack) genotypes under varying soil moisture conditions including biological nitrogen fixation for cowpea. Both crops were grown under field conditions and the following four moisture levels (averages per level) were applied: well-watered (ca. 420 mm), moderately well-watered (ca. 350 mm), medium stress (ca. 290 mm), and severe stress (ca. 220 mm).

The triticale experiment evaluated the agronomic performance and the spectral response of triticale to water stress under semi-arid conditions. The results showed a significant ($P < 0.05$) influence of moisture levels on the spectral reflectance, as well as on biomass and grain yield performance of triticale. However, these measured parameters did not significantly ($P > 0.05$) respond to genotypes probably due to the pre-screening of the genotypes or the lack of distinct genetic diversity in the studied parameters. Under well-watered conditions, triticale produced a grain yield of 3.9 t ha^{-1} in 2013 and 4.9 t ha^{-1} in 2014. These yields were however, found to be low when compared to other studies. Even though, no statistical differences were observed among the genotypes, Agbeacon showed a tendency of higher performance compared to the other genotypes. Of the four spectral indices tested, water based indices i.e. the water index (WI) and normalised difference water index (NDWI) were

found to be more effective in detecting leaf water status compared to greenness based indices (normalised difference vegetation index and nitrogen reflectance index). This is because NDWI and WI respond to short term changes in water content of leaves. Overall, the performance of triticale showed a good adaptation to semi-arid conditions.

The evaluation of $\delta^{13}C$ and $\delta^{18}O$ as screening traits for potential yield and water use efficiency under drought conditions produced interesting results. The findings showed that, $\Delta^{13}C$ was positive and strongly related to grain yield and thus has potential to be used as a surrogate for grain yield in triticale under water stress. We also found a negative relationship between $\Delta^{13}C$ and intrinsic water use efficiency ($WUE_{intrinsic}$), which suggests that breeding for higher $WUE_{intrinsic}$ in triticale may not necessarily yield the desired improved grain yield. Measured grain $\Delta^{13}C$ and flag leaf $\Delta^{13}C$ suggested minimum contribution of pre-anthesis assimilates to grain filling under water stress, contrary to what is reported in literature. However, for concrete conclusions on the source of assimilates to the grains under drought conditions, further studies are still needed. Combining $\delta^{13}C$ and $\delta^{18}O$ provided more information on the physiological responses of triticale to varying moisture levels. The $\delta^{13}C$ and $\delta^{18}O$ were used to test the dual isotope model by Scheidegger et al 2000 and the results showed that, vapour pressure deficit (VPD) of air plays an important role in the operation of the model. The study indicated that the model worked only under high VPD when stomatal conductance limits transpiration rate but failed to work when VPD was relatively low and limiting transpiration rate.

In order to address the declining soil fertility in the smallholder farming sector as well as the predicted loss in productivity of the commonly grown dry bean, this PhD study evaluated cowpea genotypes for biomass yield, grain yield and biological nitrogen fixation (BNF) under varying moisture levels. The results showed that soil moisture levels indeed affect biomass production, grain yield, nodule formation and the ability of cowpea to fix atmospheric nitrogen. BNF and nodule formation were the most sensitive to water stress compared to the other parameters. Severe water stress reduced BNF by 57% relative to well-watered conditions while nodule mass was reduced by 80% for the same soil moisture levels. Genotype TV4607 was superior in most of the parameters determined except for grain yield. As a result, TV4607 produced the highest biomass and returned the most nitrogen back to the soil compared to the other genotypes. However, IT00K-1263 emerged as the superior

genotype due to its ability to produce the optimum balance of biomass, grain yield and BNF. Stomatal behaviour of cowpea under varying moisture levels was also investigated and the results showed sensitivity of stomatal conductance to soil moisture levels. As expected, stomatal conductance was high under well-watered conditions compared to water stressed conditions. Genotypic variation in stomatal conductance was only observed at early stages of cowpea growth i.e. at 47 and 54 days after planting (DAP).

In conclusion, this PhD has shown that $\delta^{13}C$ has potential to be used in breeding for drought resistance in triticale and probably other small grain crops. This study also revealed that there is minimum contribution of pre-anthesis assimilates to grain filling under water stress, contrary to what is reported in literature and hence more research is needed.The evaluated genotypes of triticale and cowpea showed tolerance to drought stress under semi-arid conditions. Agbeacon for triticale and IT00K-1263 for cowpea were identified as the most promising genotypes and hence their adoption in the smallholder farming system could be a step towards adapting to future warmer and drier climates

List of Symbols and Abbreviations

%Ndfa	Percentage nitrogen derived from atmosphere
‰	per mil
°C	Degrees Celsius
^{15}N	Stable isotope of nitrogen with mass 15
ANOVA	Analysis of variance
A	Photosynthetic rate
BNF	Biological nitrogen fixation
C	Carbon
Ca	atmospheric CO_2 concentration
Ci	intercellular CO_2 concentration
DAP	Days After Planting
E	transpiration rate
GDD	Growing Degree Days
gs	Stomatal Conductance
IPCC	Intergovernmental Panel on Climate Change
IT1	IT00K-1263
IT2	IT99K-1122
LTAS	Long Term Adaptation Scenarios
kg	kilogram
mm	millimetres
MS	Moderate Stress
MW	Moderately well-watered
N	Nitrogen
NDVI	Normalised Difference Vegetation Index
NDWI	Normalised Difference Water Index
NRI	Nitrogen Reflectance Index
PEPC	Phosphoenolpyruvate carboxylase
rH	Relative humidity

Rubisco	Ribulose 1,5 biphosphate carboxylase
SS	Severe Stress
SSA	Sub-Saharan Africa
STI	Stress Tolerance Index
t ha^{-1}	tonnes per hectare
TV1	TVu14632
TV2	TVu4607
VPD	Vapor Pressure Deficit of air
WI	Water Index
WUE	Water Use Efficiency
WW	Well-watered
YR	Yield Reduction
YSI	Yield Stability Index
$\delta^{13}C$	Natural abundance of ^{13}C per mil
$\delta^{18}O$	Natural abundance of ^{18}O per mil
$\delta^{15}N$	Natural abundance of ^{15}N per mil

1 Chapter 1. General Introduction

1.1 Introduction

Climate change is a global problem that has attracted considerable attention of policy makers and researchers. This is because climate change affects almost every sector of our society. Chief among the sectors is agriculture where climate change is threatening food security. The threat on food security is further exacerbated by the projected increase in human population in the coming decades. World population is expected to reach nine billion by 2050 (Godfray et al., 2010, Kearney, 2010) with most of the population increase occurring in developing countries, mainly in Asia and Africa (Cleland, 2013). However, Africa is the most vulnerable continent to climate change due to its low adaptive capacity (Boko et al., 2007). Population growth in Sub-Saharan Africa (SSA) is expected to grow by 1.9% per year in the period towards 2050 while that of the world would be around 0.55% (Alexandratos and Bruinsma, 2012).

Climate change is defined by the Intergovernmental Panel on Climate Change (IPCC) as, "*a change in the state of the climate that can be identified (e.g., by using statistical tests) by changes in the mean and/or the variability of its properties, and that persists for an extended period, typically decades or longer*" (IPCC, 2014b). The main driver of climate change is anthropogenic emission of greenhouse gases mainly CO_2 into the atmosphere leading to global warming. The concentration of CO_2 in the atmosphere has risen from pre-industrial revolution concentrations of about 270 ppm to the current 400 ppm (World Meteorological Organisation, 2016). It continues to increase and is expected to surpass 500 ppm by 2050 depending on the emission scenario (IPCC, 2014a). The major threats of climate change on agriculture and more specifically on crop productivity is temperature increase in and the decrease in precipitation. Temperatures are expected to rise by 1.4 to 5.8 ˚C, depending on the location, (Urruty et al., 2016) while precipitation is expected to increase in some areas and decrease in others. Furthermore, the occurrences of extreme events like droughts, floods, and heat waves are also on the rise (Lipper et al., 2014).

The question therefore is, how is humankind going to deal with the foreseen challenges? According to the IPCC (2014b), the impacts and risks related to climate change and variability can be reduced and managed through mitigation and adaptation. Mitigation refers to the efforts to reduce or prevent emission of greenhouse gases (IPCC, 2014c) while adaptation refers to the adjustment to actual or expected climate and its effects (IPCC, 2014b). These two are intimately linked, as mitigation reduces the magnitude of global warming, thus increases the time available for adaptation processes. In agriculture, particularly in crop production, one of the main challenges is how to adapt crops to future warmer and drier climates in many parts of the world. Fig. 1.1 shows the global distribution of future changes in drylands which clearly shows a world-wide increase in drylands particularly in Sub-Saharan Africa.

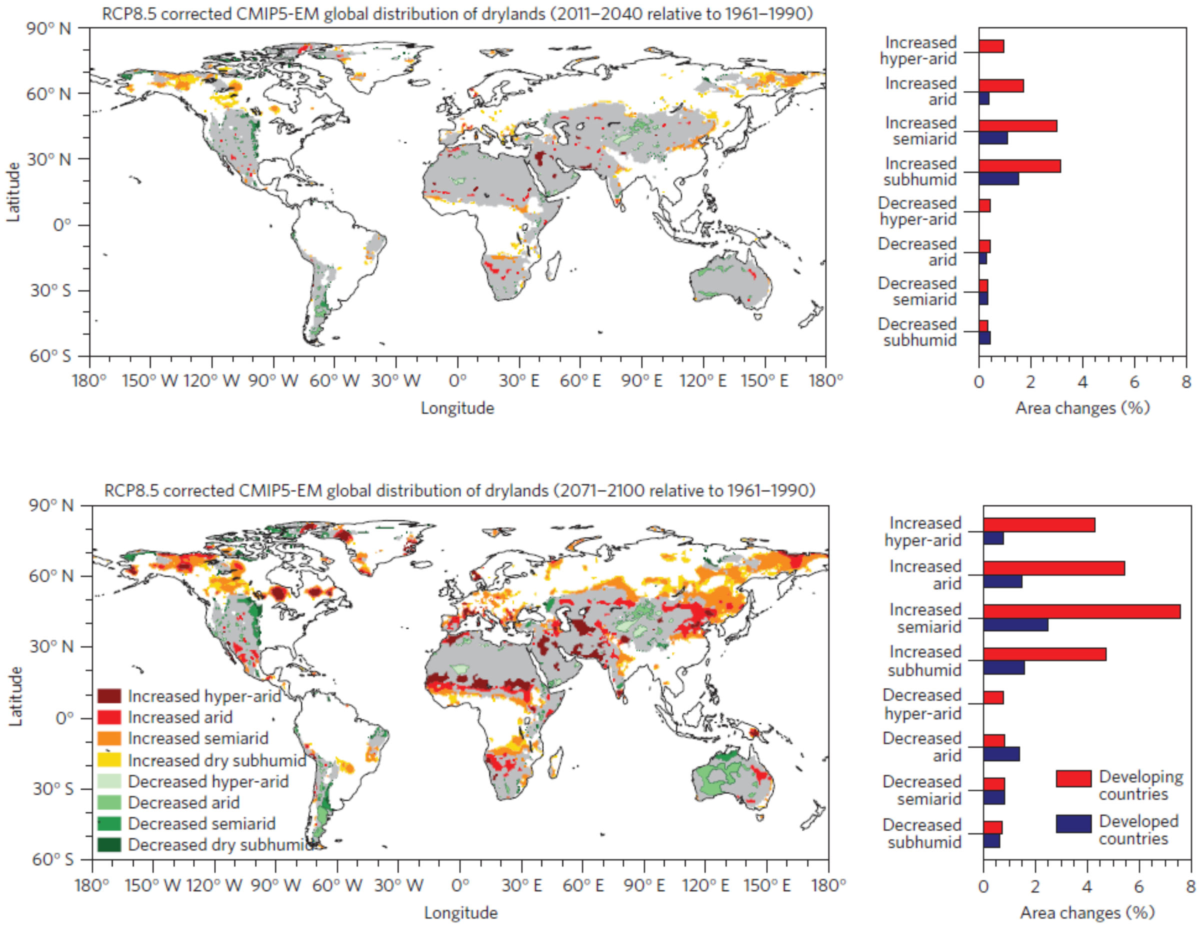


Fig. 1.1: Global distribution of future changes in the dryland subtypes. Representative Concentration Pathways (RCP8.5) are shown relative to the baseline period (1961–1990) for 2011–2040 (**top map**), and 2071–2100 (**bottom map**) with the corresponding area changes (units: percentage of global land area) in developing and developed countries. The grey shading denotes the baseline drylands in 1961–1990. Changes include any transition from adjacent and nonadjacent subtypes. **Maps adopted from Huang et al. (2015)**.

Adapting crops to future warmer and drier environments entail, among others, improvement in drought resistance of the crops. Hence, this study focusses mainly on the improvement of genotypes through screening traits for drought tolerance.

Screening for drought tolerance requires a good understanding of the physiological processes linked to drought tolerance and finding ways of manipulating or improving them. Plant breeding is one way that can be used to improve crops' tolerance to drought but is often difficult and slow (Shiferaw et al., 2011, Wahid et al., 2007), as it usually involves selecting high yielding cultivars under both well-watered and water limited conditions. However, yield is a quantitative trait that displays high interaction between environment and genotypes (Farooq et al., 2009, Araus et al., 2008). Surrogate traits provide better and relatively faster ways of screening cultivars for drought resistance. Drought resistance is strongly linked to higher water use efficiency (WUE) in crops but screening for higher WUE in the field is laborious (Tardieu, 2011). Identification of reliable, fast and easily measurable traits is important in adapting crops to drier future climates. As such, one of the main objectives of this study was focused on assessing the use of $\delta^{13}C$ as a screening trait for drought resistance in triticale.

Feeding nine billion people in 2050 also requires identification of 'future crops'. Cereals such as wheat (*Triticum* spp.), maize (*Zea mays*) and rice (*Oryza sativa*) are at present the major sources of carbohydrates in the human diet (Lafiandra et al., 2014). A substantial percentage of the world population relies on legumes like dry bean (*Phaseolus vulgaris*) for their protein uptake. However, climate change is threatening the productivity of the very crops that people rely on. For example, global wheat production is projected to drop by 6% for every 1^0C increase in temperature (Asseng et al., 2015). Global maize production is also predicted to drop by 10% by the year 2055 (Jones and Thornton, 2003). In SSA, 30% of the current maize producing areas are expected to become unsuitable in the same period due to heat and drought stress (Rippke et al., 2016). Similarly, 60% of the current drybean producing areas in SSA are also projected to become unsuitable for drybean production by the end of the century (Rippke et al., 2016). Rippke et al. (2016), suggest that farmers might need to shift towards more drought-tolerant cereals like millet (*Pennisetum glaucum*) and sorghum (*Sorghum bicolor*) in place of maize but alternatives for wheat and beans are also needed as they are also under threat. Currently, triticale (x. *Triticosecale* Wittmack) offers a better alternative for wheat even though the bread making quality is still inferior to that of wheat. Triticale is a hybrid of wheat and rye (*Secale* spp.) which inherited the yield potential of wheat and the resistance to unfavourable conditions of rye (Mergoum and Macpherson, 2004). Hence, triticale performs much better than wheat under unfavourable conditions such as drought, extreme temperature and pH. In the case of legumes, cowpea could be a better alternative to drybean as it is more drought tolerant.

Due to the vulnerability of the smallholder farming sector in the SSA region, more attention is needed to improve their adaptive capacity and to build resilience to climate change and variability. The vulnerability of smallholder farmers in the region is heightened by their dependence on the already marginalized natural resource base for their livelihoods (Ziervogel et al., 2008). In SSA, drought and heat stress are not the only threats to food security, but also the continued decline in soil fertility. Low soil fertility, has for a long time, been the major constraint to crop productivity in the smallholder farming sector of this region (Pypers et al., 2011, Vanlauwe et al., 2015). Smallholder farmers in SSA are mostly resource poor and farming on soils that are inherently infertile (Vanlauwe et al., 2015). Fertilizer usage by farmers, particularly nitrogen is far below the world average. According to Vanlauwe et al. (2014), farmers in the sub-Saharan region apply nearly 15 times less fertilizer per hectare compared to the rest of the world.

Legume intercropping, which has been practiced by smallholder farmers for a long time, is a key farming practise that could be improved to enhance productivity in the smallholder farming sector. Legumes do not only contribute to improving soil fertility when residues are retained but are also a key source of protein, particularly in rural communities. Common legumes already being incorporated in farming system include cowpea (*Vigna unguiculata* L. Walp.), groundnut (*Arachis hypogaea* L.), drybean, and bambara groundnut (*Vigna subterranean* L. Verdc.) (Nyemba and Dakora, 2010). But, as reported earlier, climate change is already affecting land suitability for the production of most of the legumes listed (Rippke et al., 2016). The incorporation of drought tolerant legumes including cowpea which have the capacity to fix substantial amounts of nitrogen (N) under water-limited conditions will go a long way in improving soil fertility in the smallholder sector.

In some parts of Sub-Saharan Africa such as South Africa, the effect of climate change and variability on the agricultural sector has been explored (Benhin, 2008, Gbetibouo and Hassan, 2005, Gbetibouo et al., 2010, Ziervogel et al., 2014). South Africa, is advanced in terms of research, observation, and climate modelling when compared to most African countries and hence has managed to downscale climate projections from regional to local projections under a project known as the Long Term Adaptation Scenarios (LTAS) (Ziervogel et al., 2014). The LTAS project has shown that mean annual temperatures in South Africa have increased by at least 1.5 times more than the global average of 0.65°C over the past five decades. LTAS also projects increase in flooding and drought events across the country. The projected increase in drought poses risks to the water resources and food security of the country. South Africa, just like many other southern African countries, is a water scarce country with average annual rainfall of 450 mm compared to the global average of 840mm. In addition, upto 64% of its water resources are already being used for agriculture (Benhin, 2008). This level of consumption applies to many other water scarce countries (Morison et al., 2008). Thus, climate change will significantly affect agriculture particularly field grown crops which constitute 80% of the total cultivated land in South Africa (Gbetibouo and Hassan, 2005).

Gbetibouo et al. (2010), analysed the vulnerability of South African agriculture to climate change and variability and found that there is variation in vulnerability to climate change across the nine provinces of South Africa (Fig 1.2). The variation was attributed to the diverse social, economic and political environment. Limpopo Province together with Kwazulu Natal and the Eastern Cape were found to be the most vulnerable provinces due to their low adaptive capacity. Interestingly, the study found Limpopo province to have a lower exposure to climate change and variability but is nevertheless more sensitive to climate change due to its lower adaptive capacity. This lower adaptive capacity could be attributed to the significantly higher number of resource poor smallholder farmers in the province (Tshiala and Olwoch, 2010) who depend heavily on rain fed agriculture for their livelihood. Hence, this study is focused on improving the adaptive capacity of smallholder farmers in semi-arid regions such as the Limpopo Province, through: 1) crop improvement by screening for drought resistance traits under semi-arid conditions, and; 2) the evaluation for drought resistant cereal and legume genotypes.

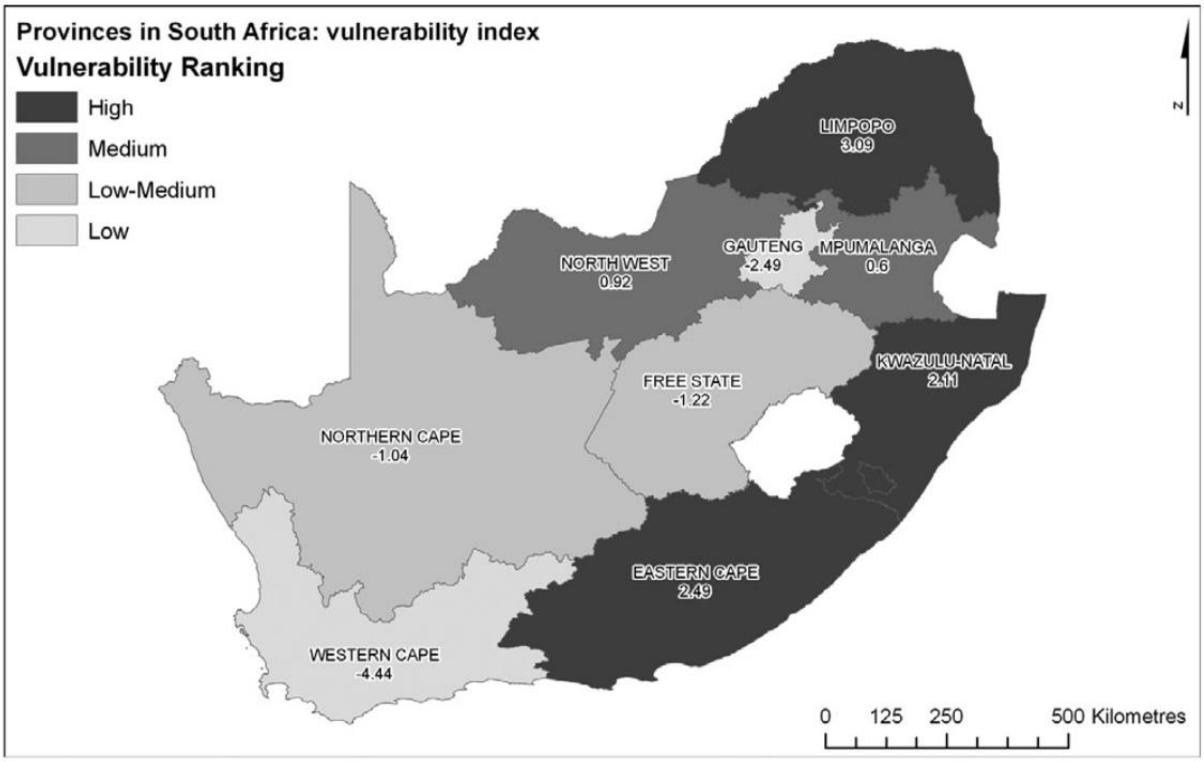


Fig. 1.2: Map of vulnerability indices across South Africa's nine provinces. Adopted from Gbetibouo et al. (2010).

1.2 Problem statement

Climate change and variability is threatening the livelihood of smallholder farmers in arid and semi-arid regions particularly in SSA. Smallholder farmers are more vulnerable to climate change due to their low adaptive capacity which is compounded by their reliance on rain fed agricultural systems (Kotir, 2011). Furthermore, many parts of SSA (Southern, East and West Africa) are expected to become drier and warmer due to climate change. As a result, the suitability of many crops in the region is under threat (Rippke et al., 2016), posing risk to food security. In southern Africa, South Africa is the major source of food (Benhin, 2008) hence adverse effects of climate change in South Africa could destabilise the whole region.

Water resources are also projected to decline as result of the predicted decline in precipitation and hence stream flow. The continued reduction in precipitation means food production will rely more on irrigated agriculture. However, at present, more than 70% of fresh water resources are already being used in irrigated agriculture (Morison et al., 2008, Blignaut et al., 2009). Such rates of water consumption are not sustainable, particularly in water scarce countries like South Africa. In addition, water resources are also under immense pressure from industrial and domestic needs.

Therefore, there is a need for water use efficient crops to cope with decreased precipitation and water supplies. However, the measurement and breeding for high WUE is not easy and hence the need for surrogate traits.

Drought and heat stress are not the only threat to food security in SSA, but also a decline in soil fertility. Smallholder farmers in SSA are resource poor and are cultivating on degraded soils, yet their fertilizer application rates remain very low (Vanlauwe et al., 2015). Due to these challenges crop productivity in SSA is predicted to decrease by more 50%, again threatening food security in the region. This, therefore, calls for cheaper and sustainable alternatives to improve crop productivity in the smallholder sector.

1.3 Rationale

Limpopo province is a dry area and climate change predictions are that the province will become even drier. Precipitation and soil moisture are expected to decrease so are water resources in the province. Therefore, to ensure food security there is need to build resilience in the smallholder sector by improving crop water use efficiency. But the challenge is that conventional methods of determining water use efficiency are laborious. Hence there is need to identify traits that can be used as surrogate for WUE. In addition, the small holder farmers in Limpopo are resource poor and in need of sound alternatives to improve crop productivity in the face of climate change. To improve crop productivity, there is a necessity to incorporate drought resistance legumes in their farming system.

1.4 Aim and Objectives

1.4.1 Overall aim

The main aim of the study was to evaluate the use of $\delta^{13}C$ and $\delta^{18}O$ as proxies for WUE and drought tolerance in triticale genotypes whilst at the same time screening cowpea genotypes for high biological nitrogen fixation and suitability in semi-arid regions.

1.4.2 Specific objectives

The specific objectives of the study were:

i. to evaluate the agronomic performance of triticale genotypes in the semi-arid conditions of the Limpopo Province under varying soil water conditions

ii. to assess the potential use of $\delta^{13}C$ and $\delta^{18}O$ isotope signatures as screening traits for higher WUE and drought resistance in triticale under varying moisture levels
iii. to identify superior cowpea genotype(s) with high BNF, and grain yield under drought conditions for improved soil fertility and nutrition in the smallholder farming sector
iv. to investigate the stomatal behaviour of cowpea genotypes grown under varying moisture levels

1.5 Hypotheses

It was hypothesised that:

i. triticale is a suitable crop for biomass and grain production under semi-arid conditions of the Limpopo Province
ii. $\delta^{13}C$ and $\delta^{18}O$ isotope signatures can be used as screening traits for higher WUE and drought resistance in triticale under varying moisture levels
iii. BNF and grain yield of cowpea genotypes differ in response to varying soil moisture levels
iv. there is a variation in stomatal behaviour of cowpea genotypes grown under varying moisture levels

1.6 Thesis outline

It is against the background above that this thesis is outlined as follows:

Chapter 2 provides a review of the relevant literature.

Chapter 3 evaluates the yield performance of four triticale genotypes in a hot semi-arid area of the Limpopo province. The four spring type genotypes were selected from preliminary study carried out with eight genotypes. The study was aimed at assessing the possibility of growing triticale in a semi-arid environment like the Limpopo region whilst simultaneously assessing a rationing irrigation scheme so as to identify an irrigation scheme that produces reasonable grain and biomass yields with the lowest amount of water possible. The study also explored remote sensing techniques in monitoring water stress in triticale.

Chapter 4 examined the combined use carbon-13 and oxygen-18 in assessing the physiological responses of triticale under varying moisture levels. In addition, the study evaluates the use of ^{13}C as screening trait for higher WUE and drought resistance in triticale while at the same time testing the capability of the dual isotope model to explain the variation of carbon isotope composition in leaves.

Chapter 5: In order to improve crop productivity in the smallholder farming sector and in the face of climate change, alternative cheaper forms of N are needed. Hence this chapter is focused on screening potential cowpea genotypes for BNF and grain yield under drought conditions. The four genotypes used in the study were selected from a pool of 90 lines obtained from the International Institute of Tropical Agriculture (IITA). The screening of the genotype will help in building resilience in the smallholder farming sector.

Chapter 6 investigates the stomatal behaviour of cowpea genotypes grown under varying moisture levels. The opening and closing of the stomata is one of the most important physiological process that simultaneously control carbon assimilation and water loss in plants. Hence, it plays a significant role in the drought tolerance of crops.

Chapter 7: This chapter provides a summary and general conclusions of the most important findings in this study. It also presents grey areas for further research.

2 CHAPTER 2 - Literature review

2.1 Climate change and drought

Agricultural production in the smallholder sector of many African countries is expected to be compromised by climate change and variability (Müller et al., 2011). A report by Müller et al. (2011) suggest that yields could be reduced by as much as 50% in the rain fed farming systems of Africa. However, vulnerability to climate change is not uniform across the whole of Africa (Müller et al., 2011), but in East and Southern Africa vulnerability is compounded by the large number of smallholder farmers that depend on marginalised land for livelihood (Ziervogel et al., 2008). Thirty percent of the world land area is arid and semi-arid (Fang and Xiong, 2015) and in Africa this proportion of arid and semi-arid lands is expected to increase by 5-8% by 2080 (Boko et al., 2007). According to the IPCC (2014b) report on regional aspects, most parts of Southern Africa will experience a downward trend in precipitation. In addition, intra-seasonal variation in precipitation particularly the onset, duration, frequency of dry spells and rainfall intensity are all expected to change. Accurate prediction of precipitation trends in Africa have nevertheless been hindered by lack of sufficient observational data (IPCC, 2014b). However, South Africa is quite advanced in terms of research, observation, and climate modelling when compared to most African countries but it still lacks a robust national system that provides spatially extensive climate data (Ziervogel et al., 2014).

Reports show that droughts have been very frequent in many arid and semi-arid areas. In South Africa, droughts have been occurring every three to six years in the last two decades (Boone et al., 2004, Thomas et al., 2007). Predictions in South Africa thus show increases in temperatures of 2.3 to 9.6˚C and precipitation decreases of 2 to 8% by 2100 (Benhin, 2008). Benhin (2008), also reported the vulnerability of the South African agricultural sector as due to: (i) the semi-arid nature of the country with increased farming on marginal lands, (ii) increased frequency of droughts and (iii) the scarcity of water, which is exacerbated by a high spatial variability of rainfall.

2.2 Drought: effects and management

Drought is defined by the IPCC (2014a) as *'period of abnormally dry weather long enough to cause hydrological imbalance*.' Drought is however regarded as a relative term and is defined relative to an activity. As such, there are different types of droughts i.e. agricultural drought, hydrological drought and meteorological drought (Blum, 2011b). Meteorological drought occurs when there is a prolonged period with less than average precipitation and it usually precedes the other kinds of drought. Hydrological drought occurs when the water reserves available in aquifers, lakes, and reservoirs are below average. Agricultural drought is when there is insufficient moisture for maximum growth of crops, range or plantations. This study focuses mainly on agricultural drought which impact crops due to moisture deficit in the root zone (Trenberth et al., 2014). The amount of moisture in the root zone depends on precipitation and the amount of water infiltrating into the soil. Hence, soil moisture in many semi-arid regions is expected to decrease due to climate change. Soil moisture in drier parts of southern Africa is expected to decrease by more than 10% (IPCC, 2014a).

The effects of drought on crops were extensively reviewed by Farooq et al. (2009). Fig. 2.1 shows a summary of the causes of drought, its effects and symptoms in plants, plant responses to drought as well as the strategies for drought management. The effects of drought on crops can be variable and depend mainly on: (i) the severity of the drought (ii) the growth stage of the crop and (iii) drought resistance of the crop. If the drought is too severe it stimulates the senescence of the whole plant, no matter the growth stage or the resistance. However, when the drought is mild, the effects can manifest itself in different ways and the severity depends on the growth stage and resistance of the crop. Productivity in many crops is severely reduced when drought occurs during development of generative organs and also during seed filling (Farooq et al., 2014). However, when it occurs during the vegetative stages many crops may recover to give reasonable yields. This ability to quickly recover from a dry spell is a desirable physiological trait for drought resistance in crops (Rivas et al., 2016).

Plants exhibit different strategies for resistance against drought. The response may occur at leaf level or at the whole plant level (Chaves et al., 2002). At the leaf level, plants commonly respond to water stress by closing their stomata to reduce water loss. As a result, carbon gain (photosynthesis) and cell growth are affected (Araújo et al., 2015). Studies have however shown that cell expansion and cell division are reduced by mild drought stress, before photosynthesis is affected (Blum, 2011b, Prasad et al., 2008). If water stress continues, leaf size is reduced, leaf rolling occurs and plants may align their leaves parallel to the sun rays (Agbicodo et al., 2009, Fang and Xiong, 2015). At the plant level, some of the responses may include; flowering early to escape drought, decrease shoot growth, increase root growth for more efficient water uptake (Farooq et al., 2014, Hossain et al., 2016). Other phenological effects of drought on plants include increased root to shoot ratio due to increased assimilate allocation to roots, resulting in improved water uptake (Hossain et al., 2016, Pask and Reynolds, 2013).

Mild drought stress can be managed mainly in two different ways: the first is the improvement of crop genotypes (adapting crops to drought stress) and the second is adjusting the management practices. Management practices that can be improved include: sowing dates, planting density and soil and water management. This study focusses mainly on the improvement of genotypes through screening traits for drought tolerance. Some of the common traits that have been identified for improving drought resistance include: high WUE, vigorous root growth, higher biomass partitioning into harvest product, early maturity, stomatal control, delayed leaf senescence, among others (Araújo et al., 2015). Therefore, there is need for traits that can be measured easily in a consistent way and which allow high throughput measurements. The need for surrogate traits is also necessitated by the difficultness to make observations more reliable and by the need to speed up the breeding process (Shiferaw et al., 2011, Wahid et al., 2007).

There are several mechanisms which plants use to respond to, adapt to and survive drought stress. These response mechanisms include morphological, physiological, biochemical, cellular and molecular changes (Farooq et al., 2009, Fang and Xiong, 2015). Morphological changes relate to changes in the plant’s structure e.g. reduction in leaf area or increase in root/shoot ratio; physiological responses relate to the functioning of the plant during stress such as the closing of the stomata; while biochemical responses refer to the production of reactive oxygen species. The mechanisms enable plants to grow and produce satisfactory yields under water stress. The four mechanisms of drought resistance in plants are: drought avoidance, drought tolerance, drought escape, and drought recovery (Fang and Xiong, 2015). Drought avoidance refers to the ability of the plant to maintain high water status under dry conditions either by water uptake or reduction in water loss mainly through closing their stomata (Luo, 2010). Drought tolerance is the ability of the plant to sustain a certain level of physiological activities under severe drought stress while drought escape refers to the adjustment of the growth period or planting time in the growing season to avoid seasonal drought. Lastly, drought recovery refers to the ability of the plant to recover and resume growth after exposure to drought stress (Luo, 2010, Fang and Xiong, 2015).

2.3 Effect of increasing CO_2 concentration on crop productivity

The effect of climate change is not all gloomy, as projected increases in atmospheric CO_2 are expected to enhance productivity of C_3 crops (like small grain cereals) (Deryng et al., 2014). C_4 plants, will not benefit from the CO_2 fertilization as their photosynthesis is already near CO_2 saturation and hence will not respond to elevated CO_2 (Ghannoum, 2009, Vanuytrecht et al., 2012). The high CO_2 concentration in the atmosphere is expected to have different effects on plants depending on their photosynthetic pathways as observed in free-air CO_2 enrichment (FACE) experiments (Leakey et al., 2009). In such experiment C_3 crops (e.g. wheat, soybean, cowpea) are expected to gain through increases biomass accumulation due to CO_2 fertilization while the increase in CO_2 is expected to have no effect on C_4 crops like maize (Vanuytrecht et al., 2012). Modelled data show that the enhancement of productivity due to increased CO_2 in C_3 crops is due to reduced water loss via reduction in stomatal conductance (Deryng et al., 2016). Productivity will be enhanced by the elimination of photorespiration and thus, improved WUE. Empirical evidence however shows a strong interactive effect between CO_2 concentration and soil moisture (Amthor, 2001, Ewert et al., 2002, Kang et al., 2002, Wu and Wang, 2000, Madhu and Hatfield, 2015). Some studies show a general decrease in crop performance under water stress even with high CO_2 (Amthor, 2001, Ewert et al., 2002, Wu and Wang, 2000) while others show improved performance under water stress with high CO_2 (Kang et al., 2002).

A recent study on soybean has actually shown that C_3 crops will not benefit much from elevated CO_2 concentrations under drought conditions (Gray et al., 2016). The explanation for this could also be due to the fact that while elevated CO_2 enhances photosynthesis and reduces water loss due to reduced stomatal conductance, it also enhances leaf area index resulting in increased water use thus offsetting the benefits of elevated CO_2 (Fatichi et al., 2016). Nevertheless, there is a common agreement that increased CO_2 will improve crop performance under unlimited water supply. In as much as elevated CO_2 enhances photosynthesis (under well-watered conditions), the negative effects of increased atmospheric CO_2 concentration outweigh the positives.

Elevated CO_2 results in higher temperatures which speed up crop development rate; thus reducing time to photosynthesise and build vegetative parts. Consequently, seed filling period is shortened resulting in lower yields. Also, increased temperatures causes heat stress in plants (Wahid et al., 2007). High temperatures also lead to high evaporative demand which also leads to increased water loss through evaporation and transpiration (Lobell et al., 2013).

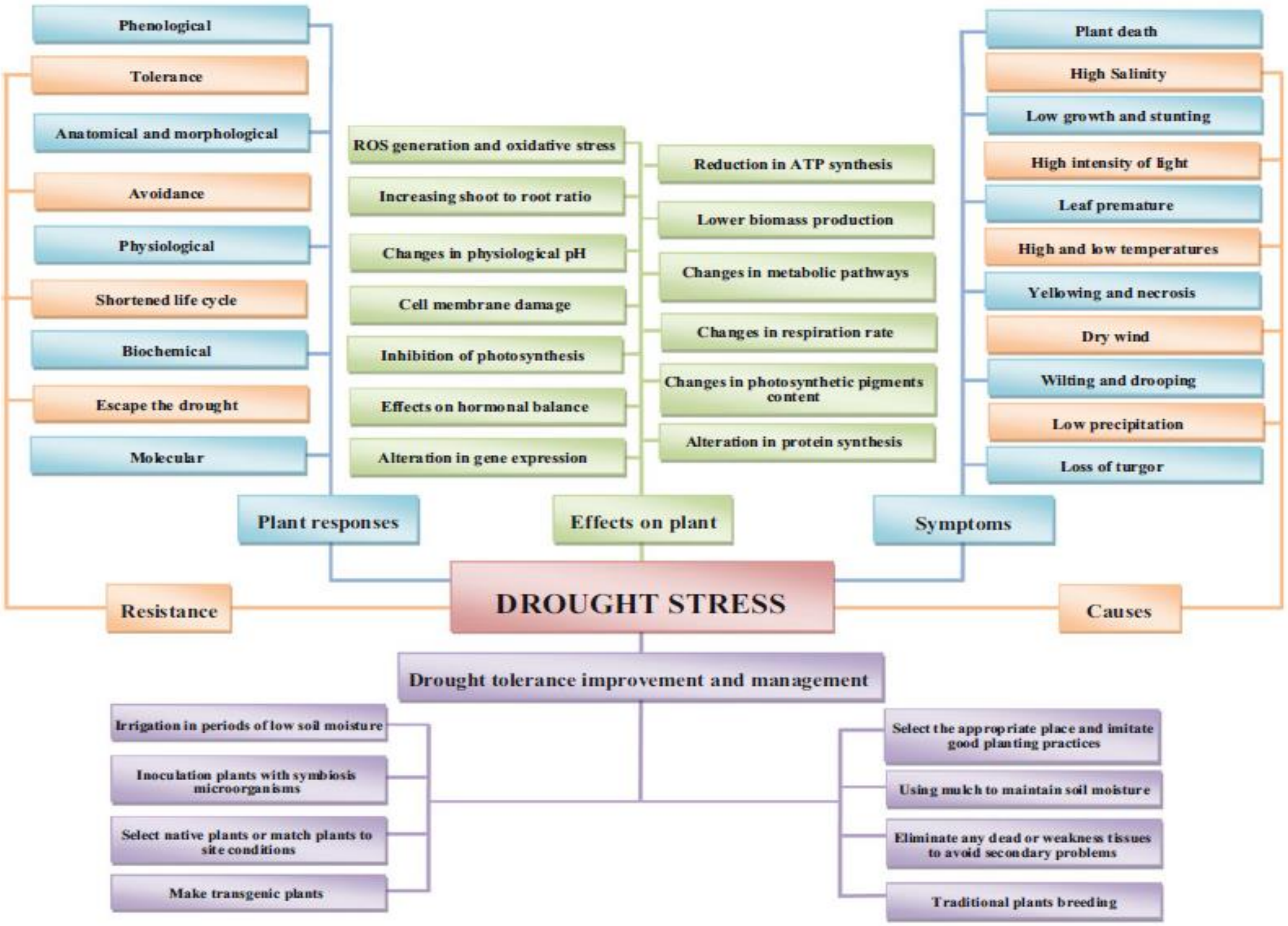


Fig. 2.1: Drought stress: Its causes, symptoms, effects on plants, responses to drought and the mechanisms involved. (Adopted from Hossain et al. (2016)).

2.4 Carbon and oxygen stable isotopes

Carbon has two stable isotopes: ^{12}C and ^{13}C and oxygen has three stable isotopes: ^{16}O, ^{17}O and ^{18}O. Studies examining stable isotopes at natural abundance level are usually reported in delta (δ), values given in per mil (‰) and represents the ratio of $^{13}C/^{12}C$ or $^{18}O/^{16}O$ (R value) in a sample relative to the value of the same ratio in an international standard. The standard for ^{13}C is the limestone Vienna Pee Dee Belemnite (VPDB) and that for ^{18}O is Vienna Standard Mean Ocean Water (VSMOW). The R value is given as:

$$R = \frac{^{13}C}{^{12}C} \text{ or } R = \frac{^{18}O}{^{16}O} \qquad (1.1)$$

and

$$\delta^{13}C / \delta^{18}O = \left(\frac{R_{sample}}{R_{s\tan dard}} - 1 \right) x1000 \qquad (1.2)$$

Some studies report carbon isotope content as carbon isotope discrimination ($\Delta^{13}C$), which is the measure of $\delta^{13}C$ value in plant material relative to that of air on which plants feed. The $\delta^{13}C$ of plants is usually less than that of air due to isotope fractionation occurring during CO_2 diffusion through stomata and mesophyll layers until its photosynthetic assimilation (carboxylation)(Harley et al., 1992, Kodama et al., 2011). The fractionation also differs with the photosynthetic pathway of the plant (C_3 and C_4) due to the different enzymes used for carboxylation. C_3 plants use ribulose bisphosphate carboxylase (Rubisco) which has a higher ^{13}C discrimination compared to C_4's phosphoenolpyruvate carboxylase (Cabrera-Bosquet et al., 2009). The discrimination is calculated as follows (Farquhar et al., 1989):

$$\Delta^{13}C = \frac{R_{air}}{R_{plant}} - 1 \qquad (1.3)$$

and may be calculated as

$$\Delta^{13}C = \frac{\delta^{13}C_{air} - \delta^{13}C_{plant}}{1 + \delta^{13}C_{plant}} . \qquad (1.4)$$

$$\Delta^{18}O = \frac{\delta^{18}O_{plant} - \delta^{18}O_{IrrWater}}{1 + \delta^{18}O_{IrrWater}} \qquad (1.5)$$

2.5 Applications of ^{13}C and ^{18}O in plants studies

Stable isotope techniques have been used in many studies which include paleoclimate, biogeochemistry, identification and quantification of sources of water and nutrients used by plants, carbon allocation in plants, partitioning sources of plant or soil respiration, determination of sources of carbonates, comparing chemical or physical properties of soil organic matter and many other studies(Fry, 2007). Carbon and oxygen stable isotope signatures have proved to be a vital tool in identifying medium to long term effects of environmental factors on CO_2 and H_2O gas exchanges in plants (Ripullone et al., 2009).

The carbon isotope composition ($\delta^{13}C$) of leaf organic matter reflects the fractionation processes occurring during the diffusion of $^{12}CO_2$ and $^{13}CO_2$ (Ripullone et al., 2009). In C_3 plants like wheat, triticale and cowpea the ^{13}C discrimination is related to diffusional fractionation in air (4.4‰) and discrimination against $^{13}CO_2$ by ribulose 1,5 bisphosphate carboxylase (Rubisco) (30‰) and the ratio of intercellular to ambient partial pressure of CO_2 (Farquhar et al. 1982) while in C_4 plants this discrimination is due to Phosphoenolpyruvate carboxylase (PEPC) estimated at -5.7‰ at 25^{o}C (Dercon et al., 2006). The difference in carbon isotope composition between C_3 and C_4 therefore results from the differences in the isotopic fractionation of Rubisco activity in C_3 plants and the PEPC activity in C_4 plants (Monneveux et al., 2007). Rubisco discriminates more again ^{13}C compared to PEPC hence C_4 plants normally have high $\delta^{13}C$ values compared to C_3.

Oxygen isotope composition ($\delta^{18}O$) has also proved to be very essential in plant studies as $\delta^{18}O$ of leaf organic matter also reflects the source of plant water and processes that have been occurring in the plant. The $\delta^{18}O$ value of organic matter therefore is mainly determined by the isotopic composition of the soil water, leaf water enrichment due to transpiration, and biochemical fractionations (Scheidegger et al., 2000). Leaf water enrichment in turn depends on the ratio of intercellular vapour pressure to that of the atmosphere. Thus a low relative humidity results in an increase of $\delta^{18}O$ values in the leaf water (Scheidegger et al., 2000).

Observations of ^{13}C isotope discrimination have been an important tool for evaluating the impact of water stress on crop performance and estimation of WUE in plants (Dercon et al., 2006, Farquhar and Richards, 1984, Zhao et al., 2004). However, its inability to differentiate whether the changes in WUE are driven by stomatal conductance (*gs*) or CO_2 assimilation has led to the development of a conceptual model combining both $\delta^{13}C$ and $\delta^{18}O$ values (Ripullone et al., 2009, Scheidegger et al., 2000). Hence, a combination of both $\delta^{13}C$ and $\delta^{18}O$ values provides more information and better understanding of the response of plants to water stress.

2.6 Water use efficiency

Water is arguably the most limiting abiotic factor affecting crop productivity in the world (Farooq et al., 2014, Tambussi et al., 2007a). The availability of water, particularly in semi-arid regions is increasingly becoming threatened by frequent droughts and increased evapotranspiration rates leading to over-reliance on irrigation to meet food demand. Currently, about 80% of world fresh water is used for irrigation (Morison et al., 2008) and in many dry areas such levels of consumption are unsustainable (Condon et al., 2004) as water resources are also under increased pressure from industrial and domestic users. Water supply is mostly critical in rain fed farming areas located in semi-arid regions like SSA. Such areas are the most vulnerable to water stress due to the reliance on rain fed agriculture which is highly sensitive to climate variability and the low adaptive capacity of the smallholder farmers (Kotir, 2011).

Due to the low rainfall and the continued threat of climate change on water resources, there is a need for improved soil water management and improved crop water use (Barnabás et al., 2008). Improved crop water use entails the improvement in water use efficiency (WUE). According to Foley et al. (2011), improving WUE is one of the main targets of crop research particularly in arid and semi-arid environments, with the aim of finding sustainable ways of increasing crop productivity while at the same time reducing water losses. However, one of the major bottlenecks to produce “more crop per drop” has been the lack of or the evaluation of appropriate plant traits (Araus et al., 2008). Direct measurement of WUE under field conditions remains a big challenge due to the large work load (Tardieu, 2013) and has stalled the use of the WUE trait in crop improvement programs. There is, therefore, a need to identify reliable proxies of WUE that can be measured quickly; are correlated to yield and that can also provide the highest repeatability and heritability.

WUE can be determined at different levels (e.g. leaf, canopy, terrestrial level) and also at different temporal scales hence can be measured in several ways (Abbate et al., 2004, Tambussi et al., 2007a). Katerji et al. (2008), proposes two approaches to determine WUE. The first approach is eco-physiological, which is based on the instant relationship between photosynthesis (CO_2 assimilation) and transpiration per leaf unit area. The second being agronomical approach (more classical), measured as the ratio of biomass or yield per amount of water transpired or used. The different definitions of WUE were summarized by Tambussi et al. (2007a) as shown in Fig. 2.2.

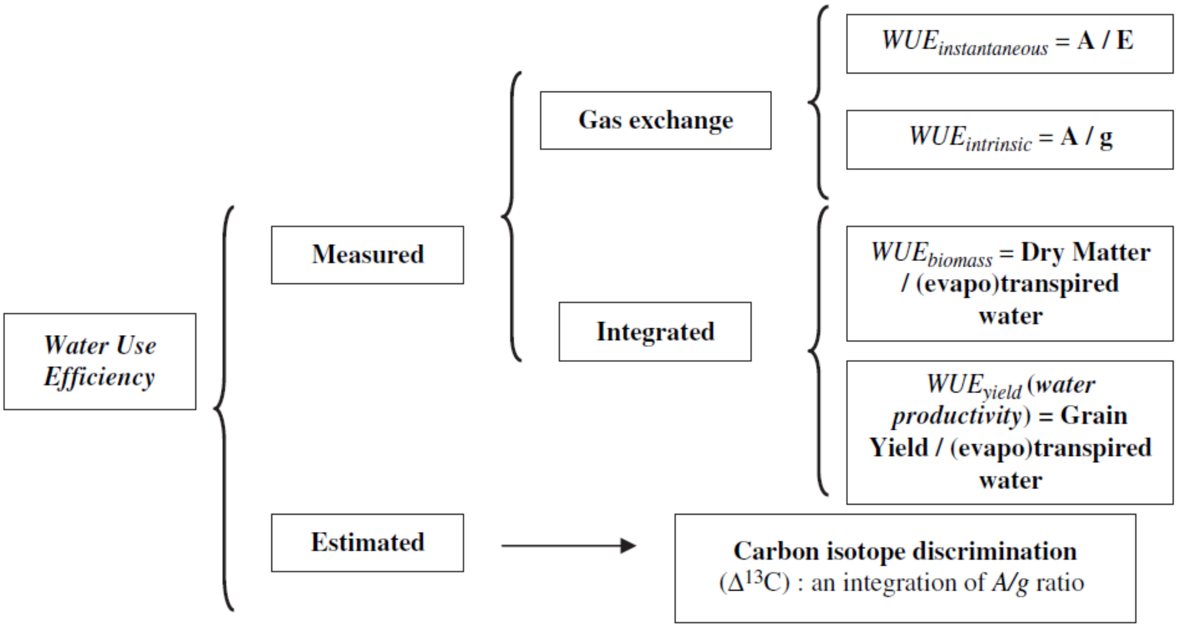


Fig. 2.2: Flow chart showing the different methods of measuring and estimating WUE in plants. *A* is net photosynthetic rate (µmol m^{-2} s^{-1}); *E* is transpiration rate (mmol m^{-2} s^{-1}); *g* is stomatal conductance (mol m^{-2} s^{-1}) and $\Delta^{13}C$ is carbon isotope discrimination (‰). Adopted from Tambussi et al. (2007a)

Fig. 2.2 shows that WUE can be measured as gas exchange at the leaf level as instantaneous WUE ($WUE_{instantaneous}$) or as intrinsic WUE ($WUE_{intrinsic}$). At the plant level, WUE can be measured in terms of biomass ($WUE_{biomass}$) or as yield (WUE_{yield}). WUE can also be estimated through carbon isotope discrimination ($\Delta^{13}C$). The ratio between CO_2 concentrations in the intercellular spaces to that of the ambient CO_2 has allowed for the successful use of $\delta^{13}C$ values in estimating WUE in plants. The relationship between $\delta^{13}C$ and WUE exists because the isotope discrimination of plants was found to be linearly linked to the partial pressure of CO_2 in the leaf intercellular spaces (*ci*) and that of the ambient air (*ca*) (Farquhar et al., 1982, Scheidegger et al., 2000).

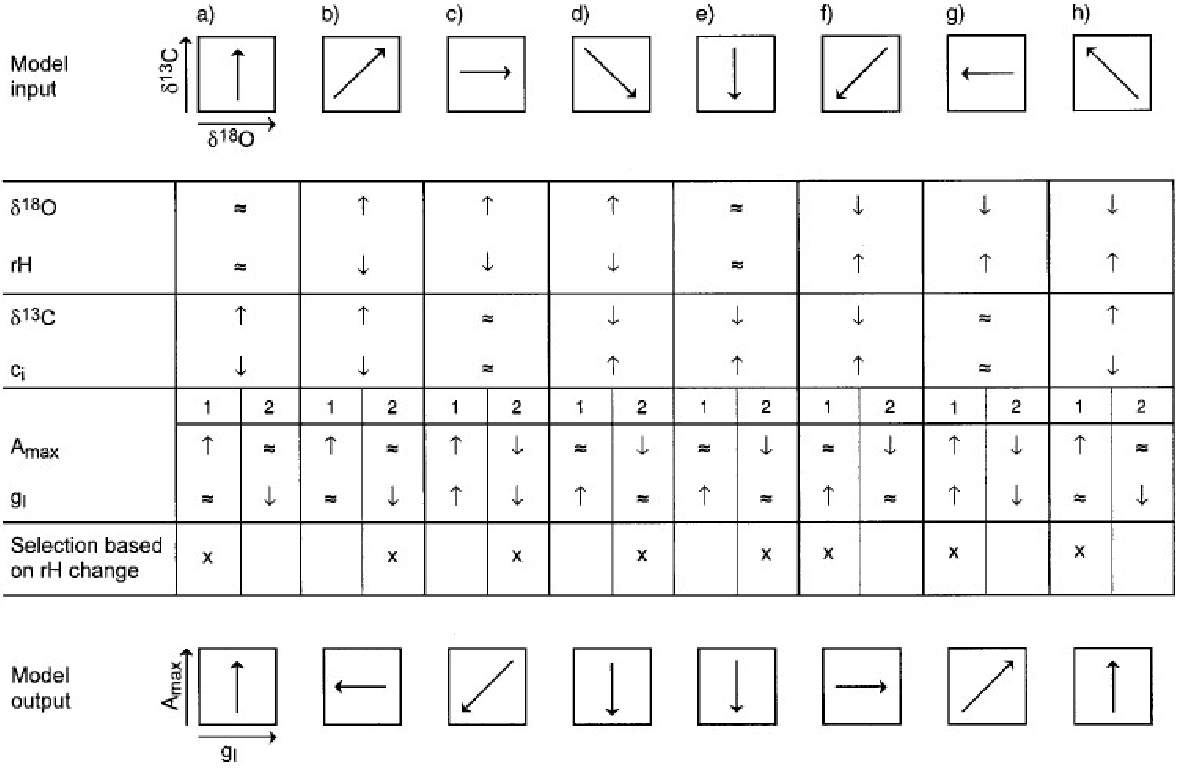
Studies that have explored the use of $\delta^{13}C$ in estimating WUE have largely been more successful with C_3 than with C_4 plants (Dercon et al., 2006, Monneveux et al., 2007, Farquhar, 1983) mainly due to the lower ^{13}C discrimination by C_4 compared to C_3. Nevertheless, some studies with C_4 plants like sorghum have been successful especially in differentiating genotypes in terms of $WUE_{instantaneous}$ and yield (Hammer et al., 1997; Henderson et al., 1998). Instead of using $\delta^{13}C$ alone, oxygen isotope enrichment has been used for both C_3 and C_4 plants due to its independence of the photosynthetic processes, and has therefore been proposed as an indirect measure of transpiration, differences in stomatal conductance and yield (Araus et al., 2008, Cabrera-Bosquet et al., 2009b, Cernusak et al., 2008). A negative relationship between $\delta^{18}O$ and $WUE_{instantaneous}$ has been found in some studies and was found to be influenced by the stomatal conductance which in turn is affected by vapour pressure differences between intercellular spaces and ambient air (Barbour, 2007, Ripullone et al., 2009).

2.7 Dual isotope conceptual model

The dual isotopic model was developed by Scheidegger et al., (2000) to explain the changes in $\delta^{13}C$ occurring as a response to changing environmental conditions. The model is semi-quantitative and assesses effects of the environment on CO_2 and H_2O gaseous exchanges at leaf level. The model allows for the estimation of intercellular CO_2 concentration (*Ci*) from $\delta^{13}C$, and air relative humidity (*rH*) from $\delta^{18}O$. The estimated *Ci* and *rH* are then used to determine stomatal and photosynthesis responses to the environmental conditions. Thus, the output is given as the relationship between photosynthesis capacity (*Amax*) and stomatal conductance (*gs*). *rH* and temperature influence vapour pressure deficit of the air (VPD), which is the driving force for transpiration (see equation 1.6, adopted from (Zha et al., 2017)). A reduction in *rH* results in increased VPD i.e. high evaporative demand and thus causes an increase in $\delta^{18}O$ in leaf water, resulting in an inverse relationship between *rH* and $\delta^{18}O$ as shown on Fig. 2.3.

$$\mathrm{VPD} = \left(1 - \frac{\mathrm{RH}}{100}\right) \times 0.611e^{\left(\frac{17.27\mathrm{T}}{\mathrm{T}+237.3}\right)} \qquad (1.6)$$

To use the model, leaves sampled from plants growing in differing environments are first analysed for their $\delta^{13}C$ and $\delta^{18}O$ signatures. A change in *rH* is then derived from the change in δ¹⁸O. The change in δ¹⁸O is assumed to be primarily due to a change in leaf water enrichment caused by different air humidity (Scheidegger et al., 2000). According to Ripullone et al., (2009), there is a link between δ¹⁸O and isotopic fractionation of water during transpiration in leaves. During transpiration, molecules of water containing lighter isotopes ($H_2{}^{16}O$) tend to diffuse faster from the site of evaporation to the atmosphere (Farquhar et al., 2007). In this way, water becomes enriched in ^{18}O, compared to water coming from the soil.



	a)		b)		c)		d)		e)		f)		g)		h)	
δ18O	≈		↑		↑		↑		≈		↓		↓		↓	
rH	≈		↓		↓		↓		≈		↑		↑		↑	
δ13C	↑		↑		≈		↓		↓		↓		≈		↑	
ci	↓		↓		≈		↑		↑		↑		≈		↓	
	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
Amax	↑	≈	↑	≈	↑	↓	≈	↓	≈	↓	≈	↓	↑	↓	↑	≈
gl	≈	↓	≈	↓	↑	↓	↑	≈	↑	≈	↑	≈	↑	↓	≈	↓
Selection based on rH change	x			x		x		x		x	x		x		x	

Fig. 2.3: The dual isotopic model, adopted from Scheidegger et al. (2000)

In the second stage, the change in *Ci* is derived from the change in δ¹³C. Plants discriminate against ^{13}C during photosynthesis through the ratio of *Ci* to atmospheric CO_2 concentration (*Ca*)(Farquhar et al., 1982). A lower ratio results in an increased discrimination against $^{13}CO_2$ (Ripullone et al., 2009). On the third stage, possible causes of a change in *Ci* are split into cases denoted by 1 and 2 in Fig. 2.3. The change in *Ci* may be due to changes in *Amax* and *gs*. Taking for example scenario b) in Fig. 2.3, a decrease in *Ci* can be interpreted as 1) due to an increase in *Amax* with a constant *gs,* or, 2) a decrease in *gs* and a constant *Amax*. So, to differentiate between these two cases, a selection is made based on the *rH* change. Thus, the *rH* data is important in the application of the model. In this case *rH* was decreasing hence with a decrease in *rH*, the stomatal conductance is expected to decrease since plants tend to close their stomata in dry air, thus case 2 is chosen (decrease in *gs* with a constant *Amax*) as causing the changes in $\delta^{13}C$ and $\delta^{18}O$ (Scheidegger et al., 2000). Nevertheless, the output does not mean that there was absolutely no change in *Amax* but just that the change in *gs* was more pronounced than the change in *Amax*.

The dual isotope model has been applied in several studies to interpret the variation in $\delta^{13}C$ (Sullivan and Welker, 2007, Roden and Farquhar, 2012, Ripullone et al., 2009). Most of these studies have mainly been carried out on perennial C_3 plants. The model is however not without limitations and its operation has been questioned (Roden and Siegwolf, 2012). Major concerns on the model have been on the interpretation of $\delta^{13}C$ and $\delta^{18}O$ values. For instance, the model output depends on the relationship between $\delta^{13}C$ and $\delta^{18}O$ values but there is no recommended standard scaling for the relationship. Also, the model interprets changes in $\delta^{18}O$ as influenced primarily by stomatal conductance. That means environmental influences on evaporative enrichment have to be constant (e.g. source of water, relative humidity, etc.) (Roden and Siegwolf, 2012). The evaporative enrichment of $\delta^{18}O$ is a debatable issue as there are contrasting views on how the enrichment occurs (Barbour and Farquhar, 2000, Ferrio et al., 2012, Sheshshayee et al., 2005).

2.8 Origins, production and uses of triticale

Triticale (×*Triticosecale* Wittmack) is the hybrid between the female parent wheat (*Triticum* spp.) and the male parent rye (*Secale* spp.). The present varieties are hexaploid containing the A and B genome of wheat and the R genome of rye It inherited the high yielding potential of wheat together with the resistance to pathogens and adaptability to marginal growing conditions such as drought, extreme temperature, salinity, extreme pH of rye (Mergoum and Macpherson, 2004). Triticale is also suitable for low-input farming systems due to its higher tolerance to pests and diseases and its extensive root system that can efficiently absorb nutrients (Oettler, 2005, Mergoum and Macpherson, 2004). However, triticale did not inherit the good baking qualities of wheat and therefore, is mostly used as a livestock feed and for poultry (McGoverin et al., 2011). Nevertheless, with the rate at which triticale research is growing, the bread making quality of triticale is expected to catch up soon through the insertion of fragments of D chromosomes of wheat. The D chromosome found in wheat controls the endosperm hardness and the gluten quality of wheat dough. This chromosome gives the bread making quality of wheat. In triticale bread making quality is controlled by the R chromosome inherited from rye. Thus, replacing the R with the D in triticale will improve is bread making quality (Lukaszewski, 2006, McGoverin et al., 2011).

Due to its robustness, triticale often out-yields wheat in unfavourable conditions (Bassu et al., 2011). In South Africa, spring triticale is reported to produce grain yields of up to 40% higher than wheat in low potential soils (Botes and Saul, 2009). Thus triticale has often been grown in areas that are not suitable for wheat e.g. drought stressed regions and soils with acidity or alkalinity problems (Salmon et al., 2004). In addition, studies that have compared small grain cereals (wheat, barley, triticale etc.) have consistently obtained higher yields in triticale both under favourable and unfavourable conditions (Estrada-Campuzano et al., 2012, Motzo et al., 2013). Fig. 2.4, adopted from FAOSTAT 2015 also shows that average triticale grain yield has always been higher than of wheat. Triticale is therefore, the cereal of the future due to the predicted increase in marginal lands as a result of climate change and variability.

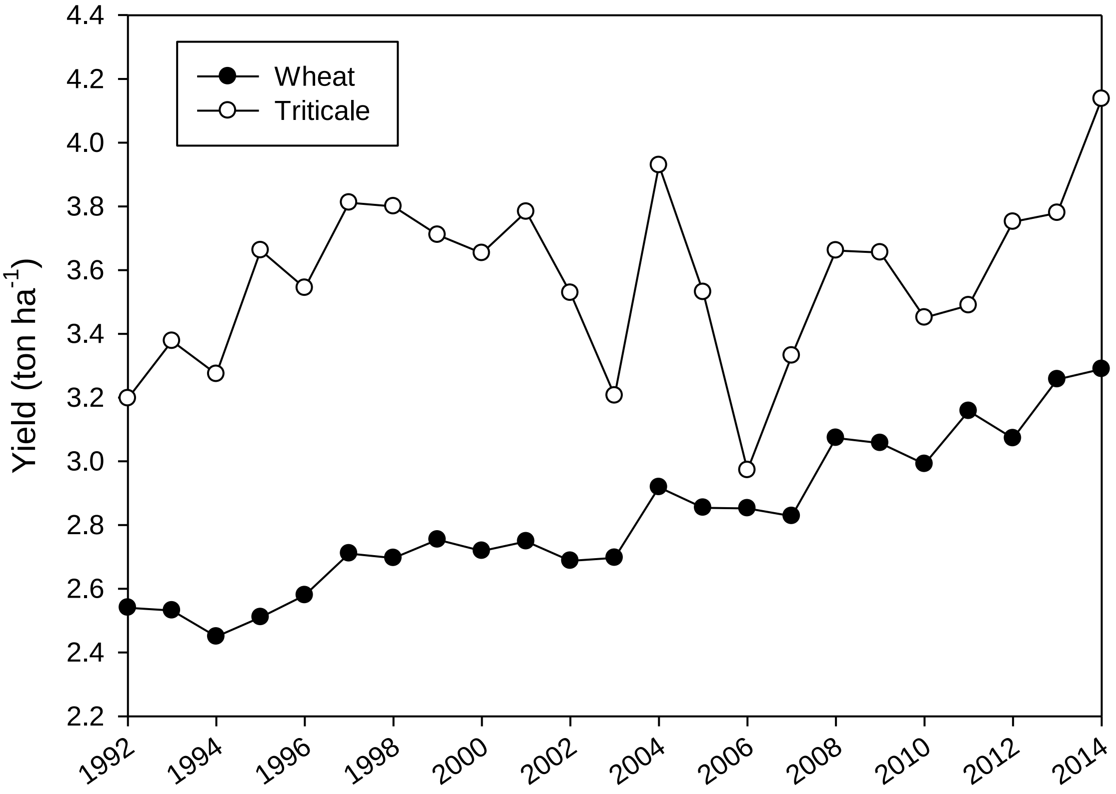


Fig. 2.4: Comparison of world wheat and triticale yields (hg/ha) from 1992 to 2014. Replotted from FAO (2015) data.

Triticale varieties can be classified into spring, winter, and facultative types based on their vernalisation requirements. Spring types have limited vernalisation requirements to go from vegetative to reproductive stages while winter types require a period of several weeks with temperatures between -1°C and 8°C.

2.9 Origins, production and uses of cowpea

Cowpea is one of the most commonly cultivated legumes in the tropics and subtropics. It is particularly grown in less developed countries of Sub-Saharan Africa (SSA), Asia, Central and South America due to its relatively wide adaptation to drought and low nutrient environments. Cowpea is believed to have originated in Africa (Coulibaly et al., 2002), with its domestication occurring mainly in West Africa, in countries like Ghana, Nigeria and Niger.

According to FAO (2015), global production of cowpea stood at 5.5 million tonnes in 2014, more than 90% of which was produced in Africa. The average cowpea yield is reported to be about 450 kg ha^{-1} (Abate et al., 2012). This yield level is the lowest of all tropical legumes, mainly due to cowpea being produced on marginal soils (Abate et al., 2012, Hall, 2012). However, under optimal conditions, cowpea can produce high yields of up to 3 000 kg ha^{-1} (Hall, 2012). Cowpea is an integral part of traditional cropping systems by smallholder farmers in arid and semi-arid areas which often experience droughts. It is also commonly grown as an intercrop with cereals like maize and sorghum (Labuschagne et al., 2008) and is rarely produced in sole cultures. However, in countries like Senegal more cowpea is grown in mono cultures due to increased demand and commercialization of its production (Ehlers and Hall, 1997, Hall, 2012). Cowpea is traditionally grown by resource poor farmers in dry areas, hence, its grain and fodder yields are generally low. Singh and Tarawali (1997) attribute the low yields mainly to low densities and shading by cereals, drought stress and low soil fertility as well as pests and diseases.

Cowpea is a multiple end-use crop. Its young leaves, green pods and green seeds are used as vegetables (Singh et al., 2003). The grains have almost the same nutritional value as beans (*Phaseolus vulgaris*) (Ehlers and Hall, 1997) and are a good source of protein. Antova et al. (2014), reported cowpea protein content ranges of 22.5 to 25.6% and starch of 28.3 to 36.2%. Cowpea is also important as a nutritious fodder and is also used as green manure (Ehlers and Hall, 1997). According to Singh et al. (2003)

mature cowpea haulms are cut and rolled in bundles whilst still green and used as feed supplements. In addition, cowpea is an efficient N_2 fixer(Giller, 2001), fixing most of its N requirement thereby reducing N fertilizer requirement. Its capacity is fix substantial amounts of N even under stress conditions, makes it a strategic crop important in cereal-legume intercropping or rotation systems, particularly in dry regions of SSA (Singh et al., 2003).

2.10 Biological Nitrogen Fixation

Biological nitrogen fixation (BNF) is a natural process whereby atmospheric nitrogen (N_2) is reduced to ammonia in the presence of the enzyme *nitrogenase* (Postgate, 1998). BNF is the biological equivalent of the energy and cost intensive Haber Bosch process that is used industrially in the production of N fertilizers (Sheokand et al., 2012). The enzyme *nitrogenase* is found naturally in microorganisms such as the symbiotic *Rhizobium* spp and also in free-living *Azospirillum* spp and *Azotobacter* spp. Therefore, BNF can occur through free-living soil microorganisms or through symbiotic associations of microorganisms with leguminous plants. Leguminous plants fix atmospheric nitrogen in symbiosis with rhizobia, which live in the root nodules. The rhizobia infect root hairs of the leguminous plants and produce the nodules which become home to the bacteria where they obtain energy from the host plant and take free nitrogen from the soil air and process it into combined nitrogen. The infection process is illustrated on Fig. 2.5. In return, the plant receives the fixed N from nodules and produces food and forage protein (Mulongoy, 1992). When the leguminous plants die, it decays adding fixed N to the soil.

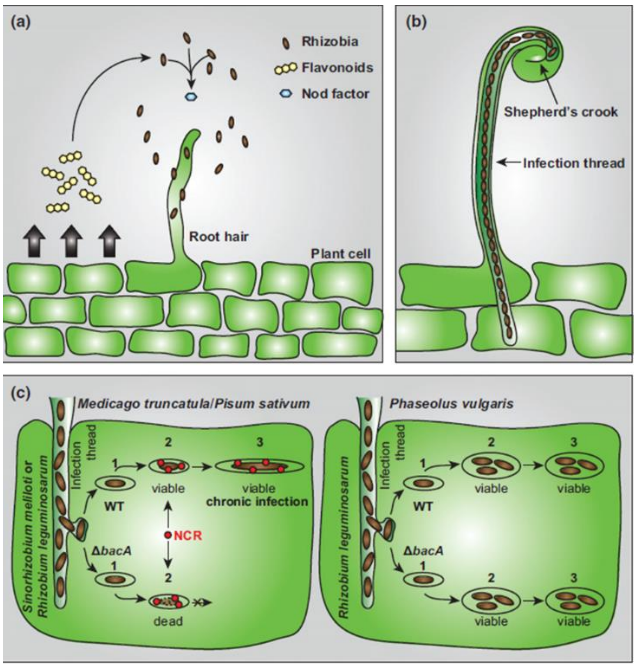


Fig. 2.5: Rhizobia interacting with legumes. (a) The legume secretes flavonoids which induce the rhizobia to produce Nod factors and attract them to the plant root hair cells. (b) Nod factor signalling triggers several developmental changes, including root hair curling which traps the rhizobia in Shepherd's crooks. (c) The rhizobia escape the infection thread and are taken into the host cell via an endocytosis-like process (Haag et al., 2013)

Even though the rhizobium is naturally available in ecosystems, in many areas the rhizobia are relatively ineffective (Postgate, 1998) and hence good effective rhizobium are cultured and made available in different media. These cultures are then inoculated on the seed at planting to promote inoculation. Many legumes are specific to certain rhizobium strains and will not be nodulated by any rhizobium but there are also promiscuous legume species that can be inoculated by many different strains of rhizobium (Thuita et al., 2012). However, when a legume plant is growing in a soil with high mineral N, the symbiosis is affected as the legume resists infection and nodulation by the rhizobium (Postgate, 1998). This is mainly because the symbiosis will be of no advantage to the legume plant because of high costs of supplying substrates and maintaining the symbiosis, yet there will be an easily available N source in the soil. Hence, rhizobium nodulation is in a way controlled by among other factors, soil N status.

2.11 Environmental factors affecting BNF

BNF is an efficient, economic and environmentally friendly source of N. It is key to sustainable agricultural systems in poor soils, which are inherently deficient in N (Hungria and Vargas, 2000). Total annual input of N by BNF is believed to be in the range 50 to 70 Tg with symbiotic associations in arable lands contributing 21 Tg (Lindström et al., 2010). However, BNF contributions in arable lands are not without constraints particularly in SSA. A significant proportion of legumes are cultivated in marginal soils where several abiotic stresses limits N_2 fixation. The factors affecting BNF can be classified into three groups: edaphic factors, climatic factors (mainly temperature and light) and biotic factors (pests, defoliation of host plant and crop completion). Any environmental factor that significantly influences growth of rhizobia or the host plant may strongly affect BNF (Hakeem et al., 2016). As alluded to earlier, BNF is also influenced by the proportion of assimilates that is allocated to the roots (Fenta et al., 2012). Environmental factors affecting BNF are comprehensively dealt with in several studies (Hungria and Vargas, 2000, Rao et al., 2002, Weisany et al., 2013, Zahran, 1999, Zengeni et al., 2006). This section will highlight some of the edaphic factors constraining BNF as they are predominant in SSA.

- **Water stress** is believed to be the major constraint of BNF. It affects both the plant and rhizobial growth. It also affects the formation and longevity of nodules.
- **High temperature** constrains BNF through its effect on rhizobial survival and symbiotic establishment. High temperatures inhibit root-hair formation thus reducing the number of sites for nodulation (Hungria and Vargas, 2000). However, some strains are known to survive soil temperatures of up to 44°C.
- **Salinity** is a serious threat to agriculture in arid and semi-arid regions of the world. Salinity affect N_2 fixation by inhibiting infection of root hairs by rhizobia (Rao et al., 2002). High salt concentrations may affect soil microbial populations through direct toxicity as well as through osmotic stress (Zahran, 1999).
- **Soil acidity** is a major problem in highly leached and weathered soils (Hungria and Vargas, 2000) and also in sandy soils (Zengeni et al., 2006). Such soils are usually inherently infertile and incapable of supporting high rhizobial populations. They also often contain toxic levels of aluminium and manganese. The optimum pH for rhizobial growth is between six and seven.

3 CHAPTER 3 - Yield performance, carbon assimilation and spectral response of triticale to water stress

Published in *Experimental Agriculture* as:

Munjonji, L., Ayisi, K. K., Vandawalle, B., Dhau, I., Boeckx, P. & Haesaert, G. 2016. Yield performance, carbon assimilation, and spectral response of triticale to water stress *Experimental Agriculture*. First View: 1-18. http://dx.doi.org/10.1016/j.fcr.2016.06.001

Abstract

Water stress is arguably the most limiting factor affecting cereal productivity in the world and its effects are likely to increase due to climate change. It is therefore imperative to have a thorough understanding of water stress effects on crop physiological processes so as to better manage, improve and adapt crops to future climates. Triticale is a relatively new crop with great potential to adapt to future climates. A field study was carried out in a steppe (arid) climate in the Limpopo Province of South Africa, to investigate the influence of four moisture levels on: 1) flag leaf CO_2 assimilation and flag leaf carbon content; 2) the utility of flag leaf spectral reflectance to monitor leaf water status and as an indicator of biomass and grain yield and 3) biomass and grain yield performance of four spring triticale genotypes in a dry winter environment (steppe, arid climate). The experiment was carried out in a factorial arrangement of four moisture levels (well-watered (WW), moderately well-watered (MW), moderate stress (MS) and severe water stress (SS)) and four spring type triticale genotypes. Soil moisture levels significantly influenced biomass accumulation, grain yield, CO_2 assimilation, flag leaf carbon content and spectral reflectance. Grain yield levels ranged from 0.8 to 3.5 t ha^{-1} in 2013 and 1.8 to 4.9 t ha^{-1} in 2014. CO_2 assimilation was significantly higher under WW conditions (9.92 $\mu mol\ m^{-2}\ s^{-1}$ in 2013; 11.64 $\mu mol\ m^{-2}\ s^{-1}$ in 2014) and decreased gradually with moisture level to 1.82 and 4.74 $\mu mol\ m^{-2}\ s^{-1}$ under SS in 2013 and 2014, respectively. Flag leaf carbon content was significantly higher under water limited conditions compared to the well-watered treatments. Normalised Difference Vegetation Index (NDVI), Normalised Difference Water Index (NDWI) and Water Index (WI) were significant and positively correlated to biomass and grain yield. WI was particularly strongly correlated to biomass (r^2 = 0.72***) and grain yield (r^2 = 0.55***). However, no clear varietal effects were detected. This study revealed that carbon tends to accumulate in flag leaves under water stress and that flag leaf carbon content is influenced more by the export capacity of the flag leaves than by the CO_2 assimilation rate. WI was found to be a superior index in monitoring water stress in triticale compared to NDVI and NDWI. Above all, spring triticale proved to be adaptable to the steppe (dry) climate of Limpopo and that

livestock farmers in the province can successfully grow triticale for silage under moderate stress conditions.

3.1 Introduction

Water stress is arguably the most limiting factor affecting cereal productivity in the world (Farooq et al., 2014, Ambavaram et al., 2014). Water stress is likely to increase in many dry areas as climate change is expected to decrease precipitation and increase evapotranspiration (Lobell et al., 2008). Drier areas are expected to become drier while wet areas are projected to become wetter. The effects of water stress will be exacerbated by the projected increase in temperatures (IPCC, 2014a). Rising temperatures and high evapotranspiration rates will increase reliance on irrigation to meet food demand thus putting more pressure on water resources. It is therefore imperative to have a wide-ranging understanding of water stress effects on crop physiological processes so as to better manage, improve and adapt crops for future climates.

Triticale (x. *Triticosecale* Wittmack), a hybrid of wheat (*Triticum* spp.) and rye (*Secale* spp.), is a robust and utility crop with great potential for food and feed production in future environments. It inherited the high yielding potential of wheat together with the resistance to pathogens and adaptability to marginal growing conditions such as drought, extreme temperature, salinity, extreme pH of rye (Mergoum and Macpherson, 2004, Tohver et al., 2005). In addition, triticale is also suitable for low input farming systems due to its tolerance to pests and diseases and its extensive root system that can efficiently absorb nutrients under poor soil fertility conditions (Mergoum and Macpherson, 2004). Triticale is a relatively new crop but its production has more than tripled in the past two decades from 4.5 million tonnes in 1990 to 14.5 million tonnes in 2013 (FAO, 2015). The production is concentrated in Europe with Poland being the highest producer having produced more than 3 million tonnes in 2013 (FAO, 2015). Triticale is however less known in Africa, and is only produced by a few countries mostly Tunisia and South Africa.

Triticale is used mainly as livestock feed but has potential in bread making and bioethanol production (McGoverin et al., 2011). The importance of triticale continues to grow due to the versatility of its utilization as it can be used in all its forms as grain, forage, whole plant silage, hay and straw (Motzo et al., 2013, McGoverin et al., 2011). In South Africa triticale produces grain yields of up to 40% higher than wheat in low potential soils (Botes and Saul, 2009). Cultivar improvements in the country are targeted at its value-added end use as livestock feed and improving its productivity in the winter rain fed (Mediterranean type) of the Western Cape Province (McGoverin et al., 2011). However, the stress tolerance and utility of triticale as livestock feed would be more suitable to the dry winter areas (Steppe, arid) of Limpopo and Eastern Cape Provinces, which have low crop potential but are more suitable for livestock production. These areas experience poor livestock condition due to low quantity and quality of available forages during the winter dry season (Matlebyane et al., 2010, Mapiye et al., 2009).

The major challenge of producing triticale in semi-arid (dry winter and hot, wet summer) environments is the availability of water (no winter rainfall) and heat stress that may occur during the reproductive stages of triticale. The combined effect of heat and water stress on yield is stronger than the effects of each stress type alone (Lipiec et al., 2013). Yield losses under water stress are essentially due to reduction in CO_2 assimilation (Hassan, 2006, Petridis et al., 2012, Roohi et al., 2013), hindrance to the export of assimilated carbon (Sevanto, 2014, Tausz and Grulke, 2014), suppressed leaf growth (Araus et al., 2008, Blum, 2011b) and accelerated leaf senescence (Farooq et al., 2014, Penfold and Buchanan-Wollaston, 2014).

In recent years, remote sensing has become important in monitoring crop water status, nutrient deficiencies and predicting crop characteristics like grain yield and aboveground biomass (Chandrasekar and Sesha Sai, 2015, Gao, 1996). The normalized difference vegetation index (NDVI) is currently the most common spectral reflectance index used in such applications. For example, some farmers use NDVI through simple instruments like the Green Seeker Handheld Crop Sensor (Trimble, USA) to monitor crop vigour. However, according to Chen et al. (2005), the index has limited success when used to estimate crop water status. NDVI is a greenness based index which is more responsive to changes in chlorophyll. The use of water based indices like the normalised difference water index (NDWI) (Gao, 1996) and water index (WI) (Peñuelas et al., 1997) which respond to changes in vegetation water content, could provide more information. Water stress can also be monitored by physically measuring soil and leaf water content but these methods are usually destructive, tedious and difficult to measure on a large scale. Hence, precise spectral reflectance characterisation at leaf level would allow for improved determination and management of water stress in field crops.

Though triticale is known for its competitiveness in stress environments, its performance, just like of any other crop, is influenced by environmental factors. Therefore, research efforts are still needed to improve current genotypes. A better understanding of yield performance, carbon fixation, and spectral response of triticale to water stress is critical for improving its: adaptation to water limited environments, management practices, and adoption by farmers. It is against this background that this study was carried out to investigate the effect of water stress on: 1) flag leaf CO_2 assimilation and flag leaf carbon content; 2) the utility of flag leaf spectral reflectance, not only as a tool to sense leaf water status but also as an indicator of biomass and grain yield in triticale; and 3) biomass and grain yield performance of four spring triticale genotypes in a dry winter environment (steppe, arid climate) characterised by high post anthesis temperatures.

3.2 Materials and Methods

3.2.1 Study site and experimental design

The study was carried out at the University of Limpopo experimental farm, Syferkuil (23°50’ S; 029°41’ E), Limpopo Province, South Africa during two winter seasons; June to October in 2013 and July to November in 2014. The area receives rainfall, ranging from 400 to 600 mm per annum (Benhin, 2006), 85% falling in summer, between November and March. Average minimum and maximum temperatures are respectively; 4 to 20°C in winter and 17 to 27°C in summer (calculated six-year average; 2008 to 2013). According to the Köppen-Greiger climate classification, the climate falls under BSh (arid, steppe, hot) (Kottek et al., 2006).

The experimental design was a randomised complete block design with 4 replications in factorial arrangement of moisture levels and genotypes. The four moisture levels were as described below and the amounts are shown in Table 3.1.

- Well-watered (WW): 25% soil moisture depletion before recharging to field capacity (FC);
- Moderately well-watered (MW): 50% soil moisture depletion before recharging to FC;
- Moderate stress (MS): 75% soil moisture depletion before recharging to 50% of FC;
- Severe stress (SS): moisture was allowed to dry out from detectable first node, with 40 mm supplementary irrigation being applied later in the season

In most parts of this report the simple notation of WW, MW, MS and SS will be used to describe the moisture levels. The moisture levels were applied after crop establishment corresponding to Zadoks Growth Stage 31 (GS31) (Zadoks et al., 1974). Under SS, 40 mm of supplementary irrigation was applied later in the season to avoid permanent wilting of the crop. Soil moisture was measured regularly using Diviner 2000 (Campbell Scientific, Australia) from access tubes installed at the center of each plot. Irrigation was applied when half of the plots under the same irrigation level reached the threshold value. Soil moisture was measured three to four times a week. The study was carried out in the dry winter season June to November when moisture levels were easily controlled without rainfall interference.

A preliminary study was carried out in 2012 with eight spring type genotypes and three moisture levels (MW, MS and SS). Four commercial genotypes from South Africa; Agbeacon, Bacchus, Rex, and US2007 were selected based on aboveground biomass yield. Triticale was planted in rows, 25 cm apart using a tractor drawn planter at a density of 200 plants m^{-2}. The plot sizes were 10 m x 10 m and irrigation was by Rain Bird sprinklers (Rain Bird, USA), fitted with Arad M20 water meters (Arad, Israel) to record the amount of water applied. In addition, rain gauges were installed at the center of each plot.

The soil was classified as a Chromic Luvisol (Hypereutric) (WRB, 2014). The soil depth ranges from 60 - 70 cm, with sandy clay loams overlaying sandy clays and an available water capacity (AWC) of 80 mm. The AWC was estimated using the hydraulics properties calculator (Saxton and Rawls, 2006). Fertilization was adapted to local practice: Nitrogen (N) was applied at a rate of 50 kg N ha^{-1}, phosphorus (21 kg P ha^{-1}) and potassium (12 kg K ha^{-1}) in both seasons.

3.2.2 Agronomic and climate measurements

Aboveground biomass was measured at milking stage (GS71) and harvest maturity (GS92). Sampling was done from the middle rows in the plot at GS71 and GS92. Plants were harvested at 10 cm aboveground and dried to a constant weight at 65˚C. Leaf area was measured destructively using an AM300 leaf area meter (ADC BioScientific, UK). Harvest Index (HI) was calculated as percentage of grain yield of total aboveground biomass at GS92. Growing Degree Days (GDD) were calculated as (Tmax + Tmin)/2 – Tb, where Tb is base temperature, taken as 0˚C (Santiveri et al., 2002). Weather data was collected from an automatic weather station installed about 500 m from the research plot.

3.2.3 Carbon assimilation

CO2 assimilation was measured using a LCi-SD Ultra Compact Photosynthesis System (ADC BioScientific, UK), on clear sunny days between 11h00 and 13h00. Measurements were taken on the mid part of the abaxial side of flag leaves. Leaf measurements were recorded manually by pressing the record button when the value of Ci (intercellular CO2 concentration) had stabilized. The measurement time was about two minutes per leaf. To stay within the recommended period of measurement, one leaf was measured per plot. Even though one leaf was measure it was representative enough since all treatments were replicated four times. The system has CO2 measurement range of 0-2000ppm, H2O range of 0-75mbar, PAR range of 0-3000 µmols m^{-2} sec^{-1} and temperature range of -5 to 50°C.

3.2.4 Layout of the experimental plot

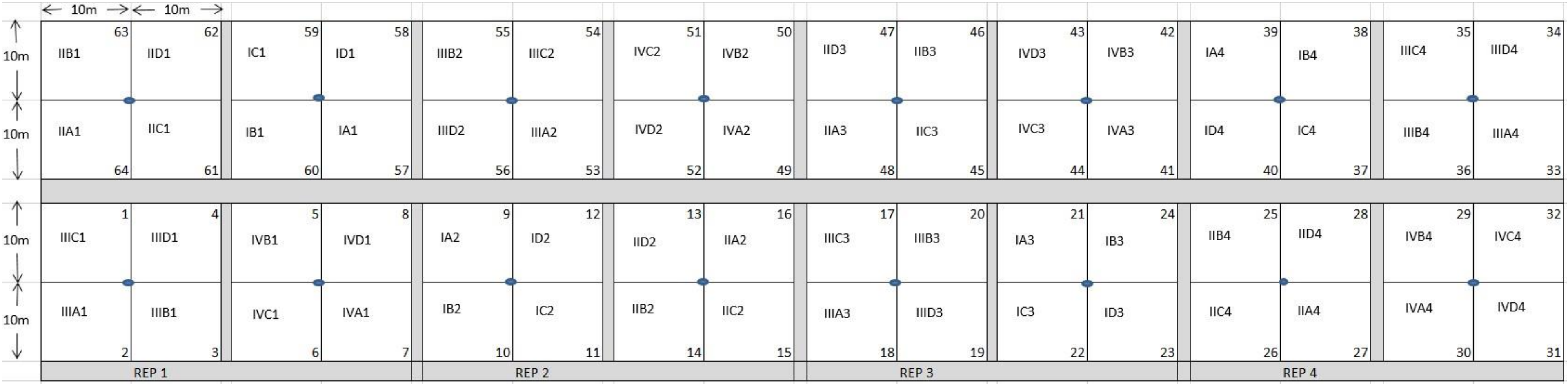


Fig. 3.1: Field layout of the experimental plot. Where the letters A, B, C and D represent genotypes. Roman numerals represent moisture regimes i.e. I = WW; II = MW; III = MS and IV = SS. Arabic numerals represent replication number as shown by REP1, REP2, REP3 AND REP4.

The above field plot plan template was used for both crops (triticale and cowpea) since in both experiments four genotypes and four moisture levels were used and all were replicated four times. Both moisture regimes and genotypes were however, randomized each year. Dots on the layout show position of the sprinklers. Rain gauge and Diviner 2000 access tubes were installed at the center of each plot (64 in total). Figure 9.1 in the appendix shows a photograph of the plot.

3.2.5 Drought tolerance indices

Three indices were calculated to give a measure of the drought resistance of the genotypes. The indices were: Yield Reduction (YR), Stress Tolerance Index (STI), and Yield Stability Index (YSI).

$YR = 100(Yp - Ys)/Yp$ *(3.1)*

$STI = (Yp \times Ys)/\bar{Y}p^2$ (Fernandez, 1993) *(3.2)*

$YSI = Ys/Yp$ (Bouslama and Schapaugh, 1984) *(3.3)*

Where Yp is yield of genotype under WW; Ys = yield of genotype under SS conditions; and $\bar{Y}p$ is the mean yield of all four genotypes under WW. YR was also calculated to give a measure of grain yield decrease due to 25% (WW – MW, YR25), 50% (WW – MS, YR50) and 75% (WW – SS, YR75) decrease in soil moisture from optimal.

3.2.6 Hyperspectral reflectance data

The hyperspectral reflectance of single flag leaves was measured using a portable Analytical Spectral Device (ASD) Spectroradiometer (ASD, USA) that detects reflectance in the 350 to 2500 nm spectral region with a 10 nm resolution. Leaf reflectance was measured by clipping the leaf with a leaf clipper that has an embedded background to measure the reflectance. The spectral reflectance data was measured on clear sunny days between 11h00 and 13h00 during two campaigns, at GS31 and GS71. The measured data was processed using the ViewSpec Pro (ASD, USA) software before being subjected to statistical analyses. Four vegetation indices were calculated: Normalised Difference Vegetation Index (NDVI) (Rouse et al., 1974); Normalised Difference Water Index (NDWI) (Gao, 1996); Water index (WI) (Peñuelas et al., 1997); and Nitrogen Reflectance Index (NRI) (Schleicher et al., 2001). The indices were calculated using the following formulas:

$NDVI = (R_{805} - R_{657}) / (R_{805} + R_{657})$ *(3.4)*

$NDWI = (R_{857} – R_{1241}) / (R_{857} + R_{1241})$ *(3.5)*

$WI = R_{900}/R_{970}$ *(3.6)*

$$NRI = (R_{555} - R_{657}) / (R_{555} + R_{657}) \quad (3.7)$$

where R is the reflectance value at the given band

3.2.7 Statistical Analyses

Analysis of variance was performed to calculate the effects of soil moisture reduction and genotype on the studied parameters. Mean comparisons were done using least significance difference (LSD) and where denoted by *, ** or *** for significance levels $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$, respectively. All data were analyzed using the SPSS 17.0 statistical package (SPSS, USA).

3.3 Results

3.3.1 Weather condition and irrigation levels during growing seasons

Figure 2.1A shows the daily maximum and minimum temperatures recorded on station at Syferkuil in 2013 and 2014 growing seasons. Daily maximum temperatures during the reproductive months (September to November) of triticale reached as high as 35˚C in both years (Fig. 3.1A, double arrow). The same Fig. 3.1A also shows an upper control temperature of 25˚C and lower control temperature of 12˚C representing optimum temperatures for spring crops (Hossain and Da Silva, 2012). The mean maximum and minimum temperatures in Fig. 3.1B show that mean temperatures were slightly higher in 2013 compared to 2014 during the reproductive stages of the crop. Mean monthly temperatures were within the optimum range of 12 to 25˚C. Monthly total rainfalls for the two years are shown in Fig. 3.1B. The total amount of rainfall received during the whole of 2013 growing season (June to October) was only 10.9 mm while 35 mm was received in 2014 (July to November). Figure 3.3 shows daily average air vapour pressure deficit (VPD) calculated from crop emergence until flowering stage in the growing seasons. The VPD was calculated using equation 1.6. VPD was found to be significantly higher during the 2014 growing season compared to the 2013 growing season.

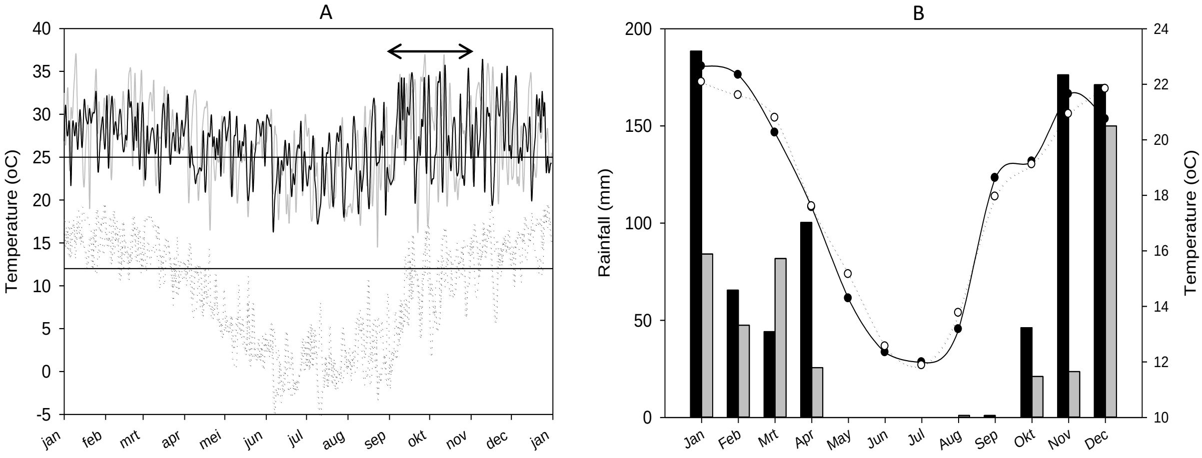


Fig. 3.1A: Daily maximum and minimum temperatures in the two seasons (2013 maximum = solid grey; 2013 minimum = dashed grey; 2014 maximum = black solid; and 2014 minimum = dashed black). The double arrow shows the reproductive growth period. Fig. 3.1B: Mean monthly temperatures for Syferkuil in 2013 (black shaded circles and line) and 2014 (open circles and dashed line). Fig. 3.1B also shows monthly total rainfall for 2013 (black bars) and 2014 (gray bars).

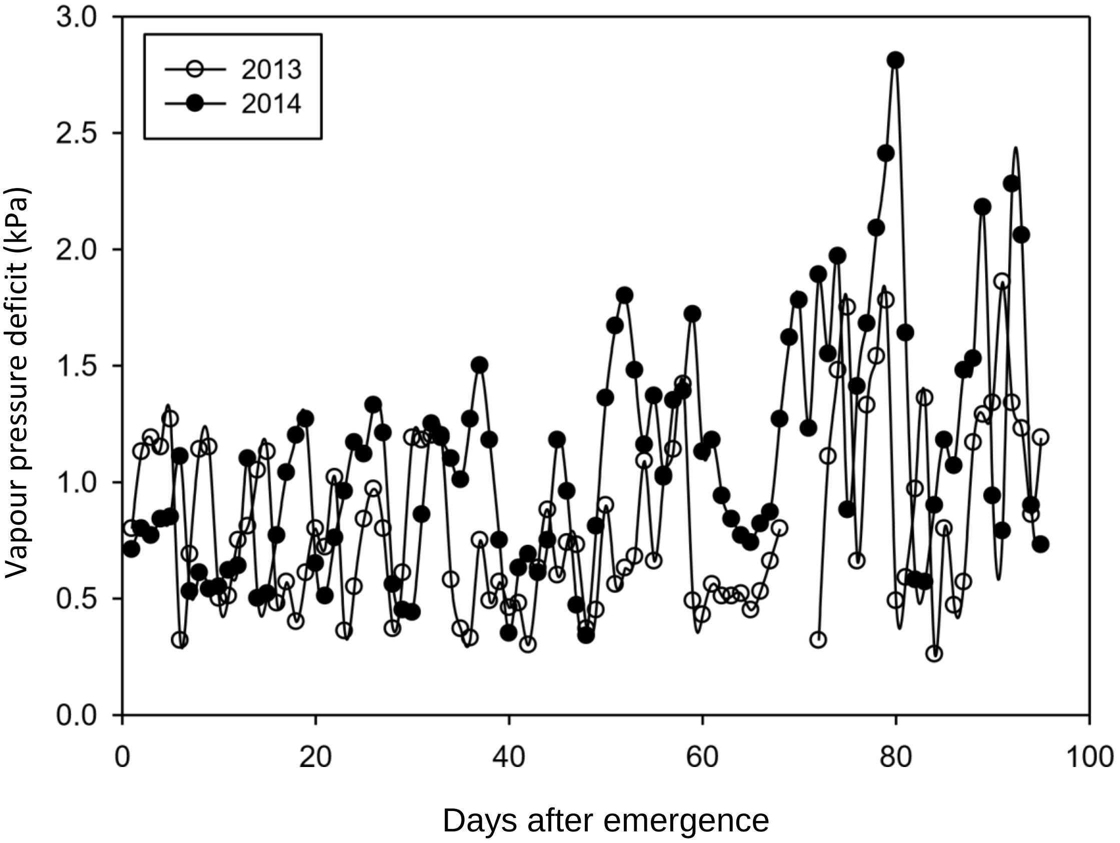


Fig. 3.3: Daily average air vapour pressure deficit during the 2013 and 2014 growing seasons for the period from emergence to flowering.

3.3.2 Aboveground biomass, grain yield and yield components

Soil moisture had a strong influence on biomass accumulation, grain yield, leaf area, 1000 kernel weight and HI but had no influence on ear length in both seasons (Table 3.1). Neither genotypic differences nor interaction effects were observed ($P > 0.05$) in the measured parameters though Bacchus showed a tendency of performing better than the other genotypes (Table 3.3). Severe water stress reduced grain yield by more than 75% in both seasons with grain yield ranging from 0.8 to 3.9 t ha^{-1} in 2013 and 1.8 to 4.9 t ha^{-1} in 2014. In 2013, biomass was not significant between WW and MW conditions but was significant between MS and SS conditions. Also, WW and MW were significantly different to both MS and SS. In 2014, biomass was different for all moisture levels. Grain yield and ear weight were not significantly different between WW and MW in both seasons but were significantly different to grain yield observed under MS and SS conditions. In 2013, differences in grain yield and ear weight were observed between MS and SS conditions but these differences were not observed in 2014. Leaf area did not differ under higher moisture levels (WW and MW) but was significantly lower under MS and SS in 2013 In 2014, WW, MW and MS leaf areas were similar but differed from SS. In the first season (2013), kernel weight and HI were similar under WW, MW and MS conditions. Only under SS were kernel weight and HI significantly lower. In the second season (2014), HI did not show any differences across all the four moisture levels.

The total amount of water received (including rainfall) by the crop for the different moisture levels in the two seasons is shown in Table 3.1. The amount of water received under SS was almost half of that received under WW in both seasons.

Table 3.1: Aboveground biomass at GS71; and GS92, grain yield, ear weight, ear length, leaf area, 1000 kernel weight, harvest index and the amount of water applied under four irrigation levels.

			Biomass		Grain	Ear	Ear	Leaf	Kernel	Harvest
Year	Moisture	Water	GS71	GS92	Yield	Weight	Length	Area	Weight	Index
	Level	mm	---------------------t ha^{-1}-------------				cm	mm^{2}	g	
2013	WW	450	12.5^{a}	13.6^{a}	3.5^{a}	5.6a	11.5^{a}	1867^{a}	39.1^{a}	25^{a}
	MW	346	11.9^{a}	13.6^{a}	3.9^{a}	6.0a	10.6^{a}	1949^{a}	41.3^{a}	29^{a}
	MS	322	9.0^{b}	9.6^{b}	2.4^{b}	3.5b	10.4^{a}	1419^{b}	42.3^{a}	24^{a}
	SS	226	6.3^{c}	6.9^{c}	0.8^{c}	2.0c	10.4^{a}	861^{c}	24.5^{b}	12^{c}
		-	***	***	***	***	ns	*	***	***
2014	WW	426	13.6^{a}	15.0^{a}	4.9^{a}	5.9^{a}	9.8^{a}	1400^{a}	42.2^{a}	32^{a}
	MW	364	11.0^{b}	12.2^{b}	4.0^{ab}	5.0^{ab}	9.1^{a}	1323^{a}	46.8^{b}	34^{a}
	MS	289	8.3^{c}	9.5^{c}	3.1^{bc}	4.4^{bc}	9.9^{a}	1424^{a}	36.9^{c}	32^{a}
	SS	247	5.8^{d}	6.8^{d}	1.8^{c}	3.1^{c}	9.2^{a}	988^{b}	36.8^{c}	26^{a}
		-	***	***	***	***	ns	***	***	ns

*Letters represent significant differences. Significance levels: *$P < 0.05$, ***$P < 0.001$, ns = not significant. WW = well-watered; MW = moderately well-watered; MS = moderate stress; SS = severe stress*

3.3.3 Growing season effect

No growing season effect was observed on the amount of biomass accumulated at GS71 and GS91 for all four moisture levels (Table 3.2). The effect of growing season on grain yield and kernel weight was observed only under SS where both grain yield and ear weight were higher in 2014 compared to 2013. The ears were longer in 2013 compared to 2014 under WW, MW and SS conditions but were not different under MS conditions. Leaf area under WW and MW conditions was higher in 2013 compared to 2014 but no seasonal effect was observed under MW and SS conditions. No growing season effect was observed for kernel weight under WW conditions but it was significant for the other three moisture levels. HI tended to be higher in 2014 compared to 2013 but was only significant under MS and SS conditions. When grown under WW conditions, triticale required 1962 and 2027 GDD to reach physiological maturity in 2013 and 2014, respectively. Fewer GDD were required for the other three moisture levels as the crop matured earlier. For instance, under SS, the crop reached physiological maturity after 1767 and 1812 GDD in 2013 and 2014, respectively.

Table 3.2: Growing season effect on biomass, grain yield, ear weight, ear length, leaf area, 1000 kernel weight, and harvest index

		Biomass		Grain	Ear	Ear	Leaf	Kernel	Harvest
Irrigation	Year	GS71	GS91	Yield	Weight	Length	Area	Weight	Index
		-------------------t ha^{-1}---------------------				cm	mm^2	g	
WW	2013	12.5	13.6	3.5	5.6	11.5	1867	39	25
	2014	13.6	15.0	4.9	5.7	9.8	1400	42	32
		ns	*ns*	*ns*	*ns*	*	*	*ns*	*ns*
MW	2013	11.9	13.6	3.9	6.0	10.6	1949	41	29
	2014	11.0	12.2	4.0	5.2	9.1	1323	47	34
		ns	*ns*	*ns*	*ns*	**	***	***	*ns*
MS	2013	9.0	9.6	2.4	3.6	10.4	1419	42	24
	2014	8.3	9.5	3.1	4.0	9.9	1401	37	32
		ns	*ns*	*ns*	*ns*	*ns*	*Ns*	*	*
SS	2013	5.4	6.1	0.8	1.7	10.3	798	25	13
	2014	5.7	6.9	1.9	3.2	9.1	982	38	27
		ns	*ns*	**	**	***	*Ns*	***	***

*Significance levels: *P < 0.05, **P < 0.01, ***P < 0.001, ns means not significant*

Table 3.3: Aboveground biomass (at GS71 and GS92), grain yield, ear weight, ear length, leaf area, 1000 kernel weight, and harvest index of the four genotypes under four moisture levels averaged for the two seasons

		Biomass		Grain	Ear	Ear	Leaf	Kernel	Harvest
Irrigation	Genotype	GS71	GS91	Yield	Weight	Length	Area	Weight	Index
		-------------------t ha^{-1}---------------------				cm	mm^2	g	
WW	Agbeacon	12.3	15.0	3.9	5.4	11.7	1582	40.3	26
	Bacchus	14.0	16.2	4.6	6.4	9.8	1613	41.1	27
	Rex	13.7	14.1	5.0	5.7	10.4	1818	41.4	35
	US2007	12.2	12.1	3.2	5.2	10.7	1522	39.9	26
		ns	ns	ns	ns	ns	ns	ns	ns
MW	Agbeacon	11.3	13.0	4.0	5.5	9.8	1512	43.6	31
	Bacchus	12.1	14.7	4.0	5.9	10.3	1731	73.7	28
	Rex	12.1	12.2	3.8	5.9	9.5	1819	45.4	31
	US2007	10.2	11.7	4.1	5.1	9.7	1482	43.5	36
		ns	ns	ns	ns	ns	ns	ns	ns
MS	Agbeacon	8.9	10.1	2.5	3.8	10.5	1336	39.2	24
	Bacchus	9.9	10.7	3.5	3.7	9.9	1496	40.8	32

	Rex	7.9	8.3	1.8	3.0	9.8	1311	37.7	22
	US2007	7.9	8.9	3.0	4.1	10.5	1515	40.9	33
		ns	ns	ns	ns	ns	ns	ns	ns
SS	Agbeacon	5.9	7.3	1.4	2.5	10.0	1001	29.2	19
	Bacchus	6.4	6.8	1.4	2.6	9.5	964	31.8	19
	Rex	5.7	6.0	1.5	2.6	9.7	936	32.4	24
	US2007	4.5	6.1	1.3	2.1	9.7	734	32.5	20
		ns	ns	ns	ns	ns	ns	ns	ns

ns = not significant. WW = well-watered; MW = moderately well-watered; MS = moderate stress; SS = severe stress

3.3.4 CO_2 assimilation and flag leaf carbon content

Flag leaf CO_2 assimilation rate and flag leaf carbon content were significantly influenced by soil moisture content (Table 3.4). CO_2 assimilation rate decreased with decreasing soil moisture level. In contrast, flag leaf carbon content increased with decreasing soil moisture level. CO_2 assimilation rates were not different under WW and MW conditions but were higher than the rates observed under MS and SS conditions. In 2013, CO_2 assimilation rates were different between MS and SS but were not different in 2014. Flag leaf carbon content did not differ between WW and MW conditions and also between MS and SS except for GS92 in 2013 where carbon content under SS was significantly higher than under MS conditions. Mean flag leaf carbon content at GS71 was significantly higher than the mean flag leaf carbon content at GS92 in both seasons (Fig. 3.2). Mean flag leaf carbon content was the highest at GS71 in 2014 and no seasonal differences were observed in flag leaf carbon content at GS92. Also, a significant negative relationship (-0.34**) was observed between flag leaf carbon content and grain yield (Fig. 3.3). The relationship in Fig. 3.3 is for combined data of the two seasons measured at GS92.

Table 3.4: Flag leaf CO_2 assimilation rate and carbon content at GS71 and GS92

		CO_2	Flag Leaf Carbon Content (%)	
		Assimilation	Milk	Harvest
Year	Irrigation	*(μmol m^{-2} s^{-1})*	GS71	GS92

2013	WW	9.92^{a}	37.2^{b}	35.1^{c}
	MW	8.01^{ab}	37.2^{b}	34.3^{c}
	MS	6.68^{b}	39.2^{a}	36.9^{b}
	SS	1.82^{c}	39.8^{a}	39.7^{a}
		***	***	***
2014	WW	11.64^{a}	39.4^{c}	34.6^{b}
	MW	11.15^{a}	40.1^{c}	35.2^{b}
	MS	6.11^{b}	41.7^{a}	38.3^{a}
	SS	4.74^{b}	41.2^{a}	38.5^{a}
		***	**	**

Letters represent significant differences. Significance levels: **P < 0.01; ***P < 0.001, ns = not significant. WW = well-watered; MW = moderately well-watered; MS = moderate stress; SS = severe stress

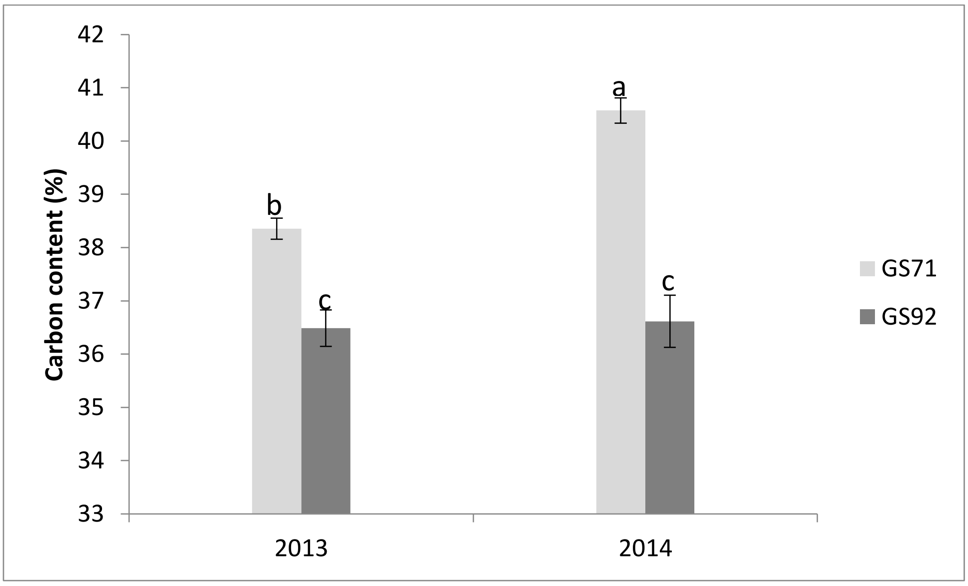


Fig. 3.2: Average flag leaf carbon percentage at GS71 and GS92 for 2013 and 2014 growing seasons; Letters show significant differences.

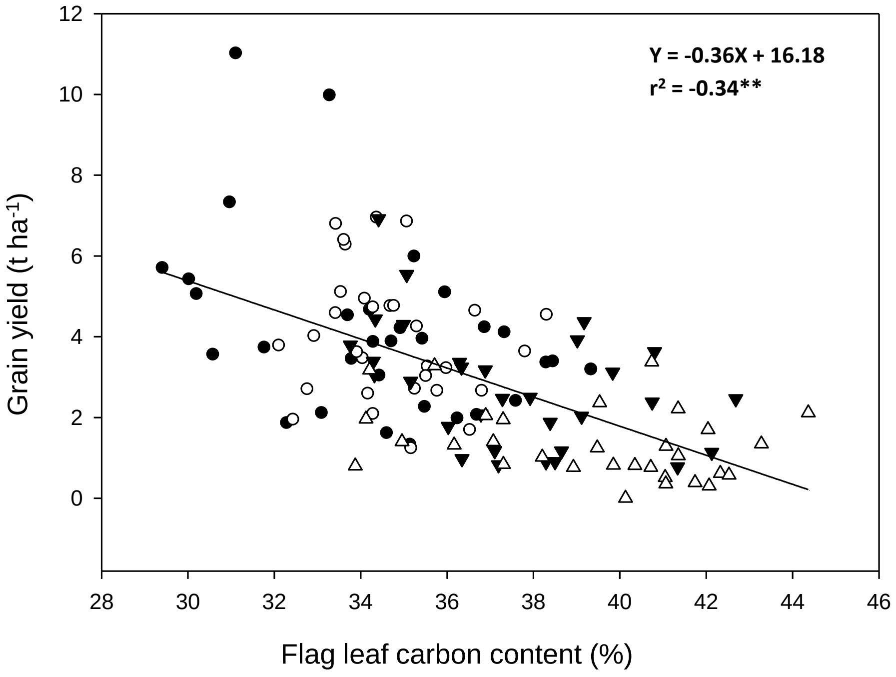


Fig. 3.3: Regression between grain yield and flag leaf carbon content at GS92. WW (solid circles); MW (open circles); MS (solid triangles); and SS (open triangles)

3.3.5 Yield reduction and drought tolerance indices

Fig. 3.4 shows grain yield reduction percentages resulting from 25% (WW vs. MW, YR25), 50% (WW vs. MS, YR50) and 75% (WW vs. SS, YR75) decrease in soil moisture. The percentage grain yield loss corresponded with the percentage decrease in soil moisture. For instance, 25% decrease in soil moisture resulted in less than 20% decrease in grain yield for all genotypes in the two growing seasons. A 75% moisture decrease resulted in almost similar decrease in grain yield in 2013. In 2014, 75% decrease in soil moisture resulted in about 60% decrease in grain yield. In 2013, US2007 had very low grain yield under WW conditions (2.1 t ha^{-1}, Table 3.5) which resulted in negative reduction percentages which were not computed. Though not significant, Agbeacon had the lowest yield reduction percentage at 25% moisture decrease. Bacchus and Rex were the two genotypes most affected by severe water stress compared to Agbeacon and US2007.

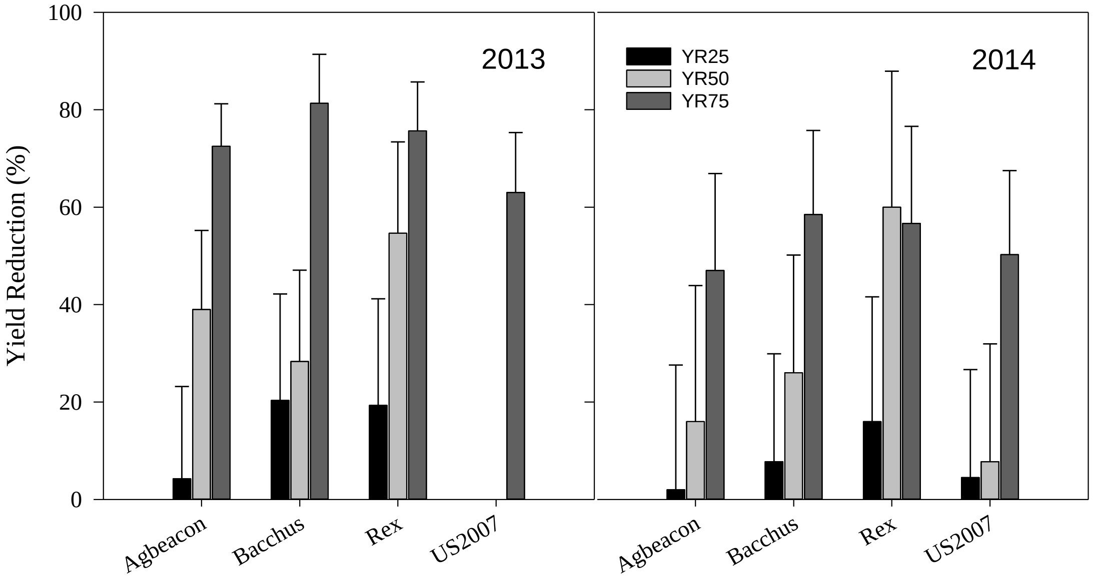


Fig. 3.4: Yield reduction of four triticale genotypes when moisture level is reduced by 25% (WW vs. MW, YR25), 50% (WW vs. MS, YR50) and 75% (WW vs. SS, YR75) for the two seasons (2013 and 2014). The missing data on US2007 is due to the extremely low grain yields observed under WW conditions that resulted in negative yield losses.

The performance of the four triticale genotypes for potential yield (Yp) and yield under severe water stress conditions (Ys) is shown in Table 3.5. Table 3.5 also shows the stress tolerance index (STI) and yield stability index (YSI) of the genotypes in the two seasons. Genotypes did not differ significantly in the parameters measured over the two seasons except in 2013, where US2007 had significantly lower potential yield relative to the other genotypes. As expected potential grain yield was higher compared to grain yield under water stress. Even though genotypes did not differ in YSI, Agbeacon had the highest YSI in the two seasons corresponding to a lower yield reduction observed in Fig. 3.4. STI was not consistent in the two seasons with Rex having the highest STI in 2013 and Bacchus in 2014.

Table 3.5: Yield reduction (YR), Stress Tolerance Index (STI), and Yield Stability Index (YSI) of the four genotypes calculated using potential yield (Yp) and water stressed yield (Ys)

Year	Genotype	Yp	Ys	STI	YSI
2013	Agbeacon	4.1^a	1.1	0.25	0.28
	Bacchus	4.0^a	0.8	0.20	0.21
	Rex	3.7^a	0.9	0.30	0.25
	US2007	2.1^b	0.6	0.10	0.27
		**	**ns**	**ns**	**ns**

2014	Agbeacon	3.7	1.8	0.29	0.62
	Bacchus	5.2	1.9	0.44	0.49
	Rex	5.1	1.9	0.42	0.43
	US2007	4.3	1.9	0.37	0.50
		ns	**ns**	**ns**	**ns**

*Yp = grain yield under optimal and Ys =grain yield under severe stress. Letters represent significant difference. **P < 0.01; ns – not significant*

3.3.6 Spectral reflectance indices

Four regions on the reflectance curve showed differences in the reflectance of the flag leaves as affected by the moisture levels. The regions were: visible (533 to 660 nm); near infrared (758 to 1270 nm); middle infrared (1430 to 1850 nm) and far infrared (1913 to 2500 nm) (see Fig. 3.5). Figure 3.5 shows the differences in the spectral reflectance of triticale between WW and SS conditions. The measurements were taken at GS 71. At GS31, the stage at which the moisture levels were started, soil moisture effect was not significant for all four indices. However, the indices had higher values at this stage compared to those measured later at GS71. The NDVI, NDWI and WI were strongly influenced by moisture level at GS71 (Table 3.6) with higher values being observed under high moisture levels compared to low moisture levels. None of the indices showed significant differences between MS and SS conditions. Differences in NDWI and WI were mainly found under WW, MW, and the two lowest moisture levels (MS and SS) while for NDVI differences were only found between the two groups; well-watered conditions (WW and MW) and water stressed conditions (MS and SS). Thus, NDVI could not separate between WW and MW conditions. NRI was not affected by moisture level at GS31 and GS71.

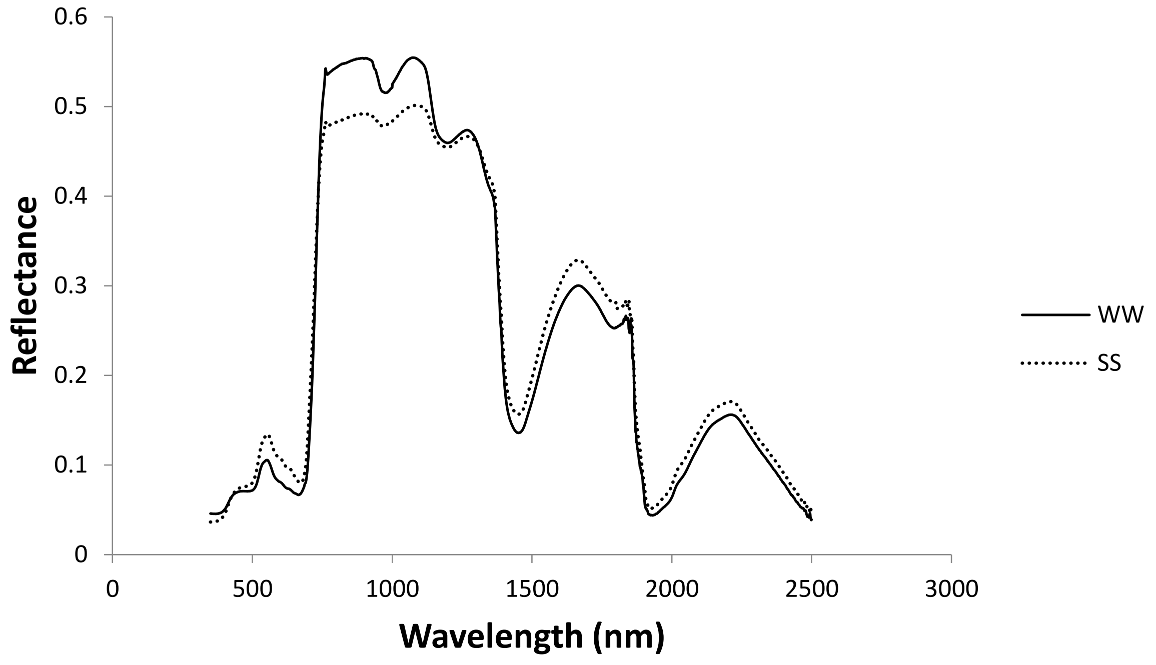


Fig. 3.5: Spectral reflectance curve of triticale plants grown under well-watered (WW) and severe stress (SS) conditions

Table 3.6: Normalised Difference Vegetation Index (NDVI), Normalised Difference Water Index (NDWI), Water Index (WI) and Nitrogen Reflectance Index (NRI) calculated from reflectance measured at GS31 and GS71 in 2014

	NDVI		NDWI		WI		NRI	
Irrigation	GS31	GS71	GS31	GS71	GS31	GS71	GS31	GS71
WW	0.83^{a}	0.78^{a}	0.089^{a}	0.082^{a}	1.085^{a}	1.073^{a}	0.29^{a}	0.23^{a}
MW	0.81^{a}	0.77^{a}	0.090^{a}	0.062^{b}	1.083^{a}	1.055^{b}	0.26^{a}	0.22^{a}
MS	0.82^{a}	0.70^{b}	0.088^{a}	0.039^{c}	1.086^{a}	1.031^{c}	0.28^{a}	0.21^{a}
SS	0.81^{a}	0.70^{b}	0.084^{a}	0.028^{c}	1.082^{a}	1.026^{c}	0.26^{a}	0.23^{a}
	ns	**	ns	***	ns	***	ns	ns

*Letters represent significant differences. Significance levels: *P < 0.05; **P < 0.01; ***P < 0.001, ns means not significant. WW = well-watered; MW = moderately well-watered; MS = moderate stress; SS = severe stress*

CO_2 assimilation rate was significantly correlated to NDVI, NDWI and WI (Table 3.7) but the correlation was stronger with WI (0.63***). Flag leaves with higher water content at GS71 also had higher CO_2 assimilation rates. The three indices (NDVI, NDWI and WI) were also significantly correlated with biomass and grain yield and again WI had stronger correlations with biomass (0.72***) and grain yield (0.46***) than any other index. Flag leaf carbon content (both at GS71 and GS92) were inversely related to the other parameters.

Table 3.7: Bivariate correlations of biomass (GS71), grain yield, leaf area, CO_2 (CO_2 assimilation), C_GS71 (flag leaf carbon content at GS71), C_GS92 (flag leaf carbon content at GS92), NDVI, NDWI, WI and NRI

	Biomass	Grain Yield	Leaf Area	CO_2	C_GS71	C_GS92	NDVI	NDWI	WI
Grain Yield	0.64***								
Leaf Area	0.47***	0.42***							
CO_2	0.48***	0.33*	0.25						
C_GS71	-0.47***	-0.29*	-0.24	-0.37**					
C_GS92	-0.29*	-0.38**	-0.03	-0.46***	0.37**				
NDVI	0.46***	0.42**	0.32**	0.52***	-0.19	-0.28*			
NDWI	0.56***	0.43**	0.34**	0.56***	-0.40**	-0.22	0.61***		
WI	0.72***	0.55***	0.39**	0.63***	-0.49***	-0.41**	0.74***	0.88***	
NRI	0.22	0.21	0.16	0.05	-0.05	-0.20	0.73***	0.21	0.07

*Significance levels: *P < 0.05; **P < 0.01; ***P < 0.001. NDVI= normalized difference vegetation index, NDWI = normalized difference water index, WI = water index, NRI = nitrogen reflectance index*

3.4 Discussion

3.4.1 Aboveground biomass, grain yield and yield components of triticale

The average grain yield (3.9 t ha^{-1} in 2013 and 4.9 t ha^{-1} in 2014) obtained under well-watered conditions, are relatively low compared to those obtained in other studies(Dogan et al., 2009, Estrada-Campuzano et al., 2012). Dogan et al. (2009) reported yields above 6 t ha^{-1} in Turkey. A plausible reason for the relatively low yields under well-watered conditions (WW and MW) is the low plant density and N fertilizer applied. Grain cereals respond positively to N fertilization and in this study only 50 kg N ha^{-1} was applied compared to the recommended rates of 80 to 100 kg N ha^{-1} or even higher rates as reported in some studies (Cabrera-Bosquet et al., 2009a, Motzo et al., 2013). A low plant density of 200 plants m^{-2} was used compared to higher plant densities of more than 300 plants m^{-2} used in other studies (Estrada-Campuzano et al., 2012, Motzo et al., 2013). Lower N rates were used because triticale is known to perform well even under low soil fertility (Mergoum and Macpherson, 2004) and also because poor farmers in Limpopo do not have financial resources to apply high doses of fertilizer. Low densities were used to improve water use by the crop as it is recommended to use lower plant densities under water limiting conditions (Tokatlidis, 2013).

While the yield may look small compared to the above-mentioned yields, they are similar to the world average of 3.8 t ha^{-1} in 2013 and higher than the 2.3 t ha^{-1} reported in Tunisia (FAO, 2015). Tunisia is one African country that consistently produces triticale. The grain yield observed under SS conditions (0.8 t ha^{-1} in 2013 and 1.8 t ha^{-1} in 2014) were low when compared to 3.6 t ha^{-1} reported by Schittenhelm et al. (2014) under similar moisture conditions, albeit under humid, warm temperate climate. In this study, under steppe (arid) climate, an extra 40 mm of water was required to avoid total desiccation of the crop under SS. This shows that it is virtually impossible to grow a spring crop to maturity on residual moisture in a steppe (arid) environment due to the amount of water that is lost through evapotranspiration. The relatively lower biomass and grain yield can also be attributed to a shortened grain filling stage resulting from terminal heat stress experienced during the reproductive and grain filling stages (Fig. 3.1A) (Barnabás et al., 2008, Day and Atkin, 1984, Dias and Lidon, 2009). High temperatures negatively affect CO_2 assimilation (Farooq et al., 2011) even under WW conditions. Low yields observed under water limited conditions (MS and SS) may have been due to accelerated leaf senescence (Nawaz et al., 2013) and/or kernel abortion (Hays et al., 2007) caused by either water stress, heat stress or a combination of the two. In this study grain filling occurred between October and November when daily maximum temperatures exceeded 30^{o}C reaching highs of 35^{o}C (Fig. 3.1A), well above 25^{o}C considered to be the optimum (Hossain et al., 2012, Farooq et al., 2011). Supraoptimal temperatures occurring post anthesis are known to reduce kernel cytokinin levels, a hormone that promotes endosperm cell proliferation (Banowetz et al., 1999) resulting in lower kernel weight. Also, plants under water stress produce abscisic acid (ABA), which in turn inhibits endosperm cell division (Cheikh and Jones, 1994). However, it suffices to say that the grain yield levels observed in this study showed a good thermo tolerance of the triticale cultivars.

The HI and kernel weight are important parameters that determine yield in grain crops. Higher yields are realized in crops that convert a significant amount of accumulated assimilates into grain. The HI observed under SS was extremely low in 2013 (12%) which is less than half of what is reported in many studies (Aggarwal et al., 1986, Bassu et al., 2011) but in 2014 (26%) the HI was relatively high and similar those reported by Estrada-Campuzano et al. (2012). The kernel weights observed in both seasons fell within the expected range for triticale of 35 to 55 g (Erekul and Köhn, 2006, Manley et al., 2011) except under SS in 2013. A common response of plants to water stress is reducing leaf area to restrict water loss (Chaves et al., 2002). Smaller flag leaves were observed under SS indicating that low soil moisture levels affected leaf growth, reducing photosynthesising area and subsequently the biomass accumulated. Leaf expansion is known to decline under limited soil moisture due to a decrease in turgor pressure of newly formed leaf cells (Bacon, 1999). Table 3.7 shows strong significant correlations between leaf area and grain yield and with biomass, which is consistent with literature where dry matter production linearly increases with the amount of solar radiation intercepted by the leaf area (Bacon, 1999).

The lack of genotypic differences under well-watered conditions could be because the genotypes could not express themselves as much as they would due to the heat stress experienced. Under water stress, the lack of genotypic differences maybe because the genotypic diversity for drought tolerance was too small as shown by the lack of differences in STI and YSI. This lack of distinct genetic diversity in grain yield in some triticale genotypes was also reported by Dogan et al. (2009). Blum (2014), stated that "Any given triticale cultivar or selection cannot be taken a priori as being stress resistant''.

3.4.2 Growing season effect

The lack of differences in biomass accumulation between 2013 and 2014 for both GS71 and GS92 could be an indication of the similarities in environmental conditions in both seasons. Grain yield and kernel weight tended to be higher in 2014 compared to 2013 and was significant under SS. This can be attributed to the differences in assimilate partitioning as reflected by HI, kernel weight and GDD. More assimilates were portioned to the grain in 2014 compared to 2013 as shown by higher HI and kernel weight. It was also observed that average flag leaf carbon content at GS71 in 2014 was significantly higher than at GS71 in 2013. The flag leaf carbon content at GS92 was however, similar in both years. This shows that more assimilates were exported to the grains in 2014. According to Khaliq et al. (2008), flag leaves are a major contributor of assimilates for grain filling.

3.4.3 Flag leaf CO_2 assimilation and carbon content

According to Blum (2011b), cereal grain filling depends on two main carbon sources which are; current assimilates from post-anthesis photosynthesis and reserve carbohydrates stored mainly in the stems. Grain filling under water limited condition such as the SS treatment will depend on stored assimilates from the vegetative stage while assimilates for grain filling under well-watered conditions (WW and SS) would be expected to come from current photosynthesis assimilates (Plaut et al., 2004). In this study, there was on average 70% reduction in grain yield (77% in 2013 and 63% in 2014; Table 3.1 and Fig. 3.4) between WW and MW conditions which corresponded well with an average of 70% reduction in CO_2 assimilation rate (81% in 2013 and 59% in 2014; Table 3.4). The low CO_2 assimilation rate observed under SS conditions suggests that assimilates for grain filling may have come from reserves rather than from current photosynthesis. Blum (2011b) reported increased utilization of stored assimilates for grain filling, if photosynthesis is diminished by stress. However, as alluded to earlier, the shortened grain filling period resulting from high temperatures could have resulted in more assimilates staying in the stems instead. The decreasing CO_2 assimilation rate with soil moisture may have resulted from decreased stomatal conductance. As soil moisture decreases, roots produce ABA which signal stomata closure (Saradadevi et al., 2014) thus reducing CO_2 diffusion into the leaf resulting in less carbon fixation. Intuitively, high flag leaf CO_2 assimilation rate would be expected to correspond to high carbon content in the flag leaves but the reverse was true in this study. This is mainly because under favourable conditions up to 80% of assimilated carbon by mature leaves is exported to sinks (Lemoine et al., 2013). However, sugars may also accumulate in leaves in case of growth limitation (Hummel et al., 2010) and hindrance to assimilate export. These results reveal that flag leaf carbon content is influenced more by the export capacity of the flag leaves than by CO_2 assimilation rate.

3.4.4 Spectral reflectance indices

Spectral reflectance indices were more effective at detecting leaf water status at wavelengths between 758 and 1270 nm. When soil moisture was constantly available, spectral reflectance values were higher an indication of good crop vigour but water stress reduced the values and the vigour also. Similar findings have been reported (Claudio et al., 2006, Schittenhelm et al., 2014). The strength of the relationship between the indices (NDVI, NDWI and WI) with grain yield and biomass followed the order WI > NDWI > NDVI, showing the superiority of WI in monitoring leaf water status in triticale. Accordingly, the results concur with the findings of Gutierrez et al. (2010). Even though water stress results in loss of leaf greenness, NDVI could not separate the moisture levels better than NDWI and WI. This is because NDWI and WI respond well to short term changes in water content of leaves compared to NDVI and NRI. WI also showed great predictive capacity for CO_2 assimilation rate in triticale flag leaves as shown by the correlation (0.63***) in Table 3.7. This correlation is of great ecological significance as it gives a measure of the photosynthetic activity of plants. WI has potential to be used in estimating CO_2 sequestration especially in forests were direct physical measurement is a challenge. Other studies on WI which are related to photosynthetic activity have focused mainly on its correlation to leaf area index (Roberts et al., 1998). According to Diker and Bausch (2003), NRI can be used to estimate N status in plants and its spatial variability in soils. In this study no differences in NRI were observed both at GS31 and GS71 showing that there was no variability in N status in the field. Hence, the differences observed particularly in the greenness based NDVI where solely due to moisture treatments. The advantages of using spectral reflectance indices in monitoring water stress is not only that they are rapid and non-destructive, but can be quantitative for example, significant correlations have been found between these spectral reflectance indices and leaf water potential (Ramoelo et al., 2015). Leaf water potential is a measure of plant water status. The lack of genotypic differences in spectral response to soil moisture alludes to the earlier notion that the four genotypes used in this study may not have been screened for drought tolerance because drought tolerant genotypes are characterised by a stay green flag leaf (Foulkes et al., 2007) which would have given higher NDVI on tolerant genotypes compared to the susceptible ones.

3.5 Conclusion

The local spring triticale cultivars showed good adaptability to the steppe (arid) environment of the study area (Limpopo Province). They particularly showed tolerance to post anthesis high temperatures by producing reasonable yields even when maximum temperatures were above optimal. Even though the study showed that triticale cannot be grown to maturity on residual moisture alone, it performed reasonably well under MS conditions. At this moisture level, livestock farmers can successfully grow triticale for whole plant silage by harvesting it at milk stage and should expect aboveground dry biomass yields of more than 8 t ha^{-1}. The study also revealed the superiority of water based indices, particularly the water index (WI) over green based NDVI in monitoring leaf water status in triticale. WI also showed potential in predicting photosynthetic activity in triticale.

4 Chapter 4 - Combining carbon-13 and oxygen-18 to unravel triticale grain yield and physiological response to water stress

Published in Field Crops Research as:

Munjonji, L., K.K. Ayisi, B. Vandewalle, G. Haesaert, and P. Boeckx. 2016b. Combining carbon-13 and oxygen-18 to unravel triticale grain yield and physiological response to water stress. *Field Crops Research*. 195:36-49.
http://dx.doi.org/10.1017/S0014479716000107

Abstract

Water availability in semi-arid regions is increasingly becoming threatened by erratic rains and frequent droughts leading to over-reliance on irrigation to meet food demand. Improving crop water use efficiency (WUE) has become a priority but direct measurements remain a challenge. There is a need to identify reliable proxies and screening traits for WUE. Carbon isotope discrimination ($\Delta^{13}C$) offers potential as a proxy for WUE, but its application is hindered by environmental factors and thus varies greatly among different studies. A two-year study was carried out with four moisture levels, ranging from well-watered (430 to 450 mm) to severe stress (SS) (220 to 250 mm), combined with four commercial triticale genotypes grown under field conditions in a hot, arid, steppe climate of Limpopo in South Africa. The study tested the use of $\Delta^{13}C$ as a proxy of intrinsic WUE and grain yield of triticale. Secondly, $\delta^{13}C$ and $\delta^{18}O$ in combination with measured gas exchanges were used to test the functionality of the dual isotope model to interpret causes of variation in carbon isotope composition. Thirdly, grain filling carbon assimilate sources were inferred from measured flag leaf and grain $\Delta^{13}C$.

The results showed that moisture levels significantly influenced grain yield, intrinsic WUE and $\Delta^{13}C$ in triticale. Well-watered conditions resulted in higher grain yields when compared to other moisture levels. Grain yield ranged from 3.5 to 0.8 t ha^{-1} and 4.9 to 1.8 t ha^{-1} in 2013 and 2014, respectively. $\Delta^{13}C$ was also high under well-watered conditions and decreased with decreasing moisture level while $WUE_{intrinsic}$ increased with decreasing moisture level. The relationship between $\Delta^{13}C$ and grain yield was positive ($P < 0.01$), but only significant under water stressed conditions, indicating dependence of the relationship on moisture level. The relationship between $\Delta^{13}C$ and $WUE_{intrinsic}$ did not depend on the moisture level but showed a negative relationship when data for all moisture levels were combined. $\delta^{13}C$ showed a negative relationship with photosynthetic rate (A), while the relationship between stomatal conductance (gs) and $\delta^{18}O$ was variable. Hence, the dual isotope model could only predict that variation observed in $\Delta^{13}C$ and thus intrinsic water use efficiency was due to a concomitant decrease in both A and gs when transpiration was not limited by evaporative demand. Flag leaf $\Delta^{13}C$ measured under SS at GS71 in the 2014 growing season, was significantly higher (2.2 to 3.6‰) than grain $\Delta^{13}C$, also measured under SS, suggesting minimal contribution of flag leaf photosynthesis to grain filling. No genotypic differences were observed in $\Delta^{13}C$, grain yield and $WUE_{intrinsic}$, indicating a probable lack of diversity in the studied genotypes.

The results of this study show that carbon isotope discrimination could be useful as a predictor of triticale grain yield in drought prone areas. $\Delta^{13}C$ also offers potential as a proxy for $WUE_{intrinsic}$ and that breeding for lower $\Delta^{13}C$ values could result in varieties with higher $WUE_{intrinsic}$ in triticale. Flag leaf photosynthesis and pre-anthesis assimilates contribute much less carbon to grain filling under water stress than previously thought. Lastly, our results show that the dual isotope model is operational, but it is not all encompassing and should be applied together with vapor pressure deficit data.

Key words: grain yield, triticale, intrinsic water use efficiency, $\Delta^{13}C$

4.1 Introduction

Water availability in semi-arid regions is increasingly becoming threatened by erratic rains, frequent droughts, rising temperatures and evapotranspiration rates leading to over-reliance on irrigation to meet food demand. Currently, about 80% of world fresh water is used for irrigation (Morison et al., 2008) and in many dry areas such levels of consumption are unsustainable (Condon et al., 2004) as water resources are also under increased pressure from other users. To feed the projected nine billion people by 2050 (Cleland, 2013), crop management, particularly of cereals, must adapt to climate variability through the use of varieties that use water efficiently (Barnabás et al., 2008). Improving cereal water use efficiency (WUE) has for a long time been one of the main targets of crop research particularly in arid and semi-arid environments, with the aim of finding sustainable ways of increasing crop productivity while reducing water losses (Foley et al., 2011). Crop WUE plays an important role in the exchange of water between the biosphere and the atmosphere and thus has an effect on the global water cycle (Seibt et al., 2008). However, one of the major bottlenecks in cereal breeding to produce “more crop per drop” has been the lack of or the evaluation of appropriate traits (Araus et al., 2008). Direct measurement of WUE under field conditions remains a big challenge due to the large amount of work (Tardieu, 2013) and has stalled the use of the WUE trait in crop improvement programmes. There is, therefore a need to identify reliable proxies of WUE that can be measured quickly; that are correlated to yield and that can also provide the highest repeatability and heritability.

Stable isotope ratios of plant material are a powerful tool in ecological research as they indicate key environmental and physiological processes (Barnard et al., 2012). Carbon isotope composition ($\delta^{13}C$) or the discrimination value ($\Delta^{13}C$) have frequently been used as a time-integrated measure of the intrinsic water-use (Barbour et al., 2011, Farquhar et al., 1989, Cabrera-Bosquet et al., 2009b). Intrinsic water use efficiency ($WUE_{intrinsic}$) is the ratio of photosynthetic rate relative to stomatal conductance (*A/gs*). Stomatal conductance plays an important role in the trade-off between water conservation and carbon assimilation as it controls both CO_2 uptake and water loss (Araya et al., 2010). Changes in stomatal conductance result in changes in leaf $\delta^{13}C$ and in turn in crop WUE (Farquhar and Richards, 1984). However, according to Seibt et al. (2008), the relationship between $\delta^{13}C$ and WUE is not direct due to the influence of external biotic and abiotic factors on the ratio of intercellular CO_2 to that of the atmosphere (*Ci/Ca*) (the primary determinant of discrimination against ^{13}C-CO_2 in leaves). It is argued that WUE at leaf level depends on evaporative demand, which does not directly affect $\delta^{13}C$ (Seibt et al., 2008). Thus, WUE and $\delta^{13}C$ can vary independently of one another, making the use of $\delta^{13}C$ as a proxy for WUE questionable (Seibt et al., 2008).

According to Farquhar et al. (1982), the relationship between $\delta^{13}C$ and WUE exists because isotope discrimination of C_3 plants is linearly linked to *Ci/Ca* ratio. A reduction in *Ci/Ca* could be the result of either a greater *A* at a constant *gs* or a lower *gs* at a constant *A* or even to changes in both factors (Condon et al., 2004). In order to decipher which of the two (*gs* or *A*) is causing changes in $\delta^{13}C$, oxygen isotope composition ($\delta^{18}O$) is used in a dual isotope model proposed by Scheidegger et al. (2000). Isotopic fractionation of water during transpiration in leaves determines $\delta^{18}O$ (Ripullone et al., 2009) and is therefore a proxy of the evaporative flux and is modified by *gs* and not by *A* (Roden and Siegwolf, 2012). In the dual model, relative humidity is assumed to be a major factor influencing *gs*, whereby lower humidity gives rise to lower *gs* (Scheidegger et al., 2000) and thus higher $\delta^{18}O$. Overall, the dual isotope model has potential in evaluating various stress factors in plants (Scheidegger et al., 2000). Therefore, by measuring $\delta^{13}C$ and $\delta^{18}O$ in the same material, $\delta^{18}O$ allows to assess which factor (*A* or *gs*) drives the variation in $WUE_{intrinsic}$ (i.e. variation in $\delta^{13}C$) under varying growing conditions (e.g. drought). $\delta^{13}C$ would be useful information for cultivar improvement and breeders could then use this trait screening approach in breeding programmes. $\delta^{18}O$ has been used in several small grain crop studies (Cabrera-Bosquet et al., 2011, Cabrera-Bosquet et al., 2009b, Ferrio et al., 2007). It has been used to assess long-term transpiration performance of genotypes (Cabrera-Bosquet et al., 2009b, Sheshshayee et al., 2010) as well as a grain yield predictor (Ferrio et al., 2007), but to our knowledge, less is known about the applicability of the dual isotope model in annual crops. The dual isotope model has mainly been tested for trees (Barnard et al., 2012, Ripullone et al., 2009, Roden and Farquhar, 2012).

The measurement of carbon isotope discrimination in plant material offers a powerful means of evaluating WUE at leaf level as it can provide repeatability and heritability required for a selecting trait (Condon and Richards, 1992). Plants are sensitive to changes in soil moisture (Davies and Gowing, 1999) and $\delta^{13}C$ measured in plant material is capable of detecting subtle changes in *Ci/Ca* resulting from small soil moisture fluctuations (Farquhar et al., 1989). Even though, ^{13}C shows potential, not many small grain cultivars have been selected for high WUE using this tool. We are only aware of a single study by Rebetzke et al. (2002) which reported the selection of wheat cultivars using Δ^{13}C as a selecting trait and there are currently no reports on triticale selection via Δ^{13}C. There is also very limited information on δ^{13}C variation in triticale genotypes. The available literature are mainly comparison studies between triticale and other small grain cereals (e.g. Motzo et al. (2013) and Yousfi et al. (2010)). Triticale was selected for this study as it out-yields wheat in both favourable and unfavourable conditions (Bassu et al., 2011, Estrada-Campuzano et al., 2012). It is also believed that triticale will become more important in the future than wheat if grain quality is improved (Blum, 2014). Its importance will arise due to: climate change; the spreading of agriculture in marginal lands and the need to feed the ever increasing population under harsh conditions.

The main purpose of the study was to test if stable isotopes of carbon and oxygen can be used for screening drought stressed triticale genotypes. Specifically, we aimed to: 1) test the use of carbon isotope discrimination as a proxy of intrinsic WUE and grain yield in field grown triticale; 2) test if ^{18}O and ^{13}C data can be used to assess whether changes in δ^{13}C are due to changes *A* and/or *gs* of field grown triticale and 3) explore the use of carbon isotope discrimination to infer sources of carbon assimilates to grain filling.

4.2 Materials and Methods

4.2.1 Study site and experimental design

The study was carried out at the University of Limpopo experimental farm, Syferkuil (23^{o}50’ S; 029^{o}41’ E), Limpopo Province, South Africa during two winter seasons; June to October in 2013 and July to November in 2014. The experimental conditions were exactly the same as described under subsections 2.2.1 and 2.3.1 and the experiment was laid out at shown in Fig. 3.1.

4.2.2 Agronomic measurements

Aboveground biomass was measured at early milk stage (GS71) and at harvest maturity (GS92) and grain yield at GS92. Sampling was done from two, 1.7 m long middle rows, covering an area of 0.85 m2 (170 plants) by cutting the plants at 10 cm aboveground and drying them to constant weight at 65°C. Biomass sampling was done at two stages to determine dry matter available for silage, hay or straw.

4.2.3 Leaf gas exchanges

Leaf gas exchange measurements were taken on a single leaf using LCi-SD Ultra Compact Photosynthesis System (ADC Bio Scientific, UK). The measurements were taken three times each season on clear sunny days from 11h00 to 13h00. The specific data collected included: photosynthetic rate (*A*), stomatal conductance (*gs*), transpiration (*E*), intercellular CO_2 concentration (*Ci*), and atmospheric CO_2 (*Ca*). These measurements were taken on flag leaves as described on section 2.2.3.

4.2.4 Isotope Analyses

Flag leaves for isotope analyses were sampled at GS71 and GS92 and grain samples at GS92. Samples were dried at 65˚C to constant weight and ground to a fine texture using a ZM200 mill (Retsch, Germany). The $^{13}C/^{12}C$ isotope ratio (R_{sample}) of both leaf and grain samples were analyzed using an Automated Nitrogen Carbon Analyser – Solid and Liquids (ANCA-SL, SerCon, UK) interfaced with an Isotope Ratio Mass Spectrometer (IRMS) (20-20, SerCon, UK). The isotope composition was reported as $\delta^{13}C$ in ‰ using Vienna Pee Dee Belemnite (V-PDB) as international standard ($R_{standard}$) and calculated using the formula below:

$$\delta^{13}C_{\mathrm{sample}} = \left(\frac{\mathrm{R_{sample}}}{\mathrm{R_{standard}}} - 1 \right) \mathrm{x}\ 1000 \qquad \textit{(4.1)}$$

The ^{13}C discrimination ($\Delta^{13}C$) was then calculated following Farquhar et al. (1982) from $\delta^{13}C_{sample}$ as follows:

$$\Delta^{13}C = \frac{\delta^{13}C_{air} - \delta^{13}C_{sample}}{1 + (\delta^{13}C_{sample} / 1000)} \qquad (4.2)$$

Where $\delta^{13}C_{air}$ and $\delta^{13}C_{sample}$ are the carbon isotope compositions of air and plant samples (leaves or grain), respectively. $\delta^{13}C_{air}$ was fixed at -8.15‰ (CDIAC, 2015)

The $^{18}O/^{16}O$ ratio in the same samples was analyzed using Thermal Conversion Elemental Analyzer (TC-EA-IRMS) (SerCon, UK) interfaced with IRMS (20-20, SerCon, UK). A composite water sample was collected from the sprinklers and analyzed for ^{18}O isotope composition using a Cavity Ring-Down Spectrometer (CRDS), (L2130-i, Picarro, USA) coupled with a vaporizing module (A0211 high-precision vaporizer, Picarro, USA) and a micro combustion module (MCM, Picarro, USA). The isotope composition was reported as $\delta^{18}O$ ‰ and calculated as shown below using Vienna Standard Mean Ocean Water (VSMOW2) an international standard.

$$\delta^{18}O_{\mathrm{sample}} = \left(\frac{\mathrm{R_{sample}}}{\mathrm{R_{standard}}} - 1 \right) \mathrm{x}\, 1000 \qquad (4.3)$$

The ^{18}O isotope discrimination ($\Delta^{18}O$) was then calculated as follows:

$$\mathrm{D}^{18}O = \frac{d^{18}O_{sample} - d^{18}O_{irrigation\ water}}{1 + (d^{18}O_{irrigation\ water} / 1000)} \qquad (4.4)$$

Where $\delta^{18}O_{sample}$ and $\delta^{18}O_{irrigation\ water}$ represent the oxygen isotope compositions of the plant sample and irrigation water, respectively. The measured $\delta^{18}O_{irrigation\ water}$ was -6.44‰.

4.2.5 WUE determination

Integrated WUE was calculated as the ratio of aboveground dry biomass to total amount of water used ($WUE_{biomass}$) and also as the ratio of grain yield against total amount of water used (WUE_{grain}). The total amount of water added was obtained from the summation of the rain gauge recordings over the growth period.The latter WUE_{grain} was calculated because it responds well to variation in water supply (Katerji et al., 2008). At leaf level, intrinsic WUE ($WUE_{intrinsic}$) and instantaneous WUE (WUE_{inst}) were calculated as follows: $WUE_{inst} = A/E$ and $WUE_{intrinsic} = A/gs$ where A is photosynthetic rate, E is transpiration rate, and gs is stomatal conductance.

4.2.6 Statistical Analyses

A two-way analysis of variance (ANOVA) was performed to calculate the effects of moisture level and genotype on the studied parameters. Post Hoc multiple comparisons for observed means was done using Tukey and different means were denoted by *, ** or *** for significance levels $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$, respectively. All data were analyzed using the SPSS 20 statistical package (SPSS, USA).

4.3 Results

4.3.1 Grain yield, aboveground biomass and integrated WUE

Table 4.1 shows the effect of moisture levels on grain yield, total aboveground dry biomass at GS92, $WUE_{biomass}$ at GS71 and GS92 and WUE_{grain}. Moisture levels significantly influenced grain yield and aboveground biomass in both seasons (Table 4.1). As expected, triticale performed better under non-limited water supply compared to water limited conditions with yields ranging from 0.8 t ha^{-1} under SS to 3.5 t ha^{-1} under WW in 2013 and 1.8 to 4.9 t ha^{-1} in 2014. Differences were also observed for $WUE_{biomass}$ at GS71 and GS92 and for WUE_{grain}. The general trend observed was a decreasing WUE with decreasing soil moisture. In 2013 the WUE was significantly higher under MW compared to the other moisture levels while in 2014, WUE was significantly higher under WW and MW compared to MS and SS. However, WUE_{grain} was only significant between WW and SS in 2014.The performance of the genotypes in terms of biomass accumulation, grain yield and WUE averaged over the two seasons is shown in Table 4.3. Neither genotypic differences nor the interaction of genotype and moisture levels were observed.

Table 4.1: Grain yield, Total dry biomass, Integrated WUE ($WUE_{biomass}$ at GS71, $WUE_{biomass}$ at GS92, and WUE_{grain}) for four moisture levels in 2013 and 2014

		Grain Yield	Total dry Biomass	Integrated WUE		
Year	Irrigation	GS92	GS92	$WUE_{biomass}$ GS71	$WUE_{biomass}$ GS92	WUE_{grain} GS92
		-------t ha^{-1}-------		-----------*kg ha^{-1} mm^{-1}*------------		
2013	WW	3.5^{a}	13.6^{a}	31.2^{b}	31.0^{b}	8.1^{b}
	MW	3.9^{a}	13.6^{a}	37.0^{a}	40.9^{a}	11.7^{a}
	MS	2.4^{b}	9.6^{b}	29.9^{b}	29.8^{b}	7.3^{b}
	SS	0.8^{c}	6.9^{c}	26.9^{b}	29.7^{b}	3.4^{c}
		***	***	*	*	***
2014	WW	4.9^{a}	15.0^{a}	39.2^{a}	39.4^{a}	11.7^{a}
	MW	4.0^{ab}	12.2^{b}	31.8^{a}	34.0^{a}	10.1^{a}
	MS	3.1^{bc}	9.5^{c}	28.3^{b}	32.5^{b}	9.5^{b}
	SS	1.8^{c}	6.8^{d}	23.5^{b}	27.6^{b}	7.4^{b}
		***	***	*	*	*

*Significance levels: *P < 0.05, **P < 0.01, ***P < 0.001, ns means not significant. Different letters in the same column indicate significant differences*. WW = well watered, MW = moderately well watered, MS = Medium stress, SS = severe stress

4.3.2 Flag leaf gas exchanges at GS71

Transpiration rate, stomatal conductance, photosynthetic rate and $WUE_{intrinsic}$ were highly influenced by moisture level (Table 4.2) but genotypes did not show any differences (Table 4.3). Moisture level did not significantly affect *Ci*/*Ca* and WUE_{inst} in both seasons. Higher moisture levels (WW and MW) increased *E, A,* and *gs* while lower moisture levels (MS and SS) decreased them. The average measured atmospheric CO_2 concentration (*Ca*) above the triticale canopy was 379.7 ± 3.9 ppm for both years. While most gas exchanges significantly decreased with decreasing soil moisture, $WUE_{intrinsic}$ increased with decreasing soil moisture level. Comparing the two seasons, 2013 had relatively higher transpiration rates and $WUE_{intrinsic}$ compared to 2014. $WUE_{intrinsic}$ ranged from 104.5 to 194.1 µmol mol^{-1} in 2013 and 89.7 to 129.3 µmol mol^{-1} in 2014. Stomatal conductance and photosynthetic rates were however higher in 2014 than in 2013. In the 2014 season, measurements tended to be grouped in two, were WW and MW had significantly higher values than MS and SS. This could be due to higher subsoil moisture availability under WW and MW compared to MS and SS.

Table 4.2: *Ci/Ca*, transpiration rate (E), stomatal conductance (gs), photosynthetic rate (A), WUE_{inst}, and $WUE_{intrinsic}$ as affected by moisture level and genotype; *Ci/Ca* is the ration of intercellular CO_2 concentration and average atmospheric CO_2 concentration

Year	Irrigation	*Ci/Ca*	Transpiration rate (*E*) *mmol $m^{-2}s^{-1}$*	Stomatal conductance (*gs*) *mol m^{-2} s^{-1}*	Photosynthetic rate (*A*) *µmol m^{-2} s^{-1}*	WUE_{inst} *µmol $mmol^{-1}$*	$WUE_{intrinsic}$ *µmolmol $^{-1}$*
2013	WW	0.46^a	4.39^a	0.10^a	9.92^a	2.27^a	104.5^c
	MW	0.39^a	3.45^{ab}	0.07^b	8.01^{ab}	2.27^a	139.6^b
	MS	0.42^a	3.03^b	0.06^b	6.68^b	2.01^a	114.9^{bc}
	SS	0.44^a	0.95^c	0.01^c	1.83^c	2.29^a	194.1^a
		ns	***	***	***	ns	***
2014	WW	0.52^a	2.67^a	0.13^a	11.64^a	4.4^a	89.7^b
	MW	0.49^a	2.61^a	0.12^a	11.15^a	4.2^a	96.2^b
	MS	0.51^a	1.67^b	0.06^b	6.11^b	3.5^a	101.3^{ab}
	SS	0.47^a	1.26^b	0.04^b	4.74^b	3.8^a	129.3^a
		ns	***	***	***	ns	*

*Significance levels: *P < 0.05, **P < 0.01, ***P < 0.001, ns means not significant. Different letters in the same column refer to significant differences.* WW - well watered, MW - moderately well watered, MS - Medium stress, SS - severe stress

Table 4.3: Grain yield, water use efficiencies, and leaf gas exchanges of the four genotypes under four moisture levels averaged over the two seasons

			--------------------------------WUE-------------------------------				---------------------Leaf gas exchanges-----------------			
Irrigation	Genotype	Grain Yield	$WUE_{biomass}$	WUE_{grain}	$WUE_{Intrinsic}$	WUE_{inst}	*E*	*gs*	*A*	*Ci/Ca*
		t ha^{-1}	------------kg ha mm^{-1}-----------		*µmol mol* $^{-1}$	*µmol mmol*$^{-1}$	*mmol m*$^{-2}$*s*$^{-1}$	*mol m*$^{-2}$ *s*$^{-1}$	*µmol m*$^{-2}$ *s*$^{-1}$	
WW	Agbeacon	3.9(1.7)	35.7(5.5)	8.8(2.8)	86.3(9.9)	3.86(0.59)	3.38(0.82)	0.15(0.04)	13.00(4.03)	0.51(0.06)
	Bacchus	4.6(2.7)	40.6(17.8)	11.3(7.5)	89.4(2.3)	4.09(1.29)	3.25(1.12)	0.15(0.07)	12.63(5.58)	0.50(0.10)
	Rex	5.0(2.6)	37.4(13.7)	12.5(5.6)	87.0(15.3)	4.58(1.05)	3.25(0.99)	0.17(0.05)	14.38(3.39)	0.49(0.07)
	US2007	3.2(1.3)	27.1(8.9)	7.0(3.4)	70.5(17.3)	3.82(1.16)	3.13(0.80)	0.18(0.05)	12.50(3.11)	0.58(0.07)
		ns	ns	ns	ns	ns	ns	ns	ns	ns
MW	Agbeacon	4.0(1.7)	40.5(17.1)	11.8(5.1)	85.9(18.7)	4.08(0.60)	3.38(0.65)	0.17(0.05)	13.50(1.84)	0.50(0.07)
	Bacchus	4.0(1.6)	40.3(17.8)	10.2(3.8)	106.9(29.9)	4.40(0.99)	3.13(1.04)	0.14(0.05)	13.63(4.95)	0.42(0.13)
	Rex	3.8(1.4)	36.1(12.0)	10.7(4.7)	84.0(19.5)	3.80(1.20)	3.13(1.14)	0.14(0.06)	12.00(5.39)	0.53(0.11)
	US2007	4.1(1.7)	32.9(7.2)	11.0(4.3)	95.0(26.5)	3.93(0.76)	2.88(0.83)	0.13(0.05)	11.50(3.54)	0.49(0.12)
		ns	ns	ns	ns	ns	ns	ns	ns	ns

MS	Agbeacon	2.5(1.5)	33.4(8.1)	8.1(2.8)	124.6(37.9)	3.93(0.64)	1.63(0.55)	0.05(0.02)	6.63(2.59)	0.44(0.13)
	Bacchus	3.5(1.8)	33.2(1.2)	10.2(4.3)	121.7(57.3)	3.77(0.99)	1.88(0.89)	0.08(0.06)	7.38(3.53)	0.47(0.13)
	Rex	1.8(1.1)	27.8(6.9)	6.0(2.9)	129.4(57.9)	3.17(1.17)	1.43(0.50)	0.04(0.02)	4.57(2.57)	0.48(0.20)
	US2007	3.0(1.6)	29.7(9.5)	9.4(4.2)	119.7(61.2)	3.61(0.84)	1.88(0.82)	0.07(0.04)	6.63(3.79)	0.48(0.19)
		ns	ns	ns	ns	Ns	ns	ns	ns	ns
SS	Agbeacon	1.4(0.6)	30.2(6.7)	5.5(1.9)	128.8(72.2)	4.00(1.33)	1.50(1.14)	0.06(0.04)	5.38(4.06)	0.49(0.14)
	Bacchus	1.3(1.0)	28.0(4.4)	5.4(3.2)	138.8(39.8)	4.16(1.30)	1.86(0.96)	0.06(0.04)	7.14(3.84)	0.38(0.22)
	Rex	1.4(1.0)	27.2(8.1)	5.9(4.0)	124.4(51.1)	4.29(1.01)	2.38(1.40)	0.09(0.08)	9.00(5.50)	0.42(0.14)
	US2007	1.2(1.1)	29.1(1.1)	4.8(4.2)	110.6(47.7)	3.56(1.12)	1.86(1.03)	0.08(0.06)	6.89(3.47)	0.48(0.18)
		ns	ns	ns	ns	ns	ns	ns	ns	ns

$WUE_{biomass}$ - biomass, WUE_{grain} - grain, $WUE_{intrinsic}$ - Intrinsic, and WUE_{ins} – instantaneous, E- transpiration, gs – stomatal conductance, A – photosynthetic rate, Ci/Ca – ratio of intercellular CO_2 to atmospheric CO_2. WW - well watered, MW - moderately well watered, MS - Medium stress, SS - severe stress. Values in parentheses are standard deviations

4.3.3 Effect of moisture levels on $\Delta^{13}C$ and $\Delta^{18}O$

Moisture levels strongly influenced both flag leaf $\Delta^{13}C$ and grain $\Delta^{13}C$ (Table 4.4). There was a significant interaction effect between irrigation and year for $\Delta^{13}C$ and $\Delta^{18}O$ and as such the data for the two years were analysed separately. Triticale discriminated more against ^{13}C under well-watered conditions compared to water limited conditions as evidenced by higher $\Delta^{13}C$ values under WW and MW at the two sampling stages. A general decrease in $\Delta^{13}C$ was observed from WW to MS in both leaf and grain samples over the two study seasons. In 2013 at GS71, all moisture levels had significantly different $\Delta^{13}C$ values, but flag leaves sampled later in the season at GS92 showed no differences between WW and MW. SS resulted in the lowest $\Delta^{13}C$ values at both stages. Similar to the 2014 season, SS plants produced the lowest $\Delta^{13}C$. However, WW and MW did not differ at both stages. Across the two seasons and sampling stages, flag leaf $\Delta^{13}C$ ranged from 17 to 20.6‰. A T-test performed on the 2013 data showed that ^{13}C discrimination values were on average 0.65‰ higher ($P < 0.001$) at GS71 compared to at GS92 while in 2014 no differences ($P > 0.05$) were observed even though GS71 tended to have slightly higher values than GS92. In addition, T-test performed on 2014 data between flag leaf $\Delta^{13}C$ at GS71 and grain $\Delta^{13}C$ showed that; under SS, flag leaf $\Delta^{13}C$ values were on average 2.9‰ (with 95% confidence interval ranging from 2.2 to 3.6‰) higher than grain $\Delta^{13}C$ while under WW, the average difference in $\Delta^{13}C$ values between the two was 1.7‰ (95% confidence interval: 0.71 to 2.8‰).

Table 4.4: Flag leaf $\Delta^{13}C$, $\Delta^{18}O$ and carbon content measured at early milk stage (GS71) and harvest maturity (GS92) under four moisture levels in the two growing seasons

		$\Delta^{13}C$ (‰)			$\Delta^{18}O$ (‰)		Carbon content (%)	
Year	Irrigation	GS71	GS92	Grain	GS71	GS92	GS71	GS92
2013	WW	20.6^{a}	20.0^{a}	n.a.	30.9a	28.4a	37.2b	35.1c
	MW	20.0^{b}	19.7^{a}	n.a.	31.3a	24.3a	37.2b	34.3c
	MS	19.1^{c}	18.6^{b}	n.a.	28.8b	21.2b	39.2a	36.9b
	SS	18.1^{d}	17.0^{c}	n.a.	29.5b	22.9b	39.8a	39.7a
	LSD	***	***		**	**		
2014	WW	19.0^{a}	19.0^{a}	17.4^{a}	26.7^{b}	30.7^{b}	39.4^{c}	34.6^{b}

MW	18.7^{ab}	18.5^{ab}	16.7^{a}	26.8^{b}	30.8^{b}	40.1^{c}	35.2^{b}
MS	18.4^{b}	18.3^{b}	15.2^{b}	30.0^{a}	33.1^{a}	41.7^{a}	38.3^{a}
SS	17.9^{c}	17.7^{c}	14.9^{b}	30.7^{a}	34.3^{a}	41.2^{a}	38.5^{a}
LSD	**	***	***	***	***	**	**

*Significance levels: *P < 0.05, **P < 0.01, ***P < 0.001, ns means not significant. Different letters in the same column refer to significant differences. WW - well watered, MW - moderately well watered, MS - Medium stress, SS - severe stress; n.a. = not available*

Significant differences were also observed between flag leaf $\Delta^{13}C$ and grain $\Delta^{13}C$ under MW (1.9‰) and MS (3.3‰). Flag leaf carbon content was also significantly affected by moisture level at both GS71 and GS92 (Table 4.4). The carbon content was higher under water-limited conditions compared to unlimited water supply conditions. At both GS71 and GS92 carbon content was more than two percentage points higher at SS compared to WW. Also, carbon content was found to be significantly higher ($P<0.05$) at GS71 compared to GS92.

Moisture levels also had a strong influence on the oxygen isotope enrichment of triticale flag leaves. Unlike ^{13}C, ^{18}O isotope enrichment behaved differently over the two years. In 2013 $\Delta^{18}O$ values were higher under well-watered conditions (WW and MW) compared to water limited conditions (MS and SS). In 2014, it was the reverse, $\Delta^{18}O$ values were lower under well-watered conditions (WW and MW) compared to water limited conditions (MS and SS). Table 4.4 also shows that over the two seasons, there were no differences in $\Delta^{18}O$ between WW and MW, and also between MS and SS. $\Delta^{18}O$ values in 2013 ranged from 28.8 to 30.9 at GS71 and 22.9 to 28.4 at GS92, while those in 2014 ranged from 26.7 to 30.7 at GS71 and 30.7 to 34.3 at GS92.

Carbon isotope discrimination, oxygen isotope enrichment ($\Delta^{18}O$) and flag leaf carbon content failed to separate the genotypes as has been observed with other parameters. Due to this lack of genotypic differences, Table 4.5 shows average values calculated for all moisture levels combined.

Table 4.5: Flag leaf $\Delta^{13}C$, $\Delta^{18}O$ and flag leaf carbon content measured at early milk stage (GS71) and harvest maturity (GS92) for the four genotypes averaged across all moisture levels

		$\Delta^{13}C$ (‰)			$\Delta^{18}O$ (‰)		Carbon content (%)	
Year	Genotypes	GS71	GS92	Grain	GS71	GS92	GS71	GS92

2013	Agbeacon	19.5(1.1)	18.9(1.3)	n. a.	29.9(1.7)	23.8(5.3)	38.7(1.5)	36.4(2.4)
	Bacchus	19.4(1.5)	18.8(1.8)	n. a.	29.8(1.5)	24.3(3.1)	37.8(1.7)	36.5(3.0)
	Rex	19.4(1.1)	18.7(1.2)	n. a.	30.5(2.1)	24.0(3.5)	38.8(5.0)	36.9(3.0)
	US2007	19.6(1.0)	18.9(1.1)	n. a.	30.2(2.0)	24.8(4.3)	38.1(3.6)	36.2(2.7)
		ns	ns		ns	ns	ns	ns
2014	Agbeacon	18.5(0.8)	18.5(0.8)	15.7(1.6)	28.4(3.6)	31.8(2.6)	40.8(1.6)	37.1(3.9)
	Bacchus	18.7(1.0)	18.5(1.0)	16.3(1.5)	28.4(2.9)	32.5(2.0)	39.6(2.1)	36.0(3.7)
	Rex	18.3(0.9)	18.3(1.0)	16.1(1.5)	29.4(3.1)	32.0(3.2)	41.0(1.3)	37.4(4.8)
	US2007	18.5(0.9)	18.5(0.8)	16.2(1.5)	29.1(3.1)	32.5(2.2)	40.9(2.3)	36.1(3.3)
		ns	ns	ns	ns	ns	ns	ns

Values in parentheses are standard deviation; n. a. = not available

4.3.4 Relationship between different traits

Fig. 4.1 shows how the four genotypes varied in $\mathrm{WUE}_{\mathrm{intrinsic}}$ and $\Delta^{13}C$. Genotypic variation in both $\mathrm{WUE}_{\mathrm{intrinsic}}$ and $\Delta^{13}C$ tended to be higher under dry conditions (SS and MS) compared to wetter conditions (MW and WW). Bacchus had the highest $\mathrm{WUE}_{\mathrm{intrinsic}}$ and also the lowest discrimination values under SS. It showed the highest discrimination values under WW. Agbeacon had the lowest $\mathrm{WUE}_{\mathrm{intrinsic}}$ under SS but had the highest $\mathrm{WUE}_{\mathrm{intrinsic}}$ under WW. Even though statistical differences could not be found among the genotypes, error bars on Fig. 4.1 suggest differences in Agbeacon and Bacchus performance under SS. Bacchus performed far much better in $\mathrm{WUE}_{\mathrm{intrinsic}}$ compared to Agbeacon under SS with Rex and US2007 having $\mathrm{WUE}_{\mathrm{intrinsic}}$ in-between Bacchus and Agbeacon.

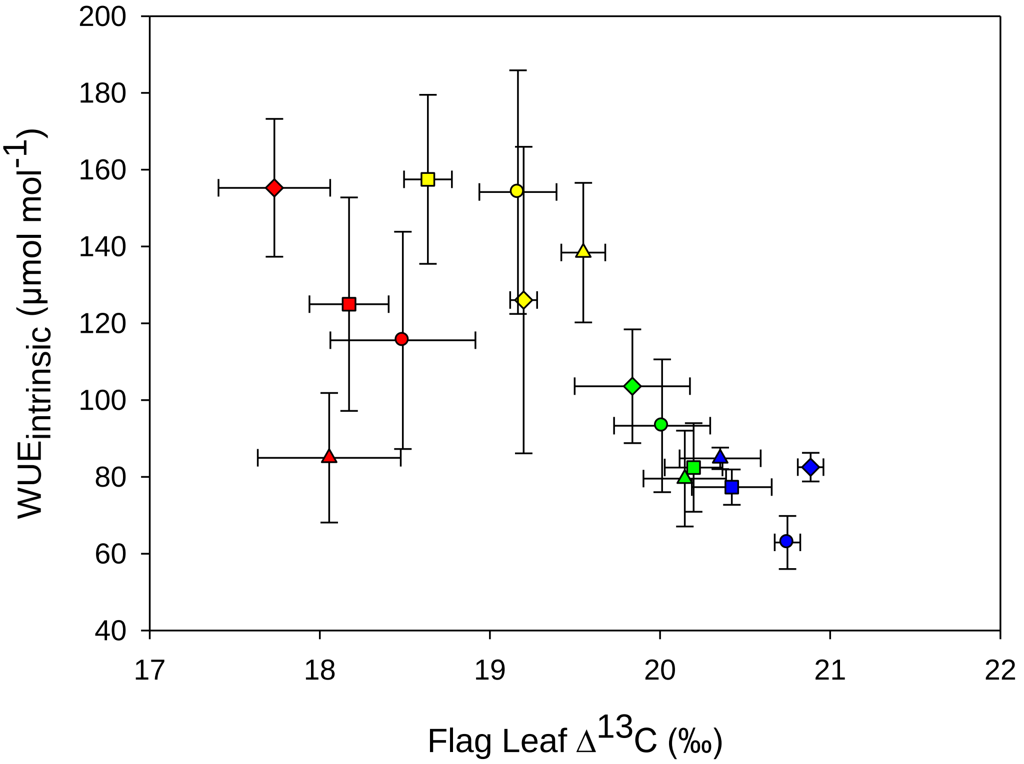


Fig. 4.1: Relationship between flag leaf Δ^{13}C at GS71 and WUE$_{intrinsic}$ of triticale genotypes under four moisture levels in the 2013 season. Triangles = Agbeacon, diamonds = Bacchus, rectangles = Rex, circles = US2007. Different colours represent different moisture levels: red = SS, yellow = MS, green = MW and blue = WW. Error bars represent standard errors.

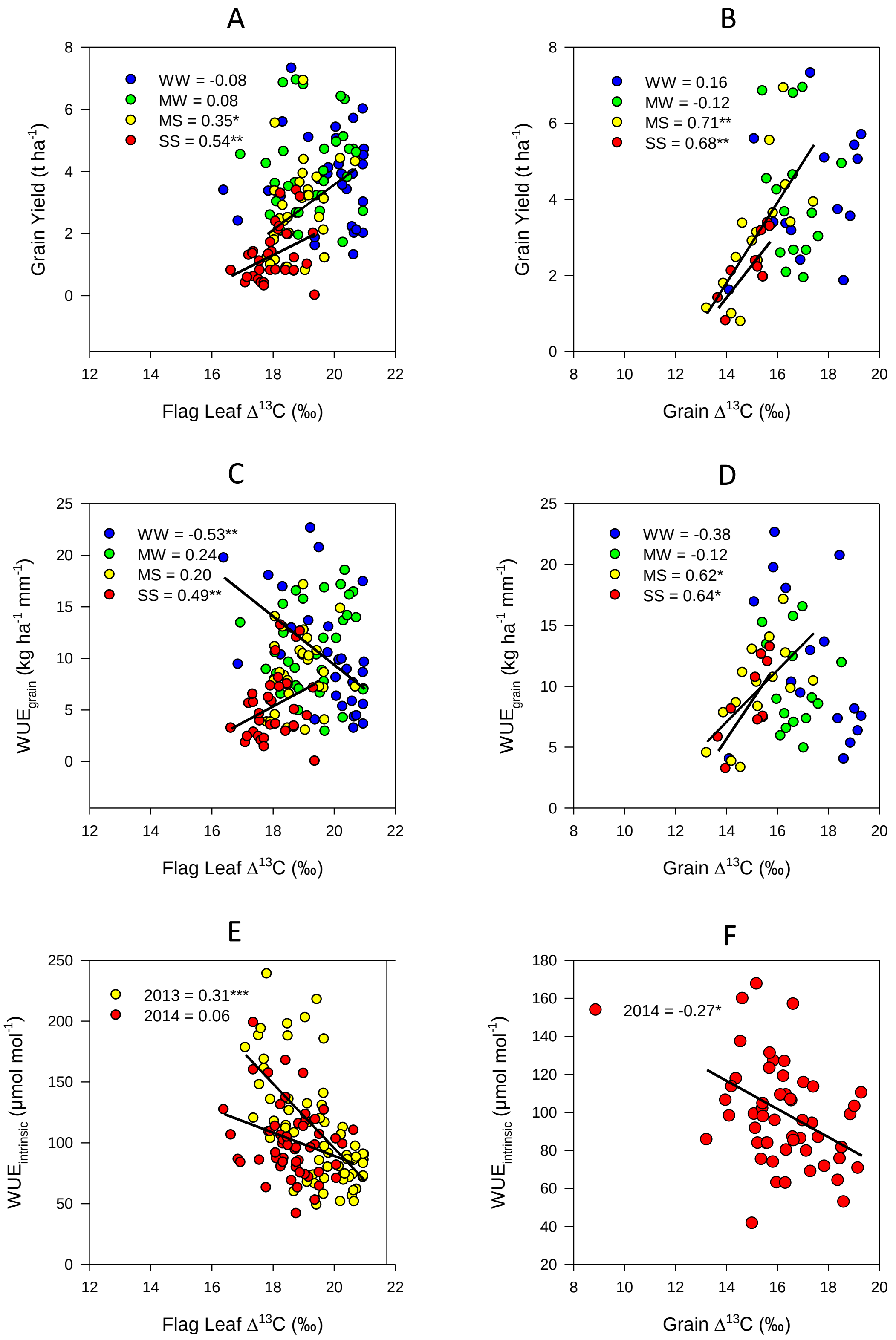


Fig. 4.2: Correlation plots: grain yield and flag leaf Δ^{13}C (A); grain yield and grain Δ^{13}C (B); grain WUE and flag leaf Δ^{13}C (C); grain WUE and grain Δ^{13}C (D); WUE$_{intrinsic}$ and leaf Δ^{13}C (E); WUE$_{intrinsic}$ and grain Δ^{13}C (F). WW, MW, MS, and SS are moisture levels representing: well-watered, moderately well-watered, medium stress, and severe stress, respectively followed by correlation coefficient values. 2013 and 2014 on E and F, represent the year. Lines were

fitted for significant correlations only. Data for flag leaf $\Delta^{13}C$ was measured at GS71 over the two seasons while grain $\Delta^{13}C$ was for GS92 in 2014.

Grain yield was significant and positively correlated to flag leaf $\Delta^{13}C$ under SS (0.54**) and MS (0.34*) (Fig. 4.2A) but the correlations were not significant under WW and MW. Similar results were observed with grain $\Delta^{13}C$ (Fig. 4.2B). The correlation coefficient values were higher with grain $\Delta^{13}C$ than flag leaf $\Delta^{13}C$. WUE_{grain} showed a significant positive correlation with flag leaf $\Delta^{13}C$ under SS (0.49**) but was negatively correlated to flag leaf $\Delta^{13}C$ under WW (0.53**) (Fig. 4.2C). WUE_{grain} was positively correlated to grain $\Delta^{13}C$ under SS (0.64*) and MS (0.63*) and showed non-significant negative correlations to MW and WW (Fig. 4.2D). The correlations between $WUE_{intrinsic}$ and flag leaf $\Delta^{13}C$ and between $WUE_{intrinsic}$ and grain $\Delta^{13}C$ were not significant when the data were separated according moisture level. However, when data for all moisture levels were combined, negative correlations were observed (Fig. 4.2E and 4.3F). A stronger correlation was observed in 2013 (0.31***) compared to 2014 (0.06*). Other important significant correlations observed in this study were between $WUE_{intrinsic}$ and WUE_{grain} (-0.33** in 2013 and -0.15^{ns} in 2014) as well as between $WUE_{intrinsic}$ and grain yield (-0.38** in 2013 and -0.20* in 2014). $WUE_{intrinsic}$ was negatively related to both grain yield and WUE_{grain} and was significant in both seasons for grain yield but was only significant in 2013 for WUE_{grain}.

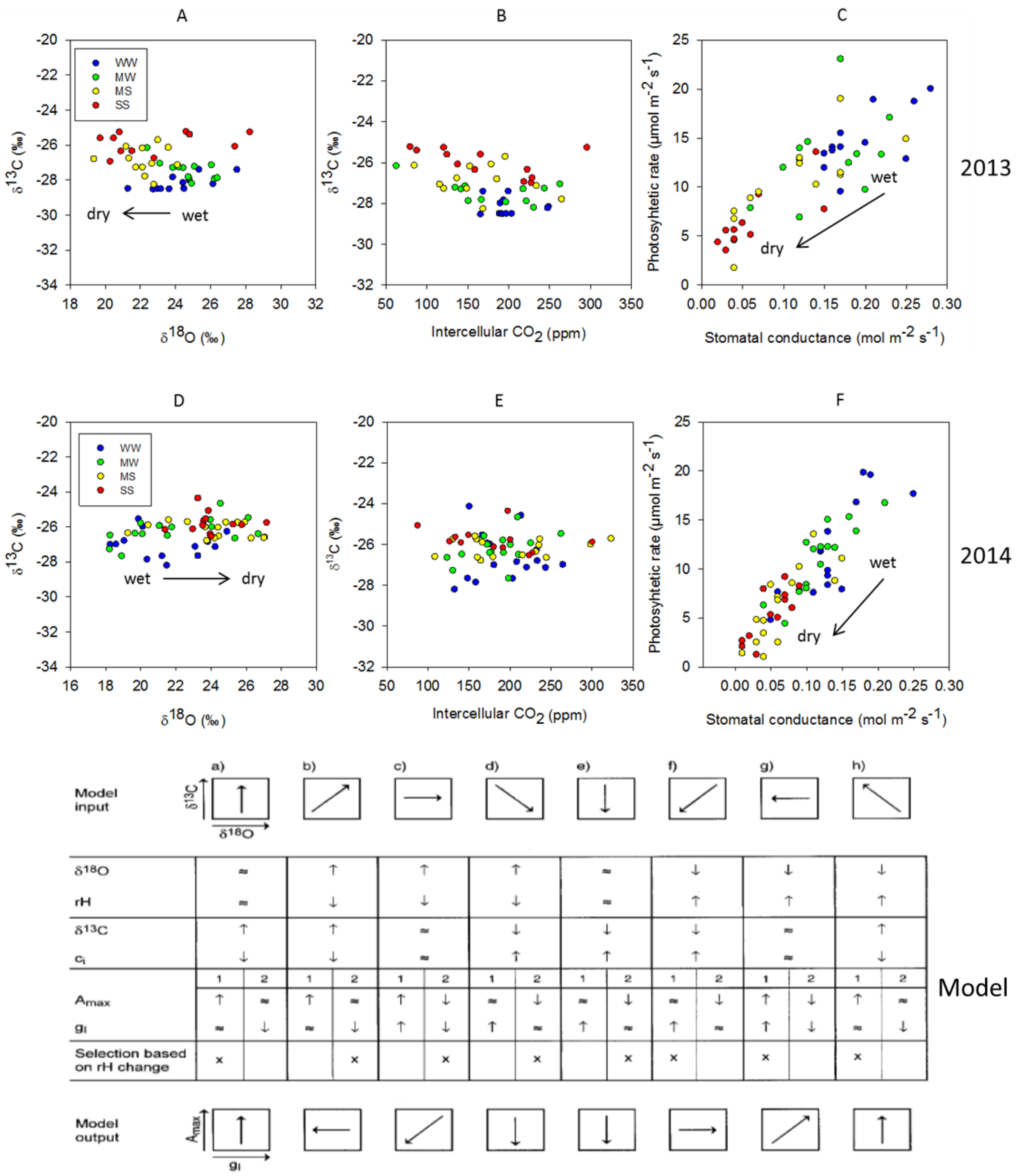
4.3.5 Testing of the dual isotope model

Isotope and gas exchange data measured at GS71 in both years (2013 and 2014) were used to test the applicability of the dual isotope model. The top row of Fig. 4.3 (A, B and C) shows the relationship between measured parameters in 2013, the second row (D, E, F) shows measured data for 2014, while the bottom plot is dual isotope conceptual model developed by Scheidegger et al. (2000). The model was tested by observing the relationships that occur when triticale growing conditions are gradually changed from well-watered (wet) to water limited environment (dry). Fig. 4.3A and Fig. 4.3D show the relationships between $\delta^{13}C$ and $\delta^{18}O$ in 2013 and 2014, respectively. These relationships are used as input for the model. Both Fig. 4.3A and Fig. 4.3D show a relatively constant $\delta^{13}C$ against a varying $\delta^{18}O$. However, a difference is observed in the direction of the arrows as conditions change from well-watered (wet) to water stress (dry). In 2013 (Fig. 4.3A), the direction of the arrow suits scenario ‘g’ of the model input while in 2014 (Fig. 4.3D), the arrow suits scenario ‘c’.

Constant $\delta^{13}C$ and a varying $\delta^{18}O$ were assumed from the recommendations of Roden and Siegwolf (2012), who recommended an equal axis scaling for $\delta^{13}C$ and $\delta^{18}O$, such that a 1‰ change in $\delta^{13}C$ corresponds to 1‰ change in $\delta^{18}O$ for an unbiased interpretation of the direction of the arrows. In addition, non-significant correlations were observed between $\delta^{13}C$ and $\delta^{18}O$ in both years.

Fig. 4.3B and Fig. 4.3E show relationships between $\delta^{13}C$ and intercellular CO_2 (*Ci* in the model) for 2013 and 2014, respectively. The plots do not show a clear shift in intercellular CO_2 concentration as affected by moisture level (WW to SS) as all moisture levels (WW, MW, MS, SS) had similar *Ci* ranges from about 100 to 300ppm. Table 4.2 also shows no statistical differences in *Ci/Ca,* thus considering that *Ca* was constant, then *Ci* should not have varied as well, hence a constant *Ci* was assumed. A constant $\delta^{13}C$ was also assumed, consistent with an earlier decision made in Fig. 4.3A and 4.3D. However, the relationships between $\delta^{13}C$ and *A* were negative in both years (Fig. 4.4B and 4.4D). Assuming constant *Ci* and $\delta^{13}C$ agrees with both scenarios 'g' and 'c' (see model).

According to the dual isotope model, scenario 'g' as observed in 2013 results in a model output of increase in both *A* and *gs* while scenario 'c' as observed in 2014, assumes a decrease in both *A* and *gs*. Scheidegger et al. (2000) and Ripullone et al. (2009) provide a detailed description on how these outputs are arrived at. The question that comes up is whether the model outputs agree with the measured data? For 2013, it does not (Fig. 4.3C) but for 2014, it does (Fig. 4.3F). The measured data show that in both years (2013 and 2014), there were decreases in both *A* and *gs* as growing conditions changed from well-watered (wet) to water-limited (dry) conditions. Yet, the model is telling us that in 2013, both *A* and *gs* increased.



	a)		b)		c)		d)		e)		f)		g)		h)	
Model input	↑		↗		→		↘		↓		↙		←		↖	
δ18O	≈		↑		↑		↑		≈		↓		↓		↓	
rH	≈		↓		↓		↓		≈		↑		↑		↑	
δ13C	↑		↑		≈		↓		↓		↓		≈		↑	
ci	↓		↓		≈		↑		↑		↑		≈		↓	
	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
Amax	↑	≈	↑	≈	↑	↓	≈	↓	≈	↓	≈	↓	↑	↓	↑	≈
gl	≈	↓	≈	↓	↑	↓	↑	≈	↑	≈	↑	≈	↑	↓	≈	↓
Selection based on rH change	×			×		×		×		×	×		×		×	
Model output	↑		←		↙		↓		↓		→		↗		↑	

Fig. 4.3: Plots A, B, C, show the relationships for data measured at GS71 in 2013 while D, E and F, show data for 2014: A and D: show relationship between $\delta^{18}O$ and $\delta^{13}C$; B and E: intercellular CO_2 concentration and $\delta^{13}C$; C and F: stomatal conductance and photosynthetic rate. Red circles (SS) = severe stress, yellow circles (MS) = medium stress, green circles (MW) = moderately well-watered, and blue circles (WW) = well-watered. The bottom plot is the dual isotope conceptual model developed by Scheidegger et al. (2000). The arrows (wet to dry) indicate change in environment from well-watered (WW) to severe stress (SS).

The model also assumes that changes in $\delta^{18}O$ are mainly due to changes in *gs* that is, an increase in $\delta^{18}O$ (^{18}O enrichment) is associated with a decrease in *gs.* The assumption was based on theories described in a review by Yakir (1992) and explored further by many other authors (Farquhar et al., 2007, Ripullone et al., 2008). This assumption was duly met by the measured data of 2014 (Fig. 4.4C) but not with 2013 data (Fig. 4.4A). In 2013, $\delta^{18}O$ increased with increasing *gs.*

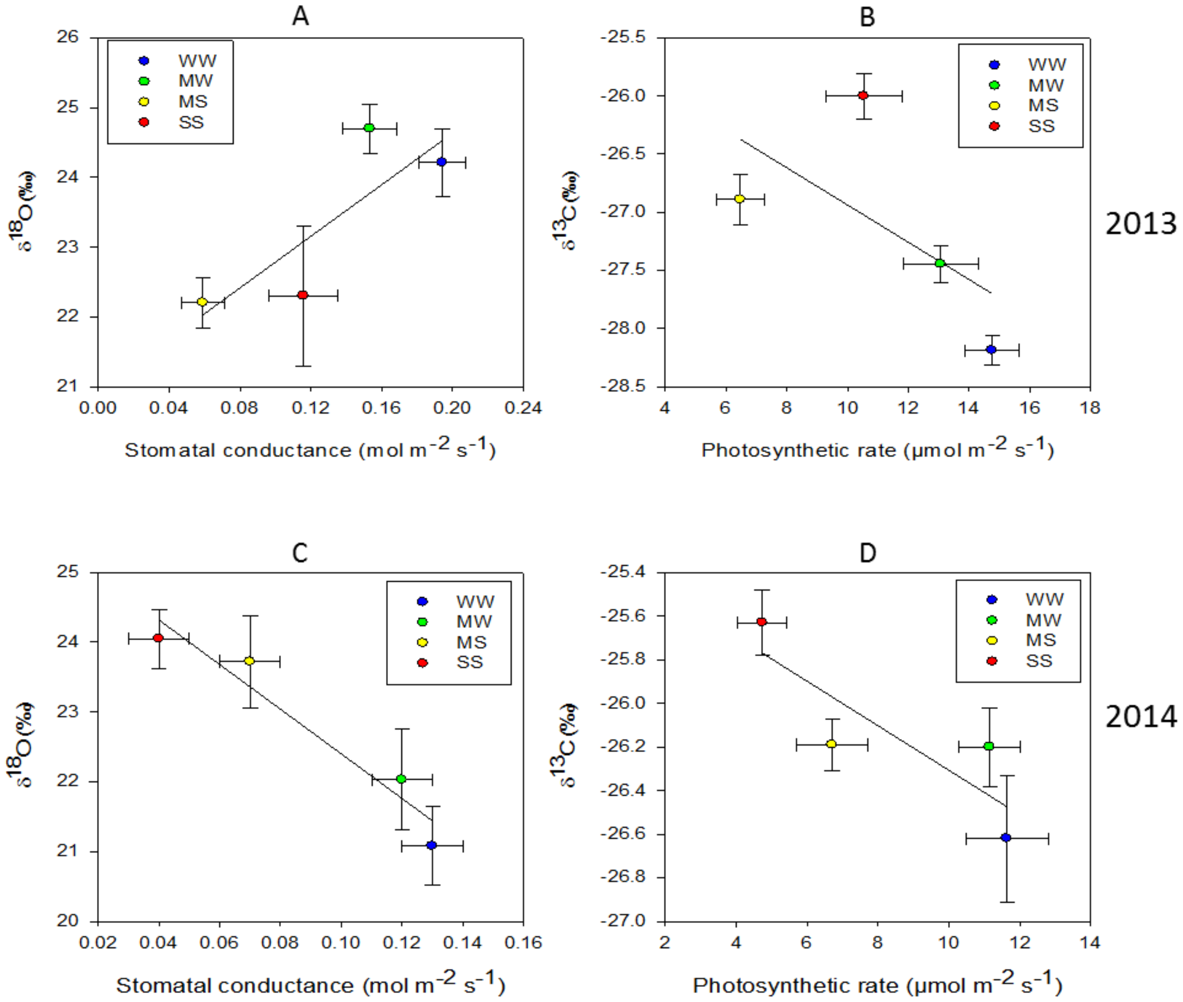


Fig. 4.4: The relationship between $\delta^{18}O$ and stomatal conductance (A) and the relationship between $\delta^{13}C$ and photosynthetic rate (B). Red circles (SS) = severe stress, yellow circles (MS) = medium stress, cyan triangles (MW) = moderately well-watered, and blue triangles (WW) = well-watered. Each bullet is the average of four genotypes and the error bars represent standard errors.

4.4 Discussion

4.4.1 Grain yield, biomass accumulation and integrated WUE

Grain yield, aboveground biomass, $WUE_{biomass}$ (at GS72 and GS92) as well as WUE_{grain} responded to moisture levels in the two study seasons. Higher grain yield and aboveground biomass observed under high moisture levels was expected since an improved water status results in higher levels of transpiration and therefore higher rates of plant growth (Cabrera-Bosquet et al., 2009b). Low grain yield and biomass were observed under SS and MS due to water stress' limitation to photosynthesis (Tezara et al., 1999) through reduction of *gs*. The 2014 growing season was better compared to the 2013 growing season particularly in grain yield and this was attributed to differences in assimilate partitioning to the grain (Munjonji et al., 2016) and also to the differences in photosynthetic rates (Table 4.2). The differences in assimilate transport and photosynthetic rates arose also from higher heat stress experienced during the reproductive stage in 2013 compared to 2014 (Fig. 3.1) which may have shortened the grain filling period (Barnabás et al., 2008). Integrated WUE ($WUE_{biomass}$ and WUE_{grain}) decreased with decreasing moisture level which was rather contrary to the findings of some studies on sugar beet were decreasing soil moisture resulted in increased integrated WUE (Bloch et al., 2006). However, the results are not uncommon as they corroborate with the findings of other studies (Cabrera-Bosquet et al., 2007, Erice et al., 2011). Under water stress (e.g. SS and MS) photosynthesis is inhibited through decreased ribulose bisphosphate supply (Tezara et al., 1999) thereby retarding biomass accumulation, and in turn WUE. Furthermore, high water loss via evaporation during the early growth stages could be another plausible reason for the observed low WUE particularly under SS where irrigation was stopped at GS31.

The negative relationship observed between $WUE_{intrinsic}$ and WUE_{grain} imply that breeding for higher $WUE_{intrinsic}$ may not always translate to better grain yield or WUE_{grain} (Condon et al., 2004). Improvement in $WUE_{intrinsic}$ may result in improved WUE_{grain} in situations where integrated WUE increases with water stress as reported by Bloch et al. (2006). The lack of consistency in the relationship between $WUE_{intrinsic}$ and grain yield in studies is not surprising as gas exchanges represent only snapshot measurements, which may fail to detect daily and seasonal variations (Impa et al., 2005). For example, measurements taken under MW would show several variations depending on the time of measurement. If stomatal conductance measurements are taken soon after an irrigation event (moisture close to FC), the values would be high compared to readings taken days later (close to refill point). Such variation could affect $WUE_{intrinsic}$. In this study measurements were taken when moisture levels were most representative of their level.

4.4.2 Carbon isotope discrimination in grain and flag leaves

The observed influence of soil moisture on $\Delta^{13}C$ is consistent with findings of other studies (Wang et al., 2013, Cernusak et al., 2013, Erice et al., 2011, Cabrera-Bosquet et al., 2011, Barbour et al., 2011, Cui et al., 2009, Cabrera-Bosquet et al., 2009b, Dercon et al., 2006). According to Davies and Gowing (1999), plants are very sensitive to small differences in soil moisture and they respond to these moisture fluctuations by regulating their stomatal conductance, which in turn may affect the *Ci/Ca* ratio, which is a major determinant for ^{13}C discrimination in leaves. When moisture is freely available in the soil as under WW, more water is absorbed by the guard cells, which expand, opening the stomata thus allowing CO_2 to diffuse into the leaf (Aliniaeifard et al., 2014). Increased *gs* at a constant *A* increases the *Ci/Ca* ratio consequently allowing for more discrimination of ^{13}C by the CO_2 fixing enzyme Ribulose bisphosphate carboxylase-oxygenase (Rubisco) and the reverse is true. Accordingly, many other studies have attributed higher $\Delta^{13}C$ values to a reduction *Ci/Ca* (Farquhar et al., 1989, Ripullone et al., 2009).

In this study, however, a constant *Ci/Ca* was found across all four soil moisture levels (Table 4.2), implying a parallel decrease of both photosynthetic rate and stomatal conductance with soil moisture, thus keeping *Ci/Ca* constant (Fig. 4.3). When stomata are wide open (e.g. under WW), more CO_2 diffuses into the stomatal cavities which may result in increased photosynthesis and likewise lower *gs* may result in decreased *A.* In C_3 plants discrimination by Rubisco is the major determinant of $\Delta^{13}C$ variation as controlled by *gs* (CO_2 supply side). Hence, the variation in $\Delta^{13}C$ observed in flag leaves under the four moisture levels arose because of the differences in the level of discrimination by Rubisco as affected by CO_2 supply. Higher discrimination values observed under WW conditions compared to SS result due to the high CO_2 supply (high *gs*) compared to the restricted CO_2 supply (low *gs*) under SS. A negative relationship between $\delta^{13}C$ and *A* was observed (Fig. 4.4B). A similar relationship can also be expected between $\delta^{13}C$ and *gs* due to the strong positive relationship observed between *A* and *gs* (Fig. 4.3C).

$\Delta^{13}C$ was measured at two different growth stages (GS71 and GS92) and in 2014 the measurement were also done on grain samples. The measurements were done at two growth stages in order to identify the stage that gives better correlation with grain yield and WUE. Earlier measurements (GS71) indicate early response of triticale to moisture stress while later measurements are an integration of the entire season. Though both growth stages were not far apart (ca. 25 days), the results showed that earlier measurements were less enriched in ^{13}C than those measured later in the season. In 2013 it was also observed that early season measurements were more efficient in separating moisture levels than later measurements (Table 4.4). In the same season, flag leaves sampled at GS71 were on average of 0.65‰ less enriched (high discrimination) than those sampled at GS92. In the following season (2014), $\Delta^{13}C$ values between the two growth stages were not significantly different though GS71 had slightly higher $\Delta^{13}C$ values than GS92. However, flag leaf $\Delta^{13}C$ measured at GS71 in 2014 were on average 2.9 and 1.7‰ less enriched compared to that of the grain under SS and WW, respectively. The decrease in $\Delta^{13}C$ values in grain samples and in flag leaves measured at GS92 can be attributed to the increase in evaporative demand occurring later in the growing season (Condon and Richards, 1992, Smedley et al., 1991). This is also confirmed by the increasing temperature and VPD in the months September to November (Fig. 3.2).

In several studies (Anyia et al., 2007, Cabrera-Bosquet et al., 2011), grain $\Delta^{13}C$ has also been found to be lower than leaf $\Delta^{13}C$, which is consistent with the results of this study. Grain $\Delta^{13}C$ has also been found to be lower under water stress conditions compared to well-watered conditions. Using isotope signatures alone, our results and those of the above-mentioned studies seem to suggest that most of grain filling assimilates are coming from post-anthesis photosynthesis and this is regardless of the moisture level, as evidenced by significantly higher flag leaf $\Delta^{13}C$ values compared to grain $\Delta^{13}C$ under all four moisture levels. If the suggestion is true, it is contrary to the common belief that assimilates for grain filling under drought conditions are exported from stored pre-anthesis assimilates (Blum, 2011a, Xue et al., 2014, Álvaro et al., 2008). If the majority of assimilates were exported from pre-anthesis stages, grain $\Delta^{13}C$ would be expected to be higher or similar to flag leaf $\Delta^{13}C$ measured under WW at GS71 because the assimilates would have been produced in the absence of water stress. In fact, the difference between flag leaf $\Delta^{13}C$ and grain $\Delta^{13}C$ was even greater under severe stress (2.9‰) suggesting less contribution of pre-anthesis assimilates. Flag leaves are believed to be the major contributors to grain filling (Khaliq et al., 2008, Guóth et al., 2009, Santiveri et al., 2004), hence isotope signatures of grain would be expected to closely resemble those in flag leaves. It is however, clear that other leaves and organs contribute to assimilate production but their discrimination levels would still be expected to be in the same range of flag leaves as the growing conditions would have been the same. The difference in $\Delta^{13}C$ between the flag leaves and grain is too wide to suggest major contribution of pre-anthesis assimilates. This, therefore, means that most of the assimilates for grain filling in triticale particularly under SS are probably from other sources like ear photosynthesis. Ear photosynthesis is a reasonable source since empirical evidence has also shown higher tolerance of ear photosynthesis to water stress compared to flag leaf (Tambussi et al., 2005) hence ear photosynthesis would be expected to contribute more to grain filling than flag leaves. Also, transport of assimilates by the phloem from the source (leaves or stems) to the sink (grain) is likely to be hindered under water stress. In the study of Araus et al. (1993), ear $\Delta^{13}C$ closely resembled grain $\Delta^{13}C$ more than flag leaf $\Delta^{13}C$ and in a more recent study to identify contributions of different organs to grain filling in durum wheat, it was also found using $\Delta^{13}C$ that ears contributed more to grain filling than flag leaves (Sanchez-Bragado et al., 2014b, Merah and Monneveux, 2015). The inference of the sources of assimilates are based on single season data of 2014 due to missing grain $\Delta^{13}C$ data for 2013, hence some caution should be given here. However, (Sanchez-Bragado et al., 2014a, Sanchez-Bragado et al., 2014b) arrived at similar conclusions using one season of data. But, if indeed, the assimilates for grain filling under drought are exported from stored assimilates then there is a high ^{13}C fractionation during phloem transport.

Carbon 13 discrimination failed to separate genotypes used in this study (Table 4.5). The studied genotypes were initially chosen for their higher biomass and grain yield and not for their differences in $\Delta^{13}C$ as applied in some studies (Chen et al., 2011, Read et al., 1993). The lack of genotypic differences was not only observed in $\Delta^{13}C$ but also in other parameters. The lack of genotypic differences observed in gas exchanges particularly *Ci/Ca*, stomatal conductance and photosynthetic rate (Table 4.3) indicates that genotypic differences in ^{13}C isotopic discrimination could not be expected. The results also suggest that the genotypes may not have been bred for high WUE (low $\Delta^{13}C$). In agreement with our findings, Araus et al. (1992), one of the few studies to evaluate $\Delta^{13}C$ variation in triticale genotypes, also found no genotypic variation. However, in both studies, only four genotypes were used. Variation in genotypic performance could have been more distinct if genotypes with a more different genetic background would have been included.

4.4.3 Flag leaf gas exchanges

Stomatal conductance (*gs*), transpiration rate (*E*) and photosynthetic rate (*A*) decreased with decreasing moisture level. These results agree with the findings of other authors (Wang et al., 2013, Cabrera-Bosquet et al., 2011). However, reports by Cabrera-Bousquet et al (2009a), suggested that the effect of moisture level particularly on *E* and *gs* also depends on other factors like nitrogen (N) fertilization. In their study they found no influence of soil moisture level on *E* under low N, but only under high N fertilization. $WUE_{intrinsic}$ and WUE_{inst} were affected differently by soil moisture levels. While $WUE_{intrinsic}$ varied with soil moisture level, WUE_{inst} did not (Table 4.2).

When plants encounter water deficit, they respond by lowering *gs* to reduce water loss. The decrease in *gs* eventually results in decrease in *A* (Table 4.2 and Fig. 4.3C). This decline in *A* due to a reduction in *gs* has also been reported by Erice et al. (2011). While soil moisture significantly influenced *A, E* and *gs*, it had no effect on *Ci/Ca* (Table 4.2). The non-responsiveness of *Ci/Ca* to soil moisture is corroborated by Cabrera-Bousquet et al (2009a) but it is also contradicted by Wang et al. (2013), who found *Ci/Ca* to decrease with decreasing soil moisture. As alluded to in the introduction, the reduction in *Ci/Ca* may be due to either a greater *A* at a constant *gs*, a lower *gs* at a constant *A* or changes in both *A* and *gs* (Condon et al., 2004, Farquhar and Richards, 1984). The constant *Ci/Ca* observed in this study is attributed to a parallel decrease in both *A* and *gs* (from WW to SS, Fig. 4.3C) see also Ripullone et al. (2009). Also, changes in *Ci/Ca* can be predicted from the relationship between $\delta^{13}C$ and $\delta^{18}O$ in the dual isotope model (Fig. 4.3).

4.4.4 Relationship of $\Delta^{13}C$ and grain yield, grain yield WUE, intrinsic WUE

Most breeding programmes target for high grain yields or traits that are highly correlated to it. Under water limiting conditions integrated WUE would be the most sought out trait as it indicates higher yield potentials under drought. Several studies with cereals, particularly with wheat have shown positive correlations between grain yield and $\Delta^{13}C$ under drought conditions (Monneveux et al., 2005, Wahbi and Shaaban, 2011). In Australia some wheat cultivars were selected for high biomass and grain yield using $\Delta^{13}C$ as a selection tool (Rebetzke et al., 2002). Accordingly, our results concur with these findings as $\Delta^{13}C$ was positively related to grain yield under SS and MS (Fig. 4.2A and 4.2B). These positive correlations were observed both for flag leaf $\Delta^{13}C$ and grain $\Delta^{13}C$ under SS and MS but were non-significant under MW and WW. However, Condon et al. (2004), suggest the use of leaf $\Delta^{13}C$ measured before anthesis because grain yield is strongly related to grain number more than grain size and grain number is determined before anthesis. In addition, early leaf $\Delta^{13}C$ is said to offer more repeatability and heritability. The lack of a significant relationship between grain yield and $\Delta^{13}C$ under well-watered conditions is congruent with the findings of Monneveux et al. (2005). Under WW, CO_2 supply (*gs)* and CO_2 demand (*A)* will probably be very high such that the effect of high CO_2 demand on *Ci/Ca* is counteracted by high CO_2 supply, resulting in minimal variation in $\Delta^{13}C$, hence the lack of a meaningful relationship between $\Delta^{13}C$ and grain yield. Farquhar et al. (1989) also mentioned the unpredictability of the relationship between $\Delta^{13}C$ and dry matter under well-watered conditions.

The relationship between flag leaf $\Delta^{13}C$ and WUE_{grain} depended on moisture level (Fig. 4.2C). Under SS the relationship was positive (0.49*) and strongly negative (-0.53**) under WW. In comparison, grain $\Delta^{13}C$ showed significant positive correlations to WUE_{grain} under SS and MS only. The observed positive correlation suggests that high yielding genotypes have a higher stomatal conductance (i.e. high discrimination) than low yielding genotypes under dry conditions. This means relatively more CO_2 is fixed under dry conditions when *gs* is high. The negative relationship observed with leaf $\Delta^{13}C$ under WW is common because according to Condon et al. (2004), under well-watered conditions, genotypes with lower $\Delta^{13}C$ tend to grow slower than genotypes with higher $\Delta^{13}C$, resulting in lower grain yield. Some studies (Anyia et al., 2007, Khazaei et al., 2008, Misra et al., 2006, Mohammady et al., 2009, Wahbi and Shaaban, 2011) have also reported negative relationships between $\Delta^{13}C$ and WUE under WW conditions while others (Araus et al., 1998, Araus et al., 2003, Kumar et al., 2011, Yasir et al., 2013) have reported positive correlations in small grain crops. There are, however, more inconsistencies regarding the relationship of $\Delta^{13}C$ with WUE and/or grain yield under WW. Thus, other traits should be used for indirect cultivar selection for higher grain yield under well-watered conditions where $\Delta^{13}C$ fails to show correlation with yield. It is generally difficult to find a stress adaptive trait that gives higher yields under both water stressed and well-watered conditions. Usually higher yields are compromised when selecting under water stressed conditions (Blum, 2011a) as the objective would be to find a crop or cultivar that can survive the drought.

The relationship between $WUE_{intrinsic}$ and $\Delta^{13}C$ (of both leaf and grain) was not significant for four moisture levels separately. The relationship was only significant when analyses were done with data of all four moisture levels combined (Fig. 4.2E and 4.2F). The lack of a significant relationship between $WUE_{intrinsic}$ and $\Delta^{13}C$ under any of the four moisture levels studied shows the independence of this relationship to moisture level. These results are in agreement with the findings of Cabrera-Bosquet et al. (2009b) who also found a negative relationship between $WUE_{intrinsic}$ and $\Delta^{13}C$ when data for all their three moisture levels were combined. However, Monneveux et al. (2006) working with durum wheat, found that $\Delta^{13}C$ was positively related to $WUE_{intrinsic}$ under irrigated conditions and negatively related under rain fed conditions while Chen et al. (2011) working with barley found a negative relationship under both well-watered and water deficit conditions. The same negative relationship between $\Delta^{13}C$ and $WUE_{intrinsic}$ was also observed for the four genotypes (Fig. 4.1). It was also observed (Fig. 4.1) that genotypes tended to vary more in $WUE_{intrinsic}$ under drier conditions (SS and MS) compared to wetter conditions (MW and WW). For triticale cultivar selection purposes, it would be sensible to select genotypes for higher $WUE_{intrinsic}$ under drought conditions. Even though genotypes did not vary that much, Bacchus had the highest $WUE_{intrinsic}$ under SS and also discriminated carbon-13 less than other genotypes (Fig. 4.1). $\Delta^{13}C$ generally reflects seasonal $WUE_{intrinsic,}$ hence measurement of $\Delta^{13}C$ would give an indication of the crop's WUE. Lower $\Delta^{13}C$ values are generally indicative of high WUE. $\Delta^{13}C$ also provides information on the growing conditions, particularly soil moisture conditions where low $\Delta^{13}C$ values are reflective of drier growing conditions.

4.4.5 Test for the dual isotope model

The dual isotope model was tested with field data of an annual crop during two growing seasons. To the knowledge of the authors this is probably the first time the model has been tested under such conditions. The results show that the model is applicable, but is not all encompassing. The model seems to hold only when there is negative correlation between $\delta^{18}O$ and *gs* i.e. when *gs* is the main factor controlling $\delta^{18}O$. Barbour (2007) reports three main sources of variation in $\delta^{18}O$ in plant organic materials, and these include 1) source water isotope composition, 2) evaporative enrichment of leaf water and 3) isotopic exchange between water and organic molecules. However, hardcore evidence of some of these theories is still lacking.

The evaporative enrichment of leaf water, which is probably the main factor affecting $\delta^{18}O$ in our study, has drawn much debate. Sheshshayee et al. (2005) argue that as transpiration increases evaporative enrichment increases, i.e. enrichment should be higher under WW compared to SS. They further provide evidence (Sheshshayee et al., 2010) that $\delta^{18}O$ increases with increasing *gs*. The findings of Sheshshayee et al. agree with the results observed in 2013 (Fig. 4.4A). On the other hand, many other authors (Barbour and Farquhar, 2000, Farquhar and Gan, 2003, Ferrio et al., 2012) argue for a decrease of ^{18}O enrichment with increasing transpiration i.e. a decrease in $\delta^{18}O$ with *gs* as observed in 2014 (Fig. 4.4C). This decrease in ^{18}O enrichment with increase in transpiration rate is attributed to the Peclet effect (Barbour and Farquhar, 2000), a process in which back diffusion of ^{18}O enriched water at the sites of evaporation is opposed by mass flow of ^{18}O depleted water. According to Farquhar et al. (2007), the findings of Sheshshayee et al. (2005) only hold when transpiration is limited by evaporative demand and when it is limited by *gs,* it does not and the Peclet effect comes into play.

Due to both sets of arguments and concurrent observations presented above, the growing conditions of 2013 and 2014 from emergence to the date of leaf sampling were evaluated. It was observed that vapor pressure deficit (VPD), which is a measure of evaporative demand, was significantly lower in 2013 compared to 2014 (Fig S1). This difference in VPD could be a contributing factor in the differences observed in $\delta^{18}O$ in both years. In 2013 the lower VPD (low evaporative demand) could have been the main factor limiting transpiration in triticale while the high VPD observed in 2014 resulted in *gs* limiting transpiration.

Overall, the model correctly predicted that the variation observed in $\Delta^{13}C$ in 2014 was due to a concomitant decrease in both *A* and *gs*. However, in 2013 the model attributes the variation in $\Delta^{13}C$ to an increase in both *A* and *gs*, which could not be supported by observed data. Overall, our data indicate that the model works when $\delta^{18}O$ is controlled by *gs,* and this is also clearly mentioned by the developers of the model (Scheidegger et al., 2000) but fails when $\delta^{18}O$ is controlled by evaporative demand as evidenced by the findings of this study and those of Sheshshayee et al. (2005).

The model also correctly predicted a constant *Ci*, which in this study was reported as a constant *Ci/Ca* ratio, whereby *Ca* is a fixed value. However, the range of *Ci* was wide for all moisture levels ranging from about 100 to as high as 300 ppm (Fig. 4.3B and 3.3D). It is not clear why there was such a wide range in *Ci*, but perhaps, the behaviour could be attributed to the instantaneity of gas exchange measurements or to patchiness (patchy stomatal conductance) (Mott and Buckley, 1998, Mott and Buckley, 2000). Patchiness results from non-uniform stomatal closure particularly in response to water stress and may result in over estimation of *Ci* (Downton et al., 1988).

4.5 Conclusion

In conclusion, the results of this study show that $\Delta^{13}C$ could be useful as a predictor for triticale grain yield in drought prone areas. $\Delta^{13}C$ also offers potential as a proxy of $WUE_{intrinsic}$. With regards to the applicability of the dual isotope model on an annual crop, the results show that the model is applicable, but is not all encompassing. The model holds only when transpiration is limited mainly by *gs* but when transpiration is limited by other factors like evaporative demand, the model does not hold. A comparison of the carbon isotope signatures of flag leaves at GS71 and GS92 to that of the grain at harvest, suggest minimal contribution of both flag leaf photosynthesis and re-mobilized pre-anthesis assimilates to grain filling. Therefore, $\Delta^{13}C$ should be further explored to elucidate the source of carbon assimilates to the grain under different growing conditions.

5 Chapter 5 - Screening cowpea genotypes for high BNF and grain yield under drought conditions

In preparation as:

Lawrence Munjonji, Kingsley K. Ayisi, Geert Haesaert, Pascal Boeckx. Screening the potential of cowpea genotypes for BNF and grain yield under drought conditions.

Abstract

Predicted increase in drought and heat stress challenges legume productivity in many regions. In sub-Saharan Africa (SSA), resource poor farmers are encouraged to incorporate legumes in their farming system to improve soil fertility. As a result, there is a need for continued improvement and identification of drought tolerant legume genotypes for sustained productivity in such farming systems. Cowpea is a commonly grown legume in SSA with a high potential to improve livelihoods. A two-year field study was carried out to identify superior cowpea genotypes with higher biological nitrogen fixation (BNF) and grain yield for improved soil fertility in the smallholder sector. Four cowpea genotypes, selected from a pool of 90 lines were grown under four moisture levels ranging from well-watered (WW) to severe water stress (SS). Grain yield, shoot biomass, nodule mass and BNF using the ^{15}N natural abundance technique were determined. There was a considerable effect of water stress on the measured parameters. Nodule mass and BNF were more sensitive to water stress compared to shoot biomass. Water stress resulted in 80% reduction in nodule biomass between WW and SS conditions. As a result, cowpea grown under SS conditions fixed 57% less N_2 compared to those under WW conditions. In comparison, shoot biomass dropped by 39%. The study also revealed significant genotypic variation in shoot biomass, grain yield and BNF. Genotype TVu4607 fixed the highest amount of nitrogen (71 kg N ha^{-1} under WW and 30 kg N ha^{-1} under SS) and also had the highest shoot biomass across all moisture levels while TVu14632 was the least performing genotype fixing 28 kg N ha^{-1} under WW and 22 kg N ha^{-1} under SS. Interestingly, TVu4607 with all the superiority in BNF and shoot biomass produced the least grain yield. The findings of this study suggest that future water scarcity will significantly affect cowpea productivity, mostly its capacity to fix nitrogen. Consequently, adaptation strategies to improve drought resistance in cowpea are needed, particularly the improvement of BNF under water stress.

5.1 Introduction

Cowpea (*Vigna unguiculata* L. Walp.) is a common grain legume crop grown by smallholder farmers in arid and semi-arid areas of Sub-Saharan Africa. Most arid and semi-arid regions experience frequent droughts and heat stress. Since cowpea is generally tolerant to drought stress due to its ability to maintain high shoot water status under drought compared to other legumes (Matsui and Singh, 2003, Hall, 2012, Rivas et al., 2016), it has been promoted in dry areas. Cowpea also generally fixes more atmospheric nitrogen (N_2) compared to other grain legumes such as soybean (*Glycine max*) and drybean (*Phaseolus vulgaris* L.) (Bado et al., 2006, Nyemba and Dakora, 2010), thus reducing the need for inorganic N fertilizers. As a legume, it does not only contribute to improving soil fertility when residues are retained but it is also a key source of protein, particularly in rural communities. Its grain protein content is reported to be in the ranges of 22-25% (Antova et al., 2014) while the leaves are a good source of vitamins, minerals and proteins (Sebetha et al., 2010). The haulms have a relatively low C:N ratio, hence are used as green manure (Giller, 2001) or as a supplementary source of protein for livestock. In mixed farming systems, cowpea also serves as a mulch, reducing water loss through evaporation, and plays an important role in weed suppression (Wang et al., 2006).

Cowpea is widely grown in West Africa where production is more commercialised because of a vibrant market (Langyintuo et al., 2003). Major world cowpea producers include Nigeria, Niger, Senegal and Ghana (FAO, 2015). In most of these countries, cowpea is produced as a single crop using high-yielding cultivars that can yield as much as 2 t ha^{-1} (Singh and Tarawali, 1997). However, in many other African, particularly southern African countries, cowpea is grown as an intercrop with staple crops like maize. It is commonly grown as a secondary crop often using poor quality seed, saved from the previous harvest (Ayisi, 2000a). It would be beneficial to the farmer if the legume crop could significantly improve the yields of the companion cereal crop in an intercropping system. More often, farmers growing cowpea for grain choose erect or semi-erect types with high harvest index, compromising on biomass yield and N contribution to the soil while those interested in fodder frequently opt for the spreading type (Singh et al., 2003). However, resource constrained smallholder farmers need a good compromise between N contribution to the soil and grain yield to meet both soil fertility and dietary needs.

Due to the high cost and poor market infrastructure in SSA, very few smallholder farmers use chemical fertilizers (Chianu et al., 2011) even though their soils are inherently infertile and hugely deficient in N. Incorporation of legumes in their traditional farming system could go a long way in improving the fertility of their soil. Many farmers already incorporate legumes in their farming system with the most common ones being cowpea, groundnut (*Arachis hypogaea* L.), drybean (*Phaseolus vulgaris* L.), and bambara groundnut (*Vigna subterranean* L. Verdc.) (Nyemba and Dakora, 2010). However, in the wake of climate change and variability, about 60% of the current drybean producing areas in SSA are projected to become unsuitable for bean production before the end of the century (Rippke et al., 2016). Consequently, transformational changes are envisaged if food production is to be maintained or increased. According to Rippke et al. (2016), transformational changes entail a total shift in production areas or shift in farming systems. Nevertheless, before the transformational changes can occur, Rippke et al. (2016) suggest adaptation phases which start with the improvement of crops and their management followed by establishment of appropriate policies and enabling environment before farmers can eventually shift crops or relocate. Meaning that farmers in SSA may be forced to shift to other crops if current crops become unviable. The shift in crops is predicted to be towards more heat and drought tolerant crops as temperature increases and precipitation decreases (Rippke et al., 2016).

Due to transformational changes foreseen for beans (and other crops), it would be expedient for researchers to start searching for alternatives grain legumes, particularly those with drought tolerant characteristics such as cowpea. Drought tolerant legumes like cowpea are particularly important for fertility restoration and crop productivity improvement in SSA. Crop productivity in southern Africa is expected to decrease by between 15 and 50% (Pye-Smith, 2011) resulting from long term nutrient mining and soil carbon decline (Vanlauwe et al., 2014, Vanlauwe et al., 2015). Hence, one of the key measures to address poor crop productivity in the face of climate change is replenishment of soil fertility, coupled with improved water management at the lowest possible cost (Sanchez and Swaminathan, 2005).

Even though cowpea is generally regarded as drought tolerant, its productivity is still governed by moisture availability during critical growth stages like flowering. Thus there is need for continuous improvement, screening and identification of high N_2-

fixing cowpea genotypes that can contribute significant amounts of biological fixed N for increased productivity in the smallholder farming sector while also producing significant grain yield. Thus the main focus of this study was to assess the performance and responsiveness of cowpea genotypes to varying moisture levels, particularly water stress, with the objective of identifying superior genotype(s) with high BNF, and grain yield for improved soil fertility and nutrition in a smallholder farming context. The study also allowed for the assessment of the effect of seasonal variability in rainfall amount and distribution on cowpea productivity.

5.2 Materials and Methods

5.2.1 Plant growth conditions and experimental design

A two-year field research was carried out at the University of Limpopo experimental farm, Syferkuil (23^{o}50’ S, 029^{o}41’ E) in the Limpopo Province, South Africa. In the first year, cowpea was planted on the 2nd of January 2015 while in the second experiment, planting was on the 22nd of December 2015. The average temperature over the growing period was 21°C in both years.

Four genotypes; IT00K-1263, IT99K-1122, TVu14632 and TVu4607 were used in the experiments and abbreviated as IT1, IT2, TV1 and TV2, respectively. The lines were sourced from the International Institute of Tropical Agriculture (IITA) in Nigeria. The lines were selected from a pool of 90 lines that were screened earlier in Limpopo over a period of two years. In the first season, all 90 lines were grown and the best eight performing lines in terms of grain yield and biomass accumulation were selected. In the subsequent season, the eight lines were further screened down to four lines with the superior desired traits. Cowpea was planted in rows, 0.9 m apart with in row spacing of 0.20 m, without inoculation and depended on the resident Bradyrhizobia population for nodulation.

The four genotypes were examined under four moisture levels in a randomized complete block design with split-plots and four replications. The main plot treatment was the irrigation level and the subplot, cowpea lines. The experiment was laid out as shown on Fig. 3.1 with genotype names represented by A, B, C and D on the figure. Moisture level was measured as described under 2.2.1 and the four moisture levels were as follows:

- Well-watered (WW): 25% soil moisture depletion before recharging to field capacity (FC);
- Moderately well-watered (MW): 50% soil moisture depletion before recharging to FC;
- Moderate stress (MS): 75% soil moisture depletion before recharging to 50% of FC;

- Rainfed (SS): the crop depended solely on rainfall. However, in the 2016 season, the plots were irrigated at the beginning to establish the experiments as the rainfall was too low at the start of the season.

Due to unexpected rainfall interferences, the moisture levels could not be maintained throughout the 2016 growing season. However, in the 2015 season, the moisture levels were maintained for most parts of the growing season due to reduced seasonal rainfall and longer drought period experienced. The amounts of water received (irrigation + rainfall) by the crop at flowering and harvesting stages for the different moisture treatments is shown in Table 5.1. The amount of water received by the crop between flowering and harvesting in 2016 was the same as no irrigation was done as there was enough moisture in the soil from rainfall. The small difference observed is attributed to variation in rain gauge recordings most probably due to wind or tilting of the rain gauges.

Table 5.1: Total amount of water received by cowpea genotypes at flowering and harvesting stages in 2015 and 2016

	Irrigation +rainfall (mm)			
	2015		2016	
Irrigation	Flowering	Harvesting	Flowering	Harvesting
WW	181	348	314	419
MW	154	267	314	418
MS	112	173	237	312
SS	73	121	216	299

The soil was classified as a chromic Luvisol (Hypereutric) (WRB, 2014) with available water capacity (AWC) of 80 mm. The AWC was estimated using the hydraulic properties calculator (Saxton and Rawls, 2006). Before the first planting, composite top soil samples (0 to 30 cm) were analysed for pH, organic carbon (OC), electrical conductivity (EC) and macronutrients. The soils had a pH (H_2O) of 8.1, 500 mg N kg^{-1}, 18.7 mg P kg^{-1}, 369 mg K kg^{-1}, 1008 mg Ca kg^{-1}, 710 mg Mg kg^{-1}, 33 mg Na kg^{-1} and OC of 0.5%. The EC measured before the start of the experiments was 82 µS cm^{-1} with an exchangeable sodium percentage (ESP) of 4.3 making it a nonsaline soil.

The soils were then fertilised with single super phosphate (10.5%) at a rate of 35 kg P ha^{-1}. The same rate of P was applied in the subsequent year.

5.2.2 Plant harvesting and sample preparation

Shoot biomass, root biomass, nodules, and leaf area were determined at 50% flowering (i.e. time when half of the plants had flowered). Biomass at 50% flowering was harvested from a 0.9 m^2 area (equivalent to four plants per plot) from all plots on the same day in each year (Fig. 5.1) while grain yield was determined at harvest maturity from four middle rows of 2 m length each (equivalent to 40 plants per plot). Grain yield was harvested on different days for the different treatments as the cowpea reached physiological maturity at different times. The time when the first harvest was done in each year is shown by the big arrows on Fig. 5.1. Shoot biomass was collected by cutting the main stem at about 3 cm above the ground, leaving small stumps. Root biomass was then sampled by first watering the soil around the stumps to avoid root hair and nodule loss before carefully digging out the stumps. The roots were then washed with water on a sieve to remove bound soil particles and collect root biomass, before separating the nodules. Dry weight of the shoot, roots and nodules were determined after separately oven drying the samples at 65^{o}C to constant weight. Leaf area was determined using a leaf area meter (AM 300, Bio Scientific, UK). Four youngest fully grown leaves were sampled from each plot and the leaf area was determined within two hours of harvesting.

5.2.3 Biological N_2 fixation using ^{15}N natural abundance method

Biological nitrogen fixation was determined based on shoot biomass. Oven-dried whole shoot samples were first shredded using Trapp hammer mill TRF 400 (Metalurgica TRAPP LTDA, Brazil) before being milled to a fine powder using a ZM200 mill (Retsch, Germany). The $^{15}N/^{14}N$ isotope ratio (R_{legume}) and total N of the samples were analyzed using an Automated Nitrogen Carbon Analyser – Solid and Liquids (ANCA-SL, SerCon, UK) interfaced with an Isotope Ratio Mass Spectrometer (IRMS) (20-22, SerCon, UK). The isotope composition was reported as $\delta^{15}N$ in per mil (‰) using AIR as international standard ($R_{standard}$) and calculated using the formula below:

$$\delta^{15}N_{Legume} = 1000 \times [(R_{Legume} - R_{Standard})/R_{Standard}] \qquad (5.1)$$

Where $R = {}^{15}N/{}^{14}N$

The percentage of nitrogen derived from air (%Ndfa) was determined using:

$$\%Ndfa = 100 \times (\delta^{15}N_{Refplant} - \delta^{15}N_{Legume}) / (\delta^{15}N_{Refplant} - B) \qquad (5.2)$$

Where the B value is the $\delta^{15}N$ of the whole cowpea grown without any external or soil N (completely depended on atmospheric N) while ‘Refplant’ and ‘Legume’ refer to the shoot of goose grass (*Eleusine coracana*) and shoot of cowpea genotypes grown in the field. The B value used for BNF calculation was 0.04‰ and was taken from literature (Ayisi et al., 2000b). A naturally growing goose grass was used as a reference plant.

Total N yield was calculated from shoot biomass and grain yield as follows:

$$\text{N yield of shoot (kg ha}^{-1}\text{) = Shoot biomass (kg ha}^{-1}\text{) x \%N in shoot} \qquad (5.3)$$

$$\text{N yield of grain (kg ha}^{-1}\text{) = Grain biomass (kg ha}^{-1}\text{) x \%N in grain} \qquad (5.4)$$

Biologically fixed N was calculated as:

$$\text{BNF (kg ha}^{-1}\text{) = Total N yield (kg ha}^{-1}\text{) x \%Ndfa/100} \qquad (5.5)$$

N returned to the soil was then calculated as the difference between total N yield of shoot biomass and total N yield exported via the grains.

5.2.4 Statistical Analyses

The analysis was conducted in accordance with the standard procedure for analysis of variance (ANOVA) of a randomised complete block design in split plot arrangement (Gomez and Gomez, 1984). The ANOVA was performed to calculate the effects of moisture level and genotype on the studied parameters. Where the interaction effect was not significant, data was split according to moisture level and analysed for the effect of genotype at each moisture level (for some parameters). Post Hoc multiple comparisons for observed means was done using Tukey, and different means were denoted by *, ** or *** for significance levels $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$, respectively. Bivariate Pearson Correlation was carried out to assess the relationship between shoot biomass and leaf area as well as between shoot biomass and root biomass. All data were analysed using the SPSS 20 statistical package (SPSS, USA).

5.3 Results

5.3.1 Rainfall distribution and amount

The distribution of daily rainfall received during the two growing seasons is shown on Fig. 5.1. Short arrows show dates of biomass collection at 50% flowering and long arrows show the first grain harvesting dates (2015-black arrows; 2016-gray arrows). Time to physiological maturity varied due to a different moisture level in both years. The total amount of rainfall received from planting to last harvesting was 164 mm in 2015 and 288 mm in 2016. No significant rains were received in the immediate period before and after planting in 2016, hence the crop was irrigated to establish the experiment. However, effective rains were received in January in both years. More rainfall was received in 2016 before biomass sampling at 50% flowering compared to 2015. At the time of biomass collection (short arrows), a total of 73 mm was received in 2015 while at the same stage 218 mm (rainfall + irrigation) had been received in 2016. Furthermore, the rainfall distribution differed between the two seasons. A longer mid-season dry period was experienced in 2015 compared to 2016. Soon after the dry period in 2016, 126 mm of rain was received in ten days when the cowpea was still flowering while much of the late rain received in 2015 came when most of the cowpea had reached physiological maturity.

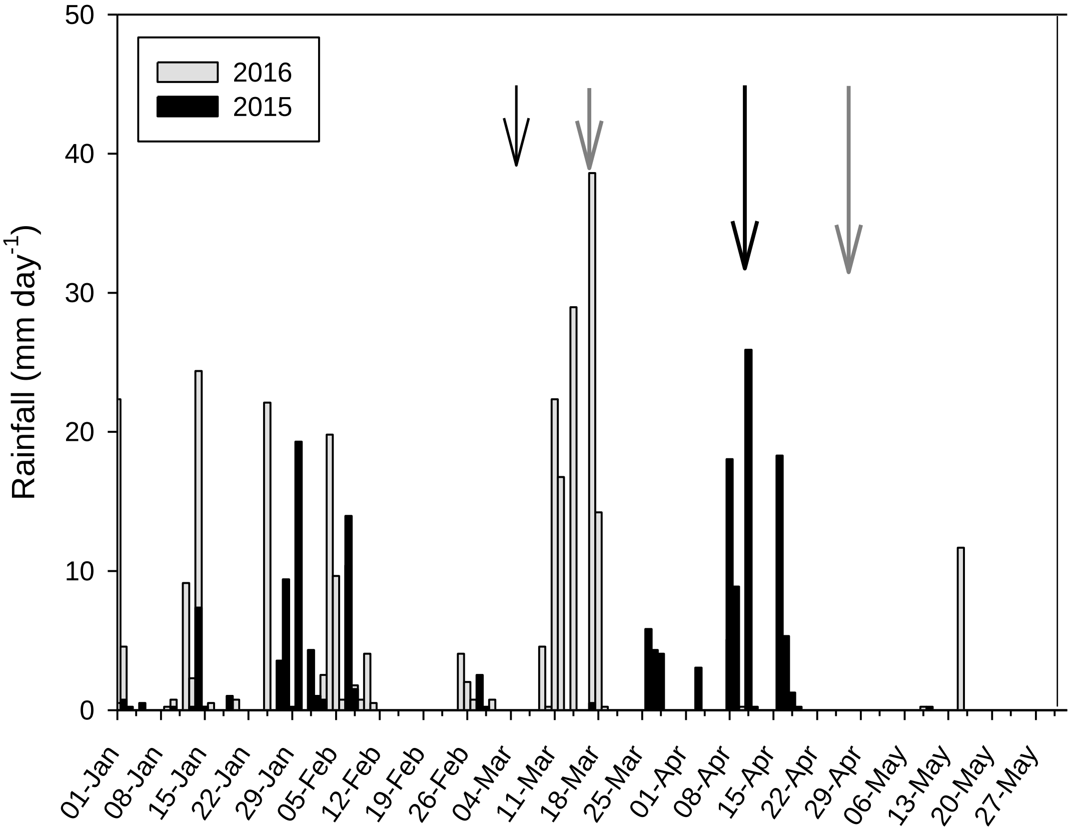


Fig. 5.1: Daily rainfall received during the two growing seasons, starting in January until harvest (2015 - black bars and 2016 - grey bars). Short arrows indicate time of biomass collection at 50% flowering and long arrows indicate first harvesting dates (2015-black arrows; 2016-gray arrows).

5.3.2 Shoot biomass and grain yield

Fig. 5.2 shows the performance of the four genotypes under the four moisture levels. Due to interactions observed between irrigation level and year on both shoot biomass and grain yield, the data are shown separately per year. Both genotype and moisture levels had a significant influence on shoot biomass and grain yield in both years save for 2016 where moisture level did not significantly affect grain yield. No significant interaction was observed between genotype and moisture level. The effect of moisture level was expressed more in 2015 compared to 2016 due to lower rainfall interference in 2015 (Fig. 5.2). The accumulated biomass in 2016 was almost double that attained in 2015, for the different moisture levels. The effect of moisture level presented as averages (solid yellow bars), shows that both shoot biomass and grain yield decreased as moisture level decreased from WW to SS. Individual genotypes responded differently to the moisture levels however the average percentage decrease in shoot biomass of the genotypes from WW to SS was 47% in 2015 and 32% in 2016. TV2 was the best performing genotype in shoot biomass accumulation across all moisture levels while TV1 was the least performing. Under SS in 2015, with only 73 mm of water received, TV2 produced the highest shoot biomass of 2.0 t ha^{-1}. In the following year, genotype IT1 produced the highest shoot biomass (4.3 t ha^{-1}) with the same water stressed treatment, albeit after receiving 216 mm of water

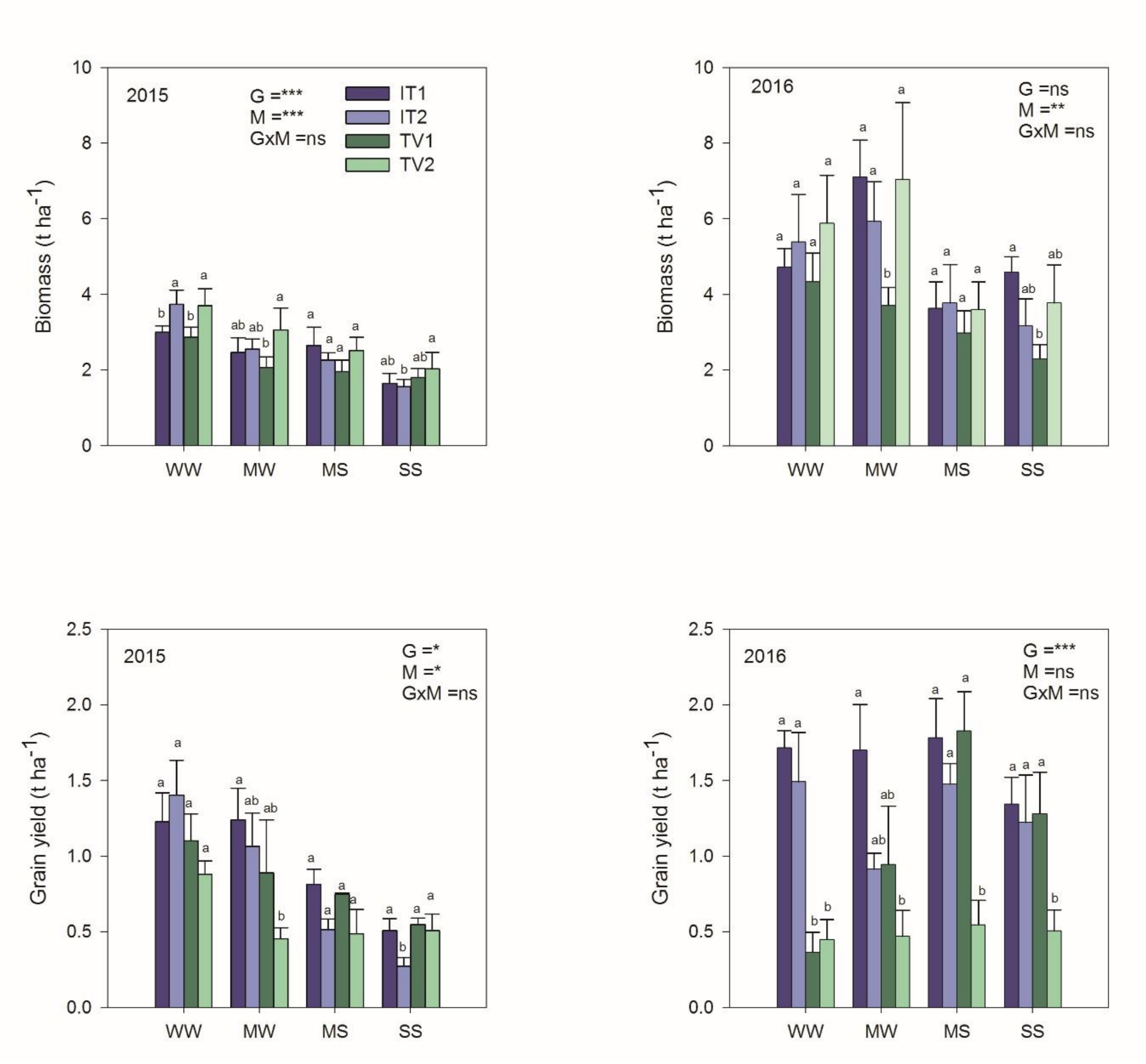


Fig. 5.2: Dry biomass of cowpea lines (IT1, IT2, TV1, TV2) at 50% flowering (top figures) and grain yield (bottom figures) as affected by soil moisture and genotype. Letters show differences among the genotypes at each moisture level (different letters represent significant differences).

Due to the observed general decrease in grain yield with decreasing moisture level, the average grain yield of the genotypes was found to have dropped by 60% from 1.15 t ha^{-1} under WW to 0.46 t ha^{-1} under SS in the first season (2015). In the second season where more rainfall was received during flowering, grain yield performances seemed to interact with moisture level though not statistically significant. Genotype TV1, yielded higher under water limited conditions compared to under well-watered conditions while the grain yield of TV2 was rather low (less than 0.5 t ha^{-1}) but stable throughout. On the other hand, IT1 performed well; yielding more than 1.5 t ha^{-1} of grain under WW, MW and MS.

The difference in grain yield performance of the genotypes was more prominent in 2016 where total rainfall received during the growing period was almost twice that received in 2015. In 2016, the IT genotypes (IT1 and IT2) produced as much as three times higher grain yield compared to the TV genotypes (TV1 and TV2) under the WW. Grain yield performance of genotype TV2 did not correspond to its shoot biomass accumulation as it had the lowest grain yield under almost all moisture levels. Its average grain yield was mostly below 0.5 t ha^{-1} in both seasons except under WW in 2015 where it yielded above 0.5 t ha^{-1}. On the other hand, genotype IT1 consistently produced higher grain yield in both seasons. In 2016, averaged grain yield across all moisture levels was above 1.60 t ha^{-1}, whilst in 2015 it averaged 1.20 t ha^{-1} for WW and MW, 0.81 t ha^{-1} under MS and 0.51 t ha^{-1} under SS. IT2 shoot biomass and grain yield were generally the most responsive to soil moisture level, particularly in 2015 where the moisture levels were less affected by rain. Grain yield of IT2 dropped sharply from WW to SS, losing 80% of its potential yield while on average the other lines only lost 50% of their grain yield.

When comparing the grain yields under rain fed (SS) treatment alone, it was observed that average grain yield in 2016 (1.09 t ha^{-1}) was 58% higher than that in 2015 (0.46 t ha^{-1}). This difference reflects the seasonal variability that can between seasons.

5.3.3 BNF and N returned to the soil

Both soil moisture levels and genotype significantly influenced BNF (Fig. 5.3). Biological nitrogen fixation generally decreased in response to decreasing moisture level. In the 2015 growing season, BNF varied across all moisture levels while in 2016, differences were only observed between well-watered conditions (WW and MW) and water-limited conditions (MS and SS). On average BNF dropped by 57% under SS compared to WW in 2015. In 2016, the reduction in BNF between WW and SS was 46%. As observed in Fig. 5.3, genotypes responded differently in terms of BNF under the four moisture levels. Notable, was the stability of BNF of TV1 over the two years averaging about 25 kg ha^{-1} and was also the least of all genotypes. Prominent also was the high amounts of N fixed by TV2 over the two seasons as well. Genotypes IT2 and TV2 were the largest N_2 fixers under WW in 2015, fixing about 60 kg N ha^{-1}. Except for TV1, BNF in 2015 was severely reduced under SS compared to WW by a magnitude of up to two thirds. In the second season (2016), only TV2 maintained a relatively high level of N_2 fixation under WW. Water stress again significantly reduced BNF to the same low level of about 20 kg N ha^{-1}. The sensitivity of BNF to moisture level did not only vary among the genotypes but it also responded to seasonal variation in the amount of water received. In the first season and as observed with grain yield, BNF of genotype IT2 was the most sensitive to water stress. Under SS, IT2 only fixed a quarter of the amount it fixed under WW. On the other hand, TV1 was the least sensitive to moisture level, with a constant N_2 fixation rate over both years. Overall, BNF did not significantly vary over both years despite the huge differences observed in shoot biomass.

The amount of N returned to the soil varied with year as evidenced by more addition of N to the soil in 2016 compared to 2015. Soil moisture did not significantly influence the amount of N returned to the soil in 2015 but had a significant influence in 2016 ($p < 0.01$). On the other hand, genotypes significantly varied mainly because of the exceptional performance of TV2. In 2016, more N was returned to the soil under well-watered conditions (WW and MW) relative to water stressed conditions (MS and SS). In both years and as observed with BNF, genotype TV2 remarkably contributed more N to the soil than any other genotype under all four moisture levels while on average TV1 contributed least. When the amount of fixed N (BNF) was compared to the total N returned to the soil, it was found that N_2 fixation was more sensitive to soil moisture level. In 2015, there was a 22% reduction in the amount of N returned to the soil between WW and SS compared to the 57% reduction observed with BNF, while in 2016 the reduction percentages were 35% for soil N addition and 46% for BNF.

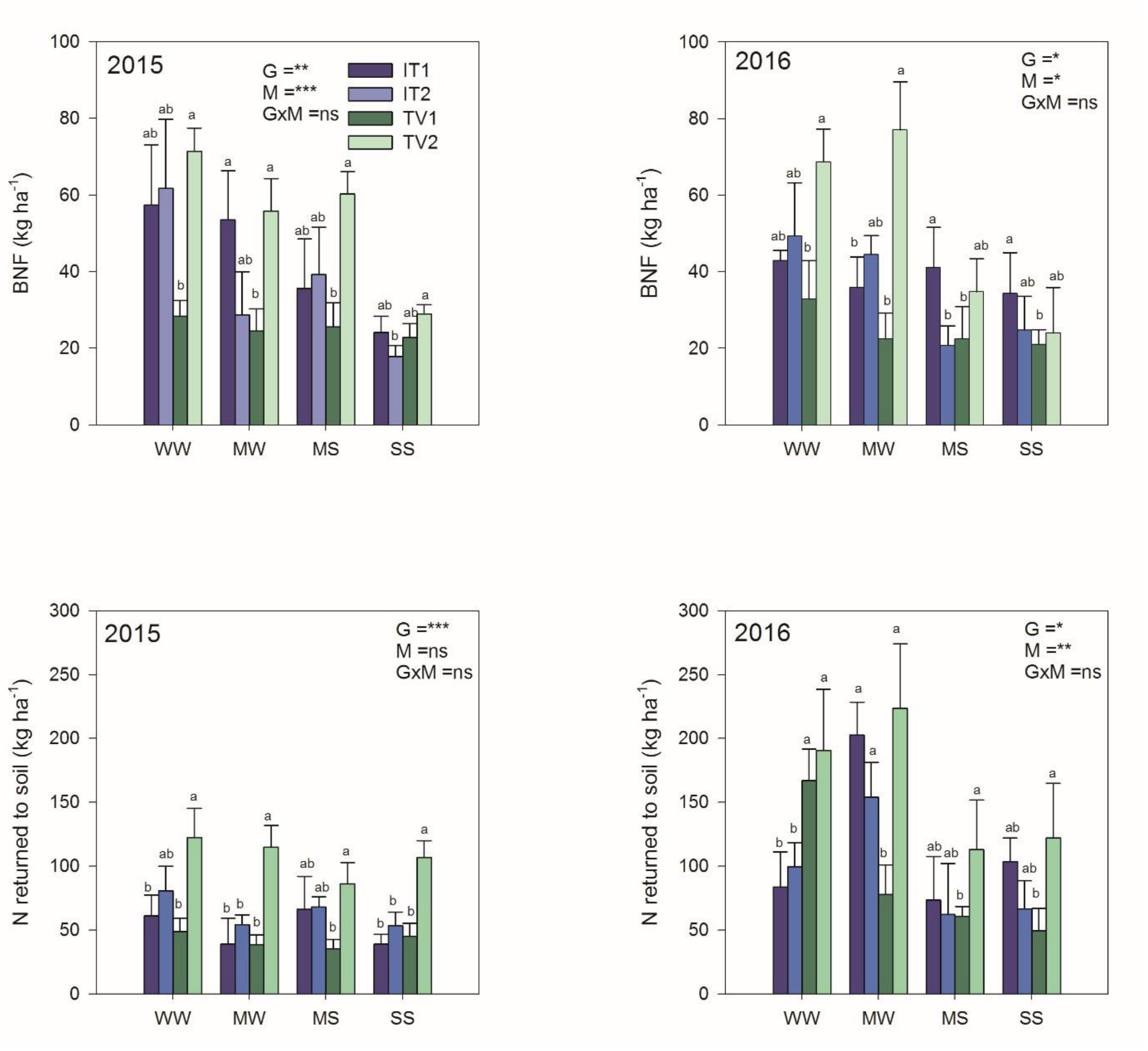


Fig. 5.3: BNF and total N yield as influenced by soil moisture level. Letters show differences among the genotypes at each moisture level (different letters represent significant differences).

5.3.4 Nodule biomass

The nodule biomass per plant also varied among the genotypes in both seasons (Fig. 5.4). In 2015, all genotypes significantly differed ($p < 0.01$) in nodule biomass. The nodule biomasses were ranked as follows: TV2 > IT1 > IT2 > TV1, with biomass ranging from 119 (genotype TV1) to 288 mg plant^{-1} (genotype TV2). Genotype TV1 with highest nodule mass also fixed the most N in 2015 as shown in Fig. 5.3. In the 2016 season, the IT genotypes (IT1 and IT2) did not show differences in nodule biomass but were significantly lower than the TV genotypes (TV1 and TV2). Nodule biomass in 2016 ranged from 100 (genotype IT2) to 216 mg plant^{-1} (genotype TV1). Surprisingly, TV1 which had the lowest nodule biomass in 2015, had the highest in

2016 mainly because of an increase in nodule numbers. Nodule biomass was also significantly influenced by moisture level in both years (Fig. 5.4, right). In the first season, nodule biomass gradually decreased with moisture level, with all moisture levels yielding different nodule mass. In the second season, nodule biomass was not different between WW and MW but was significantly higher than under MS and SS. In a drier season (2015, Table 5.1) severe water stress reduced nodule biomass by 80% from WW to SS while in a relatively wetter year the reduction was only 56%.

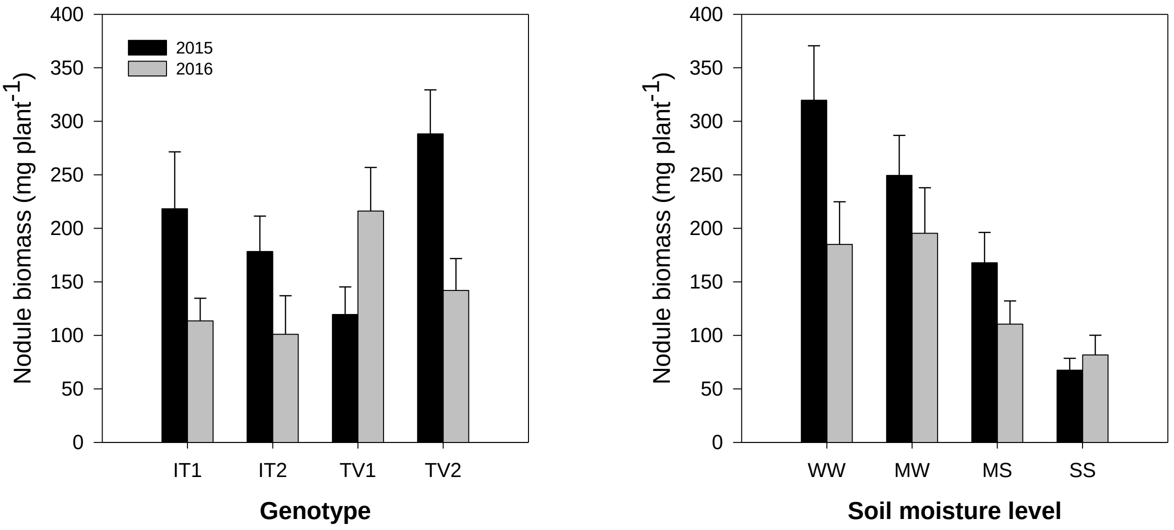


Fig. 5.4: Nodule biomass as affected by genotype (left) and moisture level (right) throughout the two years. Small letters on top of bars show differences between treatments in 2015 while capital letters show differences in 2016.

5.3.5 Leaf area and root biomass

Leaf area and root biomass varied considerably among genotypes (Fig. 5.5). In both seasons, genotype TV1 produced the smallest leaves among the four genotypes. Leaves of genotypes IT1 and TV2 were generally larger than those of IT2 and TV1 in 2015. Figure 5.5 also shows the genotypic variation observed in root biomass, over the two years. In both years, genotype TV2 produced the highest root biomass. The box plots (Fig. 5.5) show data for all moisture levels combined hence it was observed that the lowest root biomass observed under SS for genotype TV2 was greater than the highest root biomass of genotype TV1 measured under WW. The same was also observed with leaf area. In the first growing season (2015), genotypes IT2 and TV1 did not differ in root biomass while in the second season (2016) all genotypes differed in root biomass. Due to the higher amount of water received in 2016, the genotypes produced significantly higher root biomass ($P < 0.001$) in the second season compared to the first season. A strong positive relationship ($r^2 = 0.41$, $P < 0.01$) was also observed between leaf area and root biomass such that genotypes with larger leaves had higher root biomass. Figure 5.6 shows that root biomass and leaf area were all significant and positively related to shoot biomass ($r^2 = 0.60$ in 2015, $r^2 = 0.55$ in 2016; $P < 0.01$). However, the relationship between grain yield and both root biomass and leaf area was nonsignificant.

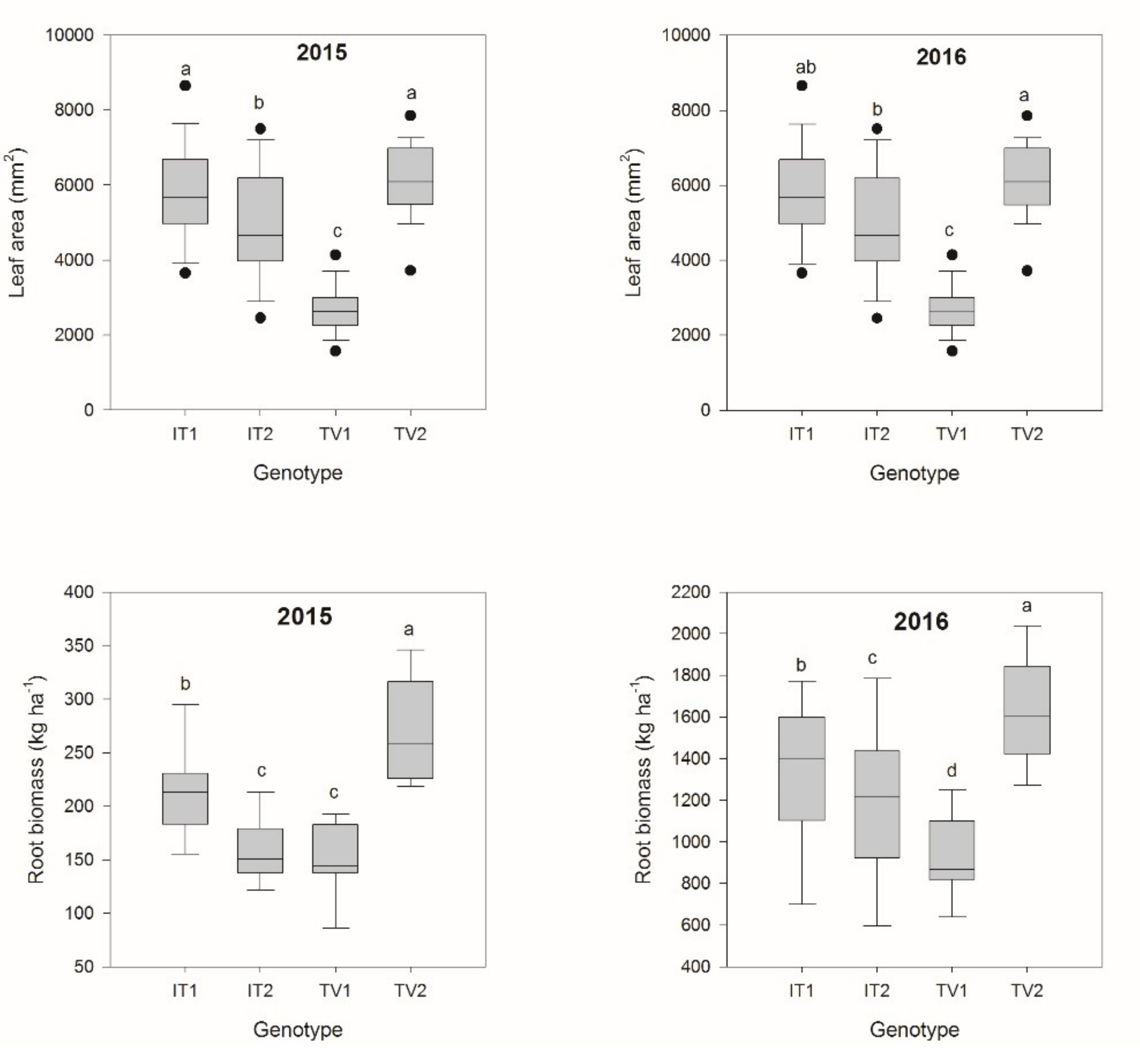


Fig. 5.5: Box plots of leaf area and root biomass of the four genotypes. Each box plot shows data for all moisture levels for each genotype. Letters show differences among the genotypes (different letters represent significant differences).

5.3.6 Root/Shoot ratio

The effect of moisture levels and genotypes on the root/shoot over the two seasons is shown in Fig. 5.7. For genotypes, the results represent an average of all the moisture level, while for moisture levels results are averages of all genotypes at each moisture level. The root/shoot ratio significantly differed among the genotypes where genotypes IT1 and TV2 had higher ratios compared to IT2 and TV1 in 2015. In 2016, only TV2 had a significantly higher root/shoot ratio compared to the other three genotypes. Moisture level also significantly influenced root/shoot ratio. The ratio was highest under SS and lowest under WW. This trend was observed over the two growing seasons. Also, the year variability in root/shoot ratio was observed whereby significantly higher ratios where observed in 2016 compared to 2015.

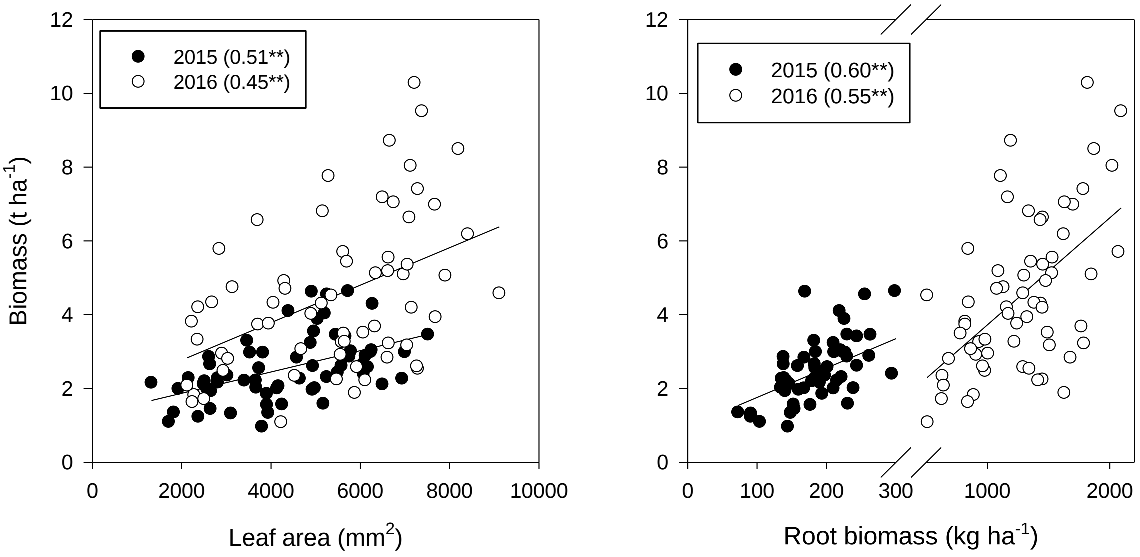


Fig. 5.6: Relationships between above ground biomass and leaf area (left), above ground biomass and root biomass (right) for the two seasons.

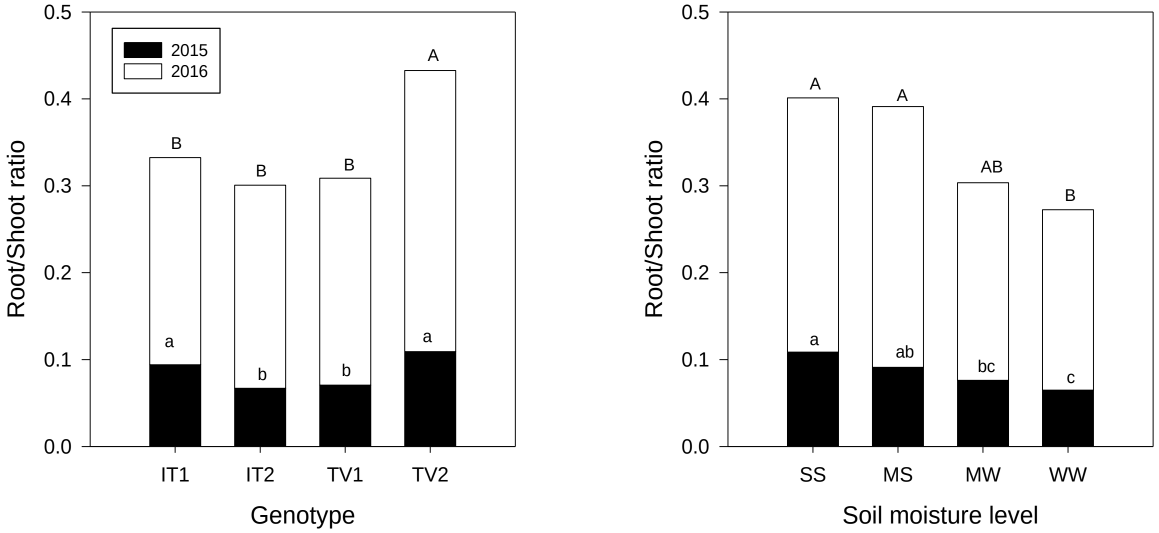


Fig. 5.7: Stacked bar graphs showing averaged values of root/shoot ratios per genotype (left) and as affected by moisture level (right). Small letters on top of bars show differences between treatments in 2015 while capital letters show differences in 2016.

5.4 Discussion

The results of this study clearly indicate the strong influence of soil moisture on cowpea biomass production, BNF and grain yield. The results highlight the considerable variation existing among cowpea genotypes as well as on rainfall distribution and amount between growing seasons. Due to the differences in rainfall distribution (Fig. 5.1), the influence of moisture was hugely subdued in the second season compared to the first season. The evidence of which is shown by the lack of differences in grain yield observed between WW and SS in 2016. The resurgence of rains in 2016, occurring at flowering stage, promoted growth, counteracting the effect of mild stress experienced earlier. The flowering stage is considered to be the most sensitive (Daryanto et al., 2015) and yield determining growth stage, hence there was less variation in grain yield among the moisture levels in 2016 due to post flowering moisture availability. Similar post flowering growth has also been reported in other legumes (Zakeri and Bueckert, 2015). However, a difference in genotypic performance was largely observed and maintained over the two years despite the interference of rain on moisture levels in 2016.

5.4.1 Grain yield, shoot and root biomass

Water stress significantly decreased grain yield, shoot and root biomass, nodule biomass as well as leaf area. These results were mostly observed in the first season when a prolonged drought and generally low rainfall was experienced. The results show that cowpea is also responsive to soil moisture levels like other crops as differences observed in moisture levels were accordingly reflected in grain yield and biomass, agreement with the findings of other studies (Bastos et al., 2011). From the results also show that even crops like cowpea, which are generally regarded as drought tolerant (Dadson et al., 2005, Sinclair et al., 2015), will also succumb to the effects of climate change and variability, particularly decreases in soil moisture and variability of water availability over the growing season. According to Ray et al. (2015), climate variability accounts for a third of observed grain yield variability. In some instances, climate variability can cause grain yield variability of up to 60%, mostly due to precipitation and/or temperature fluctuations.

Low biomass and grain yield may continue to be experienced in the Sahel, southern Africa and parts of eastern Africa if no adaptation measures are implemented as precipitation continues to decrease due to climate change (Kotir, 2011). In this study, a 60% reduction in grain yield was observed in the first season between WW and SS, scenarios which represent a wetter and a drier season, respectively. The 121 mm of water received by cowpea in 2015 under SS (Table 5.1) represents about a 25% of the mean annual rainfall (ca. 500 mm) of the area while the 299 mm received in 2016, represents about 60%. In addition, the inter-seasonal variability in rainfall as represented by the rain fed soil moisture regimes (SS) in the two seasons, accounted for a 58% difference in grain yield (0.46 t ha^{-1} in 2015 and 1.09 t ha^{-1} in 2016). Therefore, rainfall amount and distribution significantly affects cowpea grain yield levels, highlighting the importance of continuous evaluation and improvement of genotypes. The low grain yield observed under rain fed conditions, i.e. SS, indicates that cowpea is already yielding below potential in many dry areas. With climate models, predicting further decreases in precipitation particularly in dry areas, cowpea yields will therefore continue to decrease if drought tolerance and crop management is not improved. Considering that cowpea is more drought tolerant than other commonly grown legumes like groundnut and beans, the results of this study may suggest worse off scenarios for groundnut and beans. In fact, modelled data already show significant future losses in bean productive capacity of many countries not only in SSA (Rippke et al., 2016) but also in the Americas (Bouroncle et al., 2016).

Variation in genotypic sensitivity to water stress was clearly revealed by the grain yield performances of IT1 and TV2 in the first season. Whilst water stress markedly reduced grain yields of IT1 by 80%, the grain yield of TV2 was largely constant. Other genotypes also showed susceptibility to water stress by losing about 50% of their grain yield under SS compared to under WW. Similar reduction in cowpea grain yield resulting from water stress has been reported in other studies (Bastos et al., 2011, Dadson et al., 2005). The overall range of grain yield observed in this study are comparable to those found by Ayisi (2000a) at the same experimental farm. However, the highest grain yield levels realised in this study (± 1.70 t ha^{-1}) is lower than observed in other studies (Singh and Tarawali, 1997, Makoi et al., 2009, Gnahoua et al., 2016). Nevertheless, the average yield of 0.46 t ha^{-1} observed under very dry condition (SS in 2015) is comparable to reported average cowpea yield in Africa (0.43 t ha^{-1}) for 2013 (FAO, 2015). Due to the rather low cowpea utilisation in Southern Africa, compared to other regions like West Africa, this study also evaluated for high biomass yielding genotypes, which could increase soil fertility as most smallholder farmers in the region practice intercropping. High biomass yielding genotypes contribute more N to the soil as observed with genotype TV2 (Fig. 5.3) but usually compromise on grain yield.

As observed in Fig. 5.2, genotypes that produced higher shoot biomass did not necessarily result in higher grain yield. This was particularly true for genotype TV2. Thus, TV2 would be more suitable as a forage legume and/or as green manure as it constantly produced higher shoot biomass but performed dismally in grain yield. On the other hand, genotypes IT1 and IT2 had a good balance of biomass accumulation and grain yield, with genotype IT1 being slightly superior to IT2 in grain yield particularly under SS. Genotype TV1's performance in grain yield was modest and in between TV2 and, IT1 and IT2. For the subsistence farmers in dry regions of SSA, particularly drybean farmers as projected by Rippke et al. (2016) will be required to transform in the near future, genotype IT1 provides a better alternative. The genotype offers a good balance between grain yield and shoot biomass accumulation combined with the ability to produce competitive grain yield under both dry and wet conditions. In addition, the cultivar was very competitive not only in BNF, but also in other water stress tolerance related traits, like root biomass and root/shoot ratio.

Root biomass and leaf area were positive and strongly related to shoot biomass accumulation (Fig. 5.6). Genotype TV2 for instance with larger leaves and higher root biomass (TV2) produced higher shoot biomass. This could be due to improved light interception by the leaves (Weraduwage et al., 2015, Zamski and Schaffer, 1996). Likewise, genotypes with low leaf area and root biomass (TV1) produced low shoot biomass. Reduced leaf area is normally associated with improved tolerance to water stress due to reduced water loss (Agbicodo et al., 2009). This was however not reflected in genotype TV1's performance but it was observed that cowpea growing under SS had significantly lowered leaf area, in response to reduce water loss. According to Blum (2011b), abscisic acid is the hormone responsible for reduced leaf area under water stress. The same hormone also promotes root growth and root hydraulic conductivity under water stress leading to increased root/shoot ratio. Indeed, in this study higher root/shoot ratio was observed under SS and decreased with increasing moisture level. In addition, genotypes IT1 and TV2 which had relatively higher shoot biomasses under SS in 2015 and 2016, respectively, had significantly higher root/shoot ratios in the respective years.

5.4.2 BNF and N returned to the soil

The results of this study showed that, N_2 fixation by cowpea is quite sensitive to soil moisture fluctuations. On average, IT1, IT2 and TV2 fixed about 57% less N under water limited conditions (SS) compared to WW. While the amount of N fixed by TV1 was largely constant over the two years. Similar reduction in N_2 fixation have been reported in previous studies (Devi et al., 2010, Sheokand et al., 2012, Sinclair et al., 2015). Some of the reasons for such a decline in N_2 fixation under water deficit were attributed to decreased *Bradyrhizobia* populations and restricted nodule formation (Hungria and Vargas, 2000). It was indeed observed in this study that water stress, particularly under SS, significantly reduced nodule biomass. In the 2015 season, when moisture levels were well expressed, nodule biomass was reduced by 80% from WW to SS. Root growth was also restricted under SS, which could have resulted in reduced sites of attachment for the rhizobia due to reduced root hair formation (Hungria and Vargas, 2000). BNF was also found to be more sensitive to water stress than biomass. BNF has also been reported to be more sensitive to water stress than transpiration, photosynthesis and leaf growth (Giller, 2001, Serraj et al., 1999). Interestingly, resurgence of rain in 2016 did not influence BNF as much as it did for grain yield. BNF still showed differences between well-watered (WW and MW) and water-limited conditions suggesting sensitivity of BNF to water stress during the early growth stages.

Cowpea growing under SS fixed low N_2 but accumulated high N in the shoot. Shoot N content was significantly higher under SS compared to the other moisture levels (results not shown). The %Ndfa which was in the range of 25 to 34%, indicated that the studied genotypes derived most N from soil. This phenomenon is not uncommon as other studies have also reported such low levels of N_2 fixation in cowpea (Ayisi et al., 2000b, Belane and Dakora, 2009) and also in some groundnut genotypes (Mokgehle et al., 2014). Cowpea genotypes do have the capacity to fix up to 90% of their N requirement but some genotypes have a very low N_2 fixing rate (as low as 15%) (Peoples et al., 2009). However, Mokgehle et al. (2014) regard the ability of a legume to take up substantial amounts of soil N and yet contribute large amounts of fixed N as a desirable trait for agriculture and for ecosystem functioning. This ability was effectively displayed by TV2 and could be a more desirable trait on conditions where there are substantial amounts of inorganic N in the soil.

The amount of N returned to the soil via shoot residues is the amount of N that contributes to soil fertility after subtracting N exported by the grains. There were no differences in the average amount of N returned to the soil among moisture levels. This can be attributed to the parallel decrease in biomass and grain yield, for example, under SS, even though less biomass was produced, there was also very little N exported through grains. However, the amount of N returned to the soil by the genotypes varied due to the huge amount returned by TV2. The average N returned to the soil for all genotypes ranged from 44 to 64 kg N ha^{-1} but TV2's contribution alone ranged from 86 to 122 kg N ha^{-1} showing its superiority over the other genotypes. The variation could be explained by the genotypic differences observed in shoot biomass accumulation as well as in the differences observed in the shoot N concentration. Shoot biomass is believed to be a controlling factor on BNF as organic substrates from photosynthesis supply energy to the *Rhizobia* for N_2 fixation, and hence growth (Anglade et al., 2015). The importance of biomass to N_2 fixation has also been explored in other studies (Unkovich et al., 2010). In this study it was observed that, genotype TV2 which fixed more N across all moisture levels in 2015 also produced more shoot biomass.

Several reasons were identified as possible causes for the relatively lower N_2 fixation by cowpea in this study. The first cause was attributed to accessing residual N from a previously fertilised triticale crop. The presence of bioavailable inorganic N (NH_4^+ and

NO_3^-) in soils can down-regulate N_2 fixation by legumes (Menneer et al., 2003, Szpak et al., 2014). Findings in this study showed that cowpea derived quite a significant amount of N from the soil. The second reason could be attributed to the alkalinity of the soil as soil pH (in water) over the two seasons was averaging 8.1 and the electrical conductivity (EC), measured before the experiments were established, was 82 µS cm^{-1}. The optimum pH for rhizobia growth is considered to be between 6 and 7 (Hungria and Vargas, 2000). The effect of soil salinity on BNF is also well documented (Zahran, 1999). Soil salinity is a major limitation to crop production in arid and semi-arid regions, and is known to reduce nodulation by inhibiting early stages of the symbiosis (Zahran, 1991). However, the amount of N_2 fixed by the genotypes particularly by TV2 suggests a good tolerance to soil salinity considering the EC and the high pH. Lastly, the lower N_2 fixation may just have been a result of less efficient native rhizobia (Thuita et al., 2012) since the cowpea seeds were not inoculated before planting. de Freitas et al. (2012) also reported lower N_2 fixation rates for cowpeas grown without inoculation compared to inoculated ones. In contrast, Giller (2001) reports that cowpea rarely responds to inoculation due to its promiscuity.

It might be desirable for cowpea cultivars grown for fertility improvement, to produce high biomass with a lower harvest index as they would contribute more N to the soil than is exported through grains. Such a characteristic has been shown by genotype TV2, which returned more N to the soil mainly because it exported less N through grains. In some studies, e.g. de Freitas et al. (2012), cowpea had high %Ndfa (more than 40%) but the amount of N fixed was low (15.7 kg ha^{-1} for non-inoculated cowpea). In comparison, this study showed that cowpea derived only a third of its N requirement from air, with cowpea under SS deriving a quarter of its N requirement from air but the amount of N fixed was relatively higher (above 20 kg N ha^{-1} under SS) while cowpea under WW derived up to 60 kg N ha^{-1}. This shows that while it is important for a legume to have a high %Ndfa, it is equally important to consider the ultimate amount of N fixed and returned to the soil. The BNF observed in the current study could significantly improve if the genotypes are grown on N depleted soils.

5.5 Conclusion

In conclusion, the study showed that cowpea genotypes varied considerably in shoot biomass production, BNF and in grain yield. BNF was found to be more sensitive to water stress compared to biomass accumulation. The study also showed that biomass accumulation in cowpea was strongly related to leaf area as genotypes with a larger leaf area accumulated more biomass than genotypes with smaller leaves, irrespective of the moisture level. Genotypes with larger leaf area, produced more biomass and fixed more N_2, hence, are important for soil fertility improvement. Overall, all the genotypes studied showed good tolerance to drought and saline soil conditions, and thus they have great potential for improving soil fertility in the (semi) arid regions of SSA. Genotype IT1 (IT00K-1263), showed the best balance of biomass accumulation, BNF and grain yield. Genotype IT2 (IT99K-1122) also revealed superior biomass and BNF tendencies, but its grain yield was more sensitive to water stress than IT1. Genotype TV1 (TVu14632) proved to be the least suitable genotype for (semi-)dry regions as it was outperformed by other genotypes for most of the studied traits. Genotype TV2 (TVu4607), was the least performing genotype in terms of grain yield but proved to be the leading genotype for improving soil fertility through BNF and contribution to soil N pool, compared to the other genotypes. Finally, given the fact that, the commonly grown legume (drybean – *Phaseolus vulgaris*), is projected to become unsuitable in 60% of the current growing areas in Sub-Saharan Africa due to climate shifts (Rippke et al., 2016), it is critical that more drought tolerant grain legumes such as cowpea are promoted in such areas. Bulgarian farmers in areas experiencing increased occurrences of drought and heat stress are already substituting their main legume (*Phaseolus vulgaris*) with drought tolerant cowpea (Antova et al., 2014).

6 Chapter 6 - Stomatal behaviour of cowpea genotypes grown under varying moisture levels

In preparation as:

Lawrence Munjonji, Kingsley K. Ayisi, Geert Haesaert, Pascal Boeckx. Stomatal behaviour of cowpea genotypes grown under varying moisture levels.

Abstract

Low water availability is one of the major limitations to growth and productivity of crops world-wide. Plants lose most of their water through the stomata thus, the stomata play an important role in controlling transpiration and photosynthesis. One of the objectives of this study was, therefore, to assess the behaviour of stomata of cowpea grown under well-watered and water stressed conditions. Four cowpea genotypes were grown under four different moisture levels under hot semi-arid conditions in South Africa. Stomatal conductance was then measured at 47, 54, 70 and 77 days after planting (DAP). The results showed that stomatal conductance was significantly influenced by genotypes and moisture levels. Genotypes varied in stomatal conductance early in the growth stages, that is, at 47 and 54 DAP but no significant differences were observed at 70 and 77 DAP. Genotype TVu4607 had significantly higher stomatal conductance under severe stress conditions at both 47 and 54 DAP. Moisture level on the other hand, did not influence stomatal conductance at 47 and 77 DAP but strongly influenced the stomatal conductance at 54 and 70 DAP. Higher stomatal conductance was observed under well water-watered conditions and significantly decreased with decreasing moisture level. The correlation between biomass at flowering and stomatal conductance at 47 DAP was only positive and significant under severe stress and not under well-watered conditions. In conclusion, the results showed that cowpea genotypes respond differently to water stress and that they differ more at the early growth stages. In addition, the study showed that cowpea genotypes with higher stomatal conductance early in their growth stage, yield more biomass. These findings could be useful in adapting cowpea to drought conditions.

6.1 Introduction

Water use efficiency and productivity of plants depend on the stomatal control of CO_2 for photosynthesis and transpiration. The compromise between photosynthesis and transpiration depends on the stomatal response to environmental conditions (Lawson and Blatt, 2014). According to Hetherington and Woodward (2003), stomata respond to a broad spectrum of signals and timescales, that is, from intercellular signals to global climate change signals; and from minutes to millennia. As a result, stomata control global fluxes of CO_2 and water, with an estimated 440 x 10^{12} kg of CO_2 and 32 x 10^{15} kg of water vapour passing through stomata of leaves every year. Decrease in stomatal conductance in response to climate change has recently been reported. Increased CO_2 concentrations are expected to increase CO_2 assimilation while reducing water loss (Deryng et al., 2016, Keenan et al., 2013) thus improving WUE. However, the benefits of future elevated CO_2 concentrations will not be realised under intense drought conditions (Gray et al., 2016). Nonetheless, the role of stomata in controlling crop productivity and global fluxes of water and CO_2 cannot be underestimated. Therefore, understanding the behaviour of the stomata in legume crops like cowpea will provide a basis for future manipulation of this attribute to improve crop productivity particularly in drought prone areas.

The occurrence of drought periods in semi-arid areas where cowpea is commonly grown is expected to increase (Lobell et al., 2008) and thus will negatively affect cowpea productivity. To maintain high levels of crop productivity in such environments, there is a need to develop drought tolerant cultivars and water use efficient crops (Barnabás et al., 2008). The development and improvement of drought tolerance in cowpea is only possible if there is a better understanding of its physiological responses to limited water supply. One of the primary physiological responses with a significant role in drought tolerance is stomatal conductance. Stomatal conductance is also believed to be a reliable integrative indicator of water stress (Ninou et al., 2012) even though patchy stomatal conductance could present challenges (Mott and Buckley, 1998, Mott and Buckley, 2000). The opening and closing of the stomata which is mainly affected by water availability (Farooq et al., 2009), has an influence not only on the amount of CO_2 that diffuses into the leaf for photosynthesis but also on the amount of water lost through the leaf (Farquhar and Sharkey, 1982). This compromise between water loss and carbon gain determines the water use efficiency (WUE) of the crop. WUE is strongly related to drought tolerance (Hall, 2012) and can be measured as a ratio of biomass accumulation to the amount of water transpired/used. Alternatively, WUE can be measured at leaf level as the ratio of photosynthetic rate to stomatal conductance (Condon et al., 2002), commonly referred to as intrinsic WUE ($WUE_{intrinsic}$). The integration of $WUE_{intrinsic}$ over time can be estimated through carbon isotope composition (Tambussi et al., 2007a).

Carbon isotope composition of leaves provides an integrated measure of photosynthetic rate and stomatal conductance overtime (Ninou et al., 2012). Carbon isotope composition has also been shown to be related to WUE (Farquhar and Richards, 1984). During CO_2 uptake plants tend to discriminate against CO_2 that contains the heavier (^{13}C) isotope of carbon, the magnitude of which depends on the CO_2 partial pressure in the intercellular spaces. Well established theories show that lower intercellular CO_2 concentration results in reduced discrimination of ^{13}C-CO_2. The theories further show that low intercellular CO_2 concentration may result from a reduced stomatal conductance, an increased photosynthetic rate or a concomitant decrease in both (Condon et al., 2004). Hence, a greater WUE is shown as lower discrimination against ^{13}C-CO_2 (Bloch et al., 2006). Due to the relationship described above, many studies have been carried out with cowpea (Makoi et al., 2010, Sekiya and Yano, 2008), and also with cereals (Cabrera-Bosquet et al., 2009b, Anyia et al., 2007) as researchers search for surrogates of WUE. In many of such studies stomatal conductance seems to play a major role in influencing the CO_2 concentration in intercellular spaces and hence carbon isotope discrimination and photosynthetic rate. However, other scholars believe that photosynthetic rate may also be affected by non-stomatal restrictions like mesophyll conductance and other biochemical limitations (Singh and Raja Reddy, 2011). In fact, Farooq et al. (2009) reported that stomatal limitations could be smaller under drought conditions compared to non-stomatal limitations.

Other factors to consider that may have an effect on CO_2 and^{13}C-CO_2 discrimination include the isohydric and anisohydric behaviour of plants as well as the stomatal oscillations occurring during the day. Substantial differences between isohydric and anisohydric plants have been reported (Jones, 2006). Isohydric plants regulate (close) their stomata to maintain a relatively constant leaf water potential while anisohydric plants allow leaf water potential to decline under water stress (Munns et al., 2010, Saradadevi et al., 2014). In addition, cyclic oscillations in stomatal conductance have been reported (Dzikiti et al., 2007, Ellenson and Raba, 1983) which may influence CO_2 capture and the measurement of stomatal conductance.

In trying to elucidate the role of stomatal conductance in drought resistance, carbon isotope discrimination and in cowpea productivity, studies have been carried out in which water stress has been targeted at certain growth stages (Anyia and Herzog, 2004, Rivas et al., 2016). However, very few studies have looked at the progressive effect of different moisture levels on stomatal conductance and on how the resultant variation in stomatal conductance influences other gas exchange parameters like photosynthetic rate and $WUE_{intrinsic}$. The broader aim of this study was, therefore, to assess how gas exchanges, carbon isotope leaf composition and biomass production in cowpea vary along a soil moisture gradient. Recent reports (Hall, 2012) suggest that current cowpea genotypes show resistance to vegetative-stage drought and thus this study took a particular interest on how stomatal conductance in cowpea vary with time. The study also investigated whether there is early growth stage genotypic variation in cowpea stomatal conductance and whether that influences biomass production.

6.2 Materials and Methods

6.2.1 Plant growth conditions and experimental design

This study was simultaneously conducted with the previous study on screening cowpea for high BNF at the University of Limpopo experimental farm, in the Limpopo Province of South Africa. The planting dates and the growing conditions were similar to those described under subsections 4.2.1 and 4.3.1. The same four genotypes: IT00K-1263 (IT1), IT99K-1122 (IT2), TVu14632 (TV1) and TVu4607 (TV2) were used in the experiment.

6.2.2 Plant harvesting and sample preparation

Shoot biomass, was determined at 50% flowering from an area of 0.9 m2. Dry weight of the shoot was determined by oven drying the samples at 65oC to constant weight. More detailed description provided on section 4.2.2.

6.2.3 Leaf gas exchange measurements

Leaf gas exchange measurements were carried out using LCi-SD Ultra Compact Photosynthesis System (ADC Bio Scientific, UK). The measurements were taken four times in the first season on clear sunny days between 11h00 to 13h00. The measurements were taken at 47, 54, 70, and 77 days after planting (DAP) in 2015. In 2016 the measurements were taken only once at 70 DAP due to equipment failture. The measurements were done on youngest fully expanded leaves. As described on section 2.2.3, the measurement time with the photosynthesis system is about two minutes per leaf and to keep within the recommended time, only one leaf was measured per plot. However, each treatment was replicated four times.

6.2.4 Isotope Analyses

For isotope analyses, five to ten young fully grown leaves (depending on the size) were randomly sampled from different plants per plot at 50% flowering. The leaves were dried at 65°C to constant weight and ground to a fine powder using a ZM200 mill (Retsch, Germany). The $^{13}C/^{12}C$ and $^{18}O/^{16}O$ isotope compositions of the leaves were analyzed as described in Chapter 3 under section 3.2.4.

6.2.5 WUE determination

WUE of biomass ($WUE_{biomass}$) was calculated as the ratio of shoot biomass (as described under 4.2.2) to total amount of water used. The total amount of water added was obtained from the summation of the rain gauge recordings from planting to flowering. At leaf level, intrinsic WUE ($WUE_{intrinsic}$) was calculated as follows: $WUE_{intrinsic} = A/gs$ where A is the photosynthetic rate, and gs is the stomatal conductance. A and gs were obtained from the measurements of the Photosynthesis System described under 5.2.3.

6.2.6 Statistical Analyses

The analysis was conducted in accordance with the standard procedure for analysis of variance (ANOVA) of a randomised complete block design in split plot arrangement (Gomez and Gomez, 1984). The ANOVA was performed to calculate the effects of moisture level and genotype on the studied parameters. Where the interaction effect was not significant, data was split according to moisture level and analysed for the effect of genotype at each moisture level (for some parameters). Post hoc multiple comparisons for observed means was done using Tukey and different means were denoted by *, ** or *** for significance levels $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$, respectively. Bivariate Pearson Correlation was carried out to assess the relationship between gs and $\delta^{13}C$ and between gs and biomass. All data were analysed using the SPSS 20 statistical package (SPSS, USA).

6.3 Results

6.3.1 Genotypic variation in stomatal conductance with time and moisture level

Stomatal conductance varied with genotype, moisture levels and also with time (i.e. DAP) (Fig. 6.1). The interaction between genotype and moisture level was not significant throughout the period of measurement. Genotypes varied in *gs* at 47 and 54 DAP but did not differ at 70 and 77 DAP in 2015. No genotypic variations in *gs* were observed in 2016 where the gas exchange measurements were only carried out at 70 DAP. Genotypic differences were however observed at moisture levels MW, MS and SS. At MW and MS, IT1 had a significantly higher *gs* than the other genotypes while under rain fed conditions (SS), TV2 had a higher *gs*. At 54 DAP, genotypic variation in *gs* was only observed under MS where TV2 had the highest *gs* which differed from TV1 and IT2 but not from IT00K-1263. At the same moisture level IT1 also had a significantly higher *gs* compared to TV1. It was also observed that TV2 again showed higher *gs* values under SS at 54 DAP although it was not statistically different. Moisture levels did not significantly influence *gs* 47 DAP and 77 DAP but at 54 and 70 DAP, the effect was significant. At these two growth stages, a decrease in *gs* with decreasing moisture level was observed, where *gs* was higher under well-watered conditions compared to water-limited conditions. In addition, *gs* was generally lower at the late stages i.e. 70 and 77 DAP.

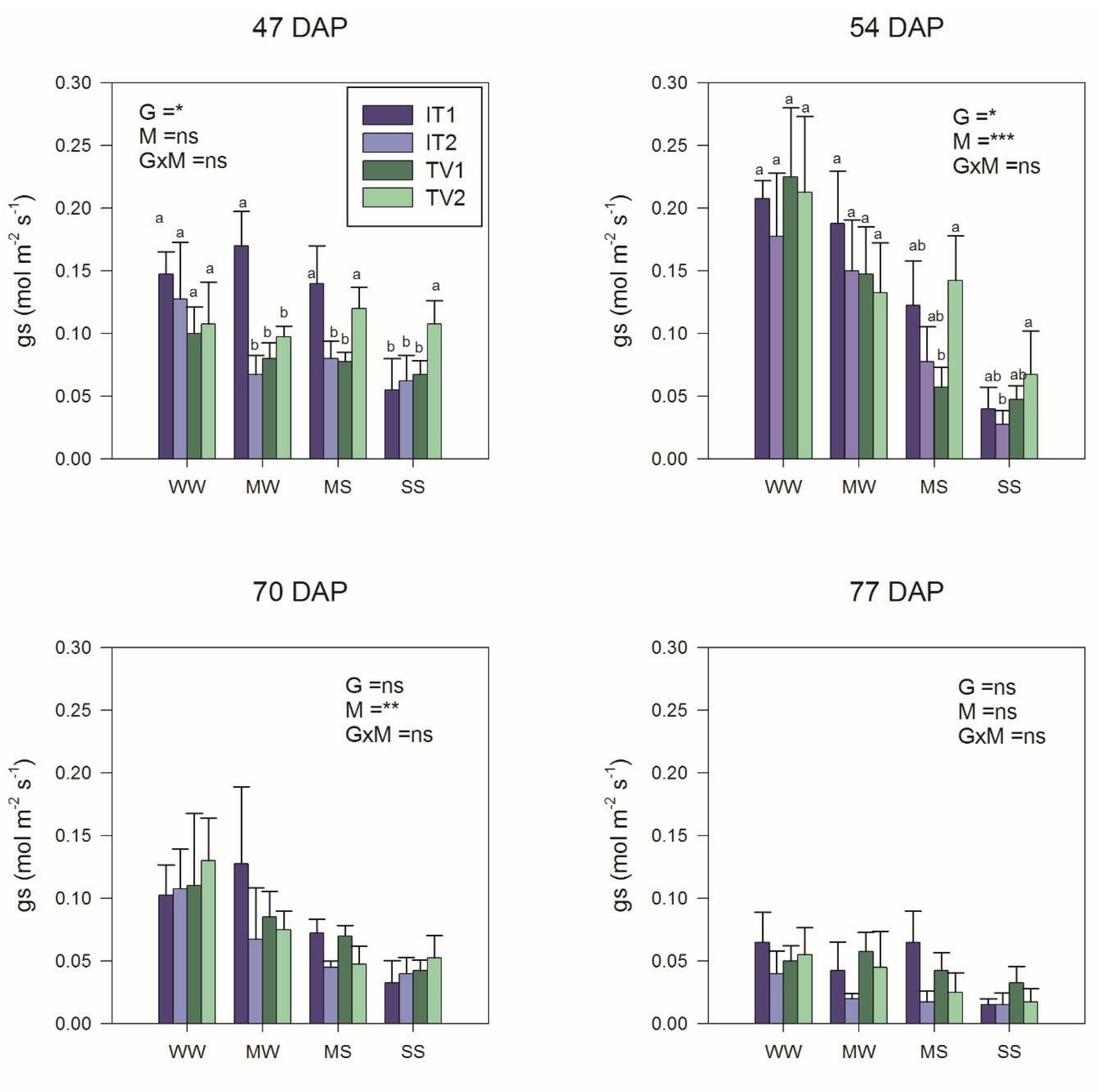


Fig. 6.1: Variation of stomatal conductance (gs) as influenced by genotype (G) and moisture levels (M) measured at different DAP in 2015 and 2016. Significant differences are indicated with *. Letters show differences among the genotypes at each moisture level (different letters represent significant differences)

6.3.2 Evolution of leaf gas exchanges with time under the four moisture levles

The progression of cowpea mean *gs, A, E* and *Ci/Ca* (averaged for the four genotypes) with DAP is shown on Fig. 6.2. Moisture levels significantly influenced the progression of measured gas exchange parameters as cowpea growing under well-watered conditions had high values of the *gs, A, E* and *Ci/Ca* compared to cowpea growing under water-limited conditions. At higher moisture levels (WW and MW), an initial surge in *gs, A*, and *E* was observed from 47 to 54 DAP followed by a gradual decrease from 54 to 77 DAP. However, E levelled off between 70 and 77 DAP. At lower moisture levels (particularly under SS), there was no initial surge in *gs, A*, and *E* but rather a steady decrease with time from the first measurement (47 DAP) to the last (77 DAP). The *Ci/Ca* also decreased with DAP for all moisture levels, except for a partial increase observed under WW at 70 DAP. The *gs* was for the greater part above 0.10 mol m^{-2} s-1 under WW and MW but was mostly below 0.05 mol m^{-2} s^{-1} under SS (see also Fig. 6.3). Differences in *gs, A*, and *E* resulting from the effect of moisture levels were more distinct at 54 DAP while differences in *Ci/Ca*, were more pronounced at 70 DAP. Significant differences in the gas exchange parameters were clear between WW and SS at all four different dates. Interestingly, it was also observed that even though high moisture levels were maintained (e.g. under WW) *gs, A*, and *E* still decreased with DAP from 54 DAP onwards. The variation of *A* and *gs* with time and moisture levels was very similar and strongly related (Table 6.1).

Figure 6.3 shows evolution of *gs* of the genotypes with DAP at each moisture levels with a control line at 0.10 mol m^{-2} s^{-1} showing the threshold value below which photosynthesis is affected as described by Flexas et al. (2004). The figure shows that under WW, *gs* was above the threshold at 47, 54 and 70 DAP and was only below the threshold at 77DAP. Under MW, only IT1 maintained *gs* at above the threshold until 70 DAP. However, under SS, all genotypes had *gs* below the threshold except for TV2 at 47DAP.

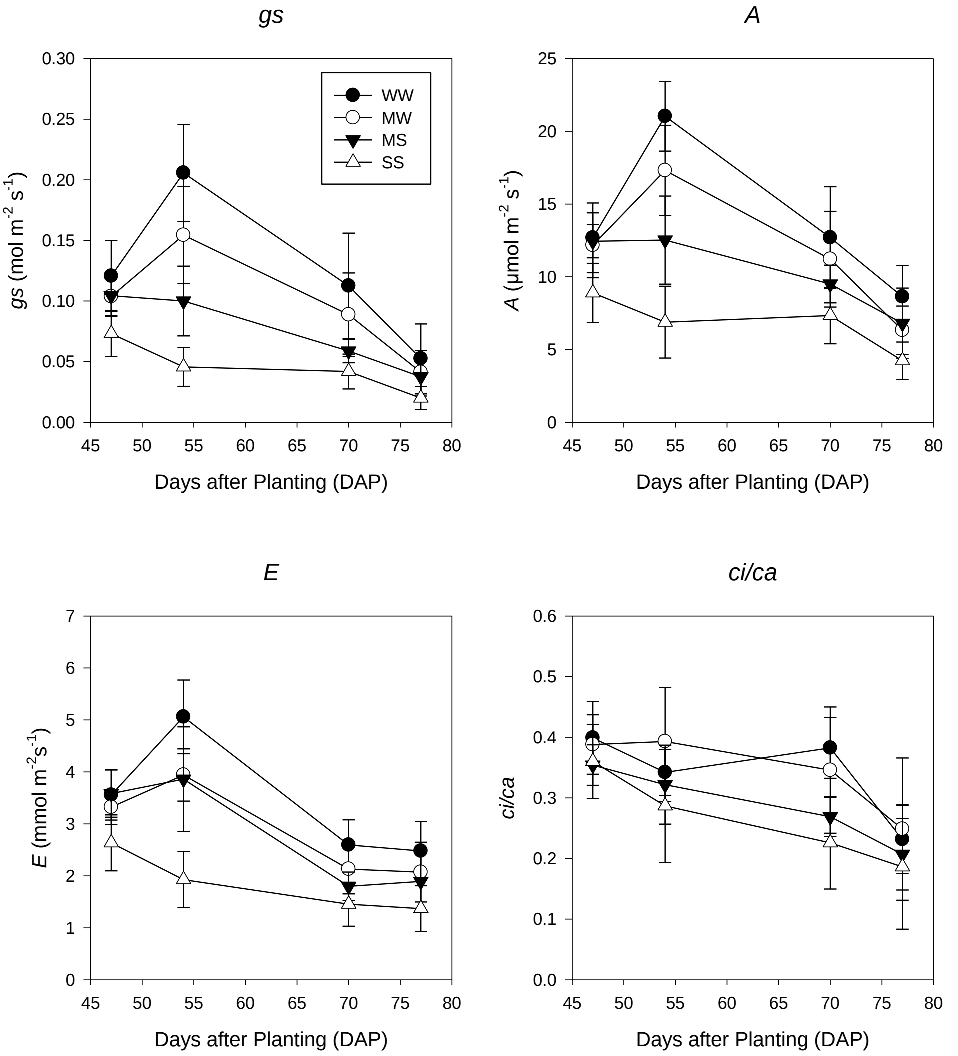


Fig. 6.2: Mean stomatal conductance (gs), photosynthetic rate (A), transpiration (E), and ratio of intercellular CO_2 concentration (ci) to ambient CO_2 concentration (ca) of four cowpea genotypes measured at 47, 54, 70 and 77 days after planting (DAP). Errors bars indicate standard error of means

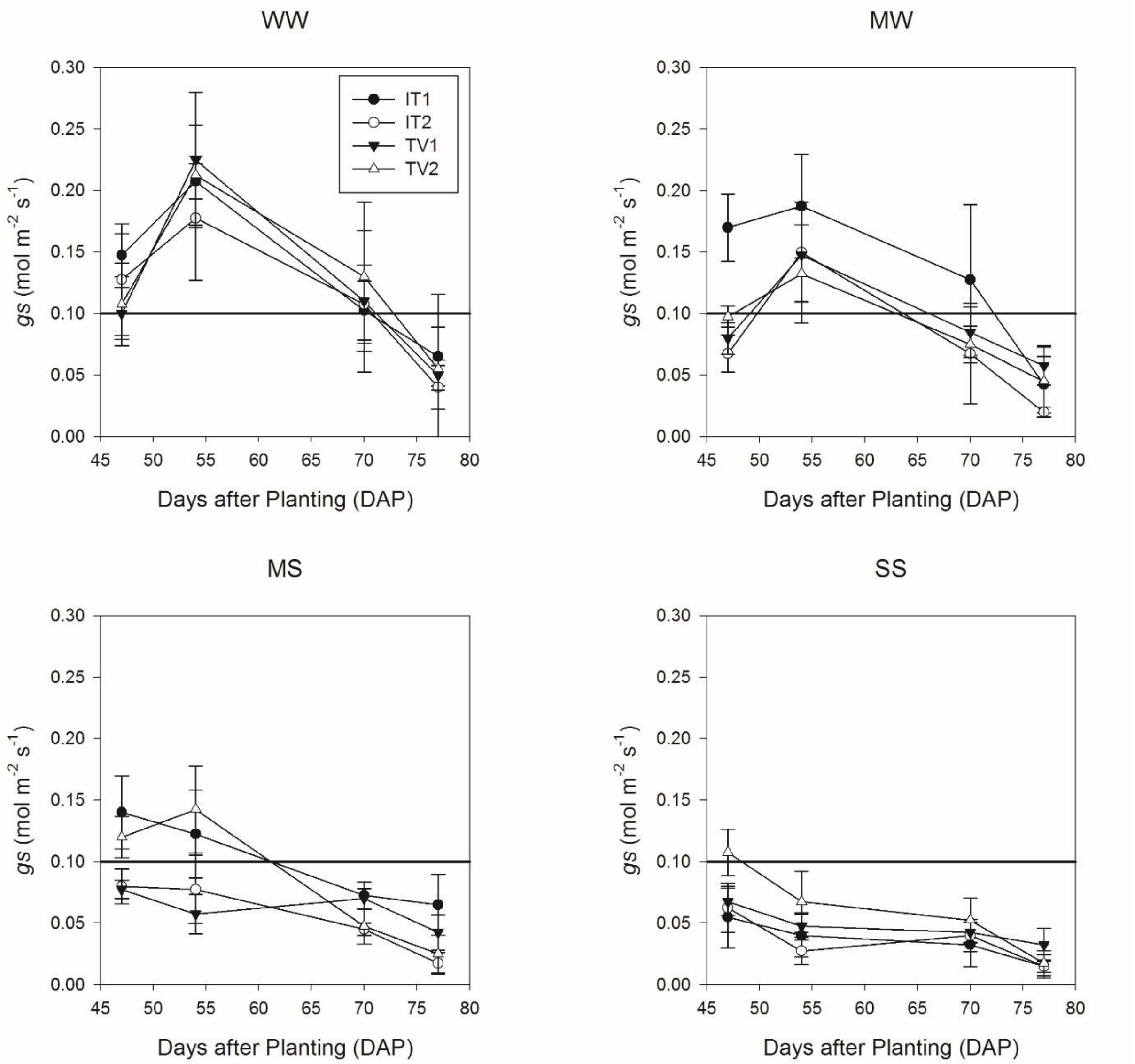


Fig. 6.3: Evolution of *gs* with DAP at different moisture levels. The line at 0.10 mol m^2 s^{-1} is a control line showing the threshold value below which photosynthesis is affected as described by Flexas et al. (2004).

6.3.3 Relationship between *gs* at 47 DAP and biomass and $\delta^{13}C$

Figure 6.4 shows the relationship between *gs* and biomass and also between *gs* and $\delta^{13}C$ during the early growth stages of cowpea (47 DAP) as affected by the four moisture levels. The relationships were analysed at 47 DAP to check whether *gs* measured at early stages is related to biomass production or $\delta^{13}C$. The results showed no significant relationships between biomass production at flowering and early *gs* measured in cowpea grown under WW, MW and MS conditions. Similar relationships were also observed with leaf $\delta^{13}C$. However, under rain fed (SS), positive relationships were observed between *gs* and biomass accumulated at flowering ($r^2 = 0.58$, $p < 0.05$) as well as between *gs* and $\delta^{13}C$ of leaves sampled at flowering stage ($r^2 = 0.6$, p <

0.05). Cowpea grain yield, which is reported in Chapter 4 was also positively related to *gs* at 47 DAP under SS ($r^2 = 0.50$, $p < 0.05$) and under WW (0.64, $p < 0.01$). It was interesting to note that a similar relationship between $\delta^{13}C$ and *gs* was also observed for the other days (54, 70 and 77 DAP). The overall relationship between $\delta^{13}C$ and *gs* was mostly negative when data for all moisture levels were combined. Table 6.1 shows this relationship for data measured at 70 DAP in both years.

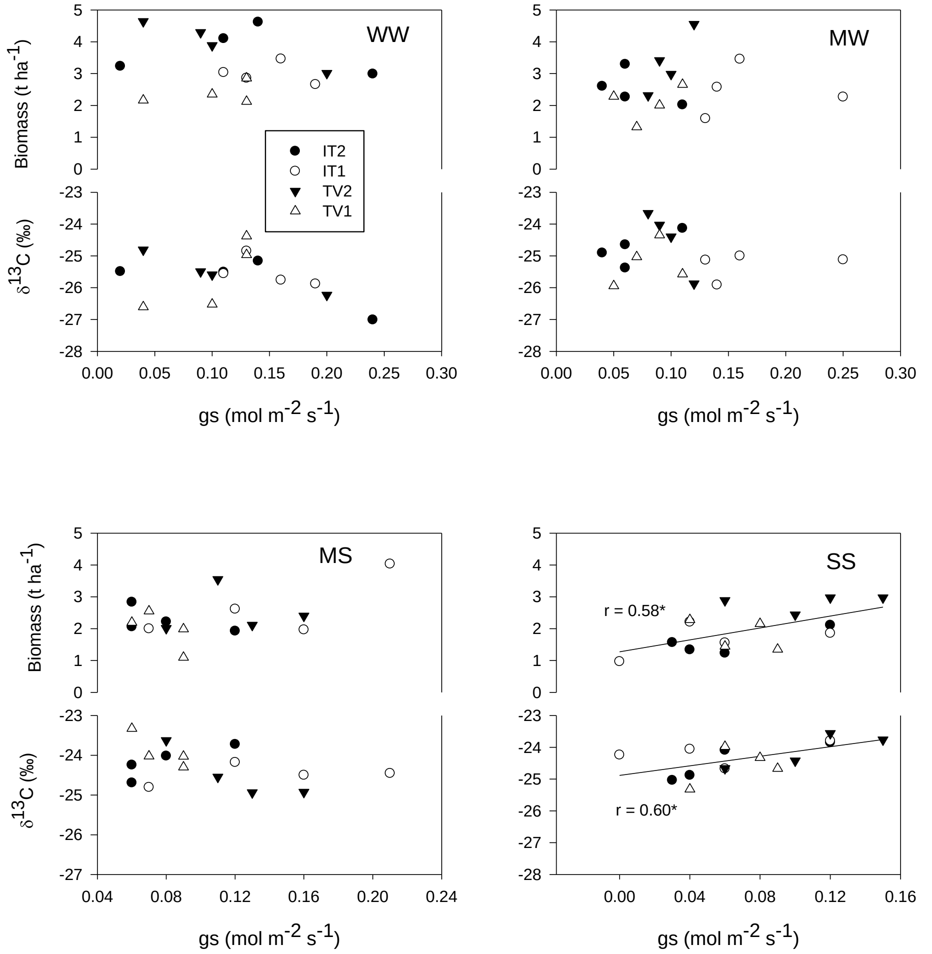


Fig. 6.4: The relationship between stomatal conductance (*gs*) measured at 47 DAP and biomass accumulated at flowering and as well leaf $\delta^{13}C$ measured at flowering.

6.3.4 Genotypic variation in $\delta^{13}C$ and $\delta^{18}O$ with moisture level

In both seasons (2015 and 2016), moisture level significantly influenced leaf $\delta^{13}C$ and $\delta^{18}O$ (Fig. 6.5) but genotypic effects on the two parameters were not observed. There were no differences in $\delta^{13}C$ values for cowpea grown under MS and SS; and this was observed in both seasons. In the first season, differences in $\delta^{13}C$ values were only observed between WW and MW. Also, in both seasons, differences in $\delta^{13}C$ were observed between water-limited conditions (MS and SS) and well-watered conditions (WW and MW). Thus, cowpea discriminated more against ^{13}C under wellwatered conditions compared to water-limited conditions, resulting in more negative $\delta^{13}C$ values being observed under well-watered conditions. In 2015, average $\delta^{13}C$ values were -25.6‰ under WW and -24.3‰ under SS while in 2016 the averages were -26.9‰ under WW and -24.7‰ under SS. Due to the higher rainfall received in 2016, cowpea discriminated more against ^{13}C in 2016 compared to 2015.

As observed with $\delta^{13}C$, $\delta^{18}O$ also responded to moisture levels but not to genotypes. Significant differences in $\delta^{18}O$ were observed between well-watered (WW and MW) and water-limited (MS and SS) conditions in both seasons. The response of $\delta^{18}O$ was similar to that of $\delta^{13}C$. Both $\delta^{13}C$ and $\delta^{18}O$ increased with decreasing moisture level. In 2015 the average $\delta^{18}O$ in the leaves was 15.6‰ under WW and 18.5‰ under SS, with a difference of 2.9‰. In the following season $\delta^{18}O$ values were almost double of those observed in 2015. The average $\delta^{18}O$ values were 33.4‰ and 36‰ under WW and SS, respectively. The difference of 2.6‰ observed between WW and SS in 2016 was similar to the difference observed between the same moisture levels in 2015 (2.9‰). However, the relative difference in percentage points was much smaller in 2016 (7.2%) compared to 2015 (15.7%).

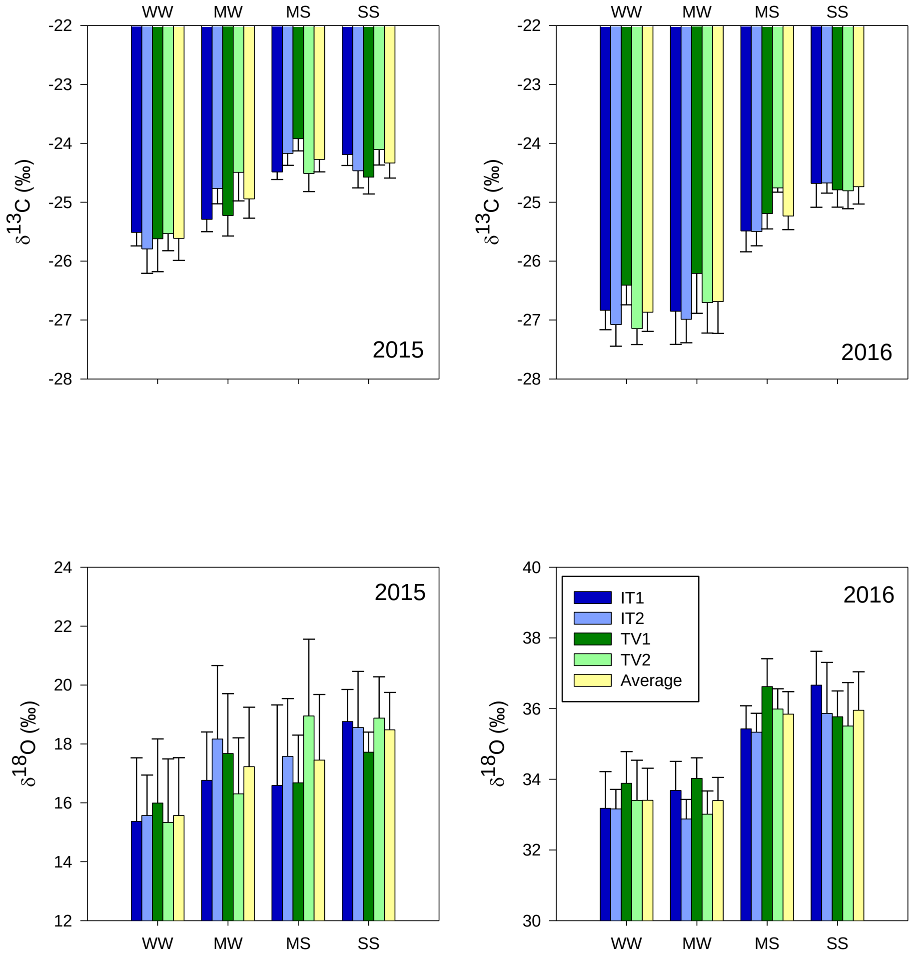


Fig. 6.5: Influence of moisture levels on genotypic δ^{13}C and δ^{18}O measured in leaves at flowering stage in 2015 and 2016. Average is mean of all genotypes at a particular moisture level.

6.3.5 WUE$_{\text{biomass}}$ and WUE$_{\text{intrinsic}}$ as influenced by moisture level

WUE$_{\text{biomass}}$ varied with moisture level in 2015 but not in 2016 (Fig. 6.6). Likewise, genotypes had a significant effect on WUE$_{\text{biomass}}$ only in the 2015 growing season and not in 2016. The interaction effect between moisture level and genotype was not significant in both years. In 2015, a general increase in mean WUE$_{\text{biomass}}$ with decreasing moisture level was observed where WUE$_{\text{biomass}}$ was higher under SS followed by MS and then MW and WW. Average WUE$_{\text{biomass}}$ of 26.8 kg ha^{-1} mm^{-1} was observed in cowpea grown under SS compared to 18.0 kg ha^{-1} mm^{-1} observed under WW. Genotypic variations observed in 2015 where only significant under WW and SS. Under SS, TV2 had the highest WUE$_{\text{biomass}}$ of 38.6 kg ha^{-1} mm^{-1} while the other three genotypes did not vary with WUE$_{\text{biomass}}$ values ranging from 21.4 to 24.8 kg ha^{-1} mm^{-1}. Under WW, TV2 mantained a higher WUE$_{\text{biomass}}$ (in magnitude) followed by IT2, while TV1 had the lowest $WUE_{biomass}$. Even though no genotypic differences were observed in the 2016, however in magnitude, TV1 had the lowest $WUE_{biomass}$ under all moisture level. On average, $WUE_{biomass}$ was significantly lower in 2016 compared to that of 2015. When data for all moisture levels were averaged, TV2 had significantly higher $WUE_{biomass}$ compared to the other three genotypes in 2015. When correlated to the measured gas exchanges, $WUE_{biomass}$ did not show any significant relationship with the measured gas exchange parameters in both seasons (Table 6.1). No significant relationship was also observed between $WUE_{intrinsic}$ and $WUE_{biomass}$.

$WUE_{intrinsic}$ was also influenced by moisture levels only in both seasons. No genotypic differences were observed and the interaction between moisture and genotype was also insignificant. $WUE_{intrinsic}$ was higher under water-limited conditions (MS and SS) compared to well-watered conditions (MW and WW). However, in 2015, differences were also observed between MS and SS. $WUE_{intrinsic}$ ranged from 120 to 200 µmol mol^{-1} in 2015 whilst in 2016 it ranged from 133 to 189 µmol mol^{-1}. Genotypes did not vary in $WUE_{intrinsic}$ at each moisture level and this was observed in both seasons. Even when data for all moisture levels were combined the genotypes still did not show any differences in $WUE_{intrinsic}$ as was observed with $WUE_{biomass}$. $WUE_{intrinsic}$ was also negatively related *to gs, A, E* and *Ci/Ca* in both seasons (Table 6.1). In addition, $WUE_{intrinsic}$ was positively related to $\delta^{13}C$.

Table 6.1: Correlation among gas exchange parameters and $WUE_{intrinsic}$, $WUE_{biomass}$, and $\delta^{13}C$ in 2015 and 2016 measured at 70 DAP

2015	*gs*	*A*	*E*	*Ci/Ca*	$WUE_{intrinsic}$	$WUE_{biomass}$
A	**0.89****					
E	**0.97****	**0.89****				
Ci/Ca	**0.30***	-0.06	**0.30****			
$WUE_{intrinsic}$	**-0.46****	-0.22	**-0.42****	**-0.67****		
$WUE_{biomass}$	-0.19	-0.18	-0.08	0.10	0.09	
$\delta^{13}C$	**-0.49****	**-0.38****	**-0.41****	**-0.23***	**0.34****	**0.31***
2016						
A	**0.90****					

E	**0.94****	**0.91****				
Ci/Ca	-0.01	**-0.35****	-0.11			
$WUE_{intrinsic}$	**-0.60****	**-0.31****	**-0.54****	**-0.54****		
$WUE_{biomass}$	0.11	0.15	0.09	-0.02	-0.05	
$\delta^{13}C$	**-0.78****	**-0.72****	**-0.74****	-0.04	**0.48****	**-0.26***

*Bold values show significant correlations. Asterisks show significance: * for $p < 0.05$ and **for $p < 0.01$. Data averaged for all moisture levels*

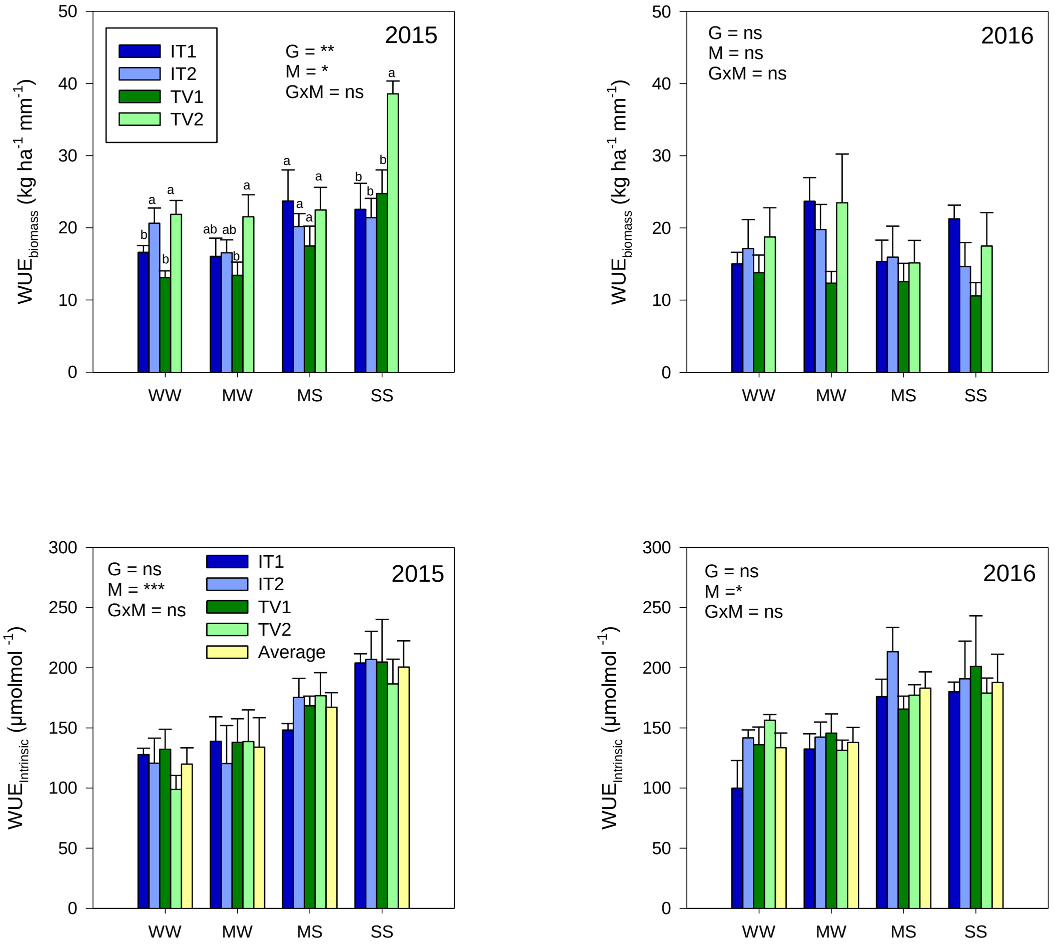


Fig. 6.6: The effect of moisture levels on biomass WUE and Intrinsic WUE of the four studied genotypes measured in 2015 and 2016. Letters show differences among the genotypes at each moisture level (different letters represent significant differences)

6.4 Discussion

6.4.1 Variation in leaf gas exchange with genotype, moisture level and DAP

The study has shown that soil moisture significantly influences leaf gas exchanges in cowpea. Stomatal conductance*, A* and *E* were found to be high under higher moisture level compared to lower moisture levels. Such results were expected and agree with the findings of similar studies (Ninou et al., 2012). It was also interesting to note that genotypes only varied in *gs* during the early growth stages but not in later stages. A plausible explanation for such a variation could be differences in root establishment, where genotypes that establish their roots faster would have much more access to water, resulting in high leaf water content and hence higher *gs*. Genotypes that showed higher early *gs* in this study also had higher root biomass and root/shoot ratio (see Chapter 4). For example, TV2 had the highest root/shoot ratio in both years (Fig. 4.5) and this was also reflected in the *gs* of TV2 (Fig. 5.1 and Fig. 5.3). In support, Agbicodo et al. (2009) reported that plants that maintain higher tissue water potential under low water availability are able to do so by developing efficient root system that maximises water uptake. Accordingly, it can be postulated that the high *gs* of TV2 under SS may have resulted from a stronger and a more efficient rooting system that enabled it to tap water. Interestingly, at both 47 and 54 DAP genotypes did not vary in *gs* under WW conditions but showed much variation under lower moisture levels (MW, MS and SS). This could probably be due to the easily available moisture that allowed the genotypes to absorb enough moisture to keep stomata open. Under MW and MS, IT1 and TV2 consistently showed higher *gs* compared to the other two genotypes but under SS, only TV2 maintained a relatively higher *gs*. The results suggest that TV2 probably has the capacity to establish an efficient rooting system very early in the season. In Chapter 4, TV2 was found to have significantly higher root biomass compared to the other genotypes.

Genotypic differences observed in *gs* under low water availability could be due to differences in drought resistance or to differences in the response mechanism of the genotypes to water deficit. Genotypes with higher capacity to develop efficient rooting system, show drought tolerance by maintaining relatively higher *gs*, *E* an *A* while those with less efficient rooting system, may show drought avoidance by closing their stomata, hence low *gs*, *E* and *A*. TV2 displayed a strong drought tolerance under moderate stress (MS) at 54 DAP. The *gs* of TV2 under MS and MW were similar while other genotypes had significantly lower *gs* at MS compared to MW. Stomata of cowpea are known to be sensitive to soil drying (Hall, 2012), and thus enabling the plant to exhibit drought avoidance mechanism (Anyia and Herzog, 2004, Singh and Raja Reddy, 2011). The sensitivity of the stomata to soil moisture could explain the variation in *gs* across all moisture levels exhibited by the other three genotypes at 54 DAP.

While genotypic differences in *gs* were observed at early growth stages (47 and 54 DAP), the soil moisture effect was only observed at mid-season growth stages i.e. 54 and 70 DAP (Fig. 5.1). The lack of moisture level effect in the early growth stage is difficult to explain but could have resulted from relatively higher *gs* displayed by TV2 and IT1 even under lower moisture levels. The lack of differences in the effect of soil moisture level on *gs* at 77 DAP was probably due to leaf aging. Stomatal conductance values observed at 77 DAP were significantly low, mostly below 0.05 mol m^{-2} s^{-1} (Fig. 5.1 and Fig. 5.3). Figure 5.1 shows a gradual decrease in *gs* with time from 54 DAP to 77 DAP, while Fig. 5.2 shows *gs* decreasing with DAP even under WW which all support the idea of leaf and plant aging. In addition, stomata are generally known to be more open early in the season than at its end (Austin et al., 1990). When moisture levels significantly influenced *gs* (54 and 70 DAP), values were higher under well-watered conditions compared to water-limited conditions agreeing with the findings of Bloch et al. (2006) in sugar beet.

The strong correlation observed between *gs* and *A* ($r^2 = 0.90$, $p < 0.01$) suggests that variation in CO_2 assimilation in cowpea was mainly due to *gs* and less likely due to non-stomatal effects. However, the water stress may not have been severe enough to disturb other photosynthetic processes. The control of *gs* on *A* is also confirmed by the identical evolution of *A* and *gs* with time (Fig. 5.2). Similarities in the evolution of *A* and *gs* have also been reported in other studies with legumes (Liu et al., 2005). *E* also showed strong dependence on *gs* ($r^2 = 0.97$, $p < 0.01$, 2015 season). According to Flexas et al. (2004), photosynthesis is only affected when *gs* goes below 0.10 - 0.15 mol m^{-2} s^{-1}. In this study, cowpea grown under rain fed conditions (SS) and also under MS had *gs* values lower than 0.10 mol m^{-2} s^{-1} for most of their growing period (Fig. 5.2 and Fig. 5.3). This, therefore, suggests that, cowpea growing in semi-arid regions are likely photosynthesising at sub-optimal levels throughout its growth stages. As a result, biomass and grain yield observed in 2015 (Fig. 4.2) were significantly lower under SS, an indication of the supressed photosynthesis rate. Thus, improvement in cowpea stomatal conductance could increase productivity of cowpea under arid conditions. TV2 and IT1 where the only genotypes that managed to maintained *gs* values above 0.10 mol m^{-2} s^{-1} under moderate stress (MS) until 54 DAP. Consequently, IT1 was found to be the most suitable genotype in the studied environment.

$\delta^{13}C$ which provides an integration of the environmental conditions during the period of CO_2 assimilation (Ninou et al., 2012) differed with moisture level. $\delta^{13}C$ values were more negative under well-watered conditions compared to water-limited conditions which corroborates the findings of many other studies on C_3 plants (Bloch et al., 2006, Cabrera-Bosquet et al., 2009b, Wang et al., 2013). Less negative $\delta^{13}C$ values under SS were attributed to lower ^{13}C discrimination due to the partial closure of the stomata occurring under soil drying. The influence of *gs* and of *A* on $\delta^{13}C$ is confirmed by the strong negative relationship between $\delta^{13}C$ and the two gas exchange parameters (*A* and *gs*) (Table 5.1). The relationship between *gs* at 47 DAP and $\delta^{13}C$ showed a positive relationship under SS only (Fig. 5.4). Furthermore, a similar relationship was observed with *gs* measured in the later growth stages of cowpea. The positive relationship suggests genotypic variation in $\delta^{13}C$ of cowpea grown under SS conditions, whereby genotypes that showed higher *gs* would be expected to have less negative $\delta^{13}C$ values. However, the observed results did not show significant genotypic variation in $\delta^{13}C$ under SS (Fig. 5.5). Figure 5.1 shows that TV2 had significantly higher *gs* under SS at 47 DAP but this did not translate to significantly higher $\delta^{13}C$ (Fig. 5.5). Biomass also showed a similar positive relationship with *gs* under SS at 47 DAP (Fig. 5.4) meaning that TV2 with higher *gs* was also expected to have higher biomass at flowering. Unlike the observation with $\delta^{13}C$, TV2, did show higher biomass at flowering.

6.4.2 Relationships of $WUE_{biomass}$ and $WUE_{intrinsic}$ as influenced by moisture level

Higher WUE is regarded as one of the strategies that can be used to improve crop performance under water-limited conditions (Araus et al., 2002). WUE can be measured or estimated in several ways as described by Tambussi et al. (2007a). In this study WUE was measured in terms of: biomass accumulation ($WUE_{biomass}$); as gas exchange ($WUE_{intrinsic}$) and estimated using $\delta^{13}C$ data. The results showed that both $WUE_{intrinsic}$ and $WUE_{biomass}$ were influenced by moisture level except for $WUE_{biomass}$ in 2016. $WUE_{intrinsic}$ was higher under water-limited conditions in both seasons while

$WUE_{biomass}$ was only higher under low-water availability in 2015. In similar studies, (Bloch et al., 2006) also found higher WUE under water limited conditions. However, other studies are contrary to such findings (Erice et al., 2011). The lack of differences in $WUE_{biomass}$ in 2016 may be attributed to the relatively higher rainfall received in that year. Higher $WUE_{biomass}$ under water limited conditions results from the crop assimilating carbon only when evaporative demand is low, hence loses less water per carbon gained (Bloch et al., 2006). Under well-watered conditions plants continue to assimilate carbon even when evaporative demand is very high resulting in higher losses of water per carbon gained, leading to lower $WUE_{biomass}$. As observed on Fig. 6.5, TV2 had a strikingly high $WUE_{biomass}$ under SS in 2015 which as discussed earlier maybe attributed to the high root biomass allowing the plant to tap water more efficiently. Unfortunately, the high $WUE_{biomass}$ of TV2 only translated into into high biomass and not grain yield.

$WUE_{intrinsic}$ was high under water stressed conditions because of the differences in the rate of decrease between *A* and *gs*. When stomata partially close, the photosynthetic rate does not immediately decrease resulting in higher $WUE_{intrinsic}$ (Ninou et al., 2012). In addition, the CO_2 gradient across the stomatal pore is weaker than the H_2O gradient hence a mild stomatal closure under drought stress suppresses H_2O loss more than CO_2 gain resulting in increased WUE (Bacon, 2004). Also, $WUE_{intrinsic}$ increases when *A* increases at a constant *gs* (Singh and Raja Reddy, 2011). There was no significant relationship between $WUE_{biomass}$ and $WUE_{intrinsic}$, probably because $WUE_{intrinsic}$ is an instantaneous measurement that reflects conditions prevailing at the instant of measurement while $WUE_{biomass}$ is an integration of the conditions from sowing to the time of biomass sampling. However, $\delta^{13}C$ provides an integration of $WUE_{intrinsic}$ overtime as shown by the positive and strong relationship between $\delta^{13}C$ and $WUE_{intrinsic}$ in both seasons (Table 6.1). The strong relationship shows that $\delta^{13}C$ can be used as a surrogate of $WUE_{intrinsic}$ in cowpea. Similar conclusions have also been drawn in other studies.

6.5 Conclusion

In conclusion, the findings of this study showed that *gs* responded to genotypic variation, moisture levels and to the time of measurement. Genotypes varied at early growth stages and mostly under water limited conditions with genotype TVu4607 showing significantly higher *gs* under SS. In Chapter 4, TVu4607 also had higher biomass under SS suggesting that genotypes with higher *gs* under dry conditions produce higher biomass. The $\delta^{13}C$ and $\delta^{18}O$ values varied with moisture level but did not respond to genotypic variation. Highly significant and positive correlations observed between $\delta^{13}C$ and *gs* under SS suggest that $\delta^{13}C$ could be useful as a screening trait for higher *gs* in cowpea under drought conditions. Similarly, $\delta^{13}C$ and $WUE_{intrinsic}$ were positively related in both years indicating that $\delta^{13}C$ can also be a possible surrogate of $WUE_{intrinsic}$ in cowpea. However, there is need for further assessment of these traits with a large genotypic pool, as the four genotypes used in this study did not vary in both $\delta^{13}C$ and $WUE_{intrinsicc}$.

7 Chapter 7. General conclusions and future perspectives

7.1 General Conclusion

Drylands are home to more than 38% of the world population and are sensitive to climate change. In addition, dryland areas are projected to increase by up to 23% by the end of the century compared to the baseline of 1961-1990 (Huang et al., 2015). Due to these projected changes, the suitability of many crops under such areas is under threat and so is food security if no adaptation measures are taken. In this PhD, drought tolerant traits of cowpea and triticale were evaluated. We also assessed the performance of the genotypes under semi-arid conditions. As a legume, cowpea was further assessed for its ability to contribute to soil fertility through N_2 fixation under dry conditions.

Triticale is a man-made crop that has gained importance mainly as a forage crop because of its high yield potential and the versatility of its utilization (Motzo et al., 2013). Triticale has great potential for food and feed production in future environments due to its adaptability to marginal growing conditions such as drought, extreme temperature, salinity etc. Due to the poor grain quality of triticale compared to wheat, triticale is currently being used mainly as a livestock feed. Though it's a relatively new crop and widely unknown in Africa, farmers need to be aware of the potential role of triticale in sustainable farming systems. Hence, the study in Chapter 2 evaluated the performance of four spring-type triticale cultivars under semi-arid conditions as well as under varying soil moisture levels given the fact that the major challenge of producing spring triticale in a semi-arid environment is the unavailability of water and heat stress that may occur during the reproductive stages.

The findings of the study showed that the local spring triticale cultivars (Agbeacon, Bacchus, Rex, and US2007) were well adapted to the semi-arid conditions. They particularly showed tolerance to post-anthesis high temperatures by producing reasonable yields even when maximum temperatures where above optimal. The observed triticale grain yield ranged from 0.8 to 3.5 t ha^{-1} in the first year and from 1.8 to 4.9 t ha^{-1} in the following year. The study also showed that under semi-arid conditions, as those experienced in the Limpopo Province, triticale cannot be grown under residual moisture. Triticale could also not complete its life cycle even when irrigation was stopped at stem elongation stage as it needed an extra 40 mm of irrigation to reach maturity. However, it is possible to grow triticale under moderate water stress and produce reasonable yields. Under moderate stress, livestock farmers could successfully grow triticale for whole plant silage by harvesting it at milk stage and should expect aboveground dry biomass yields of higher than 8 t ha^{-1}. Of the four genotypes studied, Agbeacon proved to be the most tolerant genotype by consistently having a higher yield stability index over both years.

Chapter 2 also assessed the potential use of spectral reflectance indices in monitoring water stress in triticale. Remote sensing has become important in monitoring crop water status, nutrient deficiencies and predicting crop characteristics such as grain yield and aboveground biomass (Chandrasekar and Sesha Sai, 2015, Gao, 1996). Remote sensing therefore enables the monitoring of crops and the prediction of yields without physical contact with crops. Monitoring of plant water status can be carried out at any growth stage before physiological maturity and can also help identify stressed crops. However, for yield prediction, mid-season measurements could provide more reliable estimates of the yield. The technique is, therefore, very useful under large scale commercial farming systems. The study evaluated four indices to identify the best index for monitoring water stress in triticale. Water based indices particularly the water index (WI) and NDWI were found to be superior to green based indices like NDVI and NRI in monitoring leaf water status in triticale.

Chapter 3 addressed the core objective of this PhD which was to test the use of carbon isotope discrimination as a proxy of $WUE_{intrinsic}$ and grain yield in field grown triticale. As reported in Chapter 1, climate change and variability present a major challenge to food security particularly in arid and semi-arid areas. Dryland areas are predicted to increase, enhancing the risk of land degradation and desertification (Huang et al., 2015). Moreover, dryland expansion is occurring in areas where most of the population growth is expected. To meet the food demand for this increasing human population more food would be expected to come from marginal lands. Some major crops e.g. maize and soybean are already predicted to be unsuitable in some of these areas (Rippke et al., 2016). Therefore, there is need to adapt crops to future warmer and drier conditions and one of the ways of doing that is to screen for quicker reliable traits indicating improved water use efficiency in crops.

The study also tested the use of $\Delta^{13}C$ as a proxy of grain yield and $WUE_{intrinsic}$ showed that $\Delta^{13}C$ could be useful as a predictor for triticale grain yield in drought prone areas. Highly significant and positive correlation were observed between flag leaf $\Delta^{13}C$ and grain yield as well as between grain $\Delta^{13}C$ and grain yield under drought conditions. Since triticale is a C_3 small grain cereal, these findings can also be extrapolated to other C_3 small grain crops like wheat, rice and barley. $\Delta^{13}C$ was further found to be a potential surrogate for $WUE_{intrinsic}$. The correlation between the two was strongly negative implying that breeding for higher $WUE_{intrinsic}$ may not necessarily yield the desired improved grain yield.

Assimilates for grain filling under water stress are commonly reported to come from stored pre-anthesis assimilates (Blum, 2011a, Xue et al., 2014, Álvaro et al., 2008). However, contrary results, under water stress, have been found in this study, using a non-destructive method. The findings of this study strongly suggest that most assimilates for grain filling under water stress actually come from post-anthesis photosynthesis. The conclusion was arrived at due to the differences observed in the ^{13}C signatures of the flag leaves sampled around anthesis and those of the grain at harvesting. If the assimilates for grain filling, came from pre-anthesis assimilates the carbon 13 signatures of the grain would closely resamble those of the flag leaves sampled around anthesis. Instead, the study found differences of up to 2.9‰ under water stress thus casting doubt on the suggestion that most assimilates are from reserves.

Chapter 3 also tested the applicability of the dual isotope model (Scheidegger et al., 2000) on an annual crop. The model was developed to help interpreting variations that are observed in $\delta^{13}C$. This study found that the model can be used to interpret $\delta^{13}C$ variations observed in triticale, but only when transpiration rates are limited by *gs*. The scenario occurs when evaporative demand (VPD) is relatively high such that *gs* becomes the limiting factor for transpiration rate. However, when VPD is relatively low, *gs* ceases to be the main factor limiting transpiration rate, but rather the evaporative demand itself limits transpiration. In our study the model worked perfectly in 2014 where a negative relationship was observed between $\delta^{18}O$ and *gs*. In the same year also, VPD was found to be higher relative to 2013. Due to the lower VPD observed in 2013 and the positive correlation observed between $\delta^{18}O$ and *gs*, the model failed to correctly predict the source of $\delta^{13}C$ variation in triticale leaves.

In Chapter 4, the potential of cowpea for BNF and grain yield under semi-arid conditions was evaluated. A total of 90 lines were screened, reduced to eight and then to the final four cultivars used in the study. The genotypes were tested under four varying moisture levels but in the second year the moisture levels were basically reduced to two due to the interference of rainfall. The results showed that the genotypes performed differently in terms of shoot biomass, root biomass, root/shoot ratio, BNF and in grain yield. Soil moisture levels were also found to have a significant influence on the studied parameters where BNF and nodule biomass were found to be more sensitive to water stress compared to biomass production. On average the studied genotypes fixed about a third of their N requirement, meaning that they derived substantial amounts of N from the soil.

Chapter 5 investigated the variation in stomatal conductance (*gs*) and other gas exchanges as affected by soil moisture and time of measurement (DAP-days after planting) as well as how the variation influenced cowpea biomass production and carbon isotope discrimination in cowpea. As expected, we found a significant influence of soil moisture level on *gs* where higher *gs* was observed under well-watered conditions and lower *gs* under water-limited conditions. Genotypes also varied in *gs* but the variation only occurred at the early growth stages, i.e. at 47 and 54 DAP. In addition, the observed genotypic variation only occurred under lower moisture levels specifically under SS.

Overall, our findings in Chapter 4 and 5 showed that genotype TV4607 was superior to the other genotypes in terms of biomass production (both below and above ground), nodule mass, BNF and in *gs* under drought conditions. However, TV4607 did not do well with respect to grain yield and was found to be very late maturing. Genotype IT00K-1263, was found to be the best genotype as it had the best balance of shoot biomass, BNF and high grain yield, while genotype TVu14632 was the worst performing genotype in terms of biomass, BNF, nodule mass and grain yield. IT99K-1122 had a relatively high grain yield and average BNF but was found to be very susceptible to aphids.

7.2 Future perspectives

7.2.1 Triticale performance in a semi-arid region and spectral response under varying moisture levels

Triticale is a robust crop which is tolerant to diseases and extreme temperatures and is adapted to less favourable soils and climates (Mergoum and Macpherson, 2004). Triticale should, therefore, be promoted in areas that are not suitable for most crops due to abiotic stresses. Triticale has so far been successfully grown in different environments around the world e.g. Europe (Schittenhelm et al., 2014), South America (Estrada-Campuzano et al., 2012), USA (Gibson et al., 2007), Australia (Milgate et al., 2015) and also in Africa (Manley et al., 2011). Triticale has, therefore, shown wide adaptability to a wide range of climates. Spring triticale used in this study needs limited vernalisation to transit from vegetative to reproductive stages. Therefore, it would be interesting to evaluate the performance of spring triticale in a summer season under rain fed conditions.

Also, in the current study spectral reflectance indices were calculated from spectral field measurements using a portable Analytical Spectral Device (ASD) Spectroradiometer (ASD, USA) with similar bands to those found on Hyperspectral Imaging Satellites. Further studies are therefore necessary to evaluate the spectral reflectance indices calculated from Hyperspectral Imaging Satellites. It would also be interesting to use satellite images to identify and monitor common triticale diseases like powdery mildew and brown leaf rust. The use of satellite images to monitor triticale diseases can start by by using field spectrometry to identify wavelengths that discriminate between diseased and healthy triticale leaves and then use the identified wavelengths to monitor diseases.

7.2.2 The use of carbon 13 as surrogate of grain yield and WUE

In this study the genotypes were initially screened for high biomass yield and the best four cultivars out of eight were selected for the study. This meant that the performance of the genotypes was not far apart. This was also confirmed in the study as most parameters showed no significant differences among the studied genotypes. However, for better calibration of the tested proxies, further studies with many genotypically different cultivars is needed. In addition, the selection of the genotypes may need to be based on known differences in WUE and $\delta^{13}C$. The use of carbon 13 as a surrogate of grain yield is currently limited in C_4 crops due to the low discrimination of ^{13}C by phosphoenolpyruvate carboxylase. Attempts have been made to explore the use of $\delta^{13}C$ in C_4 crops like maize (Cabrera-Bosquet et al., 2009c, Dercon et al., 2006, Monneveux et al., 2007) but the results have so far been not very promising. Nevertheless, $\delta^{18}O$ has shown potential as a secondary trait for yield potential in maize (Cabrera-Bosquet et al., 2009d). To make sure that the search of surrogate traits does not lag behind for important cereals like maize, further investigation is needed to explore the use of $\delta^{18}O$ in C_4 crops.

Regarding the source of assimilates for grain filling under drought conditions, more work still needs to be done. Currently, there is little information available on the use of $\delta^{13}C$ to trace assimilates to grains under drought conditions. The available information (Merah and Monneveux, 2015, Sanchez-Bragado et al., 2014a, Sanchez-Bragado et al., 2014b, Sanchez-Bragado et al., 2016) has been obtained from experiments under less severe water stress conditions and the information so far (from $\delta^{13}C$ analyses) shows a high contribution of the ears to grain filling. Considering the high drought tolerance of ear photosynthesis compared to the flag leaves (Tambussi et al., 2005, Tambussi et al., 2007b), one can infer a significant contribution of ears to grain filling under drought. However, to clearly understand this source-sink relationship under drought conditions, studies are needed that simultaneously compare different methodologies i.e. the destructive methods, leaf shading and the use of $\delta^{13}C$, as described by Merah and Monneveux (2015) and Sanchez-Bragado et al. (2016).

7.2.3 Testing of the dual isotope model

The process of oxygen isotope enrichment in leaves remains a debatable issue. A review of the literature reveals two schools of thought on how the leaves become enriched in ^{18}O. The first school of thought suggest that ^{18}O enrichment in leaves increases with increasing transpiration (Sheshshayee et al., 2005) while the second school of thought is contrary (Barbour and Farquhar, 2000, Farquhar and Gan, 2003, Ferrio et al., 2012). Therefore, further research is needed to elucidate explicitly all factors controlling oxygen enrichment in leaves and this could also help in modifying the dual isotope model so that it becomes all encompassing. There are also several concerns (about 10) regarding the operation of the model that were raised by Roden and Siegwolf (2012). Some of the concerns include the need to have a homogenous environment with regards to factors that influence evaporative enrichment i.e source water, humidity and $\delta^{18}O$ of water vapor. Other factors include the need to have a standard scaling for δ^{1c}C and δ^{18}O as well as for *A* and *gs.* If the concerns are addressed, they also could lead to the smooth operation of the model which is clearly beneficial to researchers.

7.2.4 BNF in cowpea under varying moisture levels

While BNF observed in the current study was satisfactory considering the growing conditions i.e. drought and saline soils, there are opportunities for further improvement. For example, it is unclear whether BNF would be improved if cowpea seeds were inoculated with a commercial rhizobium before planting. Also, the adoption of new genotypes is always a challenge in the smallholder farming sectors, hence there is need to promote the best performing genotype (IT00K-1263) under farmer managed trials. In the current study, BNF was determined from shoot biomass alone but as observed in Chapter 4 the genotypes produce substantial amounts of root biomass and hence BNF might have been underestimated. Future studies maybe necessary to assess the contribution of the roots and of the nodules to the total amount of N fixed by cowpea under arid conditions. Also, considering that after harvesting, the roots remain underground, contributing to soil organic matter, it would also be important to assess how water stress affect the N content of the roots as well. For example, similar moisture levels can be applied and then monitor how root length, architecture as well the proportion of fine roots varies with moisture level. This will provide more information on response of plants to water stress. Furthermore, roots are rarely, if ever used as phenotyping traits in plant studies. It would also be interesting if more research is put in trying to find root traits that can be used as surrogate traits for e.g. WUE.

In addition, the studied genotypes produced significant amounts of shoot biomass which when left as residues on the field will contribute to the nutrition of the subsequent crop through decomposition. However, it is known that decomposition rates in arid and semi-arid areas are generally slow hence it would be interesting to determine the residence time of the residues and how much of its N is made available to the subsequent crop? This kind of study can be done through the use of isotopes as tracers. Alternatively, incubation tests mimicking arid and semi-arid soil environments can be carried out and then measure the amount of N mineralised over a season.

7.2.5 Stomatal responds to varying moisture levels

There is no doubt that stomatal conductance plays a significant role in drought tolerance of cowpea and it, therefore, needs to be explored further. In the current study *gs* was determined at four occasions starting at 47 DAP but the intervals were not consistent. For a better understanding of the seasonal evolution of *gs* with time it would be interesting to start the measurements very early i.e. 14 DAP and also for the measurements to be more frequent e.g. weekly intervals. Also, it would be interesting to test the recovery of *gs* and other gas exchange parameters after exposure to severe stress. For example, it would be interesting to know how quickly and to what level gas exchanges recover after being exposed to severe drought. This kind of information is very important because often in semi-arid regions, crops are exposed to mid-season droughts that vary in severity and for many crops it is not known how quickly leaf photosynthesis recovers after a rain event.

Also, stomatal conductance responds to signal coming from roots in the form of abscisic acid (Mott and Buckley, 2000). Thus roots have a role to play in the assimilation of CO_2 as well as in the loss of water through transpiration. Further studies are therefore needed to understand how cowpea roots contribute to the drought resistance of the crop. Further investigations maybe needed to assess the capacity of cowpea roots to produce abscisic acid under water stress and also on whether this capacity can be manipulated to improve drought tolerance. In Chapter 4 we found that the root/shoot ratio increased with water stress but we not sure how this happens. Further studies are needed on the architecture of the roots to understand whether the roots grow deeper in search of water or they produce finer roots to maximise water absorption. In addition, it may also be important to assess in terms of proportion the sensitivity of both shoot growth or root growth under water stress.

This PhD has shown that $\delta^{13}C$ has potential to be used in breeding for drought resistance in triticale and probably other small grain crops. The evaluated genotypes of triticale and cowpea showed tolerance to drought stress under semi-arid conditions. Agbeacon for triticale and IT00K-1263 for cowpea were identified as the most promising genotypes and hence their adoption in the smallholder farming system could be a step towards adapting to future warmer and drier climates

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9 Appendix 1: Fig. 9.1

Fig. 9.1: Photo taken from the field showing the rain gauges, sprinklers and Diviner 2000 access tubes