Agronomic, Physiological, and Proteomic Characterization of Three Improved Varieties of Maize (*Zea mays* L.)

A Contribution to Maize Breeding Programs of Mozambique

Dizimalta dos Santos Fernando Miquitaio

Dissertation presented to obtain the Ph.D degree in Molecular Biosciences

Instituto de Tecnologia Química e Biológica António Xavier | Universidade Nova de Lisboa

Oeiras,
May, 2020

Oeiras, May, 2020

Agronomic, Physiological, and Proteomic Characterization of Three Improved Varieties of Maize (*Zea mays* L.)

A Contribution to Maize Breeding Programs of Mozambique

Dizimalta dos Santos
Fernando Miquitaio

ITQB-UNL | Av. da República, 2780-157 Oeiras, Portugal
Tel (+351) 214 469 100 | Fax (+351) 214 411 277

www.itqb.unl.pt

Agronomic, Physiological, and Proteomic Characterization of Three Improved Varieties of Maize (*Zea mays* L.)

A Contribution to Maize Breeding Programs of Mozambique

Dizimalta dos Santos Fernando Miquitaio

Dissertation presented to obtain the Ph.D degree in Molecular Biosciences
Instituto de Tecnologia Química e Biológica António Xavier | Universidade Nova de Lisboa

Oeiras, May, 2020

Ph.D. Supervisors:

Dr. Isabel Alexandra Aguiar de Abreu (Supervisor)

Principal Investigator

Head of the Proteome Regulation in Plants Lab (ITQB NOVA)

Prof. Maria Margarida Oliveira (Co-Supervisor)

Associate Professor with Habilitation at ITQB NOVA

Head of the Plant Functional Genomics Lab

Ph.D. Thesis Advisory Board:

Dr. Corina Carranca

Instituto Nacional de Investigação Agrária e Veterinária (INIAV)

Prof. Joaquim Miguel Costa

Instituto Superior de Agronomia (ISA)

The work presented in this Thesis was mainly performed at:
Proteome Regulation in Plants Lab (PRPLANTS)
Instituto de Tecnologia Química e Biológica António Xavier
Universidade NOVA de Lisboa
Avenida da República, Estação Agronómica
2780-157, Oeiras
Portugal
Mass spectrometry tests were performed at:
UniMS ITQB-iBET
Avenida da República, Estação Agronómica
2780-157, Oeiras
Portugal
Field experiments were performed at:
Estação Agrária de Sussundenga, Manica province, Mozambique and
Estação Agrária de Rotanda, Manica province, Mozambique
Instituto de Investigação Agrária de Moçambique (IIAM)
Centro Zonal Centro, Estrada Nacional nº6, Chimoio, Manica province,
Mozambique

The student, **Dizimalta dos Santos Fernando Miquitaio**, developed is Ph.D. studies with the financial support of Fundação para Ciência e Tecnologia (FCT) through the Ph.D. fellowship:
SFRH/BD/128391/2017

The Ph.D. candidate also received financial support from the PGCD (Graduate Programme Science for Development) during the initial stages of his Ph.D. classes in Cabo-Verde.

“Happy is the man who finds wisdom (…). To gain it is better than gaining silver, and having it as profit is better than having gold”

(Proverbs 3:13,14 *New World Translation of the Holy Scriptures*)

To Cândida, Liliane, and Aléxia

Agradecimentos (Acknowledgements)

Começar e acabar o doutoramento foi provavelmente o desafio mais marcante da minha vida. Por isso gostaria de agradecer a todos os que directa ou indirectamente me apoiaram ao longo destes quatro anos.

- Em primeiro lugar agradeço ao meu Deus, Jeová, a quem devo a minha vida e tudo o que consegui alcançar.

- À minha orientadora, Isabel Abreu, por ter-me aceitado incondicionalmente no seu laboratório. Obrigado por tudo o que me ensinaste ao longo destes anos. Por teres acreditado em mim, por teres sido uma orientadora sempre presente e acima de tudo compreensiva. Se tivesse de repetir tudo, escolhia-te de novo como orientadora!

- À minha co-orientadora, Professora Margarida Oliveira, pelos conselhos e orientação que me deste na escolha do grupo. Mesmo muito ocupada, a tua porta sempre esteve aberta para ajudar-me no que fosse necessário. Contigo aprendi que os desafios da vida não nos devem impedir de sorrir sempre.

- À Universidade Pedagógica em Moçambique, por terem autorizado a continuação dos meus estudos em Portugal.

- Ao Dr. Magalhães Amade Miguel, investigador principal do IIAM, pelo apoio material e técnico para a materialização dos ensaios de campo.

- Ao José Simbe, técnico do IIAM, pela total entrega e dedicação na condução dos ensaios de campo. O teu profissionalismo foi simplesmente brilhante.

- À Inês Luís. Nem sei por onde começar. Tu ensinaste-me praticamente tudo o que sei sobre as técnicas da proteómica. Não me esqueço das inúmeras vezes em que gastaste dias inteiros a ensinar-me as técnicas e os fundamentos teóricos por detrás delas. Dei-te imenso trabalho!

- Ao Bruno. Foi com a tua ajuda que a espetrometria de massa deixou de ser “um bicho de sete cabeças” para mim. Obrigado pela tua amizade e pelos muitos conselhos e dicas que me deste para o meu progresso científico.

- À Valéria, por teres sido companheira de batalha. Obrigado pela força e pelas coisas que me ensinaste.

- À Margarida Rosa, pela amizade, simpatia, e pelos contributos para a melhoria do meu trabalho. Fizeste-me sentir bem-vindo ao laboratório desde o primeiro dia.

- À Helena Sapeta, pelas excelentes sugestões para a melhoria do meu trabalho. Abriste-me os olhos para muitas coisas.

- Aos demais colegas do GPlants, pelas várias sugestões e pela força. Eu sei que nem sempre me compreenderam, por isso obrigado por mesmo assim me terem apoiado sempre que precisei de ajuda.

- Ao pessoal técnico e administrativo do ITQB NOVA. Sem o vosso apoio não chegaria ao fim desta batalha. Um obrigado especial ao Hugo Matias, não apenas pelo apoio na montagem dos ensaios, mas também pelas boas sugestões técnicas. A tua veia de Engº Florestal foi fundamental.

- Aos membros do comité de tese, a Drª. Corina Carranca pelas contribuições científicas tão valiosas. Ao Prof. Joaquim Miguel Costa, pelo seu tempo valioso gasto a ensinar-me a usar o LI-COR, pelas ricas sugestões, e por me teres tratado como um amigo e não como aluno, embora eu o fosse.

- À coordenação do PGCD, particularmente a Joana Sá, Patrícia Beldade, Carla Semedo, e Inês Pires, pelo apoio incondicional.

- Aos colegas do PGCD, em particular aos da terceira edição. Foi convosco que reaprendi a ser aluno, a respeitar as diferenças de opinião, a trabalhar em equipa e a ser “mano”.

- Aos meus familiares e amigos em Moçambique, o meu muito obrigado pelo constante apoio moral.

- Aos meus amigos em Portugal, pelo apoio emocional e os bons momentos que passamos juntos. Espero poder recebê-los em Moçambique um dia destes!

- À minha família, em particular a minha esposa, Cândida. Eu sei o quanto foi difícil para ti largar a nossa casa em Moçambique para me apoiar nesta batalha em Portugal. Obrigado por teres resistido até ao fim ao meu lado. À minha filha Liliane, pelos abraços e beijinhos fartos que me deste ao longo destes anos. À minha filha Aléxia, por trazeres mais luz à minha vida. Tú és o verdadeiro triunfo deste doutoramento 😊.

Resumo geral

O milho é a cultura alimentar mais importante em Moçambique. Contudo, a sua produção é muito baixa e ciclicamente afectada por stress abiótico tais como secas recorrentes principalmente em zonas áridas e semi-áridas, assim como por cheias e ciclones. O stress biótico causado por pestes e doenças também afecta a produção de alimentos. Todos estes constrangimentos levam à constante insegurança alimentar e a necessidade de apoio humanitário por parte de entidades locais ou internacionais para o fornecimento de alimentos.

Há várias estratégias em curso em Moçambique para se ultrapassar este problema sendo uma das prioridades, a produção de variedades de milho mais resilientes à seca.

O trabalho apresentado nesta Tese Doutoral teve por objectivo gerar conhecimentos para apoiar os programas nacionais de melhoramento do milho em Moçambique. O trabalho foi desenvolvido como um complemento do esforço nacional para gerar variedades de milho mais resistentes à seca. Para este efeito, combinámos três abordagens de caracterização das plantas: ao nível agronómico, fisiológico e proteómico, tendo como foco explorar características relacionadas com a resistência à seca em três variedades Moçambicanas de milho, de polinização aberta, nomeadamente, Matuba, ZM309 e ZM523 e ainda incluindo o genótipo B73 como controlo.

Numa primeira fase fez-se a análise da produção de grão em condições edafo-climáticas locais por dois anos consecutivos com ensaios agronómicos em duas Estações Agrárias localizadas no distrito de Sussundenga, província de Manica. Apenas as variedades de Moçambique foram analisadas nesta fase. Foram testados dois diferentes sistemas produção, nomeadamente, a monocultura e a consorciação com feijão frade, *Vigna unguiculata* L. (localmente

conhecido por feijão “nhemba”), ambos com ou sem aplicação de fertilizante mineral (NPK). No geral, verificou-se que, em comparação com a monocultura simples, a consorciação e a aplicação de fertilizante mineral tendencialmente contribuíram para o aumento e estabilidade da produção de milho. Estes resultados realçaram a necessidade de reforçar-se o investimento na fertilização e na optimização de práticas agrícolas locais como a consorciação, que em determinadas condições podem contribuir para aumentar e estabilizar a produção ao longo do tempo.

Em paralelo com os ensaios de campo, foram realizadas experiências em ambiente controlado para avaliar respostas ao nível fisiológico e proteómico, potencialmente relacionadas com resistência a seca. Neste caso, além das variedades de Moçambique, foi introduzido o bem caracterizado milho B73 como genótipo controlo. Verificou-se que em stress hídrico as plantas reduziram a área foliar bem como a abertura dos estomas (reduções entre 42.4 a 58.6%) e enrolaram as folhas (principalmente em ZM309 e ZM523). Todas estas características são conhecidas pelo seu efeito na redução da perda de água por transpiração. Também se verificou que em seca severa, a biomassa da raiz em ZM523 era superior aos restantes genótipos, o que aumentaria a sua capacidade de captura da água existente no solo.

Uma parte importante do trabalho centrou-se na caraterização proteómica de todos os genótipos, para encontrar padrões moleculares potencialmente relacionados com a resistência a seca. Os dados de proteómica obtidos por técnicas de espectrometria de massa de última geração, revelaram que no geral os níveis das enzimas da fotossíntese C4, bem como os da RuBisCO activase, da subunidade maior da RuBisCO e das subunidades da ATP sintase (alfa, beta e gama) foram reduzidos em seca severa, facto que se harmoniza com os baixos níveis de assimilação de CO_2 decorrentes da menor abertura dos estomas.

Estas reduções também são apontadas como uma estratégia das plantas para reduzir gastos desnecessários de energia e evitar a formação de espécies reactivas de oxigénio. Um dos aspectos interessantes deste estudo é que embora o desempenho fisiológico das plantas tenha reduzido com o stress hídrico, as proteínas do PSII e PSI aumentaram. Consistente com o facto de que o stress hídrico é geralmente acompanhado do aumento da temperatura, observou-se ainda a subida do teor em proteína HSP70. O aumento em HSP70 pode ajudar a manter a estabilidade proteica em condições de secura nestes genótipos.

Os dados da presente Tese Doutoral constituem uma abordagem preliminar a estas variedades Moçambicanas de milho e naturalmente não esgotam a caracterização possível do seu perfil de resistência a seca. Assim, sugerimos um estudo mais aprofundado de caracterização fisiológica das variedades nas condições edafo-climáticas das zonas áridas e semi-áridas de Moçambique. Tal estudo poderia gerar conhecimento de mais directa aplicação por parte de melhoradores e agricultores locais.

Esta tese ainda inclui uma discussão final considerando as possíveis soluções de natureza sócio-económica para mitigar o problema da insegurança alimentar em contexto moçambicano. Entre as medidas apresentadas destacam-se o aumento do investimento no sector agrícola, por via da massificação do uso de sementes melhoradas, uso controlado de fertilizantes e implementação de estratégias de baixo custo que permitam a recolha e conservação de água, garantindo disponibilidade em épocas de crise.

Em conclusão, este trabalho abriu novas perspectivas para investigação e melhoramento no sector agrícola apoiando a caracterização dos materiais vegetais depositados no banco nacional de germoplasma de Moçambique através de abordagens fisiológicas e proteómicas.

General abstract

Maize is the most important food crop in Mozambique. However, maize production is low and is cyclically impaired due to abiotic stresses such as recurrent droughts (particularly in the arid and semi-arid areas), soil infertility, floods, and cyclones events. Biotic stresses, such as pests and diseases, also affect food production. Altogether, these constraints lead to food insecurity and the need for humanitarian food aid from local and international communities.

Several strategies are currently in place to overcome this situation, with one of the priorities being the generation of more drought-resilient maize varieties.

The work contained in this Ph.D. Thesis aimed at generating knowledge that can be incorporated in the national maize breeding programs. It was developed as part of the Mozambican national effort to deliver maize varieties that are more resistant to drought stress. To achieve this goal, we have combined agronomic, physiological, and proteomic approaches, to explore drought-resistance features in three commonly used Mozambican open-pollinated maize varieties, Matuba, ZM309, and ZM523, and further including B73 as control variety.

The work began with the assessment of production in rainfed conditions in two agronomic experimental sites of Sussundenga district in Mozambique for two consecutive years. Only Mozambican varieties were evaluated at this stage. We tested different cropping systems: monocropping and intercropping with cowpea *Vigna unguiculata* L. (locally known as “feijão nhemba”), with or without the supply of mineral fertilization (NPK). In general, intercropping together with mineral fertilizer tended to contribute to increasing maize production, as compared to monocropping. Thus, this research in field conditions supported the idea that the investment in mineral fertilization and the optimization of local

agronomic practices, such as intercropping, may contribute to increase maize yields and to stabilize production at certain conditions.

In parallel with the field tests, controlled experiments were conducted to assess the physiological and proteomic responses of four maize genotypes in different irrigation regimes, aiming at exploring features related to drought resistance. In these experiments, we included the well-characterized maize B73 as control genotype. We found at water limiting conditions, a lower stomatal opening (42.4 to 58.6% reduction), leaf rolling (mainly in ZM309 and ZM523), and reduced leaf area, as compared to well-watered. All these traits were listed as strategies to reduce water loss by transpiration. Additionally, compared to other genotypes, root biomass accumulation was higher in ZM523 under severe drought, which is associated with increased water uptake capacity.

We have further investigated the proteomic profiles of the maize genotypes, aiming at finding molecular signatures eventually related to drought resistance. Proteomic data generated through cutting-edge mass spectrometry techniques were obtained, showing that, in general, severe drought repressed the levels of C4-photosynthesis enzymes, as well as of RuBisCO activase, RuBisCO large chain, and ATP synthase subunits (alpha, beta, and gamma). These observations were consistent with the lower levels of CO_2 assimilation resulting from the reduced stomatal opening. These reductions are considered as strategies of energy conservation and ways to reduce the formation of reactive oxygen species. Interestingly, although displaying lower photosynthetic performance in severe drought, the genotypes response to this stress was to increase content in PSII and PSI proteins. Also, consistent with the fact that high temperatures often occur associated with drought, HSP70 levels were found to increase with drought. HSP70 proteins could help to maintain protein stability in drought conditions in these genotypes.

The data presented in this Ph.D. Thesis provides a preliminary characterization of these Mozambican maize genotypes, but obviously could not fully profile their drought resistance behavior. Therefore, we suggested a more exhaustive physiological characterization to take place in arid and semi-arid areas in Mozambique. This could deliver closer-to-application knowledge for local breeders and farmers.

This thesis also comprehends a final discussion considering possible solutions at the socio-economic level to overcome food insecurity in Mozambique. The increase of public and private investment in the agriculture sector by stimulating the use of improved seeds, fertilizer, and cheaper alternatives and techniques to collect and store water to use in water-scarcity periods was considered. Another example mentioned is the need to invest in nutritional education, especially targeting women.

In conclusion, this work aims to have opened new avenues of agriculture research and improvement, supporting the characterization of plant materials deposited in the national germplasm bank in Mozambique with physiological and proteomic data.

List of the most used abbreviations

ETR – electron transport rate

FC – field capacity

IIAM - Instituto de Investigação Agrária de Moçambique (Agricultural Research Institute of Mozambique)

OPV- open-pollinated variety

PSII – photosystem II

PSI – photosystem I

ROS – reactive oxygen species

RWC – relative water content

WUE – water-use efficiency

Key-words

Agronomic performance, B73, CO_2 assimilation, C4-photosynthesis, drought, fertilizer application, intercropping, maize, maize breeding programs, Matuba, physiological characterization, proteomic characterization, *Zea mays* L., ZM309, ZM523.

Table of contents

Chapter I. General Introduction 1

1.1. Maize in the world: general characterization 3

1.2. Origin and distribution of maize 5

1.3. The taxonomy and botany of maize 7

1.4. Characterization of agriculture in Mozambique: opportunities and challenges 10

1.5. Maize production in Mozambique 18

1.6. The profile of nutrition in Mozambique 21

1.7. The agroecological requirements of maize production 25

1.8. Types of maize varieties 27

1.9. Brief description of the maize genotypes used in the present work 29

1.9.1 Matuba 29

1.9.2 ZM309 29

1.9.3 ZM523 29

1.9.4 B73 30

1.10 Main goals, hypothesis and thesis outline 31

Chapter II. Characterization of the agronomic performance of the Mozambican maize varieties under study 37

Abstract 39

2.1. Introduction 41

2.2. Material and Methods 46

2.2.1 Field experiments in Mozambique 46

2.2.1.1 The 2017/2018 cropping season experiments 46

2.2.1.2 The 2018/2019 cropping season experiments 47

2.2.4 Soil sampling and analysis 48

2.2.5 Weather conditions in both cropping seasons 48

2.2.6 Statistical analysis 49

2.3. Results and discussion 49

2.3.1 Soil analysis and climatic characteristics in both cropping seasons....49

2.3.2 Crop yield assessment in Sussundenga and Rotanda in the 2017/2018 season....50

2.3.3 Crop yield assessment in Sussundenga and Rotanda in 2018/2019 season....56

2.4. Conclusion, recommendations and future studies....63

Acknowledgments....65

Supplementary Data....65

References....66

Chapter III. Characterization of maize growth and physiological responses induced by different water treatments in controlled environments.... 73

Abstract....75

3.1 Introduction....76

3.2. Material and Methods....79

3.2.1 Growth and biomass production at maturity stage in greenhouse conditions....79

3.2.2 Growth, biomass production, and physiological evaluation at seedling stage in growth chamber conditions....80

3.2.3 Growth and biomass determination....80

3.2.4 Determination of the water field capacity....81

3.2.5 Gas-exchange measurements and chlorophyll fluorescence analysis....81

3.2.6 The light response curves....83

3.2.7 Relative water content determination....83

3.2.8 Statistical analysis....83

3.3. Results and Discussion....84

3.3.1 Growth and biomass production at the maturity stage in greenhouse conditions....84

3.3.2 Growth and biomass production at seedling stage under growth-chamber conditions....86

3.3.3 Leaf water status and water loss by evapotranspiration in growth chambers 90
3.3.4 Leaf gas exchange of the instantaneous measurements 93
3.3.5 Chlorophyll *a* fluorescence parameters 97
3.3.5 Gas exchange parameters of light response curves 99
3.3.7 Conclusions 111
Acknowledgments 112
Supplementary Data 113
References 113
Supplemental figures 119
Chapter IV. Proteomic features governing drought tolerance in the Mozambican maize varieties under study 121
Abstract 123
4.1 Introduction 124
4.2. Photosynthesis: Carbon Reactions 126
4.2.1 Ecology and geological history of the C4 pathway 132
4.2.2 The biochemical subtypes of C4 photosynthesis 134
4.3. Basic introduction to Liquid Chromatography-Mass spectrometry (LC-MS) 136
4.3.1 Liquid chromatography 137
4.3.2 Mass spectrometry (MS) 138
4.3.2.1 Components of a mass spectrometer 140
4.3.2.2 Analyte ionization 140
4.3.2.3 Mass analysis 142
4.3.2.3.1 Quadrupole mass analyzer 142
4.3.2.3.2 The Triple-quadrupole instrument for MS.MS 143
4.3.2.3.3 The TOF Mass Analyzer (Time-of-flight instruments) 143
4.3.2.3.4 Hybrid instruments 144
4.3.2.4 Detectors 145

4.3.3 Data-dependent analysis (DDA) and Data-independent acquisition (DIA) and Sequential Window Acquisition of all Theoretical Fragment on Spectra (SWATH) acquisition methods.145

4.4. Material and Methods 147

4.4.1 Leaf tissue material and growth conditions 147

4.4.2 Sample preparation and protein extraction 147

4.4.3 Protein digestion using FASP protocol 148

4.4.4 Liquid Chromatography tandem MS (LC-MS/MS) analysis..150

4.4.5 SWATH data acquisition, MS/MS data and statistical analysis 151

4.5. Results and discussion 152

4.5.1 Principal component analysis 152

4.5.2 SWATH-MS quantitative proteomics analysis 160

4.5.2.1 The levels of the Photosynthesis related proteins: CO_2 assimilation enzymes..160

4.5.2.2 ROS-elimination enzymes 166

4.5.2.3 PSII and PSI Electron transport chain proteins..............170

4.5.3 Conclusion and recommendations for future studies 178

Acknowledgments 179

Supplementary Data 179

Supplemental figures 188

Chapter V. General overview, conclusions, and recommendations . 199

5.1 Introduction 201

5.2. General overview of the work and discussion.............................202

5.2.1 The agronomic performance in field conditions.....................202

5.2.2 The physiological and proteomic characterization 203

5.3. Strengthening Mozambique research to support breeders.........206

5.4 The economic and social challenges of maize production..........207

References 210

List of figures

Figure 1.1 - Morphology of teosinte and maize. Differences in plant morphology between teosinte and maize are shown in (A), while differences in ear morphology are shown in (B). Teosinte plants have several branches with multiple ears on each branch and tassel at the tip of the branch; maize plants have few branches with a single ear on each branch and tassel at the tip of the branch. Teosinte ears have few grains implanted in fruit cases, while maize ears have many grains with exposed fruit cases Source: Jian et al. (2019). 5
Figure 1.2 - Morphology of maize. Adapted from Larkins (2017). 9
Figure 1.3 – Representation of maize male (pollen grain) and female (embryo sac) gametophytes in the maize life cycle. A potential pollen tube entry path is indicated in the embryo sac image. ac (antipodal cells); int (integument); nu (nucellus); sy (synergids). Source: Larkins (2017). 10

Figure 2.1 - A legume plant root showing nodules attached to the roots. Source: Flynn & Idowu (2015). 43
Figure 2.2 - Effect of different cropping systems (monocropping and intercropping) in total grain weight per plot (A), the weight of 1000 grains (B), cob weight per plot (C), Plant fresh weight per plot (D) in the 2017/2018 cropping season. Sus (Sussundenga); Rot (Rotanda); M (monocropping); I (intercropping). Bars are an average of three plots±SE. No statistical differences among varieties were found within cropping systems and locations (one-way ANOVA, *p*<0.05). 52
Figure 2.3 - Effect of different cropping systems (monocropping and intercropping) in number of cobs (A), number of grain rows per cob (B), Plant height (C), Root length (D) in the 2017/2018 cropping season. Sus (Sussundenga); Rot (Rotanda); M (monocropping); I (intercropping). Bars are an average of three plots±SE. Different letters represent significant differences within cropping systems and locations (one-way ANOVA, Tukey test, *p*<0.05). 53
Figure 2.4 - Effect of different cropping systems and mineral fertilizer application in total grain weight per plot (A), weight of 1000 grains (B), Cob weight (C), Plant fresh weight (D) in the 2018/2019 cropping season. Sus (Sussundenga); Rot (Rotanda); M (monocropping); I (intercropping); F (fertilizer application). Bars are average of three plots±SE. No statistical differences amongst varieties were found within cropping systems and locations (one-way ANOVA, *p*<0.05). 58
Figure 2.5 - Effect of different cropping systems in a number of cobs (A), number of grain rows per cob (B), Plant height (C), Root length (D) in the 2018/2019 cropping season. Sus (Sussundenga); Rot (Rotanda); M (monocropping); I (intercropping); F (fertilizer application). Bars are an average of three plots±SE. No statistical differences amongst varieties were found within cropping systems and locations (one-way ANOVA, *p*<0.05).. 60

Figure 3.1 - Growth and biomass production of the maize genotypes in different watering regimes in greenhouse conditions. The aboveground fresh weight (A), belowground fresh weight (B), aboveground dry weight (C), belowground dry weight (D), plant height (E), and plant stem diameter (F) are shown. The bars are means ±SE (n=7). Different letters indicate statistical significance of the differences among genotypes within the treatments (Tukey test, $p<0.05$). 85
Figure 3.2 - The impact of different watering regimes in plant biomass, leaf area, and leaf water status under growth chamber conditions. Shoot fresh weight (A), root fresh weight (B), shoot dry weight (C), root dry weight (D), 3rd leaf area (E), and Relative Water Content (RWC) (F) are shown. The bars are means ±SE (n = 5, except for the leaf area that is n=10). Different letters indicate statistical significance of the differences among genotypes within treatments (Tukey test, $p<0.05$). 87
Figure 3.3 - The impact of different watering regimes in water loss by evapotranspiration. The levels of water loss under well-watered conditions (75% FC) (A), moderate drought (40% FC) (B), and severe drought (25% FC) (C) are presented. The dots are means ±SE (n=5). Different letters indicate statistical significance of the differences among genotypes within days after sowing (Tukey test, $p<0.05$). 91
Figure 3. 4 - Leaf gas exchange and chlorophyll a fluorescence parameters of the instantaneous measurements for all water treatments. Net photosynthesis rate (A_n) (A), stomatal conductance to water vapor (gs) (B), PSII photochemistry (Φ_{PSII}) (C), Electron Transport Rate (ETR) (D), Intrinsic Water-Use Efficiency (WUEi, A_n/gs) (E), and Intercellular CO_2 (Ci) (F) are shown. The bars are means ±SE (n=10). Different letters indicate statistical significance of the differences among varieties within treatments (Tukey test, $p<0.05$). The data shown are the mean results of the three independent experiments. 95
Figure 3.5 - The light response curves of all maize genotypes in well-watered conditions. The net photosynthesis rate (A_n) (A), stomatal conductance to water vapor (gs) (B), PSII photochemistry (Φ_{PSII}) (C), Electron Transport Rate (ETR) (D), Intrinsic Water-Use Efficiency (WUEi, A_n/gs) (E), and Intercellular CO_2 (*Ci*) (F) in response to light variation are shown. The dots are means ±SE (n = 3). Different letters indicate statistical significance of the differences among genotypes within light intensity (Tukey test, $p<0.05$). 100
Figure 3.6 - The light response curves of all maize genotypes in moderate drought conditions. The net photosynthesis rate (A_n) (A), stomatal conductance to water vapor (gs) (B), PSII photochemistry (Φ_{PSII}) (C), Electron Transport Rate (ETR) (D), Intrinsic Water-Use Efficiency (WUEi, A_n/gs) (E), and Intercellular CO_2 (*Ci*) (F) in response to light variation are shown. The dots are means ±SE (n = 3). Different letters indicate statistical significance of the differences among genotypes within light intensity (Tukey test, $p<0.05$). 102
Figure 3. 7 - The light response curves of all maize genotypes in severe drought treatment. The net photosynthesis rate (A_n) (A), stomatal conductance to water vapor (*gs*) (B), PSII photochemistry (Φ_{PSII}) (C), Electron Transport Rate (ETR) (D), Intrinsic Water Use Efficiency (WUEi, A_n/gs) (E), and Intercellular CO_2 (*Ci*) (F) in response to light variation are shown. The dots are means ±SE (n = 3).

Different letters indicate statistical significance of the differences among genotypes within light intensity (Tukey test, *p*<0.05). 104

Figure 4.1 **-** The representation of the carbon fixation and oxygenation reactions of RuBisCO. The C3 carbon fixation cycle is represented at the left and the photorespiratory cycle at the right. ATP, adenosine triphosphate; CH_2O, carbohydrate; RuBP, ribulose 1,5 bis-phosphate; PGA, 3-phosphoglycerate (a 3-carbon compound); PG, phosphoglycolate (a 2-carbon molecule) (source: Kellogg, 2013). 128
Figure 4.2 **-** Diagrammatic representation of the C4-photosynthetic cycle (Luis et al., 2016). 130
Figure 4.3 **-** Representation of the basic MS-based proteomics workflow. A is the Sample preparation where proteins are extracted from tissues or cells are digested into peptides using proteases such as trypsin. B: Liquid chromatography-mass spectrometry: peptides are separated by high-performance liquid chromatography (HPLC) and electrosprayed directly into the mass spectrometer. Peptide ions are measured at high resolution in a data-dependent mode: after each full MS scan, the most intense peptide ions are fragmented to generate MS/MS spectra. C: Spectra interpretation: the full MS spectra provide information about the peptide mass, intensity, presence of a PTM, and stable isotope pairs. The mass of each fragmented peptide together with its fragment ion pattern, is searched against databases (for example, Uniprot database) for peptide identification and bottom-up protein assembly (Source: Hein, 2014). 139
Figure 4.4 **-** Representation of the essential components of mass spectrometers (Wilson & Walker, 2010). 140
Figure 4.5 **-** Electrospray ionization source (ESI). The ESI creates very small droplets of a solvent-containing analyte by atomization or nebulization as the sample is introduced into the source through the fine hollow needle capillary. The solvent evaporates in the high-vacuum regions as the spray of droplets enters the source. These may have multiple charges depending on the availability of ionizable groups (Wilson & Walker, 2010). 141
Figure 4.6**-** Schematic representation of the quadrupole mass analyzer (Niessen, 2006). 142
Figure 4.7 - A triple-quadrupole instrument for MS-MS. An ion of a particular m/z value is selected in the first quadrupole, Q1. However, instead of being detected, it passes through the second quadrupole, Q2, where it is subjected to collision with the collision gas. The Q3 acts like a second quadrupole mass spectrometer, MS-2, to scan m/z to obtain a spectrum of the fragment ions. The collision cell, Q2, is frequently a radio frequency (RF)-only quadrupole containing the appropriate collision gas. No mass filtering occurs here; the RF merely constrains the ions to allow a higher number of collisions to occur. The fragmentation that takes place here is at the peptide bond and one of the fragments will retain the

charge, resulting in either a y-series or a b-series ion (Niessen, 2006; Wilson & Walker, 2010). 143
Figure 4.8 **–** Representation of the time-of-flight (TOF). The ions move into the flight tube, where the lighter ions travel faster than the heavier ions to the detector. If the ions are accelerated with the same potential at a fixed point and fixed initial time, the ions will separate according to their mass to charge ratios. Following, time of flight is converted to mass (Source: Wilson & Walker, 2010). 144
Figure 4.9 **-** The schematic representation of a Q-TOF instrument. Ranges of m/z values are selected (Q), and peptides are fragmented (Collision cell), followed by the acquisition of the fragments in a time-of-flight mass spectrometer (Adapted from Aebersold & Mann, 2016). 144
Figure 4.10 **-** Principal Component Analysis of maize genotypes under well-watered conditions, using the 221 detected proteins. The different genotypes are identified by different colors. 154
Figure 4.11 **-** Principal Component Analysis for B73 genotype, using the 221 proteins detected. Different water treatments are identified by different colors and symbols. 154
Figure 4.12 **-** Principal Component Analysis for Matuba, using the 221 proteins detected. The different water treatments are identified by different colors and symbols. 155
Figure 4.13 - Principal Component Analysis for ZM309, using the 221 proteins detected. The different water treatments are identified by different colors and symbols. 156
Figure 4. 14 **-** Principal Component Analysis for ZM523, using the 221 proteins detected. The different water treatments are identified by different colors and symbols. 156
Figure 4.15 **-** Principal Component Analysis for B73, using only CO_2 assimilation related enzymes. The different water treatments are identified by different colors and symbols. 157
Figure 4.16 - Principal Component Analysis for Matuba, using CO_2 assimilation related proteins. The different water treatments are identified by different colors and symbols. 157
Figure 4.17 **-** Principal Component Analysis for ZM309 using CO_2 assimilation related proteins. The different water treatments are identified by different colors and symbols. 158
Figure 4.18 - Principal component analysis for ZM523 using CO_2 assimilation related proteins. The different water treatments are identified by different colors and symbols. 158
Figure 4.19 - Principal component analysis for the entire set of maize genotypes under control conditions using the electron transport chain related proteins. The different genotypes are identified by different colors. 159
Figure 4.20 - Quantification of total C4-photosynthesis enzymes by SWATH-MS. Phosphoenolpyruvate carboxylase (A), NADP-Malate dehydrogenase (B),

NADP-Malic enzyme (C), pyruvate orthophosphate dikinase (D). Values represent the mean ± SE of three biological replicates. Replicates represents a pool of leaves harvested from five plants. Different letters indicate that the value is statistically significant at $p<0.05$ within field capacity. 162

Figure 4.21 **-** Quantification of total alternative C4-photosynthesis enzymes by SWATH-MS. Aspartate aminotransferase (A), Phosphoenolpyruvate carboxykinase (B). Values represent the mean ± SE of three biological replicates. Replicates represents a pool of leaves harvested from five plants. Different letters indicate that the value is statistically significant at $p<0.05$, within field capacity. 163

Figure 4.22 **-** Quantification of total RuBP carboxylation proteins by SWATH-MS. RuBisCO activase (A), RuBisCO large subunit (B), RuBisCO small subunit. Values represent the mean ± SE of three biological replicates. Replicates represents a pool of leaves harvested from five plants. Different letters indicate that the value is statistically significant at $p<0.05$, within field capacity. 164

Figure 4.23 **-** Quantification of total ROS detoxification proteins and stress-induced proteins by SWATH-MS. Catalase 2 (A), Catalase 3 (B), Peroxidase 42 (C), heat shock protein 70 kDa (D). Values represent the mean ± SE of three biological replicates. Replicates represents a pool of leaves harvested from five plants. Different letters indicate that the value is statistically significant at $p<0.05$, within field capacity. 167

Figure 4.24 – Schematic representation of the Z scheme for O_2-evolving photosynthetic organisms. The photon absorption by the reaction center chlorophylls is represented by the vertical arrows: P680 for photosystem II (PSII) and P700 for photosystem I (PSI). The P680*, which is the excited PSII reaction center chlorophyll, transfers an electron to pheophytin (Pheo). (2) On the oxidizing part of PSII, P680 oxidized by light is re-reduced by Yz, which has received electrons from the oxidation of water through the activity of the oxygen-evolving complex. (3) On the reducing side of PSII, pheophytin transmits electrons to the acceptors QA and QB, which are plastoquinones. (4) The cytochrome b6f complex transfers the received electrons to the soluble protein plastocyanin (PC), which reduces P700+ (oxidized P700). (5) The acceptor of electrons from P700* (A0), is thought to be a chlorophyll, and the following acceptor (A1) is a quinone. Then, a series of membrane-bound iron-sulfur proteins (FeSX, FeSA, and FeSB) transfers electrons to soluble ferredoxin (Fd). (6) The soluble flavoprotein ferredoxin–NADP reductase (FNR) reduces NADP+ to NADPH, which is used to fuel the Calvin cycle to reduce CO_2. The dashed line shows the cyclic electron flow around PSI (Taiz & Zeiger, 2002). 170

Figure 4.25 - Quantification of total PSII related proteins by SWATH-MS. Photosystem II CP43 reaction center protein (A), Cytochrome f (B), Photosystem II protein D1 (C), Photosystem II reaction center protein H (D), Photosystem II D2 protein (E), Cytochrome b6 (F). The Photosystem II CP43 reaction center protein binds chlorophyll and helps catalyze the primary light-induced photochemical processes of PSII. Cytochrome f and Cytochrome b6 are components of the

cytochrome b6-f complex, which mediates electron transfer between photosystem II (PSII) and photosystem I (PSI), cyclic electron flow around PSI, and state transitions. The D1/D2 (PsbA/PsbD) reaction center heterodimer binds P680, the primary electron donor of PSII, as well as several subsequent electron acceptors. The Photosystem II reaction center protein H is one of the components of the core complex of photosystem II (PSII), required for its stability and/or assembly. Values represent the mean ± SE of three biological replicates. Replicates represents a pool of leaves harvested from five plants. Different letters indicate that the value is statistically significant at $p<0.05$ within field capacity. 172

Figure 4.26 **-** Quantification of total PSI related proteins by SWATH-MS. Photosystem I reaction center subunit N (A), ATP synthase subunit alpha (B), Photosystem I P700 chlorophyll a apoprotein A1 (C), ATP synthase subunit beta (D), Photosystem I P700 chlorophyll a apoprotein A2 (E), ATP synthase subunit gamma (F). Photosystem I reaction center subunit N may function in mediating the binding of the antenna complexes to the PSI reaction center and core antenna. The Photosystem I P700 chlorophyll a apoprotein A1, and A2 binds P700, the primary electron donor of photosystem I (PSI), as well as the electron acceptors A0, A1 and FX. ATP synthase produces ATP from ADP in the presence of a proton gradient across the membrane. The ATP alpha chain is a regulatory subunit; the catalytic sites are hosted primarily by the beta subunits; the gamma chain is believed to be important in regulating ATPase activity and the flow of protons through the CF_0 complex. Values represent the mean±SE of three biological replicates. Replicates represents a pool of leaves harvested from five plants. Different letters indicate that the value is statistically significant at $p<0.05$, within field capacity. 175

Chapter I. General Introduction

1.1. Maize in the world: general characterization

Maize (*Zea mays* L.) is the most important cereal crop in the world after wheat and rice (Verheye, 2010; Fato et al., 2011; Ghatak et al., 2017). The name "maize" is originated from the Spanish word for the plant, *maiz*, but in the USA is known as *Indian corn* or corn. In South Africa, it is known as *millies*, from the Portuguese word *milho* (Verheye, 2010).

Maize is an important staple food in developing countries, particularly in Latin America and Africa, and the main ingredient for local drinks and food products. In most developing countries, maize is grown mostly as a food crop (as grain maize), while in other countries (such as USA and Brazil), this crop is important for the animal feed or ethanol production (Verheye, 2010).

The per capita consumption of maize is highest in southern Africa, where Mozambique is also included. The values reach an average of 120 kg per person per year in Lesotho, 107 kg in Malawi, and more than 80 kg in Zambia, Zimbabwe, and South Africa (Buck, 2017).

Maize crop is grown in more diverse edaphoclimatic conditions than any other crop, and vast genetic differences occur among the cultivars of maize grown in these diversified areas (Verheye, 2010).

Commercial maize is widely classified as flint, dent, flour, sweet, and pop types, basically depending on the degree of hardness of the endosperm (Verheye, 2010; Buck, 2017).

In 2017/2018, more than 1,078 million tons of maize were produced in more than 170 countries in about 191 million hectares of land, making maize an important staple food for about 1.2 billion people around the world. The top producers in 2017/2018, were the United States of America with 371.1 million tons, China with 259.07 million tons, Brazil with 82 million tons, and Argentina and Ukraine with 32 and 24 million tons, respectively. In Africa, South Africa was the largest producer, with 13.1 million tons, Nigeria was the second with 11 million tons followed by Ethiopia, Egypt, and Tanzania with 8.0, 6.4, and 5.35 million tons, respectively (USDA, 2019).

In comparison to the other continents such as North America, Asia, and Europe, maize production in Africa is very low. For example, while the average yield worldwide is approximately 5.5 tons/hectare/year, the average grain-yield in Africa is about 2.0 tons/hectare/year (Buck, 2017). Under traditional agrarian practices, maize is widely cultivated together with other (food) crops (intercropping) and is usually not fertilized (Verheye, 2010).

Maize production in Africa is continuously and severely affected by many threats, which include biotic stresses (such as insects, bacteria, viruses, nematodes, fungi), and abiotic stresses (such as drought and floods). The weaknesses also include infrastructural and socio-economic based factors such as low-quality of the seeds, low levels of mechanization, and poor post-harvest procedures (Buck, 2017).

In developing countries, tropical white maize tends to be more widely cultivated than yellow maize, which is preferred in developed countries, representing the majority of maize grown worldwide. Therefore, the world's maize production is 90% yellow maize, while in Africa, 90% of the total maize production is white maize, which highlights the preference for the last in Africa (Buck, 2017). White and yellow maize are biologically and genetically similar (Verheye, 2010). The difference in color is due to the presence of a carotene pigment in the yellow maize and its absence in tropical white maize (Anami et al., 2009).

1.2. Origin and distribution of maize

The origin of maize is controversial. It is widely accepted that modern maize evolved from teosinte (*Zea mays* ssp. *parviglumis*), originating in the Río Balsas drainage of western Mexico (Blake, 2006; Verheye, 2010).

Teosinte and the current maize differ deeply in their plant and inflorescence architecture. For example, teosinte plants typically have many long branches, each with multiple small ears along their length and tassels at their tip. On the opposite, maize plants typically have one or two short branches, each with a single ear at its tip. A teosinte plant can have several hundred ears, each with only 10 grains, while maize, in general, has only two ears, each with a vast number of grains (Fig. 1) (Jian et al., 2019).

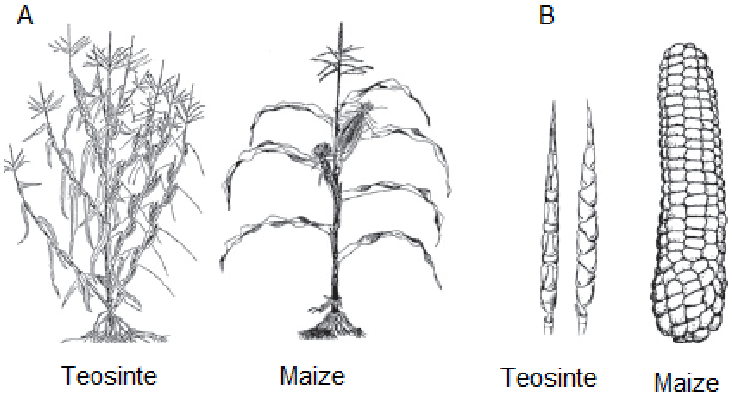

Figure 1.1 - Morphology of teosinte and maize. Differences in plant morphology between teosinte and maize are shown in (A), while differences in ear morphology are shown in (B). Teosinte plants have many branches with several ears on each branch and tassel at the tip of the branch; maize plants have few branches with a single ear on each branch and tassel at the tip of the branch. Teosinte ears have few grains implanted in fruit cases, while maize ears have many grains with exposed fruit cases. Source: Jian et al. (2019).

Teosinte and maize belong to the same genus called *Zea*, which consists of five species: 1) perennial diploid (2n = 20) *Z. diploperennis*,

2) perennial tetraploid (2n = 40) *Z. perennis*, 3) annual diploid (2n = 20) *Z. luxurians*, 4) annual diploid (2n = 20) *Z. nicaraguensis*, and 5) the annual species *Zea mays*. The latter is composed of four annual diploid (2n = 20) subspecies: (i) ssp. *mays*, the domesticated maize, (ii) ssp. *mexicana*, (iii) ssp. *parviglumis*, and (iv) ssp. *huehuetenangensis*. The *Z.m*. ssp. *mexicana* and *Z.m*. ssp. *parviglumis* are most closely related to the current domesticated maize than the others (Fukunaga et al., 2005; Trtikova et al., 2017).

Hybridization is very common between domesticated maize and *Z.m*. ssp. *parviglumis*, but gene flow does not occur in the other way around, which may explain why teosintes continue to coexist even when growing in the proximity of maize populations (Trtikova et al., 2017).

The first maize cultivators shaped the genetic transformation of teosinte (Blake, 2006). The initial domestication[1] of teosinte occurred 6,000 to 7,500 years ago, possibly when people practiced agriculture to reduce the effect of annual changes in natural conditions such as rainfall, which could affect productivity. Ancient people have tried to change the natural life cycle of teosinte by planting it in more favorable places, watering and weeding it, and selecting larger and healthier seeds for replanting. All these farming practices had eventually led to its domestication (Blake, 2006; Verheye, 2010).

During thousands of years, populations used maize for several purposes, such as for food, drinks, and also to acquire building materials. Before 2500 BC, people were initially interested in selecting maize cobs with more grains, and only later they were interested in selecting cobs with larger grains. Because of the slower changes in cob

---

[1] Domestication refers to the genetic modification of teosinte, which rendered it dependent on humans for dispersal (Blake, 2006).

morphology, people were possibly more interested in increasing the number of cobs per plant after 2500 BC[2]. Also, genetic analysis of maize suggests that people were possibly selecting for increased protein and starch quality, and that modern maize plant architecture had already been selected by 2500 BC (Blake, 2006).

Maize was introduced in Africa at the beginning of the 16th century and rapidly expanded within the continent. The main drivers of the process were the Portuguese and Arab explorers in West and East Africa, from where it spread inland through the oceanic slave-trade routes, and later to Asia (Anami et al., 2009; Verheye, 2010).

Because of its wide climatic adaptability in several edaphoclimatic conditions, maize cultivation expanded rapidly, and its grain soon became a part of the local diet as an alternative of traditional root crops (such as cassava, yams, sweet potatoes) and many other small grains (Verheye, 2010).

Several reasons may explain the expansion of maize within the African continent. First, its taste was easily accepted by the local populations and, therefore, it could rapidly substitute other traditional starchy food such as sorghum and millet. Additionally, the higher yields, the husks that give protection against birds and rain, the ease to harvest and store well if properly dried, the low hard labor requirements, and its short growing season are listed as the other reasons to be considered (Verheye, 2010; Cherniwchan & Moreno-Cruz, 2019).

1.3. The taxonomy and botany of maize

Zea mays L. is an annual monocotyledonous diploid (2n = 20) belonging to the genus *Zea* and Poaceae family (Verheye, 2010).

---

[2] BC-Before Christ

Maize is a 2-3m high grass with a single solid stem (stalk) 1.4-5 cm in diameter, with clearly defined nodes and internodes. The leaves arise from the nodes, alternately on opposite sides on the stalk (Fig. 1.2) (Fato et al., 2011).

Maize has three types of root systems: primary system, secondary system, and supporting roots. The primary roots function on the nutrient's absorption from the first to the third week after germination. The secondary roots, formed above the primary root, are permanent, and their main function is water and nutrient absorption. The supporting roots originate from the first nodes above the ground surface and are also involved in water and nutrients absorption, besides providing plant support (Fato et al., 2011).

Maize is a monoecious grass with male and female flowers rising in separate inflorescences on the same plant (Fig. 1.2) (Nannas & Dawe, 2015).

Although it is self-fertile, the plant's monoecious character and protandry (male flowers mature before female flowers) ensure cross-pollination of about 90-95%. The tassel male inflorescence is a terminal panicle, with up to 40 cm long, which expands out from the enclosing leaves at the top of the stalk. The male or staminate flowers are present within spikelets on the branches. The stamens elongate at anthesis, and the pollen is released by the anthers (Verheye, 2010).

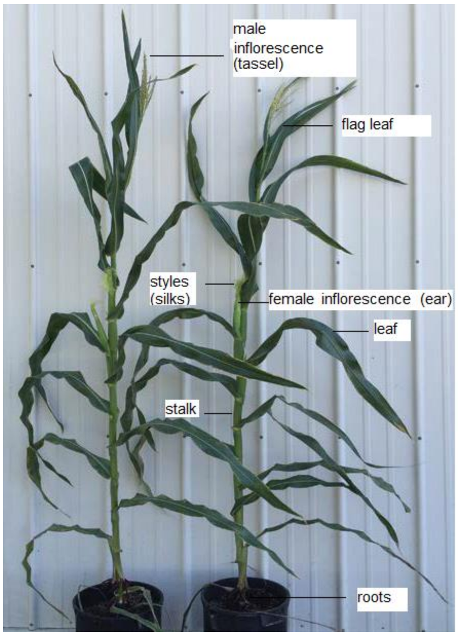

Figure 1.2 - Morphology of maize. Adapted from Larkins (2017).

Maize pollen is anemophilous, *i.e.*, dispersed by wind. Under favorable conditions, it is viable for 24 hours, while the silks are pollen receptive for a much longer time, *i.e.*, 7 to 12 days (Verheye, 2010). The female inflorescence, called an ear, develops on a short side-branch, which develops from the axil of one or more of the middle leaves (Nannas & Dawe, 2015).

An ear is a modified spike of which the central axis or cob carries paired spikelets, with one fertile flower each, in longitudinal rows. Consequently, each cob will always have an equal number of rows of grains. After pollen has fallen on receptive silk, the grain germinates and grows a pollen tube down the inside of the silk, through which pass two sperm nuclei. When they reach the ovule there, one of the two nuclei fertilizes the haploid egg to form a diploid embryo, while the second fuses with the diploid central cell to form the triploid endosperm or the nutritive tissue. Then, the ovary wall and the ovule coat fuse to form the pericarp (hull, seed-coat). The maize grain or caryopsis consists of the embryo (*i.e.,*10-13% of the grain), the endosperm, and pericarp. The grain may also differ in color, structure, and chemical composition (Fig. 1.3) (Verheye, 2010; Fato et al., 2011; Nannas & Dawe, 2015).

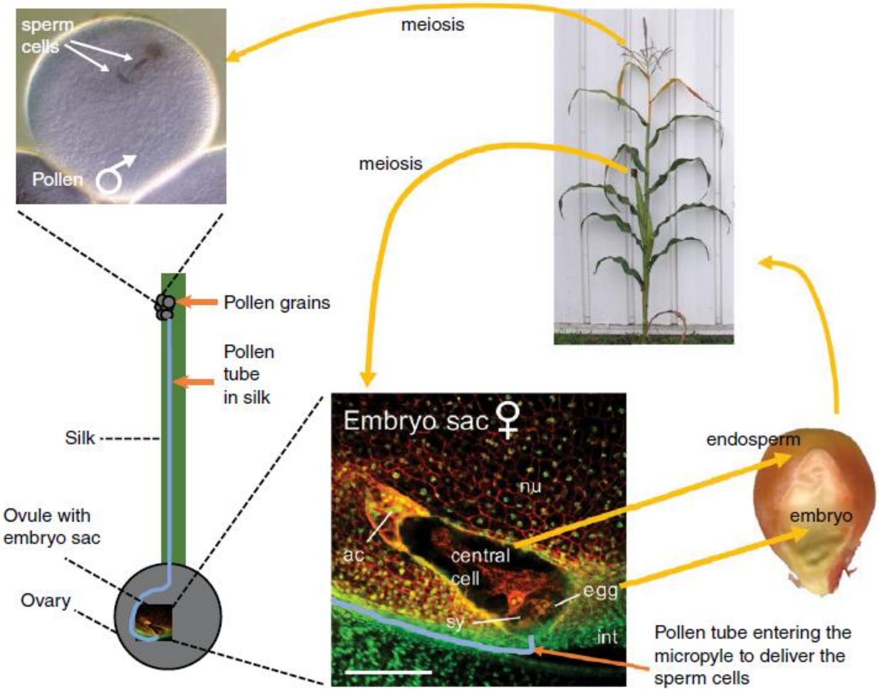

Figure 1.3 – Representation of maize male (pollen grain) and female (embryo sac) gametophytes in the maize life cycle. A potential pollen tube entry path is indicated in the embryo sac image. ac (antipodal cells); int (integument); nu (nucellus); sy (synergids). Source: Larkins (2017).

1.4. Characterization of agriculture in Mozambique: opportunities and challenges

Mozambique is a country on the eastern coast of Southern Africa that has limits: to the north, Tanzania; to the northwest, Malawi, and Zambia; to the west, Zimbabwe, South Africa, and Swaziland; to the south, South Africa; to the east, a part of the Indian Ocean designated by the

Mozambique Channel (Portal of the government of Mozambique[3], 2019).

Mozambique is a vast country with an area of 799,380 km^2, a land border of 4,330 km, and a coastline of 2,400 km with three important ports, not only for the country but also for the neighboring hinterland countries such as Zimbabwe, Zâmbia, and Malawi (PEDSA, 2011).

According to the 2017 population census, the country has a population of 27,909,798 inhabitants, of which 33.4% are living in urban areas and 66.4% in rural areas (INE[4], 2019).

Agriculture is one of the main vectors of development and is defined as the base of the economy by the Republic Constitution of Mozambique. Between 2014 to 2017, the agriculture sector contributed 23% to the Gross Domestic Product (GDP). In addition to that, agriculture employs 80% of the population, most of them in rural areas (PEDSA, 2011; FAO, 2015; Tomo & Alberto, 2016; BAD, 2018).

The agrarian sector in Mozambique has gone through several stages from the colonial era to the present. During the Portuguese occupation, Portuguese settlers were granted credit for the establishment of farmers in Mozambique, which mobilized local people as salariat workers and the introduction of new production technologies such as animal traction and plow. Large agrarian companies were established mainly in the central region, and they were dedicated to the production and exportation of sugar, tea, cotton, sisal, and copra. Peasants not involved in the companies were primarily engaged in family subsistence

---

[3] http://www.portaldogoverno.gov.mz/ (accessed in September, 2019)

[4] National Institute of Statistics of Mozambique, http://www.ine.gov.mz/ (accessed in September, 2019)

production and the surplus was used for sale. In 1970, local small scale farmers production accounted for 70 to 75% of total production (ex: maize, cassava, cotton, cashew nut) and more than 90% of primary food production of the country (Mosca, 1996). Moreover, the work system in Portuguese plantations and small and medium-sized companies depended on the forced recruiting of large quantities of seasonal and cheap labor to compensate for the weak mechanization. This forced recruitment was carried out through coordinated actions by the colonial police, local administrators and traditional authorities (Castel-Branco, 1994)

After independence, in 1975, Portuguese settlers abandoned the country, and the agrarian private sector almost disappeared, which originated breaks in the national economy. The government replaced the private sector with the establishment of public companies based in Socialist models to circumvent the dark scenario. Also, cooperatives of production based on common land ownership and collective work were created. However, deficient management and organization contributed to the failure of these sectors, and the income of the majority of the population decreased (Mosca, 1996). For example, between 1974 to 1977, national agronomic production decreased by 13% globally (Castel-Branco, 1994).

In 1985, Mozambique adopted the system of the World Bank and the International Monetary Fund, and a “shy” privatization of the companies has begun. In 1987, the government began an economic reform called “Programa de Reabilitação Económica or PRE” (Economic Rehabilitation Program). The goal was to rescue the past production levels, through intervention in the private sector, price policies, and allocation of production means (Castel-Branco, 1994).

Although the above-mentioned reform, agricultural production had fallen in Mozambique in the 1980’s, mainly because of the civil war between the regular Arm and RENAMO (Resistência Nacional Moçambicana) that devastated the country over 16 years (1976-1992). For example, agricultural production fell 30% from 1980 to 1990. The southern Africa drought of 1992/1993 further reduced crop production in Mozambique. In both situations, the deficit in production was covered by food aid sent by the international community (Tschirley et al., 1996; Brück, 1998).

The large scale supply of food aid also brought some disadvantages such as weakening production incentives, the market prices, and the government’s fiscal position, as well as enriching some people involved in food distribution and increasing the gap between rural and urban areas (Brück, 1998).

From early 1994, the supply of food aid in the market began to diminish due to the end of civil war and the opening of more significant possibilities of exploring the agricultural potential of all agroecological zones of the country (Tschirley, et al., 1996).

From the agricultural point of view, Mozambique has ten agroecological zones with different characteristics, which are defined mainly by the precipitation, evapotranspiration, and soil type (FAO, 2015; PEDSA, 2011; Silici et al., 2015). The arid and semi-arid agro-ecologic areas are found in the southern region and parts of the Zambézia and Tete provinces. The sub-humid to humid zones are mainly located in medium to high altitude areas in the central and northern part of the country where soils are more fertile. Therefore, the central and northern regions have better conditions to produce the majority of crops under the most common practices, rainfed conditions with low or non-use of fertilizers than the southern region (Tomo & Alberto, 2016).

According to the 2007 forest inventory, the country forest covers an estimated 54.8 million hectares, which corresponds to 70% of its surface area. Of this area, 26.9 million hectares are proactive forests, 13.2 million are located in forest reserves, and the remaining areas are occupied by multiple-use forests. The country also has 15 large river basins with massive potential to fostering production and productivity, nine of them shared with neighboring countries (PEDSA, 2011).

Despite all this agricultural potential, Mozambican agriculture is predominantly of the subsistence type, characterized by low levels of production and productivity (PEDSA, 2011). Also, the agricultural sector is constrained by low investments. At least in 2016, less than 10% of the country's budget was allocated to the agrarian sector (Tomo & Alberto, 2016). Also, only 10% of more than 36 million hectares of arable land is used for agriculture (PEDSA, 2011; FAO, 2015; Silici et al., 2015).

The levels of productivity are stagnant, with an increase in production levels, mainly due to the expansion of production areas. The country also recorded significant increases in production levels of emerging crops such as sesame and soybeans that have been stimulated by the increased demand in the international market (Tomo & Alberto, 2016).

More than 80% of the total cultivated area is used for primary food crops, with maize, cassava, and beans accounting for about 60% of the total area under cultivation. Horticulture occupies only 5%, and yield crops like sugar cane, cotton, tobacco, tea, and oilseeds are produced in only 6%. Most of this production is for self-consumption, and production is very low oriented to the market (PEDSA, 2011).

Many other factors are contributing to the vulnerability of the agriculture sector in Mozambique, besides the sub-use of its natural resources. Primary food production is subjected to wide variations due to the uncertain climate and recurrent droughts, particularly in semi-arid areas, and cyclones, that lead to food insecurity (Hellmuth, 2007; PEDSA, 2011), and raises the urgency of development capabilities to minimize the impacts of climate change (FAO, 2015). Although flooding events are quite often in some parts of the country, no flood-resistant crop varieties exist in Mozambique. Therefore, farmers use early-maturing varieties to circumvent the risk of crop loss due to flooding (Mabaya, 2017).

Climate change is not the only contributor to the general low production in Mozambique. It is a combination of factors. Socio-economic related challenges such as low use of fertilizers (4-5%), low use/overuse of chemicals, poor post-harvesting procedures, limited use of animal traction (11.3%), low use of improved seeds (only 10% in case of maize OPV´s, 1.8% in case of rice, less than 5% for overall hybrid varieties), fake seeds, limited production area, and biotic stresses such as pests and diseases are added to the equation (PEDSA, 2011; FAO, 2015; Come & Neto, 2017; Mabaya, 2017).

Soil degradation caused by erosion, reduction in natural soil fertility, increase in soil salinization, especially in coastal zones, and the very limited capacity for water storage in rain season for later use in scarcity season are also limiting the overall goal of increasing production (FAO, 2015).

In order to circumvent the above-mentioned challenges, the government adopted different strategies, some of them as policies. Most of these strategies were oriented to respond to the current food crisis, which raised a need for a long-term strategy for fostering agricultural development. One of the policies that called much attention was the Action Plan for the Reduction of Absolute Poverty (PARPA I&II, 2001-2009), in which one of the main pillars was to increase food production through increased access to agricultural inputs, access to bank credit especially for women living in rural areas, improvement of post-harvesting procedures, amongst others. However, by the end of its implementation, almost 10 million people were under severe poverty, food insecurity, and unemployment (PEDSA, 2011).

In 2007, the government also began the elaboration of “The Strategic Plan for the Development of the Agrarian Sector” (PEDSA), which includes a vision with a short, medium, and long-term horizon of the development of agricultural production for ten years (2011-2020). The strategy comprises four basic pillars: an increase in production and productivity; access to the market; sustainable use of resources, such as land, water, forests, and fauna; and the creation of strong agrarian institutions (PEDSA, 2011).

Some of the examples of on-ground support to agriculture development based on the above strategy took place in 2016 when the government subsidized about 720 tons of maize seed (16% of total maize seed sales), 120 tons of cowpea seed (33% of total seed sales), and three tons of rice seed (less than 1% of total rice seed sales) mostly to smallholder and emerging farmers. There were also about 3,035 agrarian extensionists in 2017 trained to deliver their knowledge to contribute for developing the agriculture sector (Mabaya, 2017).

Between 2005 to 2014, the government decided to allocate investment funds for districts (basically in rural areas) in order to create jobs, increase production, wealth, and reduce poverty. These funds were called “Fundo de Investimento a Iniciativas Locais” (Local Initiative Investment Fund) and comprised 7 million Meticais per district per year (around US$107,000). The fund management was allocated to the district governments in strict coordination with the Advisory Councils which ensured transparency in the project’s evaluation, selection, and monitoring. However, constraints regarding ambiguities in funds distributions, deficient access to the funds by women, and poor monitory, weakened the investment (Cumbe et al., 2010).

Currently, one subsidy program is promoted by the Ministry of Land, Environment and Rural Development that is called “SUSTENTA”. The program aims at stimulating the rural economy together with environmental conservation. The project has a budget of US$40 million, and it is intended to be implemented in 10 districts of Nampula and Zambézia provinces and directly benefit 125.000 rural families. With this project, the farmers receive technical assistance and means of production (fnds.gov.mz, access date: 15th of January 2020).

Also, the government is putting effort into increasing the investment to the agriculture sector, which was 3.6% of the government’s budget in 2010 (Global Nutrition Report, 2014) up to 10% in the next five years.

All the above-mentioned strategies can be considered well designed but still possess weaknesses, identified among public and private agrarian institutions, for instance, lack of capable human resources to implement the strategies, and weak coordination among all the sectors involved (PEDSA, 2011; Carrilho et al., 2013).

It is very important for the government to review all the policies, being more than just a list of intentions, put them together, and guarantee its consistency and implementation. This may include improvement of infrastructures, the agro-processing industry, expansion of irrigation technologies including the capacity of water storage, increase of incentives to private investment and access to bank credit, human capital development, and strong farmers associations (PEDSA, 2011; Carrilho et al., 2013; FAO, 2015).

More commitment effort also needs to be put in critical areas such as research and breeding, not only increasing the investment in seed and research companies but also increasing the number of breeders. These will allow an increase in the availability of better seeds that are resistant to drought, pests, and diseases, eliminate fake seeds and reduce the time currently needed for releasing new varieties. Also, the creation of the Plant Breeders' Rights law and seed policy are highlighted to incentive more researchers to join the local breeding programs (Mabaya, 2017).

One vision that is also currently often debated and defended by many agrarian economists is the need for strengthening agribusiness by better bridging the agro-industry and the farmers/farmers associations. In that partnership, the industries would supply the farmers with all the needed inputs to produce raw material, and its price would be fairly negotiated by the two parts. Also, it is necessary to strengthen the mechanisms of credit repayment by the farmers, as several cases of non-repayment occur, complicating the allocation of funds to other farmers.

Finally, infrastructural problems such as the absence or deteriorate roads and railways, lack of irrigation and storage systems, and insufficient electricity supply that makes the irrigation systems dependent on the high-cost fossil fuels, requires particular attention. This will not only foster production but also generate food trade and income, especially among small scale farmers (PEDSA, 2011).

1.5. Maize production in Mozambique

Maize is the most important cereal crop in Mozambique (Tomo & Alberto, 2016). It is produced in almost all regions of the country, being the Central and Northern regions producing a surplus. Due to recurrent drought events, the Southern region often produces less than the amount required; as a result, it has to rely on supplies from other areas, mainly outside the borders such as from South Africa (Tschirley & Abdula, 2007).

Production of maize is currently estimated to be around 0.3 - 0.9 tons per hectare, while the average of the southern African countries, where Mozambique is located, is 4.9 tons per hectare (Stevens & Winter-Nelson, 2008; Tomo & Alberto, 2016). In general, the majority of the small-scale farmers produce maize exclusively for household needs (or subsistence production), with a little surplus to sell in the vast local market (Stevens & Winter-nelson, 2008; Dias, 2013; Silici et al., 2015).

Maize is also the most common seed imported by Mozambique. The seeds are mainly imported from Zimbabwe and South Africa (Mabaya, 2017).

Maize represents about 75% of the total value of small farmers production, and 90% of this production occurs on farms under 2ha in size, with yields generally low and highly variable. Maize is generally grown under rainfed conditions, with limited use of purchased inputs such as pesticides and mineral fertilizers, as well as improved seeds (Silici et al., 2015). The land allocated to maize is about 45.9%, and most of the maize land is occupied with landraces (Kassie et al., 2012).

In 2017/2018, the Ministry of Agriculture estimated maize production at about 2.45 million tons (Source: fews.net, September 2019). In terms of market share, about 57% of the total amount of maize consumed in the country comes from the local production, 20% is imported, 11% is sold in local markets, 7% is destinated to animal feed, and 5% is exported (Tomo & Alberto, 2016).

In general, one main cropping season occurs per year, which starts in October (in south/center regions) and November (in the north region) and ends in June/July (in South/North regions). In some areas of the country, mainly in the southern and central regions, there is a second season of maize production between May and September that can be used by the farmers, particularly if the main season fails (Tomo & Alberto, 2016).

In Mozambique, maize is primarily grown for human consumption with a small percentage for animal feed. In industry, maize is used in the manufacture of starch, cornflakes, flour, sweeteners, syrups, beer, alcohol as well as in baking. The main way that maize is used is through flour used to make a type of porridge, usually called *xima, uva, uthua, ugali, sadza*, etc. Maize is also consumed in the form of *maçaroca* (cooked or roasted cob), and when dry, it can be consumed roasted. Part of the production can also be used in the preparation of beverages, which can be sweet (*Maheu*) or fermented (*uputshu, xidangwana, bwadua*, *moa, doro,* etc.) (Fato et al., 2011).

Maize production in Mozambique is impaired by the same agricultural challenges discussed above. Particular focus is being given to drought stress. For example, cereal production in the 2015/16 cropping season was below the average, particularly in drought-affected areas in southern and central Mozambique, which doubled the price of maize and also subjected 1.5 million people to a food crisis scenario (WFP, 2016).

In 2019, due to recurrent water scarcity in the southern region and the two cyclones (Idai and Kenneth) that devastated the center and some northern parts of Mozambique, food insecurity demanded more victims (fews.net, 2019).

In order to overcome constraints related to climate changes, deficient soil nutrients, and biotic stresses, science and technology are also starting to contribute with solutions, as can be seen by the increasing number of research projects that aim to address the constraints in maize production. These actions resulted in the release of maize varieties with high yield potential adapted to different agro-ecological zones (Fato et al., 2011).

Also, as discussed above, food security assurance in Mozambique depends on several developing strategies that must be implemented in a well-coordinated manner. The input of fertilizers and irrigation, and the annual production of two or more crops per plot, are possible strategies to increase maize production.

1.6. The profile of nutrition in Mozambique

Despite the central role of agriculture within the population, the nutritional situation in Mozambique remains precarious, with 44% of children under the age of five affected by chronic malnutrition due to chronic diseases and poor diet. Food insecurity also affects 35% of the population (Global Nutrition Report, 2014; FAO, 2018).

Micronutrient-deficiency, also called hidden hunger, is a serious issue in Mozambique, affecting particularly the most vulnerable groups, women and children, respectively. The prevalence of vitamin A deficiency was estimated to be 69% for under five-year-old children in 2005 (Harvestplus, 2012).

The reasons for chronic malnutrition are many and complex. Besides the previously-mentioned challenges of agricultural production in Mozambique, there is a lack of proper infant and child-feeding practices coupled with poor nutritional education coverage (UNICEF[5], 2018).

Consumption of milk and milk products is deficient, especially in rural areas. Additionally, in rural-area households normally have only two

---

[5] Source: https://www.unicef.org/mozambique/en/nutrition

meals a day (lunch and dinner), while in urban areas, three meals are common (FAO, 2011). Curiously, some of these affected people in rural areas are cattle breeders.

Maize as a staple food is widely consumed in vast areas of the country, particularly in the provinces of Tete, Manica, Niassa, and Gaza but much less in Nampula (Silici et al., 2015). Maize's average annual per capita consumption was about 80kg in 2015, which highlights the importance of this crop for to feed the population (fews.net, 2016). With the exception of green legume leaves, that often accompany staple foods, the consumption of micronutrient-rich foods (other vegetables, fruits, and animal-derived foods) is extremely low. Cereals, roots, and starchy tubers represent almost 80% of the energy consumed in the diet. The reasons behind this situation include the low diversity of production, low access (physical and financial) to nutritious foods, the poor level of knowledge about nutrition, and limitations that affect the treatment and feeding of children, such as women's work overload (PEDSA, 2011).

After maize, the second most important staple crop in the country is cassava. Although cassava is cultivated in all provinces, it is relatively more important in Nampula, Zambézia, Inhambane, and Cabo Delgado, where more than 70% of smallholders involved in its production. Rice ranks as the third most important staple food in Mozambique and is the second most important cereal crop after maize, representing 8.73% of the total area allocated to food crops. Rice is mostly produced in Sofala, Zambézia, and under irrigated systems in Chókwè and Xai-Xai (Silici et al., 2015).

Although most consumed in Mozambique, the white maize lacks provitamin A and the important amino acids lysine and tryptophan (Buck, 2017). On the other hand, yellow maize that is less consumed is rich in vitamin A (Verheye, 2010). However, due to the past recurrent food aid, with yellow maize as a major component, its consumption, at least in 1990s, had a negative connotation and was associated to poverty and absence of means to buy “elite” food such as rice and white maize (PSAM, 1997).

Vitamin A is an essential micronutrient for human health, and its deficiency can be very dangerous since it limits growth, weaken immunity, can cause xeropthalmia and blindness, and ultimately increase mortality among populations (Harvestplus, 2012; Stevens et al., 2008)

Regarding the goal of reducing vitamin A deficiency, there is a widespread program led by different NGO’s aiming at producing and distribute orange-fleshed sweet potato biofortified with vitamin A. Within this program, breeders produced varieties of orange-fleshed sweet potato with a content of beta-carotene (that is converted to vitamin A in the human body) ranging from 30 – 100 ppm, much higher than local varieties with 2ppm, which is contributing to the reduction of malnutrition and improving overall people’s health (Harvestplus, 2012).

Biofortification of staple crops has many benefits over other approaches to improve micronutrient consumption (Stevens et al., 2008).

Throughout the years, several projects/programs were put on the ground in order to alleviate the problem of chronic malnutrition. For instance, from 2007 to 2009, the HarvestPlus disseminated orange-fleshed sweet potato to 14000 farming households in Zambézia province to reduce Vitamin A deficiency among children and women of childbearing age. Additionally, the World Vision and Helen Keller International (HKI) collaborated through supervision and nutrition training, while the International Potato Center (CIP) was responsible for providing cropping systems and farm extension related to the production. The project led to an increase of 68% in orange-fleshed sweet potato adoption and Vitamin A intake doubled by the project's end (HarvestPlus, 2012).

Regarding biofortified food acceptance in urban areas, a study on development and introduction of orange maize with high levels of provitamin A carotenoids with the support of HarvestPlus (scientists at the University of Illinois) was reported by Stevens et al. (2008). In this study, they analyzed the consumer acceptance of provitamin A-biofortified maize in Maputo (Capital city of Mozambique) and concluded that the existence preferences for white maize do not exclude the acceptance of biofortified orange maize.

Biofortification can be achieved either through genetic modification or through conventional breeding. The above-mentioned maize and sweet potato varieties have been biofortified with provitamin A through conventional breeding (Stevens et al., 2008).

Following, in 2013, the government launched the National Program of Food Fortification[6], which includes the obligation of fortifying maize flour, wheat, cooking oil, sugar, and salt, except for maize flour produced by small scale farmers for household consumption. For instance, salt is fortified with iodine, cooking oil with Vitamin A, maize, and wheat flour with iron, folic acid, zinc, and complex "B" vitamins. Projections indicate that up to 13 million people would benefit from the fortification strategy (Boletim da República, Serie I., N. 16, 2016).

In 2013, the NGO ADPP (From People to People Development Aid, "Ajuda de Povo para Povo", in Portuguese), began the implementation of a Food School Project called "Comida para o saber" (Food for the

---

[6] Food fortification: addition of one or more micronutrients to the food product.

Knowledge), with the finance support of USDA. The project is being implemented in four districts of Maputo province, reaching 74.000 children through the distribution of daily school meals in 265 selected primary schools. The project aims at reducing food insecurity, increase academic performance, and student retention at schools. The implementation of the project also comprises school gardens that directly benefit 18.497 children through the products collected (ADPP, 2016).

Currently, the Food and Agriculture Organization (FAO), with the finance support of the European Union (EU), is developing a nutritional education program that integrates home gardens as a source to improve food and nutritional security. It's expected to reach 30.000 women in seven districts in Zambézia, Manica, and Sofala provinces that are seriously affected by chronic malnutrition (FAO; 2018).

The government, through the collaboration of UNICEF and Scale-up Nutrition/SUN Movement[7], has committed at the highest level within the government to improve nutrition programs and reduce chronic malnutrition in children under the age of five until 35% by 2020 (UNICEF, 2018).

1.7. The agroecological requirements of maize production

Several factors affect the growth and development of maize crops, such as precipitation, temperature, radiation, and photoperiod. In Mozambique, the most important factors are precipitation and temperature. For its good development and productivity, maize crops need a good distribution of rain ranging between 450 and 600 mm during

---

[7] A Civil Society Network towards a world free from malnutrition in all its forms by 2030.

its cycle (Verheye, 2010; Fato et al., 2011). Tropical maize is also adapted to a day length of 12–13 hours (Anami et al., 2009).

Growth and vegetative development of the plant are optimal at temperatures between 22° to 30° C. The crop cannot be cultivated if the mean minimum temperature during plant development falls below 10°C, or if day temperature rises above 45°C for long periods. The critical phase of the maize development to ensure good yields is the flowering and filling stage of the grain. At this stage, the crop requires sufficient moisture and temperatures between 15 °C and 30 °C (Fato et al., 2011).

At an average daily temperature below 20^{o}C, maize takes another 10 to 20 days to reach maturity at each decrease of 0.5^{o}C. The optimum temperature for the germination of the corn is between 18 to 20^{o}C, and for the vegetative growth and flowering, the temperatures must vary between 25 to 30^{o}C (Fato et al., 2011).

The average annual temperatures favor maize cultivation in Mozambique. The country presents monthly average temperatures above 25^{o}C during the rainy period and a minimum between 19^{o}C to 22^{o}C (Fato et al., 2011).

As a C4-plant, maize is a water-efficient crop, but still, its water requirement is high, mainly because it produces larger amounts of biomass in a short period. Water requirements vary between 2 to 3mm/day for young plants, and up to 6 mm/day at or close to anthesis depending on atmospheric conditions (Verheye, 2010).

Regarding the substrate, maize can be grown on a variety of soils from the sandy to the clayey. However, maize grows better in deep, medium-textured soils with a high organic matter content, with good water conservation and drainage capacity, and good nutrient content necessary for all plant developmental stages. The optimum pH ranges from 5 to 7. Alkaline soils prone to floods and saline soils negatively affect maize grain yield (Verheye, 2010).

1.8. Types of maize varieties

There are two main types of maize varieties: open-pollinated varieties (OPVs) and hybrid varieties. Open-pollinated varieties are those that, at the time of seed production, offer no control over the (free) pollination process. The resulting grain production can be used as a seed in the following two or three cropping seasons, continuously, without replacement. To maintain the purity of the seed, it is necessary to isolate the field of production (CIMMYT, 1999; FAO, 2010).

An OPV is "different" if it possesses traits that distinguish it from other known varieties and define its identity (CIMMYT, 1999; FAO, 2010). The advantages of OPV are mainly related to the low cost of seed and no need for the annual substitution of the seed, as they maintain a high degree of stability for several generations. The disadvantages are related to low yields and lack of plant uniformity when compared to hybrids seeds (CIMMYT, 1999; FAO, 2010).

Hybrid varieties are the result of crossing two different breeding lines. Hybrids are produced by the cross-pollination of unlike parents of the same crop. Parent plants are selected for certain desirable traits and are self-pollinated for several generations to produce what is called "inbred lines". These inbred lines are then cross-pollinated to produce the F1 generation, which is known as a hybrid. Because the parents are genetically different, the F1 will have "hybrid vigor" (which is the opposite of consanguinity). The process results in a strong and vigorous plant with greater yields under good edaphoclimatic conditions (Arncken & Dierauer, 2005; FAO, 2010).

The main disadvantage is that the produced grain resulting from a hybrid seed cannot be used as a seed in the following season, making them more expensive. The advantages are high yield, uniformity of plants, and higher response to fertilizers (CIMMYT, 1999; FAO, 2010).

In Mozambique, under the formal seed system, IIAM and CIMMYT provide pre-basic seed, mainly hybrids, to some selected seed companies, independently. Then, the seed companies produce basic and/or certified seed and sell it in the market (Tomo & Alberto, 2016; Mabaya, 2017).

The central base of the formal system is that there is a clear distinction between “seed” and “grain.” On the other hand, this distinction is not so clear in the informal system that is most common (Sperling et al., 2013). In the informal (or traditional) seed system, farmers are responsible for seed multiplication, storage, processing, and trade, while in the formal system, specialized organizations with distinct roles provide new varieties (Howard et al., 2001).

Desirable traits for the maize varieties that are mostly selected among farmers are good yield and stability, tolerance to drought, efficient nutrients use, resistance to main pests and diseases, high-Quality Protein Maize (QPM), early maturation, and hard grain, the latter contributing for the good storability conditions (Bartolomeu et al., 2016; Mabaya, 2017).

1.9. Brief description of the maize genotypes used in the present work

1.9.1 Matuba

Matuba is the most popular maize OPV in Mozambique, and it was released in 1995 by IIAM/CIMMYT (Tomo & Alberto, 2016; Mabaya, 2017). This variety has a very short cycle, which means less risk of crop loss, and has good yields (5 to 6 tons/ha) (Come & Neto, 2017).

Although there is no uniform Matuba variety in Mozambique since OPV's are developed from landraces, this variety was the best available choice for a traditional control variety in the present project. It is a white, quick flint variety that is suitable for coastal regions. It has good Downey mildew resistance and good storability. It is not susceptible to weevil damage because it is a flint grain (Thierfelder et al., 2018).

1.9.2 ZM309

ZM309 is a white semi-flint, early maturing OPV (110-120 days to mature at mid-altitudes and 90-110 days in hot low lands) with high yield potential in drought-prone areas. ZM309 has good resistance to maize streak virus, grey leaf spot, and rust as well as tolerance to low-nitrogen stress. It has a yield potential of 5000 kg/ha, which is high when compared to other OPV's of the same maturity (Setimela & Thierfelder, n.d.).

1.9.3 ZM523

ZM523 is an early maturing drought tolerant OPV (120-130 days). It has a white semi-dent grain with 14-16 grain rows per cob, an average plant height of 180- 185 cm, and is classified as tolerant to drought, nitrogen stress, and low soil pH. It has a yield potential of 6000 kg/ha, which is high when compared to other OPVs of the same maturity. It is described as having some resistance to biotic stresses, namely moderate to good levels of resistance to maize streak virus, gray leaf spot (*Cercospora zeae-maydis*), common rust (*Puccinia sorghi*) and northern leaf blight (*Exserohilum turcicum*) (Setimela & Thierfelder, n.d.).

The ZM309 and ZM523 varieties were developed by IIAM/CIMMYT through crossing local varieties with germplasm from CIMMYT (Aslam et al., 2015; Tomo & Alberto, 2016). Mother-baby trials were used for farmers' participation in the selection, adoption, and selection of the OPVs (Aslam et al., 2015). The "mother" trial is replicated within-site to test a range of technologies and research hypotheses under researcher or breeder management. This trial is either localized on a research station or on-farm. The "baby" trial contains a number of satellite trials (each trial is one replicate) of large plots under farmer management and farm resources. Each trial compares one to four technologies (usually a subset of those tested in the mother trial chosen by the farmer or researcher) with farmers' technologies/cropping systems. Researchers indicate the best management for each proposed technology, they supervise actual farmer practice, and record farmer perceptions and ranking (Snapp, 1995).

The Mozambican maize genotypes used in the present work were all certified seed sent by the Instituto de Investigação Agrária de Moçambique (Agricultural Research Institute of Mozambique).

1.9.4 B73

B73 is a purely homozygous inbred line, developed by continued self-crossing. It is not indicated for field production as the above-described varieties but is an essential resource for plant breeding and other studies in maize genetics. It is the current representative reference genome for maize. It has a nucleotide sequence of the 2.3-gigabase in the genome and over 32,000 genes (Schnable et al., 2012).

In the present work, B73 was used as a control variety specifically for the physiological and molecular characterization and was multiplied in the ITQB NOVA greenhouse.

1.10 Main goals, hypothesis and thesis outline

The present work was developed as part of Mozambique's national effort to generate maize varieties that are better adapted to cope with climatic changes particularly water scarcity. This work intends to open new avenues of research through the generation of knowledge that can be incorporated in the national maize breeding programs. In our research, we tried to integrate the agronomic, physiological, and molecular perspectives to contribute to elucidating drought-resistance traits of three Mozambican open-pollinated maize improved varieties. We expect that different combinations of these traits will contribute to increase the resistance of Mozambique's maize germplasm to drought and improve its water-use efficiency and ultimately increase maize yield in water-limiting environments.

Particular focus was given to the molecular aspects, aiming at providing potential molecular markers that can assist the choice of maize varieties to be grown in specific environments in Mozambique. Our key hypothesis is that the levels of enzymes involved in photosynthesis (such as PEPC, PPDK, NADP-ME, Rubisco, and RubisCO activase) can be potentially correlated with the photosynthetic capacity of maize plants under drought stress.

To test this hypothesis, we have planned the work in several steps, briefly outlined as follows:

Thus, adding to this introductory **Chapter I**, this thesis comprises four more chapters that focus on the used methodology, the results obtained, the discussion of the results, and the main conclusions.

Chapter II. Characterization of the agronomic performance of the Mozambican varieties under study.

In this chapter, we evaluated the agronomic performance of three Mozambican maize varieties under the local agroecological conditions and practices such as intercropping with grain legumes and mineral fertilization, both in rainfed conditions. To achieve this, field experiments were performed for two consecutive years in two Agronomic Stations in Manica province in the Central region of Mozambique. The data obtained were also used to support the experiments done in controlled environments.

Chapter III. Characterization of maize growth and physiological responses induced by different water treatments in controlled environments.

In this chapter, we describe the results obtained from ambient controlled experiments. Growth and physiological features of the varieties under different watering regimes are presented and discussed, with particular focus on drought stress. Some of the physiological traits targeted in this part of the work are net photosynthesis rate, stomatal conductance to water vapor, and PSII photochemistry.

Chapter IV. Proteomic features governing drought tolerance in the Mozambican maize varieties under study.

In this chapter, we describe how the levels of photosynthetic enzymes (such as PEPC, PPDK, NADP-ME, Rubisco, and RubisCO activase) can be correlated with the photosynthetic capacity of the varieties, with particular focus on drought treatments. The levels of proteins related to PSII integrity and the antioxidant machinery are also included in this discussion. We expect to bring new insights into the characterization of the Mozambican maize varieties by adding the proteomic perspective to the maize breeding programs.

Chapter V. General overview, conclusions, and recommendations.

In the final chapter, we aim at integrating the agronomic, physiological, and proteomic data and give an overall exposition on the characterization of the three Mozambican maize varieties under study. We also discuss how this knowledge can be integrated into the national maize breeding programs. The Autor also provides his perspective on how agriculture, specifically how maize production can reach acceptable yield levels for the household needs, internal trade, and ultimately exportation.

References

ADPP (Ajuda de Povo para Povo). (2016). Projecto de Alimentação Escolar. Maputo.

Anami, S., Block, M. De, Machuka, J., & Lijsebettens, M. Van. (2009). Molecular Improvement of Tropical Maize for Drought Stress Tolerance in Sub-Saharan Africa. *Critical Reviews in Plant Science*, 28, 16–35. https://doi.org/10.1080/07352680802665305

Arncken, C., & Dierauer, H. (2005). Hybrid Varieties for Organic Breeding for Organic Production (Vol. 41). A Report

Aslam, M., Maqbool, M. A., & Cengiz, R. (2015). Drought Stress in Maize (Zea mays L .) Effects, Resistance Mechanisms*, Global Achievements and Biological Strategies for Improvement*. https://doi.org/10.1007/978-3-319-25442-5

Bartolomeu, A., Bento, A., Fernando, I., Greenberg, S., Muianga, D., & Simbe, N. (2016). Mapping Farmer Seed Varieties in Manica, Mozambique: Report on initial investigations into agricultural biodiversity. Johannesburg.

Blake, M. (2006). Dating the Initial Spread of Zea mays. In *Histories of Maize* (pp. 1–678). California: *Academic Press*.

Brück, T. (1998). Guerra e Desenvolvimento em Moçambique. *Análise Social*, *xxxiii*(149), 1019–1051.

Buck, S. De. (2017). Maize in Africa. *Gent: VIB International Plant Biotechnology Outreach.*

Carrilho, J., Abbas, M., Júnior, A., Chidassicua, J., & Mosca, J. (2013). Desafios para a Segurança Alimentar e Nutrição em Moçambique. Maputo.

Castel-Branco, N. (1994). Problemas Estruturais do Desenvolvimento Agrário. In *Moçambique Perspectivas Económicas* (pp. 31–85). Maputo: *Faculdade de Economia da Univ. Eduardo Mondlane e Fundação Friedrich Ebert*.

Cherniwchan, J., & Moreno-cruz, J. (2019). Maize and Precolonial Africa. *Journal of Development Economics*, *136*(March 2018), 137–150. https://doi.org/10.1016/j.jdeveco.2018.10.008

CIMMYT (1999). Development, Maintenance, and Seed Multiplication of Open-Pollinated Maize Varieties (2nd ed.). Mexico. *CIMMYT*.

Come, F., Neto, F., & Matuba, M. (2017). Adoção da Variedade de Milho Matuba pelos Pequenos Produtores do Distrito de Sussundenga, Moçambique. *Revista Em Agronegócio e Meio Ambiente*, *10*(4), 977–994.

Cumbe, E., Dumangane, S., & Lencastre, M. (2010). Análise do Acesso ao Fundo de Desenvolvimento Distrital (FDD) na Óptica de Género. 1–51.

Desenvolvimento), B. (Banco A. de. (2018). Resumo dos Resultados *2018* - Moçambique. Abidjan.

Dias, P. (2013). Analysis of Incentives and Disincentives for Maize in Mozambique. Rome.

FAO. (2010). Seeds in Emergency. Rome: FAO.

FAO. (2011). Nutrition Country Profile Republic of Mozambique.

FAO. (2015). Programa da FAO em Moçambique (2012-2015).

Fato, P., Mulima, E., Senete, C. T., & Nhamucho, E. (2011). Manual de Produção de Milho. Maputo: *Instituto de Investigação Agrária de*

Moçambique.

Fews.net. (2016). Regional Maize Market Fundamentals in Southern Africa.

Fukunaga, K., Hill, J., Vigouroux, Y., Matsuoka, Y., G, J. S., Liu, K., Doebley, J. (2005). Genetic Diversity and Population Structure of Teosinte. *Genetics*, *169*(April), 2241–2254. https://doi.org/10.1534/genetics.104.031393

Ghatak, A., Chaturvedi, P., & Weckwerth, W. (2017). Cereal Crop Proteomics: Systemic Analysis of Crop Drought Stress Responses Towards Marker-Assisted Selection Breeding. *Frontiers in Plant Science*, *8*(June), 1–25. https://doi.org/10.3389/fpls.2017.00757

HarvestPlus. (2012). Disseminating Orange-Fleshed Sweet Potato: Mozambique Country, Report. In *Washington, D.C.: HarvestPlus*.

Hellmuth, M. (2007). Climate risk management in Africa: Learning From Practice. New York. *The International Research Institute for Climate and Society (IRI)*.

Howard, J., Low, J., Jeje, J. J., & Boughton, D. (2001). Constrangimentos e Estratégias para o Desenvolvimento do Sistema de Sementes em Moçambique. Maputo.

Jian, C., Fernando, L., Bradbury, P. J., Olukolu, B. A., Xue, W., & York, A. M. (2019). The Genetic Architecture of Teosinte Catalyzed and Constrained Maize Domestication. *PNAS*, *116*(12), 5643–5652. https://doi.org/10.1073/pnas.1820997116

Kassie, G. T., Erenstein, O., Mwangi, W., Larovere, R., Setimela, P., & Langyintuo, A. (2012). Characterization of Maize Production in Southern Africa: Synthesis of CIMMYT/DTMA Household Level Farming System Surveys in Angola, Malawi, Mozambique. *CIMMIYT*.

Larkins, B. (2017). Maize Kernel Development. Boston. *CAB International.*

Mabaya, E. (2017). Mozambique Brief 2017 - The African Seed Access Index.

Mosca, J. (1996). Evolução da Agricultura Moçambicana no Período Pós-Independência. *Instituto Superior de Agronomia.*

Nannas, N. J., & Dawe, R. K. (2015). Genetic and Genomic Toolbox of *Zea mays*. *GENETICS*, *199*(March), 655–669. https://doi.org/10.1534/genetics.114.165183

PEDSA. (2011). Plano Estratégico Para o Desenvolvimento do Sector Agrário *2011-2020*.

PSAM (Projecto de Segurança Alimentar de Moçambique) (1997). A disponibilidade de productos e o poder de compra dos consumidores: grãos de milho e arroz.

Report, G. N. (2014). Nutrition Country Profile-Mozambique.

Boletim da República (2016). *Imprensa nacional de Moçambique*. pp. 237–241

Schnable, P., Pasternak, S., Liang, C., Zhang, J., Fulton, L., Graves, T. A., Kumari, S. (2012). The B73 Maize Genome: Complexity, Diversity, and Dynamics. *Science*, *326*(November 2009), 1112–1115.

Setimela, P., & Thierfelder, C. (n.d.). Variedades de Milho de Polinização Aberta (OPVs). Vol. 258, pp. 1–2. Maputo. *Instituto de Investigação Agrária de Moçambique.*

Silici, L., Bias, C., & Cavane, E. (2015). Sustainable agriculture for small-scale farmers in Mozambique, A Scoping Report. London.

Snapp, S. (1995). Quantifying Farmer Evaluation of Technologies: The Mother and Baby Trial Design.

Sperling, L., Boettiger, S., & Barker, I. (2013). Integrated Seed Systems.

Stevens, R., & Winter-nelson, A. (2008). Consumer Acceptance of Provitamin A-Biofortified Maize In Maputo, Mozambique. *Food Policy*, *33*, 341–351. https://doi.org/10.1016/j.foodpol.2007.12.003

Thierfelder, C., Rusinamhodzi, L., Setimela, P., Walker, F., Eash, N. S., Mp, P. O. B., & Pleasant, M. (2018). Conservation Agriculture and Drought-Tolerant Germplasm: Reaping The Benefits of Climate-Smart Agriculture Technologies In Central Mozambique. *Agronomy for Sustainable Development*, *38*(5), 414–428. https://doi.org/10.1017/S1742170515000332

Tomo, A., & Alberto, M. (2016). Mozambique Early Generation Seed Study.

Trtikova, M., Lohn, A., Binimelis, R., Chapela, I., Oehen, B., Widmer, A., & Hilbeck, A. (2017). Teosinte in Europe – Searching for the Origin of a Novel Weed. *Scientific Reports*, *7*(January), 1–7. https://doi.org/10.1038/s41598-017-01478-w

Tschirley, D., & Abdula, D. (2007). Toward Improved Marketing and Trade Policies to Promote Household Food.

Tschirley, D., Donovan, C., & Weber, M. (1996). Food Aid and Food Markets: Lessons From Mozambique. *Food Policy*, *21*(2), 189–209.

USAID. (2016). Mozambique Early Generation Seed Study.

USDA (United States Department of Agriculture) (2019). World Agricultural Production.

Verheye, W. (2010). Growth and Production of Maize: Traditional Low-Input. Oxford. *UNESCO-EOLSS Publishers*.

WFP. (2016). Milho Em Grão Duas Vezes Mais Caro do que em Julho De 2015.(https://documents.wfp.org/stellent/groups/public/documents/ena/wfp286617.pdf)

Chapter II. Characterization of the agronomic performance of the Mozambican maize varieties under study

The experimental work in field conditions presented in this Chapter was mostly performed with the collaboration of IIAM researchers based in Manica province, Mozambique (see acknowledgments section)

Abstract

Maize is the most important staple food in Mozambique. Maize production employs about 75% of the population, most of them producing maize mainly for household needs. Thus, given the importance of maize production to feed the population, there is a need to continuously improve the different cropping systems that exist locally. The present study aimed at evaluating the agronomic performance of three commonly used open-pollinated maize varieties, namely, Matuba, ZM309, and ZM523. The cropping systems studied included: maize as a monoculture and maize intercropped with cowpea *Vigna unguiculata* (L.) Walp., growing in either natural culture conditions or with supplementing inorganic fertilization.

In general, the varieties performed similarly within the cropping systems, with none of the varieties standing out over the other. In the first cropping season (2017/2018), where only monocropping and strip intercropping were evaluated, the maize grain yield, plant fresh biomass, weight of 1000 grains, cob weight, plant height, and root length were tendentially lower in intercropping in Sussundenga than monocropping. These reductions indicated that intercropping did not favor maize yield as we expected. On the other hand, values of yield parameters were similar between monocropping and intercropping in Rotanda, suggesting that Sussundenga conditions favored competition between crops more than the ones in Rotanda.

In the second season (2018/2019), the application of mineral fertilizer contributed positively to the overall maize yield. Also, relay intercropping (*i.e.,* desynchronization of sowing times) was introduced and found to reduce competition for soil resources, increasing maize yield over the monocropping, including in Sussundenga.

In Rotanda, fertilizer application in monocropping contributed to a 55 to 130% increase in total grain weight per plot as compared to control, while in intercropped blocks, fertilizer justified 68 to 130% increase. As previously observed in Rotanda, in the absence of fertilization, monocropping, and intercropping systems performed similarly.

Maize yield in intercropping appeared more stable in Rotanda in both seasons than in Sussundenga, where yield reduction was visible in intercropping in the first cropping season. This feature in Rotanda was mainly attributed to the higher levels of mineral content such as calcium and magnesium and the slight lower soil acidity, which could positively impact the attachment of rhizobia to root hairs. In spite of the reduced number of seasons analyzed with these data, and considering production stability, Rotanda may be a better choice for the use of intercropping than Sussundenga.

We concluded that intercropping may be considered as a good practice to improve grain yield, although proper optimization of the practices, such as using distinct sowing times, should be considered. In general, fertilizer application had a positive impact on yield, and can be recommended to improve productivity. To enhance intercropping performance, the application of nutrients was also considered crucial. However, further studies on fertilizer optimization in intercropping, specifically in the agroecological zones in Mozambique, are needed so that fertilizer overuse/underuse can be prevented, the use of resources maximized, and the environment protected.

2.1. Introduction

In Mozambique, maize is mostly cultivated as a subsistence crop (Silici et al., 2015), and usually grown in rainfed conditions, with no or limited access to irrigations systems, improved seeds, and inputs such as pesticides or inorganic fertilizers (Tomo & Alberto, 2016) (see chapter I for further details on Mozambican Agriculture).

Since agricultural development and innovation imply much experimental work in different edapho-climatic conditions, agronomists and breeders are especially concerned with the scientific validity of their assays and corresponding translation to actual agrarian practices (Maat, 2011). Thus, in the present work, field experiments were designed to maximize relevance to the local farmers and to facilitate implementation within the farming practices used in Mozambique.

Most farmers in Mozambique lack financial conditions to improve their farming systems and implement strategies for alleviating soil constraints such as low fertility. Among the fertilizers, N is the most important nutrient for maize development (Fato et al., 2011; Jiang et al., 2018), while for global agroecosystems P is probably the most limiting mineral nutrient for plant growth (Rusinamhodzi et al., 2012; Yan et al., 2014).

To circumvent low soil fertility, many farmers in Central Mozambique cultivate maize intercropped with grain legumes as a strategy to increase food security and income. Within local populations, maize–legume intercropping has the potential to reduce the risk of crop failure, improve production and income, and increase food security (Rusinamhodzi et al., 2012).

Intercropping is a farming practice or system involving two or more crop species, or genotypes, growing together and coexisting for a time in different field configurations patterns as following explained (Yan et al., 2014; Brooker et al., 2015).

There are three types of Intercropping, namely: mixed intercropping, where two or more crop species are grown together at the same time in a field lacking any spatial configuration; the strip intercropping, in which two or more crop species are grown in separate, but in adjacent rows at the same time; and finally the relay intercropping, that involves the staggered planting of two or more crops together in a way that only parts of their life cycles overlap (Bybee-finley & Ryan, 2018). The relay intercropping can be applied with or without any particular spatial configuration of crops (Raza et al., 2019).

In intercropping legume/cereal, rhizosphere effects promote phosphorus and nitrogen assimilation in maize roots and thus enhance maize growth and nutrient uptake, which is particularly important in nutrient-poor soils (Yan et al., 2014).

Legumes are crucial to alleviate the constraints related to assimilable nitrogen (N) limitations in the soil and to improve cereal crop productivity such as maize. They also have an advantage in covering the soil surface and thus reducing soil erosion, in repressing weed development, and reducing pests and diseases (Gianoli et al., 2006; Festus & Jonas, 2010; Rusinamhodzi et al., 2012; Yan et al., 2014). Additionally, legumes enhance efficient land-use and resources, such as sunlight, moisture, and soil nutrients (Gianoli et al., 2006; Brooker et al., 2015). In water-deficient conditions, legumes may also increase water-use efficiency, primarily attributed to the improved water capture through the complementary root distribution and reduced surface run-off (Brooker et al., 2015; Tsujimoto et al., 2015). Intercropping can also contribute to the crop yield stability or production consistency throughout the seasons by decreasing the risk of crop failure (Bybee-finley & Ryan, 2018).

The nitrogen fixation by legumes mentioned above is the result of the symbiosis between a bacterium and a plant. Biological nitrogen fixation is the process that converts inert N_2 into biologically useful ammonia (NH_3). This important process that improves soil quality is mediated in nature only by N-fixing Gram-negative rhizobia bacteria (*Rhizobiaceae*). Nitrogenase catalyzes the reduction of N_2 to NH_3 in the energetically costly reaction as follows:

$$N_2+8H^++8e^-+16ATP \rightarrow 2NH_3+H_2+16ADP+16P_i.$$

In legumes and a few other plants, the bacteria live in small nodules that grow on the roots after colonization (Fig. 2.1) (Flynn & Idowu, 2015; Geddes & Oresnik, 2016).

Figure 2.1 - A photograph of a legume plant root showing nodules attached to the roots. Source: Flynn & Idowu (2015).

Legume nitrogen fixation starts with the formation of a nodule. The rhizobia bacteria in the soil invade the root and multiply within the cortex cells. The plant works supplying the necessary nutrients and energy for the bacteria. Within a week after infection, small nodules began to be visible. In the field, these small nodules can be seen 2–3 weeks after planting, depending on the legume species and germination conditions. When nodules are young and not fixing nitrogen, they are typically white or gray inside. As nodules increases in size, they gradually change to a pink or reddish color, which indicates that nitrogen fixation has begun (Flynn & Idowu, 2015). The pink or red color is originated by leghemoglobin (an oxygen transporter that is similar to hemoglobin in blood) that regulates oxygen flow to the bacteria. Since nitrogenase is sensitive to oxygen, leghemoglobin seems to transport enough oxygen to allow the rhizobia respiration while avoiding nitrogenase inhibition. The heme-protein is mutually produced by the legume and the bacteria; the legume producing the apoprotein and the bacteria being responsible for producing the heme (porphyrin ring bound to an iron atom) (Wagner, 2011).

Legume nodules that are no longer fixing nitrogen usually turn green and may be removed by the plant. Environmental factors such as temperature and water availability may not be controlled by the farmer, but nutrients deficiencies (especially phosphorus, potassium, zinc, iron, molybdenum, and cobalt) can be corrected with mineral fertilizers and may have an impact in nitrogen fixation capacity (Flynn & Idowu, 2015).

Legume/maize intercropping can partially compensate deficiencies in soil nutrients and increase maize productivity. This feature is due to the below-ground interactions, the rhizosphere effects, and spatial effects (Yan et al., 2014; Brooker et al., 2015). Through these effects, legume/maize intercropping not only enhances the P nutrition of maize but also changes the chemical and microbiological profile of the rhizosphere.

Rhizosphere effects improve the availability of P to maize due to rhizosphere acidification that occurs via the release of organic acids and protons from legume roots. P is transported from the soil into root cells by specific transporter proteins within the plasma membrane. The identified plant P transporters have been classified into three families: PHT1, PHT2, and PHT3. P uptake is particularly dependent on the high-affinity transporters of the PHT1 family. Five genes of the maize PHT1 family (Pht1;1, Pht1;2, Pht1;3, Pht1;4, Pht1;6) are expressed in maize roots (Yan et al., 2014). It is also known that phosphate starvation induces the expression of these genes. After being transported into root cells, inorganic P is assimilated and incorporated in the general metabolism through oxidative phosphorylation in root tissues. This P can then be used for the growth and development of maize plants (Yan et al., 2014).

Another way in which plants can mobilize P is by increasing the release of extracellular phosphatases that mineralize soil organic P in the rhizosphere, making it available to be uptaken by the plants (Franke et al., 2018).

The importance of maize in Mozambique highlights the need for the continuous improvement of the cropping systems that are used locally, intercropping included. Therefore, in the present study, carried out in two sites of Manica province in the Central region of Mozambique, we evaluated the agronomic performance of three maize varieties under local agroecological conditions and practices. Maize was grown either as a monoculture or intercropped with cowpea *Vigna unguiculata* (L.) Walp., with and without fertilizer addition, to assess the impact of different cropping systems in maize yield. Thus, we hypothesized that maize–cowpea intercropping is more productive than maize production as a monoculture and that fertilizer application would increase yield as compared to non-fertilized fields. We also hypothesized that intercropping is an option to alleviating low soil fertility constraints and stabilize grain yield over the cropping seasons.

We planned this work, testing different cropping systems and locations to contribute to maximizing maize yield and economic resources, thus supporting the local agrarian decision-makers, farmers, and plant breeders. We also intended to explore agronomic features that could be explained by the physiological and proteomic data (see chapters III and IV for details on maize physiological and proteomic characterization).

2.2. Material and Methods

2.2.1 Field experiments in Mozambique

For the assessment of productivity, field experiments were conducted consecutively in 2017/2018 and 2018/2019 in two sites of Manica province, namely, the Agronomic Stations of Sussundenga (19° 19'02.00" S, 33° 14'25.24" E, 620m above sea level) and Rotanda (19.50000 Latitude 32.91667 longitude, 966m above sea level), with the collaboration of the Institute of Agricultural Research of Mozambique (IIAM). These stations belong administratively to the Sussundenga district in Manica province (Supplemental Figure S2.1) and are located about 40km apart.

The climate of Sussundenga district is predominantly Tropical Wet Savanna – AW (Köppen Climate Classification) with two distinct seasons, wet and dry. The annual average precipitation is about 1.171 mm, while the evapotranspiration is about 1.271 mm. The rain season ranges from November to March, varying significantly in quantity and distribution. The maximum and minimum annual averages temperatures are 29.5 and 17.6 ^{o}C. The district is composed of different groups of soils, such as red clayey, red sandy, and medium texture reds, and lytic rocks (MAE, 2012). Although belonging to the same district, the two agronomic stations differed in soil properties and also in the levels of precipitations (see section 2.3.1 for more details).

2.2.1.1 The 2017/2018 cropping season experiments

The first field experiments were conducted between November 2017 and March 2018, using certified improved maize varieties Matuba, ZM309, and ZM523 in rainfed conditions. A completely randomized blocks design experiment with three replicates was prepared (Davis et al., 2017) and two cropping systems, namely monoculture (M) and intercropping (I) with cowpea *Vigna unguiculata* (L.) Walp., variety IT17

(IIAM) (locally known as “feijão nhemba”). In both sites of production, three blocks (each with 20x6m of area) and a total of nine plots (each with 6x6m of area) in each cropping system were used (see Supplemental Figure S2.2 for a general overview of the experimental design). The experimental units (plots) were composed of eight maize rows and were surrounded by a 1m border of bare ground. Rows at the edge of the plots were considered border rows and were not included in the data collection. Twenty-four maize seeds were sown in each row, with 80 cm spacing between the planting rows and 25 cm within the rows, corresponding to 192 plants per plot (equivalent to 53,333.333 plants/ha). In the intercropped plots, cowpea was planted between the maize rows, totalizing seven rows in each plot spaced 80 cm and 30 cm within the rows, and a total of 20 plants in each cowpea row. Monocropping and intercropping blocks were separated 3m apart. The maize plants were thinned three weeks later, leaving one plant per hole. Maize and bean were sown simultaneously, 5-12 cm deep, and always after the beginning of rainfall to ensure proper germination and plant establishment. Fields were prepared by tractors (tillage), hand sowing was carried out, and hand/hoe weeding was performed when needed. In summary, the treatments were: monocropping (M); intercropping (I) with cowpea (*Vigna unguiculata* L. (Walp). Soil acidity correction was not performed in any of the cropping seasons.

2.2.1.2 The 2018/2019 cropping season experiments

The second season of field experiments was similar to the previous one, but with the addition of mineral fertilizer application (NPK, 14.76-28.76-14.76, these values are %, supplier: OMNIA) and different sowing times between the two species (or relay intercropping). For the fertilized blocks, a basal application method of 0.64kg (178kg per hectare) of NPK fertilizer in each plot before sowing was used. The main objective of the fertilizer application at sowing time was to was to distribute the fertilizer over the plots uniformly and to mix it with soil (Fato et al., 2011). In the second phase, a top-dressing method was applied 30 days after sowing, with 0.32kg (89kg per hectare) of urea in each plot of the fertilized blocks, aiming to supply the growing plants with a readily available form of nitrogen (Fato et al., 2011). In summary, the treatments were: monocropping (M); intercropping (I) with cowpea (*Vigna unguiculata* L. (Walp); and fertilizer application (F) either in monocropping or intercropping. In 2018/2019 cowpea was sown six weeks after maize sowing to minimize competition between the two species (Kimaro et al., 2009; Rusinamhodzi et al., 2012)

2.2.4 Soil sampling and analysis

In the 2017/2018 season, the soil was sampled and analyzed twice, at sowing and at harvesting time. Three soil depths were considered, 0 to 15 cm, 15 to 30 cm, and 30 to 45 cm, respectively, in both sites. Soil samples were analyzed in the Regional Laboratory of Soil and Plant Analysis in Nampula province in Mozambique using the Mehlich-1 method[8]. Data on soil analyses from the 2017/2018 season are presented in supplemental Tables S2.1 and S2.1. Soil-parameters were not analyzed in the second cropping season since no significant differences were expected at, least in the non-fertilized blocks.

2.2.5 Weather conditions in both cropping seasons

Climatic data were obtained from the weather stations located in both sites of experiments. Details are presented in Supplemental Figures S2.3 and S2.4.

---

[8] Mehlich-1: method used to evaluate soil extractable P. Labile P is extracted by acid dissolution, which preferentially attacks P pools associated with calcium compounds and, to a less extent with aluminum and iron compounds (Gatiboni et al., 2010).

2.2.6 Statistical analysis

Data of maize grain yield, whole plant fresh biomass, the weight of 1000 grains, number and weight of cobs, number of grain rows per cob, plant height and root length were analyzed with one-way ANOVA and Tukey test to compare means of varieties within the cropping systems and locations. Differences were considered significant at $p<0.05$. Statistical analysis was performed using SigmaPlot 11.0 software package (Systat Software Inc., Chicago, IL, USA).

2.3. Results and discussion

2.3.1 Soil analysis and climatic characteristics in both cropping seasons

Regarding the soil analysis, we observed that soil acidity was, in general, slightly higher in Sussundenga (tendentially pH<6). The values of coarse sand were also higher in Sussundenga, but values of silt, clay, humidity, Ca, Mg, organic matter, sum of exchangeable cations of negligible acidity, capacity of cation exchange, and rate saturation were higher in Rotanda. The values of fine sand, extractable phosphorus, and soil density were fluctuant in both locations (Supplemental Tables S2.1 and S2.2).

The soil pH indicates the amount of acidity present in the soil and is considered as a standard and routine in soil analysis. This is because soil pH affects the solubility and availability of many elements as well as the microbial activity. In general, a soil pH of 6.0 to 7.0 is ideal for most food crops such as maize (*Zea mays* L.), soybean (*Glycine max* L. Merr.), and wheat (*Triticum aestivum*). Soil acidity correction, which is usually done by liming or adding calcium amendments to the soil (Carter & Gregorich, 2006), was not performed in our experiments.

In the 2017/2018 season, the maximum mean air temperature in Sussundenga over the cropping season was ~28.0°C and the total precipitation was 2135,5 mm. Rotanda recorded ~28.0°C of maximum temperature and 1056 mm of total precipitation, basically half the amount of rain that was recorded in Sussundenga. In the 2018/2019 season, Sussundenga recorded maximum temperatures rounding ~29.0°C, and 1654 mm of precipitation, respectively, while in Rotanda 700 mm and ~29.0°C was recorded for precipitation and maximum temperature (Supplemental Figures S2.3 and S2.4).

2.3.2 Crop yield assessment in Sussundenga and Rotanda in the 2017/2018 season

In the 2017/2018 season, we decided to perform field experiments as much similar to small farmer's practices as possible. Therefore, we implemented blocks with maize growing as monoculture and blocks with maize growing intercropped with cowpea, in both cases without any addition of mineral fertilizer.

Additionally, in the first season, we obtained an equivalent grain yield (in kg/ha) that ranged between 2410 to 3304kg/ha in monocropping in Sussundenga, and 962 to 1283Kg/ha in intercropping. In Rotanda, we recorded grain yield that ranged between 1996 to 2310kg/ha in monocropping, and 1478 to 2034kg/ha in intercropping. All these values were above the local estimated yield of 300 - 900 kg/ha (Stevens & Winter-Nelson, 2008; Tomo & Alberto, 2016). Although not applying fertilizer at this time, the use of improved seeds in the study may have contributed to a greater yield over the local yield average per hectare, with the majority of the small farmers not using improved seeds or fertilizer.

In general, we found no statistical differences amongst varieties within cropping systems in the 2017/2018 season (one-way ANOVA, Tukey test, $p<0.05$). However, ZM309 variety tended to have the highest total grain weight per plot, the weight of 1000 grains, cob weight, and root length in monocropping in Sussundenga (Fig. 2.2 and 2.3D). Also, the total grain weight, whole plant fresh biomass, the weight of 1000 grains, cob weight, plant height, and root length were tendentially lower in intercropped blocks as compared to monocropping blocks (Fig. 2.2, and 2.3C and D). For example, a 50-60% reduction was recorded in grain yield. These reductions indicate that in the tested conditions intercropping did not improve yield over monocropping.

In Rotanda, slightly lower values of grain yield, the weight of 1000 grains, and cob weight in intercropped blocks compared to monocropping were also recorded but were less obvious than observed in Sussundenga. Therefore, whichever factor has contributed negatively to the overall maize yield in intercropped blocks in Sussundenga, it had a much less impact on yield in intercropped blocks in Rotanda.

Also, there was a significant investment of the plants in biomass production in Rotanda that was not proportional to what would be expected for the grain yield and weight of 1000 grains. Therefore, this feature may be related to the higher nitrogen recovery efficiency (NRE), which reflects the ability of aboveground plant parts to recover N from the soil, while nitrogen internal efficiency (NIE), which is the capacity of the plants to transform nitrogen absorbed by the crop into the grain, was possibly lower (Yu, et al., 2015; Wang et al., 2019). Thus, specific environmental factors there during the grain filling period may have affected the translocation efficiency of nitrogen and sugars accumulated during the pre-anthesis stage (Barbottin et al., 2005; Zhou et al., 2018). The above-described tendency was also observed in the second cropping season (2018/2019).

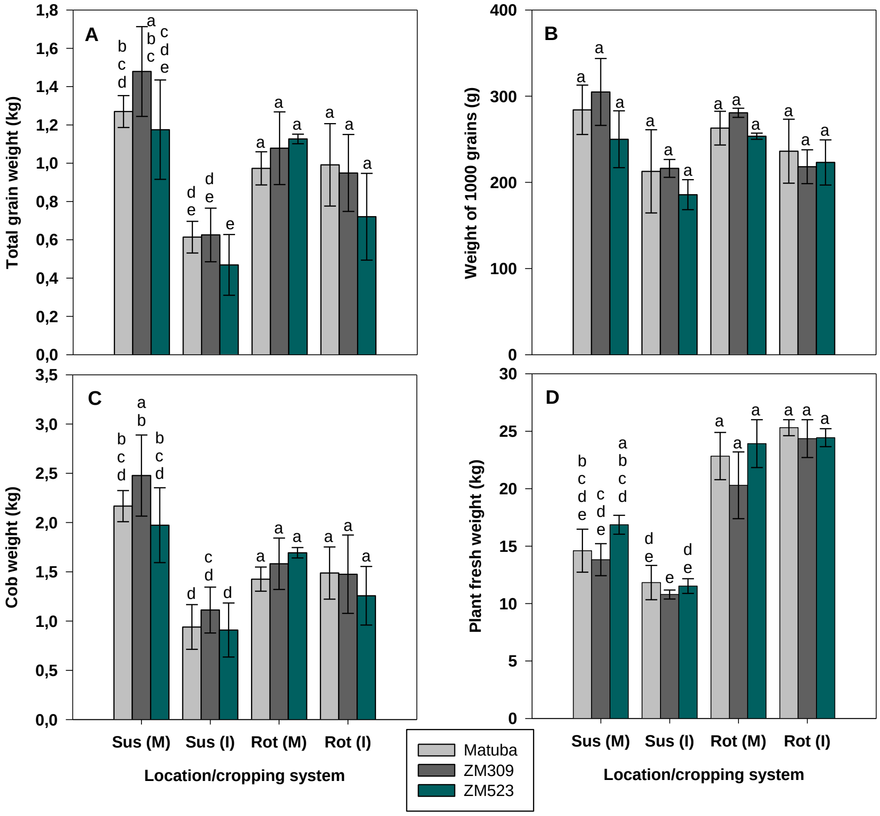

Figure 2.2 - Effect of different cropping systems (monocropping and intercropping) in total grain weight per plot (A), the weight of 1000 grains (B), cob weight per plot (C), Plant fresh weight per plot (D) in the 2017/2018 cropping season. Sus (Sussundenga); Rot (Rotanda); M (monocropping); I (intercropping). Bars are an average of three plots±SE. Different letters represent significant differences within cropping systems and locations (one-way ANOVA, Tukey test, p<0.05).

On the other hand, monocropping in Sussundenga performed slightly better regarding yield parameters compared to the same treatment in Rotanda. This slight improvement could be due to a possible better rainfall distribution and higher water abundance in Sussundenga, namely at the reproductive stage (Supplemental Figure S2.3). Also, the nitrogen internal efficiency of the plants was possibly higher in Sussundenga compared to Rotanda (Wang et al., 2019).

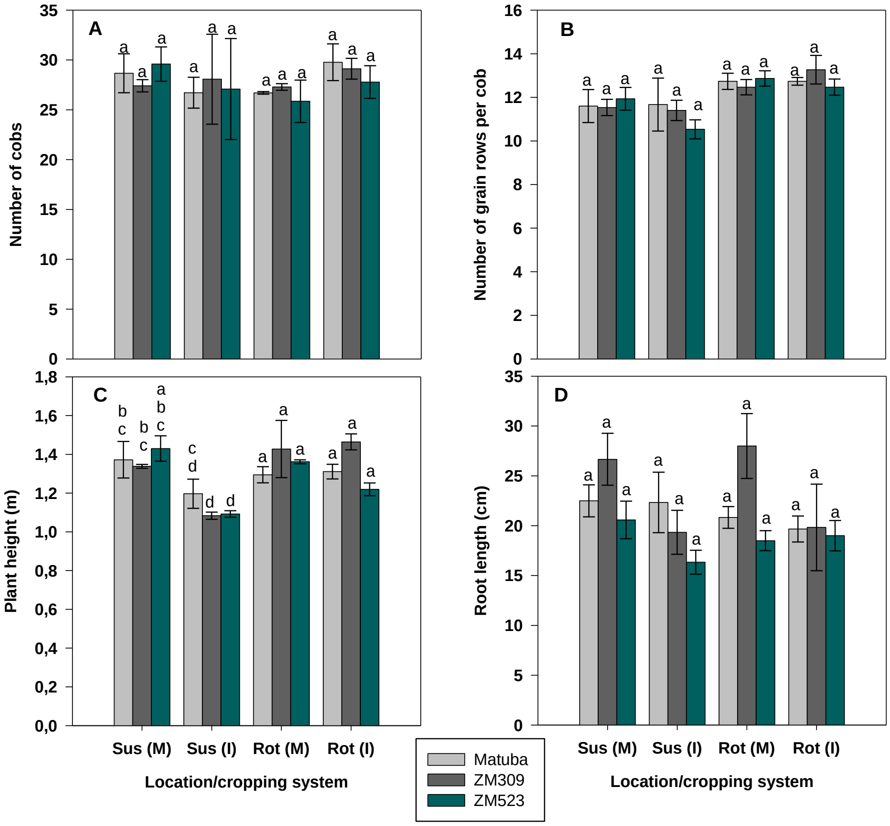

Figure 2.3 - Effect of different cropping systems (monocropping and intercropping) in number of cobs (A), number of grain rows per cob (B), Plant height (C), Root length (D) in the 2017/2018 cropping season. Sus (Sussundenga); Rot (Rotanda); M (monocropping); I (intercropping). Bars are an average of three plots±SE. Different letters represent significant differences within cropping systems and locations (one-way ANOVA, Tukey test, *p*<0.05).

In these experiments, using maize OPV's without any fertilizer addition, values of the number of cobs, number of grain rows, and weight of 1000 grains were similar to those observed in different maize hybrids grown under local conditions in Pakistan (Tahir et al., 2008). Also, values of 1000 grains weight were higher compared to those obtained for example with the hybrid "Oba super I" supplemented with different levels of poultry manure in Nigeria (Akongwubel et al., 2012) and hybrids Cera 410 and Cera 450 supplemented with different combinations of N and P (Băşa et al., 2016) in Romania, meaning that the varieties used in our experiments can be considered as good material to improve maize production by agronomic techniques and help farmers in the Mozambican context.

Regarding the prominent yield reduction in intercropping in Sussundenga, several explanations were considered. The reduction can be associated with competition between the two species for soil resources (interspecific competition), with cowpea possibly dominating over maize. The sandy soil in Sussundenga highly susceptible to soil surface run-off would also contribute to the loss of nutrients, and increased competition, and consequently to the low productivity in maize. Gianoli et al. (2006) pointed out that the potential benefits of the plant species within a cropping system must be balanced against the costs in terms of reduced productivity of the focal crop (in this case was maize) due to plant competition for resources.

Also, not all intercropping systems are described as providing benefits in terms of all possible metrics. When intercropping benefits do occur, they are the result of full exploitation of resources, such as solar radiation, water, soil, and fertilizers. The uneven distribution of capture organs such as roots, is likely to induce dominance relationships between plants, affecting their agronomic performance (Brooker et al., 2015).

Another reason for the notably lower yield in intercropping in Sussundenga is the possible poor cowpea nitrogen fixation due to soil nutrients dynamics there. Although fixing nitrogen from the air when there is a lack of nitrogen in the soil is a major success factor, the legume crops cannot overcome the yield gap entirely without the use of other fertilizers. In work developed by Velasco et al. (2008), where they analyzed the effects of phosphorus application on biological nitrogen fixation on herbaceous legumes, they observed a higher number of nodules and total dry weight matter in phosphorus treatment when compared to the control, which confirmed the positive effects of phosphorus application on the formation and activity of nodules, and that the addition of P fertilizer is often essential for good nitrogen fixation by legumes (Franke et al., 2018). Also, Zhao et al. (2019), found that intercropped maize and pea had significant advantages on carbon and nitrogen accumulation in plant tissues as compared with corresponding monoculture crops, and these advantages were further enhanced with increased nitrogen fertilization.

The amount of nitrogen fixed also depends on the genetic potential of the legume, the rhizobia, and the symbiosis, and on the ability of legumes to establish their symbiosis, which depends on the environment and soil management (Franke et al., 2018).

We also suggested that the beneficial effects of intercropping, which makes nitrogen more available and is very important at flowering and grain filling stages (Fato et al., 2011), were lost due to nitrogen wash or its predominant use in the vegetative stage rather than in the reproductive. In other words, nodule senescence had possibly occurred early before the grain filling stage.

For the last scenario, relay intercropping could minimize this gap. Relay intercropping is a kind of intercropping in which two or more crops grow simultaneously during only part of their life cycles (*i.e.*, partial overlap of life cycles). In that case, nitrogen-fixing legumes could be introduced between silking and physiological maturity of maize (Niringiye et al., 2005; Velasco et al., 2008). In this case, the legumes can continue to grow after maize harvesting. Later, as farmers prepare the land for the next season, they can incorporate the legume biomass into the soil (Velasco et al., 2008). This staggering of the planting times in the intercropped system can reduce interspecific competition. Staggered planting is also may also contribute to reducing the risk of total crop failure when rainfall is erratic (Rusinamhodzi et al., 2012). Additionally, in another study, relay intercropping was important to compensate nitrogen demand in the later stage of growth in maize and considered as a sustainable and efficient system of nitrogen-use under reduced fertilizer application (Du et al., 2019).

The smaller effect on maize yield from intercropping, in Rotanda, can probably be explained by the specific soil properties there. For instance, values of clay, the sum of exchangeable cations, the capacity of cation exchange, the rate of base saturation, Mg, K, and Ca were higher in Rotanda than in Sussundenga. Also, the pH levels in Sussundenga were, in general, lower than that recorded in Rotanda (Tables S2.1 and S2.2.). These features corroborate the observation made in previous soil characterization studies made by Wijnhoud (1997), which described the soils of Sussundenga Station as acidic with dominant features such as the presence of exchangeable aluminum and low amount of exchangeable hydrogen. Soil acidity is known as a cause of serious soil infertility problems, with calcium and magnesium uptake by plants being replaced by aluminum, resulting in nutrient deficiencies and reduced yields. Also, the type of clay in Sussundenga is characterized by a low cation exchange capacity (Wijnhoud, 1997).

All these observations reinforce that soils with a predominantly clayey texture have good nutrient and water retention capacities, while sandy soils are poorly fertile (Wijnhoud, 1997). Thus, by growing in soil conditions of Sussundenga, maize plants could not capture enough nutrients when exposed to interspecific competition.

2.3.3 Crop yield assessment in Sussundenga and Rotanda in 2018/2019 season

Based on the data obtained in the 2017/2018 season, where intercropping did not improve maize yield in Sussundenga, we have planned a second field trial introducing blocks with mineral fertilizer to assess its effects on maize yield and eventually identify variations in yield performance associated to different varieties. This is important because, although fertilizer adoption in Mozambique is still low, there is an increasing effort from the government to increment the use of inorganic fertilizer among small-scale farmers through direct financing.

Additionally, based on the hypothesis we previously rose that the competition for soil nutrients would be contributing to the adverse effects in intercropping in Sussundenga, we decided to desynchronize the sowing time of the two species (or relay intercropping). According to Niringiye et al. (2005), greater yield advantages due to complementary use of resources (e.g., light, water, and nutrients) are realized from crop mixtures when maturity differences of the target species are large enough. Thus, we sow cowpea six weeks after maize sowing, as suggested by Rusinamhodzi et al. (2012).

In general, fertilizer application improved grain yield over the nonfertilized plots. In the second season, we obtained an equivalent grain yield (in kg/ha) that ranged between 1100 to 1809kg/ha in monocropping in Sussundenga, and 2898 to 3958Kg/ha in intercropping, both without fertilizer. The monocropped plots with fertilizer yielded 3966 to 4777kg/ha, and for the intercropped plots, 6580 to 7726kg/ha was recorded. In Rotanda, we recorded grain yield that ranged between 1980 to 3077kg/ha in monocropping, and 2613 to 3100kg/ha in intercropping, in both without the use of fertilizer.

The monocropped plots with fertilizer yielded 4639 to 5925kg/ha, and for the intercropped plots, 5214 to 7213kg/ha was recorded. These values agreed with previous studies reporting the performance of the tropical improved maize varieties under monocropping, intercropping, and fertilizer use (Rusinamhodzi et al., 2012; Tamene et al., 2018; Kiwia et al., 2019), and were above the local average yield.

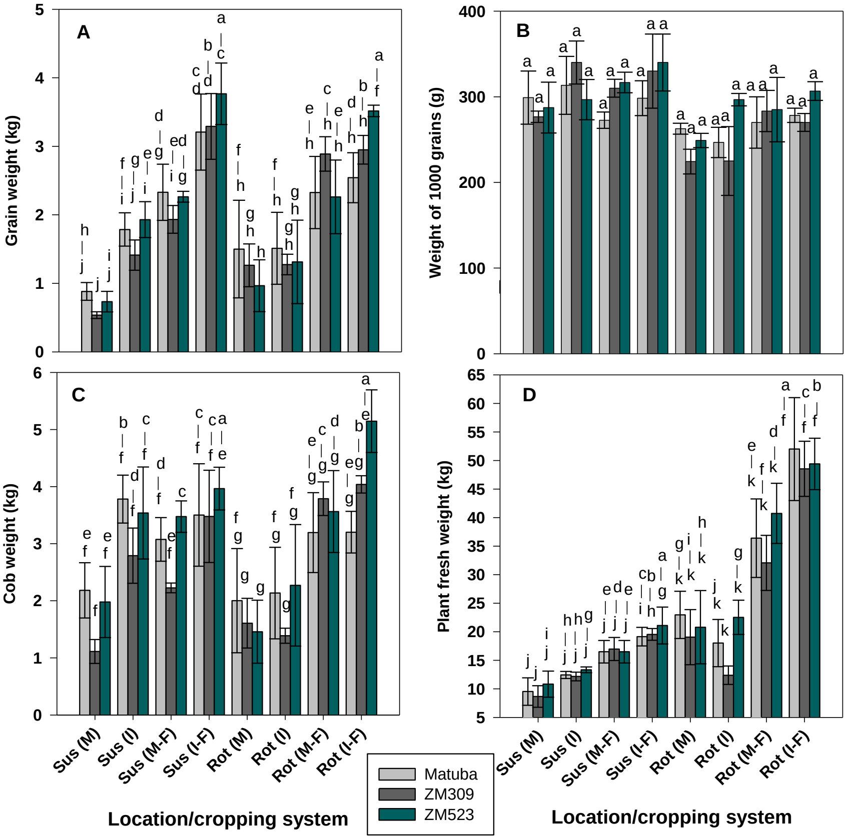

Figure 2.4 - Effect of different cropping systems and mineral fertilizer application in total grain weight per plot (A), weight of 1000 grains (B), Cob weight (C), Plant fresh weight (D) in the 2018/2019 cropping season. Sus (Sussundenga); Rot (Rotanda); M (monocropping); I (intercropping); F (fertilizer application). Bars are an average of three plots±SE. Different letters represent significant differences within cropping systems and locations (one-way ANOVA, Tukey test, $p<0.05$).

Reports on the performance of maize varieties regarding agronomic parameters were previously described (Tahir et al., 2008; Akongwubel et al., 2012; Băşa et al., 2016; Maqbool et al., 2016; Marković et al., 2017), with intercropping and fertilizer application indicated as positively impacting maize yield as compared to monocropping and non-fertilized fields.

Both in Sussundenga and Rotanda, we found no statistical differences among varieties within treatments in all parameters (one-way ANOVA, $p<0.05$). In Sussundenga, there was a tendency for a slight increase in grain yield, cob weight, fresh biomass, and cob number, as we move from the monocropping blocks to intercropping and fertilized blocks (Fig. 2.4 and 2.5A). Intercropping recorded at least a two-fold increase in grain yield in all varieties when compared to monocropping. These data suggest that in the second season, intercropping contributed to higher productivity over monocropping in Sussundenga. Also, the combination of intercropping and mineral fertilizer application increased maize yield as compared to sole maize, to intercropping without fertilizer, and to monocropping with fertilizer, except for plant height and root length (Fig. 2.4 and 2.5).

Fertilizer application in Sussundenga contributed to smaller root length in the second cropping season if compared to the first season. Therefore, the good nutrient availability in the soil through fertilizer addition may have impacted the root ability to penetrate deeper into the soil.

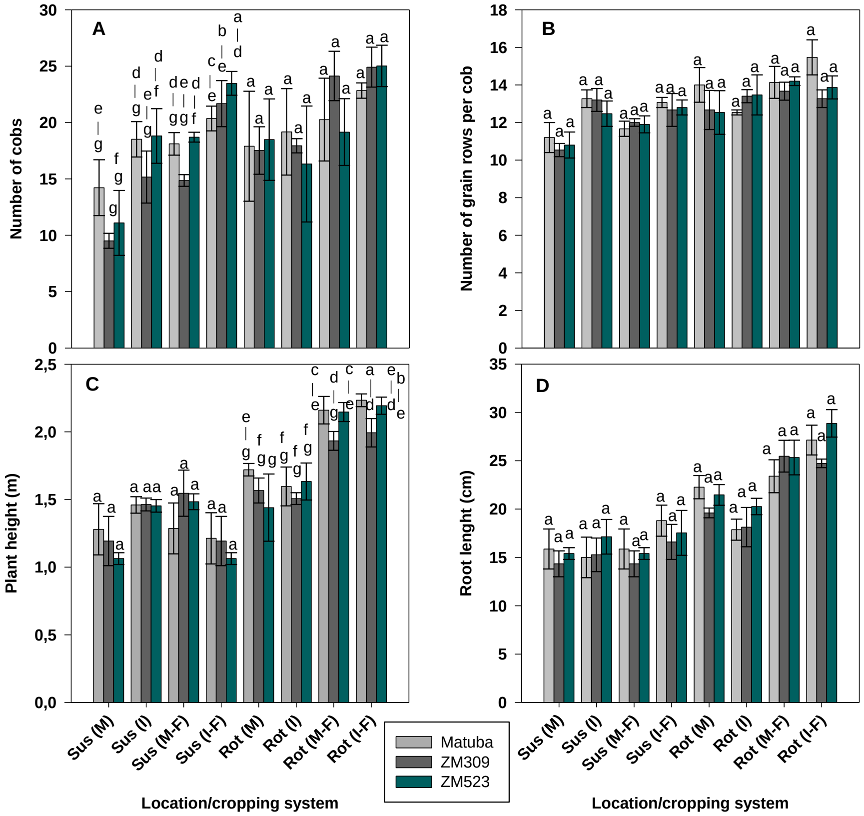

Figure 2.5 - Effect of different cropping systems in a number of cobs (A), number of grain rows per cob (B), Plant height (C), Root length (D) in the 2018/2019 cropping season. Sus (Sussundenga); Rot (Rotanda); M (monocropping); I (intercropping); F (fertilizer application). Bars are an average of three plots±SE. Different letters represent significant differences within cropping systems and locations (one-way ANOVA, Tukey test, $p<0.05$).

The better performance of intercropped blocks in Sussundenga in the 2018/2019 season could have the contributions of several factors. Although the sandy soil characteristics in Sussundenga have not changed (continued soil surface run-off), the fertilizer application and different rainfall patterns may have minimized the loss of nutrients.

Another possibility is that cowpea could more successfully fix nitrogen in year two than in the first year. Also, and more likely, relay intercropping may have supplied maize plants with enough nitrogen and phosphorus in the reproductive stage and significantly reducing interspecific competition for soil resources.

In Rotanda, monocropping and intercropping without fertilizer performed similarly. The data were consistent with what we observed in the previous season. Also, a notable increase was observed for most of the parameters in all fertilized blocks, with intercropping showing slightly higher values (Fig. 2.3 and 2.4). Raza et al. (2019) also reported relay intercropping systems producing equal or higher grain yield than sole maize yield. Therefore, based on the data collected in the two cropping seasons, Rotanda can be recommended as better for intercropping practice than Sussundenga. In Rotanda, for both strip and relay intercropping, there was a balance between monocropping and intercropping and a relatively more stable grain yield over the cropping seasons. The relatively higher levels of calcium and lower soil acidity in Rotanda might contribute to the production stability in the intercropped blocks. For example, the plant-available calcium supply is critical for nitrogen fixation. The step in which bacteria of the genus *Rhizobium* penetrate a legume root and, in response, the plant forms a nodule, depends on Ca supply. Also, the Inhibition of nodulation is described as a major limiting factor in nitrogen fixation of many legume species cultivated in acid mineral soils (Hart et al., 2013; Weisany et al., 2013).

Fertilizer application in monocropping contributed to a 56% to 135% increase in grain yield as compared to the non-fertilized blocks, and a 68% to 167% increase in fertilized intercropped blocks. Plant fresh biomass was the parameter that recorded the highest increase in fertilized blocks, being almost two-fold or higher than in non-fertilized blocks. Another study that also aimed at investigating the impact of inorganic fertilizers in sustainably intensifying intercropping systems (cereal-pigeon pea) was done in Mozambique, Kenya, and Tanzania. In this study, fertilizer in the intercropping system improved cereal yields by 71–282%, increased benefit-cost ratios by 10–40%, and reduced variability in cereal yields by 40–56% as compared to unfertilized intercrops (Kiwia et al., 2019).

We also found a notable increase of around four centimeters in root length in fertilized blocks as compared to the non-fertilized ones in Rotanda. Due to the higher nutrient availability and probably the low need for growing deeper, we were expecting an opposite phenotype, but this increase can be attributed to a high nutrient-use efficiency such as high Nitrogen-Use Efficiency (NUE). For example, in the study of Yu et al. (2015), maize with smaller root/shoot values had lower NUE, while the maize with larger root/shoot values had higher NUE values.

NUE is defined as the ratio of grain yield to plant N and is a good indication of the response of reproductive sink capacity and growth to the uptaken N. Grain number is the main trait that is sensitive to N utilization and is highly correlated with maize grain yield response to N supply (Kriz & Larkins, 2009).

Regarding the effect of nutrients on root biomass, there is a general consensus that P deficiency leads to higher root/shoot ratios. However, regarding the impact of P on root biomass length the debate is more controversial. Several studies reported by Mollier & Pellerin (1999) indicated that P fertilization increases root length and biomass on a wide range of species and experimental conditions. For example, Hajabbasi & Schumacher (1994), reported that the addition of P increased the relative growth rate of roots in two maize genotypes, CM37 and W153R, and this increase was attributed to the enhanced P-uptake capacity in these varieties. Bruce et al. (1994) studied the effects of P supply on root growth in a different species, cucumber, and also observed an increase in root length.

On the other hand, we observed that for the treatments without fertilizer application, root length in 2017/2018 was higher than in the 2018/2019 season. This could be related to different nutrient and water availability/-dynamics in both cropping seasons.

In both Sussundenga and Rotanda, intercropping with fertilizer performed better than intercropping without fertilizer addition. This is also supported by Niringiye et al. (2005), that claimed that the intercrop system yield may not be sustained without external inputs and that it is very important to implement studies on the effect of fertilizer on the performance of this cropping system.

The overall increase in maize yield with fertilizer application was also observed in several studies (Rusinamhodzi et al., 2012; Băşa et al., 2016; Jiang et al., 2018), where grain yield and biomass were significantly higher in all N application treatments as compared to the control, thus highlighting the positive impact of proper fertilization.

2.4. Conclusion, recommendations and future studies

Our agronomic data suggests that under sufficient rainfall amounts, Matuba, ZM309, and ZM523 generally performed similarly in both Rotanda and Sussundenga, with none of them standing out over the other. The main reasons behind the differences between the two locations may be related to the particular dynamics of belowground features such as soil nutrient composition and also the rainfall amount and distribution throughout the cropping seasons.

Intercropping was considered as a good practice to alleviate soil fertility constraints. However, under certain conditions, proper optimization of the practices, such as the planting date would be necessary. Niringiye et al. (2005) also recommend that before planting legumes, all maize leaves below the ear should be stripped off, to increase the amount of solar radiation reaching the legumes.

Maize yield obtained from intercropped blocks were more consistent in Rotanda in the two seasons, as we observed that in Sussundenga, significant yield reduction was observed in intercropped blocks in the first cropping season. Taking these present data into account, and using the rationale of production stability, Rotanda can be considered the best choice for the implementation of intercropping.

Fertilizer application impacted positively in yield over the non-fertilized blocks and is recommended for the improvement of maize productivity, also in intercropping conditions. However, further studies on fertilizer optimization in intercropping, specifically when considering the agroecological zones in Mozambique, are needed to avoid incorrect use of fertilizer. Yu et al. (2015) also recommend that to improve NUE in maize, breeding programs targeting specifically the root traits are essential.

Although not included in the present study, crop rotation, is strongly recommended over continuous monocropping practices. Rotation of maize with legume can result in more effective sub-soil nitrate and water utilization than maize as a single monoculture, which makes this practice as a good alternative on the Mozambican context (Festus et al., 2010). Also, crop rotation may contribute to reduced pest and disease within cereal crops (Franke et al., 2018).

We also recommend a future study on the use of intercropping with fertilizer trees such as *Gliricidia* that can be more effective in pumping soil nutrients rather than a legume-maize rotation system, although, farmers without permanent ownership of land (which is common in Mozambique) would prefer species with shorter life cycles like legumes (Festus et al., 2010).

Finally, taking into consideration that besides agriculture, chicken farming is also widespread in Mozambique, the high cost of inorganic fertilizers can also be circumvented by the use of organic fertilizer such as poultry manure to ensure an adequate supply of nutrients and increase crop yield and profitability as suggested by Akongwubel et al. (2012).

Acknowledgments

Field data acquisition and experiments monitoring: Dr. Magalhães Amade Miguel and João Simbe, both from IIAM-Centro Zonal Centro, Mozambique.

Meteorological data acquisition: David Mutongoreca and Januário Virgílio João, both from IIAM-Centro Zonal Centro.

Supplementary Data

Supplemental Tables

Table S2.1 - Soil sample analysis data of the 2017/2018 cropping season at sowing time.

Table S2.2 - Soil sample analysis data of the 2017/2018 cropping season at harvesting time.

Supplemental figures

Figure S2.1 - Administrative division of Mozambique

Figure S2.2 - Schematic representation of the experimental design

Figure S2.3 - Meteorological data of 2017/2018 cropping season

Figure S2.4 - Meteorological data of 2018/2019 cropping season

References

Akongwubel, A. O., Ewa, U. B., Prince, A., Jude, O., Martins, A., Simon, O., & Nicholas, O. (2012). Evaluation of Agronomic Performance of Maize (*Zea mays* L .) under Different Rates of Poultry Manure Application in an Ultisol of Obubra, Cross River State, Nigeria. *International Journal of Agriculture and Forestry*, *2*(4), 138–144. https://doi.org/10.5923/j.ijaf.20120204.01

Barbottin, A., Lecomte, C., & Jeuffroy, M. (2005). Nitrogen Remobilization During Grain Filling in Wheat. *Crop Science*, (May), 1141–1150. https://doi.org/10.2135/cropsci2003.0361

Băşa, A., Ion, V., Dumbravă, M., Temocico, G., Epure, L., & Ştefan, D. (2016). Grain Yield and Yield Components at Maize under Different Proceding Crops and Nitrogen Fertilisation Conditions. *Agriculture and Agricultural Science Procedia*, *10*, 104–111. https://doi.org/10.1016/j.aaspro.2016.09.025

Brooker, R. W., Bennett, A. E., Cong, W., Daniell, T. J., George, T. S., Hallett, P. D., White, P. J. (2015). Improving Intercropping: A Synthesis of Research in Agronomy, Plant Physiology And Ecology. *New Phytologist*, 107–117.

Bruce, A., Smith, S., & Tester, M. (1994). The Development Of Mycorrhizal Infection In Cucumber: Effects of P Supply on Root Growth, Formation of Entry Points and Growth of Infection Units. *New Phytol.*, *127*, 507–514.

Bybee-finley, K. A., & Ryan, M. R. (2018). Advancing Intercropping Research And Practices in Industrialized Agricultural Landscapes. *Agriculture*, *80*(8), 1–24. https://doi.org/10.3390/agriculture8060080

Carter, M. R., & Gregorich, E. G. (2006). Soil Sampling and Methods of Analysis. New York: Taylor & Francis Group.

Davis, R., Harris, G., Roberts, P., & Macdonald, G. (2017). Designing Research and Demonstration Tests for Farmers' Fields (pp. 1–8). pp. 1–8. *UGA Extension*.

Du, Q., Zhou, L., Chen, P., Liu, X., Song, C., & Yang, F. (2019). Relay-Intercropping Soybean With Maize Maintains Soil Fertility and Increases Nitrogen Recovery Efficiency by Reducing Nitrogen Input. *The Crop Journal*. https://doi.org/10.1016/j.cj.2019.06.010

Fato, P., Mulima, E., Senete, C. T., & Nhamucho, E. (2011). Manual de Produção de Milho. Maputo. *Instituto de Investigação Agrária de Moçambique.*

Festus, K. A., Ajayi, O. C., Silesh, G., Chirwa, P. W., & Jonas, C. (2010). Review Article Fertiliser Trees For Sustainable Food Security In The Maize-Based Production Systems Of East And Southern Africa . A review. *Agronomy*, *30*, 615–629.

Flynn, R., & Idowu, J. (2015). Nitrogen Fixation by Legumes. NM State University.

Franke, A. C., Brand, G. J. Van Den, Vanlauwe, B., & Giller, K. E. (2018). Agriculture, Ecosystems and Environment Sustainable Intensification Through Rotations With Grain Legumes in Sub- Saharan Africa: A review. *Agriculture, Ecosystems and Environment*, *261*(September 2016), 172–185. https://doi.org/10.1016/j.agee.2017.09.029

Gatiboni, L. C., Menotti, C., Oliveira, B. De, Roberto, P., & Jaime, E. A.

(2010). Soil Available Phosphorus by Mehlich-1, Mehlich-3 and Anion Exchange Resin and its Sensitivity to Soil clay content.

Geddes, B. A., & Oresnik, I. J. (2016). The Mechanism of Symbiotic Nitrogen Fixation. *Advances in Environmental Microbiology*, (December), 69–97. https://doi.org/10.1007/978-3-319-28068-4

Gianoli, E., Ramos, I., Alfaro-Tapia, A., Valdéz, Y., Echegaray, E., & Yábar, E. (2006). Benefits of A Maize – Bean – Weeds Mixed Cropping System in Urubamba Valley, Peruvian Andes. *International Journal of Pest Management*, *52*(September 2016), 283–289. https://doi.org/10.1080/09670870600796722

Hajabbasi, M., & Schumacher, T. (1994). Phosphorus Effects on Root Growth and Development in Two Maize Genotypes. *Plant and Soil*, 39–46.

Hart, J. M., Sullivan, D. M., Anderson, N. P., Hulting, A. G., Horneck, D. A., & Christensen, N. W. (2013). Soil Acidity in Oregon: Understanding and Using Concepts for Crop Production. *Oregon State University.*

Jiang, C., Lu, D., Zu, C., Zhou, J., & Wang, H. (2018). Root-Zone Fertilization Improves Crop Yields And Minimizes Nitrogen Loss in Summer Maize in China. *Scientific Reports*, (2018), 1–9. https://doi.org/10.1038/s41598-018-33591-9

Kimaro, A. A., Timmer, V. R., Chamshama, S. A. O., Ngaga, Y. N., & Kimaro, D. A. (2009). Agriculture, Ecosystems and Environment Competition Between Maize and Pigeonpea in Semi-Arid Tanzania: Effect on Yields and Nutrition of Crops. *Agriculture, Ecosystems and Environment*, *134*, 115–125. https://doi.org/10.1016/j.agee.2009.06.002

Kiwia, A., Kimani, D., Harawa, R., Jama, B., & Sileshi, G. W. (2019). Sustainable Intensification with Cereal-Legume Intercropping in Eastern and Southern Africa. *Sustainability*, *11*, 1–18.

Kriz, A. L., & Larkins, B. A. (2009). Biotechnology in Agriculture and Forestry (Vol. 63).

Maat, H. (2011). The history and future of agricultural experiments. *NJAS - Wageningen Journal of Life Sciences*, *57*(3–4), 187–195. https://doi.org/10.1016/j.njas.2010.11.001

MAE (Ministério da Administração Estatal, Moçambique) (2012). Perfil Do Distrito de Sussundenga Província De Manica.

Maqbool, M., Tanveer, A., Ali, A., Abbas, M. N., Imran, M., Quayyum, M. A., Abid, A. A. (2016). Growth and Yield Response of Maize (*Zea mays* L.) to Inter and Intra -Row Weed Competition under Different Fertilizer. *Planta Daninha*, *34*, 47–56.

Marković, M., Josipović, M., Šoštarić, J., & Jambrović, A. (2017). Response of Maize (*Zea mays* L.) Grain Yield and Yield Components to Irrigation and Nitrogen Fertilization. *Journal of Central European Agriculture*, *18*(1), 55–72. https://doi.org/10.5513/JCEA01/18.1.1867

Mollier, A., & Pellerin, S. (1999). Maize Root System Growth And Development As Influenced By Phosphorus Deficiency. *Journal of Experimental Botany*, *50*(333), 487–497.

Niringiye, C., Ssekabembe, C., & Kyamanywa. (2005). Effect of Plant Population on Yield of Maize and Climbing Beans Grown in Intercropping System. *African Crop Science Society*, *13*, 83–93.

Raza, M. A., Hayder, M., Khalid, B., Xia, Z., Feng, L. Y., Khan, I., Yang, W. (2019). Effect of Planting Patterns on Yield, Nutrient Accumulation And Distribution in Maize and Soybean under Relay Intercropping Systems. *Scientific Reports*, (August 2018), 1–14. https://doi.org/10.1038/s41598-

019-41364-1

Rusinamhodzi, L., Corbeels, M., Nyamangara, J., & Giller, K. E. (2012). Field Crops Research Maize – Grain Legume Intercropping is an Attractive Option for Ecological Intensification that Reduces Climatic Risk for Smallholder Farmers in Central Mozambique. *Field Crops Research*, *136*, 12–22. https://doi.org/10.1016/j.fcr.2012.07.014

Silici, L., Bias, C., & Cavane, E. (2015). Sustainable Agriculture for Small-Scale Farmers in Mozambique. A scoping report. London.

Stevens, R., & Winter-nelson, A. (2008). Consumer Acceptance of Provitamin A-biofortified Maize in Maputo, Mozambique. *Food Policy*, *33*, 341–351. https://doi.org/10.1016/j.foodpol.2007.12.003

Tahir, M., Tanv, A., A, A., & W, A. (2008). Comparative Yield Performance of Different Maize (Z*ea mays* L.) Hybrids under Local Conditions of Faisalabad-Pakistan. *Pakistan Journal of Life and Social Sciences*, *6*, 118–120.

Tamene, D., Anbessa, B., Ta, L., & Dereje, G. (2018). Refining Fertilizer Rate Recommendation for Maize Production Systems in Assosa, North Western Ethiopia. *Advanced Techniques in Biology & Medicine*, *6*(1), 1–9. https://doi.org/10.4172/2379-1764.1000253

USAID. (2016). Mozambique Early Generation Seed Study.

Velasco, V. V., Salgado, S., & Cardenas, R. (2008). Influence of P Fertilization on Biological Nitrogen Fixation in Herbaceous Legumes Grown in Acid Savannah Soils from the Tabasco State, Mexico Influence of P Fertilization on Biological Nitrogen Fixation in Herbaceous Legumes Grown in Acid Savannah Soils. *Journal of Sustainable Agriculture ISSN:*, *0046*, 25–42. https://doi.org/10.1300/J064v31n03

Wagner, S. (2011). Biological Nitrogen Fixation. *Nature Education Knowledge* 3(10):15.

Wang, Z., Bao-luo, M., Yu, X., Gao, J., Sun, J., Su, Z., & Yu, S. (2019). Physiological Basis of Heterosis for Nitrogen Use Efficiency of Maize. *Scientific Reports*, *18708*(9), 1–11. https://doi.org/10.1038/s41598-019-54864-x

Weisany, W., Raei, Y., & Allahverdipoor, K. H. (2013). Role of Some of Mineral Nutrients in Biological Nitrogen Fixation. *Bulletin of Environment, Pharmacology and Life Sciences*, *2*(March).

Wijnhoud, J. D. (1997). Solos e Outros Recursos Naturais da Estação Agrária de Sussundenga. pp. 1–41. *Série Terra e Água.*

Yan, S., Du, X., Wu, F., Li, L., Li, C., & Meng, Z. (2014). Proteomics Insights Into the Basis of Interspecific Facilitation For Maize (*Zea mays*) in Faba Bean (*Vicia faba*)/Maize Intercropping. *Journal of Proteomics*, *109*, 111–124. https://doi.org/10.1016/j.jprot.2014.06.027

Yu, P., Li, X., White, P. J., & Li, C. (2015). A Large and Deep Root System Underlies High Nitrogen-Use Efficiency in Maize Production. *PLos One*, 1–17. https://doi.org/10.1371/journal.pone.0126293

Zhao, Y., Fan, Z., Hu, F., Yin, W., Zhao, C., & Yu, A. (2019). Source-to-Sink Translocation of Carbon and Nitrogen is Regulated by Fertilization and Plant Population. *Frontiers in Plant Science*, *10*(July), 1–15. https://doi.org/10.3389/fpls.2019.00891

Zhou, B., Serret, M. D., Pie, J. B., & Shah, S. S. (2018). Relative Contribution of Nitrogen Absorption, Remobilization, and Partitioning to the Ear During Grain Filling in Chinese Winter Wheat. *Field Crops Research*, *9*(September), 1–11. https://doi.org/10.3389/fpls.2018.01351

Supplementary data

Supplemental tables

Table S2.1- Soil sample analysis data of 2017/2018 cropping season at sowing time.

Site	Depth	pH		EC	Dr	Ds	Hr	Ha	Granulometry (%)			
	(cm)	(1:2,5)		(1:2,5)								
		H_2O	**KCl**	**µS/cm**	**g/cm^3**	**g/cm^3**	**%**		**CS**	**FS**	**silt**	**clay**
Sus	0-15	6.73	4.75	30	2.98	1.48	0.15	3.09	61	23	10	5
Sus	15-30	6.26	4.41	30	2.54	1.48	0.90	3.73	52	30	13	5
Sus	30-45	5.95	4.45	31	2.63	1.40	1.25	5.54	63	28	6	3
Rot	0-15	5.97	5.32	29	2.60	1.26	2.40	3.84	50	39	7	4
Rot	15-30	6.00	5.48	31	3.14	1.28	4.36	4.66	40	40	12	8
Rot	30-45	6.00	5.53	20	2.50	1.32	2.52	6.21	60	30	5	5
		K	**Ca**	**Mg**	**H+Al**		**S**	**T**	**V**	**O.M.**		**P**
		-------------------$Cmol_c/kg$------------------						-----------%------------				*ppm*
Sus	0-15	0.47	0.35	0.15	0.70		0.97	1.67	58	0.42		8
Sus	15-30	0.37	0.45	0.30	0.60		1.12	1.72	65	0.52		8
Sus	30-45	0.32	0.35	0.20	0.90		0.87	1.77	49	0.49		0
Rot	0-15	0.60	1.75	0.95	1.00		3.30	4.30	77	0.40		1
Rot	15-30	0.47	1.35	0.50	2.35		2.32	4.67	50	0.70		1
Rot	30-45	0.37	1.20	0.45	0.85		2.02	2.87	70	1.37		1

EC (electrical conductivity); Dr (real density); Ds (soil density); Hr (residual humidity); Ha (absolute humidity); CS (coarse sand); FS (fine sand); Sus (Sussundenga), Rot (Rotanda); S (sum of exchangeable cations of negligible acidity); H+Al (potential acidity) T (capacity of cation exchange); V (rate saturation); O.M (organic matter); P (extractable phosphorus); $Cmol_c/kg$ (centimoles of charge/kg); µS/cm (microsiemens/cm).

Table S2.2. Soil sample analysis data of the 2017/2018 cropping season at the harvesting time.

Site	Depth (cm)	pH (1:2,5)		EC (1:2,5)	Dr	Ds	Hr	Ha	Granulometry (%)			
		H_2O	**KCl**	**μS/cm**	**g/cm^3**	**g/cm^3**	**%**		**CS**	**FS**	**silt**	**clay**
Sus (M)	0-15	5.9	4.37	48	2.35	1.36	1.14	1.42	44	28	5	24
Sus (M)	15-30	4.52	4.37	58	2.38	1.27	1.34	1.63	43	24	6	27
Sus (M)	30-45	4.55	4.41	62	2.70	1.19	1.66	2.25	33	23	8	37
Sus (I)	0-15	4.94	4.54	43	2.39	1.41	0.70	0.91	53	27	4	16
Sus (I)	15-30	4.59	4.29	93	2.96	1.33	0.86	1.42	51	26	4	18
Sus (I)	30-45	4.24	4.30	106	2.86	1.22	1.22	174	45	21	5	28
Rot (M)	0-15	5.47	5.39	62	2.74	1.19	2.92	6.44	15	25	34	27
Rot (M)	15-30	5.47	4.43	58	3.36	1.21	0.75	6.50	15	23	30	32
Rot (M)	30-45	6.78	5.73	74	2.39	1.34	2.43	7.12	9	18	30	43
Rot (I)	0-15	5.56	5.69	65	2.37	1.32	2.73	4.93	16	23	33	28
Rot (I)	15-30	6.91	5.51	82	2.46	1.30	2.77	5.32	13	20	32	35
Rot (I)	30-45	6.61	5.73	58	2.29	1.34	2.95	5.65	8	17	28	47
		K	**Ca**	**Mg**	**H+Al**		**S**	**T**	**V**	**O.M.**		**P**
		-------------------*$Cmol_c/kg$*------------------						-----------*%*------------				*ppm*
Sus (M)	0-15	0.37	1.00	0.25	1.98		1.62	3.60	45	0.61		9
Sus (M)	15-30	0.26	1.00	0.25	1.65		1.51	3.16	48	0.58		7
Sus (M)	30-45	0.26	1.00	0.50	1.73		1.76	3.49	50	0.58		3
Sus (I)	0-15	0.22	0.75	0.25	1.40		1.22	2.62	47	0.51		6
Sus (I)	15-30	0.30	1.00	0.00	1.57		1.30	2.87	45	0.47		5
Sus (I)	30-45	0.41	0.75	0.25	1.82		1.41	3.23	44	0.44		4
Rot (M)	0-15	0.30	4.25	1.75	1.82		6.30	8.12	78	0.81		8
Rot (M)	15-30	0.22	3.25	2.00	1.90		5.47	7.37	74	3.05		6
Rot (M)	30-45	0.41	4.00	1.75	1.40		6.16	7.56	81	0.61		6
Rot (I)	0-15	0.22	4.50	2.00	1.57		6.72	8.29	81	0.88		7
Rot (I)	15-30	0.26	4.00	1.50	1.49		5.76	7.25	79	0.75		7
Rot (I)	30-45	0.30	3.25	1.75	1.24		5.30	6.54	81	0.68		6

Sus (M)-Sussundenga monocropping; Sus (I)-Sussundenga intercropping; Rot (M)-Rotanda monocropping; Rot (I)-Rotanda Intercropping.

Supplemental Figures

Figure S2.1- Administrative division of Mozambique. Sussundenga district is highlighted in red. Source: United Nations (2016), edited.

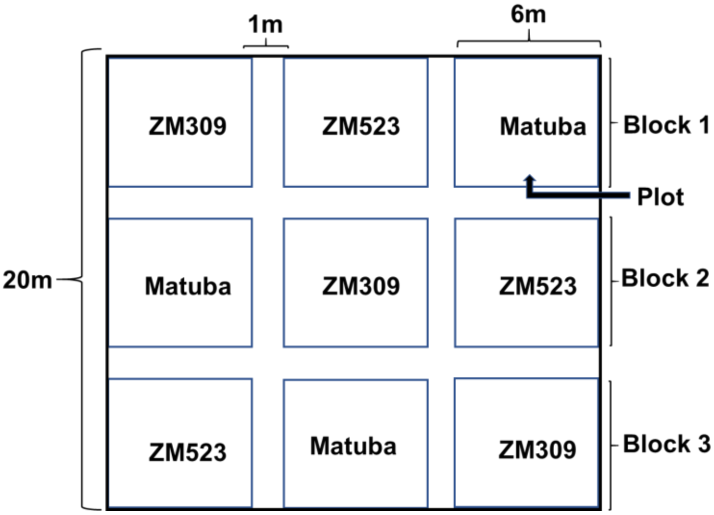

Figure S2.2 – schematic representation of the experimental design. Each block is composed of three plots (blue squares), each representing a different treatment or maize variety.

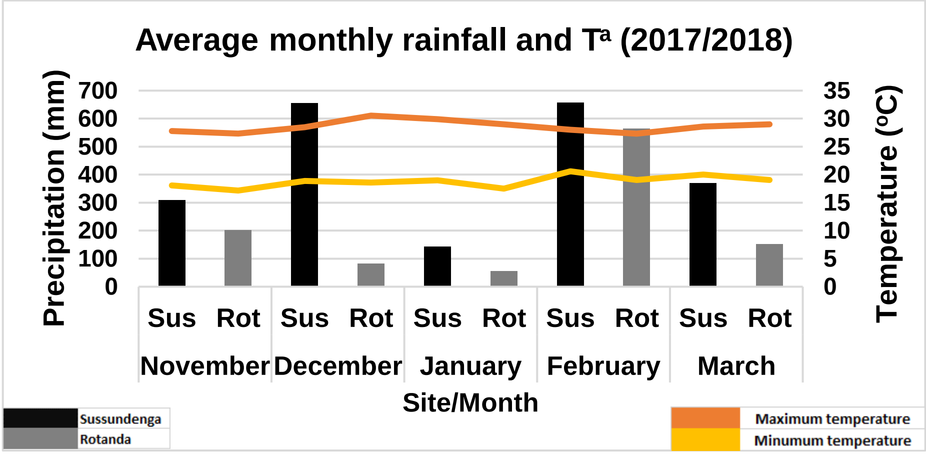

Figure S2.3 – Meteorological data of 2017/2018 cropping season for Sussundenga (Sus) and Rotanda (Rot) regions.

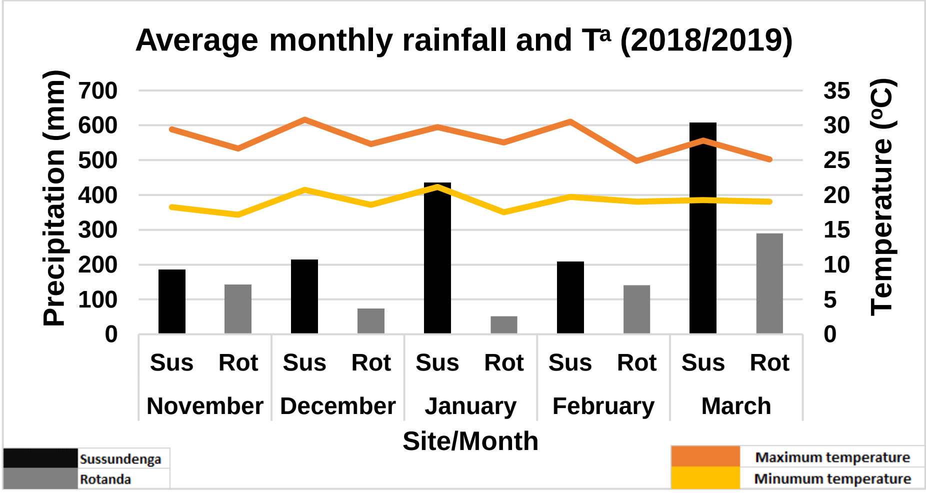

Figure S2.4 – Meteorological data of 2018/2019 cropping season Sussundenga (Sus) and Rotanda (Rot) regions.

Chapter III. Characterization of maize growth and physiological responses induced by different water treatments in controlled environments

The work presented in this Chapter was entirely performed by Dizimalta dos Santos Fernando Miquitaio

Abstract

Mozambique is a country that is severely affected by climate change, namely with recurrent drought episodes that affect the production of staple crops such as maize. Thus, it is essential to continue generating and delivering knowledge about different strategies that may contribute to crop drought resistance and be used in breeding programs to develop improved seeds.

In this work, we aimed at exploring morphological and physiological strategies within drought resistance in three Mozambican open-pollinated maize varieties, namely, Matuba, ZM309, and ZM523. The well-characterized maize inbred line B73 currently in use in the host lab was included as a control. All genotypes were tested in the controlled environment of greenhouse and growth chambers in three watering regimes (75%, 40%, and 25% of field capacity), corresponding to well-watered, moderate drought, and severe drought conditions.

Water limitations negatively affected growth and biomass production, as well as photosynthetic capacity in all genotypes. We could observe some of the drought resistance characteristics, such as the reduced stomatal opening in all the genotypes, more pronounced reduction of leaf area in Matuba and B73, and more visible leaf rolling in ZM523 and ZM309. These features are known to be associated with reduced water loss by transpiration and increased water-use efficiency (WUE). Also, in severe drought, root biomass was higher in ZM523 as compared to the other genotypes, which could allow this variety to uptake more water. However, at shoot level, ZM523 was the variety with lower photosynthetic performance in severe drought conditions, particularly visible when testing the response to light intensity variations. Despite all the differences observed in drought resistance strategies, the overall water status of the plants was very similar across genotypes.

We have further observed a reduction of Electron Transport Rate (ETR) between PSII and PSI in all drought-stressed genotypes. ETR reduction is a way to reduce ROS formation in chloroplasts in low CO_2 availability, as well as a mechanism of energy conservation under harsh conditions, but it also limits ATP and NADPH synthesis.

In this work, we also hypothesized that molecular mechanisms, such as ROS elimination, less degradation of PSII proteins (such as D1), and an adequate pool of carotenoids could play a pivotal role in the drought tolerance success, particularly for protection and repair of the photosystems' reaction centers.

All the observations mentioned above led us to the conclusion that it is necessary to describe all the specific features that make a particular variety listable as a "drought-resistant" one. Also, since Mozambican varieties may display different strategies to cope with drought, it is of use for the breeding programs to aggregate all types of information on well-organized plant-feature and performance descriptions, so that more robust maize seed may be developed and provided to farmers either being open-pollinated or hybrid varieties.

3.1 Introduction

As described in chapter II, the Mozambican varieties were first tested in local field conditions in the Manica province, which offered the required water levels for normal maize development. Therefore, our next goal was to test the morpho-physiological performance of the varieties, including a control genotype (B73 was selected), in water-limiting conditions.

The present study is critical because water scarcity is one of the major limiting factors for crop productivity in Mozambique, affecting particularly staple crops such as maize (PEDSA, 2011) (a detailed discussion of the challenges of Mozambican agriculture is presented in chapter I).

Recurrent drought events are a consequence of the global climate change that is impacting the earth's crust resulting in infrequent and erratic precipitations, higher temperatures, and expansion of areas affected by flooding or water limitations (Lamaoui et al., 2018).

Drought and heat can negatively impact crop productivity, thus reducing the income for farmers. Drought stress occurs when soil and atmospheric humidity are low if compared to the ambient air temperature that is high. This results in an imbalance between the evapotranspiration and water uptake from the soil (Lamaoui et al., 2018), which in severe drought conditions may even cause embolism (gas bubbles) or collapse of xylem cells (Minorsky, 2003).

Plants as sessile organisms must respond to drought stress via several complex biochemical, physiological, morphological, and anatomical adaptations, resulting in short and long-term developmental and growth-related changes (Aslam et al. 2015; Gururani et al., 2015; Ghatak et al., 2017).

Resistance strategies to cope with drought in plants are multiple and mostly related (Riccardi et al., 2004). Resistance to drought can be described as less reduction in yield (for example, maize grain yield) under drought conditions in comparison to the normal water levels. In fact, the resistance can guarantee certain yield levels within farmers even in harsh conditions. Resistance can take place in three main ways, namely, in the form of escape, avoidance, and tolerance (Aslam et al., 2015).

Drought escape is achieved via completing plant life cycle before severe water stress occurs (e.g., early flowering), drought avoidance takes place via enhancing intaking water capacity (e.g., developing root systems or conserving water by reducing transpiration such as closure/reduction of stomata and leaf area), while drought tolerance occurs via improving osmotic adjustment, increasing cell wall elasticity to maintain tissue turgidity, water circulation (for example through aquaporins), ROS elimination, protection or degradation of proteins (chaperones, dehydrins, proteases, and antiproteases) or different cellular structures, through the synthesis of abscisic acid (ABA) that is crucial in stomata closure, via carbon and nitrogen metabolism, and signal transduction (Riccardi et al., 2004; Ghatak et al., 2017).

Plant drought tolerance is a complex feature requiring a global view to completely understand its mechanisms. Its study is build up by combining data obtained through different approaches, including physiology and phenotypic data (Abreu et al., 2013; Wang et al., 2016). Also, germplasm evaluation in areas where severe drought largely fluctuates should be carried out comparing well-watered conditions and different levels of drought stress (Zaman-Allah et al., 2016).

Hence, our first attempt to explore biological strategies of maize genotypes under water scarcity conditions used semi-controlled greenhouse conditions. We explored the growth and biomass traits in well-watered conditions *versus* moderate drought. These watering regimes were chosen to allow the completion of the maize life cycle.

For further characterization, we tested the genotypes in a fully controlled environment in growth chambers, further including severe drought. Drought stress was intended to be severe enough so that features that become important for plant survival could be distinguished from those that affect plants in well-watered conditions (Zaman-Allah et al., 2016).

We hypothesize that stress treatments would decrease overall physiological performance and growth of the maize genotypes, and that different strategies to cope with the stress would associate with particular genotypic backgrounds. The molecular assessment of these experiments is detailed in Chapter IV.

3.2. Material and Methods

3.2.1 Growth and biomass production at maturity stage in greenhouse conditions

Growth and biomass production were assessed through experiments in greenhouse conditions at ITQB NOVA. Experiments were conducted from May to November 2018, until plant maturity stage, using maize B73 as control and three maize varieties from Mozambique, Matuba, ZM309, and ZM523, respectively. The Mozambican maize genotypes were certified seed provided by the Agricultural Research Institute of Mozambique (IIAM), and the inbred line B73, currently in use in the host Lab, was multiplied at the ITQB NOVA greenhouse.

Plants were grown in 75% field capacity (FC) (well-watered) and 40% FC (moderate drought) in 15L round pots with approximately 32 cm diameter at the top, 25 cm diameter at the bottom, and 31 cm depth. The sunlight intensities ranged within 300–800 µmol photons m^{-2} s^{-1}, ~13h light/11h dark on average, and air temperature ranged ~30ºC/25ºC (day/night). Pots were filled with about 7kg of a substrate composed of a mixture of peat and soil 3:1 (v/v). Seven plants were used in each of the eight treatments, totalizing 56 plants that were kept at the set field capacity by weighting at least twice per week, adding water when necessary. Pots were also randomly moved once per week to minimize position-dependent effects. To guarantee continuous nutrient supply to the plants, a total of 21g of slow-release fertilizer (Osmocote, NPK 16-9-12) was added to each pot, 15g at the sowing stage and 7g two months later.

At the end of the plant life cycle, fresh and dry biomasses were assessed. Briefly, plants were separated into below and aboveground fractions, weighted and then oven-dried at 70°C for 72h before a second weighing for dry biomass determination. Stem diameter was taken at the middle point of the plants using a caliper, and plant height was assessed from the ground level up to the basis of the panicle using a measuring tape.

3.2.2 Growth, biomass production, and physiological evaluation at seedling stage in growth chamber conditions

For the characterization of the physiological responses, maize seedlings were also tested under different water treatments. Pots used were 9.5 cm height, 9.5 cm wide at the top, and 7 cm wide at the bottom. Pots were filled with 300g of a 3:1 (v/v) ratio mixture of peat and soil. Controlled experiments were conducted in a growth chamber at ITQB NOVA (FITOCLIMA 10000 HP, 3.305mx1.600mx1.950m, Aralab). A long-day photoperiod of 16/8h with photosynthetic photon flux density (PPFD) of ~320 to 340 μmol photons m^{-2} s^{-1} at 3rd/4th leaf plant height, was applied, with 28/22°C of air temperature, and 60% of air relative humidity. Seeds were sown, and during seedling development three different watering regimes were applied: 75%, 40%, and 25% FC, corresponding to well-watered conditions (control), moderate drought, and severe drought, respectively, totalizing 12 treatments (*i.e.*, 4 genotypes x 3 watering regimes). Seedlings were developed until the 3rd leaf was expanded, and photosynthetic measurements were made, usually at the beginning of the 4th leaf emergence.

3.2.3 Growth, biomass, and leaf area determination

For the fresh and dry biomass determinations, seedlings were separated into root and shoot fractions, weighted (fresh-weight), and then oven-dried at 70 °C for 72h before the second weighing (dry-weight). For the leaf area determination, 3rd leaf length and breadth at the broadest point were taken using measuring tape and ruler. Leaf area (in cm^2) was calculated as the product of the leaf length and breadth, *i.e.*, Leaf Area = lamina length x maximum width x k, (where k is the coefficient determination of leaf area, that for maize is 0.75) (Mollier & Pellerin, 1999; Tanko & Hassan, 2016; Wei et al., 2016)

3.2.4 Determination of the water field capacity

Field capacity (FC) in all controlled experiments was determined according to Medeiros et al. (2012), using the gravimetric method. A 100% field capacity consists of the weight difference between wet soil (after saturation and at least 24h free drainage under dark), and completely dry soil, and indicates the amount of water that can be retained in soil (Medeiros et al., 2012; Al-Shaheen et al., 2018). Different levels of drought stress were imposed, manipulating the amount of water available in the soil (eg., 25% FC is equivalent to only 25% of the water present in fully wet soil).

After determining the amount of water needed to define each FC selected, the maintenance of the water treatments was made by daily weighing of the pots and replacing the water lost by evapotranspiration using a precision scale. The reduction of pot weight was used to estimate relative water losses (Medeiros et al., 2012; Klukas et al., 2015). Pots under growth chamber conditions were also randomly moved every day to minimize position-dependent effects.

3.2.5 Gas-exchange measurements and chlorophyll fluorescence analysis

Leaf gas exchange measurements were done in the 3rd expanded leaves of 10 plants per treatment. The third leaf is the first leaf, that in contrast to the first and second leaf already has a C4-photosynthetic configuration (Pick et al., 2011). Also, at the three-leaf stage, seedlings can grow in soil with sufficient water (Zhao et al., 2019) when large energy reserves are depleted in seeds (Chen et al., 2016).

Photosynthetic measurements were performed with a portable Infrared Gas Analyzer (IRGA) LI-6400 (LI-COR, Inc., Lincoln, Nebraska USA), equipped with a light chamber fluorometer (LI-6400-40, LI-COR Inc.). The leaf chamber was set to a block temperature of 25 ^{o}C, 400 ppm of CO_2 reference concentration, 330 μmol photons m^{-2} s^{-1} of PPDF, 10% blue light to ensure stomatal opening, and an airflow rate of 300 μmol s^{-1}.

The assessed photosynthetic parameters were: CO_2 assimilation ($A_{n,}$ μmol m^{-2} s^{-1}), stomatal conductance to water vapor (*gs*, mol H_2O m^{-2} s^{-1}), and intercellular CO_2 concentration (*Ci*, μmol CO_2 mol air^{-1}).

By simultaneously measuring leaf gas exchange and Chl*a* fluorescence it was also possible to evaluate the photosystem II operating efficiency (Φ_{PSII}), which translates photochemistry efficiency or the rate of linear electron transport from PSII to PSI for a light-adapted leaf ($\Phi_{PSII}=(F'_m-F_s)/F'_m$) (Perdomo et al., 2016). F_s is the steady-state fluorescence yield and F'_m, the maximum fluorescence yield obtained with a light-saturating pulse. Φ_{PSII} was used for the calculation of the linear rate of electron transport (ETR, *J*,): ETR = $\Phi_{PSII} \cdot PPFD \cdot \alpha \cdot \beta$. (μmol electron m^{-2} s^{-1}), where α is the fraction of absorbed light by the measured leaf, and β is the fraction of the absorbed light energy distributed to PSII. Leaf α is usually around 0.84 and β=0.5 under steady-state photosynthesis (Flexas, 2010; Florez-Sarasa et al., 2016; Perdomo et al., 2016). Intrinsic Water-Use Efficiency was calculated as WUEi = A_n/gs, (μmol CO_2 mol^{-1} H_2O) (Medrano et al., 2015).

All instantaneous measurements always started at least 3h after the beginning of the photoperiod and within a maximum of 6 hours per day to avoid overloading the equipment.

3.2.6 The light response curves

To obtain additional insights on the photosynthetic performance, rapid light response curves were obtained to study the effects of gradients in light absorption in the photosynthetic capacity of the plants (de Sousa et al., 2017).

For the generation of light–response curves using the 3rd expanded leaf, light chamber fluorometer conditions were set as follows: 25 °C of leaf temperature, 60% of air relative humidity, 400 ppm of CO_2 concentration, and a gradually decreasing PPFD from 2000 to 0 µmol photons m^{-2} s^{-1} in 10 steps (2000, 1500, 1000, 500, 250, 150, 100, 50, 25, 0), with 180 seconds intervals of acclimation. Data were taken after the infrared gas analyzer parameters reached a steady-state value following the onset of the respective PPFD (Dohleman et al., 2009).

3.2.7 Relative water content determination

Leaf relative water content (RWC) was determined using the 3rd leaf. For this purpose, a leaf cut (3 to 5 cm^2) was taken from the middle of the leaves, and the fresh weight was determined, followed by floating on distilled water for up to 24h under dark. The turgid weight was then recorded and the cut leaf was subsequently oven-dried at about 70°C for 48h for dry weight assessment. Five plants were analyzed per treatment.

The relative water content (RWC) of the leaf was calculated as follows: RWC (%) = [(FW−DW)/(TW−DW)] ×100, where FW, DW, and TW are fresh, dry, and turgid weight, respectively (Benešová et al., 2012; Medeiros et al., 2012).

3.2.8 Statistical analysis

Data of all morphophysiological traits were analyzed using one-way ANOVA and Tukey test to compare means of genotypes within treatments. Differences were considered significant at *p<0.05*. Statistical analysis was performed using SigmaPlot 11.0 software package (Systat Software Inc., Chicago, IL, USA).

3.3. Results and Discussion

3.3.1 Growth and biomass production at the maturity stage in greenhouse conditions

In the present study, water restrictions negatively affected plant traits at the maturity stage by decreasing plant biomass, plant height, and stem diameter, as previously described (Fig. 3.1) (Aslam et al., 2015; Hussain et al., 2019). B73 was the genotype that was more affected by drought, with a reduction of all the above parameters much stronger than observed in any other of the genotypes (Fig. 3.1). For example, the fresh aboveground biomass reduced 80% under drought conditions in B73, while for the other genotypes, 25 to 70% reductions were observed. Similar tendencies were also found for the belowground biomass. Interestingly, in the greenhouse experiment, B73 was the genotype to first complete its life cycle, *i.e.*, around 15-20 days earlier than the Mozambican genotypes. Therefore, B73 possibly uses drought escape as a mechanism of drought resistance.

Under well-watered conditions, Matuba and ZM523 had both the highest values of aboveground biomass compared to both ZM309 and B73, but at moderate drought, the Mozambican varieties showed no statistical differences (Tukey test, *p*<0.05) (Fig. 3.1A and C).

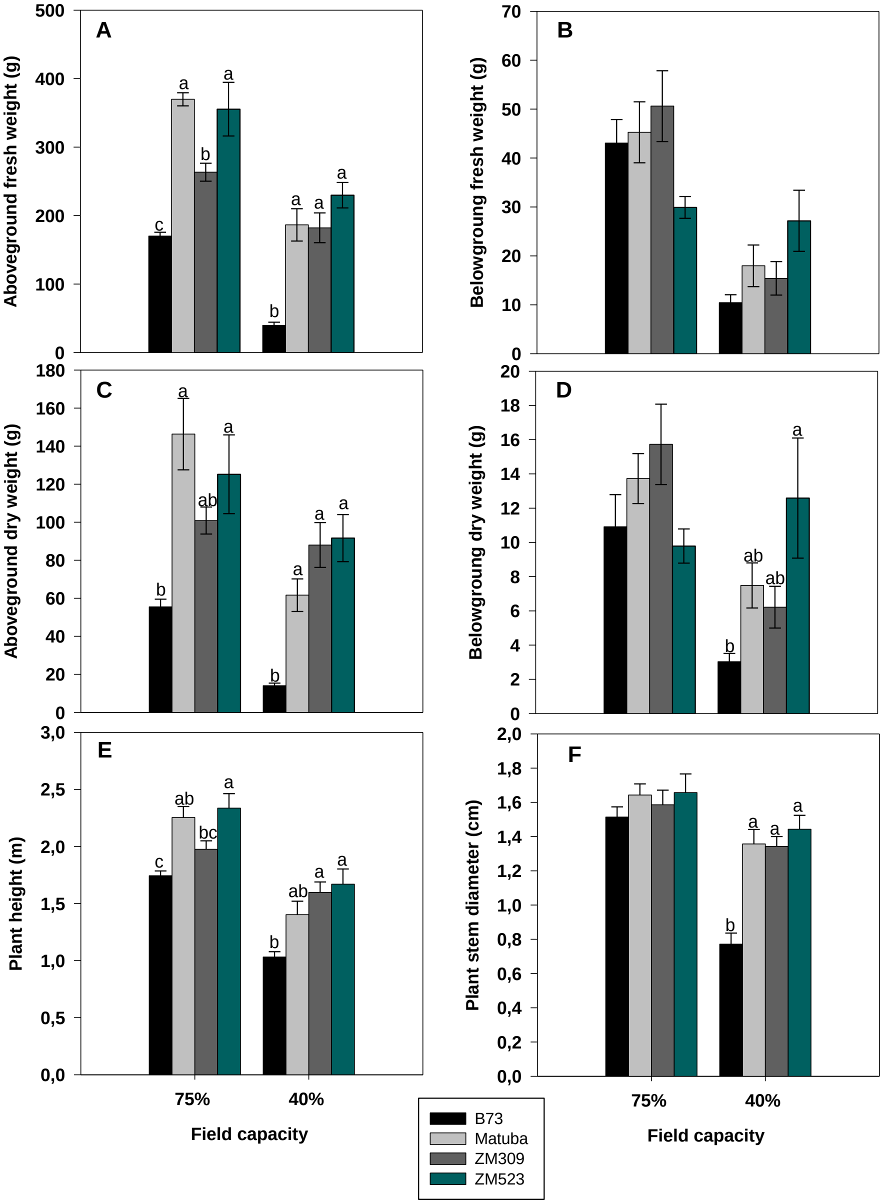

Figure 3.1 - Growth and biomass production of the maize genotypes in different watering regimes in greenhouse conditions. The aboveground fresh weight (A), belowground fresh weight (B), aboveground dry weight (C), belowground dry weight (D), plant height (E), and plant stem diameter (F) are shown. The bars are means ±SE (n=7). Different letters indicate statistical significance of the differences among genotypes within the treatments (Tukey test, *p*<0.05).

Despite not statistically significant, ZM523 tendency was to be the one with lower belowground biomass amongst Mozambican varieties in well-watered conditions. The opposite phenotype was observed in drought stress, where ZM523 maintained root biomass if compared to well-watered conditions, likely representing an adaptive tendency to enhance water uptake in water scarcity conditions. Thus, deep-root systems are probably beneficial when water is available deep in the soil, but not so much in well-watered conditions (Lopes et al., 2011).

The similarities amongst Mozambican varieties concerning the vegetative traits such as plant fresh weight and height were also observed in field experiments. Thus, these data suggest that the general performance of the Mozambican varieties may be identical in well-watered environments.

The lower aboveground biomass, height, and stem diameter with drought is consistent with previous studies (Ge et al., 2016; Riboldi et al., 2016; Sun et al., 2016; Hussain et al. 2019; Voronin et al., 2019). These reductions can be due to the inhibition of plant growth due to the reduction in cell division and enlargement. The inhibition of growth is also known to be one of the earliest responses to water deficit (Voronin et al., 2019).

3.3.2 Growth and biomass production at seedling stage under growth-chamber conditions

Drought affected plant traits at not only the maturity stage but also the seedling stage in the experiments conducted in growth chambers (Fig. 3.2A, B, C, and D). For example, leaf fresh biomass decreased 47% (in B73) to 72% (in ZM309) from well-watered to moderate drought. Under well-watered conditions, B73 displayed the lowest leaf biomass within the analyzed genotypes. This tendency was already observed at the maturity stage (see section 3.3.1 for details).

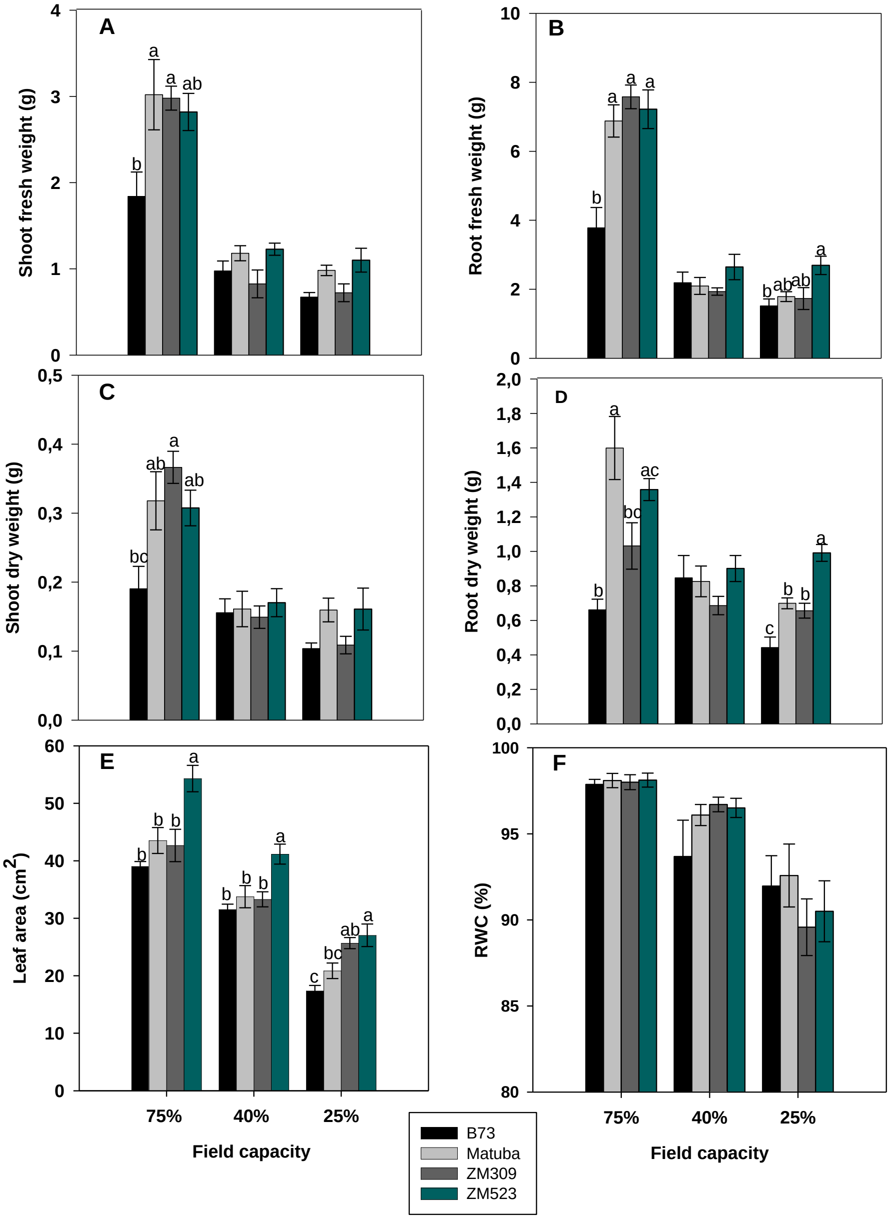

Figure 3.2 - The impact of different watering regimes in plant biomass, leaf area, and leaf water status under growth chamber conditions. Shoot fresh weight (A), root fresh weight (B), shoot dry weight (C), root dry weight (D), 3rd leaf area (E), and Relative Water Content (RWC) (F) are shown. The bars are means ±SE (n = 5, except for the leaf area that is n=10). Different letters indicate statistical significance of the differences among genotypes within treatments (Tukey test, $p<0.05$).

Curiously, B73 lower biomass should not directly result from lower levels of photosynthesis since no statistical differences were observed with the genotypes under well-watered conditions (see a detailed discussion on photosynthesis in section 3.3.4). Also, despite not statistically significant, in moderate and severe drought conditions, Matuba and ZM523 tended to have higher leaf biomass as compared to ZM309 and B73 (Fig. 3.2A and C).

Water restrictions also tended to decrease root biomass in all genotypes (3.2B, and D). Roots have critical importance for plant survival because they are the primary detectors or sensors of drought stress. Root density, volume, and the number of roots are reduced under mild and severe drought stress (Aslam et al., 2015). However, ZM523 tendency was to have the highest root biomass under drought conditions, while the other genotypes achieved root biomass up to almost twofold lower compared to well-watered conditions. The adequate root density could allow this variety to uptake more water under drought stress. Hence, the enhanced root growth observed in ZM523 under stress conditions can be considered a defense against drought (Ghatak et al., 2017). In this case, carbon allocation to roots can be considered a good trait for drought tolerance (Lavinsky et al., 2015).

Although not statistically significant as compared to Matuba and ZM309, a similar tendency of having a higher root-to-shoot ratio was also found in ZM523 and ZM309 in water-limited conditions (Supplemental Fig.S3.1). The increase in the root-to-shoot ratio can be considered a characteristic of drought response and an indication that shoot growth was proportionately more affected than root growth (Benjamin et al., 2014; Ahmad et al., 2016). Although not applying severe drought, this tendency of increasing root-to-shoot ratio was also observed in the greenhouse for ZM523, but not for ZM309. Therefore, in ZM523, root growth is possibly more important as a strategy to uptake more water than in the other genotypes (Supplemental Fig.S3.2).

The leaf area was also affected by water restrictions, with ZM523 being the least affected and B73 the most affected, while ZM309 and Matuba showed intermediated size (Fig. 3.2E). Reductions in leaf area and biomass with drought stress were also reported in other studies (Sun et al., 2016; Nelissen et al., 2018; Hussain et al., 2019) as well as different growth responses between the targeted maize genotypes. The reduction in leaf area was also attributed to diminished cell division and cell expansion (Shao et al., 2008; Nelissen et al., 2018).

Reduction in leaf area under drought conditions is considered an adaptive strategy and an early response to water deficit in maize, allowing plants to reduce water loss by transpiration (Riccardi et al., 2004). A smaller leaf area leads to reduced plant transpiration, conserving the limited water supply in the soil over a longer period. Thus, reduction in leaf area can be considered a first line of defense against drought, increasing the probability of plant survival in water scarcity conditions, with chlorophyll content, chloroplast amount, and photosynthetic activity being reduced (Aslam et al., 2015). Additionally, larger leaf in a water-restricted environment can negatively affect water availability for the plants (Shao et al., 2008). However, a reduction in leaf area by water stress is also considered a significant cause of yield penalty due to the reduction in CO_2 assimilation (Shao et al., 2008).

We also observed that the leaf area did not always match plant leaf biomass accumulation. ZM309 showed this tendency, under stress conditions in growth chamber experiments (Fig. 3.2), with similar values of leaf area as those observed in Matuba, and lower leaf biomass. This observation corroborates Weraduwage et al. (2015) that found that the relationship between leaf area and plant biomass was inconsistent and possibly attributed to changes that occurred in carbon partitioning to various organs during the transition from one growth phase to another. Therefore, growth and leaf mass can result from an increase in the area and also in thickness. They reported that area-based relative growth rate tends to considerably underestimate mass-based relative growth rate during all growth phases.

Inconsistencies regarding biomass accumulation and net photosynthesis, particularly under well-watered conditions, were also observed within our genotypes. This was particularly notable for B73, that, although performing similar net CO_2 assimilation rates as the Mozambican varieties, had lower shoot and root biomasses. Thus, its genetic profile may have an impact on its general growth, for instance, the capacity to uptake nutrients (such as nitrogen) and incorporate them in the metabolism. Also, although biomass production depends on photosynthesis, other critical steps such as sucrose synthesis and transport through phloem and carbon partitioning throughout the plant can have a significant impact on plant growth and development (Hofius & Börnke, 2007).

3.3.3 Leaf water status and water loss by evapotranspiration in growth chambers

The water stress imposed led to changes in the water status of the maize plants. However, the Relative Water Content measured was not statistically significant (Fig. 3.2F). The drop amplitude was more pronounced from control conditions to severe drought where values ranged from ~6 to ~8 percent units, with ZM309 and ZM523 being the genotypes with more visible variations in amplitude.

Regarding water loss by evapotranspiration, an overall increase was observed with time in all treatments, as the plants increased in size. Also, water deficit limited the amount of water lost by evapotranspiration compared to the well-watered treatments (Fig. 3.3C). A similar tendency was also observed by Ge et al. (2016), where the plants in the control group of two inbred maize genotypes, B73 and FFMM-A, showed significantly higher daily water consumption than the drought group ones.

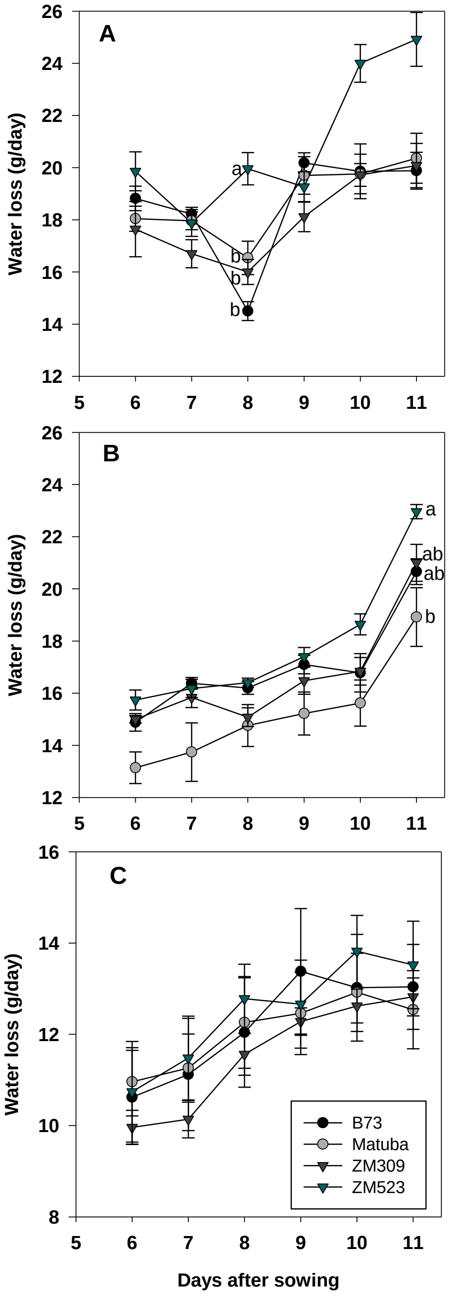

Figure 3.3 - The impact of different watering regimes in water loss by evapotranspiration. The levels of water loss under well-watered conditions (75% FC) (A), moderate drought (40% FC) (B), and severe drought (25% FC) (C) are presented. The dots are means ±SE (n=5). Different letters indicate statistical significance of the differences among genotypes within days after sowing (Tukey test, *$p<0.05$*).

Although mostly without statistical differences, ZM523 was the variety that recorded the maximum levels of water loss, which was consistent with its largest leaf area (Fig. 3.3). Also, except for ZM523, a notable decrease in water loss was observed after 8 days of sowing. After nine days of sowing, a general tendency of stabilizing water loss was observed except in moderate drought. This stabilization can be considered as an indication that the plants were close to achieving their maximum growth (for the well-watered conditions) or they were not capable of uptaking sufficient water due to the limited soil water content, and they were progressively restricting stomata aperture, thus saving water (applicable for the severe drought). Under moderate drought, the relation between the rate of plant growth/water availability and demands had possibly allowed continuous water-use by the plants.

Consistent with the previous studies (Ge et al. 2016; Riboldi et al. 2016), daily water consumption (or evapotranspiration) was largely determined by the water treatments and genotypes. Thus, the larger the leaf area, the increased water loss.

We also observed a more pronounced leaf rolling in ZM309 and ZM523 under severe drought (Supplemental Fig. S3.3). Leaf rolling was also previously reported in maize plants grown under drought conditions (Voronin et al., 2019; Riboldi et al., 2016). Leaf rolling was classified as an adaptive morphological trait to restrict transpiration and promote water conservation within leaf tissues (Voronin et al., 2019). Leaf rolling was also described as one of the mechanisms adopted in drought avoidance strategies in dry areas (Kadioglu et al., 2012; Lamaoui et al., 2018).

Leaf rolling is listed as a typical response to water deficit in numerous species such as rice, maize, wheat, and sorghum (Kadioglu et al., 2012), is considered as a good indicator of drought tolerance (Amelework et al., 2016), and used to select drought-tolerant maize germplasm

(Lavinsky et al., 2015). However, leaf rolling can be used to build a score for injury by drought stress conditions (Chen et al., 2010; Riaz et al., 2013). Therefore, under relatively mild stress, delaying leaf rolling may be important to maintain plant growth and production. Additionally, under severe drought and heat stress, enhanced leaf rolling may be associated with better chances for recovery after rewatering (Blum, 1992).

Kadioglu et al. (2012), have also reported that leaf rolling prevented structural and functional damages at the level of pigment antenna complexes and reaction centers of PSII, as well as at the level of electron transport between PSII and PSI in wheat leaves under short-period of high temperatures.

In the present study, leaf area and root growth are presented as important players in water conservation and water uptake capacity of the maize genotypes. ZM523 has the highest leaf area and root biomass observed, the latter being crucial under water deficit. However, with enhanced leaf area, ZM523 should become more susceptible to drought and show detrimental effects due to excessive transpiration. Hence, the enhanced root growth and increased water uptake coupled with leaf rolling could balance water loss potential in ZM523, while the other genotypes such as Matuba and B73 rely on reducing water loss through a more pronounced reduction of leaf area.

3.3.4 Leaf gas exchange of the instantaneous measurements

In this part of the work, we investigated the photosynthetic leaf features in response to different water treatments.

Consistent with the data of growth and biomass, the overall photosynthetic performance also tended to decrease in water-limiting conditions (Fig. 3.4). Under well-watered conditions and moderate drought, all the genotypes showed very similar levels of CO_2

assimilation, reaching maximum average values between 9.46 (Matuba) to 7.48 µmol CO_2 $m^{-2}s^{-1}$ (ZM309), respectively. We have only detected statistical differences, under severe drought, with Matuba showing higher levels of CO_2 assimilation as compared to ZM523. Despite not showing statistically significant differences, Matuba also had higher levels of CO_2 assimilation than B73 or ZM309 (Tukey test, $p<0.05$) (Fig.3.4A).

Regarding the performance of photosynthesis, we observed a reduction from 9.1 to 6.03 µmol CO_2 $m^{-2}s^{-1}$ (33% drop) when comparing 75% and 25% FC in the B73 genotype. For the other genotypes, following reductions was observed: Matuba, 9.5 to 6.37 µmol CO_2 $m^{-2}s^{-1}$ (33% drop), ZM309, 8.8 to 5.0 µmol CO_2 $m^{-2}s^{-1}$ (43% drop), ZM523, 9.3 to 4.7 µmol CO_2 $m^{-2}s^{-1}$ (49% drop), respectively (Fig.3.4A).

The stomatal conductance (*gs*) also performed as net photosynthesis, with drought limiting stomata opening. We observed 40%, 48.8%, decrease in *gs* from 75% FC to 40% FC and from 75% to 25% FC, respectively in B73. Matuba recorded 37.6%, 42,4% drops, for ZM309 were 48.7%, 58.6%, and for ZM523, 41.3%, 55.4% reductions, respectively (Fig.3.4B).

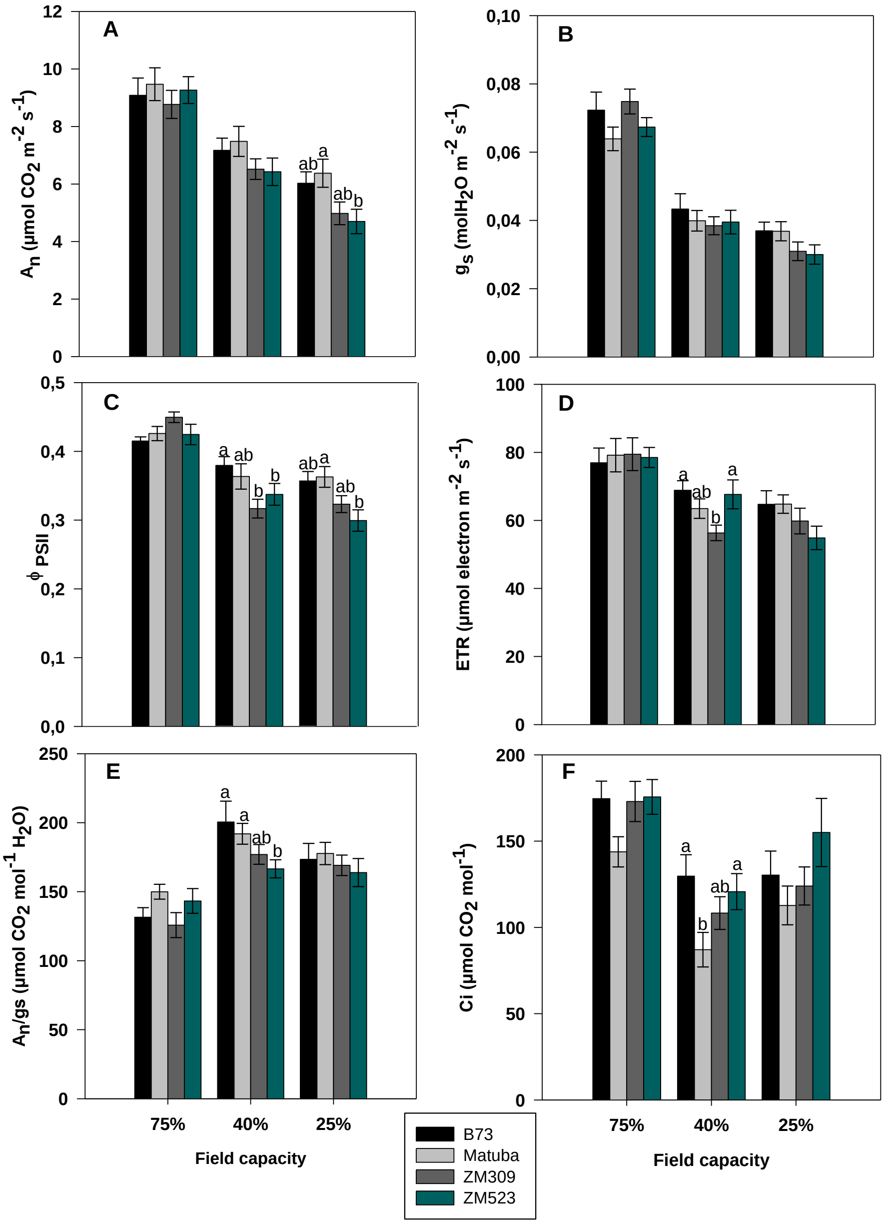

Figure 3. 4 - Leaf gas exchange and chlorophyll a fluorescence parameters of the instantaneous measurements for all water treatments. Net photosynthesis rate (A_n) (A), stomatal conductance to water vapor (gs) (B), PSII photochemistry (Φ_{PSII}) (C), Electron Transport Rate (ETR) (D), Intrinsic Water-Use Efficiency (WUEi, A_n/gs) (E), and Intercellular CO$_2$ (*Ci*) (F) are shown. The bars are means ±SE (n=10). Different letters indicate statistical significance of the differences among varieties within treatments (Tukey test, $p<0.05$). The data shown are the mean results of the three independent experiments.

The tendency of dropping in photosynthesis and stomatal conductance due to drought imposition is in agreement with the previous reports in maize (Ibarra-Laclette et al. 2009; Zhang et al., 2015; Riboldi et al., 2016; Xin et al., 2018). The strong relationship existing between photosynthesis and stomatal conductance, as we observed in our genotypes, means that the reduction in photosynthesis is regulated by stomatal closure, while a weak relationship indicates that the reduction in photosynthesis is a result of regulation by non-stomatal factors (Sun et al., 2016).

Reduction in CO_2 assimilation and stomatal conductance enhanced intrinsic Water-Use Efficiency (WUEi) in all genotypes (Fig. 3.4E). WUE is widely defined as the amount of carbon fixed as biomass in photosynthesis per unit of water used or transpired by the crop (Lawson & Blatt, 2014; Hatfield & Dold, 2019). The increase in WUE under stress treatments can be attributed to the decrease in the net photosynthetic rate associated with stomatal closure (Lamaoui et al., 2018), but in this case, the reduction in A_n is less than the reduction of *gs* (Hatfield & Dold, 2019). Also, reducing stomatal opening is thought to have a stronger inhibitory effect on water transpiration than on CO_2 diffusion through the leaf tissues (Lamaoui et al., 2018).

According to Lawson & Blatt (2014), in general, higher WUE has been observed in plants with lower *gs* and, consequently, lower stomata opening. However, the gains in saving water are usually achieved with a cost in a reduction of A_n and slower plant growth. On the other hand, plants with higher *gs* have higher rates of CO_2 assimilation and grow faster under well-watered conditions, but they generally show lower WUE.

The tendency for reducing stomatal aperture under drought conditions is important for reducing transpiration and save water, as well as to increase WUE particularly under moderate drought but not under severe drought, where stomatal closure becomes harmful (Aslam et al., 2015). Reducing of stomata opening of also inhibits the supply of CO_2 to the RuBisCO which leads to the formation of reactive oxygen species (ROS), and to a drop of intercellular CO_2 concentration (*Ci*) (Aslam et al., 2015; Benešová et al., 2012; Ghatak et al., 2017).

Previous reports have shown that stomatal response is correlated to the soil water content, which suggests that chemical signals in root-shoot communication, *e.g.*, abscisic acid (ABA), influence stomatal performance (Benešová et al., 2012; Ghatak et al., 2017). Also, ABA accumulation is increased in the leaves in response to drought-induced signals, which reduces stomatal opening (Aslam et al., 2015).

A decrease in A_n and *gs* also accompanied a decrease in the intercellular CO_2 (*Ci*) from control conditions to severe drought, but their levels did not necessarily match within treatments. Overall, Matuba and ZM309 had the lowest values. The simultaneous drops of A_n and *Ci* were an indication that a drop in net photosynthesis might be mainly caused by stomatal limitations (Wang et al., 2018). On the other hand, cases of increased *Ci* with drought can be attributed to non-stomatal rather than stomatal factors.

Despite having lower *gs* under severe drought as the other genotypes, ZM523 was the variety losing more water through evapotranspiration (Fig.3.3 and Fig. 3.4B). This can be attributed to the increased leaf area, and or higher stomata density.

3.3.5 Chlorophyll *a* fluorescence parameter

The data obtained through chlorophyll *a* fluorescence can be obtained in a rapid, highly sensitive, and non-invasive way and can be used to distinguish different responses to water limitations in a controlled environment, among different genotypes (de Sousa et al., 2017). The flow of electrons between the photosystem II and photosystem I are good indicators of the overall state of photosynthesis in plants growing under several conditions (Maxwell & Johnson, 2000).

During the photosynthetic light reactions, electrons released from water in PSII via the oxygen evolving complex are ultimately transferred to $NADP^+$ through PSI, forming the so-called acyclic electron flow. On the other hand, the electrons accumulated in the forward transport chain between PSII and PSI can be transported back to the reaction center of PSII, forming a backward electron flow. Additionally, a cyclic electron flow (CEF) around PSI also takes place, leading to ATP synthesis without the parallel generation of NADPH. However, it is not clear how drought stress affects these above types of electron flow and the concomitant ATP synthesis (Zhou et al., 2019).

In our study, drought also reduced the amount of electron transfer between PSII and PSI, illustrated by the values of PSII photochemistry (Φ_{PSII}) and ETR (Fig. 3.4C, and D). This reduction follows the tendency observed similarly to the ones observed for photosynthesis and stomatal conductance. Under severe drought, the Φ_{PSII} tendencies throughout the genotypes were similar to those observed for the CO_2 assimilation, with Matuba reaching higher values than ZM523 (Tukey test, $p<0.05$).

A decrease in electron transport was also observed in studies reported by de Sousa et al. (2017), where stressed plants showed lower Φ_{PSII} values compared to those under control conditions. This decline of photosynthesis and ETR activity also affected C3 plants like apple trees resulting in the shortage of adenosine triphosphate (ATP) and limitation of the regeneration of RuBP. This inhibition of ETR activity is frequently attributed to the humper of D1 protein turnover due to ROS accumulation (Wang et al., 2018). However, since a decline in photosynthesis in C4 plants such as maize may differ from that of C3 plants in drought conditions, the decrease in ETR would also have a different pattern in C4 plants.

However, unexpectedly our molecular data shows that under severe drought, the levels of the PSII proteins, such as the D1 protein, were higher than found in well-watered conditions. Thus, since drought is also described as damaging not only the donor and acceptor sides of PSII but also the PSII reaction center and the acceptor side of PSI (Zhou et al., 2019), we hypothesize that the increased levels of D1 protein in drought may be a strategy to enhance overall PSII protection when plants wait for a possible increase in soil water content (find a more detailed discussion in Chapter IV).

3.3.5 Gas exchange parameters of light response curves

To deepen the understanding of the differences among genotypes regarding physiological performance, we tested their photosynthetic response to light intensity variation, using rapid light response curves.
In light response curves, low light conditions account for limited photosynthesis with A_n increasing linearly with increasing light intensity. Therefore, the photons are absorbed by the leaf with great efficiency. At higher light intensities, light absorption increases accordingly, but the carbon reactions start to saturate, and A_n reaches a plateau. At low PPDF, photosynthesis is limited by the rate of electron transport while at high PPDF, photosynthesis is frequently limited by the activity of RuBisCO (ribulose-1,5-bisphosphate carboxylase-oxygenase). The result is that A_n displays a hyperbolic relationship with light intensity. The inflection point and maximum A_n level depends on the photosynthetic capacity (Cendrero-Mateo et al., 2015). Additionally, different from what occurs in C3 plants, the light response curves in the C4 photosynthetic pathway can also be affected within activities of C4-enzymes such as PEPC (Sun et al., 2012).

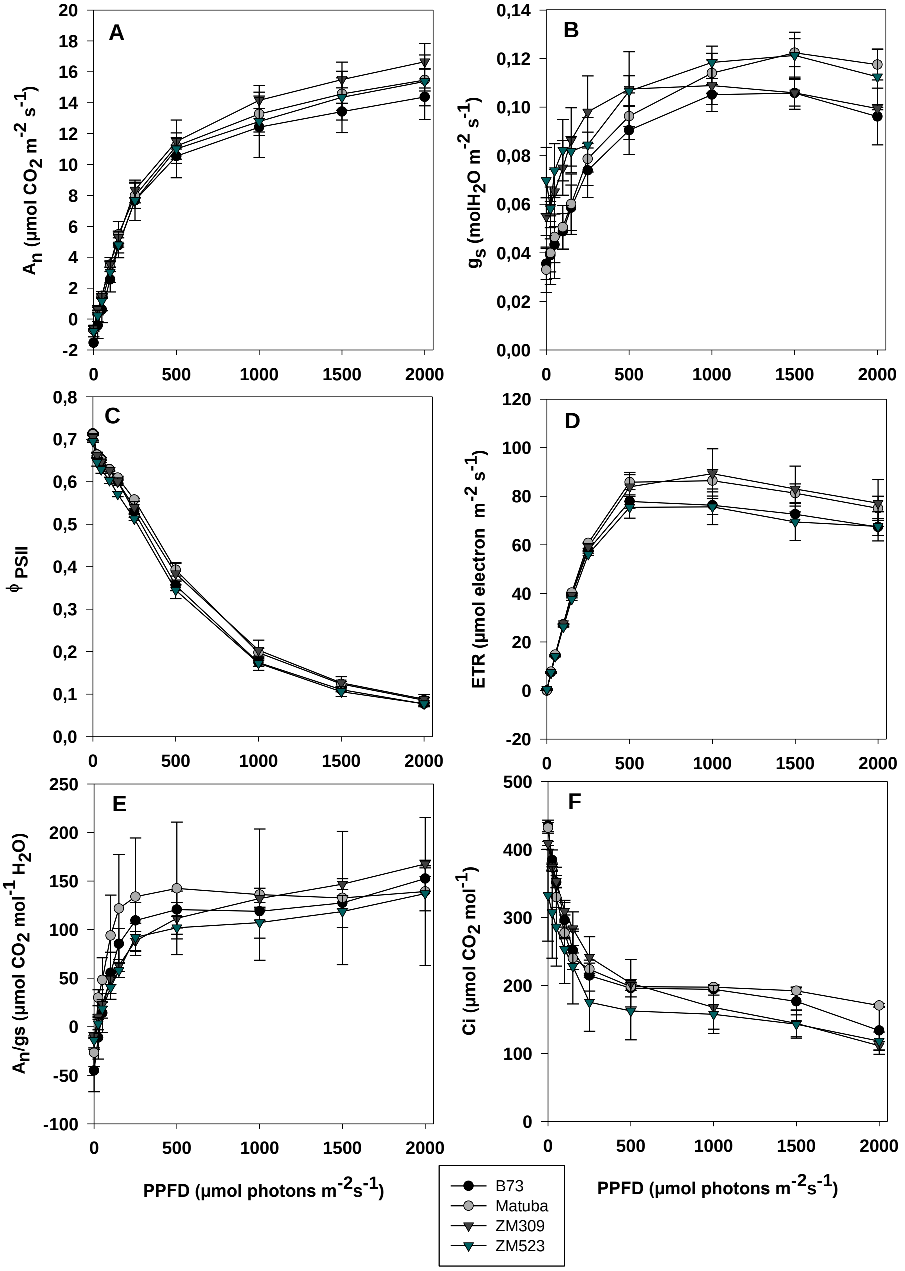

Figure 3.5 - The light response curves of all maize genotypes in well-watered conditions. The net photosynthesis rate (A_n) (A), stomatal conductance to water vapor (gs) (B), PSII photochemistry (Φ_{PSII}) (C), Electron Transport Rate (ETR) (D), Intrinsic Water-Use Efficiency (WUEi, A_n/gs) (E), and Intercellular CO_2 (*Ci*) (F) in response to light variation are shown. The dots are means ±SE (n = 3). Different letters indicate statistical significance of the differences among genotypes within light intensity (Tukey test, $p<0.05$).

All four maize genotypes showed similar tendencies regarding the light-response curves of gas exchange parameters. The net photosynthesis rates increased with light intensity until plateauing (Fig. 3.5A, 3.6A, and 3.7A). In general, drought reduced net photosynthesis rates in all genotypes. Under well-watered conditions, all the genotypes displayed similar tendencies of CO_2 assimilation throughout different light intensities, and they clustered together. The light compensation point (the irradiance at which photosynthesis equals respiration) was achieved below the light intensity of 25 μmol photons m^{-2} s^{-1} and the light saturation (the irradiation above which light is no longer the limiting factor of photosynthesis) was 1000 μmol photons m^{-2} s^{-1} in all maize genotypes (Fig. 3.5A). The tendency for *gs* response to light intensity variation was similar to that of A_n, with the genotypes also clustering together (Tukey test, $p<0.05$) (Fig. 3.5B).

The intrinsic Water-Use Efficiency (WUEi), also increased with light intensity, but above 250 μmol photons m^{-2} s^{-1}, the curves tended to stabilize (Fig. 3.5E). The intercellular CO_2 (*Ci*) showed a downward tendency with light intensity. (Fig. 3.5F). The RuBisCO and the C4-cycle enzyme activities may eventually determine the decreased tendency of *Ci* with light intensity observed in all treatments.

Under moderate drought, the tendencies were the same as those observed in well-watered conditions, but CO_2 assimilation and stomatal conductance were slightly lower (Fig. 3.6A and 3.6B).

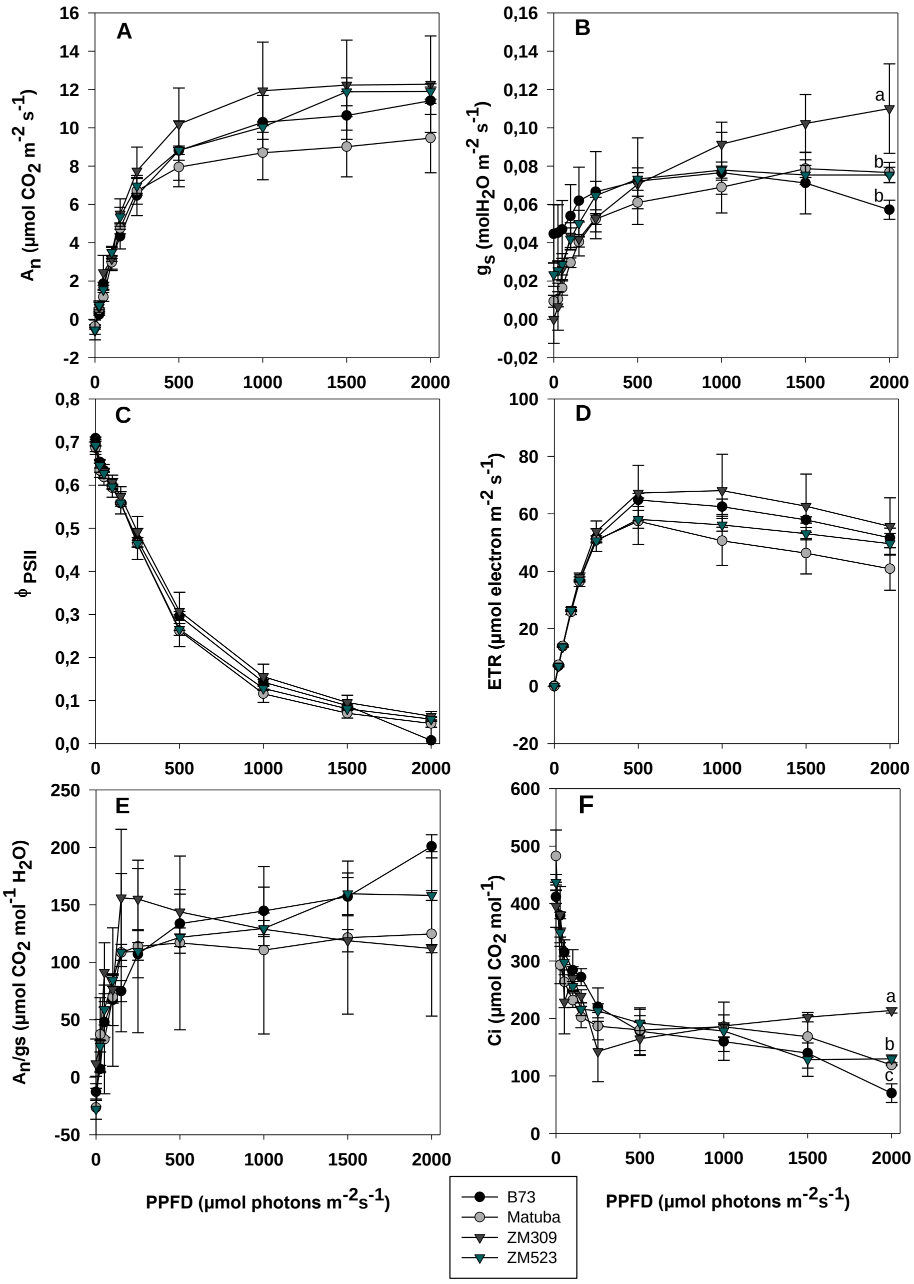

Figure 3.6 - The light response curves of all maize genotypes in moderate drought conditions. The net photosynthesis rate (A_n) (A), stomatal conductance to water vapor (gs) (B), PSII photochemistry (Φ_{PSII}) (C), Electron Transport Rate (ETR) (D), Intrinsic Water-Use Efficiency (WUEi, A_n/gs) (E), and Intercellular CO$_2$ (*Ci*) (F) in response to light variation are shown. The dots are means ±SE (n = 3). Different letters indicate statistical significance of the differences among genotypes within light intensity (Tukey test, p<0.05).

The WUEi values reached peak values at different light intensities among genotypes, which indicates that drought imposition began to exacerbate differences among these genotypes (Fig. 3.6E). ZM309 reached a plateau at 250 µmol photons m^{-2} s^{-1} followed by a downward tendency, while B73 achieved a plateau at a maximum light intensity, Matuba at 500, and ZM523 at 1500 µmol photons m^{-2} s^{-1} of light intensity, respectively. The intercellular CO_2 decreased with light intensity, reaching a stable state between 500 and 1500 µmol photons m^{-2} s^{-1}, which is consistent with limitations in CO_2 assimilation (Fig. 3.6F).

Severe drought also weakened maize leaves' capability to assimilate CO_2. The peak values were mostly achieved at 1500 µmol photons m^{-2} s^{-1}. The ZM309 variety reached the highest values of A_n (11.73 µmol CO_2 m^{-2} s^{-1}), while ZM523 showed the lowest (2.75) amongst the genotypes (Tukey test, $p<0.05$) (Fig. 3.7A).

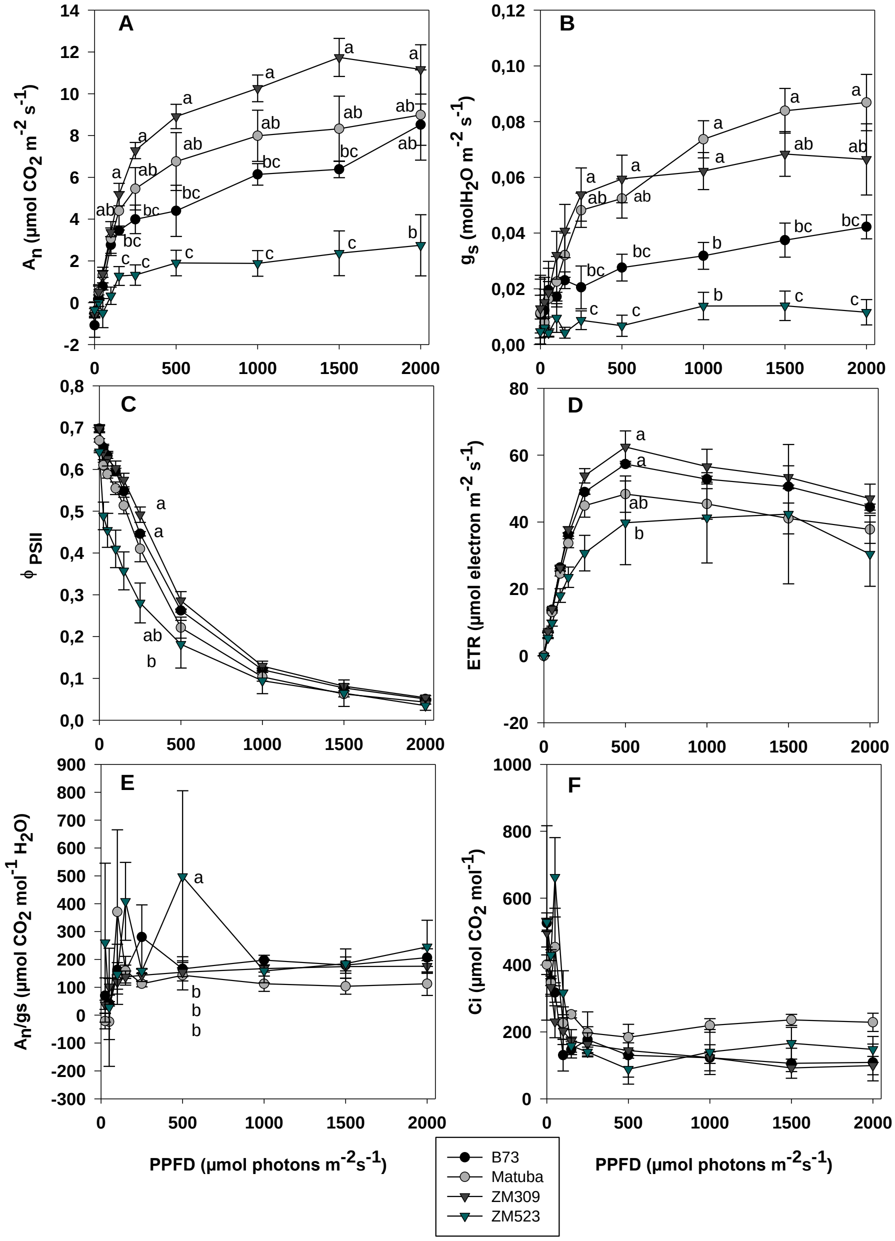

Figure 3. 7 - The light response curves of all maize genotypes in severe drought treatment. The net photosynthesis rate (A_n) (A), stomatal conductance to water vapor (gs) (B), PSII photochemistry (Φ_{PSII}) (C), Electron Transport Rate (ETR) (D), Intrinsic Water Use Efficiency (WUEi, A_n/gs) (E), and Intercellular CO_2 (*Ci*) (F) in response to light variation are shown. The dots are means ±SE (n = 3). Different letters indicate statistical significance of the differences among genotypes within light intensity (Tukey test, $p<0.05$).

Severe water deficit decreased stomata aperture, being more significant for ZM523, limiting CO_2 availability to the chloroplasts (Fig. 3.7.B). The drop amplitude of A_n and *gs* of ZM523 from well-watered to severe drought was larger than that observed for any other genotype. Also, the response of *gs* in ZM523 was basically insensitive to the light intensity increase, indicating that the stomata had possibly lost the regulation effects (Li et al., 2017).

WUEi oscillated for ZM523, achieving the highest values until 500 µmol photons m^{-2} s^{-1} followed by a constant tendency. The enhanced WUEi in ZM523 at least at light irradiance <500 µmol photons m^{-2} s^{-1}, is consistent with its lower *gs* when compared to the other genotypes. The intercellular CO_2 curves differed from those of the net photosynthesis rate, decreasing with the higher light intensity and stabilizing after 500 µmol photons m^{-2} s^{-1} in all genotypes.

As discussed in section 3.3.4, reducing stomatal aperture is a water-saving mechanism and a drought-resistance feature. It was also mentioned that to function efficiently under harsh conditions, plants must balance the gas exchange through leaf epidermis to maximize CO_2 uptake while minimizing water loss by transpiration (Lawson & Blatt, 2014). Benešová et al. (2012) suggested that genotypes displaying early stomatal-closure should have a good tolerance in conditions of long or severe water deficit. This is because they could decrease hydraulic gradients and save soil water for a longer time than plants with high *gs*. The drawback, in this case, would be a lower growth capacity and lower biomass accumulation after ending the drought period, as stomata closure affects photosynthetic capacity and, consequently, biomass production. Although the above-mentioned, in the study developed by Benešová et al. (2012), the more tolerant maize genotype

CE704, did not display such behavior and maintained open stomata and efficient transpiration. This condition probably led to an increased water loss but, at the same time, allowed the maintenance of efficient photosynthesis. In that study, in contrast to CE704, the sensitive genotype showed a slight decrease in A_n caused by drought. In another study with maize in water deficit, tolerant genotypes DKB390 and BRS1055 also showed higher A_n and *gs* as compared to the sensitive genotypes 2B710 and BRS1010 (Lavinsky et al., 2015). From the studies of Benešová et al. (2012) and Lavinsky et al. (2015), in some maize genotypes, drought resistance is characterized by the capability of maintaining stomata opening, allowing CO_2 intake while also keeping a higher canopy cooling and maintenance of the carbon metabolism (Lavinsky et al., 2015). Probably, this behavior is only possible if there is at least some water availability in the soil (soil water tension was -138 kPa in water deficit conditions in that study).

On the other hand, the risky strategy of maintaining stomata open even under drought conditions would be beneficial under moderate water deficit or in conditions where periodical rewatering occurs, as the plant would be able to retain a relatively normal (possibly only slightly diminished) growth capacity. Thus, although low stomatal conductance is usually considered as a general response of plants to drought conditions and as a trait associated with drought tolerance since it allows water conservation, it probably functions as that way only under severe drought conditions, whereas under moderate drought, maintaining stomata open would be more beneficial (Benešová et al., 2012).

Matuba is generally described by the seed companies in Mozambique as less drought-tolerant than ZM309 and ZM523. Nevertheless, in our work, under severe drought, Matuba maintained relatively higher photosynthesis and stomatal conductance as compared to ZM523. This tendency in Matuba may be attributed to a possible particular set of osmotic conditions, for instance, increased concentration of osmolytes activity (Fathi & Tari, 2016). At the molecular level, proteomics also showed detrimental effects due to drought in ZM523, such as lower levels of PSII proteins that play an important role in the protection of the reaction's centers in drought conditions (See chapter IV for a discussion on levels of PSII proteins).

The above-mentioned observations led us to conclude that it can be worthful to describe the specific features that allow listing a variety as drought-tolerant or resistant. Also, since Mozambican varieties may display different strategies to cope with drought, it would be useful to design breeding programs targeting multiple features so that the drought-resistance trait could be strengthened and more robust maize genotypes may be delivered to farmers and increase maize yield.

3.3.6 Chlorophyll *a* fluorescence of the light response curves

In drought conditions, adjustments in leaf gas exchange parameters occur in parallel and interconnected with changes in chlorophyll fluorescence, both being used to evaluate the overall photosynthetic state of the plant (de Sousa et al., 2017).

A common trend in fluorescence light-response curves was also found in all genotypes and treatments (Fig. 3.5C, D; Fig. 3.6C, D; Fig 3.7C, D). The ETR and Φ_{PSII} in well-watered conditions showed no statistical differences among genotypes (Tukey test, $p<0.05$). With drought stress intensification, the PSII quantum efficiency in maize genotypes also slightly decreased. A similar observation was made by Liu et al. (2018), who found that moderate drought stress reduced the photochemical activity of PSII from primary quinone acceptor (QA) to plastoquinol (PQH2). The entire electron transport chain from the donor side of PSII to PSI-end electron acceptors was inhibited under severe drought stress. In addition, in maize, the photochemical activity of PSII was more sensitive to drought stress than PSI.

In the present study, ETR values increased with light intensity reaching a plateau at 500 µmol photons m^{-2} s^{-1}, followed by a downward or steady tendency. At the same time, Φ_{PSII} reduced with light intensity in all treatments. In general, the drop observed in the ETR amplitude was more prominent from control conditions to moderate stress. In control conditions, maximum Φ_{PSII} values ranged from 0.69 (ZM523) to 0.71 (B73) and for ETR ranged from 75.4 (ZM523) to 85.79 µmol electron $m^{-2}s^{-1}$ (Matuba), but no statistical differences were found. In moderate stress, Φ_{PSII} and ETR showed similar tendencies as observed in control conditions, but ETR exhibited slightly lower values (Fig. 3.5C, D; Fig. 3.6C, D)

Although not statistically significant, in severe drought, ZM523 had the lowest values of Φ_{PSII} as well as of ETR, while in B73 and ZM309, the highest values were observed, and Matuba showed intermediate values (Fig 3.7C, D). This was considered an indication that the overall state of PSII in ZM523 was more affected than in the other varieties, and that the negative impact of water scarcity was probably more severe in ZM523. This observation was consistent with the lower levels of PSII proteins in this variety (see chapter IV for more details). Li et al. (2017), also concluded that the decrease in ETR and Φ_{PSII} in serious drought stress was an indication of PSII system damage. Therefore, it is suggested that the decrease of Φ_{PSII} could be one of the reasons causing reductions in A_n by non-stomatal limitations (Murata et al., 2007; Florez-Sarasa et al., 2016; Wang et al., 2018).

The electron flow is a good indicator of the overall rate of photosynthesis and gives the potential to estimate photosynthetic performance at a specific condition, whether good or evil. Damage to PSII in the leaf is considered the first manifestation of stress in plants (Maxwell &

Johnson, 2000). Higher electron transport rates and actual photochemical efficiency even under harsh conditions are considered photosynthetic features of stress resistance (Li et al., 2017).

Production of Reactive Oxygen Species, that occurs within the ETR between PSII and PSI in light reactions in chloroplasts, can be incremented during drought conditions when carbon dioxide and ATP synthesis is diminished. The damaging effects of ROS within chloroplasts include the inhibition of *de novo* synthesis of the D1 protein (also known as photosystem b A or PsbA), which is necessary for PSII repair. The effects can also include the suppression of ROS responsive chloroplast enzymes and the disarrangement of thylakoid architecture (Gururani et al., 2015). Consistent with the inhibition of D1 synthesis, the electron transport chain between photosystems can also be downregulated in drought conditions (Murata et al., 2007; Florez-Sarasa et al., 2016; Wang et al., 2018).

Taking into consideration the relation between CO_2 assimilation and ETR, the overall reduction in A_n in severe drought stress can be attributed not only to the stomatal limitations but also to other limitations, such as PSII damage (which may affect ETR), deficient mechanism of PSII repair, and lower capacity of ROS detoxification (Cendrero-Mateo et al., 2015). The limitation of CO_2 photosynthetic fixation can decrease NADPH use, resulting in a decline of $NADP^+$ levels. Since $NADP^+$ is a major acceptor of electrons in PSI, its reduction increases electron transport from PSI to molecular oxygen, with the generation of H_2O_2 via $O_2^{\bullet -}$ (Wang et al., 2018).

When comparing the photosynthetic efficiency of maize genotypes made with instantaneous measurements in light intensities similar to those of growing conditions, we observed that despite not being always statistically significant, ZM309 showed slightly lower values of net photosynthesis in severe stress than Matuba or B73. However, under light intensity variation, photosynthesis responses showed a different profile, with ZM309 having the highest performance. Thus, we hypothesize that the particular features observed for ZM309 maize may be related to a lower degradation of the D1 protein, to its phosphorylation, and to an adequate amount of carotenoids, such as xanthophyll, protecting PSII from photoinhibition through improved dissipation of excess energy. Also, the turnover of PSI core proteins PsaA and PsaB can play a role in protecting the photosynthetic machinery from photoinhibition (Maxwell & Johnson, 2000; Romanowska et al., 2016; Tikkanen et al., 2019).

Additionally, the better integrity of PSII (that ensures high light use capacity) would actively regulate the electron transport rate and the photochemical efficiency, as well as preventing the damage caused by excessive light energy to other systems through heat dissipation (Li et al., 2017).

Sousa et al. (2017), also observed that the xanthophyll cycle was efficient in giving vent to excess electron flow generated by the increase in light intensity in the chloroplasts and suggested that maize plants submitted to drought maintained their capacity to regulate the dissipation of light energy despite the stressful conditions.

On the other hand, a lower light intensity, such as the one we used for the instantaneous measurements, sometimes does not allow the expression of the maximum potential differences that can exist among genotypes. Thus, as suggested by Sousa et al. (2017) by increasing the light intensity, we might be able to discriminate maize responses to drought stress better.

3.3.7 Conclusions

Drought stress negatively affected growth and biomass production, as well as photosynthetic performance, in all the tested maize genotypes. Our data suggest that the Mozambican varieties behave similarly in well-watering conditions and only differ after drought imposition, displaying different drought resistance features. Despite showing values of CO_2 intake similar to those observed in the Mozambican varieties, growth and biomass production of the maize B73 genotype was in general lower. Differences regarding RuBisCO activity, RuBP regeneration, and uptake capacity of soil nutrients, might be related to this tendency.

We could observe some of the drought avoidance traits described in the literature, such as reduced stomata aperture in all the maize genotypes. A more pronounced reduction of leaf area was observed in Matuba and B73, and leaf rolling in ZM523 and ZM309.

Higher root biomass was observed in ZM523, likely allowing more water uptake than the other genotypes under severe drought. However, ZM523 variety had the lowest photosynthetic performance in severe drought, particularly when evaluating its response to light intensity variation.

Despite the above-mentioned differences in drought avoidance strategies, the overall water status (RWC) of the maize plants was similar across genotypes.

We observed a reduction of ETR between PSII and PSI under drought conditions in all genotypes. This reduction could contribute to reducing ROS formation in chloroplasts in low CO_2 availability, thus limiting ATP synthesis, which is also described as an energy conservation mechanism in harsh conditions. However, in C4-plants this may not be linear, because they have different chloroplasts in mesophyll and bundle-sheath cells.

In this work, we also hypothesized that molecular mechanisms, such as ROS elimination, lower degradation of PSII proteins such as D1 protein, and the adequate pool of carotenoids could play a pivotal role in the drought tolerance profile observed, particularly in the protection and repair of the photosystems.

Considering that one of the main effects of climate change in Mozambique is the irregular rainfall and the increased frequency of drought stress and recovery episodes, future studies on the physiological characterization of the Mozambican maize varieties will have to take these aspects into account. Based on the present characterization, we believe that rehydration will impact differently on the recovery success of the Mozambican maize varieties.

Also, maize physiological characterization in field conditions in arid and semi-arid regions of Mozambique (characterized by very short wet seasons) would be more meaningful for the farmers who depend on rainfall to decide the sowing and harvesting dates.

Finally, maize breeders´ efforts to deliver maize seeds that better cope with drought should be directed to maize genotypes with combined stress tolerance features. This will help local farmers to increase maize grain production and meet the population’s food demand.

Acknowledgments

Training on the use of a portable photosynthesis system LI-6400: Prof. Joaquim Miguel Costa (Instituto Superior de Agronomia)

Support on experiments setup in growth chambers and greenhouse: MSc. Hugo Matias (ITQB NOVA)

Supplementary Data
Supplemental figures

Figure S3.1 - The impact of different watering regimes in root-to-shoot ratio at maturity stage in greenhouse conditions

Figure S3.2 - The impact of different watering regimes in root-to-shoot ratio at seedling stage in growth chamber conditions

Figure S3.3 - Maize plants showing leaf rolling in severe drought conditions

References

Abreu, I. A., Farinha, A. P., Negrão, S., Gonçalves, N., Fonseca, C., Rodrigues, M., Saibo, N. J. M., Oliveira, M. M. (2013). Coping With Abiotic Stress: Proteome Changes for Crop Improvement. *Journal of Proteomics*, *93*, 145–168. https://doi.org/10.1016/j.jprot.2013.07.014

Ahmad, N., Malagoli, M., Wirtz, M., & Hell, R. (2016). Drought Stress in Maize Causes Differential Acclimation Responses of Glutathione and Sulfur Metabolism in Leaves and Roots. *BMC Plant Biology*, *16*, 1–15. https://doi.org/10.1186/s12870-016-0940-z

Al-Shaheen, M. R., Al-Shaheen, M. A., & Al-Shaheen, M. R. (2018). Effect of Biological Treatment under Water Stress Conditions on Maize. *7*(1), 1–4. https://doi.org/10.4172/2329-6674.1000164

Amelework, B., Shimelis, H., & Laing, M. (2015). Genetic Variation in Sorghum as Revealed By Phenotypic and SSR Markers: Implications for Combining Ability and Heterosis or Grain Yield. *Plant Genetics Resources*, (March), 1–13. https://doi.org/10.1017/S1479262115000696

Aslam, M., Maqbool, M. A., & Cengiz, R. (2015). Drought Stress in Maize (*Zea mays* L.) Effects, Resistance Mechanisms*, Global Achievements and Biological Strategies for Improvement*. https://doi.org/10.1007/978-3-319-25442-5

Benešová, M., Holá, D., Fischer, L., Jedelsky, P. L., Hnilicka, F., Wilhelmova, N., Rothová, O., Kocová, M., Procházková, D., Honnerová, J., Fridrichová, L., Hnilickov, H. (2012). The Physiology and Proteomics of Drought Tolerance in Maize: Early Stomatal Closure as a Cause of Lower Tolerance to Short-Term Dehydration? *PLos One*, *7*(6). https://doi.org/10.1371/journal.pone.0038017

Benjamin, J. G., Nielsen, D. C., Vigil, M. F., Mikha, M. M., & Calderon, F. (2014). Water Deficit Stress Effects on Corn (Zea mays, L.) Root:Shoot Ratio. *Open Journal of Soil Science*, *4*(April), 151–160.

Blum, A. (1992). Comparative Productivity And Drought Response Of Semi-Tropical Hybrids And Open-Pollinated Varieties of Sorghum. *Journal of Agricultural Sciences*, *118*(February), 29–36. https://doi.org/10.1017/S0021859600067976

Cendrero-Mateo, M. P., Carmo-Silva, A. E., Porcar-Castell, A., Hamerlynck, E. P., Papuga, S. A., & Moran, M. S. (2015). Dynamic Response Of Plant Chlorophyll Fluorescence To Light, Water And Nutrient Availability. *Functional Plant Biology*, (January). https://doi.org/10.1071/FP15002

Chen, J., Xu, W., Burke, J. J., & Xin, Z. (2010). Role of Phosphatidic Acid in High Temperature Tolerance in Maize. *Crop Science*, *50*(December), 2506–2515. https://doi.org/10.2135/cropsci2009.12.0716

Chen, Y., Wang, D., Ge, X., Zhao, B., Wang, X., & Wang, B. (2016). Comparative Proteomics of Leaves Found at Different Stem Positions of Maize Seedlings. *Journal of Plant Physiology*, *198*, 116–128. https://doi.org/10.1016/j.jplph.2016.03.022

Dohleman, F. G., Heaton, E. A., Leakey, A. D. B., & Long, S. P. (2009). Does Greater Leaf-Level Photosynthesis Explain the Larger Solar Energy Conversion Efficiency of Miscanthus Relative to Switchgrass?. *Plant Cell & Environment*, 1525–1537. https://doi.org/10.1111/j.1365-3040.2009.02017.x

Fathi, A., & Tari, D. B. (2016). Effect of Drought Stress and its Mechanism in Plants. *International Journal of Life Sciences*, *10*(1), 1–6.

Flexas, J., & Gallé, A. (2010). Gas-Exchange and Chlorophyll Fluorescence Measurements in Grapevine Leaves in the Field. https://doi.org/10.1007/978-90-481-9283-0

Florez-Sarasa, I., Noguchi, K., Araújo, W. L., Garcia-nogales, A., Fernie, A. R., & Flexas, J. (2016). Impaired Cyclic Electron Flow around Photosystem I Disturbs High-Light Respiratory Metabolism. *Plant Physiology*, *172*(December), 2176–2189. https://doi.org/10.1104/pp.16.01025

Ge, Y., Bai, G., Stoerger, V., & Schnable, J. C. (2016). Temporal Dynamics Of Maize Plant Growth, Water Use, and Leaf Water Content Using Automated High Throughput RGB And Hyperspectral Imaging. *Computers and Electronics in Agriculture*, *127*, 625–632. https://doi.org/10.1016/j.compag.2016.07.028

Ghatak, A., Chaturvedi, P., & Weckwerth, W. (2017). Cereal Crop Proteomics: Systemic Analysis of Crop Drought Stress Responses Towards Marker-Assisted Selection Breeding. *Frontiers in Plant Science*, *8*(June), 1–25. https://doi.org/10.3389/fpls.2017.00757

Gururani, M. A., Venkatesh, J., & Tran, L. P. (2015). Regulation of Photosynthesis During Abiotic. *Molecular Plant*, *8*(9), 1304–1320. https://doi.org/10.1016/j.molp.2015.05.005

Hatfield, J. L., & Dold, C. (2019). Water-Use Efficiency: Advances and Challenges in a Changing Climate. *Frontiers in Plant Science*, *10*(February), 1–14. https://doi.org/10.3389/fpls.2019.00103

Hofius, D., & Börnke, F. A. J. (2007). Photosynthesis, Carbohydrate Metabolism and Source – Sink Relations.

Hussain, H. A., Men, S., Saddam, H., Chen, Y., Ali, S., Zhang, S., Xu, Q., Liao, C., Wang, L. (2019). Interactive Effects Of Drought And Heat Stresses On Morpho- Physiological Attributes, Yield, Nutrient Uptake and Oxidative Status In Maize Hybrids. (February), 1–12. https://doi.org/10.1038/s41598-019-40362-7

Ibarra-Laclette, E., Herrera-estrella, L., Hayano-kanashiro, C., Caldero, C., & Simpson, J. (2009). Analysis of Gene Expression and Physiological Responses in Three Mexican Maize Landraces under Drought Stress and Recovery Irrigation. *PLos One*, *4*(10), 1–19. https://doi.org/10.1371/journal.pone.0007531

Kadioglu, A., Terzi, R., Saruhan, N., & Saglam, A. (2012). Current Advances In The Investigation of Leaf Rolling Caused By Biotic and Abiotic Stress Factors. *Plant Science*, *182*, 42–48. https://doi.org/10.1016/j.plantsci.2011.01.013

Klukas, C., Melchinger, A. E., Meyer, R. C., Riewe, D., & Altmann, T. (2015). Optimizing Experimental Procedures For Quantitative Evaluation of Crop Plant Performance in High Throughput Phenotyping Systems. *Frontiers in Plant Science*, *5*(January), 1–21. https://doi.org/10.3389/fpls.2014.00770

Lamaoui, M., Jemo, M., Datla, R., & Bekkaoui, F. (2018). Heat and Drought Stresses in Crops and Approaches for Their Mitigation. *Frontiers in Chemistry*, *6*(February), 1–14. https://doi.org/10.3389/fchem.2018.00026

Lavinsky, A. O., Magalhães, P. C., Ávila, R., Gomes-jr, C. C., & Carneiro, N. P. (2015). Advances in Crop Science and Technology Analysis of Maize Photosyntheis Parameters and Whole Plant Oxidative Damage Under Long-term Drought. 1–6. https://doi.org/10.4172/2329-8863.S1-007

Lawson, T., & Blatt, M. R. (2014). Stomatal Size, Speed, and Responsiveness Impact on Photosynthesis and Water Use Efficiency. *Plant Physiology*, *164*(April), 1556–1570. https://doi.org/10.1104/pp.114.237107

Li, J., Cang, Z., Jiao, F., Bai, X., Zhang, D., & Zhai, R. (2017). Influence of Drought Stress On Photosynthetic Characteristics And Protective Enzymes of Potato at Seedling Stage. *Journal of the Saudi Society of Agricultural Sciences*, *16*(1), 82–88. https://doi.org/10.1016/j.jssas.2015.03.001

Liu, J., Guo, Y. Y., Bai, Y. W., Camberato, J. J., Xue, J. Q., & Zhang, R. H. (2018). Effects of Drought Stress on the Photosynthesis in Maize. *Russian Journal of Plant Physiology*, *65*(6), 849–856. https://doi.org/10.1134/S1021443718060092

Lopes, M. S., Araus, J. L., Heerden, P. D. R. Van, & Foyer, C. H. (2011).

Enhancing Drought Tolerance in C4 Crops. *Journal of Experimental Botany*, *62*(9), 3135–3153. https://doi.org/10.1093/jxb/err105

Maxwell, K., & Johnson, G. N. (2000). Chlorophyll Fluorescence — A Practical Guide. *51*(345), 659–668.

Medeiros, D. B., Ciriaco, E., Rafael, H., Santos, B., Pacheco, C. M., Musser, S., … Custódio, M. (2012). Physiological and Biochemical Responses to Drought Stress in Barbados Cherry. *Brazilian Society of Plant Physiology*, *24*(3), 181–192.

Medrano, H., Tomás, M., Martorell, S., Flexas, J., Hernández, E., Rosselló, J., … Bota, J. (2015). From Leaf To Whole-Plant Water Use Efficiency (WUE) In Complex Canopies: Limitations of Leaf WUE as a Selection Target. *The Crop Journal*, *3*(3), 220–228. https://doi.org/10.1016/j.cj.2015.04.002

Minorsky, P. V. (2003). The Hot and the Classic. *Plant Physiology*, *131*, 6–7. https://doi.org/10.1104/pp.900062.6

Mollier, A., & Pellerin, S. (1999). Maize Root System Growth and Development as Influenced by Phosphorus Deficiency. *Journal of Experimental Botany*, *50*(333), 487–497.

Murata, N., Takahashi, S., Nishiyama, Y., & Allakhverdiev, S. I. (2007). Photoinhibition of Photosystem II Under Environmental Stress. *Biochimica et Biophysica Acta*, *1767*, 414–421. https://doi.org/10.1016/j.bbabio.2006.11.019

Nelissen, H., Sun, X., Rymen, B., Jikumaru, Y., Kojima, M., Takebayashi, Y., … Inz, D. (2018). The Reduction In Maize Leaf Growth Under Mild Drought Affects the Transition Between Cell Division and Cell Expansion and Cannot be Restored by Elevated Gibberellic Acid Levels. *The Plant Biotecnology Journal*, 615–627. https://doi.org/10.1111/pbi.12801

PEDSA. (2011). Plano Estratégico Para o Desenvolvimento do Sector Agrário 2011-2020.

Perdomo, J. A., Carmo-Silva, E., Hermida-Carrera, C., & Flexas, J. (2016). Acclimation of Biochemical and Diffusive Components of Photosynthesis in Rice, Wheat, and Maize to Heat and Water Deficit: Implications for Modeling Photosynthesis. *Frontiers in Plant Science*, *7*(November), 1–16. https://doi.org/10.3389/fpls.2016.01719

Pick, T. R., Braütigam, A., Schlüter, U., Denton, A. K.Colmsee, C., & Scholz, U., Fahnenstich, H., Pieruschka, R., Rascher, U., Sonnewald, U., Weber, A. P. M. (2011). Systems Analysis of a Maize Leaf Developmental Gradient Redefines the Current C 4 Model and Provides Candidates for Regulation. *The Plant Cell*, *23*(December), 4208–4220. https://doi.org/10.1105/tpc.111.090324

Riaz, A., Younis, A., Taj, A. R., Karim, A., & Tariq, U. (2013). Effect of Drought STRESS on Growth and Flowering of Marigold (*Tagetes erecta* L.). *Pak. J. Bot*, *45*(January), 123–131.

Riboldi, L. B., Oliveira, R. F., & Angelocci, L. R. (2016). Leaf Turgor Pressure in Maize Plants Under Water Stress. *Australian Journal of Crop Science*, *10*(6), 878–886. https://doi.org/10.21475/ajcs.2016.10.06.p7602

Riccardi, F., Gazeau, P., Jacquemot, M., Vincent, D., & Zivy, M. (2004). Deciphering Genetic Variations of Proteome Responses to Water Deficit in Maize Leaves. *Plant Physiology and Biochemistry*, *42*, 1003–1011. https://doi.org/10.1016/j.plaphy.2004.09.009

Romanowska, E., Buczynska, A., Wasilewska, W., Krupnik, T., Drozak, A., Rogowski, P., … Zienkiewicz, M. (2016). Differences In Photosynthetic Responses of NADP-ME Type C4 Species to High Light. *Planta*. https://doi.org/10.1007/s00425-016-2632-1

Shao, H., Chu, L., Jaleel, C. A., & Zhao, C. (2008). Water-Deficit Stress-Induced Anatomical Changes In Higher Plants. *C. R. Biologies*, *331*, 215–225. https://doi.org/10.1016/j.crvi.2008.01.002

Sousa, Antônio Carlos Ferreira De Paiva, D. S. De, Casari, R. A. N., Oliveira, N. G. De, Molinari, Bruno, H. C., Kobayashi, A. K., Magalhães, P. C., … Jr, T. (2017). A Procedure for Maize Genotypes Discrimination to Drought By Chlorophyll Fluorescence Imaging Rapid Light Curves. *Plant Methods*, 1–17. https://doi.org/10.1186/s13007-017-0209-z

Sun, C., Gao, X., Chen, X., Fu, J., & Zhang, Y. (2016). Metabolic And Growth Responses of Maize to Successive Drought And Re-Watering Cycles. *Agricultural Water Management*, *172*, 62–73. https://doi.org/10.1016/j.agwat.2016.04.016

Tanko, U., & Hassan, U. T. (2016). Leaf Area Determination for Maize (*Zea mays* L.), Okra (Abelmoschus esculentus L.) and Cowpea (*Vigna unguiculata* L.) Crops Using Linear Measurements. *Journal of Biology, Agriculture and Healthcare*, *6*(4), 103–111.

Tikkanen, M., Silveira, J. A. G., & Aro, E. (2019). Consequences Of Photosystem-I Damage and Repair on Photosynthesis and Carbon Use in *Arabidopsis thaliana*. *The Plant Journal*, 1–12. https://doi.org/10.1111/tpj.14177

Voronin, P. Y., Maevskaya, S. N., & Nikolaeva, M. K. (2019). Physiological And Molecular Responses Of Maize (*Zea mays* L.) Plants to Drought and Rehydration. *Photosynthetica*, *57*(3), 850–856. https://doi.org/10.32615/ps.2019.101

Wang, X., Cai, X., Xu, C., Wang, Q., & Dai, S. (2016). Drought-Responsive Mechanisms in Plant Leaves Revealed by Proteomics. *International Journal of Molecular Sciences*, *17*, 1–30. https://doi.org/10.3390/ijms17101706

Wang, Z., Li, G., Sun, H., Ma, L., Guo, Y., Zhao, Z., … Mei, L. (2018). Effects of Drought Stress on Photosynthesis and Photosynthetic Electron Transport Chain In Young Apple Tree Leaves. 1–9. https://doi.org/10.1242/bio.035279

Wei, S., Wang, X., Shi, D., Li, Y., Zhang, J., & Liu, P. (2016). The

Mechanisms of Low Nitrogen Induced Weakened Photosynthesis In Summer Maize (*Zea mays* L.) Under Field Conditions. *Plant Physiology and Biochemistry*, 1–10. https://doi.org/10.1016/j.plaphy.2016.04.007

Weraduwage, S. M., Chen, J., Anozie, F. C., Morales, A., Weise, S. E., & Sharkey, T. D. (2015). The Relationship Between Leaf Area Growth and Biomass Accumulation in *Arabidopsis thalian*a. *Frontiers in Plant Science*, *6*(April), 1–21. https://doi.org/10.3389/fpls.2015.00167

Xin, L., Zheng, H., Yang, Z., Guo, J., Liu, T., & Sun, L. (2018). Physiological And Proteomic Analysis Of Maize Seedling Response to Water Deficiency Stress. *Journal of Plant Physiology*, *228*(November 2017), 29–38. https://doi.org/10.1016/j.jplph.2018.05.005

Zaman-Allah, M., Zaid, P. , Trachsel, S., Cairns, J. , Vinayan, M. , & Seetharam, K. (2016). Phenotyping for Abiotic Stress Tolerance in Maize. Drought Stress, a field manual.

Zhang, R. H., Zhang, X. H., Camberato, J. J., & Xue, J. Q. (2015). Photosynthetic Performance of Maize Hybrids to Drought Stress. *BMC Plant Biology*, *62*(6), 788–796. https://doi.org/10.1134/S1021443715060187

Zhao, Y., Fan, Z., Hu, F., Yin, W., Zhao, C., & Yu, A. (2019). Source-to-Sink Translocation of Carbon and Nitrogen is Regulated by Fertilization and Plant Population. *Frontiers in Plant Science*, *10*(July), 1–15. https://doi.org/10.3389/fpls.2019.00891

Zhou, R., Kan, X., Chen, J., Hua, H., Li, Y., Ren, J., & Feng, K. (2019). Drought-Induced Changes In Photosynthetic Electron Transport In Maize Probed by Prompt Fluorescence, Delayed Fluorescence, P700 and Cyclic Electron Flow Signals. *Environmental and Experimental Botany*, *158*(November 2018), 51–62. https://doi.org/10.1016/j.envexpbot.2018.11.005

Supplemental figures

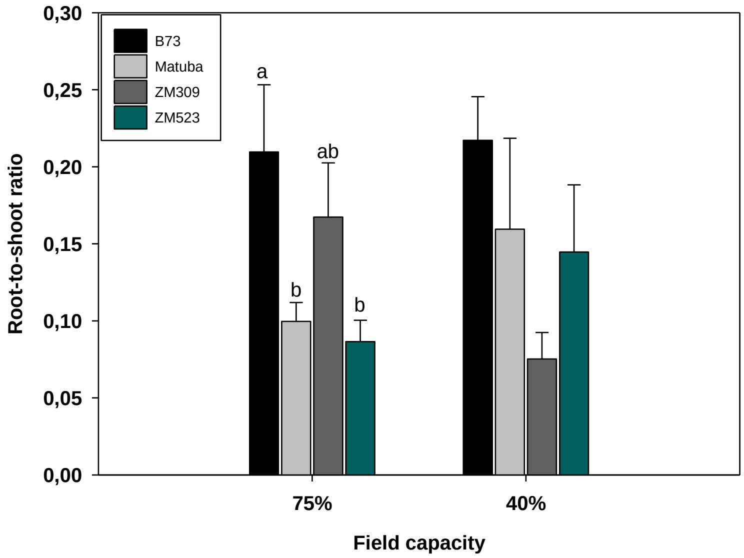

Figure S3.1 - The impact of different watering regimes in root-to-shoot ratio at maturity stage in greenhouse conditions. Data was obtained from dry biomass. The bars are means ±SE (n = 7). Different letters indicate statistical significance of the differences among genotypes within treatments (Tukey test, *p*<0.05).

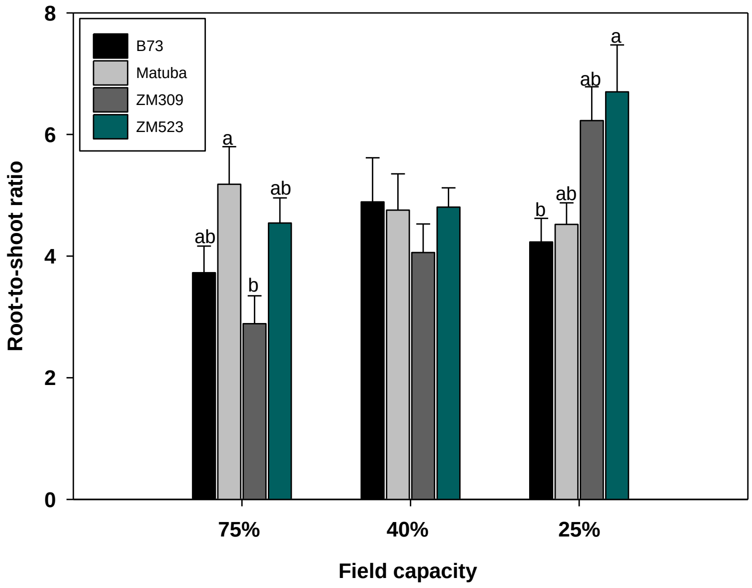

Figure S3.2 - The impact of different watering regimes in root-to-shoot ratio at seedling stage in growth chamber conditions. Data was obtained from dry biomass. The bars are means ±SE (n = 5). Different letters indicate statistical significance of the differences among genotypes within treatments (Tukey test, *p*<0.05).

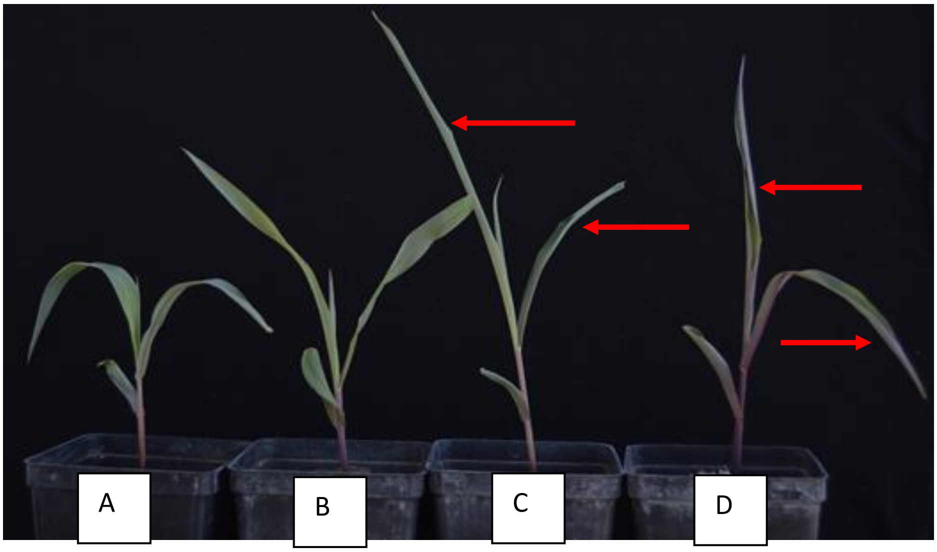

Figure S3.3 - Maize plants showing leaf rolling (red arrows) in severe drought conditions. B73 (A); Matuba (B); ZM309 (C), and ZM523 (D) seedlings are presented.

Chapter IV. Proteomic features governing drought tolerance in the Mozambican maize varieties under study

The work presented in this Chapter was mainly performed by Dizimalta dos Santos Fernando Miquitaio. Additional support was provided by Bruno Alexandre and Inês Luís (see acknowledgments section)

Abstract

The three Mozambican varieties and the control genotype B73 were grown in the controlled environment of a growth-chamber, in three watering regimes, namely, well-watered, moderate drought, and severe drought conditions. Seedlings were collected, and their proteomic profiles analyzed. We particularly focused on the analysis of the levels of the enzymes related to CO_2 assimilation, electron transport chain in photosynthesis, and ROS elimination. We tested three Mozambican maize varieties, Matuba, ZM309, and ZM523, together with the control genotype maize B73, aiming at finding molecular signatures that could be related to drought tolerance. We used the FASP digestion protocol to prepare 3rd-leaf whole protein samples for mass spectrometry analysis. IDA and DIA (SWATH) methods were used to identify and quantify the assessed proteins, respectively. We could identify differences in protein abundance that depended on water treatment. However, our data alone cannot explain differences in drought tolerance among genotypes, suggesting that multiple traits contribute to them. The levels of C4-photosynthesis enzymes were, in general repressed under severe stress conditions, particularly for NADP-ME and PPDK. A notable increase of PEPC and NADP-MDH was found in Matuba and B73 in moderate drought treatment, which may be related to stress tolerance in that specific condition. We also observed a general increase of the PSII proteins and some of the PSI under severe drought that did not match the PSII photochemistry. This increase reflects a possible strategy to protect and repair the PSII. On the other hand, maize ZM523 had, in general, the lowest levels of the PSII proteins amongst maize genotypes. The ROS elimination enzymes Catalase 3 and Peroxidase 42 accumulated in Matuba in response to drought and may thus play a role in the drought tolerance strategy of this variety; still, more studies are needed to clarify this matter.

4.1 Introduction

As described in the previous chapter, the genotypes under study, developed different phenotypic characteristics in response to imposed drought. Some of the characteristics included large root biomass that increases soil water uptake and decreased stomatal conductance and leaf area that avoided water loss by transpiration.

To complement the morphological and physiological characterizations described in Chapter III, our work also included a proteomic characterization aiming at determining molecular signatures of drought tolerance. Within the array of the molecular approaches, we chose to study protein levels since proteins are the main cell effectors of metabolic function, which changes in response to stress. To study the protein levels, we chose SWATH analysis, which is a mass spectrometry quantitative proteomic approach harbored in the analysis of whole protein extracts (see below section 4.3.3 for more details on SWATH analysis).

Proteins are the central biomolecules that collectively catalyze and control essentially all cellular processes. They form a highly structured entity known as the proteome, which is the constituent proteins that carry out their functions at specific times and locations in the cell, in physical or functional association with other proteins or biomolecules (Aebersold & Mann, 2016).

Proteomics is the study and characterization of the complete set of proteins present in a cell, organ, or organism at a given time. This technique allows qualitative and quantitative measurements of a large number of proteins that are directly involved in cellular biochemistry. Proteomic approaches provide information about protein concentrations, post-translational modification (PTMs), protein-protein interaction, regulatory functions of proteins, and structure associated with stress tolerance. They also allow for creating a complete three-dimensional (3-D) map of the cell indicating where proteins are located (Chandramouli & Qian, 2009; Mishra, 2010; Kosová et al., 2011).

Research targeting changes in plant proteome is highly informative since proteins, unlike transcripts, are direct shapers of the plant stress response (Kosová et al., 2011). Therefore, studies of plant reaction at the protein level after stress application can significantly contribute to understanding the physiological mechanisms behind plant stress tolerance. Proteomics studies may help to identify potential protein markers whose alterations in abundance can be associated with changes in some physiological parameters and evaluate the genotype's level of stress tolerance (Kosová et al., 2011). Also, comparisons between genotypes with different sensitivity towards stress (tolerance vs. susceptibility) are crucial to explaining the putative influence of differentially abundant proteins in tolerant genotypes. The positive features can then be selected for crop improvement (Abreu et al., 2013).

In this work, we aimed at assessing the levels of the C4-photosynthesis key enzymes in the different maize genotypes subjected to different water treatments. We also targeted the enzymatic systems that cope with oxidative stress. Particularly the accumulation of antioxidant enzymes such as peroxidase and catalase, as well as the proteins involved in photoprotection and electron flow through photosystems II and I. We took into account that the general tendency observed is that tolerant genotypes not only increase oxidative stress metabolism and the scavenging capacity of reactive oxygen species (ROS) but also show enhanced carbohydrate metabolism and more efficient photosynthesis (Abreu et al., 2013).

Our working hypothesis was that the levels of the photosynthetic enzymes, such as PEPC, PPDK, NADP-ME, and RuBisCO, correlate with the plant photosynthetic capacity in drought conditions.

4.2. Photosynthesis: Carbon Reactions

In this section, we describe the biochemical mechanisms of carbon dioxide concentration that allow C4-species like *Zea mays* L. to mitigate the impact of photorespiration that affects C3 plants such as rice.

Only about 3% of flowering plant species use the C4 pathway, but this relatively few species are responsible for 23% of the carbon fixed (primary productivity) in the world (Sage et al., 2012; Kellogg, 2013).

Under current atmospheric conditions, potential photosynthesis in C3 plants is suppressed by oxygen by as much as 40%. The extent of such suppression is further increased in stress conditions such as drought, high light, or high temperature, through a decline in CO_2 concentration inside leaves due to a reduction in stomatal opening (Matsuoka et al., 2001). For example, in 2013, with 380 ppm of CO_2 and summer temperatures of 30^{o}C, C3 plants would capture and store a theoretical maximum of 4.6% of the energy received in sunlight, whereas C4 plants could theoretically capture 6% (Kellogg, 2013) (CO_2 concentration is currently 414.50 ppm (March value, released by 6th April 2020, source: www.co2.earth).

Photosynthesis is the fundamental biological process of photosynthetic organisms such as plants, algae, and many species of bacteria that converts light energy to chemical energy by fixing CO_2 into sugars. Therefore, the efficiency of CO_2 fixation tends to correlate with the amount of biomass that plants can produce and, ultimately, in the case of food crops, will determine their yield (Liu et al., 2013; Tikkanen et al., 2019).

The majority of terrestrial plants, including several staple food crops such as rice, wheat, barley, soybean, and potato, assimilate atmospheric CO_2 directly through the C3 photosynthetic pathway, also known as the Calvin cycle, and these crops are classified as C3 plants. The enzyme of primary CO_2 fixation in this pathway, ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO), reacts not only with CO_2 but also with O_2 (this justifies the inclusion of 'oxygenase' in the name of the enzyme), leading to photorespiration and loss of assimilated carbon (Matsuoka et al., 2001).

The CO_2 reduction that is catalyzed by RuBisCO attaches CO_2 to carbon 2 of ribulose 1,5-bisphosphate (RuBP) (Fig. 4.1). This creates an unstable 6-carbon compound that immediately forms two molecules of 3-phosphoglycerate (3-PGA). The name "C3 photosynthesis" was originated from experiments in which the CO_2 was radioactively labeled, and firstly detectable in these 3-carbon compounds. Next, the 3-PGA molecules are reduced and used to make 6-carbon sugars, and others are recycled to regenerate RuBP (Kellogg, 2013).

When RuBisCO reacts with oxygen, O_2 enters the active site and is covalently bound to RuBP at carbon 2. The following 5-carbon product of the oxygenase reaction is unstable and rapidly produces one molecule of 3-PGA and one of 2- phosphoglycolate, with two carbons (Fig. 4.1). In the next phase, RuBP is regenerated through 3-PGA, while the 2-phosphoglycolate pass through energetically expensive and complex reactions. The phosphate group is removed, and the resulting glycolate is transported to peroxisome, where it is converted to glycine that is sent to the mitochondrion. There, through glycine decarboxylase (GDC), two molecules of glycine are combined to make one serine, which releases a molecule of CO_2 (Fig. 4.1) (Sage et al., 2012; Kellogg, 2013).

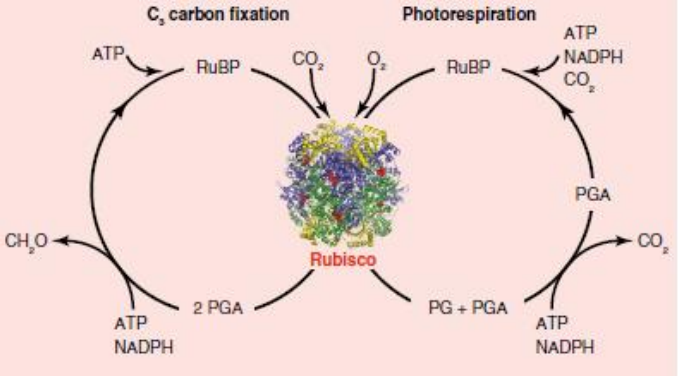

Figure 4.1 - The representation of the carbon fixation and oxygenation reactions of RuBisCO. The C3 carbon fixation cycle is represented at the left and the photorespiratory cycle at the right. ATP, adenosine triphosphate; CH_2O, carbohydrate; RuBP, ribulose 1,5 bis-phosphate; PGA, 3-phosphoglycerate (a 3-carbon compound); PG, phospho-glycolate (a 2-carbon molecule) (source: Kellogg, 2013).

In the C2 cycle, which is one of the mechanisms to reduce CO_2 loss through photorespiration, glycine decarboxylase is only expressed in the bundle sheath. The CO_2 released from serine production from glycine, pass through mesophyll cells before it can be released out of the leaf, but the majority of this CO_2 reacts with RuBisCO. Thus, C2 plants increase the efficiency of C3 even in conditions of high photorespiration (e.g., high temperature or low internal CO_2 levels). In the C2 cycle, other metabolic reactions use the serine originated from glycine decarboxylation, or this compound is sent back to the chloroplast. The C2 cycle is considered as a metabolically costly process, consuming ATP, and NADPH. Because this process releases CO_2, it is called photorespiration, but it has little to do with cellular respiration. Thus, the oxygenation of RuBP catalyzed by RuBisCO and the following cycling of glycolate clearly makes C3 photosynthesis less efficient than the C4 cycle (Kellogg, 2013).

On the other hand, C4 plants such as maize, sorghum, and sugarcane have evolved a novel biochemical mechanism to avoid photorespiration, making them photosynthetically more efficient than C3 plants (Matsuoka et al., 2001). Therefore, C4 photosynthesis resulted from a major reorganization of leaf anatomy and metabolism to allow CO_2 concentration. This mechanism in C4 plants circumvents the oxygenase activity of RuBisCO that competes with CO_2 fixation, thus increasing the photosynthetic capacity (Sage et al., 2012).

In grasses, all C4 lineages are found within the branch of the family called PACMAD (is the abbreviation for the subfamilies Panicoideae, Arundinoideae, Chloridoideae, Micrairoideae, Aristidoideae, and Danthoideae). At the family level, there are 19 families holding C4-plants, and of these, four (Poaceae, Cyperaceae, Chenopodiaceae, and Amaranthaceae) account for two-thirds of the lineages (Sage et al., 2012).

In addition to the C3 pathway, the C4-plants use the C4 photosynthetic cycle to increase the CO_2 concentration at the vicinity of RuBisCO and thus suppress its oxygenase activity. This mechanism enables C4 plants to achieve higher photosynthetic capacity particularly at higher temperatures, of up to double of that of C3 plants, producing biomass at faster rates and also performing higher water, nitrogen and light-use efficiencies (Christin et al., 2014; Kellogg, 2013; Matsuoka et al., 2001)

Leaves of C4 plants have two types of photosynthetic cells, the mesophyll cell (MC) and bundle sheath cell (BSC) (Fig. 4.2) (Matsuoka & Furbank, 2001). This spatial compartmentation is generally considered to be associated with the spatial separation of carboxylation and decarboxylation events in the two cell types (Rao & Dixon, 2016).

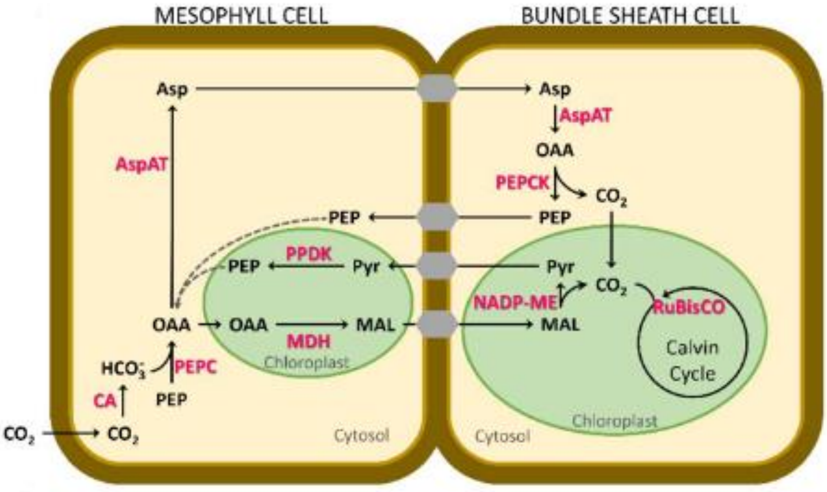

Figure 4.2 - Diagrammatic representation of the C4-photosynthetic cycle (Source: Luis et al., 2016).

In C3 plants, all the photosynthetic enzymes are localized in MCs, whereas in C4 plants, they are divided and localized in MCs and/or BSCs. Additionally, C4 plants show large venation, with a ring of BSCs surrounding each vein and an outer ring of MCs surrounding the bundle sheath. This single leaf structure, also known as Kranz anatomy (the appearance of a wreath of cells surrounding the vasculature gives rise to the term ‘Kranz’, for the German: wreath), and cell-specific compartmentalization of enzymes is critical for the operation of the C4 pathway (Fig. 4.2) (Matsuoka et al., 2001; Rao & Dixon, 2016). Also, C4 plants have veins that are much closer than what its observed in C3 plants. Photosynthetic pathway and vein spacing correlate perfectly, making it very easy to distinguish C3 and C4 plants by analyzing their leaf cross-section (Kellogg, 2013).

The initial step of CO_2 fixation in C4-plants takes place in the MC cytosol by the activity of phosphoenolpyruvate carboxylase (PEPC) that catalyzes the carboxylation of PEP (phosphoenolpyruvate) to form the C4-acid oxaloacetate (OAA) (Matsuoka et al., 2001). Because the first stable product of this pathway is a 4-carbon compound, the pathway is known as C4. Unlike RuBisCO, PEP carboxylase has only two substrates — bicarbonate and PEP — and binds to both with high affinity (Osborne & Sack, 2012; Kellogg, 2013; Paulus et al., 2013).

The OAA generated in the cytosol can have two different fates - it can be reduced to malate by the activity of NADP-malate dehydrogenase (NADP-MDH) in MCs chloroplasts, or transaminated to aspartate by the aspartate aminotransferase (AspAT) in MCs cytosol. The C4-acid generate by both reactions is transported to the BSCs and then decarboxylated to release CO_2 in the vicinity of RuBisCO. The reaction of decarboxylation is catalyzed by one or more of the three enzymes, namely, NADP-malic enzyme (NADP-ME), NAD-malic enzyme (NAD-ME), and phosphoenolpyruvate carboxykinase (PEPCK), and C4-plants are categorized in three subtypes according to their main decarboxylation enzyme (see section 4.2.2 for more details). The generated pyruvate is transported back to MCs chloroplasts to regenerate PEP through the activity of orthophosphate dikinase (PPDK) (Fig. 4.2) (Matsuoka et al., 2001).

The enzymes related to the C4 pathway were previously considered to be specific for C4 plants. This is because the activities of their homologs are low in C3 plants, and their kinetic properties are typically different from those of C4 plants. However, recent comparative studies showed that C3 plants have at least two different types of genes, one encoding enzymes with “housekeeping” function and the other that are very similar to the C4 photosynthetic genes present in C4 plants. However, the expression of the C4 genes in those C3 plants is very low and sometimes undetectable (Matsuoka et al., 2001). Also, Non-photosynthetic equivalent isoforms of PEPC, NADP-ME, and PPDK are present in all plants (Doubnerová & Ryšlavá, 2011).

Non-photosynthetic counterparts of the c4-photosynthesis enzymes PEPC, NADP-ME, and PPDK, are found in C3 plants, and non-photosynthetic tissues of all plants and they can be important, particularly in stress conditions (Doubnerová & Ryšlavá, 2011). For example, for PEPC, besides its fundamental role in the initial fixation of atmospheric CO_2 during C4 and CAM photosynthesis, this enzyme has a wide range of non-photosynthetic roles including supporting carbon-nitrogen interactions, seed formation, and germination, fruit ripening, guard cell metabolism during the stomatal opening, also delivering malate as a respiratory substrate for symbiotic nitrogen-fixing bacteroids in legume root nodules. Also, accumulating evidence has confirmed that a large number of *PEPC* genes are induced by abiotic and biotic stresses and play crucial roles in the regulation of plant tolerance to stress in C3, C4 and CAM plants. For example, overexpression of the PEPC gene in transgenic plants enhanced their tolerance to drought or salt stress, whereas its knockdown led to increased sensitivity to osmotic stress (Wang et al., 2016).

4.2.1 Ecology and geological history of the C4 pathway

Atmospheric CO_2 decreased from above 1000 ppm to levels below 700 ppm about 32 million years ago in Eocene Era, and the first C4 plants began to develop around this time. However, at that moment, the C4-plants did not cover extensive areas of the globe as they currently do. In the beginning, they were only a small part of the earth's ecosystems (Kellogg, 2013).

C4 grasses have dominated over the earth's land surface, particularly in the last 8 million years, and are currently the dominant vegetation in areas such as the Great Plains of North America, the vast African grasslands, and in large parts of Australia (Kellogg, 2013).

In the past, the expansion of the C4 grasslands was attributed to low CO_2 concentrations in the atmosphere through what is known as the “carbon starvation hypothesis” (Sage et al., 2012; Kellogg, 2013), but paleoclimatological data shows that the decrease in atmospheric CO_2 levels was far earlier than the expansion of C4 plants. This led to the hypothesis that C4 plants expansion was possibly caused by increased seasonality, with warm wet growing seasons changing with months of drought stress, with the latter conditions pointed as favoring the enhanced water-use efficiency of C4 plants (Kellogg, 2013).

Although C4 pathways are vast, the common concentrating mechanism of CO_2 in C4 plants and their higher photosynthetic efficiency in warm arid conditions gave rise to the ideas that low CO_2, high temperature, seasonal drought, and salinity are the main ecological drivers for the origins of C4 photosynthesis (Osborne & Sack, 2012).

Including high temperature as one of the selection factors for C4 photosynthesis was based on the observation that there is an increased abundance of C4 species along temperature gradients from high to low latitude and altitude and the fact that C4 grasses contribute to over 90% of the biomass of grasslands at low latitude and altitude. Clearly, the advantage of C4 photosynthesis over C3 one is evident at high temperature (Liu et al., 2013).

Drought and salinity are also listed as drivers of the evolution of C4 photosynthesis. This hypothesis is supported by the following: first, drought (physiological drought) and salinity induced the partial closure of stomata, which restricted the CO_2 supply and increased leaf temperature due to a decrease in leaf cooling through transpiration, resulting in high photorespiration, thus favoring a selection for CO_2 concentrating mechanism of C4 plants. The second reason is that due to a limited supply of freshwater under stress, C4 plants were more competitive than C3 plants because of their water-conserving strategy.

Also, low atmospheric CO_2 combined with high temperature and seasonal drought and salinity caused the excessive need for water transport, which resulted in higher stomatal conductance. In summary, C4 photosynthesis fulfilled the high-water use efficiency by allowing high rates of carbon-fixation at low stomatal conductance, even at low atmospheric CO_2. The result was a decrease in transpiration rates that allowed stomata to remain open and photosynthesis to be sustained for longer periods under drought and salinity conditions (Liu et al., 2013).

4.2.2 The biochemical subtypes of C4 photosynthesis

The three biochemical subtypes of C4 photosynthesis introduced in section 4.2 are categorized based on their principal decarboxylating enzyme found in the BS. The listed subtypes are the NADP-malic enzyme (NADP-ME), the NAD-malic enzyme (NAD-ME), and the PEP carboxykinase (PEPCK). Many C4 species also use a second decarboxylating enzyme, mainly in stress conditions. PEPCK has reduced activity in comparison to the main decarboxylase (Fig. 4.2) (Sage et al., 2012). Additionally to what occurs in stress conditions, older maize leaves can also utilize the second decarboxylation enzyme, PEP carboxykinase (PEPCK), releasing CO_2 from OAA, producing PEP that is sent to MC and can be used as the substrate of PEPC (Pick et al., 2011).

Several pieces of evidence suggest no existing pure PEPCK-type. Therefore, currently, is accepted the existence of the NAD-ME and NADP-ME subtypes, both with or without the additional reactions of the PEPCK pathway (Rao & Dixon, 2016).

The chloroplast ultrastructure of the BS tissue differs according to the biochemical subtype of C4 photosynthesis (Sage et al., 2012). For example, the light-dependent reactions of photosynthesis are not uniformly distributed in M and BS cells of NADP-ME plants (Rao &

Dixon, 2016). For example, in BS cells of the NADP-ME subtype, the number of mitochondria is low, and photosystem II is not present. On the other hand, in BS cells of the NAD-ME subtype, mitochondria, and photosystem II numbers, and grana development are high (Sage et al., 2012; Rao & Dixon, 2016). This difference in PSII numbers is because, in the NADP-ME subtype, the malate that is transported from M cells to BS chloroplasts provides NADPH, while in NAD-ME subtype, the acid aspartate does not produce NADPH (Munekage, 2016; Rao & Dixon, 2016).

The majority of NAD-ME grass lineage Chloridoideae shows an enhanced water-use efficiency if compared to the NADP-ME grasses under drought conditions. This feature is due to its leaf structure and faster leaf curling rates than the NADP-ME subtype. Although with lower water-use efficiency, plants in the NADP- ME subtype (except those in the Aristidoideae tribe) tend to have higher photosynthetic nitrogen-use efficiency under adequate or deficient nitrogen supply environments compared with other C4 grasses. This is attributed to the reduced content of nitrogen and faster RuBisCO activity in leaves found in NADP-ME grasses (Rao & Dixon, 2016).

The differences between the BS of the two subtypes are listed below. For example, in the NADP-ME subtype, the layer of cells between the BS cells and the vascular bundle is absent, and suberin is deposited in the BS cell wall (Rao & Dixon, 2016), possibly to allow the reduction in CO_2 leakage (Sage et al., 2012). Also, the BS chloroplasts have reduced grana and are arranged centrifugally in monocotyledons and centripetally in dicotyledons. Looking at the NAD-ME subtype, the vasculature is typically covered by a double sheath, consisting of the outer BS and the inner non-photosynthetic layer known as mestome sheath. Suberin is present all over in the mestome sheath instead of BS cells, and BS chloroplasts with developed grana are arranged centripetally. Differences in the origination of cell divisions may explain the loss of one layer of mestome sheath cells in the NADP-ME type. The single BS in C4 NADP-ME type has its origin in procambium, and M cells developed from the ground meristem. On the contrary, in the NAD-ME type, both BS and M cells are derived from the ground meristem, and the mestome sheath is originated from the procambium (Rao & Dixon, 2016).

In summary, malate is the dominant transported metabolite that diffuses to the BS cells in the NADP-ME subtype. In contrast, NAD-ME plants use aspartate as the major transport metabolite, which is formed by the transamination of OAA through the activity of AspAT. In the BS cells, aspartate is converted to OAA by a reductive deamination reaction. Pyruvate is also formed during the NAD-ME decarboxylation reaction, but it is partially transported back to the M cells in the form of alanine to maintain the ammonia balance between the two cell types. Alanine in the M is converted to PEP through several steps. The generated PEP then provides the precursor for a new cycle of carboxylation and decarboxylation that starts in M cells. Another particular aspect of the NAD-ME subtype is that the Aspartate is synthesized in the M cytosol, while malate formation and decarboxylation by NAD-ME occur in the BS mitochondria. In addition, the activity of the PEPCK enzyme can be found in both NAD-ME and NADP-ME subtypes, suggesting additional use of the PEPCK pathway to enhance plant adaption to several environmental conditions (Rao & Dixon, 2016).

4.3. Basic introduction to Liquid Chromatography-Mass spectrometry (LC-MS)

As reported in the introduction, Mass Spectrometry (MS) approaches were used in this work to identify and quantify the targeted proteins. Thus, in this section, we briefly describe basic principles of Mass Spectrometry (MS), known as an effective approach used to quantify known and unknown compounds and to explain the structure and chemical properties of different molecules. We also introduce liquid chromatography (LC), since that in the present work MS instrument was coupled to LC separation.

4.3.1 Liquid chromatography

Chromatography is a physical separation method in which the components of a sample to be separated are selectively distributed between two non-miscible phases: a mobile phase and a stationary phase bed. The technique is named according to what takes place after the mobile phase: gas chromatography (GC), liquid chromatography (LC), or supercritical fluid chromatography (SFC). The separation is based on the differences in distribution coefficients of the individual components in the sample (Niessen, 2006).

In Liquid Chromatography, the sample is loaded through an injection port into the mobile-phase stream delivered by the high-pressure pump and transferred within a column where the separation of the different components takes place. This separation can be monitored with a flow-through detector (Niessen, 2006).

“Most LC applications in sample separation are done with reversed-phase LC, *i.e.*, a nonpolar stationary phase and a polar mobile phase. Reversed-phase LC is ideally suited for the analysis of polar and ionic analytes” (Niessen, 2006; Dass, 2007).

Reverse-phase chromatography or hydrophobic chromatography discriminate different molecules in a sample, according to their hydrophobicity. In this type of chromatography, components with lower hydrophobicity are removed first from the column. Most of the time, the protein mixture and their subsequent elution is forced through the matrix under high pressure, which gives the designation “high performance” in the chromatographic process, making HPLC appropriate for delivering protein/peptide samples to the mass spectrometer, which is maintained under a high vacuum (Mishra, 2010).

In general, a typical mobile-phase is composed of a acetonitrile–water gradient with a fixed concentration of Trifluoracetic Acid (TFA, typically 0.05–0.5%). TFA acts as an ion-pairing agent that increases the retention of peptides and proteins but also hides secondary interactions with the silica-based stationary phase. TFA is a volatile compound, but due to its ion-pairing properties and effect on the surface tension, it may significantly inhibit the electrospray ionization (Niessen, 2006), as well as affects the stability of the electrospray resulting in decreasing sensitivity. Thus, to circumvent this, formic acid (FA) can be used and increase sensitivity (You et al., 2011).

4.3.2 Mass spectrometry (MS)

"Mass spectrometry is an analytical technique based on the production of ions, that are subsequently separated or filtered according to their mass-to-charge (*m/z*) ratio and detected" (Niessen, 2006).

The mass spectrometer consists of five steps: sample injection, sample ionization, mass analysis or separation according to *m/z*, ion or signal detection, and data processing and interpretation of the results obtained (Niessen, 2006; Dass, 2007; Wilson & Walker, 2010).

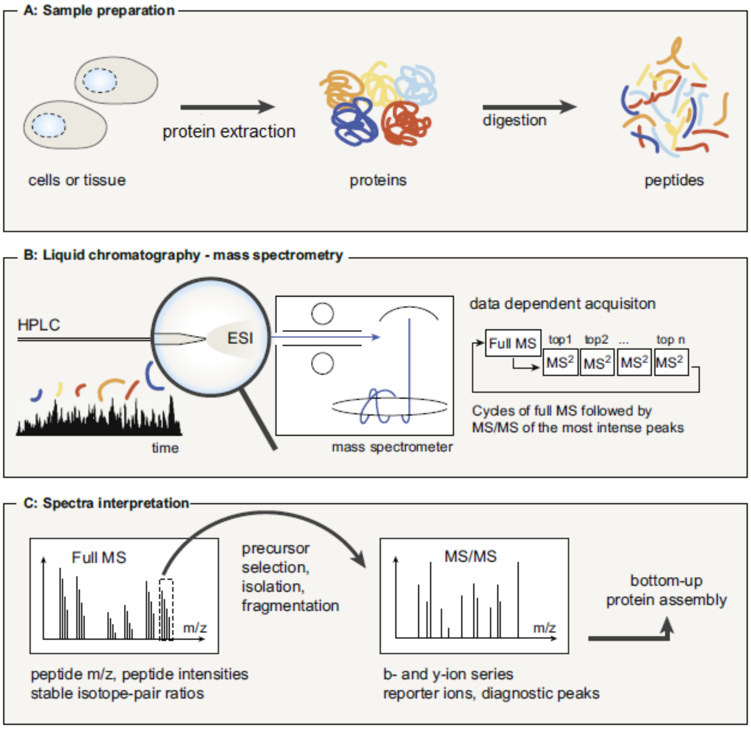

Figure 4.3 – Representation of the basic MS-based proteomics workflow. A: is the sample preparation where proteins are extracted from tissues or cells are digested into peptides using proteases such as trypsin. B: Liquid chromatography-mass spectrometry: peptides are separated by high-performance liquid chromatography (HPLC) and electrosprayed directly into the mass spectrometer. Peptide ions are measured at high resolution in a data-dependent mode: after each full MS scan, the most intense peptide ions are fragmented to generate MS/MS spectra. C: Spectra interpretation: the full MS spectra provide information about the peptide mass, intensity, presence of a PTM, and stable isotope pairs. The mass of each fragmented peptide together with its fragment ion pattern, is searched against databases (for example, the Uniprot database) for peptide identification and bottom-up protein assembly (Source: Hein, 2014).

The standard MS-based proteomics workflow follows the bottom-up principle, as described in Fig. 4.3. In that representation, proteins are first digested to peptides using a sequence-specific endoprotease, which in the present was through trypsin, which cleaves at the C-terminal of the amino acids arginine or lysine. These peptides produced are then analyzed by MS, and the proteins are reconstructed in silico making use of a different database (for example, the Uniprot database).

On the other hand, the complementary ‘top-down’ approach omits the step of enzymatic digestion and analyzes intact protein species instead (Hein, 2014; Niessen & Falck, 2015).

4.3.2.1 Components of a mass spectrometer

All mass spectrometers are similar in their main components. They basically consist of a high vacuum system, which allows ions to move freely in space without colliding or interacting with other species; an injection port, that transfers a sample into the ion source; an ion source that converts neutral species to ionic forms; a mass filter or analyzer that separates the ionic species; a detector that measures and amplifies the ion current of the ions; and finally a data system that records, processes, stores, and displays data in an easy form to be analyzed by the researchers (Dass, 2007; Mishra, 2010; Wilson & Walker, 2010).

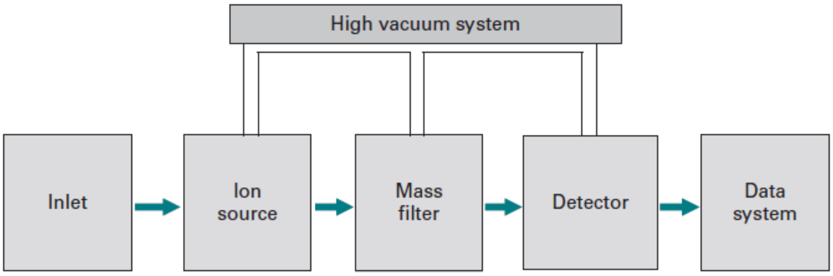

Figure 4.4 - Representation of the essential components of mass spectrometers (Source: Wilson & Walker, 2010).

4.3.2.2 Analyte ionization

Mass spectrometric techniques largely depend on converting a neutral species to a gas-phase ionic species. This is important because unlike neutral species, it is easy to manipulate the motion, direction, and detect ions (Dass, 2007).

In this section, amongst several methods, we briefly describe the most commonly used modes of ionization: MALDI and ESI.

MALDI involves inserting the analyte in a solid matrix of an organic compound, followed by transfer into the vacuum system. A laser pulse then excites the matrix molecules, leading to their desorption or removal and subsequent separation in a time-of-flight (TOF) analyzer. In contrast to MALDI, in the electrospray ionization, high voltage is applied to the emitter (that can be a fine syringe or a needle), and the liquid sample is sprayed as a charged aerosol or of charged liquid droplets. In other words, this type of ionization involves the production of ions by spraying a solution of the analyte into an electrical field (Fig. 4.5) (Dass, 2007; Mishra, 2010; Wilson & Walker, 2010).

Subsequently, the solvent molecules that accompanies the aerosol droplets rapidly evaporate, and charged analyte molecules are then transported into the vacuum of the mass spectrometer, where they finally arrive as 'naked' ions, or as a charged analyte free of solvent. The process of removing the solvent is through injecting heated neutral gas such as nitrogen (Dass, 2007; Mishra, 2010; Wilson & Walker, 2010).

The above-mentioned solvents are typically 50/50 acetonitrile (or methanol)/H_2O with 1% acetic acid or 0.1% formic acid. Ammonium hydroxide or trifluoroacetic acid (TFA, 0.02%) in 50/50 acetonitrile (or methanol)/H_2O can also be used (Niessen, 2006).

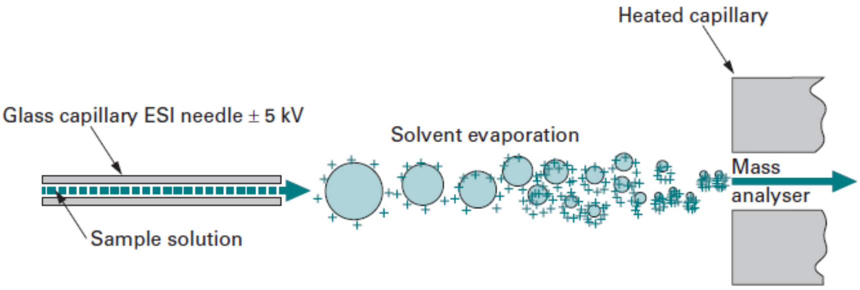

Figure 4.5 – Representation of the electrospray ionization source (ESI). The ESI creates very small droplets of a solvent-containing analyte by atomization or nebulization as the sample is introduced into the source through the fine hollow needle capillary. The solvent evaporates in the high-vacuum regions as the spray of droplets enters the source. These may have multiple charges

depending on the availability of ionizable groups (Source: Wilson & Walker, 2010).

4.3.2.3 Mass analysis

Mass analysis, *i.e.*, the separation of ions according to their *m/z* in either time or space, can take place in several ways (Niessen, 2006) as described below.

4.3.2.3.1 Quadrupole mass analyzer

The quadrupole analyzer consists of four hyperbolic or cylindrical rods that are placed parallel in a spiral array (Fig. 4.6). The opposite rods are charged by a positive or negative direct-current (DC) potential at which an oscillating radiofrequency alternating-current (RF) voltage is applied. Coupling constant and varying (radio frequency) voltages allows for the transmission of a specific range of *m/z* values along the axis of the rods (Niessen, 2006; Pitt, 2009). Following, these ions are transmitted to the detector that amplifies the electrical signal. Ions with other *m/z* have unstable trajectories (non-resonant ions) and do not pass the mass filter, because the amplitude of their oscillations becomes infinite. They are released on the rods and/or lost in the vacuum system (Dass, 2007; Mishra, 2010; Wilson & Walker, 2010).

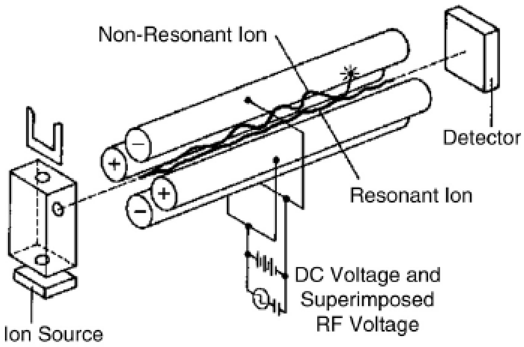

Figure 4.6- Schematic representation of the quadrupole mass analyzer (Source: Niessen, 2006).

4.3.2.3.2 The triple-quadrupole instrument for MS.MS

The second mass analyzer is the triple quadrupole instrument, in which the mass separation is performed in the first and third quadrupoles. In contrast, the second quadrupole is used as a collision cell in the RF-only mode, *i.e.*, in a Q-q_{coll}-Q configuration (Fig. 4.7) (Niessen, 2006).

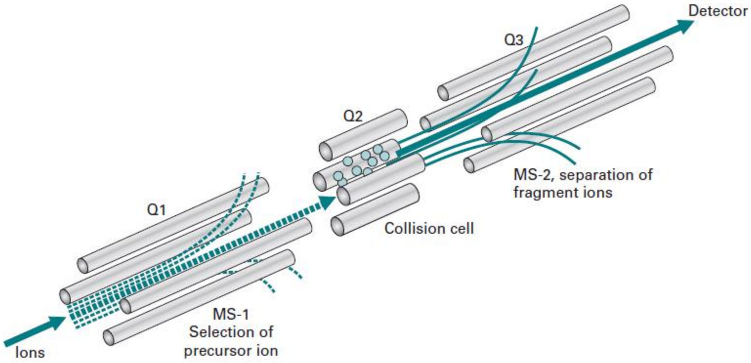

Figure 4.7 – Representation of a triple-quadrupole instrument for MS-MS. An ion of a particular m/z value is selected in the first quadrupole, Q1. However, instead of being detected, it passes through the second quadrupole, Q2, where it is subjected to collision with the collision gas. The Q3 acts like a second quadrupole mass spectrometer, MS-2, to scan m/z to obtain a spectrum of the fragment ions. The collision cell, Q2, is frequently a radio frequency (RF)-only quadrupole containing the appropriate collision gas. No mass filtering occurs here; the RF is applied to aim at constrains the ions to allow a higher number of collisions to occur. The fragmentation that takes place here is at the peptide bond, and one of the fragments will retain the charge, resulting in either a y-series or a b-series ion (Source: Niessen, 2006 and Wilson & Walker, 2010).

4.3.2.3.3 The TOF Mass Analyzer (Time-of-flight instruments)

In this type of analyzer, ions are accelerated into a field-free linear flight tube at the same energy level so that the ions with different masses take different time lengths to reach the detector and thus separated. The velocity of the moving ions together with the time taken to travel into the flight tube to reach the detector depends on their *m/z* values. Therefore, the ions with smaller mass can reach the detector faster than the ions with a larger mass (Fig. 4.8) (Niessen, 2006; Pitt, 2009; Mishra, 2010).

The flight time (t_{flight}) needed for an ion with *m/z* to reach a detector placed at a distance *d* is measured. The flight time taken by the ion is related to the *m/z*, as shown below:

$$t_{flight}^2 = \frac{m\ d^2}{2\ z\ e\ V} = \frac{m}{z}\ [\frac{d^2}{2\ e\ V}]$$

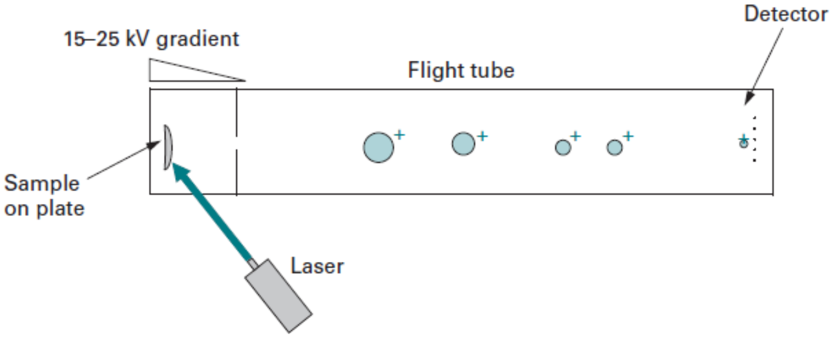

Figure 4.8 – The representation of the time-of-flight (TOF) instrument. The ions move into the flight tube, where the lighter ions travel faster than the heavier ions to the detector. If the ions are accelerated with the same potential at a fixed point and fixed initial time, the ions will separate according to their mass to charge ratios. The time of flight is then converted to mass (Source: Wilson & Walker, 2010).

4.3.2.3.4 Hybrid instruments

The 6600 TripleTOF mass spectrometers used in the present work is one example of a hybrid instrument. In these instruments (Q-TOF instrument), the third quadrupole of a triple quadrupole MS can be replaced by a TOF analyzer to produce a hybrid quadrupole time-of-flight mass spectrometer (Fig. 4.9) (Pitt, 2009).

The bigger advantage of Q-TOF in comparison to the other instruments, is the ability to perform accurate mass determination (<5 ppm) for both precursor and product ions” (Niessen & Falck, 2015).

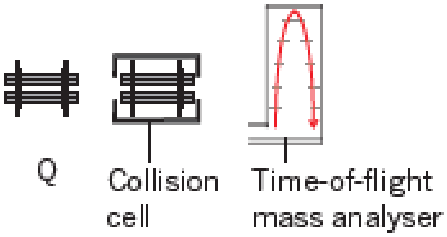

Figure 4.9 - The schematic representation of a Q-TOF instrument. Ranges of m/z values are selected (Q), and peptides are fragmented (Collision cell), followed by the acquisition of the fragments in a time-of-flight mass spectrometer (Adapted from Aebersold & Mann, 2016).

4.3.2.4 Detectors

The next step after ion separation is detection. In this process, an electric current flow is amplified and ultimately converted into a signal that is processed by a coupled computer (Wilson & Walker, 2010).

4.3.3 Data-dependent analysis (DDA) and Data-independent acquisition (DIA) and Sequential Window Acquisition of all Theoretical Fragment on Spectra (SWATH) acquisition methods

The objective of the DDA-based methods is to achieve an unbiased and complete coverage of the proteome, basically gathering mass spectra of all the ion species that co-elute at a specific point in the gradient elution (that is, precursor-ion spectra) that are recorded at the MS^1 (or full-scan) level. In that mode, the equipment changes between the acquisition of full-scan data and the acquisition of fragment-ion spectra, in which as many precursors as possible are sequentially isolated and fragmented (at the MS^2level, Fig.4.3B) (Aebersold & Mann, 2016).

The SWATH or DIA MS works differently from the conventional DDA MS, which relies on the specific selection and fragmentation of a fixed number of precursor ions in a survey scan. SWATH is based on the cyclical acquisition of precursor ions with fixed or variable isolation windows that cover the entire *m/z* range in a sample. With this approach, all ionized precursor peptides within a sample are fragmented and their fragmentation spectra collected, allowing a backward interrogation of the peptides of interest using spectral libraries previously generated by the DDA methods (Krasny et al., 2018).

SWATH-MS is a multifaceted technique and has been used in diverse applications, including the quantification of proteins in several model organisms and disease states in humans for example. SWATH-MS is also used to characterize low abundance sub-proteomes including post-translational modifications such as acetylomes and glycosylomes (Krasny et al., 2018).

The advantage of SWATH-MS if compared to DDA is that the entire range of possible precursor-ion masses can be analyzed evenly and in rapid succession, which eliminates the missing value problem of DDA (in which only a few or the most intense peptides are selected for fragmentation) (Aebersold & Mann, 2016).

An important concept in the DIA approach is the use of an LC-retention time referenced spectral ion assay library to allow peptide identification in the following SWATH step. The reference assay library previously generated should contain all the prior knowledge of the peptide components to be extracted from the SWATH data. Thus, the reference assay libraries must be species-specific and sufficiently deep in composition to enable extensive peptide identification from DIA experiments. One challenge and limitation of SWATH is the need for a reference library generation. Additionally, the peptide must be present within an assay library for it to be detected and quantified using the SWATH approach (Aebersold & Mann, 2016; Wu et al., 2016).

A common way of generating the reference assay library is through performing numerous IDA experiments, usually using fractionated samples to create library depth. However, this library building process is time-consuming and can fail in detecting less abundant proteins in some type of samples with a vast dynamic range of proteins abundances (Wu et al., 2016).

One important progress made to the SWATH method to increase protein coverage is the use of variable-sized Q1 isolation windows based on the precursor m/z density. Thus, the optimization of the size of the windows, made to be possible to cover much smaller windows of m/z densities, which improves the specificity of the data for those targeted peptides. On the other hand, larger windows can be used across lower m/z densities to ensure that proteome coverage is maintained (Hunter et al., 2012).

4.4. Material and Methods

4.4.1 Leaf tissue material and growth conditions

To better understand the proteomic responses of the genotypes to drought and how these features are related to photosynthetic capacity, the levels of C4-photosynthesis enzymes, oxidative stress mitigation enzymes, electron transport rate proteins, and stress proteins (like HSP) were assessed through proteomic techniques. For this proteomic part of the work, the four maize genotypes, Matuba, ZM309 and ZM523, and B73 as control, were tested. B73 is the variety that is currently used in the host Lab, and it has been previously targeted for proteomic characterization. Controlled experiments were conducted in a growth chamber (FITOCLIMA 10000 HP, 3.305mx1.600mx1.950m) at the Instituto de Tecnologia Química e Biológica António Xavier (ITQB NOVA) under the same conditions as previously described in Chapter III. Plants grew until the 3rd leaves were fully expanded for harvesting (normally at the emergency of 4th leaf). One-third (from leaf tip) of the 3rd fully expanded leaves were harvested five hours after light exposure and immediately frozen into liquid nitrogen and kept at -80ºC until further procedures. For each treatment, each leaf sample (replicate) consisted of a pool of leaves from five individual plants to diminish heterogenicity.

4.4.2 Sample preparation and protein extraction

Maize-leaf tissue was ground to a fine powder with mortar and pestle in liquid nitrogen. Protein extraction was performed according to Luís et al. (2016). Proteins were extracted from leaf powder samples in Lysis Buffer (LB) composed as follows: [7M Urea, 2M Thiourea, 30mM Tris, 4% (w/v) CHAPS, 4% (v/v) cOmplete, EDTA-Free Protease Inhibitor Cocktail 25x, 0.1% (v/v) Pepstatin 1mM, 10% PhosSTOP 10x, 1% (v/v) Nuclease Mix 100x]. Tubes were homogenized and put on ice for 30 min. Next, tubes were subjected to many subsequent centrifugations (4°C, 17,000 x *g*, 30 min) to remove debris. The supernatant was kept at -80ºC for further procedures.

For the total protein quantification, we used the Bradford colorimetric method with a 2-D Quant kit (GE Healthcare, USA) following the manufacturer's instructions.

4.4.3 Protein digestion using FASP protocol

To prepare samples for mass spectrometry analysis, a gel-free based protein digestion method called Filter-Aided Sample Preparation (FASP) was used. FASP is an efficient and flexible method to process protein extracts for the following bottom-up proteomic steps (Wiśniewski, 2017).

FASP works in such a way that an ultrafiltration membrane allows separation of proteins from detergents, salts, and small molecular weight reagents before enzymatic digestion. After the digestion, typically using trypsin, FASP allows the isolation of pure peptides that are free of undigested components such as nucleic acids and become ready for the next MS-based approaches (Wiśniewski et al., 2011).

The major advantage of FASP over the other sample preparation methods, such as the gel-based ones, is the high purity of the peptides produced. This is a crucial prerequisite for the following liquid chromatography fractionation and mass spectrometry steps. Another advantage is that it is particularly useful for processing very small total protein amounts and its compatibility with different detergents (Wiśniewski et al., 2011; Wiśniewski, 2017).

The FASP protein digestion was performed according to Erde et al. (2014) and Wiśniewski (2017); with modifications. First, 10kDa microcon filter units and collection tubes (Merck Millipore, Germany) were passivated by 6h incubation with 5% (v/v) TWEEN-20, followed by thorough washing in Milli-Q water, MS grade water and UA Buffer (8 M urea in 0.1 M Tris–HCl, pH 8.5). Then, 100 µg of protein sample supplemented with 12.5 pmol BSA was added to the filter, and the protein sample was reduced by the addition of DDT to a final concentration of 20 mM followed by 5 min incubation at 4ºC. Then, a series of centrifugations at 10 000 x *g* (4ºC) were carried out after the addition of different volumes of UA Buffer. The depletion of interfering substances such as excess detergent and other low-molecular-weight substances was facilitated by washing with a buffer containing 8 M urea. Urea also keeps proteins largely unfolded, which allows their derivatization and inhibits the passage of small polypeptides through the filter pores. An alkylating agent (40% Acrylamide/Bisacrylamide) was added to the sample, incubated 5 min (at 4ºC), and filter units were washed with serial centrifugations with UA Buffer and Ammonium Bicarbonate (AmB). After washing, the filter was disassembled to discard the flow-through from the collection tube and reassembled afterward. Then, 80 µL of trypsin 23 ng/µL (Promega, USA) was added to the filter units, and tubes were incubated at 37ºC for 12h with soft agitation (300 rpm in a thermal shaker, VWR). Twice, 40 µL AmB 10 mM was added to the filter unit Centrifuged for 10 min, and eluted peptides were transferred to a new Eppendorf tube. Then, 80 µL of trypsin 23 ng/µL was added to the filter units and incubated at 37ºC, 4h with soft agitation (300 rpm in thermomixer). Next, the filter was washed with 50 µL NaCl 500mM and Centrifuged 25 min. The eluted peptides were transferred to the same Eppendorf tube above mentioned and homogenized. Then, the peptide solutions were desalted using C18 tips (THERMO SCIENTIFIC, USA) according to the manufacturer's protocol and stored at -80°C.

The Pierce Quantitative Colorimetric Peptide Assay (THERMO SCIENTIFIC, USA) was used to determine the concentrations of the tryptic peptides according to manufacturer procedures. Finally, samples were dried using a speed vac concentrator prior to MS analysis.

4.4.4 Liquid Chromatography tandem MS (LC-MS/MS) analysis

Before LC-MS analyses, samples were resuspended to a final concentration of 1µg/µL in LC Buffer A (0.1% FA). We performed all MS analyses by electrospray liquid chromatography-tandem MS (LC-MS/MS).

Samples were loaded on an Eksigent NanoLC 400 HPLC system (AB SCIEX) coupled with a 6600 TripleTOF mass spectrometer (AB SCIEX). Briefly, reverse phase HPLC was performed in a trap and elution configuration using a nano cHiPLC trap column (Eksigent ChromXP C18-CL, 3-mm particle size, 120-Å pore size, 0.5 mm X 200 µm i.d., AB Sciex) and an analytical column (Eksigent ChromXP C18-CL, 3-µm particle size, 120-Å pore size,15 cm X 75 µm i.d., AB Sciex). Samples were loaded into the trap column at a flow rate of 2 µL min^{-1} for 10 min using 100% Buffer A (0.1% [v/v] formic acid in water) and eluted at a flow rate of 300 nL/min using a stepwise gradient: 0 to 1 min, 4.5% B (0.1% [v/v] formic acid in acetonitrile); 1 to 91 min, 29.7% B; 91 to 93 min, 79.2% B; 93 to 108 min, 79.2% B; 108 to 110 min, 4.5% B; 110 to 127 min, 4.5% B. Samples were run in information-dependent acquisition (IDA) mode to perform peptide and protein identification to generate a spectral library for the SWATH quantification. This spectral library was created by combining all the IDA.wiff files in unison using ProteinPilot 5.0 (AB Sciex). A UniProt database search was performed using the Paragon algorithm, which is embedded in ProteinPilot software 5.0 (AB Sciex). A Paragon search method was created with the following settings: sample type, identification; Cys alkylation, acrylamide; digestion, trypsin; instrument, tripleTOF 6600 (Sciex);

species, none; search effort, thorough; detected protein threshold, >0.05. A false discovery rate (FDR) threshold was set to below 1%.

4.4.5 SWATH data acquisition, MS/MS data and statistical analysis

Similar LC gradient and mass spectrometer settings as for the IDA acquisition described above were used to perform SWATH-MS analysis of five replicates from each one of the 12 conditions collected. SWATH-MS data were acquired using a set of 64 overlapping SWATH windows covering the precursor mass range of 400 ‑1600 m/z, a cycle time of 3.2 s, and a 50 ms survey scan.

All generated raw files were searched using ProteinPilot™ software (AB SCIEX) against a *Zea mays* database provided by the Universal Protein Resource (https://www.uniprot.org/).

Data were processed using a SWATH processing plug-in (SWATH 2.0) for PeakView 2.2 (AB Sciex). The ion peak areas exported by PeakView for each sample were normalized by total area sum. Specifically, the total sum of all ion intensities for each sample was calculated, and the maximum of those totals determined. The sum of the ion peak areas gave the relative abundance of the peptides, and the sum of the peptide areas, the relative abundance of the proteins. All loaded DIA files were exported into.txt format using an extraction window of 12 min with the following parameters: ion library mass tolerance (20 ppm); six peptides; six transitions; peptide confidence of >99%; exclusion of shared peptides.

The quantified proteins were exported to MarkerView^{TM} software version 1.2.1 (AB SCIEX) for the generation of Principal component analysis.
One-way analysis of variance (ANOVA) tests was used to determine if any significant differences between the four genotypes groups in regards to the levels (peak areas) of expressed proteins using

SigmaPlot 11.0. software package (Systat Software Inc., Chicago, IL, USA). The minimum significance was set at $p<0.05$.

4.5. Results and discussion

4.5.1 Principal component analysis

In this chapter, we present our results regarding the proteomic characterization of four maize genotypes exposed to different watering-regimes. We performed a quantitative proteomics analysis and focused particularly on the variation of the relative abundance of different groups of proteins related to photosynthesis and reactive oxygen species (ROS) detoxification. The genotypes are Matuba, ZM309, and ZM523, all Mozambican varieties, and B73 as control.

Globally we detected 221 proteins that might be playing different roles in drought tolerance. We characterized the relative abundance of the following proteins that are involved in CO_2 assimilation**:** Phosphoenolpyruvate carboxylase 1 (PEPC1), NADP-Malate dehydrogenase (NADP-MDH), Pyruvate, phosphate dikinase 1 (PPDK1), NADP-dependent malic enzyme (NADP-ME), Phosphoenolpyruvate carboxykinase (PEPCK), Aspartate aminotransferase (AspAT), Ribulose bisphosphate carboxylase/oxygenase activase (RCA1), Ribulose bisphosphate carboxylase large chain (rbcL), and Ribulose bisphosphate carboxylase small chain (RBCS).

The second group of proteins that we looked at is involved in PSII and PSI assembly and electron transport chain rate: Photosystem II D2 protein (D2), Photosystem II protein D1 (D1), Cytochrome b6 (petB), Photosystem II reaction center protein H (psbH), Cytochrome f (petA); Photosystem II CP43 reaction center protein (CP43); ATP synthase subunit beta (atpB), ATP synthase subunit alpha (atpA), ATP synthase subunit gamma, Photosystem I reaction center subunit N (PSAN), Photosystem I P700 chlorophyll *a* apoprotein A2 (psaB), Photosystem I P700 chlorophyll *a* apoprotein A1 (psaA), Chlorophyll *a*-*b* binding protein 1 (CAB1) Photosystem I assembly factor PSA3 (PSA3).

We also targeted the stress-related heat shock 70 kDa protein (HSP70) and the proteins that are involved in the oxidative stress defense, namely, Catalase isozyme 3 (CAT3); Catalase isozyme 2 (CAT2), and Peroxidase 42 (PER42).

To do the global assessment on the variations of protein levels amongst the four genotypes we used the Principal Component Analysis (PCA). Firstly, we considered the contribution of all 221 proteins detected in our samples. Therefore, we found a cluster of samples under severe drought treatment that was separated from the control and moderate stress conditions. (Supplemental Fig. S4.1). This was an indication that the identified proteins may play a role in severe drought conditions. Looking only at well-watered conditions, we found an evident separation of the B73 genotype from the Mozambican varieties (Fig. 4.10). Within the Mozambican varieties, Matuba also separated from the rest (Fig. 4.10). In contrast, within stress treatments, no evident separation was found (Supplemental Fig. S4.2 and S4.3).

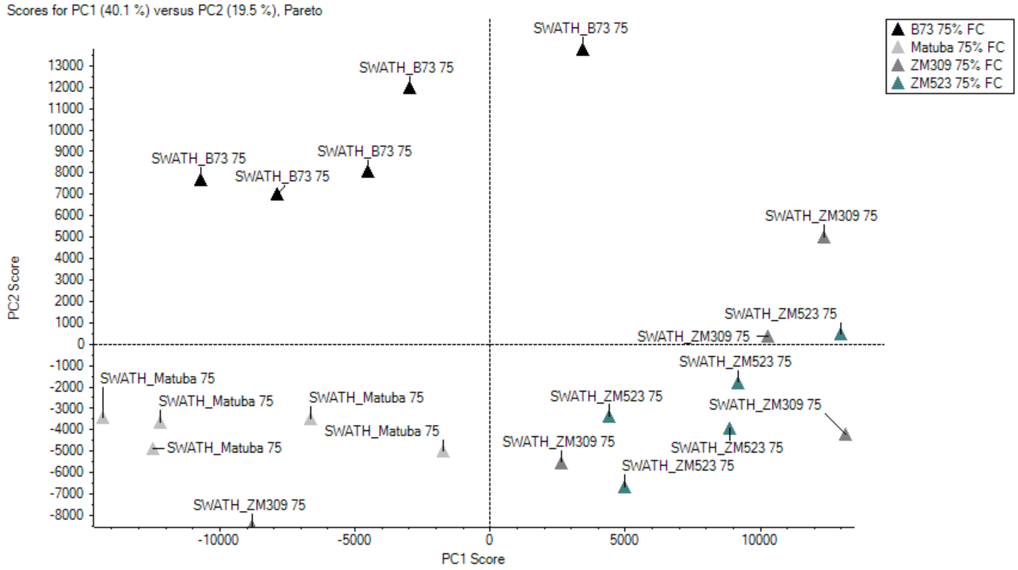

Figure 4.10 - Principal Component Analysis of maize genotypes under well-watered conditions, using the 221 detected proteins. The different genotypes are identified by different colors.

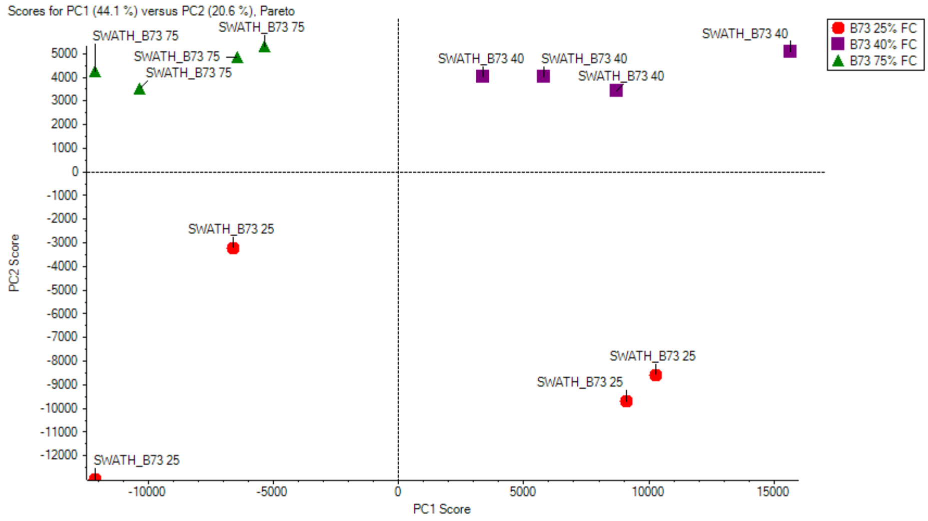

Figure 4.11 - Principal Component Analysis for B73 genotype, using the 221 proteins detected. Different water treatments are identified by different colors and symbols.

The analysis within genotypes shows that only in B73 was a clear separation of all water treatments. Within the Mozambican varieties, the condition well-watered was clearly separated from the stress treatments (Fig.4.11, 4.12, 4.13, and 4.14).

When we look only at the contribution of the proteins related to CO_2 assimilation, moderate drought treatment seems to be in the interface between severe drought and well-watered conditions. Also, severe drought is clearly separated from the well-watered treatment (Supplemental Fig.S4.4). Looking at the individual water treatments, B73 and ZM523 were found to form a different cluster from those of ZM309 and Matuba under severe drought treatment (Supplemental Fig.S4.5), but no clear distinction was found under moderate drought treatment. Under well-watered conditions, Matuba has separated apart from the other genotypes (Supplemental Fig.S4.6, and S4.7).

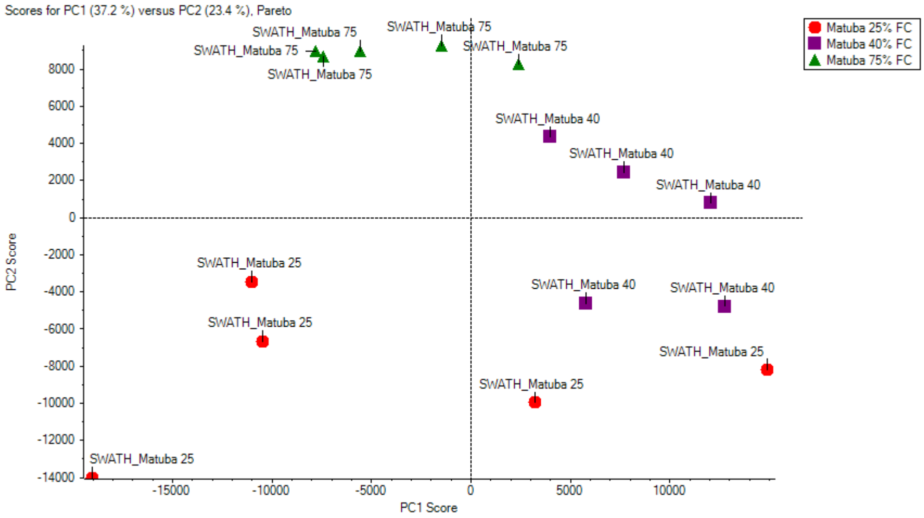

Figure 4.12 - Principal Component Analysis for Matuba, using the 221 proteins detected. The different water treatments are identified by different colors and symbols.

Figure 4.13 - Principal Component Analysis for ZM309, using the 221 proteins detected. The different water treatments are identified by different colors and symbols.

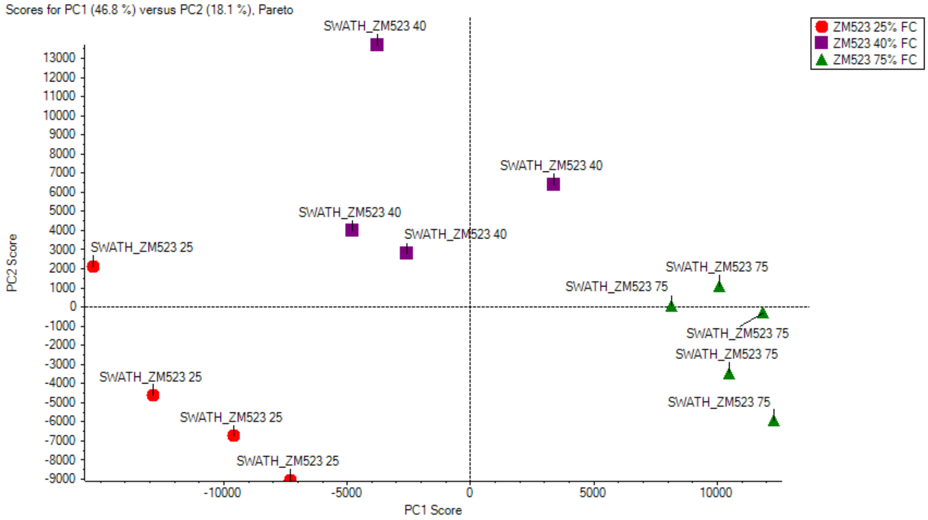

Figure 4. 14 - Principal Component Analysis for ZM523, using the 221 proteins detected. The different water treatments are identified by different colors and symbols.

The contribution of the CO_2 assimilation enzymes for the distinction of the treatments within the genotypes, shows an evident separation of the treatments in B73, Matuba, and ZM523, but less for ZM309 where severe and moderate stress treatments form a cluster (Figs. 4.15, 4.16, 4.17, and 4.18).

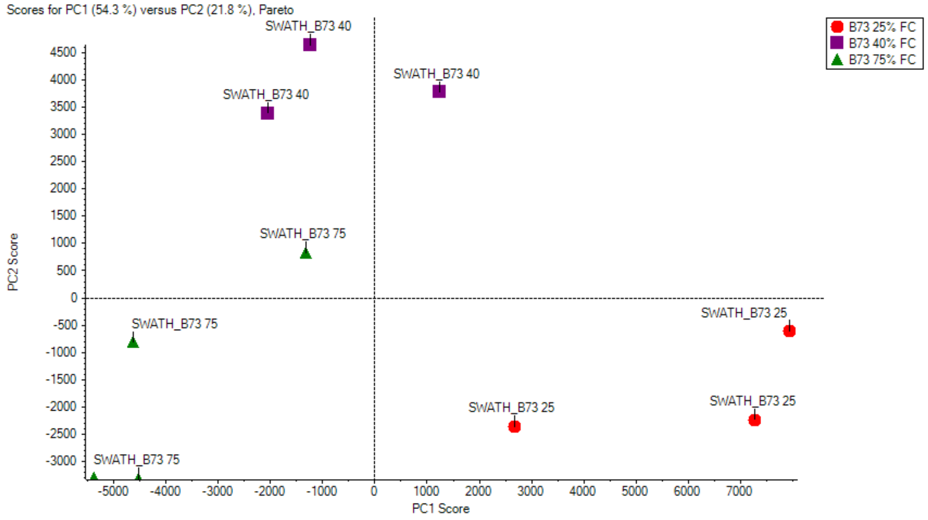

Figure 4.15 - Principal Component Analysis for B73, using only CO_2 assimilation related enzymes. The different water treatments are identified by different colors and symbols.

Figure 4.16 - Principal Component Analysis for Matuba, using CO_2 assimilation related proteins. The different water treatments are identified by different colors and symbols.

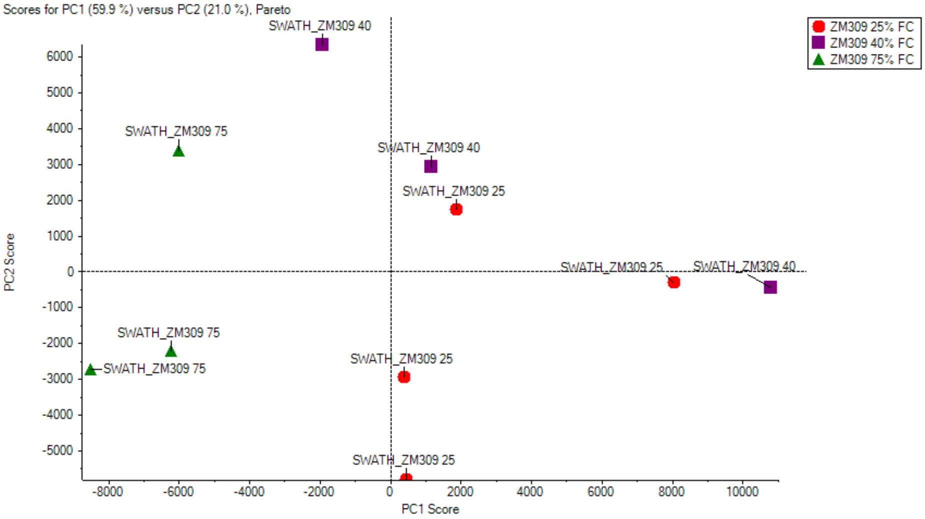

Figure 4.17 - Principal Component Analysis for ZM309 using CO_2 assimilation related proteins. The different water treatments are identified by different colors and symbols.

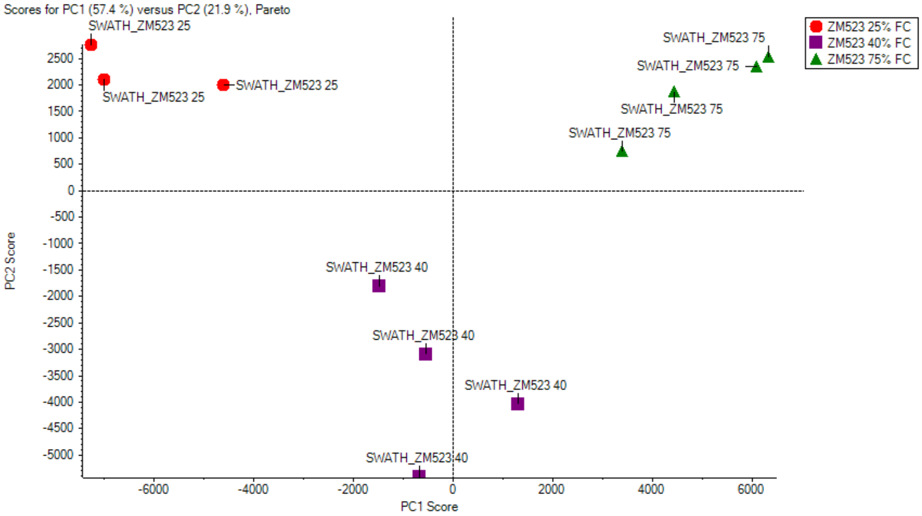

Figure 4.18 - Principal Component Analysis for ZM523 using CO_2 assimilation related proteins. The different water treatments are identified by different colors and symbols.

This observation was an indication that the CO_2 assimilation enzymes were differentially expressed throughout treatments. This means that they may play a role in stress response, or at least their levels correlate with maize drought status and thus may be seen as putative markers of that status. Sampling on a bigger population would be necessary to validate this correlation.

We also targeted the proteins related to the electron transport chain between PSII and PSI. We found that severe drought treatment tended to be separated from control conditions and moderate drought (Supplemental Fig. S4.8). This is supported by the data presented in Fig.4.25 and Fig. 4.26, which shows that higher levels of PSII and PSI proteins under severe drought treatment were detected (see a detailed discussion in the next section). Within our treatments, we found only a clear distinction between genotypes at 75% FC, with B73 and Matuba basically forming a separated cluster from ZM309 and ZM523 at PC1 (Fig. 4.19). For the other treatments, no evident separation was found (Supplemental FigS4.9 and S4.10). Within the genotypes, these proteins seem to be involved in drought response, as it can be seen that severe drought treatment is separated from moderate stress and from control conditions (Supplemental Figs. S4.11 to S4.14).

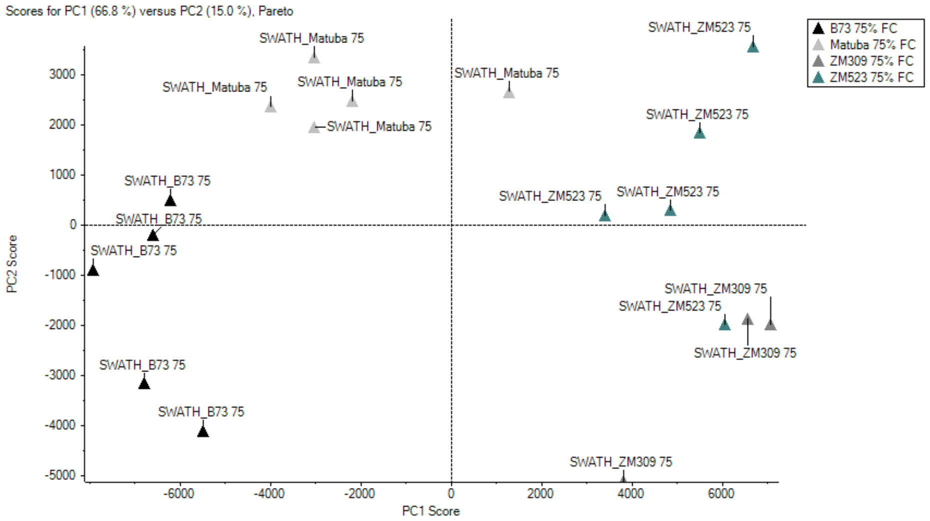

Figure 4.19 - Principal Component Analysis for the entire set of maize genotypes under control conditions using the electron transport chain-related proteins. The different genotypes are identified by different colors.

The ROS detoxification enzymes detected are apparently not playing a significant role in drought response in most of the genotypes. The PCA shows all the treatments basically clustering together (Supplemental Fig. S4.15). The assessment of the genotype's separation within treatments, shows that B73 and Matuba are separated from ZM309 and ZM523 in severe drought treatment, and Matuba is separated from the other genotypes in control conditions (Supplemental Figs. S4.16 and S4.18), while for moderate stress treatment, no evident separation was found (Supplemental Fig. S4.17). Also, within the genotypes, we found a separation of the well-watered from the stress treatments only in Matuba, which is an indication that only ROS elimination is somehow playing a role under stress conditions for this variety alone (Supplemental Figs. S4.19 to S4.22).

The separation of B73 genotypes from the Mozambican varieties in some of the PCAs mentioned above reinforced the different origins of the genotypes. This observation is also consistent with the lowest biomass accumulation observed in B73 compared to the Mozambican varieties. However, the physiological performance of B73 was similar to those observed in Mozambican varieties. This probably means that other molecular mechanisms are contributing to the differences in plant growth upfront CO_2 assimilation.

The separation of the treatments within the genotypes and also the different genotypes within the treatments are consistent with the different levels of the individual photosynthesis-related enzymes (see a detailed discussion in the next section).

4.5.2 SWATH-MS quantitative proteomics analysis

4.5.2.1 The levels of the Photosynthesis related proteins: CO_2 assimilation enzymes

The C4-photosynthesis enzymes were found to be differently expressed in the different water treatments. Tendentially, ZM523 was the genotype that showed the highest levels of these proteins under well-watered conditions (Fig. 4.20). Interestingly, higher levels of these proteins were not accompanied by visible higher photosynthetic performance, which was similar among genotypes. Also, biomass production was similar among the Mozambican varieties under well-watered conditions (see chapter III for a detailed discussion on physiological performance). This was an indication that other factors, more than just stomata aperture, might play a role in the overall photosynthetic performance of these genotypes. For instance, other critical steps, such as sucrose synthesis and transport from the mesophilic tissue (in case of C3 plants), phloem transport and carbon partitioning throughout the plant, the RUBP regeneration, the activity of the enzymes, and other factors, can exacerbate the phenotypic differences amongst genotypes (Hofius & Börnke, 2007).

The same can be applied for severe drought treatment. Although CO_2 assimilation slightly favored B73 and Matuba, the levels of the C4-photosynthesis enzymes were similar among all the genotypes. However, root biomass was notably higher in ZM523. This was an indication that non-stomatal factors may have differentially contributed to the root biomass production in this variety (Wang et al., 2018).

On the other hand, the levels of the decarboxylating enzyme PEPCK in ZM523 was maintained at lower levels throughout treatments if compared to other genotypes, suggesting a less degree of flexibility in the use of decarboxylation mechanisms (NADP-ME and PEPCK), which is particularly important in water scarcity conditions (Fig. 4.21) (Cacefo et al., 2019).

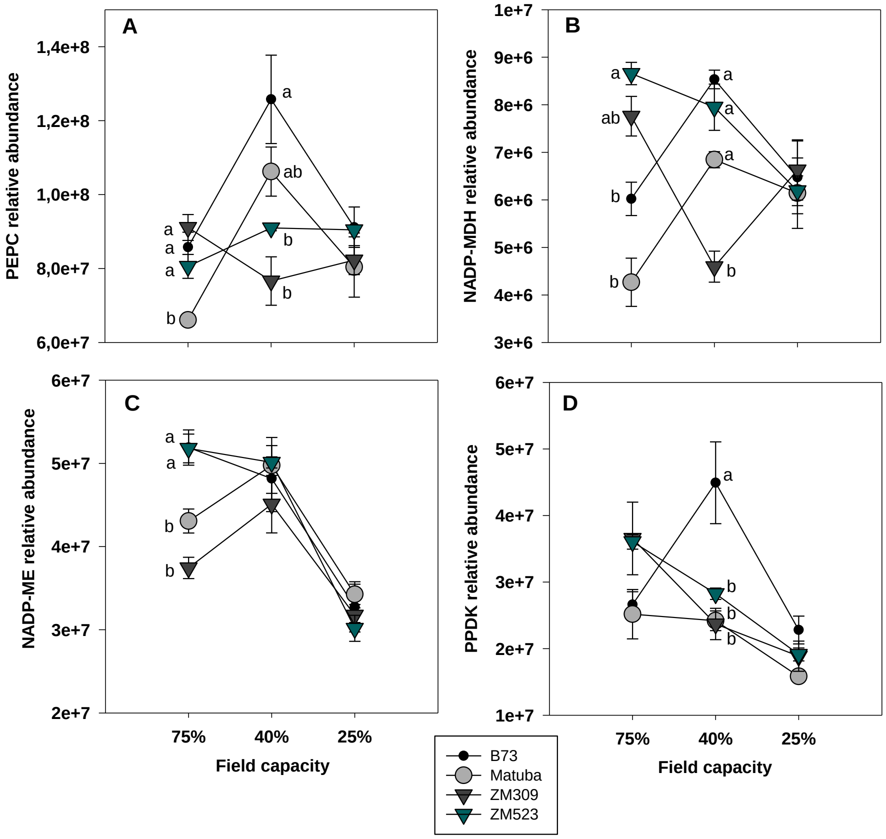

Figure 4.20 - Quantification of total C4-photosynthesis enzymes by SWATH-MS. Phosphoenolpyruvate carboxylase (A), NADP-Malate dehydrogenase (B), NADP-Malic enzyme (C), pyruvate orthophosphate dikinase (D). Values represent the mean ± SE of three biological replicates. Replicates represents a pool of leaves harvested from five plants. Different letters indicate that the value is statistically significant at $p<0.05$ within field capacity.

We also found a notable increase of PEPC and NADP-MDH in Matuba and B73 in moderate drought treatment, which suggests a possible function of these enzymes in stress tolerance in this specific condition. For example, the role of PEPC in plant stress tolerance was tested in transgenic rice (*Oryza sativa* L.) overexpressing the maize C4-PEPC gene encoding phosphoenolpyruvate carboxylase (Liu et al., 2017). These plants showed increased survival and net photosynthetic rates after 16 days of drought treatment, as compared with WT (Liu et al., 2017). Also, in work developed by Hỳsková et al. (2014), PEPC, NADP-

ME, and PPDK responded to drought by enhancing their activities. This is important because, during stress, plants require for example, a higher amount of reducing equivalents in the form of NADPH to cope with the higher production of reactive oxygen and nitrogen species. Thus, NADPH can be produced by the reaction catalyzed by NADP-ME. The NADPH is also essential for the biosynthesis of proline, which functions as an osmolyte and also as a coenzyme of antioxidant enzymes.

On the other hand, the levels of PEPC in ZM309 and ZM523 were basically similar throughout the treatments. Also, the levels of NADP-MDH under severe drought treatment in B73 and Matuba were either similar or higher as compared to well-watered conditions. Therefore, since severe drought restricted CO_2 assimilation, the activity of the C4-photosynthesis enzymes may also be lower. Also, the biochemical limitations of the net CO_2 assimilation rate can be associated with the lower RuBisCO activation by Rubisco activase (Perdomo et al., 2017).

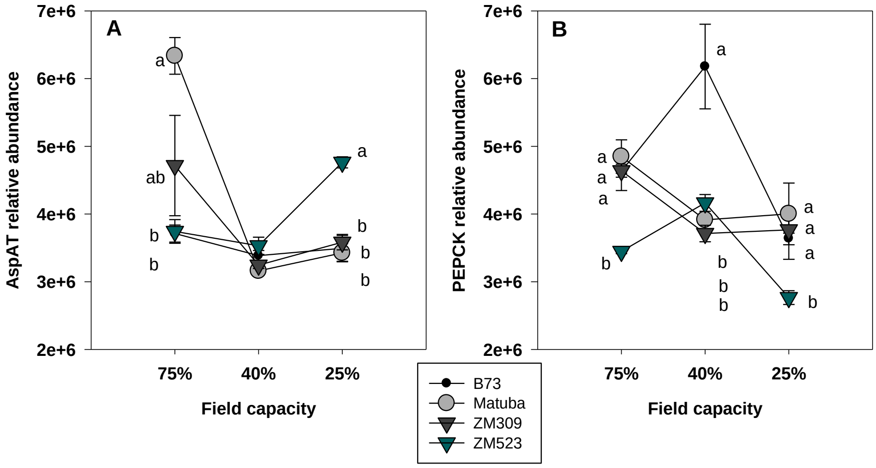

Figure 4.21 - Quantification of total alternative C4-photosynthesis enzymes by SWATH-MS. Aspartate aminotransferase (A), *Phosphoenolpyruvate carboxykinase* (B). Values represent the mean ± SE of three biological replicates. Replicates represents a pool of leaves harvested from five plants. Different letters indicate that the value is statistically significant at $p<0.05$, within field capacity.

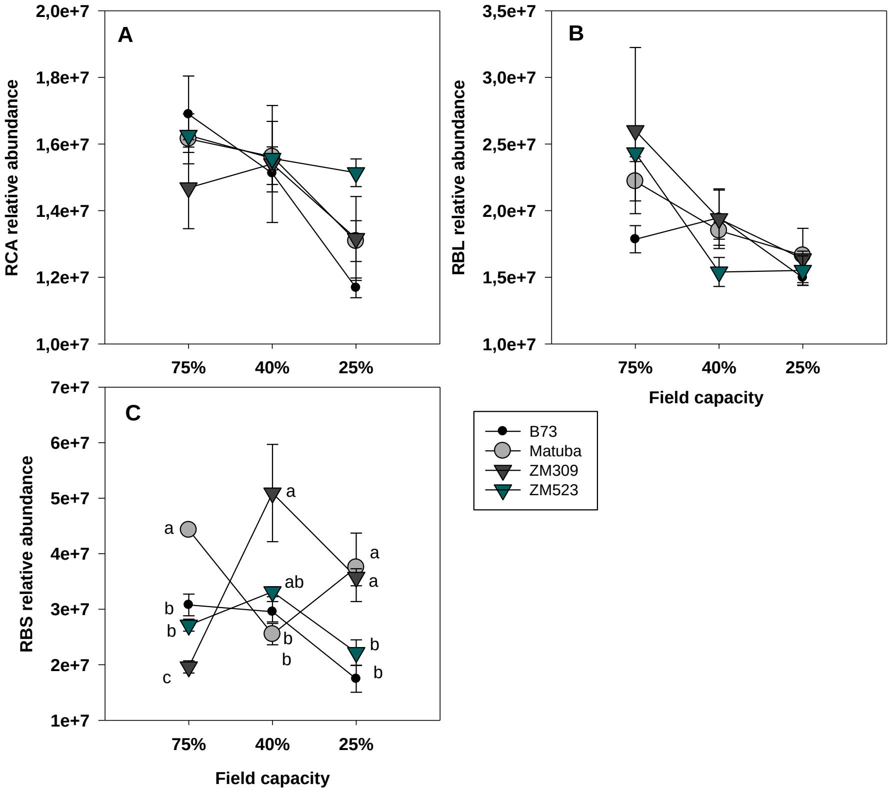

Figure 4.22 - Quantification of total RuBP carboxylation proteins by SWATH-MS. RuBisCO activase (A), RuBisCO large subunit (B), RuBisCO small subunit (C). Values represent the mean ± SE of three biological replicates. Replicates represents a pool of leaves harvested from five plants. Different letters indicate that the value is statistically significant at *p*<0.05, within field capacity.

For the proteins involved in RuBP carboxylation (RuBisCO activase, RuBisCO large and small chain), we only found statistical differences among genotypes in the levels of RuBisCO small subunit. For RCA and RBL, the genotypes were clustered together throughout treatments, and Matuba and ZM309 harbored the highest amplitude difference from control conditions to severe drought treatment when compared to B73 and ZM523 (Fig. 4.22). The overall decrease tendency of the RuBP carboxylation proteins under severe stress conditions can be associated with the biochemical limitation of the net CO_2 assimilation rates. Perdomo et al. (2017), also reported that drought reduced the levels of RCA, which is the catalytic chaperone of RuBisCO, and this would reduce the activation of the last. Thus, in that case, the lower RuBisCO activation state was related to the inhibition of photosynthetic capacity.

Also, the biochemical limitations such as the lower regeneration of RuBP, the inhibition of RuBisCO and RuBisCO activase activities, which are originated by the reduction in ATP content, can be mostly observed in longer periods of severe drought (Benešová et al., 2012).

The decreased photosynthetic activity under drought conditions can be due to reductions in stomatal conductance and RuBisCO activities, leading to lower carbon fixation that consequently results in the over reduction of components within the electron transport chain, generating ROS. In most cases, the levels of RuBisCO activase are reduced under stress; however, several salt-tolerant plant species showed protein accumulation in response to salinity. This indicates that enhanced levels of RCA may be somehow related to stress tolerance (Abreu et al., 2013).

Additionally, in our study, the levels of RBS did not match the decreased levels of RBL. For instance, for Matuba levels of RBS increased from moderate to severe drought treatments, and for ZM309, the levels under severe drought treatment were higher than those of control conditions. Thus, higher levels of RBS under water scarcity may be important to maintain RuBisCO activity. For example, although the long distance from the active site of the large subunits, a single point mutation in the small subunit caused a loss of the catalytic activity of RuBisCO (Sugawara et al., 1999). Also, looking at the structural arrangement, with the small subunit covering a substantial area at two opposite ends of the large-subunit octamer, it is assumed that it has a chaperone-like function. Thus, the small subunit might influence both holoenzyme stability and catalytic performance (Spreitzer, 2003; Andersson, 2008).

Therefore, these data suggest that our central working hypothesis stating that “the levels of the photosynthesis enzymes can be correlated with the photosynthetic capacity under drought" is not always applicable in our study. We concluded that genotype-specific accumulation of C4-photosynthesis enzymes in response to severe stress to define molecular markers of drought tolerance in these genotypes remains an open question. However, the notable increase of PEPC and NADP-MDH under mild stress possibly highlights that these proteins can somehow play a role in drought tolerance under these conditions.

4.5.2.2 ROS-elimination enzymes

The Reactive Oxygen Species (ROS) are known to accumulate during abiotic stresses, and different cellular compartments respond to them by the formation of the distinctive profiles of ROS (Jaspers & Kangasjärvi, 2010). In drought, there is an imbalance between production and elimination of the active oxygen species in plants (Li et al., 2015).

The result of this imbalance is the damage to the DNA, lipids, and proteins, also known as oxidative stress. The detrimental effects of ROS accumulation in chloroplasts also include the impairment *de novo* synthesis of the D1 protein, which is needed for PSII repair and, ultimately, for ETR between PSII and PSI (Gururani et al., 2015; Abid et al., 2018). However, ROS production should not necessarily be considered as a symptom of cellular dysfunction because it can also represent a necessary signal in adjusting the cellular machinery to the altered conditions. Additionally, ROS is responsible for modulating several signal transduction pathways, such as mitogen-activated protein kinase cascades, as well as the activity of transcription factors (Jaspers & Kangasjärvi, 2010).

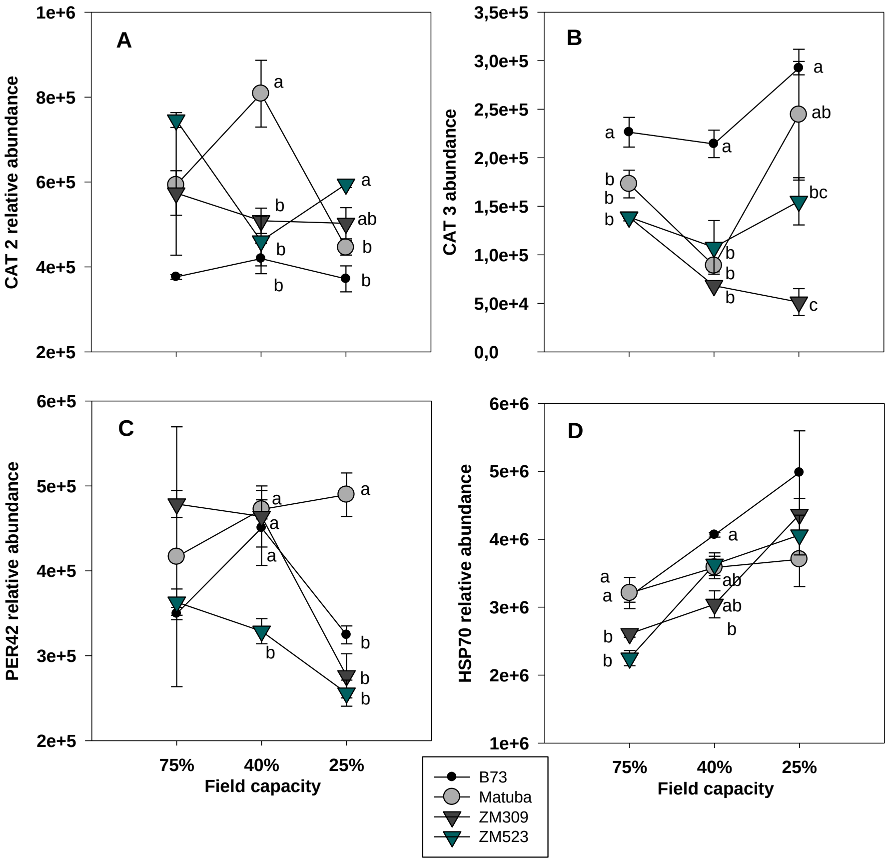

Figure 4.23 - Quantification of total ROS detoxification proteins and stress-induced proteins by SWATH-MS. Catalase 2 (A), Catalase 3 (B), Peroxidase 42 (C), heat shock protein 70 kDa (D). Values represent the mean ± SE of three biological replicates. Replicates represents a pool of leaves harvested from five plants. Different letters indicate that the value is statistically significant at $p<0.05$, within field capacity.

Additionally, since the antioxidant enzyme activity is known to increase in plants exposed to drought, the capacity of ROS detoxification has been used as an indirect selection criterion for screening drought-resistant plant genotypes (Lavinsky et al., 2015).

The reductions in carbon assimilation that occurs under drought conditions can result in an imbalance between electron excitation and its use in photosynthesis. Following, production of ROS can occur, mainly superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2). However, plants have enzymatic and non-enzymatic mechanisms to eliminate ROS. For example, Superoxide Dismutase (SOD) catalyzes the conversion of $O_2^{\bullet-}$ to H_2O_2. This H_2O_2 is later converted to O_2 and H_2O through the activity of catalase (CAT) and ascorbate peroxidase (APX) (Abid et al., 2018).

In addition to the enzymatic mechanisms, non-enzymatic antioxidants such as Glutathione (GSH) and carotenoids are involved in cellular defense from ROS. GSH protects the chloroplasts by increasing the ratio of reduced to oxidized glutathione (GSH/GSSG). Carotenoids possess a different mechanism to protect the photosynthetic apparatus via dissipating excess excitation energy through heat (Abid et al., 2018).

In our study, the individual levels of ROS-elimination enzymes varied in different water treatments. Under severe drought, Matuba displays the highest levels of CAT 3 and PER42 when compared to ZM309 and ZM523 (Fig. 4.23A, B, and C).

Differential expression of the ROS scavenging genes was also found in studies developed by Avramova et al. (2020). These studies showed that within the three CAT-encoding transcripts identified in maize, two were up-regulated (cat1 and cat2), and one was down-regulated (cat3) by drought. Additionally, the enzyme’s activity was consistent with the transcriptomic data.

Curiously, the levels of Catalase 2 and Peroxidase 42 were lower in severe drought conditions as compared to well-watered. Thus, this behavior can be related to the plants growing under stress from the beginning; *i.e.*, a possible disruption is already established between ROS production and antioxidant activity (Fig. 4.23A and C). However, drought was previously reported as having increased levels of ROS-elimination enzymes (Hayano-kanashiro et al., 2009; Lavinsky et al., 2015). This is possible considering that in these studies, plants were first subjected to well-watered conditions, then to drought conditions and recovery, while in our case, plants were subjected to the set water treatments from the beginning up to the end of the experiment. Thus, an acclimation phase and subsequent establishment of new homeostasis were possibly not observed in our case (Kosová et al., 2011; Choudhury et al., 2013). Also, lower levels of the antioxidant enzymes in severe drought conditions were possibly complemented by the non-enzymatic antioxidants such as Ascorbic Acid (vitamin C), α-Tocopherols, glutathione, carotenoids, and phenolic compounds (Ahmad et al., 2010).

The stress-induced protein HSP70 has increased its levels from well-watered to severe drought conditions in all genotypes (Fig. 4.23D). This is probably an indication that since high temperatures often accompany drought, this protein is related to drought tolerance in these genotypes.

Additionally, in most of the studies, an increase in HSP70 level was observed, making the members of the HPS protein family potential good targets for plant stress tolerance improvement (Abreu et al., 2013).

For instance, Hayano-kanashiro et al. (2009) reported that genes encoding HSP17, HSP22, HSP70, and HSP90 were induced in three genotypes under drought. These proteins protect plants by preventing protein aggregation and degradation, as well as assisting in protein refolding. The higher expression of small HSP might be preferentially activated in drought-tolerant maize genotypes in comparison to sensitive ones.

In our study, although Matuba displayed the highest levels of CAT3 and PER42, we found no evident molecular clues regarding ROS-elimination enzymes that would suggest a clear distinction of the Mozambican varieties.

4.5.2.3 PSII and PSI Electron transport chain proteins

The primary light-driven reactions of photosynthesis occur in the thylakoid membranes and are mediated by photosystem II (PSII) and photosystem I (PSI). The combination of the two light reactions in a linear electron transfer chain forms the basis of the Z-scheme (Fig. 4.24). In this scheme, the two light reactions occur in a way that electrons released from water by PSII are transferred through the plastoquinone pool, the cytochrome *b_6f* complex (Cyt*b_6f*), and plastocyanin to PSI and ultimately to ferredoxin and NADP$^+$ to produce NADPH. These electron transfer reactions are combined to proton pumping into the thylakoid lumen, and the resulting proton gradient is used to produce ATP. Following, both ATP and NADPH generated from light reactions are used in the Calvin-Benson cycle for CO_2 fixation and other assimilatory processes (Rochaix, 2011).

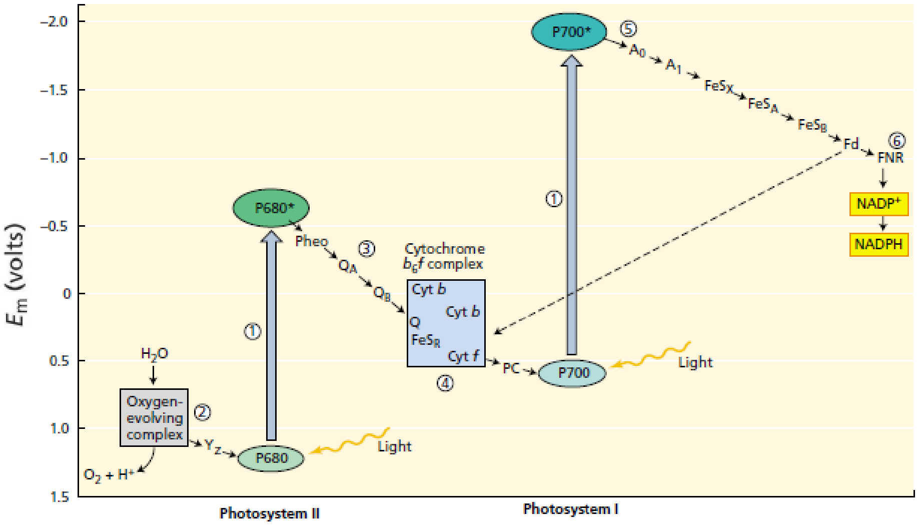

Figure 4.24 – Schematic representation of the Z scheme for O_2-evolving photosynthetic organisms. The photon absorption by the reaction center chlorophyll's is represented by the vertical arrows: P680 for photosystem II (PSII) and P700 for photosystem I (PSI). The P680*, which is the excited PSII reaction center chlorophyll, transfers an electron to pheophytin (Pheo). (2) On the oxidizing part of PSII, P680 oxidized by light is re-reduced by Yz, which has received electrons from the oxidation of water through the activity of the oxygen-evolving complex. (3) On the reducing side of PSII, pheophytin transmits electrons to the acceptors QA and QB, which are plastoquinones. (4)

The cytochrome b6f complex transfers the received electrons to the soluble protein plastocyanin (PC), which reduces P700+ (oxidized P700). (5) The acceptor of electrons from P700* (A0) is thought to be a chlorophyll, and the following acceptor (A1) is a quinone. Then, a series of membrane-bound iron-sulfur proteins (FeSX, FeSA, and FeSB) transfers electrons to soluble ferredoxin (Fd). (6) The soluble flavoprotein ferredoxin–NADP reductase (FNR) reduces NADP+ to NADPH, which is used to fuel the Calvin cycle to reduce CO_2. The dashed line shows the cyclic electron flow around PSI (Taiz & Zeiger, 2002).

The reductions of photosynthesis and activity of the ETR that occurs in drought conditions may result in the reduction of ATP and limitation of the regeneration of RuBP, which is the substrate of RuBisCO. These non-stomatal factors may contribute to a decrease in CO_2 assimilation under severe water stress (Wang et al., 2018).

The PSII, which is frequently mentioned as the engine of life on earth, is the component of the photosynthetic apparatus that is most susceptible to abiotic stresses (Gururani et al., 2015)

In our study, we observed a tendency to increase the levels of PSII proteins from well-watered to severe drought treatments in all genotypes (Fig. 4.25). This behavior might be related to drought tolerance, particularly by protecting PSII from damage. Therefore, we suggest the PSII proteins as putative markers of drought tolerance in these genotypes.

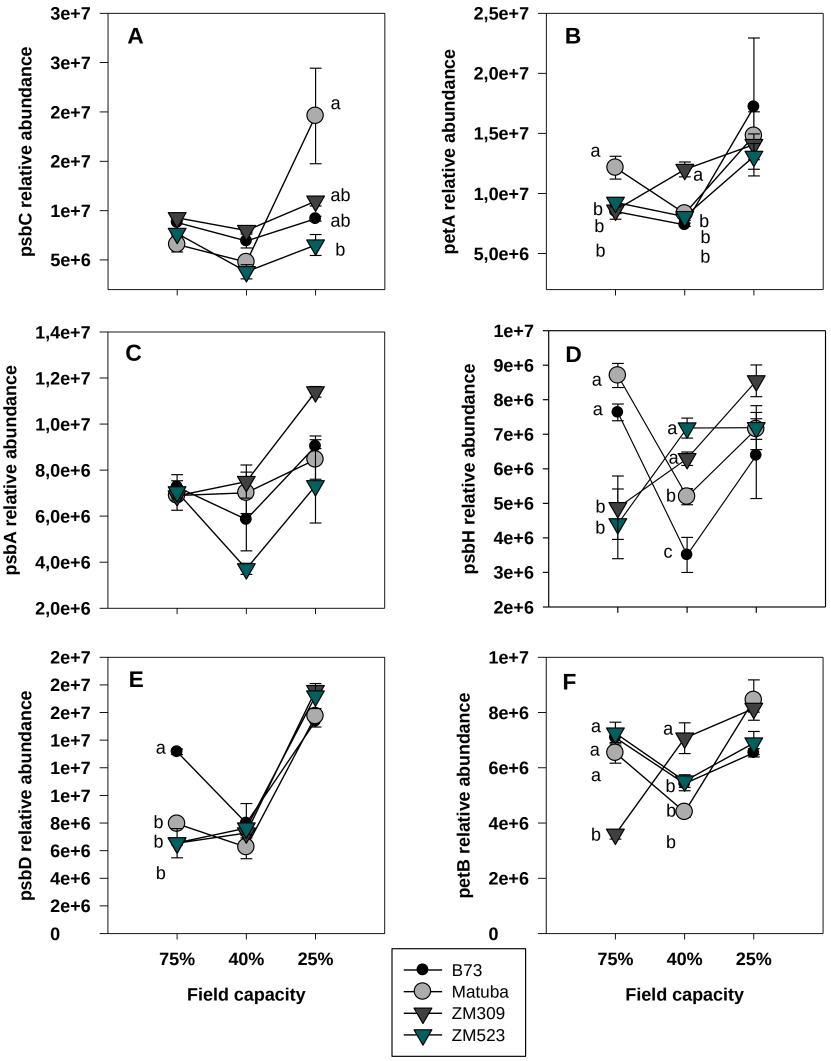

Figure 4.25 - Quantification of total PSII related proteins by SWATH-MS. Photosystem II CP43 reaction center protein (A), Cytochrome f (B), Photosystem II protein D1 (C), Photosystem II reaction center protein H (D), Photosystem II D2 protein (E), Cytochrome b6 (F). The Photosystem II CP43 reaction center protein binds chlorophyll and helps catalyze the primary light-induced photochemical processes of PSII. Cytochrome f and Cytochrome b6 are components of the cytochrome b6-f complex, which mediates electron transfer between photosystem II (PSII) and photosystem I (PSI), cyclic electron flow around PSI, and state transitions. The D1/D2 (PsbA/PsbD) reaction center heterodimer binds P680, the primary electron donor of PSII, as well as several subsequent electron acceptors. The Photosystem II reaction center protein H is one of the components of the core complex of photosystem II (PSII), required for its stability and/or assembly. Values represent the mean ± SE of three biological replicates. Replicates represents a pool of leaves harvested from five plants. Different letters indicate that the value is statistically significant at $p<0.05$ within field capacity.

ZM523 had, in general, the lowest levels of the PSII proteins as compared to ZM309, Matuba, and B73. Not surprisingly, the latter genotypes displayed higher photosynthesis under light intensity variation, which started with a saturation light of 2000 μmol photons m^{-2} s^{-1} (see chapter III for a detailed discussion on light response curves).

Also, the lower levels of D1 protein in ZM523 can be a consequence of higher levels of ROS accumulation due to decreased CO_2 assimilation in severe drought conditions (Fig. 4.25C) (Wang et al., 2018). We also suggested that the photoprotection of the PSII reaction center by xanthophyll that is involved in energy dissipation possibly occurred mainly in ZM309, Matuba, and B73, which in turn, delayed the degradation of the D1 protein (Lavinsky et al., 2015).

As introduced in chapter III, the higher levels of PSII proteins under severe drought treatment do not correlate with Φ_{PSII} levels, which were lower compared to well-watered conditions. In our case, other factors, not just the protein levels, are possibly playing a role in decreasing ETR flow. Thus, damage to the photosynthetic pigments, damage degree of the structure of thylakoid, and the phosphorylation and dephosphorylation of thylakoid membrane proteins might also be regulating the response to drought of the plant photosynthetic machinery. For instance, drought-resistant wheat genotypes were described as presenting higher phosphorylated levels of the light-harvesting complex II (LHCII), and D1 and D2 proteins, as compared to the susceptible ones (Chen et al., 2013; Chen et al., 2017).

In this same trend, a previous study reported transcripts encoding proteins for PSI and PSII and light reactions being higher under moderate and severe drought conditions (Avramova et al., 2020). This was also unexpected, given the growth inhibition and also the other studies showing downregulation of the photosynthetic activity and inhibited expression of photosynthesis genes under drought. Thus, they demonstrated that leaves developing under drought conditions increase the photosynthetic machinery to maximize photosynthesis in recovery, when the stomata are allowed to open. Also, the higher levels of PSII proteins suggests the need for enhancing its repair cycle and avoid the disruption of PSII assembly and its function in water-scarcity conditions (Gururani et al., 2015).

Shan et al. (2018), also observed an increase in PSII and PSI proteins in a different species, cassava (*Manihot esculenta* Crantz), and concluded that it was beneficial to photosystem repair and protection of the reaction centers and kept photosynthetic electron transport chain function in long-term drought resistance.

Drought tolerant maize genotypes were also described as reducing electron transport between PSII and PSI, probably to decrease the effect of photooxidation and to avoid spending energy and resources in conditions of low CO_2 availability as well as preventing the production of huge amounts of ROS (Hayano-kanashiro et al., 2009).

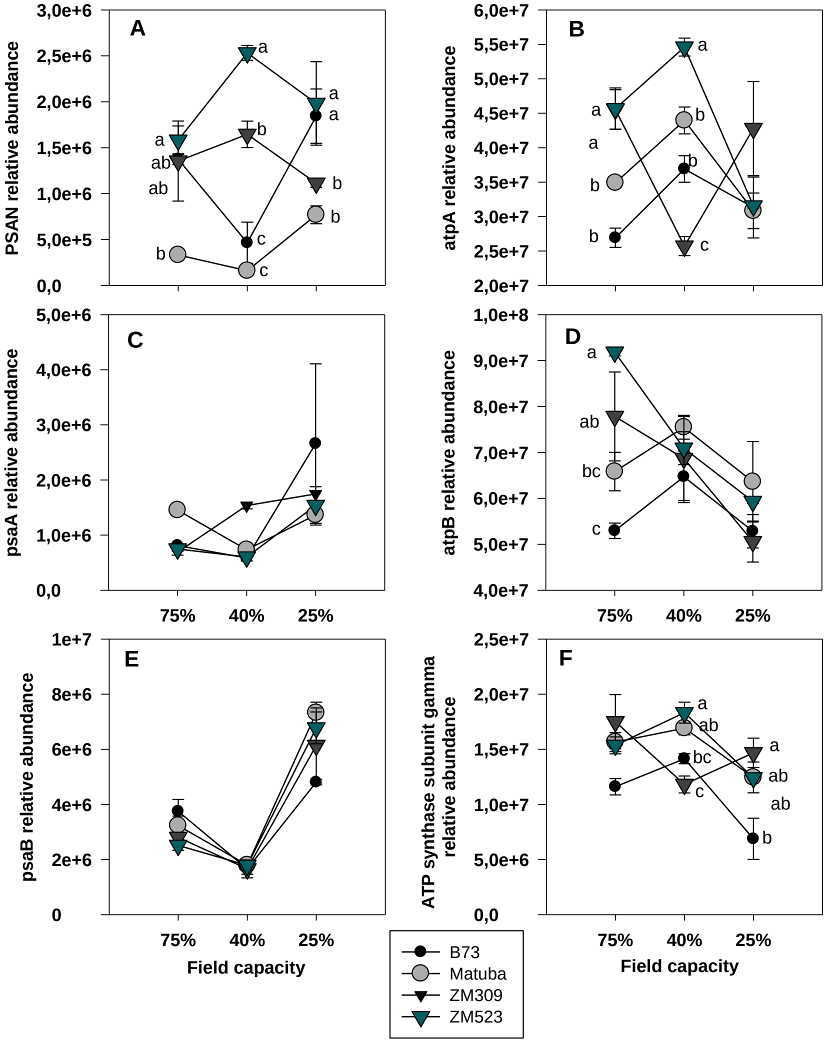

Figure 4.26 - Quantification of total PSI related proteins by SWATH-MS. Photosystem I reaction center subunit N (A), ATP synthase subunit alpha (B), Photosystem I P700 chlorophyll *a* apoprotein A1 (C), ATP synthase subunit beta (D), Photosystem I P700 chlorophyll *a* apoprotein A2 (E), ATP synthase subunit gamma (F). Photosystem I reaction center subunit N may function in mediating the binding of the antenna complexes to the PSI reaction center and core antenna. The Photosystem I P700 chlorophyll *a* apoprotein A1, and A2 binds P700, the primary electron donor of photosystem I (PSI), as well as the electron acceptors A0, A1 and FX. ATP synthase produces ATP from ADP in the presence of a proton gradient across the membrane. The ATP alpha chain is a regulatory subunit; the catalytic sites are hosted primarily by the beta subunits; the gamma chain is believed to be important in regulating ATPase activity and the flow of protons through the CF_0 complex. Values represent the mean±SE of three biological replicates. Replicates represents a pool of leaves harvested from five plants. Different letters indicate that the value is statistically significant at $p<0.05$, within field capacity.

We observed the levels of the subunits alpha, beta, and gamma that regulate the activity of ATP synthase are slightly repressed under severe water limitations compared to control conditions (Fig. 4.26B, D, F). An opposite trend is shown by the components that regulate the primary electron donor of PSI that, in general, tended to increase with water restrictions (Fig. 4.26 A, C, E). The lower levels of the ATP synthase subunits under severe drought are consistent with the reduced photosynthetic CO_2 assimilation observed (see details of the physiological characterization in chapter III).

The ATP synthase in chloroplast plays an important role in regulating electron flow. For example, enhanced activity of ATP synthase can lead to excess electron flow from PSII to PSI, which results in the production of ROS) within PSI. Contrary, when the activity of ATP synthase is strongly repressed, assimilation capacity and linear electron flow are highly reduced due to over-acidification of the thylakoid lumen. Thus, it can be concluded that the activity of chloroplastic ATP synthase is modulated by the CO_2 assimilation rate. Under low CO_2 concentrations, the activity of ATP synthase decreases to optimize the formation of proton motive force (*pmf*) and lumen acidification, which is crucial for energy dissipation at PSII to avoid ROS formation via non-enzymatic processes (Rott et al., 2011; Huang et al., 2018).

As described earlier, we observed slightly higher net photosynthesis and stomatal conductance in Matuba under severe drought compared to ZM309 and ZM523. Also, Matuba did perform higher levels of ROS detoxification enzymes PER42 and CAT 3 in severe drought as well as of the PEPC and NADP-MDH in moderate drought. Although the levels of PSII proteins increased with drought in all genotypes, ZM523 had tendentially the lowest levels of the PSII proteins. Thus, our proteomic data indicate slight differences among Mozambican varieties and suggest Matuba as possibly the most tolerant variety, but this is not entirely conclusive. Thus, the different source material used for the generation of the Mozambican varieties possibly conveyed to the different molecular and physiological profiles. However, previous descriptions put improved open-pollinated varieties ZM309 and ZM523 as the best choices for water scarcity environments. This categorization would be because Matuba was generated just through landraces (or local varieties), while for ZM309 and ZM523, landraces and germplasm from CIMMYT were used. Therefore, based on our results, we can conclude that the local maize genotypes can be considered as good source material to generate drought tolerant genotypes.

Nevertheless, there are other studies also suggesting the respective local varieties as a good source of drought tolerance traits. For instance, Mazvimbakupa et al. (2015), in South Africa, evaluated seed quality and water use characteristics of two maize landraces (GQ1 and GQ2) compared with two commercial hybrids (SC701 and PAN53). Although seed quality tests showed that hybrids had superior seed quality than landraces, the GQ2 landrace continued to perform similar to, and mostly better than the hybrid varieties, particularly under drought conditions but not GQ1 variety. This reinforced that landraces should be considered for production in drought-prone areas because of their potential to contribute to food security.

Additionally, wheat (*Triticum aestivum* L.) landraces from the Western Balkans were also considered as a potential genetic resource for drought resistance in wheat breeding. The average yield of landraces was significantly lower than that of improved varieties in both well-watered and drought conditions, and there was no evidence that yield stability of landraces was better than that of accessions. However, stress tolerance was higher in landraces than in improved varieties (Dejan et al., 2012).

Additionally, improved varieties are, in general, bred for high yield levels and are often genetically homogeneous. Consequently, there is an excess effort on obtaining high-yield, and less attention to stress tolerance traits is driven. However, local landraces are, in many cases, better adapted to local agro-ecological conditions and do not need much of agrochemical resource input as compared to improved varieties. Also, small-scale farmers typically prefer local landraces due to cultural habits such as the ones related to family traditions and cooking characteristics for traditional foods (You-zhi et al., 2012; Ficiciyan et al., 2018).

4.5.3 Conclusion and recommendations for future studies

The present molecular characterization focused on the levels of enzymes related to CO_2 assimilation, ETR, and ROS elimination in response to different water supply regimes. These enzymes were differentially expressed among genotypes within treatments, suggesting different impacts of drought and strategies to cope with it.

We concluded that under severe drought treatment, the levels of C4-photosynthesis enzymes were slightly repressed as compared to control conditions. The drop in the levels of the RuBP carboxylation proteins and ATP synthase subunits match the lower CO_2 assimilation levels.

We observed that one of the general responses to drought was the increase in the levels of PSII proteins. This might be related to the role of PSII proteins to protect and repair PSII. Among genotypes, the ZM523 tendency was to display the lowest levels of PSII and ETR proteins. This may suggest a more diminished capacity to protect and repair the PSII, in one hand, but may also represent a strategy of energy conservation in low water availability and low CO_2 assimilation.

The accumulation of the ROS-elimination enzymes was suggested to be more important in Matuba than in the other Mozambican varieties. However, more studies, for example targeting the activity of the enzymes, are needed to clarify this matter.

Additionally, changes in the contents of osmoprotectants such as proline would also give us more insights about drought tolerance features in these varieties. Finally, more studies involving stress and recovery are recommended as they could better mimic the actual scenario in field conditions.

The next steps in this work will include the use of alternative statistical tools further to extract information from our data, namely multivariate analysis. The objective will be better exploring the interactions among different factors (for example, genotype *vs.* treatment). This type of analysis will also be applied to physiological and agronomic data. Globally, the present and future analyses will allow us better to discriminate our genotype's response to different water treatments.

Acknowledgments

Training in sample preparation for MS analysis: Inês Matias Luis (Proteome Regulation in Plants Lab, ITQB NOVA)

Assistance in MS data acquisition and analysis: Dr. Bruno Alexandre (iBET)

Supplementary Data
Supplemental figures

Figure S4.1- Principal Component Analysis of the four maize genotypes, aggregating all SWATH's, all treatments, and the entire set of 221 proteins detected.

Figure S4.2 - Principal Component Analysis of the four maize genotypes, aggregating all SWATH's under moderate drought treatment using the entire set of 221 proteins detected.

Figure S4.3 - Principal Component Analysis of the four maize genotypes, aggregating all SWATH's for the severe stress treatment and the entire set of proteins detected.

Figure S4.4 - Principal Component Analysis of the SWATH's of the four maize genotypes, aggregating all 12 treatments and the CO_2 assimilation related enzymes.

Figure S4.5 - Principal Component Analysis of the SWATH's of four maize genotypes under the severe drought treatment, aggregating the CO_2 assimilation related enzymes.

Figure S4.6 - Principal Component Analysis of the SWATH's of four maize genotypes under the moderate stress treatment, aggregating the CO_2 assimilation related enzymes.

Figure S4.7 - Principal Component Analysis of the SWATH's of four maize genotypes under control conditions, aggregating the CO_2 assimilation related enzymes.

Figure S4.8 - Principal Component Analysis of the SWATH's of the four maize genotypes aggregating all 12 treatments and the electron transport chain related enzymes.

Figure S4.9 - Principal Component Analysis of SWATH's of four maize genotypes under severe drought conditions, aggregating the electron transport chain-related proteins.

Figure S4.10 - Principal Component Analysis of SWATH's of four maize genotypes under moderate drought treatment, aggregating the electron transport chain-related proteins.

Figure S4.11 - Principal Component Analysis of B73 SWATH's under all water treatments, aggregating the electron transport chain-related proteins.

Figure S4.12 - Principal Component Analysis of Matuba SWATH's under all water treatments, aggregating the electron transport chain-related proteins.

Figure S4.13 - Principal Component Analysis of ZM309 SWATH's under all water treatments, aggregating the electron transport chain-related proteins.

Figure S4.14 - Principal Component Analysis of ZM523 SWATH's under all water treatments, aggregating the electron transport chain-related proteins.

Figure S4.15 - Principal Component Analysis of the SWATH's of the four maize genotypes, aggregating all 12 treatments and the ROS elimination-related enzymes.

Figure S4.16 - Principal Component Analysis of SWATH's of four maize genotypes under severe drought conditions aggregating the ROS elimination related-proteins.

Figure S4.17 - Principal Component Analysis of SWATH's of four maize genotypes under moderate stress treatment, aggregating the ROS elimination-related proteins.

Figure S4.18 - Principal Component Analysis of SWATH's of four maize genotypes under control conditions aggregating the ROS elimination-related proteins.

Figure S4.19 - Principal Component Analysis for B73 SWATH's under all water treatments, aggregating under all water treatments using ROS elimination-related enzymes.

Figure S4.20 - Principal Component Analysis for Matuba SWATH's under all water treatments, aggregating under all water treatments using ROS elimination-related enzymes.

Figure S4.21 - Principal Component Analysis for ZM309 SWATH's under all water treatments, aggregating under all water treatments using ROS elimination-related enzymes.

Figure S4.22 - Principal Component Analysis for ZM523 SWATH's under all water treatments, aggregating under all water treatments using ROS elimination-related enzymes.

References

Abid, M., Ali, S., Qi, L. K., Zahoor, R., Tian, Z., Jiang, D., Dai, T. (2018). Physiological And Biochemical Changes During Drought and Recovery Periods at Tillering and Jointing Stages in Wheat (*Triticum aestivum* L.). *Scientific Reports*, *8*(April 2017), 1–15. https://doi.org/10.1038/s41598-018-21441-7

Abreu, I. A., Farinha, A. P., Negrão, S., Gonçalves, N., Fonseca, C., Rodrigues, M., Batista, R., Saibo, N. J. M., Oliveira, M. M. (2013). Coping With Abiotic Stress: Proteome Changes for Crop Improvement. *Journal of Proteomics*, *93*, 145–168. https://doi.org/10.1016/j.jprot.2013.07.014

Aebersold, R., & Mann, M. (2016). Mass-Spectrometric Exploration of Proteome Structure and Function. https://doi.org/10.1038/nature19949

Ahmad, P., Jaleel, C. A., Salem, M. A., Nabi, G., Ahmad, P., Jaleel, C. A., Sharma, S. (2010). Roles of Enzymatic and Nonenzymatic Antioxidants in Plants During Abiotic Stress Roles of Enzymatic and Nonenzymatic Antioxidants in Plants During Abiotic Stress. *Critical Reviews in Biotechnology*, *8551*, 161–175. https://doi.org/10.3109/07388550903524243

Andersson, I. (2008). Catalysis and regulation in Rubisco. *59*(7), 1555–1568. https://doi.org/10.1093/jxb/ern091

Avramova, V., Abdelgawad, H., Zhang, Z., Fotschki, B., Casadevall, R., Vergauwen, L., Beemster, G. T. S. (2020). Drought Induces Distinct Growth Response, Protection, and Recovery Mechanisms in the Maize. *Plant Physiology*, *169*(October 2015), 1382–1396. https://doi.org/10.1104/pp.15.00276

Benešová, M., Hola, D., Benes, M., Procha, D., Honnerova, J., & Hnilic, H. (2012). The Physiology and Proteomics of Drought Tolerance in Maize: Early Stomatal Closure as a Cause of Lower Tolerance to Short-Term Dehydration? *PLos One*, *7*(6). https://doi.org/10.1371/journal.pone.0038017

Cacefo, V., Ribas, A. F., Zilliani, R. R., Neris, D. M., Domingues, D. S., Moro, A. L., Vieira, E. (2019). Decarboxylation mechanisms of C4 photosynthesis in Saccharum spp.: Increased PEPCK Activity under Water-limiting Conditions. *BMC Plant Biology*, *19*, 1–14.

Chandramouli, K., & Qian, P. (2009). Proteomics: Challenges, Techniques and Possibilities to Overcome Biological Sample Complexity. https://doi.org/10.4061/2009/239204

Chen, Y., Cui, J., Su, Y., Zhan, C., Jie, M., & Zhan, Z. (2017). Comparison Of Phosphorylation and Assembly of Photosystem Complexes and Redox Homeostasis in Two Wheat Cultivars With Different Drought Resistance.

Scientific Reports, *7*(April 2016), 1–16. https://doi.org/10.1038/s41598-017-13145-1

Chen, Y., Zhao, Z., Zhang, H., Zeng, X., & Yuan, S. (2013). The Significance of CP29 Reversible Phosphorylation in Thylakoids of Higher Plants under Environmental Stresses. *Journal of Experimental Botany*, *64*(5), 1167–1178. https://doi.org/10.1093/jxb/ert002

Choudhury, S., Panda, P., Sahoo, L., Panda, S. K., Choudhury, S., Panda, P., … Panda, S. K. (2013). Reactive Oxygen Species Signaling in Plants under Abiotic Stress Reactive Oxygen Species Signaling in Plants under Abiotic Stress. *Plant Signaling and Behaviour*, *2324*. https://doi.org/10.4161/psb.23681

Christin, P. A. & Osborne, C. P. (2014). The Evolutionary Ecology of C4 Plants. *New Phytologist*, *204*, 765–781.

Dass, C. (2007). Fundamentals of Contemporary. New Jersey: *Wiley*.

Dejan, D., Zorić, M., Kandić, V., Perović, D., & Urlan-Momirović, G. (2012). Comparison of Responses to Drought Stress of 100 Wheat Accessions and Landraces to Identify Opportunities for Improving Wheat Drought Resistance. *Plant Breeding*, *131*, 369–379. https://doi.org/10.1111/j.1439-0523.2011.01941.x

Doubnerová, V., & Ryšlavá, H. (2011). Plant Science What Can Enzymes of C4 Photosynthesis Do for C3 Plants under Stress? *Plant Science*, *180*, 575–583. https://doi.org/10.1016/j.plantsci.2010.12.005

Erde, J., Loo, R. R. O., & Loo, J. A. (2014). Enhanced FASP (eFASP) to Increase Proteome Coverage and Sample Recovery for Quantitative Proteomic Experiments. *Journal of Proteome Research*, *13*, 1885–1895. https://doi.org/10.1021/pr4010019

Ficiciyan, A., Loos, J., Sievers-glotzbach, S., & Tscharntke, T. (2018). More than Yield: Ecosystem Services of Traditional versus Modern Crop Varieties Revisited. *Sustainability*, *10*, 1–15. https://doi.org/10.3390/su10082834

Gururani, M. A., Venkatesh, J., & Tran, L. P. (2015). Regulation of Photosynthesis During Abiotic. *Molecular Plant*, *8*(9), 1304–1320. https://doi.org/10.1016/j.molp.2015.05.005

Hayano-kanashiro, C., Ibarra-laclette, E., Herrera-estrella, L., Caldero, C., & Simpson, J. (2009). Analysis of Gene Expression and Physiological Responses in Three Mexican Maize Landraces under Drought Stress and Recovery Irrigation. *PLos One*, *4*(10). https://doi.org/10.1371/journal.pone.0007531

Hein, M. Y. (2014). A Human Interactome. *Universität München*.

Hofius, D., & Börnke, F. A. J. (2007). Photosynthesis, Carbohydrate Metabolism and Source – Sink Relations.

Huang, W., Tikkanen, M., Cai, Y., Wang, J., & Zhang, S. (2018). BBA - Bioenergetics Chloroplastic ATP Synthase Optimizes the Trade-off

Between Photosynthetic CO_2 Assimilation and Photoprotection During Leaf Maturation. *BBA - Bioenergetics*, *1859*(10), 1067–1074. https://doi.org/10.1016/j.bbabio.2018.06.009

Hunter, C., Morrice, N., & Dojahn, J. (2012). Evolution of SWATH ® Acquisition Provides Large Gains in Quantified Proteins.

Hỳsková, V. D., Miedzi, L., Dobrá, J., Vankova, R., & Ryšlavá, H. (2014). Pyruvate, Phosphate Dikinase are Involved in the Acclimation of *Nicotiana tabacum* L. to Drought Stress. *Journal of Plant Physiology*, *171*, 19–25. https://doi.org/10.1016/j.jplph.2013.10.017

Jaspers, P., & Kangasjärvi, J. (2010). Reactive Oxygen Species in Abiotic Stress Signaling. *Physiologia Plantarum*, *138*, 405–413. https://doi.org/10.1111/j.1399-3054.2009.01321.x

Kellogg, E. A. (2013). C4 Photosynthesis. *Current Biology*, *23*(14), 594–599. https://doi.org/10.1016/j.cub.2013.04.066

Kosová, K., Vítámvás, P., Tom, I., & Renaut, J. (2011). Plant Proteome Changes under Abiotic Stress — Contribution of Proteomics Studies to Understanding Plant Stress Response. *Journal of Proteomics*, *4*, 1301–1322. https://doi.org/10.1016/j.jprot.2011.02.006

Krasny, L., Bland, P., Kogata, N., Wai, P., Howard, B. A., Natrajan, R. C., & Huang, P. H. (2018). SWATH Mass Spectrometry as a Tool for Quantitative Profiling of the Matrisome. *Journal of Proteomics*, *189*(January), 11–22. https://doi.org/10.1016/j.jprot.2018.02.026

Lavinsky, A. O., Magalhães, P. C., Ávila, R., Gomes-jr, C. C., & Carneiro, N. P. (2015a). Advances in Crop Science and Technology Analysis of Maize Photosyntheis Parameters and Whole Plant Oxidative Damage under Long-term Drought. *Adv Crop Sci Tech*, 1–6. https://doi.org/10.4172/2329-8863.S1-007

Li, J., Cang, Z., Jiao, F., Bai, X., Zhang, D., & Zhai, R. (2015). Influence of Drought Stress on Photosynthetic Characteristics and Protective Enzymes of Potato at Seedling Stage. *Journal of the Saudi Society of Agricultural Sciences*, *16*(1), 82–88. https://doi.org/10.1016/j.jssas.2015.03.001

Liu, X., Li, X., Dai, C., Zhou, J., Yan, T., & Yan, J. (2017). Improved Short-Term Drought Response of Transgenic Rice Over-Expressing Maize C4 Phosphoenolpyruvate Carboxylase Via Calcium Signal Cascade. *Journal of Plant Physiology*. https://doi.org/10.1016/j.jplph.2017.08.005

Liu, Z., Sun, N., Yang, S., Zhao, Y., Wang, X., Hao, X., & Qiao, Z. (2013). Evolutionary Transition From C3 to C4 Photosynthesis and the Route to C4 Rice. *Biologia*, *68*, 577–586. https://doi.org/10.2478/s11756-013-0191-5

Luis, I., Alexandre, B. M., Oliveira, M. M., & Abreu, I. A. (2016). Selection of an Appropriate Protein Extraction Method to Study the Phosphoproteome of Maize Photosynthetic Tissue. *PLos One*, (October), 1–16. https://doi.org/10.1371/journal.pone.0164387

Matsuoka, M., Furbank, R. T., Fukayama, H., Miyao, M. (2001). Molecular Engineering of C4 Photosynthesis. *Annu. Rev. Plant Physiol. Plant Mol. Biol*, 297–314.

Mazvimbakupa, F., Modi, A. T., & Mabhaudhi, T. (2015). Seed Quality and Water Use Characteristics of Maize Landraces Compared With Selected Commercial Hybrids. *Chillean Journal of Agriculture Research*, *75*(March), 13–20. https://doi.org/10.4067/S0718-58392015000100002

Mishra, N. (2010). Introduction to Proteomics: Principles and Applications. Hoboken, New Jersey: John Wiley & Sons, Inc.

Munekage, Y. N. (2016). Light Harvesting and Chloroplast Electron Transport in NADP-Malic Enzyme Type C4 plants. *Current Opinion in Plant Biology*, *31*, 9–15. https://doi.org/10.1016/j.pbi.2016.03.001

Niessen, W. M. A. (2006). Liquid Chromatography – Mass Spectrometry (3rd ed.). New York: *Taylor and Francis*.

Niessen, W. M. A., & Falck, D. (2015). Introduction to Mass Spectrometry, a Tutorial. (i), 1–54.

Osborne, C. P., & Sack, L. (2012). Evolution of C 4 plants: a New Hypothesis For an Interaction of CO_2 and Water Relations Mediated by Plant Hydraulics. *Philosophical Transactions*, 583–600. https://doi.org/10.1098/rstb.2011.0261

Paulus, J. K., Schlieper, D., & Groth, G. (2013). Greater Efficiency of Photosynthetic Carbon Fixation Due to Single Amino-Acid Substitution. *Nature Communications*, 4–10. https://doi.org/10.1038/ncomms2504

Perdomo, J. A., Capó-bauçà, S., Carmo-Silva, E., & Galmés, J. (2017). Rubisco and Rubisco Activase Play an Important Role in the Biochemical Limitations of Photosynthesis in Rice, Wheat, and Maize under High Temperature and Water Deficit. *Frontiers in Plant Science*, *8*(April), 1–15. https://doi.org/10.3389/fpls.2017.00490

Pick, T., Denton, A. K., Colmsee, C., & Scholz, U. (2011). Systems Analysis of a Maize Leaf Developmental Gradient Redefines the Current C4 Model and Provides Candidates for Regulation. *The Plant Cell*, *23*(December), 4208–4220. https://doi.org/10.1105/tpc.111.090324

Pitt, J. J. (2009). Principles and Applications of Liquid Chromatography- Mass Spectrometry in Clinical Biochemistry. *30*(February), 19–34.

Rao, X., & Dixon, R. A. (2016). The Differences Between NAD-ME and NADP-ME Subtypes of C4 Photosynthesis: More than Decarboxylating Enzymes. *Frontiers in Plant Science*, *7*(October), 1–9. https://doi.org/10.3389/fpls.2016.01525

Rochaix, J. (2011). Biochimica et Biophysica Acta Regulation of Photosynthetic Electron Transport. *BBA - Bioenergetics*, *1807*(3), 375–383. https://doi.org/10.1016/j.bbabio.2010.11.010

Rott, M., Martins, F., Thiele, W., Lein, W., Bock, R., Kramer, D. M., Scho, M. A. (2011). ATP Synthase Repression in Tobacco Restricts

Photosynthetic Electron Transport, CO_2 Assimilation, and Plant Growth by Overacidification of the Thylakoid Lumen. *Plant Cell*, *23*(January), 304–321. https://doi.org/10.1105/tpc.110.079111

Sage, R. F., Sage, T. L., & Kocacinar, F. (2012). Photorespiration and the Evolution of C4 Photosynthesis. *Annu. Rev. Plant Biol*, *63*, 19–47. https://doi.org/10.1146/annurev-arplant-042811-105511

Shan, Z., Luo, X., Wei, M., Huang, T., Khan, A., & Zhu, Y. (2018). Physiological and Proteomic Analysis on Long-term Drought Resistance of Cassava (*Manihot esculenta* Crantz). *Scientific Reports*, *8*(November), 1–12. https://doi.org/10.1038/s41598-018-35711-x

Spreitzer, R. J. (2003). Role of the Small Subunit in Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase. *Archives of Biochemistry and Biophysics*, *414*, 141–149. https://doi.org/10.1016/S0003-9861(03)00171-1

Sugawara, H., Yamamoto, H., Shibata, N., Inoue, T., Okada, S., Miyake, C., Kai, Y. (1999). Crystal Structure of Carboxylase Reaction-oriented Ribulose 1, 5-Bisphosphate Carboxylase/Oxygenase from a Thermophilic Red Alga, *Galdieria partita*. *The Journal Of Biological Chemistry*, *274*(22), 15655–15661.

Taiz, L., & Zeiger, E. (2002). *Plant Physiology* (3rd ed.; T. Lazar, Ed.). Sinauer Associates.

Tikkanen, M., Silveira, J. A. G., & Aro, E. (2019). Consequences of Photosystem-I Damage and Repair on Photosynthesis and Carbon Use in *Arabidopsis thaliana*. *The Plant Journal*, https://doi.org/10.1111/tpj.14177

Wang, N., Zhong, X., Cong, Y., Wang, T., Yang, S., Li, Y., & Gai, J. (2016). Genome-wide Analysis of Phospho enol pyruvate Carboxylase Gene Family and Their Response to Abiotic Stresses in Soybean. *Nature Publishing Group*, (December), 1–14. https://doi.org/10.1038/srep38448

Wang, Z., Li, G., Sun, H., Ma, L., Guo, Y., Zhao, Z., Mei, L. (2018). Effects of Drought Stress on Photosynthesis and Photosynthetic Electron Transport Chain in Young Apple Tree Leaves. https://doi.org/10.1242/bio.035279

Wilson, K., & Walker, J. (2010). Principles and Techniques of Biochemistry and Molecular Biology (7th ed.). Cambridge: *Cambridge University Press*.

Wiśniewski, J. R. (2017). Filter-Aided Sample Preparation: The Versatile and Efficient Method for Proteomic Analysis. *Methods in Enzymology*, *585*, 15–28. https://doi.org/10.1016/bs.mie.2016.09.013

Wiśniewski, J., Zielinska, D., & Mann, M. (2011). Comparison of Ultrafiltration Units for Proteomic and N-glycoproteomic Analysis by the Filter-Aided Sample Preparation Method. *Analytical Biochemistry*, *410*, 307–309.

Wu, J. X., Song, X., Pascovici, D., Zaw, T., Care, N., Krisp, C., & Molloy, M.

P. (2016). SWATH Mass Spectrometry Performance Using Extended Peptide MS/MS Assay Libraries. *Molecular and Cellular Proteomics*, *15*, 2501–2514. https://doi.org/10.1074/mcp.M115.055558

You-zhi, L. I., Xian-wei, F. A. N., & Jiang-xiong, L. (2012). *Foci* of Future Studies on Abiotic Stress Tolerance of Maize in the Era of Post-Genomics. *Journal of Integrative Agriculture*, *11*(8), 1236–1244. https://doi.org/10.1016/S2095-3119(12)60120-8

You, J., Wang, L., Saji, M., Olesik, S. V, Ringel, M. D., David, M., Freitas, M. A. (2011). High-Sensitivity TFA-free LC-MS for Profiling Histones Jia. *Proteomics*, *11*(16), 3326–3334. https://doi.org/10.1002/pmic.201000445.High-Sensitivity

Supplemental figures

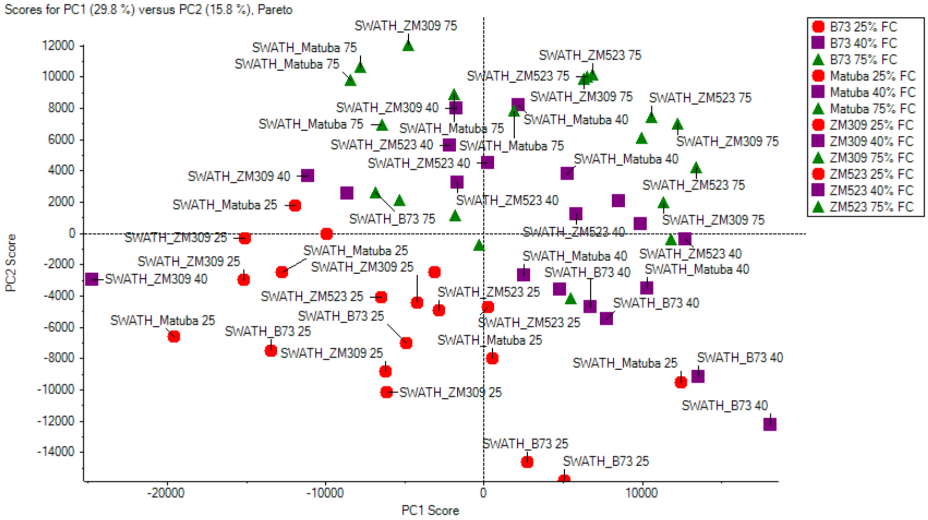

Figure S4.1- Principal Component Analysis of the four maize genotypes, aggregating SWATH's of all 12 treatments, and the entire set of 221 proteins detected. Different water treatments are identified by different colors and symbols.

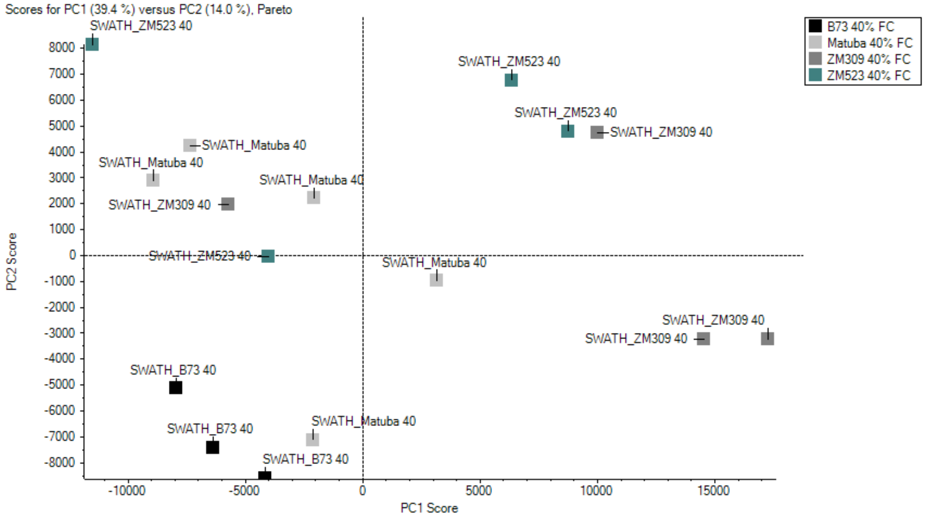

Figure S4.2 - Principal Component Analysis of the four maize genotypes, aggregating all SWATH's under moderate drought treatment using the entire set of 221 proteins detected. Genotypes are identified by different colors.

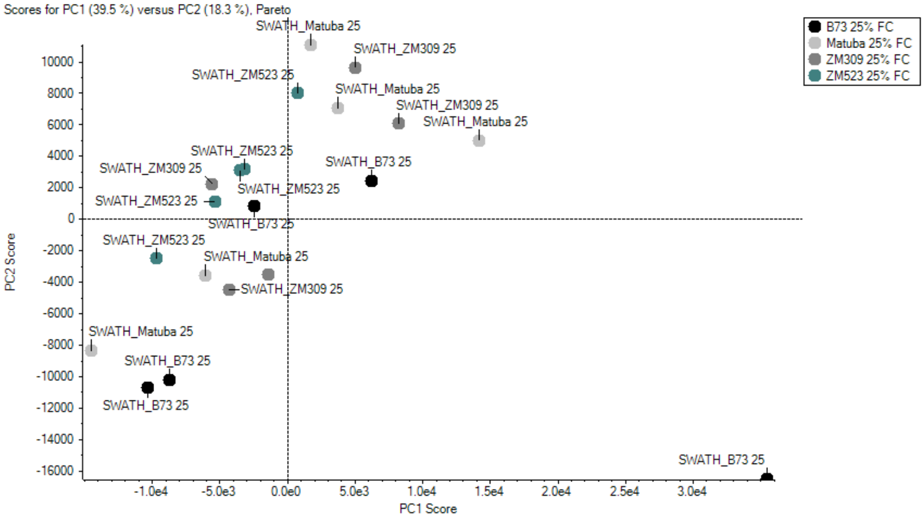

Figure S4.3 - Principal Component Analysis of the four maize genotypes, aggregating all SWATH's for the severe stress treatment and the entire set of proteins detected. Genotypes are identified by different colors.

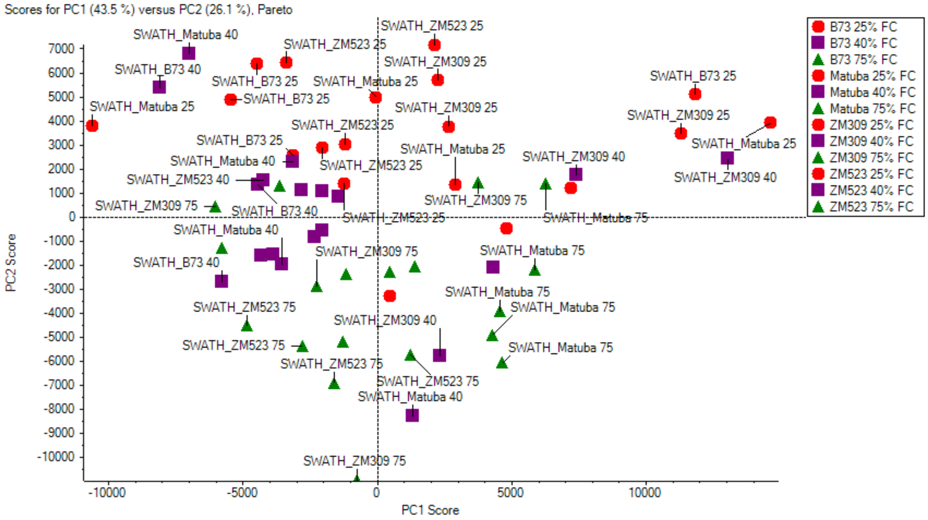

Figure S4.4 - Principal Component Analysis of the SWATH's of the four maize genotypes, aggregating all CO_2 assimilation-related enzymes. Different water treatments are identified by different colors and symbols.

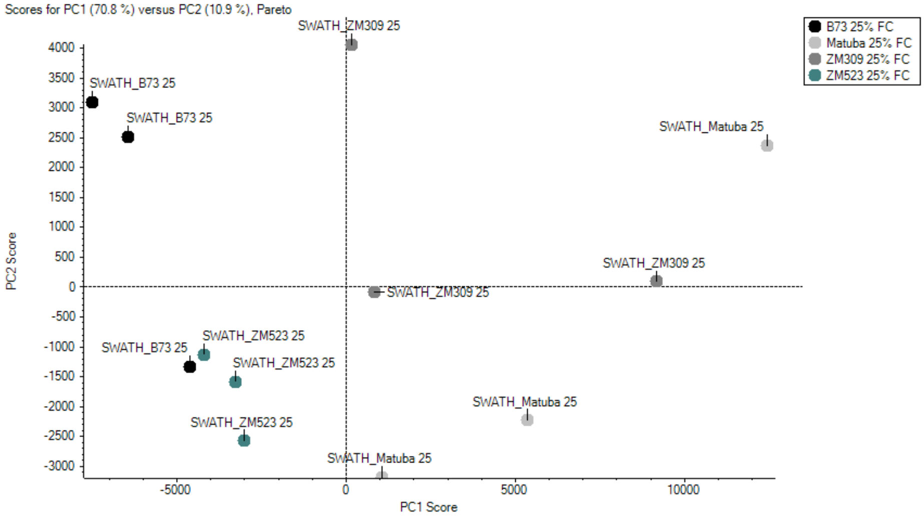

Figure S4.5 - Principal Component Analysis of the SWATH's of four maize genotypes under the severe drought treatment, aggregating the CO_2 assimilation-related enzymes. Genotypes are identified by different colors.

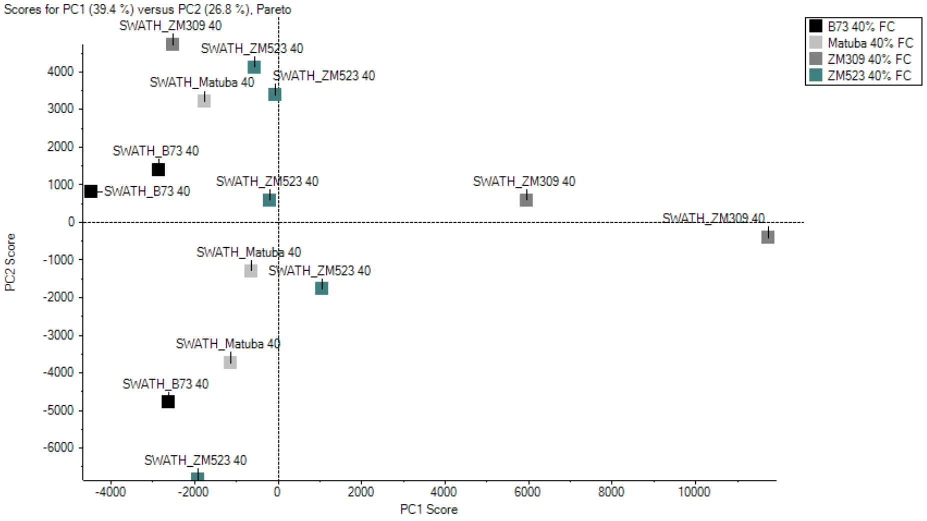

Figure S4.6 - Principal Component Analysis of the SWATH's of four maize genotypes under the moderate stress treatment, aggregating the CO_2 assimilation-related enzymes. Genotypes are identified by different colors.

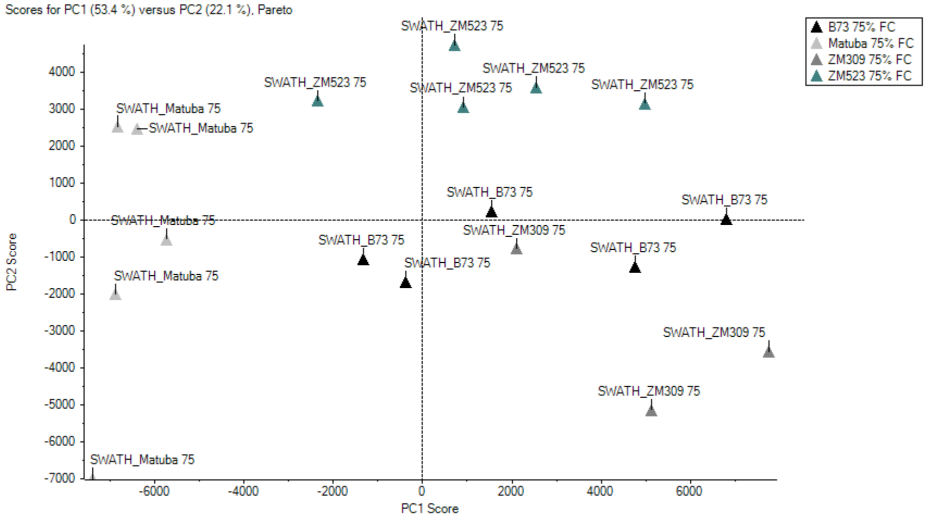

Figure S4.7 - Principal Component Analysis of the SWATH's of four maize genotypes under control conditions, aggregating the CO_2 assimilation-related enzymes. Genotypes are identified by different colors.

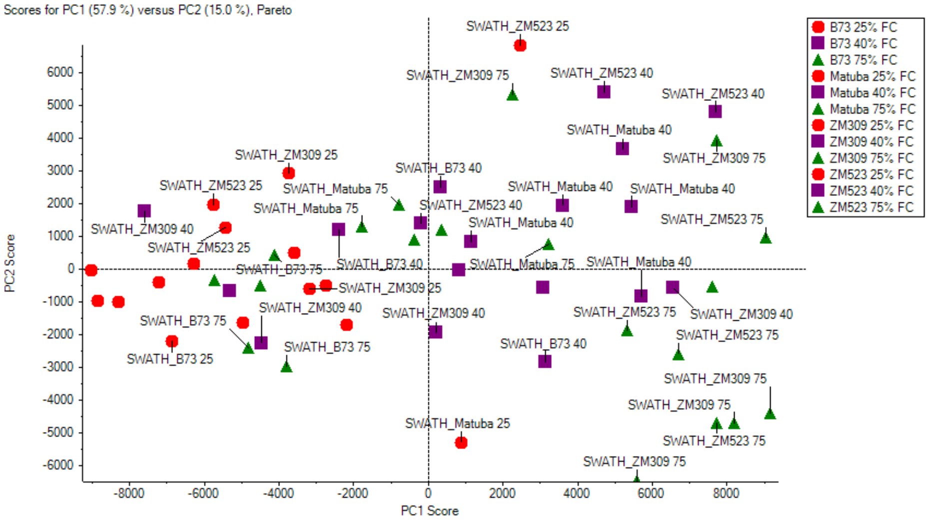

Figure S4.8 - Principal Component Analysis of the SWATH's of the four maize genotypes, aggregating all 12 treatments and the electron transport chain related-enzymes. Different water treatments are identified by different colors and symbols.

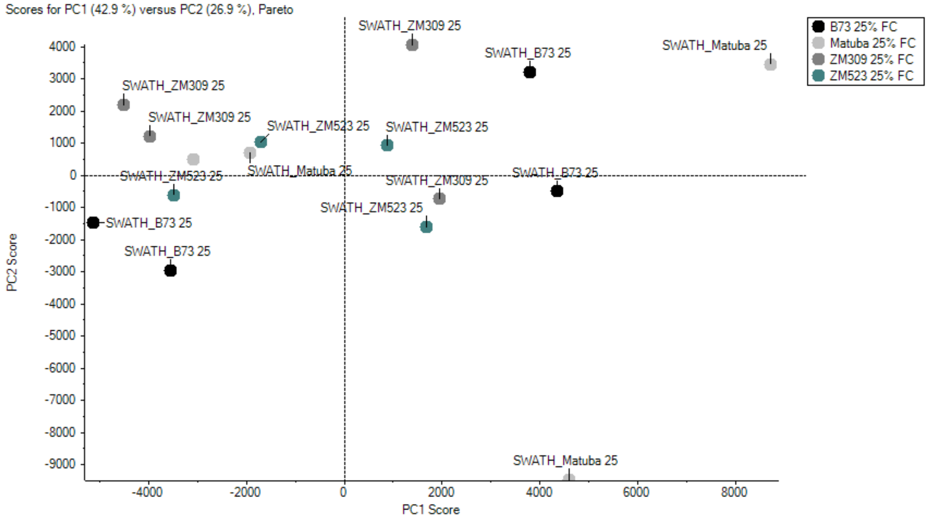

Figure S4.9 - Principal Component Analysis of SWATH's of four maize genotypes under severe drought conditions, aggregating the electron transport chain-related proteins. Genotypes are identified by different colors.

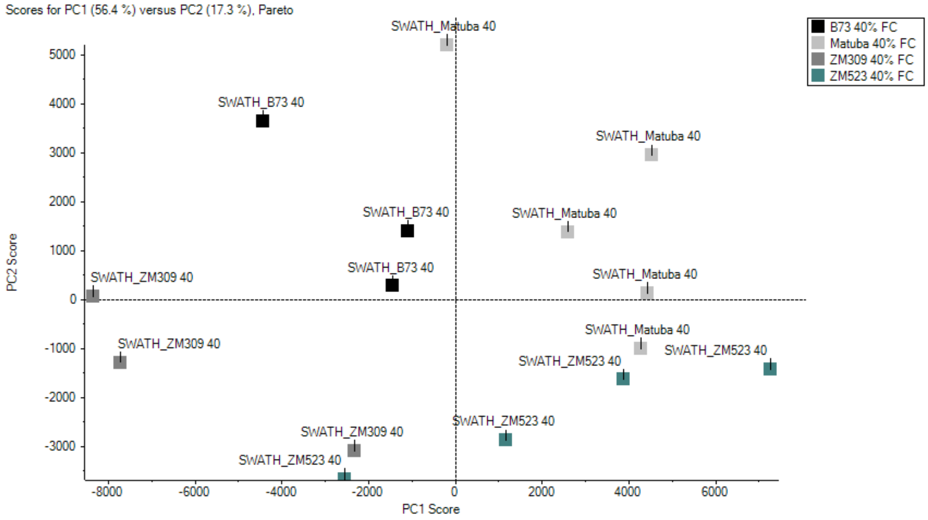

Figure S4.10 - Principal Component Analysis of SWATH's of four maize genotypes under moderate drought treatment, aggregating the electron transport chain-related proteins. Genotypes are identified by different colors.

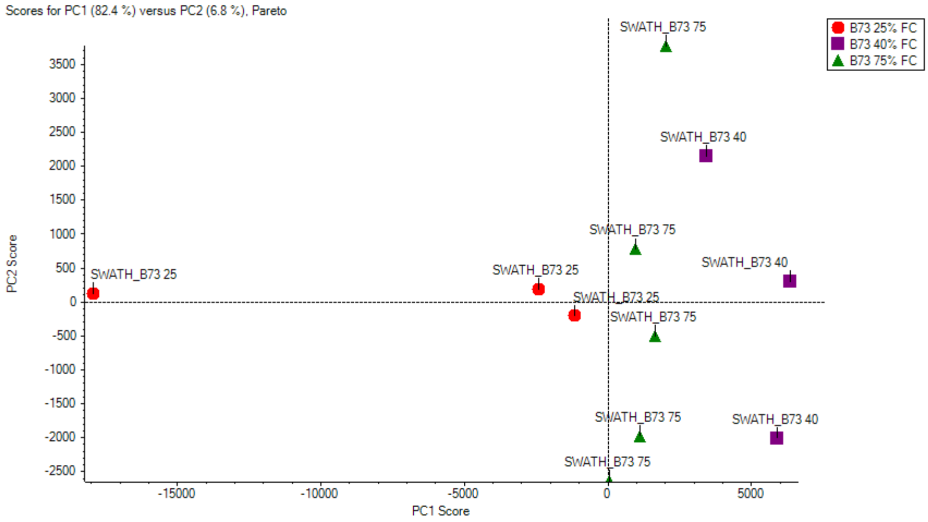

Figure S4.11 - Principal Component Analysis of B73 SWATH's under all water treatments aggregating, the electron transport chain proteins. Treatments are identified by different colors and symbols.

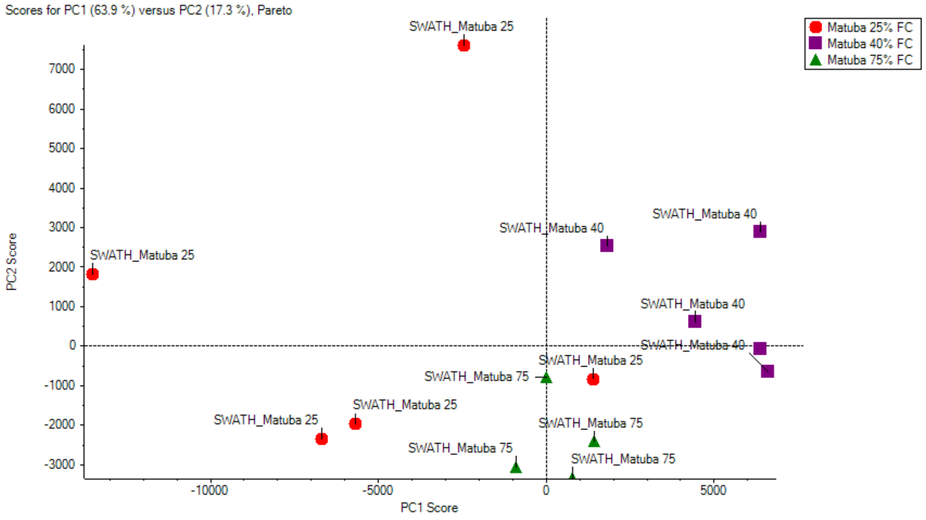

Figure S4.12 - Principal Component Analysis of Matuba SWATH's under all water treatments, aggregating the electron transport chain proteins. Treatments are identified by different colors and symbols.

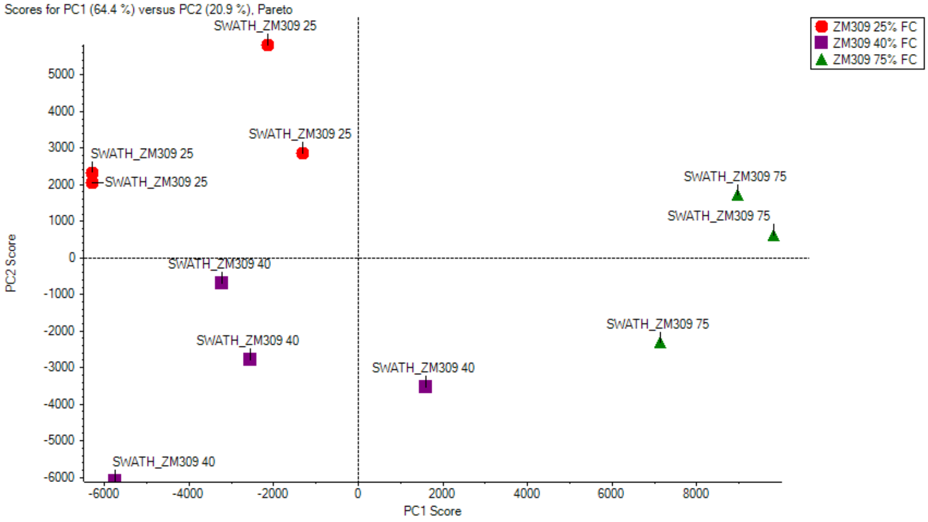

Figure S4.13 - Principal Component Analysis of ZM309 SWATH's under all water treatments, aggregating the electron transport chain proteins. Treatments are identified by different colors and symbols.

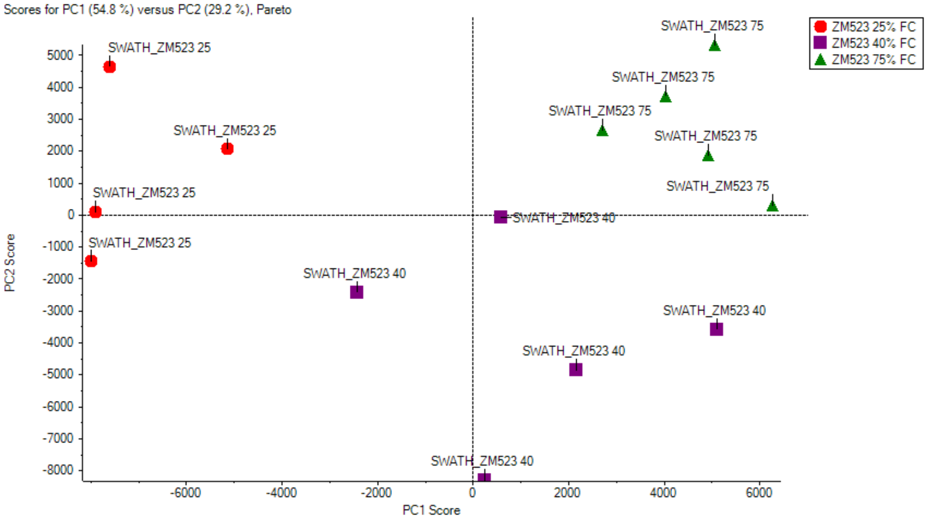

Figure S4.14 - Principal Component Analysis of ZM523 SWATH's under all water treatments, aggregating the electron transport chain proteins. Treatments are identified by different colors and symbols.

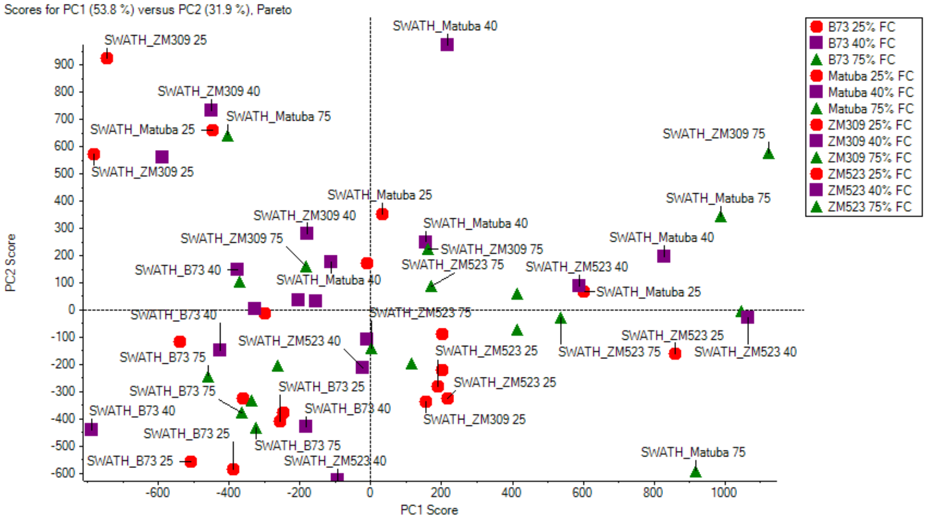

Figure S4.15 - Principal Component Analysis of the SWATH's of the four maize genotypes, aggregating all 12 treatments and the ROS-elimination enzymes. Different water treatments are identified by different colors and symbols.

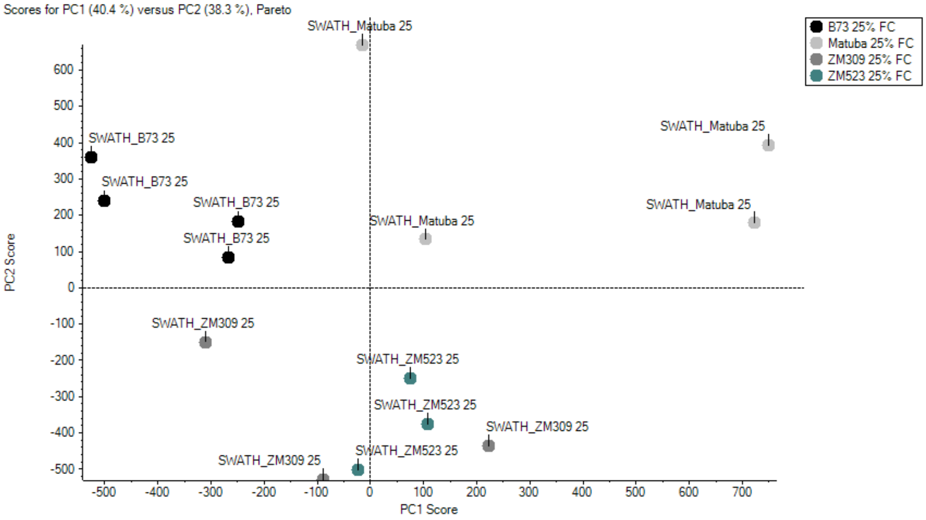

Figure S4.16 - Principal Component Analysis of SWATH's of four maize genotypes under severe drought conditions, aggregating the ROS-elimination proteins. Genotypes are identified by different colors.

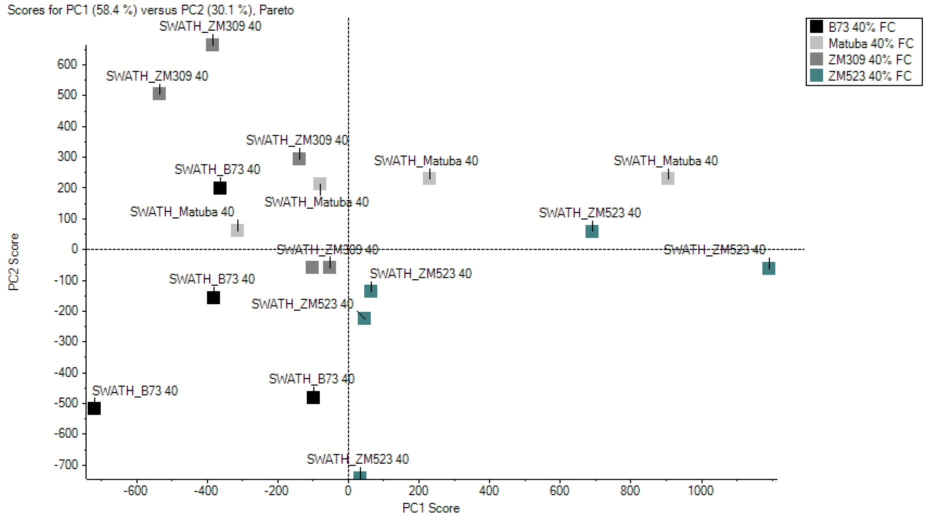

Figure S4.17 - Principal Component Analysis of SWATH’s of four maize genotypes under moderate stress treatment, aggregating the ROS-elimination proteins. Genotypes are identified by different colors.

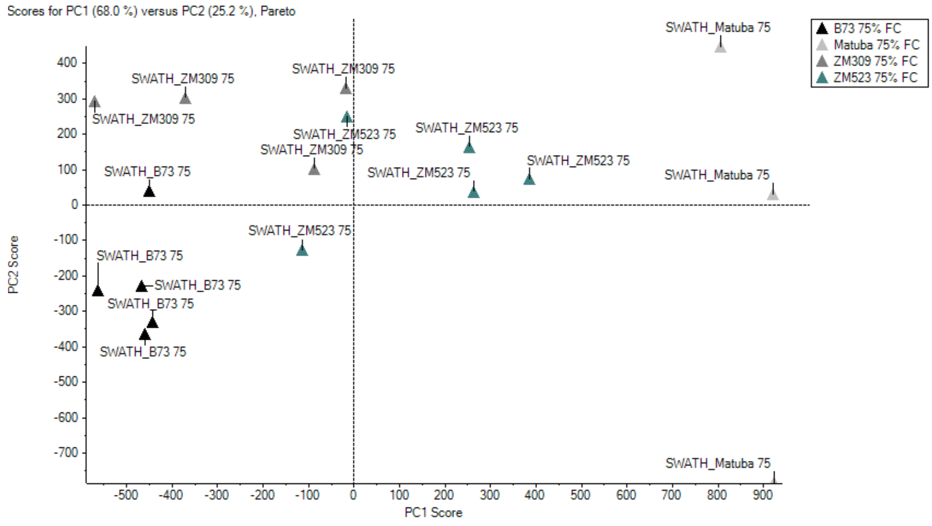

Figure S4.18 - Principal Component Analysis of SWATH’s of four maize genotypes under control conditions, aggregating the ROS-elimination proteins. Genotypes are identified by different colors.

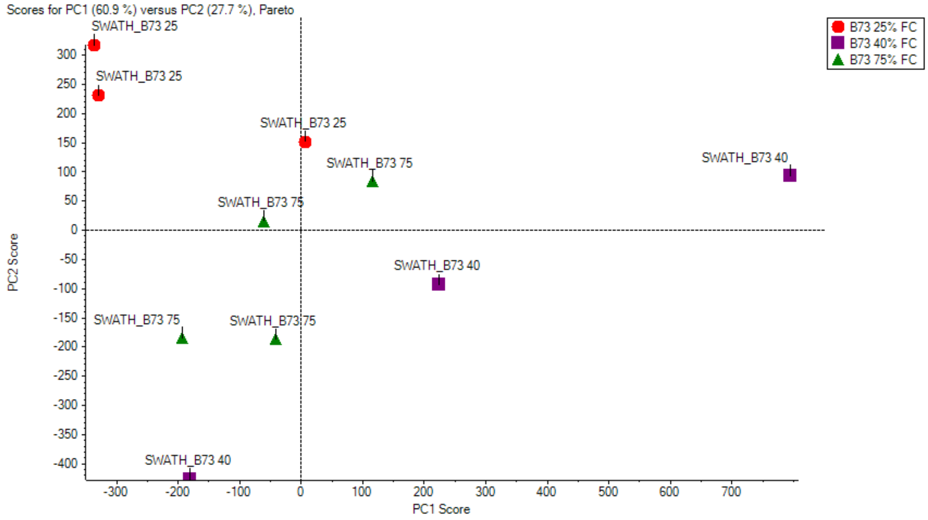

Figure S4.19 - Principal Component Analysis for B73 SWATH's under all water treatments aggregating under all water treatments, using ROS-elimination enzymes. Treatments are identified by different colors and symbols.

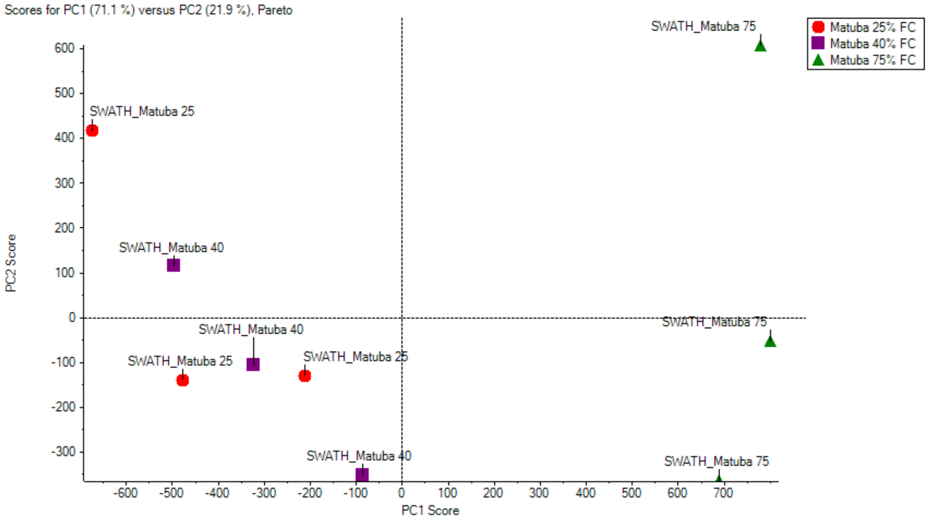

Figure S4.20 - Principal Component Analysis for Matuba SWATH's under all water treatments aggregating under all water treatments, using ROS-elimination enzymes. Treatments are identified by different colors and symbols.

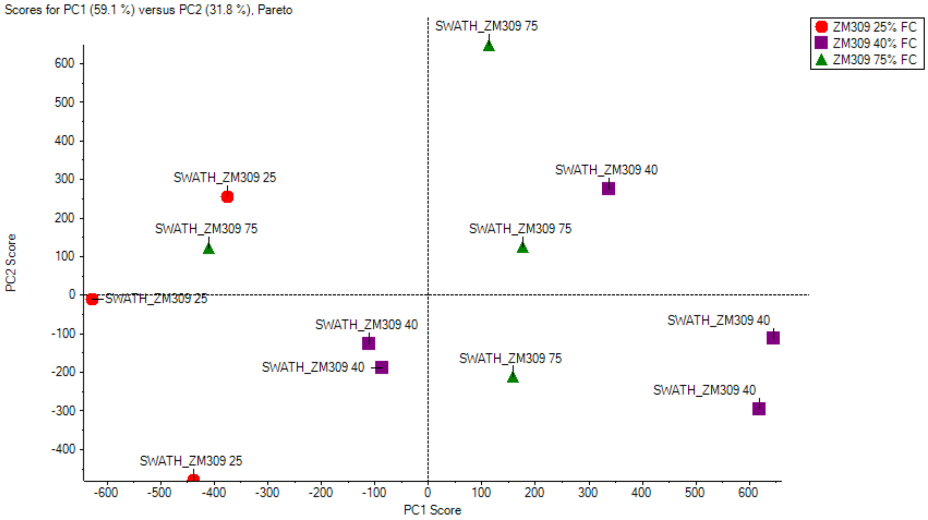

Figure S4.21 - Principal Component Analysis for ZM309 SWATH's under all water treatments aggregating under all water treatments, using ROS-elimination enzymes. Treatments are identified by different colors and symbols.

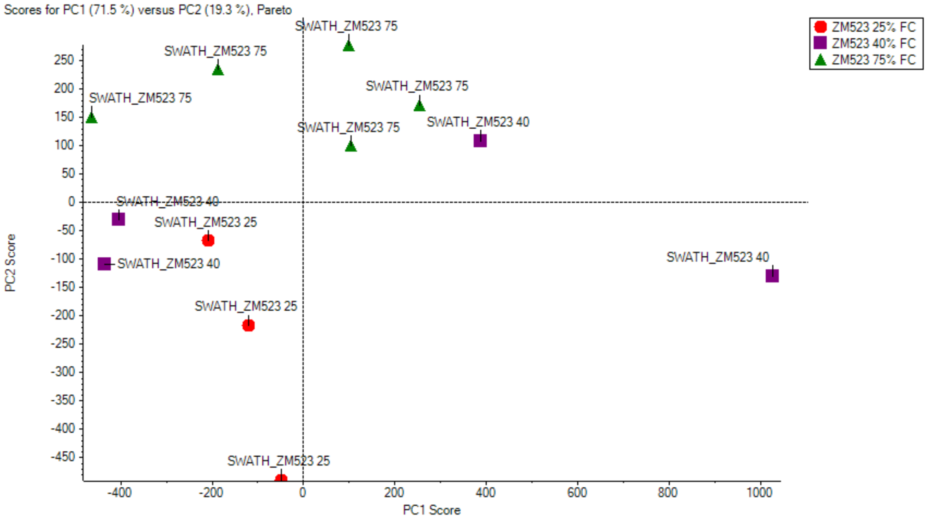

Figure S4.22 - Principal Component Analysis for ZM523 SWATH's under all water treatments aggregating under all water treatments, using ROS-elimination enzymes. Treatments are identified by different colors and symbols

Chapter V. General overview, conclusions, and recommendations

5.1 Introduction

Mozambique is one of the most successful countries in Africa, looking at its post-war reconstruction and economic recovery since the 1992's peace agreement. Despite such positive economic growth and political-social stability, Mozambique remains, unfortunately, as one of the poorest countries in the world. The country is also highly dependent on foreign aid. Chronic malnutrition, poverty, and households' vulnerability to food and nutrition insecurity due to natural disasters and other emergencies are some of the underlying challenges (FAO, 2016).

Agriculture is the largest sector of Mozambique's economy, with more than 80% of the population employed in this sector. However, challenges to increase maize production and productivity that meet people's food demands and surplus, still remain. The threats include the adverse impacts of climate change and climate variability (droughts, floods, emergent crop pests/diseases); unavailable or unaccessible quality inputs and technologies; soil degradation and low fertility; poor capacity for disease surveillance and control; insufficient extension services and poor connections between extension and research (see chapter I for further details on Mozambican Agriculture).

Drought is one of the most important challenges to the livelihoods of people in Mozambique. In general, landrace, improved open-pollinated, and hybrid maize varieties, in particular, are in permanent risk of crop failure in drought susceptible areas. This implies that either drought or associated risks may potentially have irreversible consequences to food security, particularly in poor regions, unless appropriate measures are taken. Therefore, farmers at all levels have been increasingly interested in maize varieties that have good resistance to drought, early maturity, and good performance in poor rainfall conditions (Kassie et al., 2013). This implies the need for targeted breeding programs to overcome challenges imposed by climate change.

The present work was developed as part of the Mozambican national effort to generate maize varieties that are resistant to climate changes, specifically water scarcity. Our goal was to open new avenues of research through the generation of knowledge that can be incorporated in the national maize breeding programs. Thus, we integrated agronomic, physiological and proteomic characterization to explore drought-resistance features in three Mozambican open-pollinated maize varieties, namely Matuba, ZM309, and ZM523 (see the description of these varieties in Chapter I).

5.2. General overview of the work and discussion

5.2.1 The agronomic performance in field conditions

Our first attempt at characterizing the varieties was to test the production of the Mozambican varieties in rainfed conditions in the field environment at two agronomic experimental sites of the Sussundenga district of Mozambique. The performance of the maize genotypes within the assessed cropping systems (intercropping *versus* monocropping, with or without additional fertilization) has shown to be identical. However, in general, intercropping maize with *Vigna unguiculata* L. and the application of fertilizer contributed to increase maize production and may be useful to improve soil nutrient content. The similarities found for Mozambican varieties in well-rainfed conditions in the field were consistent with the physiological and morphological data registered in well-watered conditions in the controlled environment. The varieties showed similar levels of CO_2 assimilation and biomass accumulation.

Maize yield obtained in intercropped blocks was more stable in Rotanda. This feature was mainly attributed to the higher levels of mineral content such as calcium and magnesium and the slightly lower soil acidity that may have positively affected the successful establishment of rhizobia in root hairs, improving nitrogen fixation. Additionally, strip relay intercropping seemed adequate to compensate nitrogen demand in later stages of maize growth, reducing interspecific competition for soil resources.

The use of intercropping was justified taking into consideration its beneficial impacts on soil quality regarding nitrogen and phosphorus availability to plants. Thus, intercropping is a good alternative to the use of pesticides, particularly for resource-poor farmers. Additionally, this practice is also related to decrease pests and reduce crop loss (Gianoli et al., 2006).

5.2.2 The physiological and proteomic characterization

Besides the field tests, we have also performed experiments in controlled conditions to monitor better the physiological and proteomic performance of the maize genotypes in different watering regimes, and explore features related to drought-resistance. As verified in the field assays, in well-watered conditions, the Mozambican maize genotypes behaved similarly regarding traits such as aboveground biomass or net photosynthesis. However, in water-deficit conditions, Matuba showed relatively higher levels of net photosynthesis as compared to ZM523. This was particularly interesting because in comparison with ZM523 or ZM309, the seed companies classify Matuba as “very poor” in terms of drought tolerance. We, therefore, suggest that this behavior of Matuba may be due to the lower leaf area shown, which may represent a strategy to reduce total transpiration while actually profiting from more efficient metabolic processes downstream CO_2 assimilation. Both ZM309 and ZM523 varieties exhibited more pronounced leaf rolling, a feature that is associated with reduced water loss in one hand, but on the other is listed as a score for drought injury. Although these observations make the overall debate about drought resistance somehow controversial, in the end, what matters is grain production in drought risk areas.

The observed feature that ZM523 has higher root biomass in severe stress could imply a higher water uptake ability in that water-limiting conditions in this variety. This had possibly contributed to its high scoring in the drought tolerance classification by the local breeders. Therefore, more infield studies are needed in arid and semi-arid areas of Mozambique to gain deeper insights into the physiological performance of these varieties under drought.

From the proteomic characterization, we found that the differences in levels of C4-photosynthesis enzymes observed among genotypes did not necessarily match their physiological performance or biomass production. This indicated that non-stomatal factors, such as sucrose synthesis and transport through phloem and carbon partitioning throughout the plant, as well as RuBP regeneration, or the activity of the enzymes, among other factors, are possibly contributing differently to the overall performance of the genotypes (Hofius & Börnke, 2007).

Our protein data in drought stress conditions do not point for a genotype standing over the others. The levels of C4-photosynthesis enzymes, as well as of RuBisCO activase, RuBisCO large chain, and ATP synthase subunits (alpha, beta, and gamma) were in general repressed in severe drought conditions. This was consistent with the lower levels of CO_2 assimilation in these conditions. Such reductions are also known as strategies to conserve energy and reduce the formation of reactive oxygen species.

Interestingly, opposite to the lower levels of the electron transport rate observed in drought conditions, the amount of the proteins associated with PSII and PSI tended to increase in stress (with ZM523 tendentially showing the lowest levels at PSII among genotypes). Therefore, we hypothesize that the higher levels of PSII and PSI proteins reflect the need to enhance the repair and protection of the photosystems. Also, consistent with the fact that high temperatures and lower leaf cooling often accompanies drought, HSP70 levels were found to increase with drought in all genotypes. Since, the HSP protein family prevents protein degradation and work in protein refolding under high temperature, are preferentially expressed in drought-tolerant maize genotypes in comparison to the sensitive ones (Hayano-kanashiro et al., 2009).

The ROS elimination enzymes Catalase 3 and Peroxidase 42 were found to accumulate in Matuba in response to drought, likely indicating play a role in the tolerance strategy, but requiring more studies to validate this hypothesis.

Globally, when looking at the morphological, physiological, and proteomic data collected for the four maize genotypes under study, we may conclude that the Mozambican varieties display different strategies to cope with drought stress. These include enhancing root biomass, reducing leaf area and transpiration, and increasing the levels of PSII, PSI, and HSP70 proteins.

Based on our physiological and proteomic data, we suggest that regarding drought tolerance, the classification of Matuba as “very poor” as compared to ZM309 and ZM523, should be carefully re-evaluated by the Institute of Agricultural Research of Mozambique (IIAM) and local seed companies. Moreover, we recommend more studies to evaluate the physiology of these varieties, particularly in arid and semi-arid field conditions.

This Ph.D. work can be considered an important first step towards a better characterization of the national germplasm bank of Mozambique, supported by physiological and proteomic data from a broader functional biology perspective.

Following steps towards publishing these data will also include the multivariate statistical analysis. The objective will be to cover, for instance, the interactions among different factors (for example, genotype *vs.* treatment). We will also make use of PCA combining, for example, physiological and proteomic data. Altogether, these analyses will help us to better discriminate our genotype’s response to drought.

5.3. Strengthening Mozambique research to support breeders

As previously mentioned, to address the problem of food insecurity in Mozambique, it is a priority to foster agriculture and to equip farmers and breeders with the necessary tools.

Some of the strategies that may contribute to improving maize crop yields include the use of genotypes with improved drought tolerance, coupled to the appropriate use of fertilizers, such as chemicals for pest/disease protection, and mechanization (Thierfelder et al., 2018). Also, although more challenging, enhancing tolerance to multiple stresses in plants could have an extensive impact on yields (Anami et al., 2009).

One of the studies that we suggest for Mozambique is to study the physiology of selected maize varieties exhaustively. This would support breeders with a more robust background regarding the features of local maize germplasm, in particular for the threatening drought conditions. To this end, Research Institutes and Universities would need to make some investments to acquire portable photosynthesis systems such as the Infrared gas analyzer (IRGA) or the MiniPAM to help to monitor plant behavior in varying climate conditions. Also, the organization of training sessions on how to use these devices and maximize outputs would be crucial.

The machines mentioned above are expensive, but cheaper ones such as the Multispeq can be used by plant scientists to successfully measure and analyze fluorescence-based parameters such as the photosynthetic efficiency (Φ_{II}), the linear electron transfer (LEF), and nonphotochemical quenching (NPQ). The experiments can be performed in both controlled or infield environments (more information regarding the Multispeq device can be found in https://photosynq.org/).

To generate more applicable knowledge, we strongly recommend the implementation of *in situ* field assays, preferably involving local farmers. To engage the farmers, maize landraces should be selected and included in breeding programs as it happens when performing mother-baby trials (find the explanation on this type of trial in Chapter I). All this will, of course, depend on good communication between scientists and farmers, adequate transport, and means to properly collect and send samples to the Laboratories, which in Mozambique are mostly located in the main cities.

Although we did not deeply explore root traits, we suggest that plant breeders in Mozambique should also focus on below-ground traits. This is important because drought adverse effects can be circumvented when plants invest in carbon allocation to form deeper root systems in water-limiting conditions. Additionally, the spatial configuration of the root system can shape the ability of a plant to uptake water (Lopes et al., 2011). Of course, the root system must always be analyzed in the context of whole plant development and productivity success.

5.4 The economic and social challenges of maize production

We believe that the old dream of the "green revolution" can come true in Mozambique. For example, the government and partners are putting continuous effort on irrigation projects such as the ones implemented in the districts of Boane and Magude, in Maputo province, Maganja da Costa in Zambézia province, and others in Sofala and Manica provinces (source: http://www.inir.gov.mz). This effort, combined with the presence of trained human resources, can contribute to increasing grain yields. However, unfortunately, the shortage of funding to local small-scale farmers impairs adequate access to irrigation technologies.

Since the majority of the population is involved in agriculture, we believe that the government’s focus should be there, to decrease rural poverty and vulnerability. The appropriate channels of product commercialization should also be created. For instance, creation and increase of the food processing industry (like tomato sauce production) that can be fueled with raw material from local farmers. These industries will not only give vent to the abundant raw material but also employ local people and guarantee the continued supply of the products even in out-of-season periods.

The subsidizing program of agriculture inputs such as fertilizers is recommended, but it should be well managed and conveyed to the most vulnerable farmers, without any type of nepotism. We also think that achieving the goal of the Green Revolution at mid-term will require a reduction of taxes associated with the import of agricultural inputs, at least until local investments are made in the agrarian sector by private companies and farmers associations.

João Mosca, one of the most reputed specialists in the agro-economy and rural sociology in Mozambique, has been suggesting to restructure the budget allocated to the Ministry of Agriculture to take into account research and rural extension instead of just channeling 70% of the budget to salaries as currently happening. The intervention of the government is also necessary to address the problem of bank financing, as it is widespread that the banks do not believe in agriculture, and their interest rates are very high and prohibitive. Increased bank coverage, is therefore, also essential, with at least one bank per district. With the implementation of these measures, it would be possible to support the creation of micro, small, and medium-sized agricultural companies capable of contributing to poverty alleviation and increasing economic inclusion.

Continuous investment in the formation of agrarian extensionists is also important to spread the best agrarian practices, including to remote areas. It is also necessary to do profound research on cheaper alternatives and techniques to collect and store water to make it available to farmers during scarcity periods. Local universities could play an important role in these matters, including in teaching the techniques. For example, in some locations in Manica, good examples of gravity irrigation are found which did not require huge investments.

Another aspect to be taken into consideration to guarantee food security in Mozambique is post-harvest procedures to avoid grain losses. Thus, investment in more robust storage facilities is crucial, since traditional facilities are insufficient and most often inadequate (FAO, 2010).

Traditional barns are of three main types: field barns made of wood and straw and other locally available materials, household barns built between straw roofs and fireplaces, and barns build in the yard with similar characteristics and dimensions to those in the field. All these storage facilities are rudimentary and do not offer complete protection against pests (FAO, 2010).

There are still basic infrastructural problems such as the absence or deteriorating roads and railways, and the lack or weak electricity, that compromise the irrigation systems making them dependent on the high-cost fossil fuels. These issues must also be addressed not only to foster production but also to support food trade and income, especially among small-scale farmers.

Finally, concomitant with all the above-mentioned, continuous investment, and incentives for food fortification and biofortification should be addressed. This will be particularly important for women and children’s health, currently the most affected by chronic malnutrition and still suffering from caloric as well as hidden hunger with all the negative impacts on physical and mental development and health. On that matter, nutritional education targeting women is vital, as evidence supports that women are the main players in agriculture, health and nutrition and the ones that mostly take care of children (IFPRI, 2011), thus helping to circumvent this problem and overcome intrinsic cultural barriers regarding eating habits.

We believe that by implementing these suggested measures and others raised by many agriculture specialists, civil society organizations, and local farmers, Mozambique will reduce its dependence on importations and food aid, becoming capable of increasing food exportation in quantity and quality, reducing unemployment, and ultimately meeting the basic nutritional requirements of its population.

References

INIR. National Institute of Irrigation http://www.inir.gov.mz

PHOTOSYNQ. https://photosynq.org/

Anami, S., Block, M. De, Machuka, J., & Lijsebettens, M. Van. (2009). Molecular Improvement of Tropical Maize for Drought Stress Tolerance in Sub-Saharan Africa. *Critical Reviews in Plant Science*, *28*, 16–35. https://doi.org/10.1080/07352680802665305

FAO. (2010). Continental Programme on Post-Harvest Losses (PHL) Reduction-Mozambique.

FAO. (2016). Country Fact Sheet on Food and Agriculture Policy Trends.

GIANOLI, E., RAMOS, I., ALFARO-TAPIA, A., VALDÉZ, Y., ECHEGARAY, E., & YÁBAR, E. (2006). Benefits of a Maize – Bean – Weeds Mixed Cropping System in Urubamba Valley, Peruvian Andes. *International Journal of Pest Management*, *52*(September 2016), 283–289. https://doi.org/10.1080/09670870600796722

Hayano-kanashiro, C., Ibarra-Laclette, E., Herrera-Estrella, L., Caldero, C., & Simpson, J. (2009). Analysis of Gene Expression and Physiological Responses in Three Mexican Maize Landraces under Drought Stress and Recovery Irrigation. *PLos One*, *4*(10). https://doi.org/10.1371/journal.pone.0007531

Hofius, D., & Börnke, F. A. J. (2007). Photosynthesis, Carbohydrate Metabolism and Source – Sink Relations.

IFPRI. (2011). Exploiting the Links. Washington, D.C.

Kassie, G. T., Langyintuo, A., Erenstein, O., Maleni, D., Gwara, S., & Abate, T. (2013). Drought Risk and Maize Production in Southern Africa. *Journal of Asian Scientific Research*, *3*(10), 956–973.

Lopes, M. S., Araus, J. L., Heerden, P. D. R. Van, & Foyer, C. H. (2011). Enhancing Drought Tolerance in C4 Crops. *Journal of Experimental Botany*, *62*(9), 3135–3153. https://doi.org/10.1093/jxb/err105

Thierfelder, C., Baudron, F., Setimela, P., Nyagumbo, I., Mupangwa, W., Mhlanga, B., … Gérard, B. (2018). Complementary Practices Supporting Conservation Agriculture in Southern Africa. A review. *Agronomy for Sustainable Development*, *38*, 1–22.