FACULTY OF AGRICULTURAL SCIENCES

Institute of Agricultural Sciences in the Tropics

(Hans-Ruthenberg Institute)

University of Hohenheim

Field: Crop production and Food security

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Impacts of the fungal bio-control agent *Fusarium oxysporum* f.sp. *strigae* on plant beneficial microbial communities in the maize rhizosphere

Dissertation

Submitted in fulfillment of the requirements for the degree

"Doktor der Agrarwissenschaften"

(Dr.sc.agr./ Ph.D. in Agricultural Sciences)

to the

Faculty of Agricultural Sciences

Presented by:

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Stuttgart-Hohenheim, 2016

This thesis was accepted as a doctoral thesis (dissertation) in fulfillment of the requirements for the degree "Doktor der Agrarwissenschaften" (Dr. sc.arg./ Ph.D. in Agricultural Sciences) by the Faculty of Agricultural Sciences at the University of Hohenheim, Stuttgart, Germany, on 20th January, 2016.

Date of the oral examination: 27th June 2016

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Special thanks to the Food security Center (FSC) of Universität Hohenheim for the scholarship which is part of the DAAD (German Academic Exchange Service) “Exceed program”. DAAD is supported by the German Federal Ministry for Economic Cooperation and Development (BMZ).

Dedicated to the memory of my late brother, Moses Muema Musyoki and Roseane do Socorro Gonçalves Viana of FSC

Acknowledgements

This thesis marks the achievement of a goal that was envisioned after finishing my M.Sc. Working as a doctoral student was a wonderful as well as a demanding experience. It was barely possible for me to complete this journey without the valuable support of several people who made the journey delightful despite the challenges. First of all, I thank my professor, Prof. Dr. Georg Cadisch, who has enabled me to be independent in my research and, through his excellent guiding style, I learned a lot. I also want to thank PD. Dr. Frank Rasche, my direct supervisor, for his valuable advice, constructive criticism and extensive worthwhile discussions of my research work. I am deeply grateful to Prof. Dr. Ellen Kandeler and PD. Dr. Angela Sessitsch for accepting to serve in my examination committee. I am grateful to Professor Dr. Ludwig Hölzle (Institute of Environmental and Animal Hygiene and Veterinary Medicine, University of Hohenheim) for access to his ABI 3130 Genetic Analyzer. Special thanks goes to Gabriele Kircher for her logistical support, efficient work and always going out of her way to make my working environment comfortable, as well as all my colleagues at the Institute of Plant Production and Agro-ecology in the Tropics and Sub-Tropics, an intercultural group that has enriched my life.

I wish to express my sincere gratitude towards the German Academic Exchange Service (DAAD) and the German Federal Ministry for Economic Cooperation and Development (BMZ) for providing the scholarship grant through the Exceed program. I would also like to convey my deepest appreciation to the Food Security Centre (FSC), for all the unconditional support which was accorded to me to perform my studies comfortably, especially to Prof. Dr. Manfred Zeller, Dr. Detlef Virchow, Dr. Andrea Jost, Dr. rer. nat. Brigitte Kranz and Mrs. Hélène Stauss. I am deeply grateful to, Fiat Panis foundation and Bill and Melinda gates foundation that supported my PhD field research through financial contributions. My gratitude further goes to the Real IPM Company through the department of research and development that facilitated the identification of the field sites and data collection. Special thanks go to Denis Kisasati, George Karanja and Collins Wanyama for their valuable help and company during the field activities.

I take this opportunity to thank my family, my parents, David Musyoki Kivuva and Joyce Nduku Musyoki and my brothers, Joseph Kivuva, Simon Kilonzo, Steven Musembi and the late Moses Muema. Without their support and love nothing would have been possible. In a special way, I thank my nieces, Belinda and Patience, who kept asking when I would return home and gave me the motivation to keep working hard. I thank my grandmother, Alice Nthule Kilonzo, who always encouraged me to keep chasing my dreams.

Good company in a journey makes the way seem shorter. I would like to extend huge and warm thanks to all my friends who made this journey seem shorter, despite the ups and downs; Phoebe Ogutu, Judith Zimmerman, Esther Muema, Carolin Röhl, Esther Enowashu, Juan Carlos Laso, Mike Yongha Boh, Lucy Kariuki, Patchimaporn Udomkun and Yvonne Nkwain. My eternal appreciation goes to Martin Mukong Kuma, your motivation, understanding and moral support has enriched my life in ways I would never have dreamed possible. To all other friends that I have not mentioned, not because you did not contribute much but because I am not able to include all here, I am very grateful for all the support.

I extend my special appreciation to Dr. Joseph Ogutu and Patch for their help with reviewing and proof reading my work. Lastly, but certainly, not least, I want to thank Judith Zimmermann for her translation of my summary to German and Dr. Carsten Marohn for his corrections.

Table of Contents

Acknowledgements i
Table of Contents iii
List of Tables vii
List of Figures ix
List of abbreviations xi
CHAPTER 1 1
1. General introduction 3
1.1. Background of the study 3
1.2. Statement of the problem and justification of the study 5
1.3. Potential rhizospheric interactions 6
1.4. The use of ammonia oxidizing and proteolytic microorganisms as bioindicators 8
1.5. Factors influencing microbial abundance and community structure 10
1.5.1. Soil type and texture 10
1.5.2. Climatic conditions 11
1.5.3. Crop growth stage 12
1.6. *Striga* control methods and Foxy-2 strain as a *Striga* biological control agent 13
1.6.1. Foxy-2 strain as a *Striga* biological control agent 14
1.7. Overview of the relevant techniques 18
1.7.1. Measurement of microbial community abundance 18
1.7.1.1. Quantitative Polymerase chain reaction (qPCR) 18
1.7.2. Measurement of microbial community structure 19
1.7.2.1. Terminal restriction fragment length polymorphism (T-RFLP) 19
1.7.3 Measurement of enzymatic activities 20
1.8. Guiding hypotheses 21
1.9. Guiding Objectives 21
1.10. Structure of thesis 22
CHAPTER 2 25
2. Promoting effect of *Fusarium oxysporum* [f.sp. *strigae*] on abundance of nitrifying prokaryotes in a maize rhizosphere across soil types 27

2.1. Abstract 27
2.2. Introduction 28
2.3. Material and methods 31
2.3.1. Fungal biocontrol agent 31
2.3.2. Rhizobox experiment 31
2.3.3. Quantification of prokaryotic abundance 32
2.3.4. Measurement of soil chemical parameters 33
2.3.5. Statistical analysis 33
2.4. Results 36
2.4.1. Microbial gene abundance 36
2.4.1.1. Total archaeal and bacterial communities 36
2.4.1.2. Archaeal *amoA* gene abundance 36
2.4.1.3. Bacterial *amoA* gene abundance 41
2.4.2. Treatment effects on soil chemical parameters 41
2.4.3. Linear correlations between gene abundance and soil chemical parameters 41
2.5. Discussion 42
2.5.1. Effect of "Foxy-2" on abundance of indigenous rhizosphere prokaryotes 42
2.5.2. Imperative considerations when implementing "Foxy-2" as BCA 43
2.6. Conclusions 44
2.7. Acknowledgments 45
CHAPTER 3 47
3. Soil properties, seasonality and crop growth stage exert a stronger effect on rhizosphere prokaryotes than the fungal biocontrol agent *Fusarium oxysporum* f.sp. *strigae* 49
3.1. Abstract 49
3.2. Introduction 50
3.3. Material and methods 52
3.3.1. Fungal biocontrol agent 52
3.3.2. Study site description 52
3.3.3. Field experiment and rhizosphere sampling 53
3.3.4. Microbial abundance 54

3.3.5. Microbial community composition 55
3.3.6. Soil chemical analysis 58
3.3.7. Statistical analysis 58
3.4. Results 60
3.4.1. Microbial abundance 60
3.4.2. Microbial community composition 64
3.4.3. Soil chemical properties 66
3.4.4. Correlations between microbial abundance and soil chemical properties 68
3.4.5. Regressions between microbial community composition and soil chemical properties 69
3.5. Discussion 70
3.5.1. Dominance of environmental factors over presence of BCA 70
3.5.2. Foxy-2 did not induce a resource limitation for bacteria and archaea 72
3.6. Conclusion and outlook 72
3.7. Acknowledgments 73
CHAPTER 4 75
4. Lasting influence of biochemically contrasting organic inputs on abundance and community structure of total and proteolytic bacteria in tropical soils 77
4.1. Abstract 77
4.2. Introduction 78
4.3. Materials and methods 81
4.3.1. Field experiments and soil samplings 81
4.3.2. Microbiological soil analysis 82
4.3.2.1. Soil DNA isolation 82
4.3.3. Physico-chemical soil analysis 86
4.3.3.1. Mid-infrared spectroscopic analysis 86
4.3.3.2. Total contents of soil polyphenols 87
4.3.3.3. Total soil C and N contents 88
4.3.4. Statistical analysis 88
4.4. Results 89
4.4.1. Microbial community dynamics 89
4.4.2. DRIFTS pattern interpretation 92

4.4.3. Chemical soil properties 95
4.5. Discussion 98
4.5.1. N stress compensation of proteolytic bacteria 98
4.5.2. Minor influence of soil polyphenols on entire bacterial communities 99
4.6. Conclusions and outlook 100
4.7. Acknowledgements 102
CHAPTER 5 103
5. Response of proteolytic bacteria abundance and their potential enzymatic activity to the fungal biocontrol agent *Fusarium oxysporum* f. sp. *strigae* in contrasting soils 105
5.1. Abstract 105
5.2. Introduction 106
5.3. Materials and methods 107
5.4. Results and discussion 109
5.5. Conclusion 114
CHAPTER 6 General discussion 115
6. General discussion 117
6.1. Is Foxy-2 compatible with nitrifying prokaryote communities in maize rhizospheres in contrasting soil types? 118
6.2. Natural factors are strong determinants of nitrifying prokaryotes than *Fusarium oxysporum* f.sp. s*trigae* (Foxy-2) in maize rhizospheres 119
6.3. Can proteolytic bacteria abundance and potential enzyme activity be used as bioindicators for soil monitoring under Foxy-2 inoculation in contrasting soils? 122
6.4. Concluding remarks and recommendations for future work 123
References 127
Summary 153
Zusammenfassung 157
Appendices 161
Appendix A. Additional publications 161
Appendix B. Curriculum vitae 165
Appendix C. Author's declaration 169

List of Tables

Table 1. Main advantages and disadvantages of various approaches to control *Striga* 16

Table 1. Main advantages and disadvantages of various approaches to control *Striga* (continued) 17

Table 2. Decription of primer sets, PCR ingredients and amplification details used for qPCR analysis 35

Table 3. Soil chemical parameters (mean ± standard deviation, n = 18) obtained from the rhizobox experiment 39

Table 4. Pearson's linear correlation coefficients between prokaryotic gene abundance (16S rRNA gene, *amoA* gene) and soil chemical data at 42 DAP 40

Table 5. Description of primer sets, PCR ingredients and amplification details used for qPCR and T-RFLP analysis 57

Table 6. Analysis of variance to determine significant effects of factors "Site" (ST), "Season" (SS), "Treatment" (T) and "Growth stage" (EC) and their interactions on soil chemical properties and abundance of the two assayed genes. 61

Table 7. Global R values for the main factors "Site" "Growth stage" and "Treatment" as obtained from the analysis of similarity (ANOSIM) of T-RFLP of the two studied prokaryotic groups fingerprints 64

Table 8. Dynamics of soil chemical properties as driven by factors "Site", "Season", "Treatment" and "Growth stage" and their interactions 67

Table 9. Pearson's linear correlation coefficients between the abundance of the two prokaryotic groups and soil chemical data taken at EC30 and EC90 in Homabay and Busia sites 68

Table 10. Regression analyses between community composition of the two prokaryotic groups and soil chemical data taken at EC30 and EC90 across the sites 69

Table 11. Biochemical quality parameters[a] of the three studied organic inputs 85

Table 12. Global R values for the two main factors "site" and "input type" as obtained from the analysis of similarity of T-RFLP (16S rRNA and *npr* genes) and DRIFTs fingerprints 92

Table 13. Geochemical characteristics of the differently treated soils at the experimental field sites in Embu and Machanga.....97
Table 14. Analysis of variance (ANOVA) of proteolytic bacteria abundance, enzymatic activities and soil properties as influenced by soil type (S), sampling time (ST), Treatment (TR) and their interactions.....112
Table 15. Soil chemical parameters as influenced by "Foxy-2" and "organic input" 42 days after planting 113

List of Figures

Figure 1. Conceptual model of the soil N mineralization (i.e. N cycling) pathway 9

Figure 2. Abundance of the total archaeal and bacterial community in the sandy (a and c) and clayey (b and d) soils as determined during the 6 weeks of incubation in the rhizobox experiment.....37

Figure 3. Abundance of the archaeal and bacterial *amoA* genes in the sandy (a and c) and clayey (b and d) soils as determined during the 6 weeks of incubation in the rhizobox experiment. 38

Figure 4. Monthly rainfall and temperature distribution during the short rain (SR) season (September 2012-January 2013) and the long rain (LR) season (March-August 2013). 54

Figure 5. Abundance of the total archaeal and bacterial community at Busia (a, c) and Homabay (b, d) sites as determined during the two cropping seasons. 62

Figure 6. Abundance of the archaeal and bacterial *amoA* genes at Busia (a, c) and Homabay (b, d) sites as determined during the two cropping seasons.....63

Figure 7. Canonical analysis of principal coordinates (CAP) for visual presentation of prokaryotic communities composition (TRLFP) as shaped by "Site" and "Growth stage". 65

Figure 8. Visualization of DRIFTS patterns obtained from the soils of the Embu and Machnaga experimental field sites. 87

Figure 9. Abundance of *npr* (a) and 16S rRNA (b) genes in the soils of the two experimental fields in Embu (gray columns) and Machanga (White columns). 91

Figure 10. Group average-based cluster analysis of T-RFLP (i.e., *npr* gene (a), 16S rRNA gene (b) and DRIFTS (c) fingerprints as calculated on basis of the Bray-Curtis similarity coeffients. 94

Figure 11. Areas of the two studied peaks (peak # 1(1750-1510 cm^{-1}; aromatic C = C and COO- stretchings (Smidt and Meissl, 2007; Nault et al., 2009; Demyan et al., 2012)) and peak # 2 (1450-1330 cm^{-1}; C-O of phenolic C-OH groups (Baes and Bloom, 1989))) as influenced by differently treated soil systems. 96

Figure 12. Response of *npr* gene abundance to “Foxy-2” inoculation and *T.diversifolia* in contrasting soils.....110
Figure 13. Response of *npr* gene specific enzyeme (suc-Ala-Ala.Phe-AMC) to “Foxy-2” and *T.diversifolia* in a sandy soil.....111

List of abbreviations

ABI	Applied Biosystems
ANOSIM	Analysis of similarity
ANOVA	Analysis of variance
AOA	Ammonia-oxidizing archaea
AOB	Ammonia-oxidizing bacteria
A.U.	Absorbance units
a.s.l.	Above sea level
BCA	Biological control agent
°C	Degree Celsius
C	Carbon
CA	California, USA
$CaNH_4NO_3$	Calcium ammonium nitrate
CAP	Canonical Analysis of Principal coordinates
CC	*Calliandra calothyrsus*
CIAT	International Center for Tropical Agriculture
cm	Centimetre
CON	Control
DAAD	Deutscher Akademischer Austausch Dienst
DAP	Days after planting
DGGE	Denaturing gradient gel electrophoresis
DISTLIM	Distance-based linear models
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DRIFTS	Diffuse reflectance fourier transformed infrared spectroscopy
EC30	Early leaf development stage
EC60	Flowering stage
EC90	Senescence stage
ECC	Embu *Calliandra calothyrsus*
ECON	Embu control
EOC	Extractable organic carbon

EON	Extractable organic nitrogen
ETD	Embu *Tithonia diversifolia*
EZM	Embu *Zea mays*
FAM	Carboxyfluorescein dye
FAO	Food and Agriculture Organization of the United Nations
FastDNA TM SPIN	A Kit that contains all the components needed for isolation of soil DNA.
FOS	*Fusarium oxysporum strigae*
FSC	Food Security Centre
GmbH	Gesellschaft mit beschränkter Haftung
Ha	Hactare
HEX	Carboxyhexachlorofluorescein dye
HIDI	Highly Deionized Formamide
IITA	International institute of tropical agriculture
IPM	Integrated pest management
LR	Long rains
LSMEANS	Least square means
K	Potassium
KBr	Potassium bromide
KCl	Pottasium chloride
Km	Kilometre
K_2SO_4	Pottasium sulfate
M	Molar
MA	Massachusetts, USA
MCC	Machanga *Calliandra calothyrsus*
MCON	Machanga control
$MgCl_2$	Magnesium chloride
MP	Milan Panic the Founder, President and Chief Executive of MP Biomedicals
MTD	Machanga *Tithonia diversifolia*
N	Nitrogen
npr	Neutral metaloproteases
n.s.	Not significant

NC	North Carolina, USA
NEB	New England Biolabs
NH_4^+	Ammonium
NO_3^-	Nitrates
N_t	Total nitrogen
PP	Polyphenol
PERMANOVA	Permutation multivariate analysis of variance
qPCR	Quantitative Polymerase Chain Reaction
RISA	Ribosomal intergenic spacer analysis
RNA	Ribosomal nucleic acid
ROX	Carboxy-x-rhodamine dye
SED	Standard error of the difference
SOC	Soil organic carbon
SR	Short rains
SOM	Soil organic matter
SSA	Sub-Saharan Africa
SYBR Green	An asymmetrical cyanine dye (Fluorescent DNA binding dye)
TAMRA	Tetramethylrhodine azide
T4 gene 32 protein	A nucleic acid binding protein that destabilizes the DNA helical structure
TC	Total carbon
TD	*Tithonia diversifolia*
TGGE	Temperature gradient gel electrophoresis
TRFs	Terminal Restriction Fragments
TRFLP	Terminal Restriction Fragment Length Polymorphism
μM	Micromolar
USA	United States of America
WI	Winsconsin, USA
ZM	*Zea mays*

CHAPTER 1

General introduction

1. General introduction

1.1. Background of the study

Parasitic weeds in the genus *Striga* are a significant threat to food security in smallholder maize production systems and this is expected to increase in the future given the predicted impacts of climate change (Jones and Thornton, 2003; Parker, 2014). Among the 23 described *Striga* species, *Striga asiatica* and *Striga hermonthica* (Delile) Benth. are the most economically important but also have devastating effects in Sub-Saharan Africa (Khan et al., 2014; Spallek et al., 2013). It is estimated that across the African continent annual staple cereal yield losses caused by *Striga hermonthica* range from 40 to 100%, affecting the livelihood of over 100 million resource-poor farmers (Ejeta, 2007; Teka et al., 2014; Bozkurt et al., 2015). In Western Kenya, it is estimated that 212,000 hectares of arable land is infested with *S. hermonthica*, causing up to 100% yield losses, equivalent to annual losses estimated at US$ 41 million (Hassan et al., 1995; Vanlauwe et al., 2008).

Over the years, many research efforts have been made towards the control of *Striga* and they have recommended several control options such as; crop rotation, intercropping, late planting, deep planting, transplanting of host, soil fertilization, herbicide seed dressing and biocontrol use such as *fusarium* and arbuscular mychorrhiza (Hearne, 2009). In spite of all these efforts, no one single method has been found to be optimal and wide-scale effective control of the weed remains elusive (Khan et al., 2014). Reasons for limited control of the weed range from the fact that *Striga* is highly productive with a single plant producing an enormous amount (10 000-20 000) of tiny seeds (0.3 mm x 0.15 mm) that can remain viable in the soil for over 10 years (Hearne, 2009; Atera et al., 2011). These dust-like seeds are easily dispersed by wind, water, animals and people, further enhancing the potential of its spread to non-infested areas. It also has high genetic variability within species, increasing its adaptability to different hosts and changing environments (Bozkurt et al., 2015). Moreover, its mode of action through host signaling of molecules such as strigolactones greatly complicates efforts to control its parasitism.

Due to the complexities involved in controlling the *Striga* menace, it is now widely accepted that the effective control of *Striga* requires an integration of two or more control options such as soil fertility improvement, intercropping, use of host resistant varieties and application of biological control agents (Hearne, 2009; Atera et al., 2013). The combination of biological control agents (BCAs) including *Fusarium oxysporum* f.sp.*strigae* (Fos) strains along with

tolerant varieties has shown reasonable control success under field conditions and provides environmentally friendly as well as durable strategies to combat *S. hermonthica* (Schaub et al., 2006; Venne et al., 2009; Zimmermann et al., 2015).

The fungal strain, *Fusarium oxysporum* f.sp.*strigae* ("Foxy-2") has shown significant control of *S. hermonthica* and was acknowledged to be superior in suppressing all development stages of *S. hermonthica* from germination to flowering, further proving its necessity as a critical component of an integrated *Striga* management approach (Kroschel et al., 1996; Ciotola et al., 2000; Elzein et al., 2006a; Venne et al., 2009; Ndambi et al., 2011). However, to be considered as an effective myco-herbicide component of an integrated *Striga* management for optimization, "Foxy-2" must be able to co-exist with indigenous beneficial microbial populations as well as show consistent *Striga* control in varying agroecological zones (Hearne, 2009; Avedi et al., 2014). For this to be achieved, "Foxy-2" adaptability and ability to co-exist with indigenous microbial populations in the rhizophere need to be well assessed under both controlled and field conditions.

Few studies have been carried out to investigate the effect of the biocontrol agent; "Foxy-2" on important biological resources that contribute to plant production through processes such as nitrogen (N) cycling, including nitrification and proteolysis (Avedi et al., 2014). Thus little is known on the fate of microorganisms involved in N cycing after the application of "Foxy-2" as a biocontrol agent (BCA). Given this gap in our scientific knowledge, this work is scientifically relevant as it not only addresses the risk assessment analysis in relation to important maize rhizosphere microorganisms but will also help in understanding the key factors that influence effective performance of "Foxy-2", and hence contribute towards maximizing consistency and efficacy of "Foxy-2"as a *striga* biocontrol agent for future consideration in intergrated *Striga* management.

The study was carried out as part of a project funded by Bill and Melinda gates foundation initiated by the International Institute of Tropical Agriculture (IITA), Nigeria, in cooperation with Hohenheim University, Germany. The overall goal of this project was to formulate and deploy, through strategic partnerships, an integrated approach for managing *Striga* while improving soil fertility and reducing *Striga* seed bank for sustainable increases in crop yields. In the framework of the biological control team, extended rhizosphere studies including non-target effects of "Foxy-2" on beneficial microbial communities under controlled and field were carried out.

1.2. Statement of the problem and justification of the study

In Western Kenya, as in many places in Africa, *Striga hermonthica* has led to low production of maize, the main staple crop, consequently threatening food security in the region (Khan et al., 2014). It is estimated that 76% of the land planted with maize and sorghum in Western Kenya, which is the main food basket of the country, is infestated by *Striga* (Gethi et al., 2005). Several control options have been developed to curb the infestation, yet no single method has been found to be optimal across the region (Hearne, 2009; Khan et al., 2014). Therefore, there is a continuing urgent need to develop and apply effective localized intergrated *Striga* management strategies (Vanlauwe et al., 2008; Hearne, 2009). The use of BCAs along with other control methods such as resistant varieties has been accepted as a long-term and sustainble component of an intergrated *Striga* management strategy due to its ability to reduce the *Striga* seed bank in the soil (Fravel et al., 2003; Kroschel et al., 1996; Ndambi et al., 2011). However, the large scale application of BCAs such as "Foxy-2" is often faced with resistance due to the unknown risks to the environment, especially if it is a foreign strain (Ehlers, 2009; Avedi et al., 2014). Unfortunately, the non-target effects of "Foxy-2" on beneficial microorganisms (i.e N cyling prokaryotes) are not well known although it is a prerequisite for field application.

Most studies on "Foxy-2" as a BCA, however, mainly focused on virulence, efficacy, specificity and mode of action while less is known about "Foxy-2" interactions with microorganisms involved in key soil functions such as organic matter degradation and nutrient cycling. The non-target effects of "Foxy-2" on microorganisms have been underestimated despite the fact that it as a governmental regulatory concern to ensure such risk assessment studies are carried out prior to field application of such BCAs including "Foxy-2" (Brimner and Boland, 2003; Cook et al., 1996; Avedi et al., 2014). Moreover, there has been rising concern regarding the potential of such BCAs to displace or suppress resident soil microorganisms with key functions in element cycles (e.g., nitrogen cycling (N)) due to competitive displacement (Cook et al., 1996; Gullino et al., 1997; Brimner and Boland 2003; Edel-Hermann et al., 2009; Martin-Laurent et al., 2013). Competitive displacement occurs when a BCA expels or replaces native non-target species through competition for space or nutrients (Brimner and Boland, 2003). Hence, it is essential to assess if "Foxy-2", which primarily performs in the rhizosphere, may have significant non-target effects through the reduction in abundance and/or structure of microorganisms involved in the cycling of nutrients such as N. This includes those microorganisms involved in proteolysis and

nitrification (i.e., ammonia oxidation), as they play key roles in the initial steps in soil N cycling process through the production of enzymes that metabolize nitrogenous organic bound N (proteolysis) and oxidise mineralized products (nitrification) such as ammonia (NH_4^+) to plant available compounds such as nitrates (NO_3^-) (Mrkonjic Fuka et al., 2007; Jia and Conrad, 2009; Wessén et al., 2010; Rasche et al., 2014). This may provide an understanding of the basic principles of the rhizosphere interaction between "Foxy-2" and the abundance and structure of beneficial microorganisms.

Moreover, the performance of BCAs such as "Foxy-2" is limited by effective variation in *Striga* control across regions in SSA which has been linked to biotic and abiotic factors (Avedi et al., 2014; Zarafi et al., 2015; Rebeka et al., 2013; Watson et al., 2013). For example, a recent study by Avedi et al., (2014) associated the lack of "Foxy-2" to achieve biological control of *Striga* in Western Kenya to potential antibiotic impacts by indigenous microorganisms and other abiotic factors such as soil pH, soil moisture and temperature. Despite these speculations, research on biological control of *Striga* using "Foxy-2" has not focused on the potential rhizosphere interaction effects between "Foxy-2" and native microorganisms. Therefore, this study aimed to evaluate the interactions between "Foxy-2" and microorganisms involved in key soil functions such as organic matter degradation (i.e. proteolysis) and nitrogen cycling (i.e. nitrification) in a maize rhizosphere in contrasting soil.

1.3. Potential rhizospheric interactions

It is well known that the rhizosphere, which is the biologically active zone of the soil, is a hot spot of microbial interactions (Nihorimbere et al., 2011). However, the rhizosphere is also known as a battlefield where complex rhizosphere communities, both microflora and microfauna interact and may consequently influence the outcome of the introduced BCAs ("Foxy-2") (Raajimakers et al., 2009). The indigenous beneficial microorganisms may have some antagonistic effects on "Foxy-2" by producing secondary antimicrobial metabolites, hence lowering its efficacy or through competition for water, nutrients and space since they are well adapted to the rhizosphere than the newly introduced microorganism (Fig.1, (Kubicek et al., 2001; Nihorimbere et al., 2011)).

On the other hand, "Foxy-2" may adversely affect the beneficial rhizosphere microorganisms, mostly those that have common niches such as fungal communities in the battle for establishment and persistence in the niche via strategies such as competition for space and

nutrients, Fig.1 (i.e. through its nutrients uptake (C and N) for ploliferation) (Mezzalama et al., 1997; Alabouvette et al., 2006; Raajimakers et al., 2009; Nihorimbere et al., 2011). In addition, antibiosis through the inhibition of microbial growth by diffusible antibiotics and volatile toxins from root exudates may also affect the introduced BCA ("Foxy-2") negatively or positively (Compant et al., 2005; Haas and Défago, 2005). The competitive ability of both the introduced BCA (i.e. "Foxy-2") and the indigenous beneficial microorganisms however, depends on the environmental factors and the specific niche preference of "Foxy-2" and the native microorganisms involved in organic matter degradation and nitrogen cycling (Grayston et al., 1998). It has been shown in other studies that the various groups of microorganisms involved in N mineralization and cycling have different niche preferences (Erguder et al., 2009; Mrkonjic Fuka et al., 2007). Potential niches for ammonia oxidizing archaea (AOA) involved in nitrification that have been suggested include soils with low nutrient conditions such as low ammonium, low pH as well as low organic C and N (Valentine, 2007; Erguder et al., 2009; Zhang et al., 2010). On the contrary, the counter-part ammonia oxidizing bacteria (AOB) also involved in nitrification tends to be prevalent under conditions with high ammonium, high pH and high bioavailable C and N substrate (Wang et al., 2015; Erguder et al., 2009; Höfferle et al., 2010). Recent studies have also shown that in low oxygen conditions, AOA have a greater advantage over AOB due to their mixotrophic metabolism which allows them to fix and assimilate carbon as alternative energy sources in low ammonia conditions whereas AOB can neither fix nor assimilate carborn due to the fact that they are obligate autotrophs (Zhalnina et al., 2012; Wang et al., 2015). Proteolytic bacteria involved in organic matter degradation are influenced by organic matter quantity and quality while increased protease activity in rhizosphere soils has been associated with increased rates of root exudation (rhizosphere effects) (Mrkonjic Fuka et al., 2007; Sukurai et al., 2007). Given that most proteolytic communities are saprophytic and "Foxy-2" is also saprophytic, it is likely that competition for similar nutrients may occur between "Foxy-2" and proteolytic bacteria (Sakurai et al., 2007; Ndambi et al., 2011). Under controlled conditions, poor nutrient solutions such as sorghum straw have been used to grow "Foxy-2" successfully indicating its potential to grow in poor nutrient conditions (Ndambi et al., 2011). It is therefore likely that "Foxy-2" in *Striga* infested soils, which are often degraded, may indirectly compete with AOA for the generally limited nutrient and water resources since they are both adapted to nutrient limited conditions while in nutrient rich soils (i.e. high organic matter content) AOB and proteolytic bacteria may out-compete "Foxy-2", thus

influencing its effective control of *Striga*. However, experiments need to be carried out to test this hypothesis.

Despite the potential complex interactions discussed, successful establishment and competitive colonization of the rhizosphere is a prerequisite for effective *striga* control by "Foxy-2" (Weller et al., 2002, Raaijmakers et al., 1995). An understanding of the interactions between environmental factors, "Foxy-2" and the abundance and structure of beneficial microorganisms is therefore paramount in developing a comprehensive BCA component as an integral part of an integrated *Striga* management strategy.

1.4. The use of ammonia oxidizing and proteolytic microorganisms as bioindicators

Many soil functions and services rely on soil microorganisms that drive major biogeochemical cycles including N mineralization and cycling and the use of such microorganisms as appropriate indicators for monitoring of soil perturbations has been suggested (Fig.1) (Wessén and Hallin, 2011). Based on their niche differentiation and susceptibility to environmental changes in the soil ecosystem, ammonia-oxidizing bacteria (AOB) and archaea (AOA) are among the top-scored as good bioindicators (Ritz et al., 2009; Wessén and Hallin, 2011; Pereira e Silva et al., 2013). Hence, quantifying their abundance and structural composition changes in "Foxy-2" inoculated soils may be useful to better understand the potential non-targets effects of "Foxy-2" on the N cycling process (Fig.1) (Wessén and Hallin 2011; Pereira e Silva et al., 2013). The *amoA* gene, encoding the ammonia monooxygenase that catalyzes ammonia oxidation, the first rate limiting step in nitrification, was thought to be solely found in AOB. However, recent research has shown that *amoA* gene is also found in AOA and that these two groups could be used as bioindicators for soil perturbation monitoring (Wessén and Hallin (2011).

In addition to the well-studied group of ammonia oxidizers, proteolytic bacteria play a significant role in initiating the remobilization of N from soil organic matter, paving the way for ammonia oxidizers (Fig.1) (Reichardt et al., 2001; Mrkonjic Fuka et al., 2007).

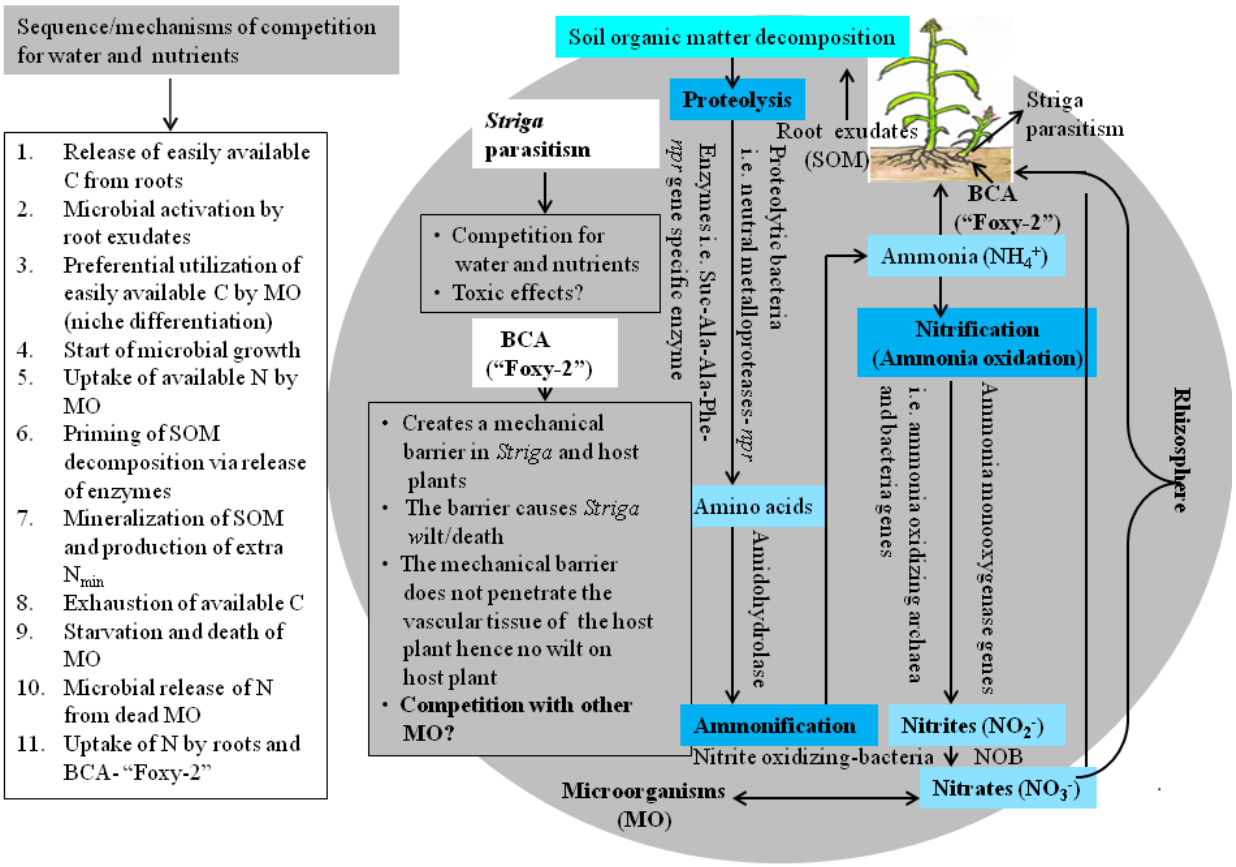

Figure 1. Conceptual model of the soil N mineralization (i.e. N cycling) pathway

Possible interactions between *Striga*, "Foxy-2"and N cyling microorganisms are shown

Arrows represent assimilation by plants and microorganisms as well as enzyme-induced transformations (adapted and modified from: Kirsten et al., 2010; Kuzyakov and Xu, 2013).

The selective inhibition of different groups of bacterial proteases have indicated that neutral metalloproteases (*npr*) are mainly responsible for protein degradation in arable soils hence their use as bio-indicators for the proteolytic processes may be useful (Bach and Munch, 2002, Kamimura, 2000). Moreover, some proteolytic bacteria such as *Bacillus subtilis* play a significant role in pathogenic interactions by producing extracellular proteases through antibiosis that have biocontrol abilities on root-pathogenic micro-fungi yet little is known about their interactions with the beneficial non-pathogenic fungal *Striga* biocontrol "Foxy-2" (Dunn and Handelsman, 2002; Cao et al., 2011). Thus investigating the interaction between "Foxy-2", proteolytic bacterial abundance and enzymatic activities may further advance knowledge on "Foxy-2" non-target effects on proteolysis, a precursory step in the nitrification process, which is crucial in N cycling.

1.5. Factors influencing microbial abundance and community structure

Most studies on AOA, AOB and proteolytic bacteria have indicated soil type, seasonal crop growth dynamics, plant species, fertilization and nutrient bioavailability as major factors determining their population sizes and structures in the rhizosphere (Reichardt et al., 2001; Ying et al., 2010). This section reviews the importance of these factors in soil microbial community dynamics and discusses potential outcomes of interactions between "Foxy-2", soil type (texture), climatic conditions, crop growth stages, nitrifying and proteolytic communities.

1.5.1. Soil type and texture

Soil type influences the size and structure of AOA, AOB, total bacteria, total archaea and proteolytic bacteria in different ways (Mrkonjic Fuka et al., 2007; Wessén et al., 2010; Wessén and Hallin, 2011). Promotion of AOB abundance and composition has been correlated with increased soil ammonium (NH_4^+) concentration while high AOA abundance has been linked to low soil nitrates, low ammonium (NH_4^+) concentration, low pH and low substrate availability, indicating that the abundances and community structures of AOA and AOB are strongly associated with different soil properties (Wessén and Hallin, 2011). Given that "Foxy-2" generally proliferates under low nutrient conditions it is likely to compete for similar niches with AOA that thrive under nutrient-limited conditions (Valentine, 2007; Ndambi et al., 2011).

Soil texture also plays a significant role in determining the microbial community abundance and structure. Clay minerals, for example, have been reported to adsorb some amino acids and peptides making them less available to the proteolytic bacteria than when they are in free solution (Chiarini et al., 1998). It is likely that the neutral metalloproteases (*npr*) responsible for proteolysis and their respective enzymatic activities would be lower in clay soils where the amino acids are bound to clay minerals hence not easily accessible than in sandy soils where their accessibility to amino acids and peptides would be easier (Chiarini et al., 1998; Mrkonjic Fuka et al., 2007). Consequently, the protective nature of clay, through its adsorption of amino acids, would reduce the effect of potential competition for resources with "Foxy-2" on the abundance of proteolytic bacteria. Moreover, clay texture has been shown to have a rhizospheric effect by limiting the availability of root exudates in soils consequently determining the microbial abundance and community structure (Chiarini et al., 1998). As such, clay minerals may limit the extent of "Foxy-2" proliferation in clay than in sandy soils

due to the limitation imposed by micro pore sizes, hence lowering the effect of potential competition for resources on the native nitrifying prokaryotes and proteolytic bacteria in the rhizosphere of clay soils. By contrast, in sandy soils where "Foxy-2" proliferation is not similarly limited, the effect of potential competition for resources on nitrifying and proteolytic prokaryotes may be greater, resulting in reduced abundance and structural diversity of these communities when inoculated with "Foxy-2" (Mezzalama et al., 1997). Moreover, sandy soils are often limited in nutrients and the competition for nutrients between "Foxy-2", proteolytic bacteria and nitrifying prokaryotes may be more in sandy than in clayey soils. It is therefore important to investigate the effects of potential competition between "Foxy-2" and nitrifying prokaryotes and proteolytic bacteria for limiting resources in contrasting soil types and texture as they play significant roles in shaping microbial abundance and structure.

1.5.2. Climatic conditions

Climatic factors play a significant role in microbial interactions with the environment through induced chemical and physical rhizosphere effects and as such may influence the effects of "Foxy-2" on proteolytic bacteria and nitrifying prokaryotes (Bagayoko et al., 2000; Marschner et al., 2002). Short-term changes in climatic conditions such as rainfall and temperature patterns (seasonality) determine soil conditions (i.e. soil temperature, soil moisture and soil pH), and organic carbon (C) through vegetation growth, consequently influencing microbial activities (Insam, 1990). For example, balanced precipitation and evaporation rates are favorable for decomposition (proteolysis) while long periods of drought and soil water saturation slow decomposition (Insam, 1990). Microbial activities, on the other hand, may be lowered by low temperatures (Insam, 1990). Low water availability, mainly influenced by rainfall and temperature patterns in oxygen deficient soils (i.e. clay soils), has been shown to affect microbial community composition by favoring fungi and proteolytic bacteria (Reichardt et al., 2001; Marschner et al., 2002). On the other hand, low water availability in aerated soils (i.e. sandy soils) has been reported to favor ammonia oxidizers such as AOB (Reichardt et al., 2001). The propagation and reproduction of *fusarium* species has been reported to be critically influenced by temperature as well as moisture, with optimal temperatures ranging from 25 to 32°C, whereas continuously moist conditions do not favour the propagation of *fusarium* species (Xu, 2003; Doohan et al., 2003). Given that the optimal temperature conditions for ammonia oxidizing bacteria and archaea ranges between 20-37 °C (Tourna et al., 2008) it is likely that in agroecologies where temperatures are higher than 32

°C, “Foxy-2” may be out-competed by indigenous nitrifying bacteria due to their wide temperature range tolerance while in less moist clay soils, fungi and proteolytic bacteria which are favoured by such conditions may influence “Foxy-2” propagation, lowering its effectiveness (Reichardt et al., 2001; Marschner et al., 2002). Overall, the interactions of “Foxy-2” with proteolytic bacteria and nitrifying prokaryotes may therefore vary depending on the local climatic conditions i.e. rainfall and temperature patterns (seasonality). Hence, further research on the influence of climatic conditions on nitrifying prokaryotes and proteolytic bacteria relative to “Foxy-2” non-target effects may contribute to a better understanding of the key drivers of the abundance and community structure of N cycling microbes in “Foxy-2” treated soils.

1.5.3. Crop growth stage

Plant physiology and vegetation growth stages influence the structure and function of the rhizosphere microorganisms through rhizodeposition (Wieland et al., 2001; Rasche et al.., 2006b) mainly through root exudates which vary in quantity and quality with crop development stages (Chiarini et al., 1998). The early development stages of maize plants, for example, have been reported to increase fast growing bacteria (r-strategists) while the later development stages such as flowering and senescence stage have been shown to favour slow growing bacteria (K-strategists) (Chiarini et al., 1998; Cavaglieri et al., 2009). These changes are mainly linked to root-induced changes in carbon availability and pH changes (Bagayoko et al., 2002). For example, approximately 50% carbon is found in young plant while in mature plants it is much less (Nihorimbere et al., 2011). Consequently, the high carbon as well as steeper pH gradients at the young stages may explain the increase in r-strategists in young stages than the flowering and senescence stage (Bagayoko et al., 2002; Chiarini et al., 1998). The adaptation of r-strategists to rapidly changing pH conditions may thus induce competition between the inoculated “Foxy-2” and the prokaryotic communities that are sensitive to pH changes, such the AOA that are more sensitive than AOB (Hofferle et al., 2010). Also, one may argue that the presence of older decaying roots at the flowering and senescence stages may contribute to increase in organic substrates (SOM decomposition) leading to the dominance of specific abundance and community structure such as AOA that have a preference for organic derived ammonia compared to their AOB counterparts (Broeckling et al., 2008; Hofferle et al., 2010)). Given that “Foxy-2” is a saprophytic fungus, it may further contribute to resource competition for organic derived N particularly with

AOA communities that prefer similar resources (Broeckling et al., 2008; Hofferle et al., 2010). However, this is a gap that needs to be further studied.

Overall, an understanding of the basic principles of rhizosphere microbial ecology including the interaction of "Foxy-2" with biotic and abiotic factors is necessary before the BCA technology can be applied in integrated *Striga* management strategies (Nihorimbere et al., 2011; Watson et al., 2013). By investigating the rhizosphere interaction effects of "Foxy-2", biotic factors (beneficial microorganisms and crop development stages) and abiotic factors (soil type, rainfall and temperature patterns), this study will not only provide the required risk assessment analysis on beneficial microbes in contrasting environmental conditions but also knowledge on the extent to which environmental factors influence the interaction between "Foxy-2" and indigenous microorganism, further deepening our understanding of the role of biotic and abiotic factors in BCAs interactions.

1.6. Striga control methods and Foxy-2 strain as a *Striga* biological control agent

Strategies to control *Striga* should focus on both preventive and management control methods; such as reducing the soil seed bank, improving soil fertility and preventing the production and spread of new seeds (Rathore et al., 2014). Many control methods used can be classified into two main groups; cultural and seed-based methods (Atera et al., 2013). Cultural control methods include non-chemical *Striga* management, ranging from manual weeding, intercropping, crop rotation, catch and trap crops, use of different planting techniques such as late or deep planting and compost application for soil fertility management (Hearne, 2009). Seed-based techniques on the other hand, often involve germplasm based-*Striga* resistance, use of herbicide coated seeds and biological control. Generally, cultural control methods are cost effective, ecologically sound, easy to practice and hence often acceptable and accessible to small-holder farmers while seed-based technologies are costly thus not easily accessed by cash-strapped farmers (Table 1). The specific limitations and advantages of each control method are summarised in Table 1.

Many studies advocate for technologies that aim at reducing *Striga* seed bank as key components of integrated *Striga* control (Oswald, 2005; Vanlauwe et al., 2008; Hearne, 2009). Biological control agents using *Fusarium* isolates may play a significant role since it has been proven to attack *Striga* at all development stages (Kroschel et al., 1996). In most countries including Kenya, BCAs, such as "Foxy-2" are subject to tests of efficacy and

assessment of risks to non-target plants and microorganisms yet, little is known on the non-target effects of "Foxy-2" on other microorganisms (Brimner and Boland 2003; Fravel et al., 2003; Avedi et al., 2014). This is a knowledge gap that needs to be filled prior to inclusion of the BCA component in an integrated *Striga* management strategy. This will not only meet the government regulatory concerns on BCAs but it will also enable easy acceptance and adaptation by users as long as "Foxy-2" is effective in controlling the *Striga* weed (Fravel et al., 2003; Ajanga and Avedi, 2013).

1.6.1. Foxy-2 strain as a *Striga* biological control agent

Ever since its isolation from a diseased *S. hermonthica* in North Ghana by Abbasher et al.,1995, extensive research efforts have been made in relation to the fungal strain (*Fusarium Oxysporum* f.sp.*strigae* ("Foxy-2")) with most studies strikingly coming to the conclusion that "Foxy-2" is a crucial component of a successful intergrated approach to *Striga* management (Beed et al., 2007; Elzein et al., 2008; Elzein et al., 2010; Ndambi et al., 2011). This has mainly been linked to its capability to attack *Striga* at all development stages and it is also considered to be environmentally friendly since it reduces use of chemical-based control of *Striga* weeds (Beed et al., 2007; Elzein et al., 2008; Elzein et al., 2010; Ndambi et al., 2011).

Taxonomic identification of the isolate was done by the Julius-Kühn-Institute, Berlin, Germany (accession number: BBA-67547-Ghana). Proper delivery systems using low cost agricultural by-products (sorghum straw and gum arabic) as inoculum seed treatment technology were optimized for efficient, accessible and increased shelf life delivery systems (Elzein and Kroschel, 2006; Elzein et al., 2006ab; Elzein et al., 2008). Cytological and host range studies also proved that "Foxy-2" does not produce mycotoxins, it is host specific as well as non-pathogenic to crops such as sorghum, pearl millet, maize, rice, cotton, groundnut, cowpea or okra (Ejeta, 2007; Elzein et al., 2010; Ndambi et al., 2011).

Risk assessment studies particularly on non-target microorganisms in varying soil types and climatic conditions remain unexplored as most studies mainly focused on the effectiveness, specificity and mode of action of "Foxy-2" (Elzein and Kroschel, 2006a, b; Elzein et al., 2008; Ndambi et al., 2011). Some efficacy studies on BCAs such as "Foxy-2" have suggested inhibitive rhizosphere microbes, climatic conditions and soil type to be linked to variable *Striga* control by BCAs (Beed et al., 2007; Avedi et al., 2014). These explanations have however, not been validated through experimental studies and there is need to understand the

interactions between “Foxy-2”, biotic and abiotic factors. This will further contribute to a better understanding of the causal factors of variable *Striga* control by “Foxy-2” since variable control has been reported as one of the major disadvantages of “Foxy-2” and other BCAs (Hearne, 2009; Avedi et al., 2014).

Table 1. Main advantages and disadvantages of various approaches to control *Striga*

Cultural methods	Advantages	Disadvantages	References
Manual weeding	Affordable, reduces seed bank and further spread.	Does not necessarily increase yield, not effective when *Striga* population is too high, labour intensive, and time consuming.	Hearne, 2009; Rathore et al., 2014.
Crop rotation	Low cost technology, simultaneously addresses the problem of soil fertility and *Striga* infestation.	Land availability, cultivation has to fit into the local cropping systems, availability and cost of intercrop seeds, varied response of *Striga* and host productivity to rotation crop.	Hearne, 2009.
Intercropping (including catch and trap cropping)	Reduces *Striga* emergence and effect on crop yield.	Additional labour through regular cutting to reduce competition, varied response of *Striga* and host productivity to intercrop, availability and cost of intercrop seed.	Hearne, 2009.
Soil fertilisation, organic and inorganic	Organic manure-causes *Striga* seed decay, inorganic N-has inhibitory effect on *Striga.*	Availability and cost of input, labour cost in application, variability in *Striga* control.	Hearne, 2009, Ayongwa et al., 2011.

Table 1. Main advantages and disadvantages of various approaches to control *Striga* (continued)

Seed-based methods	Advantages	Disadvantages	References
Herbicide seed dressing	Provides crop protection before germination and several weeks after emergence.	Dressed seeds need to be bought by farmers every season, availability and affordability of dressed seeds, farmers' seed preference.	Kanampiu et al., 2001, Hearne, 2009.
Resistant/Tolerant varieties	Reduces *Striga* densities.	High variability among commercial varieties, often expensive, hence not accessible by resource poor farmers, low public acceptance especially in cases where yields are lower than local varieties.	Ndambi et al., 2011.
Push-Pull technology	Reduces *Striga* emergence, added benefits of high quality livestock feed (i.e. Desmodium and Napier grass), improved soil fertility through biological N fixation, and reduces use of chemicals (i.e. maize stem borer moths' control).	Longer timeframe to be effective, added cost of Desmodium and Napier grass seed, reduced crop yield due to competition between maize and the Desmodium and/or Napier especially under drought stress.	Vanlauwe et al., 2008.
Biocontrol agents (i.e. *Fusarium* and mycorrhizae strains).	Reduces *Striga* seed bank and delays emergence time of *Striga*. Reduces use of chemicals.	Variable control levels and response of host productivity.	Ndambi et al., 2011; Hearne, 2009.

1.7. Overview of the relevant techniques

1.7.1. Measurement of microbial community abundance

1.7.1.1. Quantitative Polymerase Chain Reaction (qPCR)

For the past two decades, the application of qPCR in combination with the extraction of nucleic acids (DNA and RNA) has been an important analytical tool in microbial ecology (Giovannoni et al., 1990). The use of qPCR has gained interest over conventional culture–based techniques which are not only laborious but are also limited in that they can only estimate 1-10% of the microbial community (Nannipieri et al., 2003; Nihorimbere et al., 2011).

Quantitative-PCR (often referred to as real time PCR) is based on multiple amplification cycles in which template DNA/ RNA generates a mixture of microbial genes signatures present in a sample through denaturation (Rastogi and Sani, 2011). This step is followed by annealing of two oligonucleotide primers targeting specific sequences and subsequent extension of a complementary strand from each annealing primer by a thermostable DNA polymerase, resulting in an exponential increase in amplicon numbers during PCR (Smith and Osborn, 2009). The increase in amplicon numbers is recorded in real time during the PCR via detection of a fluorescent reporter i.e. SYBR Green I that indicates amplicon accumulation during every cycle (Smith and Osborn, 2009). Detailed information about the primers used and the number of cycles is presented in Chapter 2.

SYBR Green I is often used as an intercalation dye since it is more economical for real-time analysis (Giglio et al., 2003). When bound to DNA, a fluorescent signal is emitted following light excitation. However, in its unbound state, SYBR Green I does not fluoresce (Smith and Osborn, 2009).

A post–PCR dissolution (melting) curve analysis is then carried out to confirm that the fluorescence signal is generated only from a target template and not from the formation of nonspecific PCR products (Giglio et al., 2003; Gonzalez-Escalona et al., 2006). For the quantification of the unknown environmental samples, qPCR amplification from a range of serial dilutions of a known concentration of template DNA is used to construct standard curves (Smith and Osborn, 2009).

The advantages of qPCR over conventional culture-based techniques include its robustness, specific and sensitive nature to detect even minute amounts of DNA in samples (Smith and Osborn, 2009). Moreover, quantification data generated can be used to relate gene abundance

and /or level (in terms of transcript numbers) of gene expression in comparison with various abiotic or biotic factors and or biological activities and process rates (Smith and Osborn, 2009).

Even though qPCR is commonly accepted as the gold standard of the century for accurate abundance quantification, one of the major limitations of this technique is the requirement for prior knowledge of the specific target gene of interest (Smith and Osborn, 2009). To overcome this bottleneck, the combination of qPCR with other assessment methods such as terminal restriction fragment length polymorphism (T-RFLP) has been recommended for furthering biological understanding in microbial ecology (Smith and Osborn, 2009). The qPCR technique was used in Chapter 2-5 to measure the abundance of proteolytic bacteria and nitrifying prokaryotes.

1.7.2. Measurement of microbial community structure

1.7.2.1. Terminal restriction fragment length polymorphism (T-RFLP)

In contrast to PCR-detection which is a highly adapted and sensitive identification method for a single strain, molecular finger printing technologies such as denaturing or temperature gradients gel electrophoresis (DGGE/TGGE), single strand conformation polymorphism (SSCP), ribosomal intergenic spacer analysis (RISA) and terminal restriction fragment length polymorphism (T-RFLP) allow ecologists to rapidly profile whole populations in an ecosystem (Liu et al., 1997; Lefebvre et al., 2010). Within the range of these technologies T-RFLP is possibly the most utilized and preferred technique among the several existing finger printing methods (Lefebvre et al., 2010).

This stepwise approach couples PCR and rDNA extraction methods just like qPCR does. However, in T-RFLP, one of the two primers is fluorescently labeled at the 5' end. The fluorescent dye ensures that the sizes of only the terminal restriction fragment (T-RF) can be determined and the respective amount is quantified (Liu et al., 1997). Principally, rDNAs are obtained by PCR amplification by using universal or domain specific primers and the product is purified and subjected to restriction enzyme digestion with enzymes that have 4 base pair recognition sites (Liu et al., 1997; Schütte et al., 2008). Prior to electrophoresis, the digested samples are mixed with deionized formamide and a DNA fragment length standard (500 ROX™), denatured and chilled on ice. Electrophoresis is done and generates the fluorescently-labeled terminal restriction fragments which are then separated and detected on an automated DNA sequencer. The output is a series of peaks (fragments) of various sizes and heights that

represent the profile of each sample. Specific software such as Peak Scanner™ is then used to compare relative lengths of T-RFs with the internal size standard and to compile electropherograms into numeric data sets (Liu et al., 1997). Following normalization, statistical analyses can be used to determine the similarity of communities from their T-RFLP profiles (Dunbar et al., 2001).

The main advantages of T-RFLP are; it is culture-independent, rapid, sensitive and a highly reproducible method of assessing diversity of complex communities without the need for genomic sequence information (Liu et al., 1997). Although T-RFLP is more demanding in cost and labour compared to other finger printing techniques such as RISA and DGGE, the accurate sizing of the terminal restriction fragments (T-RFs) gives it an added advantage over the common gel electrophoretic systems (Widmer et al., 2006). In addition, T-RFLP analysis yields numeric data that can be further evaluated with standard statistical analyses and results may be compared with data stored in sequence databases (Schütte et al., 2008). This method is thus an easy means to assess changes in microbial community structure that occur on temporal or spatial scales or that occur in response to environmental perturbations (Liu et al., 1997). T-RFLP technique was used in Chapter 3 for assessing changes in microbial community structure in response to vegetation growth stages, soil type and treatment application ("Foxy-2" inoculation versus no "Foxy-2" inoculation and organic fertilization).

1.7.3 Measurement of enzymatic activities

Several experiments have shown that most organisms are functionally redundant and the link between their abundance and/or diversity and soil functions is important in understanding the relations between microbial community structure and function (Nannipieri et al., 2003). Metabolic reactions of living cells are catalyzed by enzymes, and their determination is important in giving a functional component to the molecular techniques. The principle of enzymatic analysis is based on enzymatic reaction with specific substrates and following the conversion product by different methods such as colorimetric, radio-labelled and fluorimetric methods (Marx et al., 2001). Among these methods, the fluorimetric method which was used in this study is probably the most commonly used. This method is based on using fluorimetrically-labelled substrates and its main advantage is that the reaction product can be measured directly in the microplate without prior extraction and purification of the product unlike many enzyme assays (Marx et al., 2001). As such, this saves time and allows a large number of soils and substrate analogues to be tested. Furthermore, the method is highly sensitive and allows small quantities of hydrolyzed substrates to be easily measured. Detailed

information on enzymatic analysis carried out in this study is presented in Chapter 5 where it was used to assess non-target effects of "Foxy-2" on *npr* protelytic potential enzyme activities.

1.8. Guiding hypotheses

The hypotheses addressed in this study were:

- Soil inoculation with "Foxy-2" increases C and N energy resource demand in the rhizosphere and induces competition effects causing reduced abundance of nitrifying prokaryotes.
- The presumed "Foxy-2" C and N resource competition effects on nitrifying prokaryotes are likely to be more detectable in a sandy than in a clay textured soil due to more limited C and N substrates in sandy than in clay soils.
- Addition of high quality organic inputs compensates the presumed "Foxy-2" competition effects through additional supply of C and N resources.
- Soil type, seasonality and crop growth stage exert stronger influences on the dynamics of nitrifying prokaryotes communities than the fungal biocontrol agent ("Foxy-2") as they have been shown to be the major determinants of microbial abundance and community structure.
- "Foxy-2" reduces the abundance and potential proteolytic bacteria activities due to competition for organic resources since both "Foxy-2" and proteolytic bacteria are saprophytic. It was further assumed that the resource competition effects would be greater in sandy than in clay soils and that application of high quality organic input would mask the competition effects by providing more C and N substrates.

1.9. Guiding Objectives

The goal of this study was to assess the non-target effects of "Foxy-2" on the abundance and community structure of total and nitrifying prokaryotes as a risk assmessment environmental measure to ensure the safety of "Foxy-2" proir to large scale field application. In order to advance our understanding on the effects of "natural" factors (i.e. seasonality, soil type and crop growth stages), the study was carried out both under controlled and field conditions. In

addition to this, the study developed and optimized protocols for quantifying proteolytic bacteria abundance and enyzmatic activities in tropical soils as a proxy to understanding "Foxy-2" effects on organic N cycling processes. The specific objectives were;

- To determine the abundance of total and nitrifying prokaryotes in a sand and clay textured soil inoculated with and without "Foxy-2", and its combination with high quality organic inputs (i.e. *Tithonia diversifolia*).
- To investigate the effects of soil type, seasonality and crop growth stages relative to the non-target effects of "Foxy-2" inoculation on the abundance and community structure of nitrifying prokaryotes.
- To optimize qPCR and T-RFLP methods for assessing the abundance and community structure of total and proteolytic bacteria in tropical soils treated with different qualities of organic inputs.
- To apply the optimized qPCR protocols to asses the non-target effects of "Foxy-2" inoculation on the abundance and enzymatic activities of proteolytic bacteria.

1.10. Structure of thesis

Subsequent to Chapter 1 that presents the general introduction, Chapter 2 of this thesis, accepted for publication in Biological Control, is entitled 'Promoting effect of *Fusarium oxysporum* [f.sp. *strigae*] on abundance of nitrifying prokaryotes in a maize rhizosphere across soil types'. This chapter mainly focuses on the undesired non-target effects of "Foxy-2" on the abundance of total archaeal and bacterial communities as well as their nitrifying counter parts under controlled conditions using two contrasting soils from Kenya. Chapter 3 of the thesis, 'Soil type, seasonality and crop growth stage exert a stronger effect on rhizosphere prokaryotes than the fungal biocontrol agent *Fusarium oxysporum* f.sp. *strigae',* submitted to the journal of Applied Soil Ecology, reports the results of a field experiment undertaken to understand the effects of soil type, seasonality and crop growth stages in relation to the non-target effects of "Foxy-2" on the abundance and community structure of nitrifying prokaryotes. This chapter explores the extent to which seasonality (i.e. short and long rains cropping seasons), soil type and vegetation growth stages influence the abundance and community structure of nitrifying prokaryotes relative to "Foxy-2" inoculation. Chapter 4 is entitled 'Lasting influence of biochemically contrasting organic inputs on abundance and community structure of total and proteolytic bacteria in tropical soils and has been published

in Soil Biology & Biochemistry. This chapter focuses on a methodological development strategy for measurement of proteolytic abundance in tropical soils and specifically examines the effect of biochemically contrasting inputs on the abundance and community structure of total and proteolytic bacteria in tropical soils. Chapter 5 which is linked to the previous study (Chapter 4) evaluated the non-target effects of "Foxy-2" on proteolytic (i.e. *npr* gene encoding neutral metalloproteases) bacteria abundance and their enzymes activity (i.e. *npr* gene specific Suc-Ala-Ala-Phe-AMC). Chapter 6 integrates all results from the previous chapters and other relevant information into a general discussion and considerations for future applications of BCAs on *Striga* control in Kenya and in SSA at large. Chapter 7 summarizes all references used in this thesis. Summaries (in English and German) and appendices (list of additional articles coauthored and published during the doctoral time frame; curriculum vitae) finalize the thesis.

CHAPTER 2

Promoting effect of *Fusarium oxysporum* [f.sp. *strigae*] on abundance of nitrifying prokaryotes in a maize rhizosphere across soil types

2. Promoting effect of *Fusarium oxysporum* [f.sp. *strigae*] on abundance of nitrifying prokaryotes in a maize rhizosphere across soil types[1]

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2.1. Abstract

The integrated application of resistant crop varieties with biological control agents (BCAs) such as the *Fusarium oxysporum* [f.sp. *strigae*] strain "Foxy-2" has shown to be effective in fighting off the weed *Striga hermonthica* which is parasitic to several cereals cultivated in Sub-Saharan Africa (Schaub et al., 2006; Venne et al., 2009). "Foxy-2" proliferates in the rhizosphere and has been mainly studied for its virulence and mode of action. Contrary, no understanding is available regarding its interactions with key rhizosphere microorganisms steering relevant nutrient cycles in soils including nitrogen (N). In this study, we tested the hypothesis that "Foxy-2" displaces indigenous prokaryotic, N cycling communities in the maize rhizosphere due to competition for organic resources. Consequently, we evaluated if the application of an N-rich organic residue (i.e., *Tithonia diversifolia* with C/N ratio = 13, lignin content = 8.9%, polyphenol content = 1.7%) compensates these presumed competition

[1] **This Chapter has been reprinted from:**
Musyoki, M.K., Cadisch, G., Enowashu, E., Zimmermann, J., Muema, E., Beed, F., Rasche, F. (2015) Promoting effect of *Fusarium oxysporum* [f.sp. *strigae*] on abundance of nitrifying prokaryotes in a maize rhizosphere across soil types, Biological Control, 83, (37-45). (DOI:10.1016/j.biocontrol.2014.12.013), with permission from Elsevier (Copyright ©2014).
The original publication is available at:
http://www.sciencedirect.com/science/article/pii/S104996441400262X

effects. In a rhizobox experiment, quantitative polymerase chain reaction was used to follow the response of rhizosphere ammonia-oxidizing archaea (AOA) and bacteria (AOB) as well as total bacteria and archaea following "Foxy-2" inoculation in two physico-chemically contrasting soils (sandy Ferric Alisol versus clayey Humic Nitisol). Soils were treated with or without "Foxy-2", *S. hermonthica* seeds, and *T. diversifolia* residues. Contrary to our expectations, we observed a distinct soil texture dependent, promoting effect of "Foxy-2" on rhizosphere prokaryotes. Abundance of AOA and total prokaryotic communities increased in response to "Foxy-2" in the sandy soil, while AOB remained unaffected. This effect on AOA was accelerated when *T. diversifolia* residues were incorporated. Further, in the clayey soil, AOA abundance was promoted when exposed to *S. hermonthica* infestation of maize. This suggested their capability to adapt to this biotic stress situation. It was concluded that ''Foxy-2'' did not pose a negative effect on targeted indigenous microorganisms, but the underlying mechanisms for the observed promoting effect of AOA abundance by ''Foxy-2'' inoculation are yet to be understood.

Key words: *Fusarium oxysporum* [f.sp. *strigae*]; *Striga hermonthica*; organic resource competition; nitrifying prokaryotes; rhizosphere; quantitative PCR.

2.2. Introduction

The weed *Striga hermonthica* represents a major constraint to food security in Sub-Saharan Africa (Midega et al., 2013; Parker, 2014). It inflicts yield losses (ranging from 30 to 90%) on staple crops such as maize, pearl millet, sorghum, and rice (Ejeta, 2007; Watson et al., 2007; Rebeka et al., 2013). In order to reduce the economic impacts caused by *S. hermonthica*, it has been widely accepted that a single control option is not effective, and, hence, a combination of sustainable multi-pronged technologies is recommended (Berner et al., 1996; Hearne, 2009; Atera et al., 2012).

Integration of biological control agents (BCAs) and tolerant crop varieties provide effective and environmentally sound benefits for *S. hermonthica* control (Beed et al., 2007; Venne et al., 2009; Rebeka et al., 2013). *Fusarium oxysporum* [f.sp. *strigae*] "Fos" strain "Foxy-2" was approved for biological control of *S. hermonthica* on maize and sorghum and it is one of the most extensively studied model strains of "Fos" (Elzein and Kroschel, 2004a; Ndambi et al., 2011; Zarafi et al., 2015). A seed treatment technology for field application of "Foxy-2" has

been developed by the University of Hohenheim (Stuttgart, Germany) in collaboration with the seed company SUET Saat-und Erntetechnik GmbH (Eschwege, Germany) (Elzein et al., 2010; Elzein et al., 2006a). "Foxy-2" was reported to be effective in reducing *S. hermonthica* germination by up to 90% under field (e.g., Benin, Burkina Faso, Nigeria) and greenhouse conditions (Ciotola et al., 2000; Elzein et al., 2006a; Venne et al., 2009). On the other hand, recent findings have indicated that "Foxy-2" did not achieve biological control of *S. hermonthica* parasitizing maize in Western Kenya further raising questions on the effectiveness and adaptation mechanisms of "Foxy-2" as a BCA (Avedi et al., 2014). Previous studies also reported that "Foxy-2" was specific to its host *S. hermonthica* (Elzein and Kroschel, 2006; Ndambi et al., 2011). Conversely, recent host range studies by Zarafi et al., (2015) found solanaceous crops (e.g., Irish potato, tomato, eggplant) to be susceptible to "Foxy-2", while biological control of *S. hermonthica* was only realized in cereal crops (e.g., sorghum, millet, maize). These contradictory findings disclose that advanced in depth understanding on rhizosphere processes are essential to understand the obvious, but underestimated interactions between "Foxy-2", crops and root associated soil microorganisms taking also the local environmental conditions such as varying soil types and climate into account (Avedi et al., 2014; Zarafi et al., 2015).

For large scale use of microbial BCAs in agricultural soils, their role and potential impact on the soil ecosystem (e.g., rhizosphere) needs to be thoroughly studied as part of ecological risk assessment (Mezzalama et al., 1997). There has been certain concern regarding the potential of BCAs to displace or suppress resident soil microorganisms with key functions in element cycles (e.g., nitrogen (N)) due to competition for resources in terrestrial ecosystems (Mezzalama et al., 1997; Edel-Hermann et al., 2009; Martin-Laurent et al., 2013). Hence, it is essential to assess if microbial BCAs such as "Foxy-2", which primarily performs in the rhizosphere, results in undesired side-effects on crop root-associated microbial communities. This includes those involved in nitrification (i.e., ammonia oxidation), a central component of soil N cycling (Hallin et al., 2009; Jia and Conrad, 2009; Wessén et al., 2010).

Previous studies on "Foxy-2" have mainly focused on virulence, efficacy, specificity and its mode of action (Elzein et al., 2004; Schaub et al., 2006; Ndambi et al., 2011; Avedi et al., 2014; Zarafi et al., 2015). But there is little information available regarding its potential ecological impacts on key rhizosphere microorganisms steering the N cycle. It has been reported that fungi draw nutrients from their host through the production of growth regulators (Strange, 2005). Accordingly, such altered nutrient availability in a crop rhizophere

inoculated with a fungal BCA such as "Foxy-2" may therefore affect the indigenous soil microbial population (Gullino et al., 1995; Mezzalama et al., 1997; Martinuz et al., 2012a). However, there remains a considerable knowledge gap on the actual influence of "Foxy-2" on ammonia-oxidizing bacteria (AOB) and archaea (AOA) which have been shown to be sensitive to soil disturbance including the exposure to fungi (Pereira e Silva et al., 2013). In addition, Wessén and Hallin (2011) showed that abundance and community structure of ammonia-oxidizing soil microorganisms were suitable bio-indicators for measuring the effect of soil perturbations and soil health.

A few years ago, the *amoA* gene, encoding the α-subunit of the enzyme ammonia monooxygenase which catalyzes ammonia oxidation, was thought to be unique to AOB (Treusch et al., 2005). Recent findings, however, indicated that AOA also play a major role in ammonia oxidation in agricultural soils (Leininger et al., 2006). Several reports suggested a terrestrial niche differentiation of both AOA and AOB (Jia and Conrad, 2009; Höfferle et al., 2010; Wessén et al., 2010). In this respect, potential niches for AOA were found in low nutrient (e.g., low ammonia substrate conditions) and low pH soils. Contrastingly, AOB responded sensitively to high ammonia concentrations (Valentine, 2007; Erguder et al., 2009; Höfferle et al., 2010).

Accordingly, it can be hypothesized that the saprophytic fitness of "Foxy-2" enabling its survival under low resource conditions may render it aggressively competitive over AOA in low nutrient soils (Elzein et al., 2010). On the other hand, AOB may be competitive against "Foxy-2" in nutrient rich soil environments fertilized with e.g., N-rich organic residues. Hence, there is a critical need to study the potential side-effects of "Foxy-2" on both AOB and AOA to underline that no negative consequences on indigenous microorganisms occur in the soil ecosystem after "Foxy-2" inoculation. The primary objective of this study was thus to assess potential impacts of "Foxy-2" on the abundance of total bacteria and total archaea as well as their ammonia oxidizing counterparts (i.e., AOB, AOA) in *S. hermonthica* affected maize rhizospheres. We compared the effects of "Foxy-2" inoculation alone and its combination with an N-rich organic resource (i.e., *Tithonia diversifolia*) on abundance of respective genes in a low (sandy Ferric Alisol) and high (clayey Humic Nitisol) nutrient resource soil taken from the central highlands of Kenya. We postulated that application of *T. diversifolia* residues redeems the presumed competition effects of "Foxy-2" via additional N resource availability in soils sustaining the abundance of ammonia oxidizing prokaryotes.

2.3. Material and methods

2.3.1. Fungal biocontrol agent

The fungal strain *Fusarium oxysporum* [f.sp. *strigae*] ("Foxy-2") used in this study was isolated from diseased *S. hermonthica* collected in North Ghana (Abbasher et al., 1995). Identification of the isolate was done by the Julius-Kühn-Institute, Berlin, Germany (accession number: BBA-67547-Ghana). However, the true identity of "Foxy-2" remains debatable as it did not cause disease on *S. hermonthica* (Avedi et al., 2014) but was pathogenic to solanaceous crops (Zarafi et al., 2015). The isolate was preserved at -80°C at the Institute of Plant Production and Agroecology in the Tropics and Subtropics, University of Hohenheim, Stuttgart, Germany.

2.3.2. Rhizobox experiment

Maize (*Zea mays* L., variety 'WH507', provided by Western Seed Company Ltd., Kitale, Kenya) was selected as the test crop as it is preferred by smallholder farmers in Western Kenya due to its tolerance to *S. hermonthica*. Maize seeds were coated with "Foxy-2" (1.15 x 10^5 colony forming units per seed) using gum Arabic (40%) as an adhesive providing a uniform inoculum coverage (Elzein et al., 2006b; SUET GmbH). After coating, coated and uncoated (controls) maize seeds were allowed to germinate on wet filter paper to ensure that only germinated seeds were used for the rhizobox experiment. Meanwhile, *S. hermonthica* seeds were surface sterilized according to Elzein et al. (2010) and germination ability of seeds (75%) was checked as described by Kroschel (2002).

Rhizoboxes (3 x 7 x 20 cm) were filled with dry soils (165 g) derived from two contrasting non *S. hermonthica* infested field sites in the central highlands of Kenya: Embu (0° 30' S, 37° 30' E; 1380 m above sea level (a.s.l.)) and Machanga (0° 47' S, 37° 40' E; 1022 m a.s.l.). Soils differed in physical properties: the Embu soil was a clayey Humic Nitisol (17% sand, 18% silt, 65% clay) derived from basic volcanic rocks, while the Machanga soil was a sandy Ferric Alisol (67% sand, 11% silt, 22% clay) derived from granitic gneisses (FAO, 1998). Each rhizobox was filled with 1 cm ground layer of vermiculite (grain size 3-8 mm) for drainage improvement. On top of this layer, the soil was added and adjusted to 50% water holding capacity.

Both soils were artificially infested with the sterilized *S. hermonthica* seeds (20 mg seeds 165 g dry $soil^{-1}$). *S. hermonthica* seeds were thoroughly mixed with the moist soils and pre-conditioned at 28°C in the dark for 7 days (Kroschel, 2002). After this step, germinated maize

seedlings were introduced into the rhizoboxes. A 1 cm layer of vermiculite was placed as a top layer after planting the maize seedling to reduce evaporation and facilitate water drainage.

Boxes were placed in an incubation chamber (12 hours artificial light (1000 µmol m^{-2} s^{-1}) and darkness at 28/21°C (day/night) for 6 weeks. Two and 4 weeks after incubation start; fertilization with inorganic liquid fertilizer (0.2% Wuxal N-P-K (8-8-6), Aglukon GmbH, Düsseldorf, Germany) was applied to avoid nutrient deficiency. In addition, a treatment with organic residues was included by incorporating air-dried and ground (particle size 1 to 3 mm) leaf and stem material of *T. diversifolia* (1 g dry matter 100 g dry $soil^{-1}$; 3.2% N) into soils. Non-fertilized treatments were included as controls.

The rhizobox experiment was arranged as a completely randomized design with 6 treatments and 3 replicates each: i) uncoated maize with *no S. hermonthica* (C), ii) uncoated maize and *S. hermonthica* (C + S), iii) coated maize with "Foxy-2" (F), iv) coated maize with "Foxy-2" and *S. hermonthica* (F + S), v) coated maize with "Foxy-2" and *T. diversofolia* (F + T), and vi) coated "Foxy-2", *S. hermonthica* and *T. diversofolia* (F + S + T).

Rhizosphere samples were taken 14, 28 and 42 days after planting (DAP). For this step, the rhizobox was opened carefully and approximately 2 g of root adhered soil was taken with care not to destruct the rooting system. Rhizosphere soil was gently scraped off with sterile forceps and transferred into sterile sampling bags. Chemical analyses were done using bulk soil samples at 42 DAP. Rhizosphere soil samples were freeze dried and stored at -20°C until molecular analysis, while bulk soils for chemical analyses were directly maintained at -20°C.

2.3.3. Quantification of prokaryotic abundance

Four hundred mg of freeze dried rhizosphere soil was used for DNA extraction from each of the 3 replicates per treatment. Soil DNA was extracted using the FastDNA® Spin for Soil Kit (MP Biomedicals, Solon, Ohio, USA) following the manufacturer's instructions. Quality of extracted DNA was checked on 1.5 % (w/v) agarose gels. DNA extracts were quantified (Nanodrop ND-1000, NanoDrop Technologies, Wilmington, USA) and stored at -20°C.

Abundance of both total and ammonia oxidizing prokaryotic communities was determined by DNA-based quantitative PCR (qPCR) using bacterial and archaeal 16S rRNA genes (total community) as well as bacterial (AOB) and archaeal (AOA) *amoA* genes as molecular markers (Table 2) (Rasche et al., 2011). For standard preparation, amplicons from each target gene were purified (Invisorb Fragment CleanUp kit, Stratec Molecular GmbH, Berlin, Germany), ligated into the StrataCloneTM PCR cloning vector pSC-A (Stratagene, La Jolla,

CA, USA) and ligation products were transformed with StrataClone Solopack competent cells (Startagene).

The qPCR assays were carried out in a 25 µl reaction containing 12.5 µl of Power SYBR® green master mix (Applied Biosystems, Foster City, CA, USA), 1 µl primer (each 0.4 µM), 0.25 µl T4 gene 32 protein (500 ng μl^{-1}, MP Biomedicals) and 10 ng template DNA. For Machanga soils, 20 ng were used as template for quantification of AOB and AOA abundance. The qPCRs were performed on a StepOnePlus™ Real-Time PCR System (Applied Biosystems) and were started with 10 min at 95°C, followed by amplification cycles specific for each target gene (Table 2). Melting curve analysis of amplicons was conducted to confirm that fluorescence signals originated from specific amplicons and not from primer-dimers or other artifacts. Each DNA sample was processed in triplicate reactions, whereas the standard curves were generated using duplicate serial dilutions of isolated plasmid DNA containing the genes studied (Rasche et al., 2011).

2.3.4. Measurement of soil chemical parameters

Total carbon (TC), total nitrogen (N_t), extractable organic C (EOC), extractable N (EON) and pH of soils were recorded using bulk soils from each rhizobox shortly after the last rhizosphere soil sampling (DAP 42). The pH analyses were conducted in a soil water ratio of 1:2.5 using a pH meter (inoLab® Labor-pH-Meter, WTW GmbH, Weilheim, Germany). TC and N_t were quantified by dry combustion (vario MAX CN analyzer, Elementar Analysensysteme GmbH, Hanau, Germany). For EOC measurement, 5 g of soil were extracted with 20 ml 0.5 M K_2SO_4, shaken horizontally (250 rpm) for 30 min and filtered (Rotilabo-Rundfilter AP55.1 (retention of 2-3 µm), Carl Roth GmbH). EOC concentration in filtered extract was measured on an Analytik Jena Multi N/C 2100 analyzer (Analytik Jena AG, Jena, Germany). Ammonium (NH_4^+) and nitrate (NO_3^-) were extracted with 1 M KCl (soil to extractant ratio (w/v) of 1:4), shaken on a horizontal shaker for 30 min at 250 rpm and filtered (Rotilabo-Rundfilter AP55.1, Carl Roth GmbH). Concentrations of NH_4^+ and NO_3^- were measured on an auto-analyzer (Bran & Luebbe, Norderstedt, Germany) (Mulvaney, 1996), while EON was determined as the difference between N_t and mineral N (NH_4^+ and NO_3^-) according to Rousk and Jones (2010).

2.3.5. Statistical analysis

Statistical analyses were performed using R software (Software R 3.0.1, R foundation for Statistical Computing, Vienna, Austria, http://www.R-project.org). To assess effects of

"Foxy-2", organic N addition (*T. diversifolia*), *S. hermonthica*, sampling time and soil type on abundance of assayed genes and soil chemical parameters, a multifactoral ANOVA was done in R. Homogeneity of variance and normality were tested on the residuals of each model. Logarithmic transformations were performed when required. Least squares means comparison between soil types, treatments and sampling time was done using the Tukey's range test ($P<0.05$). For graphical visualization, gene abundance data were shown without standard errors due to back transformation of the least square means. In addition, Pearson's linear correlation coefficients were calculated for assessing relations between abundance of studied genes with soil chemical parameters.

Table 2. Description of primer sets, PCR ingredients and amplification details used for qPCR analysis

Target group	Primer set	Thermal cycling profile	Reference
Bacterial 16 S rRNA gene	Eub338f	40 cycles	Lane (1991)
	Eub518	95°C 30s, 55°C 35s ,72°C 45s	Muyzer et al. (1993)
Archaeal 16S rRNA gene	Ar109f	40 cycles	Lueders and Friedrich (2000)
	Ar912	95°C 30s, 52°C 35s ,72°C 45s	Lueders and Friedrich (2000)
Bacterial *amoA* gene	*amoA*-1f	45 cycles	Rotthauwe et al. (1997)
	amoA-2r	95°C 45s, 57°C 60s ,72°C 60s	Rotthauwe et al. (1997)
Archaeal *amoA* gene	Arch-*amoA*F	45 cycles	Francis et al. (2005)
	Arch-*amoA*R	95°C 45s, 53°C 60s,72°C 60s	Francis et al. (2005)

2.4. Results

2.4.1. Microbial gene abundance

2.4.1.1. Total archaeal and bacterial communities

In the sandy soil (i.e., Machanga), archaeal 16S rRNA gene abundance in F and F + S treatments remained constant during the incubation period, while that in C and C + S decreased over time (Fig. 2a). Gene copies in soils treated with "Foxy-2" and *T. diversifolia* (F + S + T and F + T) were higher than treatments without *T. diversifolia* (C, C + S and F + S) in the sandy soils 28 days after planting (DAP) ($P<0.05$). In the clayey soil (i.e., Embu), "Foxy-2" treatments (F, F + S) and the addition of the N-rich organic residue (i.e., F + S + T, F + T) did not influence the abundance of archaeal 16S rRNA genes ($P>0.05$).

Total bacterial 16S rRNA gene copy numbers were promoted by F + S in comparison to C in the sandy soil ($P<0.05$; Fig. 2c). On the contrary, gene copies in treatment F under the same soil were similar to C and C+S. Treatments with *T. diversifolia* addition (F + S + T, F + T) strongly increased gene copy numbers in comparison to treatments that did not receive *T. diversifolia* (i.e., C, C + S, F, F + S). Similarly, in the clayey soil, F + S + T and F + T increased total bacterial abundance across all sampling time points although to a lesser extent ($P<0.05$; Fig. 2d). On the other hand, total bacterial abundance in C + S and C did not show any difference ($P>0.05$).

2.4.1.2. Archaeal *amoA* gene abundance

Archaeal *amoA* gene copies showed higher abundance in F compared to C and C + S in the sandy soil (Fig. 3a). Similarly, F + S + T and F + T revealed increases of archaeal *amoA* gene abundance in comparison to C, C + S and F + S ($P<0.05$). However, when combined with *S. hermonthica* (F + S), there were no significant differences between F + S, C and C + S ($P>0.05$). There were also no differences in archaeal *amoA* gene copy numbers between C and C + S ($P>0.05$).

In the clayey soil, F had higher archaeal *amoA* gene copy numbers compared to C, F + S and F + S + T ($P<0.05$; Fig. 3b). An exception was at 14 DAP, where a transient decline was observed. F + T had higher *amoA* gene abundance in comparison to C, F + S and F +S + T at the last two sampling dates ($P<0.05$). Treatment F + S + T was not different from C and F + S at 28 and 42

DAP (P>0.05). Archaeal *amoA* gene abundance remained stable in *S. hermonthica* infested soils (C + S) and was higher than C, F, F + S and F + S + T (P<0.05).

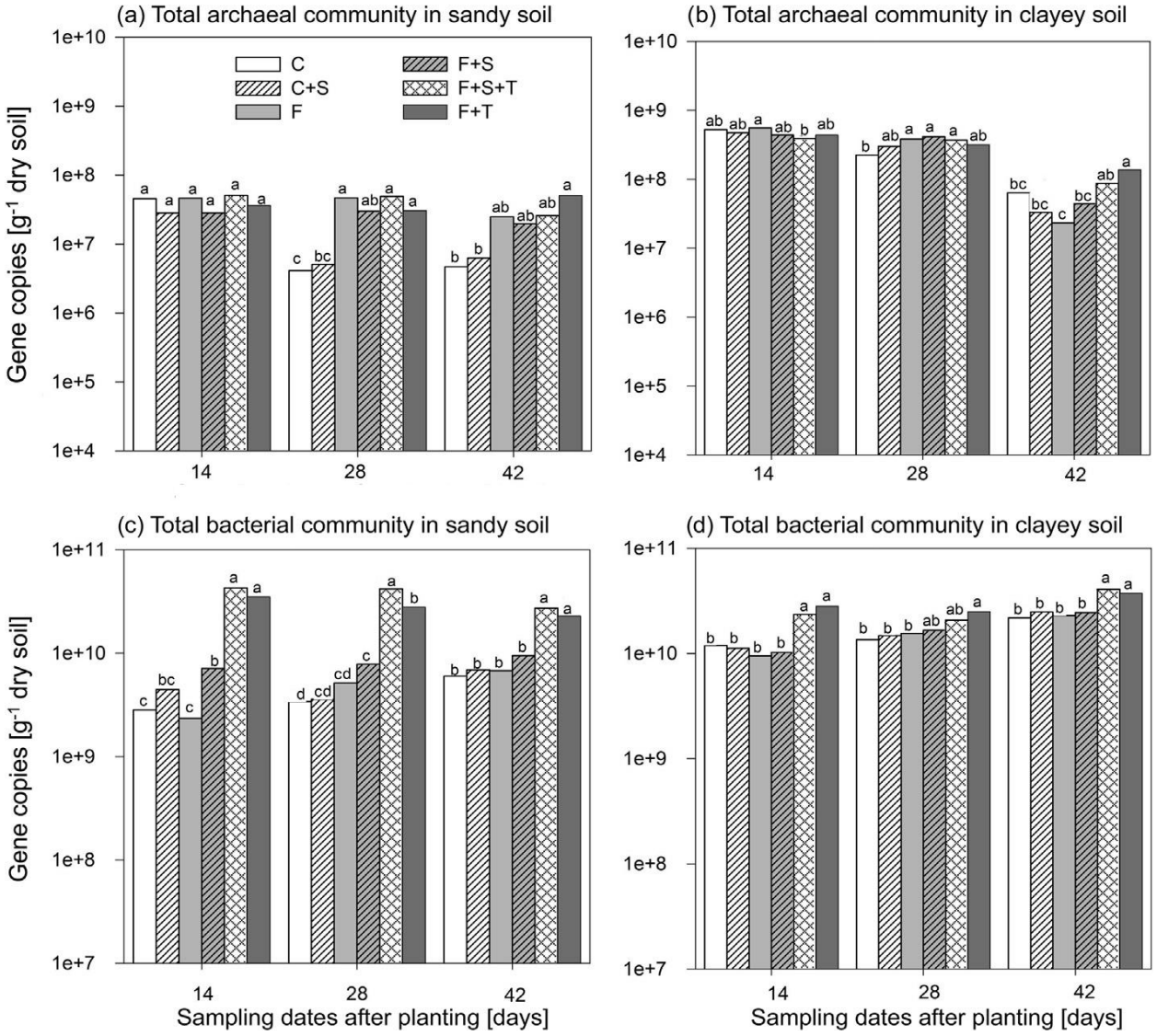

Figure 2. Abundance of the total archaeal and bacterial community in the sandy (a and c) and clayey (b and d) soils as determined during the 6 weeks of incubation in the rhizobox experiment.

Different letters within each graph at each sampling date indicate significant differences between treatments (P<0.05). Treatments are: C, uncoated maize with no *S. hermonthica*, C + S, uncoated maize and *S. hermonthica*, F, coated maize with "Foxy-2", F + S, coated maize with "Foxy-2" and S. *hermonthica*, F + T, coated maize with "Foxy-2" and T. *diversifolia*, F + S + T, coated "Foxy-2", S. *hermonthica* and *T. diversifolia*.

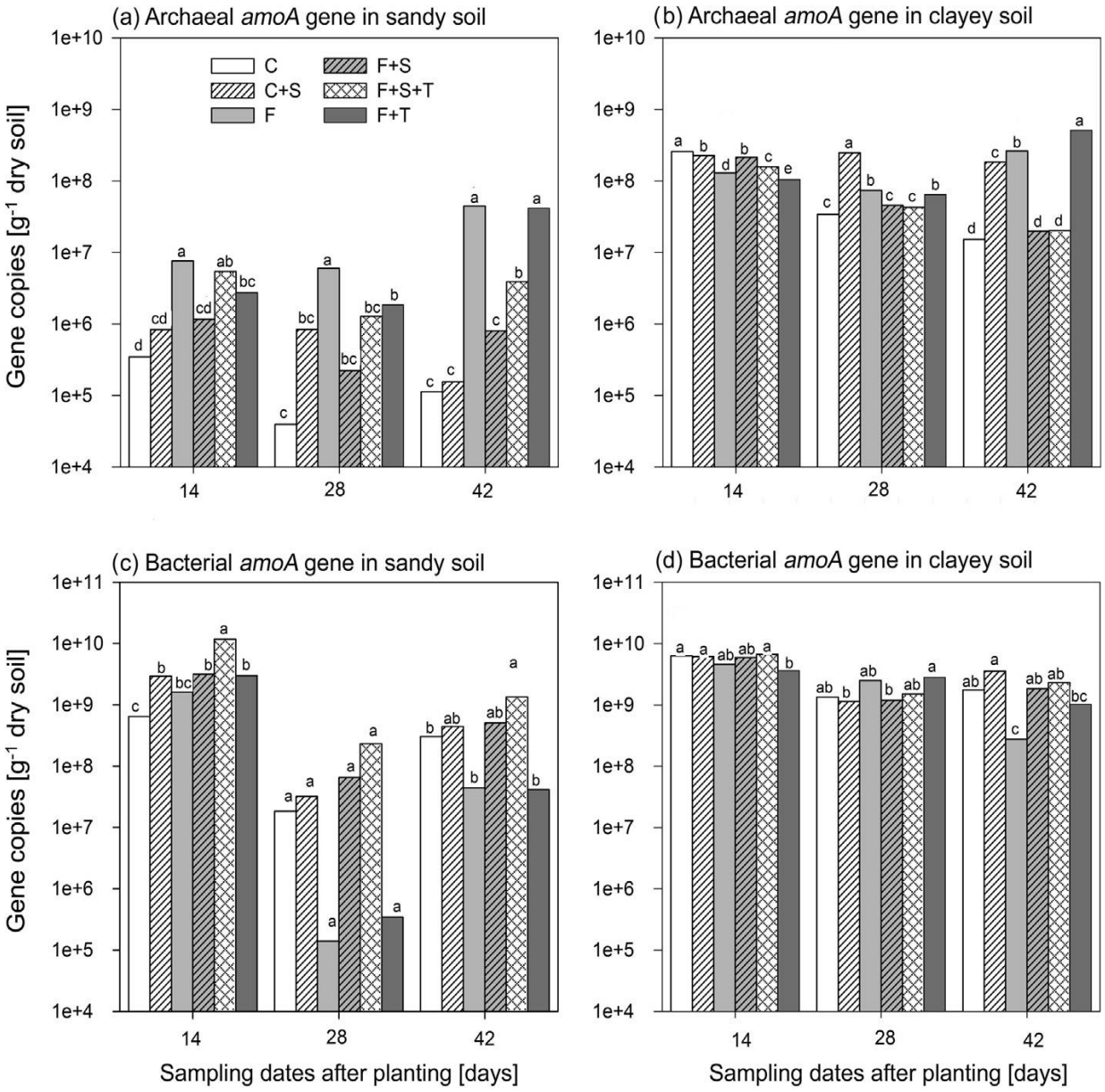

Figure 3. Abundance of the archaeal and bacterial *amoA* genes in the sandy (a and c) and clayey (b and d) soils as determined during the 6 weeks of incubation in the rhizobox experiment.

Different letters within each soil type at each sampling date indicate significant differences between treatments ($P<0.05$). Treatments are: C, uncoated maize with no *S. hermonthica*, C + S, uncoated maize and *S. hermonthica*, F, coated maize with "Foxy-2", F + S, coated maize with "Foxy-2" and *S. hermonthica*, F + T, coated maize with "Foxy-2" and *T. diversifolia*, F + S + T, coated "Foxy-2", *S. hermonthica* and *T. diversifolia*.

Table 3. Soil chemical parameters (mean ± standard deviation, n = 18) obtained from the rhizobox experiment

Soil	Treatment	TC [g kg^{-1}]	N_t [g kg^{-1}]	EOC [mg kg^{-1}]	EON [mg kg^{-1}]	NH_4^+ [mg kg^{-1}]	NO_3^- [mg kg^{-1}]	pH
Clayey soil	C	16.9 ± 0.1c	1.51 ± 0.1ab	572 ± 9.0cd	60.4± 17.2ab	3.9 ± 1.4bc	23.4 ± 2.8a	4.5 ± 0.1b
(Embu)	C + S	17.2 ± 0.1c	1.43 ± 0.1b	583 ± 15.0bcd	60.1 ± 3.1b	2.8 ± 0.2c	3.9 ± 1.6c	4.7 ± 0.1b
	F	16.9 ± 0.3c	1.47 ± 0.1ab	589 ± 9.7abc	54.9 ± 7.9b	6.8 ± 0.3a	22.0 ± 5.9a	4.6 ± 0.1b
	F + S	17.4 ± 0.2bc	1.43 ± 0.0ab	551 ± 8.3d	52.4 ± 3.3b	2.9 ± 0.1c	10.1 ± 1.7ab	4.8 ± 0.17b
	F + T	18.0 ± 0.5ab	1.57 ± 0.1a	619 ± 18.3ab	65.0 ± 8.0ab	6.4 ± 0.7ab	3.6 ± 1.9c	4.9 ± 0.2a
	F + S + T	18.6 ± 0.5a	1.59 ± 0.1a	619 ± 18.2a	71.3 ± 7.6a	5.2 ± 0.2ab	4.3 ± 2.4bc	4.9 ± 0.1a
Statistics		***	**	**	*	**	**	***
Sandy soil	C	3.70 ± 0.0b	0.35 ± 0.0b	103 ± 8.1b	15.3 ± 1.8b	3.6 ± 0.5c	1.99 ± 1.8a	4.7 ± 0.02b
(Machanga)	C + S	3.88 ± 0.2b	0.36 ± 0.0b	109 ± 5.5b	16.1 ± 0.5b	3.7 ± 1.6c	1.05 ± 0.6a	4.7 ± 0.03b
	F	3.67 ± 0.1b	0.36 ± 0.0b	100 ± 4.5b	20.3 ± 4.6b	3.5 ± 0.4c	2.07 ± 0.3a	4.7 ± 0.01b
	F + S	4.06 ± 0.2b	0.38 ± 0.0b	110 ± 16.5b	16.8 ± 0.9b	5.1 ± 1.8c	1.04 ± 0.6a	4.8 ± 0.01b
	F + T	5.64 ± 0.4a	0.57 ± 0.0a	207 ± 42.8a	28.0 ± 5.0a	15.0 ± 0.9a	3.80 ± 0.3a	5.6 ± 0.02a
	F + S + T	5.46 ± 0.6a	0.53 ± 0.0a	194 ± 41.2a	23.5 ± 4.4a	8.7 ± 1.4b	0.78 ± 0.3a	5.7 ± 0.01a
Statistics		***	**	***	**	***	n.s.	***

Abbreviations: TC, Total carbon, N_t; Total nitrogen, EOC; extractable organic carbon, EON; Extractable organic nitrogen, NH_4^+; ammonia, NO_3^-; nitrate, pH; soil pH.

Significance levels: n.s.: P>0.05; *P<0.05; **P<0.01; ***P<0.001.

Different letters within columns indicate significant differences (P<0.05)

Treatment codes: uncoated maize with no *S. hermonthica* (C), uncoated maize and *S. hermonthica* (C + S), coated maize with "Foxy-2" (F) and coated maize with "Foxy-2" and *S. hermonthica* (F + S), as well as coated maize with "Foxy-2", *S. hermonthica* and *T. diversifolia* (F + S + T) and without *S. hermonthica* (F + T).

Table 4. Pearson's linear correlation coefficients between prokaryotic gene abundance (16S rRNA gene, *amoA* gene) and soil chemical data at 42 DAP

Soil	Target gene	TC [g kg^{-1}]	N_t [g kg^{-1}]	EOC [mg kg^{-1}]	EON [mg kg^{-1}]	NH_4^+ [mg kg^{-1}]	NO_3^- [mg kg^{-1}]	pH
Clayey soil (Embu)	Total bacteria	0.84***	0.71**.	0.59*	0.73**	n.s.	n.s.	0.85***
	Total archaea	n.s.	n.s.	n.s..	n.s.	n.s.	n.s.	n.s.
	Bacterial *amoA* gene	n.s.	n.s.	n.s.	n.s.	-0.67**.	-0.52*	n.s.
	Archaeal *amoA* gene	n.s.	n.s.	n.s.	n.s.	n.s.	n.s	n.s.
Sandy soil (Machanga)	Total bacteria	0.90***	0.87***	0.91***	0.91***	0.83***	n.s	0.99***
	Total archaea	n.s.	0.52*	n.s.	0.51*.	0.61*	n.s	n.s.
	Bacterial *amoA* gene	n.s.	n.s.	n.s.	n.s.	n.s.	n.s	0.54*
	Archaeal *amoA* gene	n.s.	n.s.	n.s.	n.s.	n.s.	0.53*	n.s.

Abbreviations: TC, Total carbon, N_t; Total nitrogen, EOC; extractable organic carbon, EON; Extractable organic nitrogen, NH_4^+; ammonia, NO_3^-; nitrate, pH; soil pH.

Significance levels: n.s.: P>0.05; *P<0.05; **P<0.01; ***P<0.001

2.4.1.3. Bacterial *amoA* gene abundance

In the sandy soil, bacterial *amoA* gene abundance was not influenced by F or F + S compared to C and C + S ($P>0.05$; Fig. 3c). Overall, bacterial *amoA* gene abundance was by tendency higher in F + S + T than in C, C + S and F + S. Treatment F + T only showed higher abundance at 14 DAP in comparison to C ($P<0.05$). Only at 14 DAP, there was an abundance difference between C and C + S in bacterial *amoA* gene abundance ($P<0.05$).

In the clayey soil, bacterial *amoA* gene abundance in F and F + S treatments was not different from C and C + S ($P>0.05$; Fig. 3d). However, a different pattern was observed at incubation end with a lower abundance in the F treatment than in C, C + S, F + S and F + S + T ($P<0.05$). No difference was determined between C + S and C ($P>0.05$).

2.4.2. Treatment effects on soil chemical parameters

Addition of *T. diversifolia* residues (F + S + T and F + T) increased soil pH in both sandy and clayey soils ($P<0.001$; Table 3). In comparison to the initial soil pH (4.70 and 4.74 for the clayey and the sandy soil, respectively), soil pH remained unchanged under C, C + S, F, and F+S treatments ($P>0.05$). A similar pattern was observed for TC and N_t (Table 3). With respect to EON and EOC, F + S + T and F + T showed in both soils higher values compared to C, C+S, F and F+S (Table 3). EOC in the clayey soil was lower under F + S than F, F + T and F + S + T ($P<0.001$), but not different from both controls (C, C + S) ($P>0.05$; Table 3). NH_4^+ contents were higher in treatments F + S + T and F + T than in C, C + S, F, and F + S in the sandy soil ($P<0.001$), while in the clayey soil, F + S + T and F + T revealed higher NH_4^+ in comparison to C + S and F + S ($P<0.01$; Table 3). In the clayey soil, NO_3^- contents were higher in C and F than C + S and F + T ($P<0.01$); while no treatment effects were determined for NO_3^- in the sandy soil ($P>0.05$).

2.4.3. Linear correlations between gene abundance and soil chemical parameters

Linear correlations between gene abundance and soil chemical parameters were calculated at 42 DAP (Table 4). TC, N_t, EOC and EON were positively correlated with bacterial 16S rRNA gene copy numbers in both soils. In addition, archaeal 16S rRNA gene copies revealed a positive correlation with N_t and EON in the sandy soil. NH_4^+ correlated positively with bacterial and archaeal 16S rRNA gene abundance in the sandy soils. On the contrary, negative correlations were determined in the clayey soil between NH_4^+ and NO_3^- with bacterial *amoA* gene copy numbers, while in the sandy soil, NO_3^- was positively correlated with archaeal *amoA* gene abundance. Soil pH revealed positive correlations with bacterial

16S rRNA gene copies in both soils, while bacterial *amoA* genes only showed positive correlation with soil pH in the sandy soil.

2.5. Discussion

Fusarium oxysporum [f.sp. *strigae*] strain "Foxy-2" has been undergoing an extended evaluation for possible commercialization as a mycoherbicide in Sub-Saharan Africa. The four main components of this evaluation include (1) its ability to suppress *S. hermonthica* in varying agro-ecological conditions that was only achieved in Western Kenya (Avedi et al., 2014), (2) its non-pathogenicity to maize and other cereal crops (Avedi et al., 2014; Zarafi et al., 2015), (3) its non-pathogenicity to other food crops which is not the case for solanaceous crops that succumb to its infection (Zarafi et al., 2015), and (4) lack of negative impacts on indigenous soil microorganisms with beneficial functions colonizing the rhizosphere of the host crop, which was the subject addressed in the presented study.

2.5.1. Effect of "Foxy-2" on abundance of indigenous rhizosphere prokaryotes

Contrary to our expectations, abundance of nitrifying prokaryotes and particularly that of archaea increased in "Foxy-2" treatments in the assayed maize rhizosphere treatments. However, the observed effects differed greatly depending on soil type and organic N fertilization (i.e., *Tithonia diversifolia* residues). Further, we did not observe the hypothesized resource competition effect between the abundance of indigenous rhizosphere prokaryotes and "Foxy-2".

AOA were apparently better adapted to coexist with "Foxy-2" in the less fertile sandy soils as noted by a consequent abundance boost. This finding was supported by Valentine (2007) who showed that archaea and their nitrifying counterparts (i.e., AOA) possess a higher competitive advantage under reduced organic resource availabilities. Accordingly, we found positive correlations between AOA abundance and "Foxy-2" propagation (J. Zimmermann, personal communication).

In contrast to the sandy soil, we determined only minor differences of archaeal abundance in the clayey soil (i.e., Embu) providing a greater resource pool of soil organic matter. This was corroborated by higher TC and EOC contents in the clayey soils offering an important resource back-up sufficiently fueling both, indigenous rhizosphere archaea and also "Foxy-2" (Chivenge et al., 2011a, b; Rasche et al., 2014). Bacterial abundance increased when organic

N was added. This corresponded to other studies observing similar responses of bacterial abundance to easily available organic resources (Wessén et al., 2010).

Overall, we did not observe the hypothesized reduced abundance of indigenous rhizosphere prokaryotes by "Foxy-2" which suggested a likely niche differentiation, but not a direct resource competition (Valentine, 2007; Nicol et al., 2008, Wessén et al., 2010). On the other hand, we point out that the mechanisms driving the differences in prokaryotic gene abundance may include other, indirect drivers such as induced plant systemic resistance (i.e. production of secondary metabolites) and altered root exudation patterns of the maize crop after "Foxy-2" inoculation (Martinuz et al., 2012b; Wang et al., 2013). However, these speculative interpretations require further research to clarify the mode of actions of AOA responses to "Foxy-2" inoculation in crop rhizospheres.

2.5.2. Imperative considerations when implementing "Foxy-2" as BCA

There has been increasing concern that research on *F. oxysporum* including "Foxy-2" and other potential BCAs provided inconsistent information on their performance and safety (Ochieno, 2010; Avedi et al., 2014). Recent findings from an efficacy study on "Foxy-2" by Avedi et al. (2014) did not achieve biological control of *S. hermonthica* parasitizing maize in Western Kenya. Their report raised questions on effectiveness, mechanisms and conditions under which "Foxy-2" performs as a BCA. One of the major limitations of seed treatment with microorganisms is their narrow range of climatic or agronomic conditions as indicated in a recent review by Parker (2014). Given that the strain was originally isolated from Ghana; its inefficiency in Western Kenya may be explained by such limitations. Further adaptation studies are hence required which will contribute to advanced understanding on the behaviour of "Foxy-2" in foreign environments.

There exist also certain concerns that some misidentified strains of *F. oxysporum* could be intentionally used as "agro-terrorist weapons" against crop production around the world (Suffert et al., 2009; Avedi et al., 2014). Most recent host range studies on "Foxy-2" (Zarafi et. al., 2015) reported that this strain is also pathogenic to solanaceous crops implying that it is not highly specific to *S. hermonthica* as was earlier suggested (Ciotola et al., 1995; Elzein and Kroschel, 2004a; Ndambi et al., 2012). These contradicting results have led to further questioning of the true identity of the strain (Avedi et al., 2014). Accordingly, it was recommended to avoid intercropping of "Foxy-2" treated maize with selected solanaceous crops (Zarafi et al., 2015).

Moreover, it was suggested that "Foxy-2" undergoes further re-identification as it may not be a true forma specialis attacking *S. hermonthica* (Avedi et al., 2014). Re-identification of "Foxy-2" as well as identification of other *Fusarium* sp. strains re-isolated from diseased solanaceous crops will contribute to a better understanding on the inconsistency surrounding the specificity and identity of "Foxy-2". This will be a critical prerequisite to use this strain as BCA for *S. hermonthica*. Moreover, additional identification studies will determine if "Foxy-2" is actually suitable for release to farmers by addressing the raised concerns (Ajanga and Avedi, 2013; Avedi et al., 2014).

Based on the results of the present study, "Foxy-2" is a suitable candidate for release to farmers in Africa as we did not observe the hypothesized resource competition effect between indigenous rhizosphere prokaryotes and "Foxy-2". This implied that "Foxy-2" had no negative ecological impacts in relation to other microorganisms.

2.6. Conclusions

Based on our results, we concluded that "Foxy-2" is safe for selected members of the rhizosphere microbial community such as nitrifying prokaryotes emphasized in this study. Further, the capability of archaeal communities (total and AOA) to respond more sensitively to treatments than their bacterial counterparts supported their particular application as indicators for assessing perturbations in soils (Wessén and Hallin, 2011; Pereira e Silva et al., 2013).

Although negative effects of "Foxy-2" on non-target rhizosphere microorganisms were not determined in this study, its future application as BCA for *S. hermonthica* control has to undergo additional evaluation including a broader range of soils with contrasting physico-chemical characteristics to corroborate our assumptions. These future studies should also consider crop species other than maize (e.g., sorghum) and different cultivars of the same crop as these may pose distinct effects on microbial community dynamics in the rhizosphere (Grayston et al., 1998; Soderberg et al., 2002; Rasche et al., 2006b).

The present study was based on a short-term, controlled rhizobox experiment. To fully understand the ecological effects of "Foxy-2" and its behaviour in cropping fields and its long-term persistence in soils, similar "Foxy-2" exposure experiments should be conducted under natural field conditions to account for relevant factors such as crop development, a broader range of (organic) fertilization regimes, as well as seasonal characteristics including

rainfall and temperature patterns (Cavaglieri et al., 2009; Aira et al., 2010; Murphy and Foster, 2014).

Finally, the presented study was focused on the abundance of selected prokaryotic communities. It can be assumed that "Foxy-2" may, apart from abundance, also influence the composition of targeted prokaryotic populations. In this respect, we recommend advanced studies on potential effects of "Foxy-2" on the community composition of non-target microbial populations in crop rhizospheres using molecular fingerprinting techniques (Rasche et al., 2006a, b).

2.7. Acknowledgments

The study was financed by the Bill & Melinda Gates Foundation (project "Integrated Striga Management in Africa (ISMA) through the International Institute of Tropical Agriculture (IITA)) and Fiat Panis Foundation (Ulm, Germany). The authors thank Evonne Oyugi (Tropical Soil Biology and Fertility Institute of the International Centre for Tropical Agriculture (TSBF-CIAT), Nairobi, Kenya) for providing the soil samples from the SOM field experiments in Kenya. Ms. Musyoki is grateful to the Food Security Centre (FSC) of the University of Hohenheim funded by the German Academic Exchange Service (DAAD) for providing the scholarship. Special thanks go to Professor Dr. Hans-Peter Piepho and Dr. Juan Carlos Laso Bayas (Department of Bioinformatics, University of Hohenheim) for their support in statistical analysis.

CHAPTER 3

Soil properties, seasonality and crop growth stage exert a stronger effect on rhizosphere prokaryotes than the fungal biocontrol agent *Fusarium oxysporum* f.sp. *strigae*

3. Soil properties, seasonality and crop growth stage exert a stronger effect on rhizosphere prokaryotes than the fungal biocontrol agent *Fusarium oxysporum* f.sp. *strigae*[2]

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3.1. Abstract

Fusarium oxysporum f.sp. *strigae* (Fos) is an effective biocontrol agent (BCA) against the parasitic weed *Striga hermonthica*. It acts in the rhizosphere of several tropical cereals, where it may interfere with indigenous microbial populations. To test this impact, we assessed in a 2-season field experiment at two contrasting tropical agro-ecological sites the response of nitrifying and total indigenous prokaryotic communities in the rhizosphere of maize to the exposure of the Fos-BCA "Foxy-2". At early leaf development (EC30), flowering (EC60) and senescence (EC90) stage of maize, rhizosphere samples were obtained and subjected to community analysis of bacterial and archaeal *amoA* (ammonia monooxigenase) (AOB, AOA) and 16S rRNA genes. Abundance and community composition of all studied genes were predominantly influenced by soil type, crop growth stage and seasonality. No major effect of

[2] **This chapter has been reprinted from:**

Musyoki, M.K., Cadisch, G., Zimmermann, J., Wainwright, H., Beed, F., Rasche, F. (2016) Soil properties, seasonality and crop growth stage exert a stronger effect on rhizosphere prokaryotes than the fungal biocontrol agent *Fusarium oxysporum* f.sp. *strigae*, Applied Soil Ecology, 105, (126-136) (DOI:10.1016/j.apsoil.2016.03.021), with permission from Elsevier (Copyright © 2016). The original publication is available at: http://www.sciencedirect.com/science/article/pii/S0929139316300920

"Foxy-2" was found. Notably, total archaeal community relative to bacteria dominated in the clayey soil which was linked to its strong soil organic carbon (SOC) background. Compared to bacterial nitrifiers, domination of nitrifying archaea increased towards senescence stage which was explained by biochemical differences in organic resource availability between the crop growth stages. During the short rain season, the higher archaeal abundance was mainly driven by increased availability of organic substrates, i.e., extractable organic carbon. Our findings suggested that archaea had greater rhizosphere competence than "Foxy-2" in soils with higher clay and SOC contents. We verified that "Foxy-2" in maize rhizospheres is compatible with nitrifying prokaryotes under the given environments, in particular in clayey soils dominated by archaea.

Keywords: Maize rhizosphere; *Fusarium oxysprorum* f.sp. *strigae* inoculation; Bacterial and archaeal *amoA* gene abundance and community composition; Rhizosphere competence.

3.2. Introduction

The fungal strain *Fusarium oxysporum* f.sp *strigae* ("Foxy-2") has been acknowledged as a potent biological control agent (BCA) against *Striga hermonthica* which is parasitic to several cereals cultivated in Sub-Saharan Africa (Schaub et al., 2006; Venne et al., 2009; Elzein et al., 2010). "Foxy-2" proliferates in the crop rhizosphere and has been mainly studied regarding its virulence and mode of action (Schaub et al., 2006; Ndambi et al., 2011; Avedi et al., 2014).

The rhizosphere is a hot spot of microbial activities interacting with plants (Grayston et al., 1998), but no understanding is currently available regarding the interactions of "Foxy-2" with indigenous microorganisms colonizing the roots of cereals and environmental factors including site-specific soil and climatic conditions. This is of particular relevance since "Foxy-2" is often delivered via seed coating and proliferates subsequently along the roots where it directly interacts with other rhizosphere organisms. Thus, prior to broad-scale application of the BCA "Foxy-2" in the field, its compatibility with non-target rhizosphere microorganisms needs to be thoroughly assessed under contrasting environmental conditions (Miedaner et al., 2001; Edel-Hermann et al., 2009; Martin-Laurent et al., 2013). In the rhizosphere, "Foxy-2" must co-exist with indigenous microbial populations as well as maintain its efficacy under a range of environmental factors including seasonal alternations considering rainfall and temperature patterns, varying soil types and also crop growth stages

(Girvan et al., 2003; Beed et al., 2007; Bell et al., 2009; Raaijmakes et al., 2009; Watson, 2013).

Abundance and composition of rhizosphere microbial communities are mainly shaped by rhizodeposition which is the transfer of plant-derived carbon (C) and nitrogen (N) compounds below ground (Høgh-Jensen and Schjoerring, 2001; Kandeler et al., 2002; Rasche et al., 2006a; Wichern et al., 2008; Jones et al., 2009; Fustec et al., 2011). Rhizodeposition is influenced by external factors such as soil type, plant species and their growth stages, as well as climatic conditions (Rasche et al., 2006a; Hai et al., 2009; Hayden et al., 2010). Climatic conditions are of particular importance as rainfall and temperature control crop physiology, photosynthesis activity and consequently rhizodeposition shaping the root-associated microbial community (Waring and Running, 1998; Bell et al., 2009; Rasche et al., 2011).

It remains speculative, if an inoculation and proliferation of "Foxy-2" in the crop rhizosphere results in competition with indigenous rhizosphere for root exudates as critical energy sources and if such interactions are influenced by environmental factors. This assumption is corroborated by earlier studies showing bacterial populations affected by fungi leading to a distinct selection of competitive community members in the rhizosphere (Marschner et al, 2001; Strange, 2005; Cavagnaro et al., 2006). Musyoki et al. (2015) showed that "Foxy-2" inoculated into soils was compatible with nitrifying prokaryotes.

Many BCAs exhibit beneficial effects under laboratory set-ups, while such effects become inconsistent once they are evaluated under greenhouse or even field conditions (Lugtenberg and Kamivola, 2009, Avedi et al., 2014). Advanced understanding of the influence of "Foxy-2" on non-target, functionally relevant rhizosphere prokaryotes under contrasting field conditions is therefore not only essential to validate previous studies under controlled conditions (Musyoki et al., 2015). It is also important to evaluate the extent of a "Foxy-2" impact against acknowledged factors (e.g., seasonality, soil type, crop growth stage) that determine the dynamics of rhizosphere communities (Doohan et al., 2003; Rasche et al., 2006a; Rasche et al., 2011).

Bacteria and archaea are ubiquitous in soils and responsible for the decomposition and mineralization (i.e., N cycle) of organic matter (Widmer et al., 2006, Raybould and Viachos, 2011). A critical component of the microbial driven N cycle is the nitrification step which is catalyzed by key enzymes such as the *amoA* gene encoding the α-subunit of ammonia monooxygenase (Nicol et al., 2008; Zhang et al., 2013). It has been extensively reported that

abundance and community composition of ammonia-oxidizing prokaryotes (i.e., bacteria (AOB), archaea (AOA)) respond sensitively to environmental change including the exposure to fungi (Rasche et al., 2011; Raybould et al., 2011; Wessén and Hallin 2011; Martin-Laurent et al., 2013; Pereira e Silva et al., 2013). Although these studies acknowledge the use of AOB and AOA abundance as bioindicators for soil ecosystem disturbance surveys, the effects of BCAs (e.g., "Foxy-2") on dynamics of these functionally relevant groups in soils are yet to be understood.

The objective of this study was therefore to assess the response of ammonia-oxidizing prokaryotes to "Foxy-2" exposure and to assay these presumed effects at different growth stages of maize cultivated in contrasting environments during two cropping seasons. In addition, we have evaluated these effects against those caused by an N-rich organic input which was supposed to compensate any resource competition between "Foxy-2" and non-target rhizosphere microbial communities (Ayongwa et al., 2011; Musyoki et al., 2015). The major hypothesis was that under field conditions, natural factors such as crop growth stage, soil type and climatic conditions (i.e., rainfall and temperature patterns) expose a greater influence on the abundance and community composition of resident prokaryotic populations than the BCA "Foxy-2" in a maize rhizosphere (Buée et al., 2009; Nihorimbere et al., 2011).

3.3. Material and methods

3.3.1. Fungal biocontrol agent

The fungal strain *Fusarium oxysporum* f.sp. *strigae* ("Foxy-2") used in this study was isolated from diseased S. hermonthica collected in North Ghana (Abbasher et al., 1995). Identification of the isolate was done by the Julius-Kühn-Institute, Berlin, Germany (accession number: BBA-67547-Ghana). Since then, the isolate is being preserved at -80°C at the Institute of Plant Production and Agroecology in the Tropics and Subtropics, University of Hohenheim, Stuttgart, Germany.

3.3.2. Study site description

The field experiments were carried out in post-entry quarantine facilities (PEQ) at Agricultural Training Centre field stations in Western Kenya (Avedi et al., 2014). Two study sites (Busia, 0° 26'S-34° 15' E; 1200 m above sea level (a.s.l.); Homabay, 0° 40'-0°S and 0° 34° 50'E; 1305 m a.s.l.) were chosen because of the reported high *S. hermonthica* infestation in these areas (de Groote et al., 2005). The sites were fallow for a year before the experiment

was established. The fallow in Busia consisted of short grasses (e.g., *Digitaria scalarum*), while the fallow at Homabay consisted of grasses (*Digitaria scalarum*), and weeds such as black nightshade (*Solanum nigrum*) and thorn apples (*Datura stramonium*). The study areas have bimodal rainfall patterns with two growing seasons, the first rainy season with long rains (LR) from April to August and the second rainy season with short rains (SR) from September to January. Busia received 121 and 231 mm precipitation per month during the LR and SR season, respectively, and had a mean temperature of 27°C during both seasons (Fig. 4). Homabay received 216 and 77 mm of rainfall per month during the LR and SR season, respectively, while the mean annual temperature was 29°C in both seasons (Fig. 4). Initial soil characterization revealed that the soil at Homabay has a clayey texture (49% clay, 19% silt, 32% sand) and contained 0.22 and 2.87% total nitrogen and carbon, respectively, while Busia soil has a clay loam texture (33% clay, 22% silt, 45% sand) and contained 0.19 and 1.57% total nitrogen and carbon, respectively.

3.3.3. Field experiment and rhizosphere sampling

The study covered two seasons (first season: SR; September 2012 to January 2013; second season: LR; April 2013 to August 2013). Maize variety WH507, commonly preferred by farmers in the study area due to its less susceptibility to *S. hermonthica* and also recommended for an integrated *S. hermonthica* control, was planted in 3 x 2.7 m^2 plots with a spacing of 30 by 70 cm (Avedi et al., 2014). The experiment was laid out in a randomized complete block design (RCBD) with three replicates and comprised of three treatments: i) uncoated maize and *S. hermonthica* (C, control), ii) coated maize with "Foxy-2" and *S. hermonthica* (F+S), and iii) coated maize with "Foxy-2", *S. hermonthica* and *Tithonia diversofolia* residues as additional N source (F+S+T). Maize seeds were coated with "Foxy-2" (1.15 x 105 colony forming units per seed) as described by Musyoki et al. (2015).

Land was prepared by hand digging and two maize seeds per hill were planted at a depth of approximately 3 cm. One table spoonful of a *S. hermonthica* seed-sand mixture (1:4 ratio with approximately 1000 *S. hermonthica* seeds) was placed in every planting hole (Avedi et al., 2014). At sowing, all plots received a blanket application of 60 kg P_2O_5 ha^{-1} $season^{-1}$ as diammonium phosphate (DAP)$(NH_4)_2HPO_4$)) to avoid any phosphorus limitation. In addition, mineral N fertilizer was split applied to treatments C+S and F+S as calcium ammonium nitrate ($CaNH_4NO_3$) at a rate of 120 kg N ha^{-1} growing $season^{-1}$ with 1/3 and 2/3 added 3 and 8 weeks after sowing, respectively (Chivenge et al., 2011; Muema et al.,

2015).

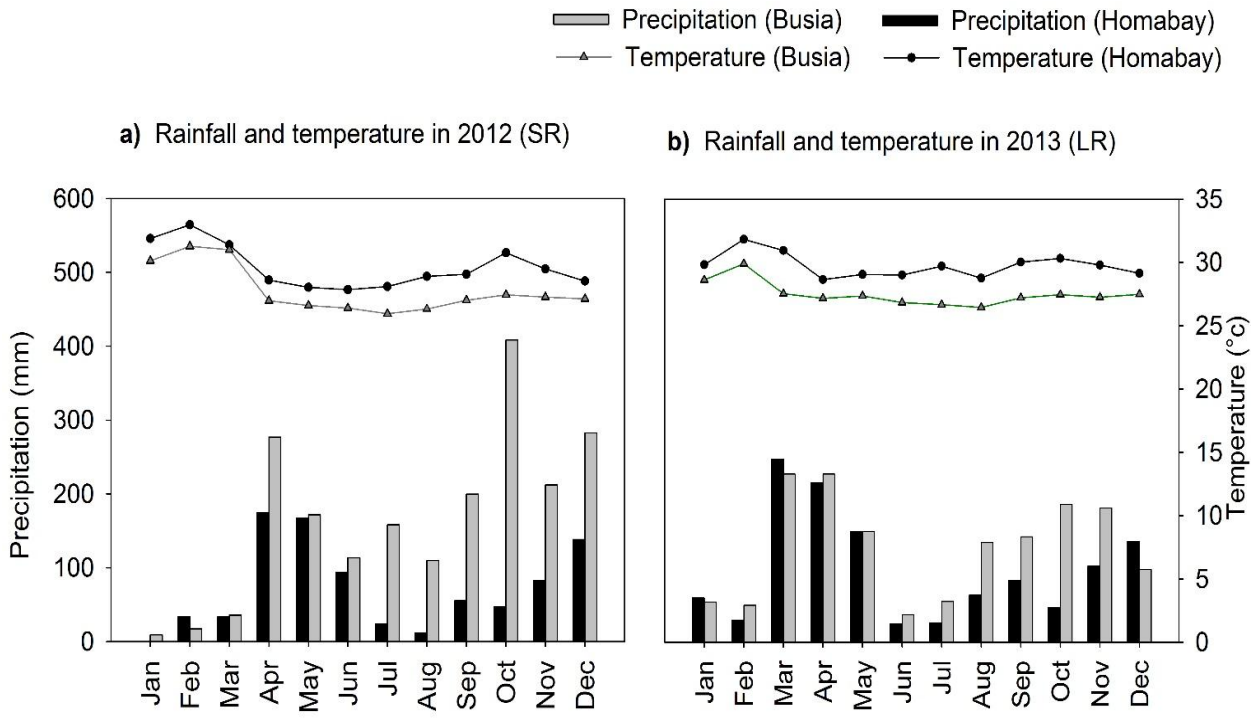

Figure 4. Monthly rainfall and temperature distribution during the short rain (SR) season (September 2012- January 2013) and the long rain (LR) season (March-August 2013).

For treatment F + S + T, N was applied as fresh *T. diversifolia* leaf and stem material (5 t dry weight ha^{-1} to supply similar levels to 120 kg of inorganic N) which was hand-incorporated to a soil depth of 0-15 cm at the onset of each rainy season. Two weeks after germination, seedlings were thinned to 1 per hole. Hand weeding was done after every 2 weeks for all weeds except *S. hermonthica*.

Rhizosphere samples (approximately 50 g) were collected according to standard procedures (Milling et al., 2004) at EC30 (early leaf development stage), EC60 (flowering stage), and EC90 (senescence stage) by shaking the roots of three plants per plot to remove non-rhizosphere soil. Rhizophere soil samples were scraped off the roots of sampled plants. The rhizosphere soil samples of the three representative plants per plot were then mixed to form one composite sample. Soils were freeze-dried and stored in a dark and dry place until further analysis.

3.3.4. Microbial abundance

Four hundred milligrams of freeze-dried rhizosphere soil was used for DNA extraction from each of the three replicates per treatment. Soil DNA was extracted using the FastDNA® Spin

for Soil Kit (MP Biomedicals, Solon, Ohio, USA) following the manufacturer's instructions. Quality of extracted DNA was checked using 1.5 % (w/v) agarose gel. DNA extracts were quantified (Nanodrop ND-1000, NanoDrop Technologies, Wilmington, USA) and stored at -20°C for further analysis.

Abundance of total and ammonia oxidizing prokaryotic communities was estimated by DNA-based quantitative PCR (qPCR) using bacterial and archaeal 16S rRNA genes (total community) as well as bacterial and archaeal genes encoding ammonia-monooxygenase (*amoA* genes) as molecular markers (Table 5). For standard preparation, amplicons from each target gene were purified (Invisorb Fragment CleanUp kit, Stratec Molecular GmbH, Berlin, Germany), ligated into the StrataCloneTM PCR cloning vector pSC-A (Stratagene, La Jolla, CA, USA) and ligation products were transformed with StrataClone Solopack competent cells (Startagene) (Rasche et al., 2011). The qPCR assays were carried out in a 25 µl reaction containing 12.5 µl of Power SYBR® green master mix (Applied Biosystems, Foster City, CA, USA), 1 µl primer (each 0.4 µM), 0.25 µl T4 gene 32 protein (500 ng µl-1, MP Biomedicals) and 10 ng template DNA. The qPCRs were performed on a StepOnePlus™ Real-Time PCR System (Applied Biosystems) and were started with 10 min at 95°C, followed by amplification cycles specific for each target gene (Table 5). Melting curve analysis of amplicons was conducted to confirm that fluorescence signals originated from specific amplicons and not from primer-dimers or other artifacts. Each DNA sample was processed in triplicate reactions, whereas the standard curves were generated using duplicate serial dilutions of isolated plasmid DNA containing the genes studied (Rasche et al., 2011). Gene copy numbers and reaction efficiencies (total bacteria 94% ± 8, total archaea 97% ± 3, AOB 96% ± 7, AOA 96% ± 9) were calculated using the Stepone software version 2.2.2 (Applied Biosystems) and presented per gram of dry soil.

3.3.5. Microbial community composition

Prokaryotic 16S rRNA and *amoA* genes were PCR-amplified as described in Rasche et al. (2011) (Table 5). Following the results of qPCR analysis (no treatment effects ("Foxy-2" and "organic inputs") over the two seasons), we conducted T-RFLP analysis only in the second season based on the hypothesis that microbial community composition is less sensitive in responding to environmental changes than community abundance (Wessén and Hallin 2011; Ritz et al., 2009). All forward primers were labeled with 6-carboxyflourescein at their 5' ends. Replicate amplicons of all two genes were pooled, purified (Sephadex G-50, GE Healthcare Biosciences, Waukesha, WI, USA) according to Rasche et al. (2006b), and 200 ng

of each purified amplicon were digested with a 5 U combination of enzymes *Alu*I and *Rsa*I (New England Biolabs, Ipswich, USA). Reactions were incubated at 37°C for 4 hours, and purified (Sephadex G-50). An aliquot of 2 µl was mixed with 17.75 µl HiDi formamide (Applied Biosystems) and 0.25 µl internal 500 ROX™ size standard (Applied Biosystems). Labeled terminal-restriction fragments (T-RFs) were denatured at 95°C for 3 min, chilled on ice and detected on an ABI 3130 automatic DNA sequencer (Applied Biosystems). Peak Scanner™ software package (version 1.0, Applied Biosystems) was used to compare relative lengths of T-RFs with the internal size standard and to compile electropherograms into numeric data set, in which fragment length and peak height >50 fluorescence units were used for profile comparison. T-RFLP profiles used for statistical analyses were normalized according to Dunbar et al. (2000).

Table 5. Description of primer sets, PCR ingredients and amplification details used for qPCR and T-RFLP analysis

Target group	Primer (reference)	qPCR	T-RFLP
All bacteria	Eub338 (Lane, 1991)	95°C 5 min	
(16 S rRNA)	Eub518 (Muyzer et al., 1993)	40 cycles: 95°C 30s, 55°C 35s,	
		72°C 45s	
	8f (Weisburg et al., 1991)		95°C 5 min
	1520r (Edwards et al., 1989)		40 cycles: 95°C 1 min, 58°C 30s,
			72°C 1 min; 72°C 10 min
All archaea	Ar109f (Lueders and Friedrich, 2000)	95°C 5 min	95°C 5 min
(16 S rRNA)	Ar912r (Lueders and Friedrich, 2000)	40 cycles: 95°C 30s, 52°C 35s,	35 cycles: 95°C 1 min, 52°C
		72°C 45s, 78°C 20s	30s, 72°C 1 min
			72°C 10 min
Ammonia oxidizing	AmoA-1f (Rotthauwe et al., 1997)	95°C 5 min	95°C 5 min
bacteria (AOB)	AmoA-2r (Rotthauwe et al., 1997)	45 cycles: 95°C 30s, 57°C 45s,	40 cycles: 94° C 30s, 53°C 30s
		72°C 45s, 78°C 20s	72°C 1 min
			72°C 10 min
Ammonia oxidizing	Arch-amoAf (Francis et al., 2005)	95°C 5 min	95°C 5 min
archaea (AOA)	Arch-amoAr (Francis et al., 2005)	45 cycles: 95°C 30s, 53°C 45s,	35 cycles: 94° C 30s, 53°C 45s,
		72°C 45s, 78°C 20s	72°C 10 min

3.3.6. Soil chemical analysis

Soil chemical analyses were performed on all soil samples taken at EC30 and EC90. Total carbon (TC), total nitrogen (N_t), extractable organic C (EOC), extractable N (EON) and pH of soils were recorded on bulk soils. The pH analysis was conducted in a soil water ratio of 1:2.5 using a pH meter (inoLab® Labor-pH-Meter, WTW GmbH, Weilheim, Germany). TC and N_t was quantified by dry combustion (vario MAX CN analyzer, Elementar Analysensysteme GmbH, Hanau, Germany). For EOC measurement, 5 g of soil were extracted with 20 ml 0.5 M K_2SO_4, shaken horizontally (250 rpm) for 30 min and filtered (Rotilabo-Rundfilter AP55.1 (retention of 2-3 µm), Carl Roth GmbH, Karlsruhe, Germany). EOC concentration in filtered extract was measured on an Analytik Jena Multi N/C 2100 analyzer (Analytik Jena AG, Jena, Germany). Ammonium (NH_4^+) and nitrate (NO_3^-) were extracted with 1 M KCl (soil to extractant ratio (w/v) of 1:4), shaken on a horizontal shaker for 30 min at 250 rpm and filtered (Rotilabo-Rundfilter AP55.1, Carl Roth GmbH). Concentrations of NH_4^+ and NO_3^- were measured on an auto-analyzer (Bran & Luebbe, Norderstedt, Germany) (Mulvaney, 1996), while EON was determined as the difference between N_t and mineral N (NH_4^+ and NO_3^-) according to Rousk and Jones (2010).

3.3.7. Statistical analysis

Statistical analyses were performed using Statistical Analysis Software program (SAS Institute, 2015). The analysis was done using a generalized linear mixed model assuming a negative binomial error distribution and a log link function. The fixed effects were; Treatment" ("Foxy-2", organic N addition (*T. diversifolia*), *S. hermonthica*, control), "Site" (soil properties), "Season" (SR 2012/2013, LR 2013), and "Growth stage" of maize (EC30, EC60, EC90) and all their two and three way interactions. The random effect included the blocking factor and replicates nested within blocks. The full model was fitted using restricted log pseudo-likelihood in the SAS GLIMMIX procedure (SAS Institute, 2015). We included a variance-covariance matrix to account for temporal auto-correlation in the residuals for all observations made within a block. The model was fitted separately for all variables across sites (soil properties) (Table 6). Due to the observed significant site effect the model was further fitted separately per site to account for site differences. Adjusted means and their associated standard errors at 95% confidence limits were estimated on the original data scale. Wherever, the two-or three-way interactions were significant, we decomposed them in terms of their simple effects slices. This enabled us to test adjusted means for significance between pairs of treatment, season or crop growth stages at fixed values of the other terms in the

interaction effect. The means were compared using the PDIFF option of the LSMEANS as well as the letters (letter display) from the SAS generalized linear mixed models procedure. For soil chemical properties, NH_4^+ and EON were log base 10 transformed, while NO_3^- was square root transformed. Therefore, no standard errors are shown for these values as back transformed data was used for comparison purposes with the non-transformed chemical data (Table 8) (Piepho, 2009).

Pearson's linear correlation coefficients were calculated for assessing the relations between abundance of nitrifying and total prokaryotes with soil chemical properties.

"Treatment", "Site", and "Growth stage" effects as well as their interaction effects on T-RFLP data sets for each gene were tested using permutation multivariate analysis of variance (PERMANOVA) (Anderson, 2013). Factor effects were further assayed based on Bray-Curtis similarity coefficients (Rees et al., 2005; Rasche et al., 2011; Rasche et al., 2014). A similarity matrix was generated for all possible pairs of samples for each target gene. The similarity matrix was used for analysis of similarity (ANOSIM) to test the hypothesis that composition of studied microbial communities was altered by factors "Treatment", "Site", and "Growth stage". ANOSIM is based on rank similarities between the sample matrix and produces a test statistic 'R' (Rees et al., 2005). A 'global' R was first calculated in ANOSIM, which evaluated the overall effect of a factor in the data set. This step was followed by a pairwise comparison, whereby the magnitude of R indicated the degree of separation between two tested communities. An R score of 1 indicated a complete separation, while 0 indicated no separation (Rees et al., 2005). For graphical visualization of the distinct effect of factor "Growth stage" and "Site" on the composition of analyzed prokaryotic communities, canonical analysis of principal coordinates (CAP) was performed on resemblance matrix data generated based on Bray-Curtis similarity coefficients (Clarke, 1993, Anderson et al., 2008; Muema et al., 2015). Moreover, to test the influence of soil chemical properties on the community composition shifts of assayed genes, distance-based linear models (DISTLM) were used (Permanova+ software package in Primer v6) (Anderson et al., 2008). This procedure calculates a linear regression between the community composition and log transformed soil chemical data using the Shannon diversity index (H') to evaluate how much of the variation in the microbial community composition is explained by variation in the soil chemical data (Legendre and Anderson, 1999; Boj et al., 2011). PERMANOVA, ANOSIM, CAP and DISTLM analyses were conducted with Primer6 for windows (version 6.1.13),

Primer-E ltd., Ply-mouth, UK) with PERMANOVA+ version 1.0.6 as add-on for Primer6 software (Anderson, 2008).

3.4. Results

3.4.1. Microbial abundance

Abundance of archaeal 16S RNA genes was influenced by factors "Site", "Season" and "Growth stage (Table 6 and Fig. 5a and b). Overall, Homabay site had higher 16S RNA archaeal gene copies than Busia site ($P<0.001$, Fig. 5a and b). A clear distinction of archaeal abundance was revealed in response to the three crop growth stages, where EC90 showed generally highest 16S RNA archaeal gene copies during the SR season, while archaeal abundance was dominating during EC60 during the LR season ($P>0.001$) (Fig. 5a and b). Furthermore, a higher archaeal 16S RNA gene abundance was found in Homabay at EC30 and EC60 in both seasons compared to Busia ($P<0.001$) (Fig. 5a and b).

Total bacterial abundance was most significantly influenced by factor "Growth stage" resulting in lowest bacterial 16S RNA gene copies at EC30 (Fig. 5c and d). Additionally, an interaction was found between factors "Season" and "Site", where higher bacterial 16S rRNA gene copies were measured at Busia during SR season ($P<0.001$) (Table 6 and Fig. 5c). The interaction of "Growth stage" and "Site" revealed a higher significant increase in bacterial 16S RNA gene copies from EC30 to EC60 and EC90 at Busia during SR season compared to LR and Homabay ($P<0.001$) (Table 6 and Fig. 5c and d).

Abundance of archaeal *amoA* gene abundance (AOA) was influenced by factors "Season" and "Growth stage" and significant interactions were found between "Growth stage" and "Site" ($P<0.05$) (Table 6). SR season showed higher AOA abundance in comparison to LR season particularly at Busia, where also highest gene copies were found at EC90 in both seasons ($P<0.01$) (Fig. 6).

Abundance of bacterial *amoA* gene abundance (AOB) was influenced by factors "Season" and "Growth stage" revealing significant interactions of "Growth stage" "Treatment" and "Site" ($P<0.001$) (Table 6). A major trend was that during the LR season, AOB revealed a constant abundance decrease from EC30 to EC90 and more strongly so at Busia (Fig. 6c). Generally, all genes tended to show a higher abundance in the treatments F + S ("Foxy-2") and its combination with *Tithonia diversifolia* (F + S + T) than the control (C) at EC30 during the LR season in both sites ($P>0.05$) (Figs.5a, b, c, d and Fig.6a, b, d).

Table 6. Analysis of variance to determine significant effects of factors "Site" (ST), "Season" (SS), "Treatment" (T) and "Growth stage" (EC) and their interactions on soil chemical properties and abundance of the two assayed genes.

Factor	Total bacteria	Total archaea	Bacterial *amoA* gene	Archaeal *amoA* gene	EOC [mg kg^{-1}]	EON [mg kg^{-1}]	NH_4^+ [mg kg^{-1}]	NO_3^+ [mg kg^{-1}]	pH [H_2O]
Season (SS)	n.s.	***	***	***	***	***	***	***	***
Treatment (T)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*	***
Site (ST)	n.s.	***	n.s.	n.s.	*	**	***	***	***
Growth stage (EC)	***	***	***	***	***	***	***	***	***
SS x T	n.s.	*	n.s.	n.s.	n.s.	*	n.s.	n.s.	***
SS x ST	***	n.s.	***	n.s.	n.s.	***	*	***	***
ST x T	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**	n.s.	***
EC x T	n.s.	n.s.	***	n.s.	**	n.s.	**	*	***
EC x ST	***	***	***	**	***	*	***	**	***
SS x T x ST	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*	***
EC x T x ST	n.s.	n.s.	***	n.s.	n.s.	n.s.	n.s.	**	***

[a]Significance levels: n.s.: P>0.05; *P<0.05; **P<0.01; ***P<0. 001.aSignificance levels: n.s.: P>0.05; *P<0.05; **P<0.01; ***P<0.001.

[b]Growth stage is nested within season.

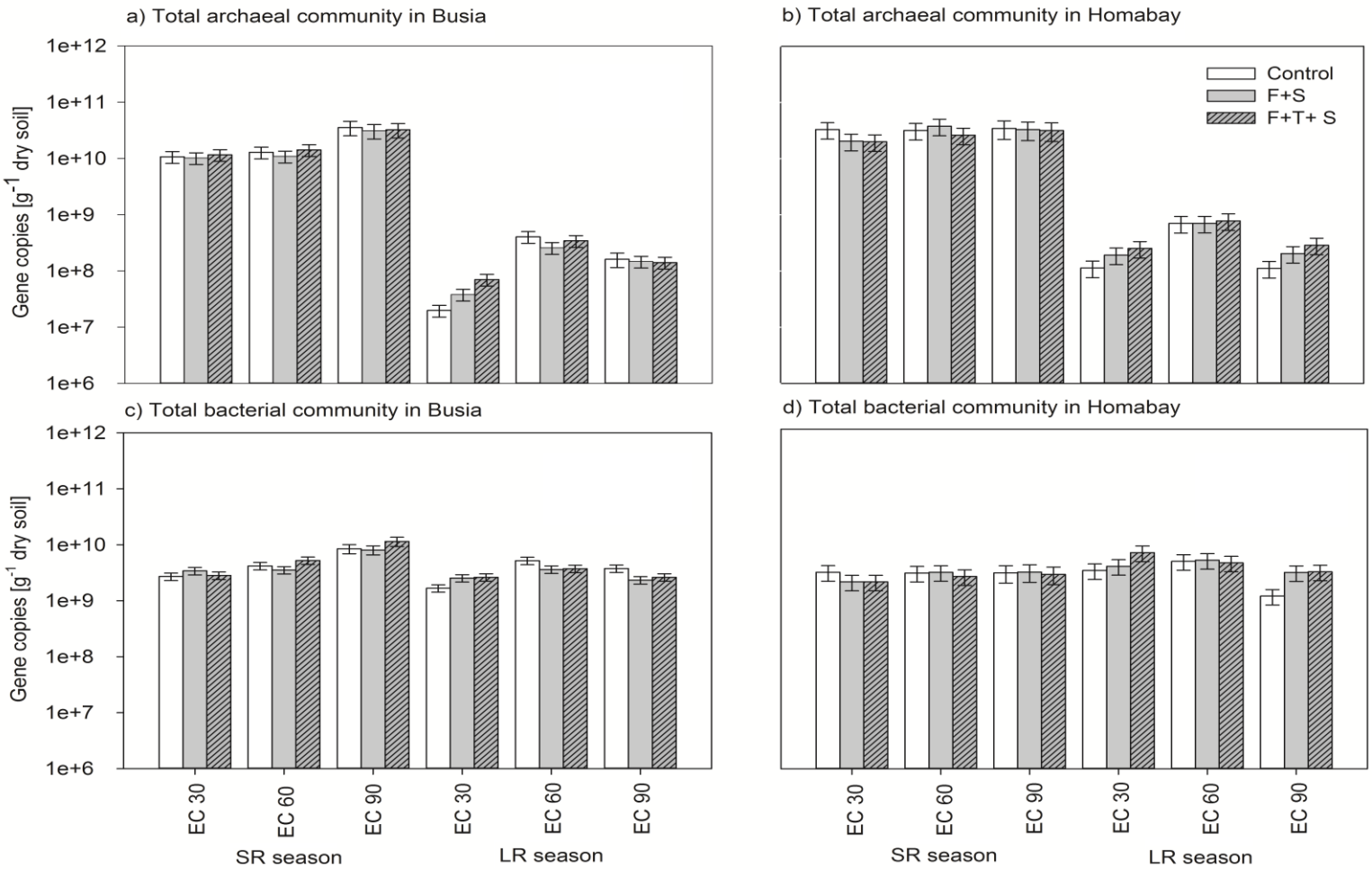

Figure 5. Abundance of the total archaeal and bacterial community at Busia (a, c) and Homabay (b, d) sites as determined during the two cropping seasons. [a]Values are given as average (n = 3) along with standard error (SE). [b]Treatments are: C+S, uncoated maize and *S. hermonthica*, F+S, coated maize with "Foxy-2" and *S. hermonthica*, F+S+T, coated "Foxy-2", *S. hermonthica* and *T. diversofolia*.

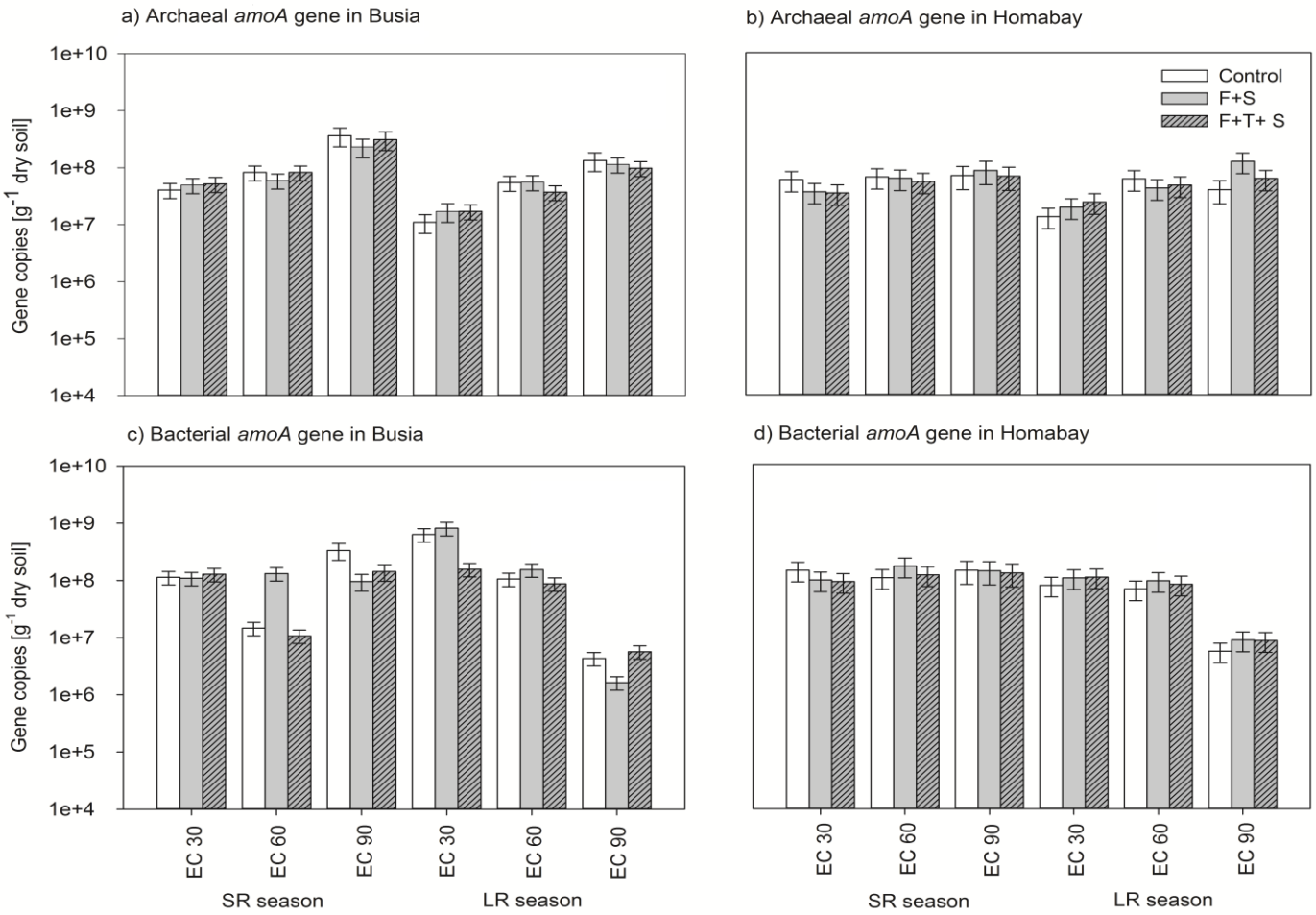

Figure 6. Abundance of the archaeal and bacterial *amoA* genes at Busia (a, c) and Homabay (b, d) sites as determined during the two cropping seasons. [a]Values are given as average (n = 3) along with standard error (SE). [b]Treatments are: C + S, uncoated maize and *S. hermonthica*, F + S, coated maize with "Foxy-2" and *S. hermonthica*, F + S + T, coated "Foxy-2", *S. hermonthica* and *T. diversofolia*.

3.4.2. Microbial community composition

Analysis of similarity (ANOSIM) of T-RFLP data revealed significant effects of factors "Site" and "Growth stage" but not "Treatment" on the community composition of all studied genes ($P<0.05$) (Table 7). A subsequent PERMANOVA analysis revealed significant interactions ($P<0.001$) as evidenced by the effect of "Growth stage" on total archaeal communities at Homabay but not at Busia site which was further proved by pairwise ANOSIM (EC30 versus EC60 ($R = 0.234$), EC60 versus EC90 ($R = 0.44$), EC30 versus EC90 ($R = 0.694$) ($P<0.01$) (Fig. 7a). For total bacterial community, the effect of "Growth stage" was more pronounced at Homabay (Fig. 7b) as was evidenced by pairwise ANOSIM (EC30 versus EC60 ($R = 0.735$), EC30 versus EC90 ($R = 0.941$) and EC60 versus EC90 $R = 0.268$). Likewise, the AOB (*amoA* gene) community composition revealed similar trends (Fig. 7d). Conversely, AOA (*amoA* gene) community composition differences by "Growth stage" were observed to a greater extent at Busia mainly between EC30 and EC90 ($R = 0.9$) ($P<0.001$) (Fig. 7c).

Table 7. Global R values for the main factors "Site" "Growth stage" and "Treatment" as obtained from the analysis of similarity (ANOSIM) of T-RFLP of the two studied prokaryotic groups fingerprints

Prokaryotic group	Factor		
	Site	Growth stage	Treatment
Total bacteria	0.791[a]***	0.780***[b]	-0.03 [n.s.]
Total archaea	0.950**	0.708***	-0.046[n.s.]
Bacterial *amoA* gene	0.172**	0.400***	-0.009[n.s.]
Archaeal *amoA* gene	0.648***	0.132**	-0.015[n.s.]

[a]R indicates the degree of separation between two populations, with a score of 1 indicating complete separation and 0 indicating no separation.

[b]Significance levels: n.s.: $P>0.05$; *$P<0.05$; **$P<0.01$; ***$P<0.001$.

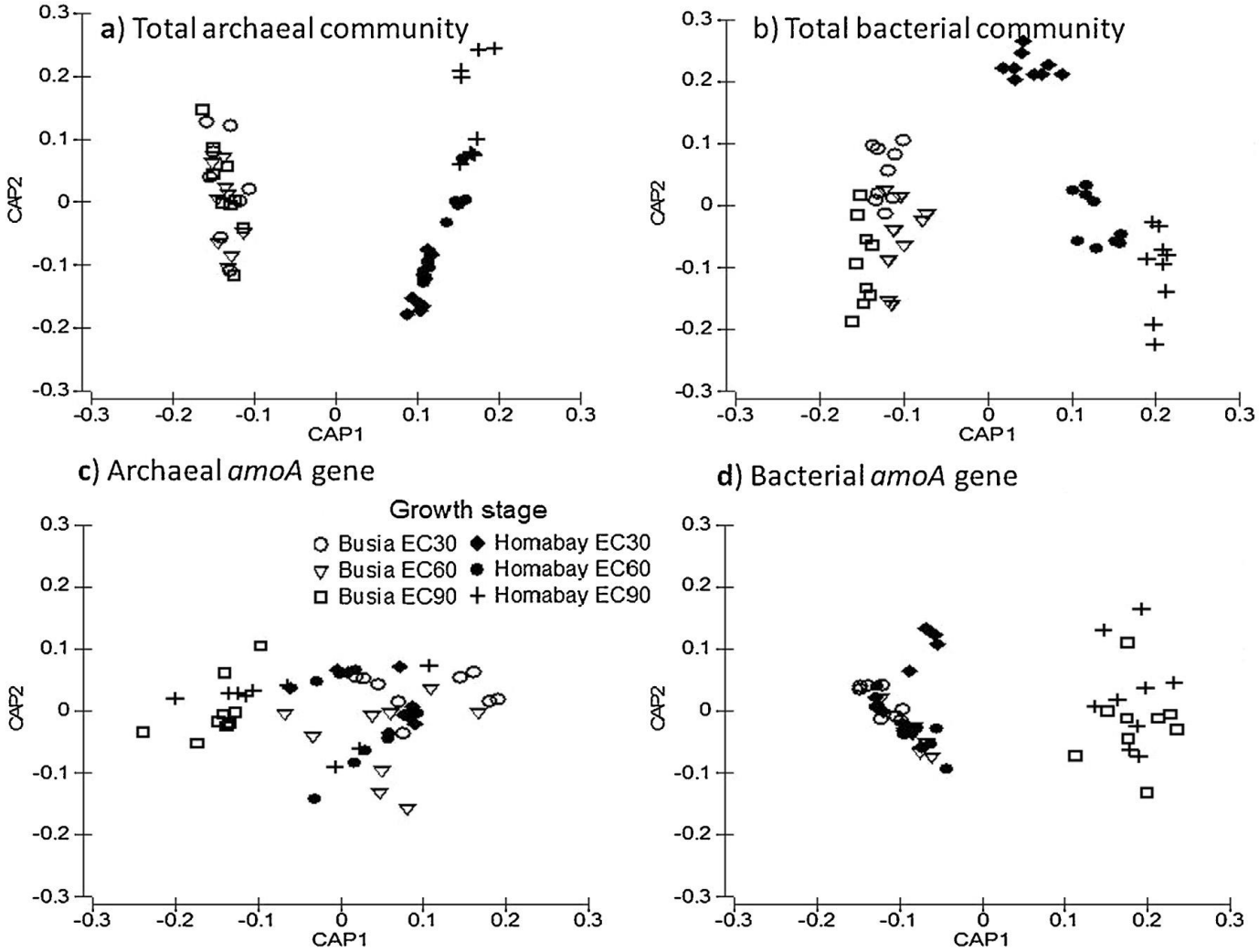

Figure 7. Canonical analysis of principal coordinates (CAP) for visual presentation of prokaryotic communities composition (TRLFP) as shaped by "Site" and "Growth stage".

[a]Prokayotic communities; a = Total archaea, b = Total bacteria, c = ammonia-oxidizing archaea and d = Ammonia-oxidizing bacteria.

3.4.3. Soil chemical properties

EOC and EON were influenced by factors “Season” “Growth stage” and “Site with significant interactions of factor “Growth stage” and “Site ($P<0.05$) (Table 6). EOC was highest at EC90 at Busia during SR season ($P<0.05$) (Table 8), while EON was largest at Homabay site at EC30 of SR season ($P<0.05$) (Table 8). EON values were highest at Busia at EC30 during LR season (Table 8). For mineral N values, seasonal effects were evidenced by higher NH_4^+ concentrations at EC30 during SR season ($P<0.001$) (Table 8). No consistent effect of factors “Treatment”, “Site” and “growth stage” on NO_3^- contents was observed due to several interactions (Table 6). Soil pH was influenced by all four factors with significant interactions ($P<0.001$) (Table 6). For example, soil at Homabay had a higher pH compared to Busia, and SR season induced mostly higher soil pH at both sites (Table 8).

Table 8. Dynamics of soil chemical properties as driven by factors "Site", "Season", "Treatment" and "Growth stage" and their interactions [a]Values are given as average (n=3) along with standard error (SE).[b]Different letters within a column show significant differences within sites and growth stages EC30 and EC90 (P<0.05). [c]Values without SE have been back transformed

Site	Growth stage	Treatment	EOC (mg kg^{-1})		EON (mg kg^{-1})		NH_{4+} (mg kg^{-1})		NO_{3-} (mg kg^{-1})		pH (H_2O	
			SR	LR	SR	LR	SR	LR	SR	LR	SR	LR
Busia	EC 30	C + S	242a	273a	22ab	118a	71a	35b	27a	40b	5.48a	5.33c
		F + S	264a	247a	14b	203a	97a	81a	29a	101a	5.49a	5.41b
		F + S + T	294a	264a	31a	132a	67a	52ab	36a	55b	5.35b	5.43a
		SE	12.54	12.43							0.02	0.02
	EC 90	C + S	281a	197b	37a	48.7a	13a	13a	0.27a	36a	5.64c	4.91b
		F + S	283a	256a	34a	92.3a	13a	21a	1.07a	15b	5.70b	4.86c
		F + S + T	301a	224ab	26a	69.9a	14a	16a	0.61a	20b	5.81a	4.99a
		SE	9.73	13.46							0.03	0.02
Homa Bay	EC 30	C + S	294a	295a	204a	148a	123a	76a	34a	18a	7.04b	6.68a
		F + S	263a	226b	142ab	150a	143a	89a	38a	14a	6.91c	6.17b
		F + S + T	266a	281ab	92b	198a	99a	82a	16b	14a	7.26a	6.11c
		SE	10.04	14.13							0.05	0.09
	EC 90	C + S	273a	156a	48a	25a	4b	3ab	9a	5a	7.35b	7.59a
		F + S	221ab	172a	33a	22a	4b	2b	5a	5a	7.93a	7.22c
		F + S + T	214b	178a	40a	29a	13a	4a	2a	5a	7.27c	7.41b
		SE	15.45	4.29							0.10	0.05

Treatments: C + S = uncoated maize + *S. hermonthica*, F + S = coated maize (with "Foxy-2") + *S. hermonthica*, F + S + T = coated maize + *S. hermonthica* + *Tithonia diversifolia*; Season: SR = short rains, LR = Long rains; Growth stage: EC30 = Early leaf development stage, EC90 = Senescence stage.

3.4.4. Correlations between microbial abundance and soil chemical properties

Extractable organic carbon (EOC) was positively correlated with total bacterial abundance and AOB abundance ($P<0.05$), whereas extractable organic nitrogen (EON) negatively correlated with AOA abundance ($P<0.001$) (Table 9). Mineral N (ammonium (NH_4^+) and nitrate (NO_3^-)) revealed a negative correlation with AOA abundance ($P<0.01$). In addition, NO_3^- correlated negatively with total bacterial abundance ($P<0.05$). Soil pH positively correlated with total archaeal abundance ($P<0.001$).

Table 9. Pearson's linear correlation coefficients between the abundance of the two prokaryotic groups and soil chemical data taken at EC30 and EC90 in Homabay and Busia sites

Prokaryotic group	EOC (mg kg^{-1})	EON (mg kg^{-1})	NH_4^+ (mg kg^{-1})	NO_3^+ (mg kg^{-1})	pH (H_2O)
Total bacteria	0.253*	n.s.	n.s.	-0.325*	n.s.
Total archaea	n.s.	n.s.	n.s.	n.s.	0.365**
Bacterial *amoA* gene	0.263*	n.s.	n.s.	0.320*	n.s.
Archaeal *amoA* gene	n.s.	-0.387**	-0.418**	-0.374*	n.s.

[a] Significance levels: n.s.: $P>0.05$; *$P<0.05$; **$P<0.01$; ***$P<0.001$

3.4.5. Regressions between microbial community composition and soil chemical properties

Variance in total bacterial community composition was explained by alterations of extractable organic carbon (EOC), extractable organic nitrogen (EON) and ammonium (NH_4^+) concentrations (Table 10). Changes in total archaeal community composition were explained by alterations of all measured soil chemical properties, with a greater variation explained by nitrate (NO_3^-) (Table 10). AOB community composition changes were explained by all measured soil chemical properties, except pH (Table 10). However, a greater percentage of AOB was explained by NH_4^+ (46%) (Table 10). Community composition changes of AOA were mainly explained by soil pH (Table 10).

Table 10. Regression analyses between community composition of the two prokaryotic groups and soil chemical data taken at EC30 and EC90 across the sites

Prokaryotic group	EOC (mg kg^{-1})	EON (mg kg^{-1})	NH_4^+ (mg kg^{-1})	NO_3^+ (mg kg^{-1})	pH (H_2O)
Total bacteria	0.280[a]***b	0.186[a]**[b]	0.383***	0.026 n.s.	0.000 n.s.
Total archaea	0.107*	0.107*	0.231***	0.316***	0.168**
Bacterial *amoA* gene	0.344***	0.245**	0.459***	0.273**	0.001n.s.
Archaeal *amoA* gene	0.000 n.s	0.009 n.s.	0.010 n.s.	0.0715n.s.	0.295**

[a]R^2 indicates the proportion of variation in Shannon diversity explained by log values of the chemical data.

[b]Significance levels: n.s.: P>0.05; *P<0.05; **P<0.01; ***P<0.001.

3.5. Discussion

3.5.1. Dominance of environmental factors over presence of BCA

Previous studies on non-target effects of beneficial *Fusarium* spp. strains as biocontrol agents (BCAs) on soil microbial communities have been restricted to controlled conditions with short investigation periods rather than field conditions (Edel-Hermann, 2009, Karpouzas et al., 2011, Musyoki et al., 2015). Generally, controlled conditions do not represent the natural conditions that prevail in the rhizopshere of crops grown in the field. In this respect, the tripartite interaction between *Fusarium* spp. BCAs, indigenous microbial communities and host crop (rhizosphere) have been suggested to vary with environmental conditions (Goh et al., 2009; Mendes et al., 2013). To account for this, we carried out field experiments during two cropping seasons to study the effects of the BCA "Foxy-2" relative to the acknowledged effects of natural factors including soil type (site), crop growth stage and seasonality (rainfall patterns) on the abundance (qPCR) and community composition (T-RFLP) of total and nitrifying prokaryotes in rhizospheres of maize grown in contrasting soils. Our results revealed that site, crop growth stage and seasonal variations controlled the abundance and community composition of prokaryotic nitrifiers to a greater extent than "Foxy-2" inoculation. This central outcome further reinforced the need for extended field studies under different conditions for better understanding of rhizospheric interaction effects between indigenous communities and potential biological control agents (Musyoki et al., 2015).

In the current study, Busia and Homabay soils had clay contents ranging from 33 to 49%, respectively, which were higher than that of the sandy soil (22%) used for a previous study by Musyoki et al. (2015), where a promoting effect nitrifying archaea was reported in a sandy but not in a clay soil. These soil texture differences may have partially masked "Foxy-2" related shifts in the abundance of total and also nitrifying prokaryotes. Notably, total archaeal abundance was higher in the Homabay soil than in that of Busia, while total bacteria, nitrifying archaea (AOA) and bacteria (AOB) were not influenced by soil type (site). This higher abundance and also a clear distinction of the composition of the total archaeal community in the clayey Homabay soil was mainly attributed to the higher soil pH and higher clay contents promoting a larger soil organic carbon (SOC) background at Homabay, an explanation which is in agreement with earlier studies (Gerzabek et al., 2002; Shen et al., 2008; Morimoto et al., 2011). Likewise, we have recently observed that clayey soils hampered the proliferation of "Foxy-2" (Zimmermann et al., 2015). It is likely that the archaeal community colonized similar resource niches like "Foxy-2" which has been reported to proliferate better in low nutrient conditions of e.g. sandy soils (Larkin and Fravel, 2002; Erguder et al., 2009; Zimmerman et al., 2015). We therefore speculated that indigenous rhizosphere archaea were involved in the suppression of "Foxy-2" as was not only corroborated by negative correlations between the BCA and indigenous total archaea (J. Zimmermann, personal communication), but also by earlier studies stating that clayey soils evolve a high natural suppression potential against microorganisms (Toyota et al., 1996; Fravel and Larkin, 2002). This hypothesized suppressive effect by archaea became obvious during the later stages of the vegetation period in both seasons (i.e., SR (short rains), LR (long rains)), where abundance of total archaea but also that of AOA increased significantly. We assumed that this archaeal dominance at the later crop growth stages increased their competitive and hence suppressive abilities over "Foxy-2". This niche-based resource competition may have been particularly evident at EC90, when "Foxy-2" was in its saprophytic stage and, similar to indigenous rhizosphere archaea, further increasing the capitalization on available indigenous SOC as central resource (Kandeler et al., 2002; Garbeva et al., 2004; Elzein et al., 2010; Musyoki et al., 2015). Moreover, advanced decomposition of organic matter derived from the fallow period prior to the field experiment setup in the SR season may have contributed to this abundance boost of AOA over AOB since AOA are generally more adapted to acquisition of organic derived nutrients over their bacterial counterparts (Wessén et al., 2010).

3.5.2. Foxy-2 did not induce a resource limitation for bacteria and archaea

Clear indications of resource limitation for bacterial and archaeal abundances were found under the tested field conditions. This was most obvious at EC30 during LR, where abundance of total and nitrifying bacteria and archaea increased with application of *Tithonia diversifolia* (TD) residues over the control treatment (C). However, this apparent resource limitation was not induced by presence of "Foxy-2" (F) as there was firstly a positive effect of the BCA on nitrifying (*amoA*) and total bacterial (16S) as well as their archaeal abundances, and the positive effect was even greater when combined with TD. This stimulating effect was not observed during the initial SR which was traced back to the decomposition of organic matter derived from the fallow period prior to the field experiment setup in the SR season. In addition, the high rainfall amount during the SR rain season (149 mm $month^{-1}$) in comparison to the lower rainfall amount during the LR season (109 mm $month^{-1}$) may have contributed to indirect changes through rhizodeposition, consequently affecting the organic resource availability in the rhizosphere (Bell et al., 2009, Rasche et al., 2011). This resource excess was reflected by overall higher total bacterial and archaeal abundances during the SR season.

Furthermore, AOB abundance dominated at the earlier growth stages, particularly at EC30 which was linked to the provision of easily degradable rhizodeposits including EOC, as well as nitrogenous metabolites such as extractable organic nitrogen (EON) and nitrates (NO_3^+) correlating positively with AOB, but negatively with AOA abundance (Hai et al., 2009; Aira et al., 2010; Neal et al., 2012). Similarly, a strong community differentiation was found between AOB and AOA at EC30 and EC90 corroborating the colonization of distinct ecological niches. In this respect, we found that ammonium (NH_4^+) contents explained more than 40% of the AOB community shift, while soil pH was the only parameter explaining the AOA community shift. Our findings agreed with earlier studies stating that niche differentiation between AOA and AOB is mainly driven by alterations of soil N and pH conditions (Valentine, 2007; Shen et al., 2008; Erguder et al., 2009; Wessén et al., 2010), which were not consistently significantly influenced by presence of "Foxy-2".

3.6. Conclusion and outlook

Our field study revealed that "Foxy-2" application did not impose a negative effect on the abundance and community composition of total and nitrifying prokaryotes. This is an important prerequisite concerning the registration of "Foxy-2" as a potential *S. hermonthica*

BCA. The major observation that crop growth stage, seasonal variations and soil type controlled total and nitrifying prokaryote abundance and community composition to a greater extent than “Foxy-2” inoculation demonstrates the need to include field studies over several seasons to obtain a more detailed, site-and seasonality-specific understanding on non-target effects of potential BCAs on indigenous microbial communities colonizing the rhizosphere of *S. hermonthica* affected crops. Notably, we found clear indications that particularly archaea and “Foxy-2” colonized similar ecological niches for organic resource acquisition in the rhizospheres of maize grown in clayey soils. In that respect, we postulate that archaeal domination in clayey soils is a considerable regulator of “Foxy-2” proliferation due to their greater rhizosphere competence over that of “Foxy-2”. It needs, however, to be tested if this potential out competition of archaea holds true under other environmental conditions than those tested in this study. Therefore, progressive studies should emphasize surveys in distinct agro-ecological zones in which not only the dynamics of indigenous rhizosphere communities under the presence of “Foxy-2” will be assayed. These studies should also consider local adaptation mechanisms of “Foxy-2” which might induce a higher resource competition potential against natural prokaryotic and also fungal communities such as sandy nutrient limited soils. Under such conditions, the use of N-rich organic residue inputs may be considered to compensate any resource competition.

3.7. Acknowledgments

The study was financed by the Bill & Melinda Gates Foundation (BMGF, Project “Integrated Striga Management in Africa (ISMA)) and Fiat Panis Foundation. Ms. Musyoki is grateful to the Food Security Centre (FSC) of the University of Hohenheim funded by the German Academic Exchange Program (DAAD) for providing the scholarship. Special thanks go to Dr. Joseph Ogutu and Dr. Juan Carlos Laso Bayas (Department of Bioinformatics, University of Hohenheim) for their support in statistical analysis. The authors are further grateful to Professor Dr. Ludwig Hölzle (Institute of Environmental and Animal Hygiene and Veterinary Medicine, University of Hohenheim) for access to his ABI 3130 Genetic Analyzer.

CHAPTER 4

Lasting influence of biochemically contrasting organic inputs on abundance and community structure of total and proteolytic bacteria in tropical soils

4. Lasting influence of biochemically contrasting organic inputs on abundance and community structure of total and proteolytic bacteria in tropical soils[3]

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4.1. Abstract

The SOM field experiments in Kenya, which have been initiated in 2002 on two contrasting soils (clayey Humic Nitisol (sand: 17%; silt: 18%; clay: 65%) at Embu, sandy Ferric Alisol (sand: 66%; silt: 11%; clay: 22%) at Machanga), were used for exploring the effect of nine year annual application of biochemically contrasting organic inputs (i.e., *Zea mays* (ZM; C/N ratio: 59; (lignin+polyphenols)-to-N ratio: 9.8); *Tithonia diversifolia* (TD; 13; 3.5); *Calliandra calothyrsus* (CC; 13; 6.7)) on the soil bacterial decomposer community. Soil samples were taken at the onset of the rainy season before application of fresh organic inputs in March 2011. We studied the abundance (quantitative PCR) and community structure (T-RFLP analysis) of the total (i.e., 16S rRNA gene) and specifically proteolytic (i.e., *npr* gene encoding neutral metalloproteases) bacteria. Alterations of the soil microbial decomposer

[3]This Chapter has been reprinted from:

Rasche, F., Musyoki, M.K., Röhl, C., Muema, E.K., Bernard Vanlauwe, B., and Cadisch, G., (2014). Lasting influence of biochemically contrasting organic inputs on abundance and community structure of total and proteolytic bacteria in tropical soils. Soil Biology and Biochemistry, 74, (204-213), with permission from Elsevier (Copyright ©2014) (DOI:10.1016/j.soilbio.2014.03.017). The original publication is available at: http://www.sciencedirect.com/science/article/pii/S0038071714001060.

community were related to differences of quantity (i.e., soil carbon (TC)) and particularly composition of SOC, where mid-infrared spectroscopic (DRIFTS) information, and contents of extractable soil polyphenol (PP) and the newly introduced PP-to-TC ratio served as SOC quality indicators. For total bacteria, effect of organic input quality was minor in comparison to the predominant influence of soil texture. Elevated soil PP content, driven by polyphenol rich organic inputs, was not suppressive for overall bacterial proliferation, unless additional decomposable C substrates were available as indicated by PP-to-TC ratios. In contrast to the total bacterial community, biochemical quality of organic inputs exposed a stronger effect on functionally specialized bacterial decomposers, i.e., proteolytic bacteria. The *npr* gene abundance was depressed in the TD treated soils as opposed to soils receiving CC, and showed a positive correlation with soil PP. It was suggested that the high presence of lignin and polyphenol relative to the N content, in organic inputs was increasing the *npr* gene abundance to counteract most likely the existence of polyphenol-protein complexes aggravating protein degradation. We concluded from our study that integration of spectroscopic, geochemical (i.e., soil PP) and molecular soil data provides a novel pathway to enhance our understanding of the lasting effect of organic input quality induced SOC quality changes on bacterial decomposers and particularly proteolytic bacteria driving soil organic N cycling.

Keywords: Organic input quality, SOC composition, bacterial decomposers, proteolytic genes, N cycling, tropical soils.

4.2. Introduction

In tropical, small-holder agro-ecosystems, microbial decomposition and mineralization of organic inputs provide a critical means to sustain soil productivity (Vanlauwe et al., 2010). In particular, the biochemical quality of organic inputs, which is mainly characterized by their content of nitrogen (N), cellulose, lignin and particularly polyphenols (Palm et al., 2001; Swift et al., 1979; Wardle and Giller, 1996), has been shown to determine the synchrony of crop nutrient supply with actual crop demand (Vanlauwe et al., 2010; Balser and Firestone, 2005). The pivotal role of soil decomposing microorganisms on crop nutrient synchronization was already earlier postulated, but the actual regulatory effect of organic input quality on

functionally relevant soil microorganisms in e.g. N cycling is, however, still poorly elucidated (Rasche and Cadisch, 2013).

Recent reports on soil microbial N cycling to date focused primarily on structural composition and abundance of functional genes responsible for prokaryotic nitrification and denitrification (e.g., Hai et al., 2009; Leininger et al., 2006; Francis et al., 2005). Comparably, the ecological significance of bacterial proteolysis (i.e., cleavage of amino acid bonds of organic input-derived proteins) has been so far mostly overlooked (Vranova et al., 2013; Weintraub and Schimel, 2005). In order to better understand N supply to crops through application of biochemically contrasting organic inputs in tropical, resource-limited agro-ecosystems, their effects on soil proteolytic microbial communities need to be, however, considered. This is justified as the transformation of organic input derived N (i.e., proteins) to mineral, plant available N is predominantly accomplished through bacterial proteolysis as the initial step in soil N cycling including nitrification (Rasche and Cadisch, 2013).

Most soil microorganisms express proteolytic activities by synthesizing an array of different proteases and peptidases (Vranova et al., 2013). Of these, neutral metalloproteases (*npr*) were shown to encompass a fundamental role in protein degradation in many agricultural soils (Sakurai et al., 2007; Bach and Munch, 2000; Kamimura and Hayano, 2000). Accordingly, Bach et al., (2002) developed an oligonucleotide set targeting neutral metallopeptidase (*npr* gene) which was used to reveal differences in abundance, expression and phylogeny of proteolytic bacterial populations in response to soil management, soil type and season (Mrkonjic Fuka et al., 2009, 2008; Sakurai et al., 2007; Bach et al., 2002). Although the presence and activity of bacterial proteases in various tropical soils were confirmed (Oseni et al., 2007; Wick et al., 2002; Insam et al., 1999), there is only limited information available to which extent abundance and diversity of bacterial proteolytic genes in tropical agro-ecosystems are controlled by biochemically contrasting organic inputs. Recently, Sakurai et al. (2007) showed on basis of denaturing gradient gel electrophoresis analysis that, in comparison to inorganic fertilizer, organic inputs (i.e., farm yard manure, rice bran) altered significantly the composition of *npr* genes in arable soils.

It has been reported for tropical agro-ecosystems that organic inputs (e.g., *Tithonia diversifolia*) rich in organic N (> 2.5 %), but poor in polyphenols (< 4 %) are subjected to fast decomposition, thus releasing a considerable amount of N in the first weeks after application to soil (Chivenge et al., 2009; Gentile et al., 2009). In contrast, organic inputs, which contain, apart from high organic N (> 2.5 %), also high amounts of polyphenols (> 4 %) (e.g.,

Calliandra calothyrsus), release N gradually so that only a small amount of organic input derived N is actually available for the succeeding crop although it remains in soil (Chivenge et al., 2009; Gentile et al., 2009). It was earlier suggested that this delayed release of protein-derived N may be the consequence of the ability of plant polyphenols to bind proteins, thus protecting these against microbial degradation (Mutabaruka et al., 2007; Handayanto et al., 1997; Schimel et al., 1996).

Consequently, the presence of organic input derived polyphenols in soils requires special attention when emphasizing the long-term effect of biochemically contrasting organic inputs on soil microbial N cycling including particularly bacterial proteolysis. The direct determination of polyphenol contents in soils via the commonly used Folin-Ciocalteu (1927) approach remains, however, difficult as the concentration of polyphenols in soils are, relative to total soil organic carbon (SOC), critically low (Kanerva et al., 2008; Suominen et al., 2003). Hence, the application of this detection method may be particularly disadvantageous for tropical soils as these are commonly highly weathered and characterized by fast proceeding microbial decomposition of organic inputs, whereby only a small proportion of applied organic matter (including polyphenols) is sequestered in the SOC pool (Oelbermann et al. 2004; Jörgensen and Castillo 2001; Fearnside 2000; Manjaiah et al. 2000).

Alternatively, diffuse reflectance Fourier transform mid-infrared spectroscopy (DRIFTS) represents compared to commonly applied physical and chemical SOC fractionation techniques (e.g., von Lützow et al., 2006)) an appropriate method to characterize the biochemical composition of SOC as altered by contrasting organic input types (e.g., Gerzabek et al., 2006; Antil et al., 2005; Haberhauer et al., 2000). DRIFTS detects vibrational bendings and stretchings of functional organic groups which are visualized in the mid-infrared spectrum ranging between 4000 to 400 cm^{-1}. DRIFTS profiles contain information of main functional groups of SOC reaching from labile (e.g., aliphatic) to recalcitrant (aromatic, phenolic) compounds (e.g., Demyan et al., 2012; Duboc et al., 2012; Tatzber et al., 2010; Janik et al., 2007; Baes and Bloom, 1989). DRIFTS has been recently used to detect functional groups (e.g., polyphenols at 1270 cm^{-1} (Janik et al., 2007)) of SOC by integrating respective peak areas of distinct spectral frequencies. Additionally, Demyan et al. (2012) used peak areas at 1620 cm^{-1} to characterize the effect of farm yard manure on aromatic compounds within the SOC pool, while Gerzabek et al. (2006) found peak heights at 2920, 1630 and 1450 cm^{-1} of soils to correlate with SOC contents of a Eutric Cambisol.

In the present study, it was our primary objective to explore if long-term application of biochemically contrasting organic inputs to two pedogenetically different tropical soils induced significant effects on the abundance and community structure of total and specifically proteolytic soil bacteria. We proposed that these alterations of total and proteolytic bacteria occurred due to organic input quality driven changes of the SOC composition considering specifically soil polyphenols as regulators of abundance and community structure of soil proteolytic bacteria harbouring the *npr* gene.

4.3. Materials and methods

4.3.1. Field experiments and soil samplings

Soil samples were obtained from the SOM field experiments which were initiated in Kenya in March 2002 to determine primarily the influence of continuous annual application of organic inputs of different biochemical quality on SOC dynamics (Gentile et al., 2008). A detailed field experiment description can be retrieved from Chivenge et al. (2009). The study sites are located in the central highlands of Kenya, Embu ("E"; 0°30´ S, 37°27´ E; 1380 m above sea level (a.s.l.)) and Machanga ("M"; 0°47´ S, 37°40´ E; 1022 m a.s.l.). Mean annual rainfall is 1200 mm in Embu and 900 mm in Machanga, which occurs in two distinct rainy seasons within the course of the year. The mean annual temperature is 20°C for Embu and 26°C for Machanga. The soil at Embu is defined as a Humic Nitisol (sand: 17%; silt: 18%; clay: 65%), while a Ferric Alisol (sand: 66%; silt: 11%; clay: 22%) is characteristic for Machanga (FAO, 1998).

The soil treatments are similar at both experimental sites including various biochemically contrasting input types according to the definition by Palm et al. (2001) (Table 11). The following organic inputs were selected for the present study: *Tithonia diversifolia* ("TD"; class I), *Calliandra calothyrsus* ("CC"; class II), and *Zea mays* ("ZM"; class III). Biochemical quality of used organic inputs was described previously in Gentile et al. (2011) (Table 11). A control with no organic inputs (CON) was also included. Organic inputs (4 Mg C ha^{-1}) are annually incorporated into the soil prior to the start of the long rains starting in March.

Soil sampling was performed at the onset of the rainy season before application of fresh organic inputs in March 2011, nine years after experiment start. For the present study, we have selected only those plots without mineral N fertilizer to exclude the effect of mineral N

on SOC and microbial community dynamics. From the three replicate plots of each treatment, ten soil cores from 0 to 15 cm were randomly taken using a soil auger with 3 cm diameter. Soil samples from each plot were bulked and transported to the laboratory in cooled boxes. Field fresh soil samples were made to pass through a 2 mm mesh, freeze-dried and shipped to University of Hohenheim for further analysis. There, the soil samples were stored under dry and dark conditions until laboratory analyses were started.

4.3.2. Microbiological soil analysis

4.3.2.1. Soil DNA isolation

Bulk soil DNA was isolated (FastDNA® Spin for Soil Kit, MP Biomedicals, Solon, Ohio, USA) and DNA extracts were quantified photometrically (Nanodrop ND-1000, Nanodrop Technologies, Wilmington, DE, USA).

4.3.2.1.1. Microbial abundance

Prior to quantification of both target genes (i.e., 16S rRNA gene (total bacteria), *npr* gene (proteolytic bacteria)), amplicons from each investigated gene were generated for standard preparation. For 16S rRNA genes, PCR cocktails of 50 µl contained 1 ng DNA template isolated from Escherichia coli DSMZ 30083T, 1 x PCR reaction buffer (Bioline GmbH, Luckenwalde, Germany), 2.5 mM $MgCl_2$, 0.15 µM of each oligonucleotide (set Eub338:Eub518; Lane, 1991; Muyzer et al., 1993), 0.2 mM of each deoxynucleoside triphosphate (dNTP), and 2 U Biotaq™ DNA Polymerase (Bioline). PCR amplifications were performed under the following conditions: initial denaturation at 95°C for 5 min, 30 cycles consisting of denaturation at 95°C for 30 s, oligonucleotide annealing at 58°C for 1 min, and polymerization for 2 min at 72°C. Amplification was completed by a final extension at 72°C for 7 min. For *npr* genes, PCR cocktails (50 µl) contained 20 ng DNA template isolated from *Bacillus cereus* DSMZ 310^T, 1 x PCR reaction buffer (Bioline), 2 mM $MgCl_2$, 0.8 µM of each oligonucleotide (set nprI: nprII; Bach et al., 2002)), 0.2 mM of each dNTP, 4 µg bovine serum albumin (Bioline) and 2.5 U Accuzyme Taq polymerase (Bioline). PCR amplifications were performed under the following conditions: initial denaturation at 94°C for 5 min, 40 cycles consisting of denaturation at 94°C for 30 s, oligonucleotide annealing at 53°C for 30 s, and polymerization at 72°C for 30 s. Amplification was completed by a final extension at 72°C for 10 min. Amplicons were checked on 1 % (w/v) agarose gels stained with GelRed™ (Biotrend Chemikalien GmbH, Cologne, Germany), purified (Invisorb Fragment CleanUp kit, Stratec Molecular GmbH, Berlin, Germany), ligated into the StrataClone™ PCR cloning

vector pSC-A (Stratagene, La Jolla, CA, USA), and ligation products were transformed with StrataClone™ SoloPack® competent cells (Stratagene). Specificity of clones used as quantitative PCR (qPCR) standards were checked via sequencing at LGC Genomics GmbH (Berlin, Germany) and BLAST analysis. Plasmid DNA was isolated (GenElute™ Plasmid Miniprep Kit, Sigma-Aldrich) and quantified as described above.

For qPCRs of both target genes, 25 µl PCR cocktails were prepared containing 12.5 µl 1 x SYBR green master mix (16S rRNA gene: Power SYBR green master mix (Applied Biosystems, Foster City, CA, USA); *npr* gene: Brilliant III Ultra-Fast SYBR qPCR master mix (Agilent Technologies Inc., Santa Clara, CA, USA)), 0.4 µM of each oligonucleotide, 5 ng template DNA, and 0.25 µl of T4 gene 32 protein (500 µg ml^{-1}, MP Biomedicals). PCR reactions were run on a StepOnePlus™ Real-Time PCR System (Applied Biosystems). Reactions were started with 95°C for 10 min to initialize polymerase activation, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 35 s and elongation at 72°C for 45 s. Melting curve analysis of amplicons was conducted to confirm that fluorescence signals originated from specific amplicons and not from primer dimers or other artifacts. Each DNA sample was processed in triplicate reactions, while standard curves were generated using duplicate 10-fold dilutions of isolated plasmid DNA. Automated analysis of PCR amplicon quality and quantity was performed with StepOne™ software version 2.2 (Applied Biosystems).

4.3.2.1.2. Microbial community structure

Amplicons of 16S rRNA and *npr* genes were subjected to T-RFLP analysis according to Rasche et al., (2011). As prerequisite for T-RFLP analysis, all forward oligonucleotides were labeled with 6-carboxyfluorescein at the 5´ end. For 16S rRNA genes, PCR conditions and amplicon purification were similar to the protocol as described above. For *npr* genes, however, three replicate PCRs were generated according to the previously described protocol, pooled and concentrated according to Rasche et al. (2006a) to achieve a sufficient amplicon quantity for digestion. Concentrated amplicons were purified with the QIAquick Gel Extraction kit (Qiagen, Hilden, Germany). For 16S rRNA genes, 200 ng of each amplicon were digested with 5 U *AluI* (New England Biolabs (NEB) Inc., Ipswich, MA, USA) restriction endonuclease at 37°C overnight. For *npr* genes, 200 ng of each amplicon were digested with 2 U *AluI* (NEB), 2 U HypCH4V (NEB), and 2 U *SacII* (NEB) restriction endonucleases at 37°C overnight. Restriction enzymes were inactivated at 65°C for 20min. Prior to T-RFLP analysis, digests were purified (Sephadex™ G-50, GE Healthcare

Biosciences, Waukesha, WI, USA) according to Rasche et al. (2006b) and 2 µl of each purified digest were mixed with 17.75 µl HiDi formamide (Applied Biosystems) and 0.25 µl internal size standard (500 ROX™ Size Standard, Applied Biosystems). Labeled terminal-restriction fragments (T-RFs) were denatured at 95°C for 3 min, chilled on ice, and subsequently detected on an ABI 3130 Genetic Analyzer (Applied Biosystems). Peak Scanner software (version 1.0, Applied Biosystems) was used to compare relative lengths of T-RFs with the 500 ROX™ size standard and to compile electropherograms into numeric data sets in which fragment length and peak height greater than 50 fluorescence units were used for fingerprint comparison. T-RFLP profiles used for statistical analyses were normalized according to Dunbar et al. (2000).

Table 11. Biochemical quality parameters[a] of the three studied organic inputs

Input type	Biochemical quality class[b]	C [%]	N [%]	C-to-N ratio	ADF-lignin [%]	Polyphenols [%]	**(**Lignin + Polyphenols)-to-N ratio
Zea mays	III	40 (1)[c]	0.7 (0.1)	59 (11)	5.4 (1.2)	1.2 (0.2)	9.8 (2.2)
Calliandra calothyrsus	II	44 (0)	3.3 (0.2)	13 (2.6)	13.0 (2.6)	9.4 (1.6)	6.7 (0.6)
Tithonia diversifolia	I	38 (1)	3.2 (0.5)	13 (2)	8.9 (0.8)	1.7 (0.8)	3.5 (0.5)

[a]Data were taken from Gentile et al., (2011).

[b]Biochemical quality classes were defined by Palm et al., (2001).

[c]Standard deviation is given in parentheses

4.3.3. Physico-chemical soil analysis

4.3.3.1. Mid-infrared spectroscopic analysis

Mid-infrared spectra of freeze-dried soil samples were recorded according to Rasche et al. (2013). Briefly, soil samples were ball-milled and maintained overnight at 32°C prior to analysis. Mid-infrared spectra of soil samples (250 mg) were recorded on a Tensor-27 Fourier transform spectrometer (Bruker Optik GmbH, Ettlingen, Germany) using a potassium bromide (KBr) beam splitter and a liquid N cooled mid-band mercury-cadmium-telluride detector. The Tensor-27 bench was mounted with a Praying Mantis diffuse reflectance chamber (Harrick Scientific Products, New York, USA) purged constantly with dry air derived from a compressor (Jun-Air International, Nørresundby, Denmark) with a flow rate of 200 l hr^{-1}. Each soil sample was analyzed in triplicate from wavelengths 3950 to 650 cm^{-1} with 16 co-added scans per sample at a resolution of 4 cm^{-1} using solid, undiluted KBr (Carl Roth GmbH) as background. The acquisition mode was double forward-backwards and the Blackman-Harris-3 apodization function was used. The spectra were recorded in absorbance units (A.U.). By using the spectral processing software OPUS version 7.0 (Bruker Optik GmbH), obtained spectra were baseline corrected using the concave rubberband correction mode and the three laboratory repetitions of each soil sample were averaged.

For interpretation of whole DRIFTS fingerprints, A.U. at 1710 data points within the range from wavelength 3950 to 650 cm^{-1} of each recorded spectrum were used for statistical analysis (e.g., analysis of similarity, cluster analysis) as described below.

For particular DRIFTS band interpretation via peak area integration, it must be considered that both mineral and organic substances may have vibration frequencies in some of the same or overlapping wavenumbers (Demyan et al., 2012). Hence, after visual inspection of DRIFTS fingerprints (Fig. 8), only those frequencies were included which could be clearly related to polyphenolic compounds. According to a literature review, the following spectral frequencies were assigned as aromatic compounds and used for further statistical purposes: peak #1 (1750 to 1510 cm^{-1}) as defined as aromatic C=C and COO- stretchings (Demyan et al., 2012; Nault et al., 2009; Smit and Meissl, 2007), and peak #2 (1450 to 1330 cm^{-1}) as defined as C-O of phenolic C-OH groups (Baes and Bloom, 1989). At these two identified DRIFTS peaks, upper and lower boundaries of wavelengths were established; a local baseline was drawn between the limits, and peak area integration was performed using the OPUS 7.0

software package to calculate the peak area (Demyan et al., 2012). DRIFTS peak areas were presented in the unit A.U.*cm^{-1}.

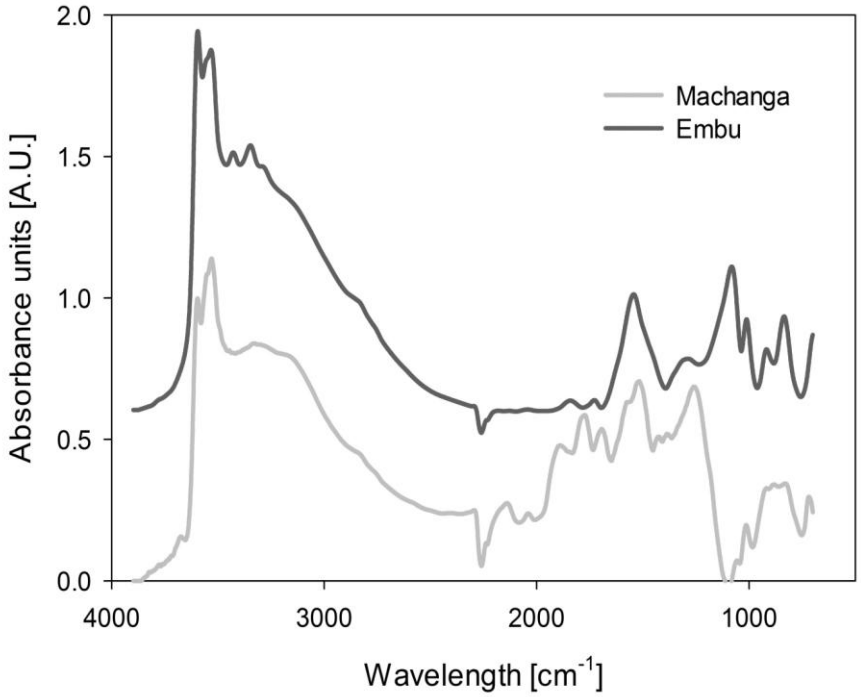

Figure 8. Visualization of DRIFTS patterns obtained from the soils of the Embu and Machanga experimental field sites.

The presented DRIFTS spectra are averaged spectra as calculated on basis of all four soil treatments (n = 12 as derived from plots CON, ZM, TD, and CC) as no significant differences were determined for the factor "input type".

4.3.3.2. Total contents of soil polyphenols

For measurement of total soil polyphenol (PP) contents, freeze-dried soil samples were first ball-milled. Five g of grounded soil were transferred into a 50 ml vessel; 10 ml of 70 % aceton (Carl Roth GmbH, Karlsruhe, Germany) were added and mixed. The vessel was placed in an ultrasonic bath (model Elmasonic S30H; Elma Hans Schmidbauer GmbH, Singen, Germany) at 70°C for 20 min. Every 5 min, the samples were mixed. Samples were centrifuged at 3000 rpm for 5 min, supernatant was transferred into a fresh 50 ml vessel and centrifugation was repeated. The supernatant was transferred into a 60 ml glass tube and placed in 70°C heating block in which the liquid was evaporated completely under a permanent N_2-gas stream using a Techne Driblock DB-2D (Bibby Scientific Limited, Staffordshire, UK). The dry extract was supplemented with 3 ml 80 % methanol (Carl Roth GmbH), mixed and incubated in the ultrasonic bath at 50°C for 5 min for better resolving.

The suspension was evaporated under the same conditions as described above until a final volume of 0.5 ml was reached. The concentrated sample was transferred into a 2.0 ml reaction vessel and centrifuged at 13000 rpm for 5 min to pellet remaining suspended particles. The purified supernatant was transferred in a new 2.0 ml reaction vessel and mixed with 0.25 ml of a diluted Folin-Ciocalteu's phenol reagent solution (2:1 with distilled water; Sigma-Aldrich, St. Louis, MO, USA) and 1.25 ml of sodium carbonate solution (20 %; Carl Roth GmbH). The 2 ml mixture was incubated in the dark at room temperature for 30 min. A calibration curve was prepared using tannic acid (Carl Roth GmbH) as reference substrate for polyphenols in known concentrations ranging from 0 to 100 mg ml^{-1}. Five hundred µl of each reaction of both, soil and calibration samples, were measured in triplicates at 725 nm on a Specord 50 photometer (Analytik Jena AG, Jena, Germany). Total tannin (here used as total soil PP) concentration in soil samples was calculated against values obtained from the calibration curve and expressed in mg polyphenol kg^{-1} dry soil.

4.3.3.3. Total soil C and N contents

Total contents of total C and N in soil samples were determined by dry combustion according to DIN/ISO 13878 (1998) using a Vario-EL III Elemental Analyzer (Elementar Analysensysteme GmbH, Hanau, Germany).

4.3.4. Statistical analysis

Normal distribution of physico-chemical and microbiological soil data was confirmed by non-parametric Kolmogorov-Smirnov tests (SPSS version 21 (SPSS 21) for Windows, IBM Corporation, Armonk, NY, USA). A two-factorial general linear model (SPSS 21) was used to determine significant treatment effects of the two factors "site" and "input type" on physico-chemical data and abundance of both investigated bacterial genes. Pearson's linear correlation coefficients were calculated for assessing significant relationships between gene abundance and physico-chemical soil parameters (SPSS 21).

Effects of the two factors "site" and "input type" on normalized T-RFLP and DRIFTS data sets were further assayed on basis of Bray-Curtis similarity coefficients (Legendre and Legendre, 1998). According to Rasche et al. (2011), a similarity matrix was generated for all possible pairs of samples of each target group. This similarity matrix was used for analysis of similarity (ANOSIM) statistics (Clarke and Green, 1988) to test if community structures of total and proteolytic bacteria as well as DRIFTS fingerprints were altered by the two factors. ANOSIM generates a test statistic, R. The magnitude of R indicates the degree of separation

between two independent communities, with a score of 1 indicating complete separation and 0 indicating no separation. For ANOSIM, a "global" R was first calculated to evaluate the overall effects of the two factors on the three individual data sets. In a second step, pairwise comparisons via ANOSIM were performed to reveal distinct effects of organic inputs on soil microbial communities and SOC composition at each study site (e.g., ECON (Embu-control) versus EZM (Embu-*Zea mays*)). In addition, Bray-Curtis similarity coefficients were used for cluster analyses using the "group average" clustering method considering the average values of the three field replications of each treatment. Calculation of similarity coefficients, ANOSIM and cluster analyses were carried out using Primer 6 for Windows (version 6.1.5, Primer-E Ltd., Plymouth, UK).

4.4. Results

4.4.1. Microbial community dynamics

Abundance of the *npr* gene was controlled significantly by the interaction of "site" and "input type" ($P<0.05$) (Fig. 9a). Similar to the abundance of the 16S rRNA gene, gene copy numbers of the *npr* gene were lower in the TD treated Machanga soil as compared to the other treatments. Abundance of the *npr* gene showed acceptable positive correlations with Nt ($r = 0.463$), soil PP ($r = 0.458$) and (lignin + polyphenols)-to-N ratio ($r = 0.549$), while negative correlations were calculated when relating to the ratios between TC-to-N_t ($r = -0.441$) and PP-to-N_t ($r = -0.408$) ($P<0.05$).

Abundance of 16S rRNA gene (total bacteria) was significantly affected by factor "site", where generally higher gene copy numbers were determined in the Embu soils ($P<0.01$) (Fig. 9b). In contrast, copy numbers of the 16S rRNA gene did not show a significant response to factor "input type", although treatment TD tended to show lower gene copy numbers as compared to the other treatments at Machanga ($P>0.05$). A positive correlation was calculated for the abundance of the 16S rRNA gene and *npr* gene ($r = 0.694$; $P<0.01$). Furthermore, 16S rRNA gene copy numbers showed positive correlations with several physico-chemical soil parameters (TC: $r = 0.576$; $P<0.01$; N_t: $r = 0.574$; $P<0.01$; soil PP: $r = 0.504$; $P<0.05$), while weak negative correlations were found with the PP-to-TC ratio ($r = -0.409$), TC-to-N_t ratio ($r = -0.409$), as well as peak #1 ($r = -0.435$) and peak #2 ($r = -0.452$) ($P<0.05$).

Analysis of similarity (ANOSIM) of T-RFLP fingerprinting data revealed significant effects of factor “site” on the community structure of both genes (16S rRNA gene (total bacteria): R = 0.361 ($P<0.01$); *npr* gene (proteolytic bacteria): R = 0.318 ($P<0.05$), while factor “input type” did not reveal any significant influence ($P>0.05$) (Table 12). The distinct effects of factor “site” were further explored by cluster analyses based on group average clustering (Fig. 10a (*npr* gene) and 10b (16S rRNA gene)). Here, it became evident that the particular effects of the contrasting organic inputs on microbial communities in the soils of the Machanga site revealed greater community responses than compared to Embu. A proof for this trend was provided by pairwise ANOSIM, which calculated for 16S rRNA gene-based T-RFLP fingerprinting a distinct community difference between the two treatments MCON versus MTD (R = 0.444), while for *npr* gene T-RFLP analysis a remarkable community difference was determined between MCC versus MTD (R = 1).

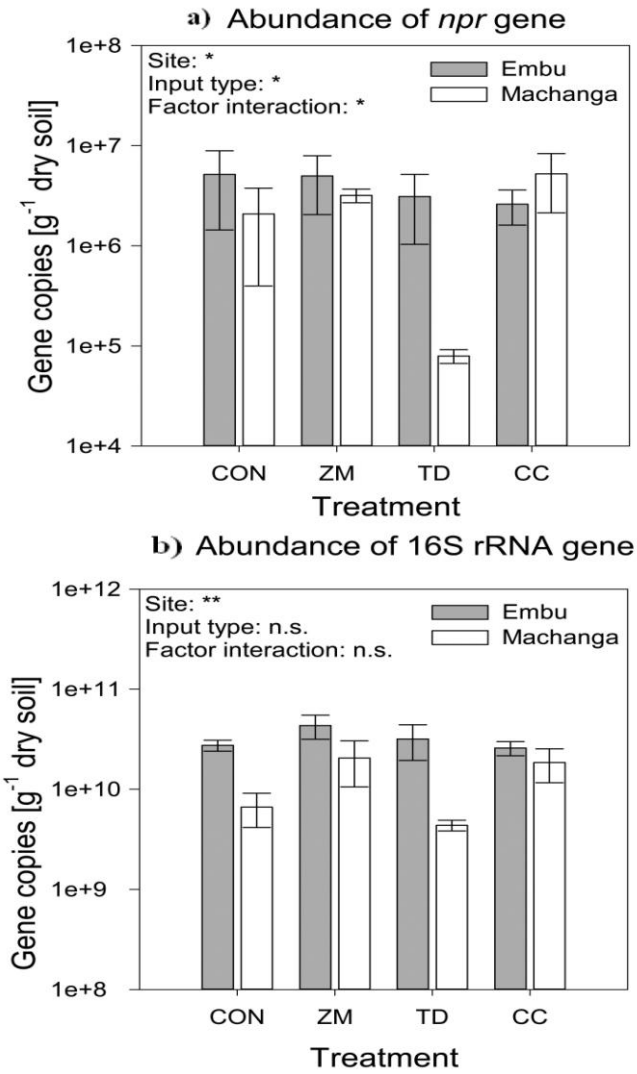

Figure 9. Abundance of *npr* (a) and 16S rRNA (b) genes in the soils of the two experimental fields in Embu (gray columns) and Machanga (White columns).

[a]Presented data are average values calculated on basis of the three field replications along with standard deviation. [b]Treatments are: CON =control with no organic inputs; ZM = *Zea mays*; TD = *Tithonia diversifolia*; CC = *Calliandra calothyrsus.*

Table 12. Global R values for the two main factors "site" and "input type" as obtained from the analysis of similarity of T-RFLP (16S rRNA and *npr* genes) and DRIFTs fingerprints

Data set	Factor	
	Site	Input type
16S rRNA gene T-RFLP analysis	0.361**[a]	n.s.
npr gene T-RFLP analysis	0.318*	n.s.
DRIFTS analysis	1.000***	n.s.

[a]Significance levels: ***: $P < 0.001$; **: $P < 0.01$; *: $P < 0.05$; n.s.: not significant ($P > 0.05$).

4.4.2. DRIFTS pattern interpretation

ANOSIM based on Bray-Curtis Similarity coefficients was used to interpret the DRIFTS spectra with respect to the effects of the two factors. A global R of 1 was calculated for factor "site" indicating a highly significant difference of the DRIFTS spectra between the soils of the two sites Embu and Machanga (P<0.001) (Table 12). The differences were particularly visible in the spectral range from wavelengths 2200 to 650 cm^{-1} (Fig. 8). The following predominant peaks related to organic compounds occurred in the DRIFTS spectra obtained from both soils: carbohydrate overtones of C-OH stretching (2171-1932 cm^{-1}; Janik et al., 2007), aromatic C=C and COO- stretchings (1717-1558 cm^{-1}; Smidt and Meissl, 2007; Nault et al., 2009; Demyan et al., 2012), C=C of aromatic groups (1544-1500 cm^{-1}; Duboc et al., 2012), aromatic skeletal (1500-1458 cm^{-1}; Smidt and Meissl, 2007), C-O of phenolic C-OH groups (1450 to 1330 cm^{-1}; Baes and Bloom, 1989), C-OH of aliphatic OH (1194-1131 cm^{-1}; Tatzber et al., 2010), C-OH of aliphatic OH (1131-954 cm^{-1}) (Stevenson, 1994), and aromatic C-H out of plane bending (944-733 cm^{-1}; Baes and Bloom, 1989; Senesi et al., 2003).

These obvious differences of the DRIFTS patterns were further confirmed by group average-based cluster analysis (Fig. 10). Contrastingly, factor "input type" did not reveal a significant global R for obtained DRIFTS spectra (P>0.05) which was also confirmed by cluster analysis (Fig. 10c). However, direct comparison of differently organic input-treated soils at the Machanga site indicated a significant effect of organic input type on DRIFTS spectra (i.e., MCON versus MZM (R = 0.778), MCON versus MCC (R = 0.852), MCON versus MTD (R = 0.778)), while no significant organic input type effects were determined in the soils at Embu.

Peak area integration was performed for peak #1 (1750 to 1510 cm^{-1}), and peak #2 (1450 to 1330 cm^{-1}) as representatives for "aromatic" compounds of studied SOC pools. Both selected peak areas were significantly affected by factor "site" ($P<0.000$), but not by factor "input type" ($P>0.05$) (Fig. 11). Generally, larger areas for both peaks were determined in the Machanga soils. Peak #1 revealed significant, negative correlations with TC ($r = -0.967$; $P<0.01$), Nt ($r = -0.876$; $P<0.01$), and soil PP ($r = -0.583$; $P<0.01$), while positive correlations were determined when relating to ratios between TC-to-N_t ($r = 0.600$; $P<0.05$), PP-to-N_t ($r = 0.723$; $P<0.01$) and particularly PP-to-TC ($r = 0.938$; $P<0.01$). A similar trend was revealed for peak #2 ($r = -0.981$ (TC), $r = -0.900$ (N_t), $r = -0.577$ (soil PP), $r = 0.633$ (TC-to-N_t ratio); $r = 0.752$ (PP-to-Nt ratio), $r = 0.950$ (PP-to-TC ratio); $P<0.05$). Both peaks did not show any correlation with biochemical quality parameters of the three organic input types ($P>0.05$).

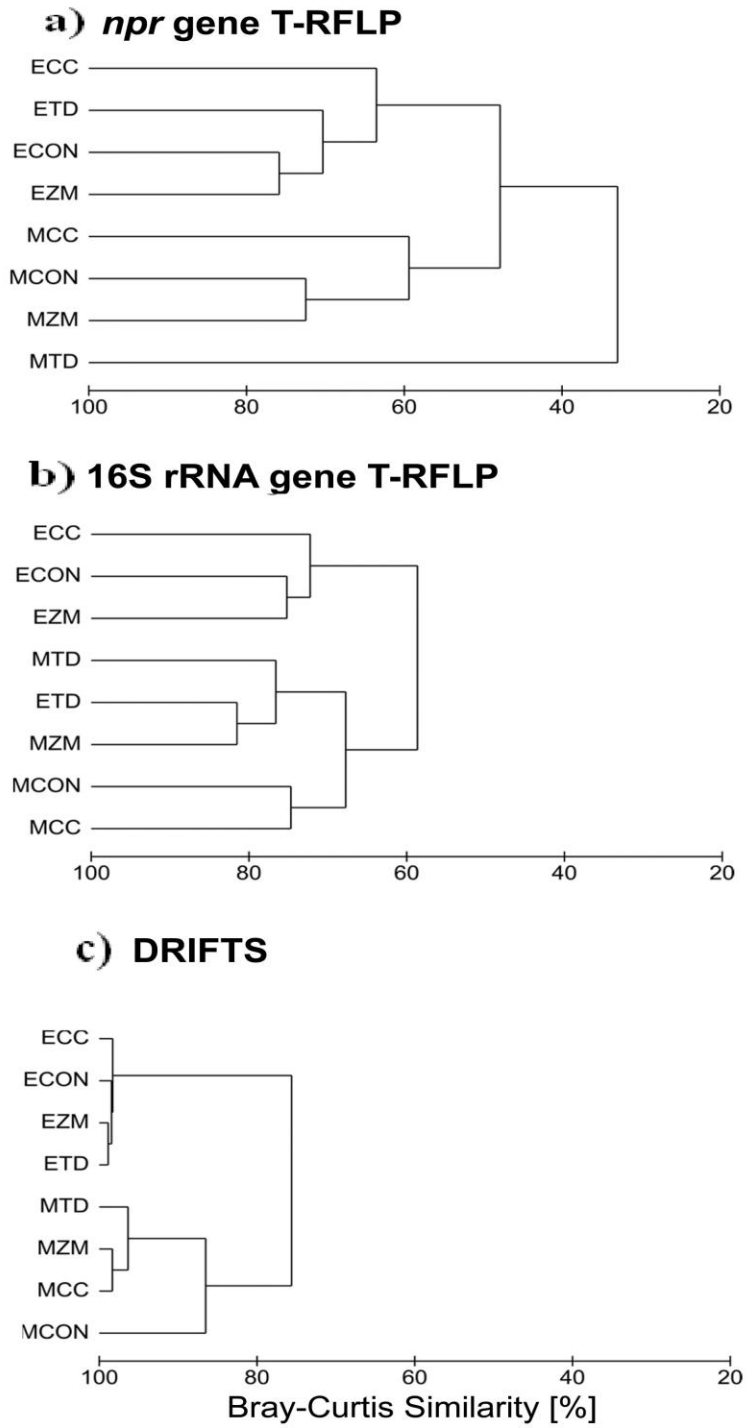

Figure 10. Group average-based cluster analysis of T-RFLP (i.e., *npr* gene (a), 16S rRNA gene (b) and DRIFTS (c) fingerprints as calculated on basis of the Bray-Curtis similarity coeffients.

[a]Treatments codes can be obtained from table 13. [b]Each treatment represents an average of n = field replications.

4.4.3. Chemical soil properties

The total contents of soil carbon (TC), soil nitrogen (N_t) and soil polyphenols (PP), TC-to-N_t ratio and PP-to-TC ratio were significantly different between the two sites Embu and Machanga ($P<0.01$), while factor "input type" did not expose any significant effect ($P>0.05$) (Table 13). A significant interaction between the two factors was determined only for TC-to-N_t ratio ($P<0.05$). There was a general trend that TC, N_t and soil PP showed higher values in the clayey Embu soils than found in the sandy Machanga soils (Table 13). In contrast, ratios of TC-to-N_t and PP-to-TC were generally lower in the Embu soils. Although the organic inputs did not change significantly the assayed soil physico-chemical parameters, it was evident that those soils treated with *Tithonia diversifolia* (TD) tended to increase TC and N_t in the soils at both study sites. No clear trend was detected for the contents of soil PP and the PP-to-TC ratio. At Embu, soil PP was generally higher in the Control (CON) and *Zea mays* (ZM) plots as compared to TD and the *Calliandra calothyrsus* (CC) plots. At Machanga, only the TD soils showed slightly higher soil PP contents than others at the same site.

TC displayed a positive correlation with soil PP ($r = 0.633$; $P<0.01$), while a negative correlation was found between TC and PP-to-N_t ratio ($r = -0.716$; $P<0.01$). TC and N_t revealed a negative correlation with the TC-to-N_t ratio ($r = -0.666$, $r = -0.829$; $P<0.01$; respectively). Soil PP showed a positive correlation with N_t ($r = 0.637$; $P<0.01$), but a negative correlation with the TC-to-N_t ratio ($r = -0.513$; $P<0.05$). No significant correlations were obtained between physico-chemical soil parameters and the biochemical quality of organic inputs ($P>0.05$).

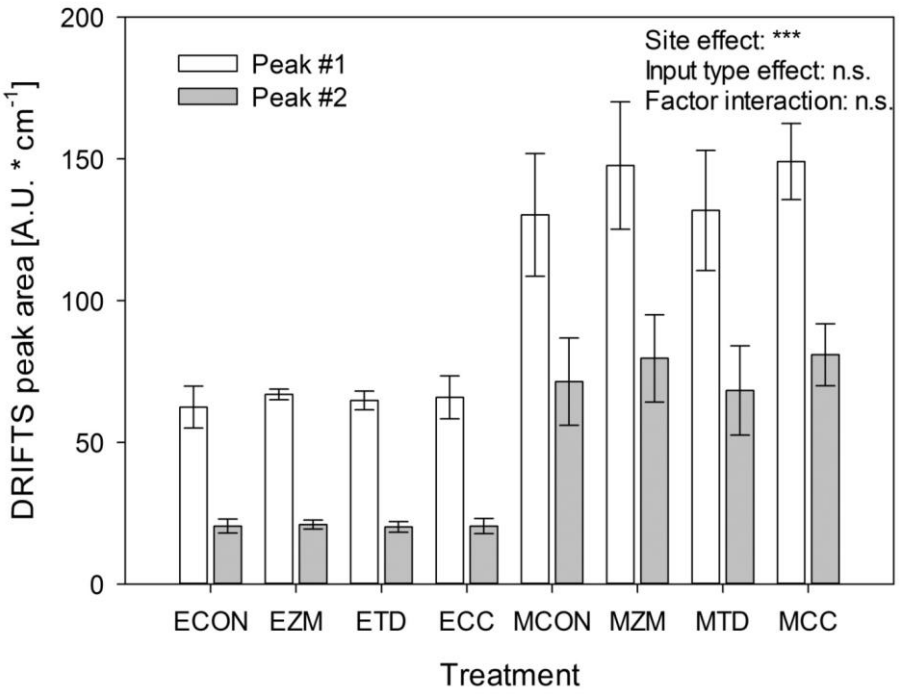

Figure 11. Areas of the two studied peaks (peak # 1(1750-1510 cm^{-1}; aromatic C = C and COO- stretchings (Smidt and Meissl, 2007; Nault et al., 2009; Demyan et al., 2012)) and peak # 2 (1450-1330 cm^{-1}; C-O of phenolic C-OH groups (Baes and Bloom, 1989))) as influenced by differently treated soil systems.

[a]Treatments codes can be obtained from Table 3. [b]Each treatment represents an average of n = 3 along with standard deviation.

Table 13. Geochemical characteristics of the differently treated soils at the experimental field sites in Embu and Machanga

[a]A two-factorial general linear model was used to detect significant effects of the two factors "site" and "input type" as well as interactions between the two factors on studied geochemical characteristics

Site	Input type	Treatment code	Total soil C (TC) [%]	Total soil N (N_t) [%]	Total soil polyphenols (PP) [mg kg^{-1}]	TC-to-N_t ratio	PP-to-TC ratio	PP-to-N_t ratio
Embu	Control	ECON	2.17 (0.32)[a]	0.26 (0.20)	27.9 (23.6)	9.4 (4.5)	12.5 (3.9)	109 (35)
	Zea mays	EZM	2.51 (0.54)	0.34 (0.13)	25.4 (16.2)	7.9 (4.4)	9.9 (2.0)	85 (37)
	Tithonia diversifolia	ETD	2.61 (0.32)	0.21 (0.05)	16.8 (5.8)	12.6 (2.7)	7.4 (0.6)	81 (15)
	Calliandra calothyrsus	ECC	2.24 (0.36)	0.20 (0.02)	16.5 (4.1)	11.4 (0.4)	6.4 (0.6)	87 (16)
Machanga	Control	MCON	0.41 (0.22)	0.02 (0.02)	11.6 (4.3)	20.4 (7.8)	29.6 (4.4)	728 (300)
	Zea mays	MZM	0.36 (0.21)	0.02 (0.02)	11.4 (6.2)	16.9 (4.8)	32.3 (1.1)	589 (178)
	Tithonia diversifolia	MTD	0.57 (0.37)	0.05 (0.03)	15.3 (4.2)	12.5 (3.1)	31.7 (2.4)	295 (37)
	Calliandra calothyrsus	MCC	0.38 (0.19)	0.03 (0.02)	11.9 (5.0)	14.5 (3.0)	29.0 (5.2)	628 (138)
Statistics[b]								
"Site"			***	***	**	***	***	***
"Input type"			n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
"Interaction"			n.s.	n.s.	n.s.	*	n.s.	n.s.

[a]Standard deviation is given in parentheses. [b]Significance levels: ***: $P < 0.001$; **: $P < 0.01$; *: $P < 0.05$; n.s.: not significant ($P > 0.05$).

4.5. Discussion

4.5.1. N stress compensation of proteolytic bacteria

The major finding of the present study was in contrast to the results obtained for the total bacterial community - the effect of organic input type on soil proteolytic bacteria which have been assayed on basis of the *npr* gene encoding neutral metalloproteases. Copy numbers of the *npr* gene were particularly depressed under the *Tithonia diversifolia* (TD) treatment at Machanga, while they were significantly higher in the soils treated with *Calliandra calothyrsus* (CC). Both organic input types had similar N contents, but differed greatly in the content of lignin and polyphenols. It has been acknowledged that high polyphenol and lignin contents reduce net residue N mineralization and hence availability of mineral N as a source for plant uptake and microbial metabolism (Handayanto et al., 1997; Contantinides and Fownes, 1994; Fox et al., 1990). Consequently, the regulative effect of lignin and polyphenols on the abundance of proteolytic bacteria was evidenced by the positive correlation between *npr* gene abundance and the residue (lignin + polyphenols)-to-N ratio substantiating the distinct response of the *npr* gene to limited N availability in the soil N pool (Handayanto et al., 1997; Contantinides and Fownes, 1994; Fox et al., 1990). Likewise, *npr* gene copy numbers revealed a positive correlation to soil PP. As PP form polyphenol-protein complexes, they further may reduce N availability and explain also the high *npr* gene abundance response of the other treatments (i.e., CON, ZM). Hence, both the presence of protein binding by polyphenols in soils as well as the high presence of lignin and polyphenol relative to N content of organic inputs of CC expressed by a high PP-to-N_t ratio in contrast to TD induced a critical N stress situation to which proteolytic bacteria responded with promoted synthesis of neutral metalloproteases to counteract the limited N availability (Triebwasser et al., 2012; Adamczyk et al., 2009; Melillo et al., 1982). This suggested that, although organic input derived N remained in the soil, proteolytic bacteria underwent a distinct promotion when soil PP was prevailing ("N stress compensation").

Similarly, a distinct shift towards fungal dominance was observed after long-term additions of polyphenol-rich residues of Peltophorum dasyrachis to soil resulting in stable polyphenol-protein complexes with reduced N availability (Mutabaruka et al., 2007). Accordingly, it could be assumed that such promotion of fungal communities might induce a stimulation of organic input dependent phenoloxidase activities as was recently shown by Kamolmanit et al. (2013). These authors reported that organic substrate-dependent N availability either

promoted or inhibited phenoloxidase supporting the ongoing controversial discussion on how N actually determines fungal decomposition activities (e.g., Edwards et al., 2011; Wu et al. 2011; Keeler et al., 2009). Hence, it could be speculated for our study that fungal phenoloxidase activities, as promoted by sufficient N supply as energy source, might have considerably contributed to the lower PP contents in the TD soils compared to the other treatments.

The proposed "N stress compensation" concept was further evidenced by the fact that both DRIFTS peaks revealed a positive correlation of 16S rRNA gene abundance with the PP-to-N_t ratio. This indicated that microbial decomposition of recalcitrant C compounds including soil PP was, apart from the availability of decomposable C as critical energy source, essentially N-dependent. This explained the significantly lower *npr* gene copy numbers observed in the Machanga soils compared to Embu. There, the low TC-to-N_t and high PP-to-N_t ratios in the TD treatment induced the low *npr* gene abundance as response to reduced N stress, e.g. higher relative N availability (Chivenge et al., 2011). It has to be considered that the presented study was based on soil samples which were obtained before application of fresh organic inputs, hence focusing clearly on longer term effects. Thus, it could be expected that the response to freshly applied inputs and availability of high protein amounts in TD might increase temporally the proteolytic activity in this treatment which may surpass other treatments including CC.

4.5.2. Minor influence of soil polyphenols on entire bacterial communities

The effect of long-term application of biochemically contrasting organic inputs on TC was more pronounced in the sandy Ferric Alisol at Machanga as compared to the clayey Humic Nitisol at Embu; the latter having a much greater background SOC due to soil type specific (e.g., heavier texture) SOC stabilization mechanisms (Chivenge et al., 2011a; von Lützow et al., 2006; Six et al., 2002). As confirmed by the positive correlation between TC content and 16S rRNA gene abundance, the higher TC content in the clayey Embu soils increased the bacterial abundance along with distinct alterations in the bacterial community structure since the start of the field experiment. It was shown earlier that soil type and specifically soil textural characteristics were critical determinants of the functional potential and community structure of soil decomposing microorganisms (Rasche et al., 2006b; Girvan et al., 2003; Blackwood and Paul, 2003). Interestingly, a positive correlation existed between 16S rRNA gene abundance and total soil PP. This indicated that extractable soil PP did not expose a suppressive effect on bacterial proliferation. On the other hand, the negative correlation

between 16S rRNA gene abundance and PP-to-TC ratio suggested that soil PP did only have an indirect effect when there was a probable prevalence of additional, less recalcitrant C substrates available which derived from the native SOC pool and/or residual, non-decomposed organic inputs ("C resource compensation effect").

The essential interaction between bacterial abundance and TC as well as soil PP content further explained the negative correlations between 16S rRNA gene abundance and integrated areas of the two selected DRIFTS peaks (peak #1: 1750 to 1510 cm^{-1}, aromatic C=C and COO- stretchings; peak #2: 1450 to 1330 cm^{-1}, C-O of phenolic C-OH groups). Although a lower content of extractable soil PP were measured, greater DRIFTS aromatic peak areas which presumably derived from the SOC pool were calculated for the Machanga compared to the Embu soils. Additionally, both DRIFTS peaks showed a positive correlation with the PP-to-TC ratio. This substantiated again our assumption that overall availability of SOC relative to the presence of soil PP was regulative for proliferation of the total decomposing bacterial biomass.

Consequently, this difference in substrate availability and particular quality of SOC along with the more sandy soil texture at the Machanga site (Gentile et al., 2008) were obviously decisive for the lower bacterial abundance. It could be assumed that a critical proportion of applied organic inputs remained partially undecomposed and thus contributed most likely to the cPOM fraction (= coarse particulate organic matter >250 µm) of the Machanga soils (Palm et al., 2001; Mafongoya et al., 1998). This assumption was supported by Samahadthai et al. (2010) and particularly Chivenge et al. (2011), where the latter report revealed an accumulation of organic input derived polyphenols in the light SOC fractions (i.e., cPOM) in the same soils explaining again the higher PP-to-TC ratios presented here. However, prospective research will have to clarify if organic input derived polyphenolic compounds in cPOM fractions as regulatory determinants of abundance of microbial decomposers contribute directly to the high increase of selected functional groups in the DRIFTS spectra obtained from the Machanga soils.

4.6. Conclusions and outlook

Long-term application of biochemically contrasting organic inputs exposed a stronger effect on functionally specialized bacterial decomposers, i.e., proteolytic bacteria, than on the total bacterial decomposer community. This effect was particularly evident in the sandy Machanga

soils, but not in the clayey soils at Embu with its presumed stronger SOC background. Hence, it could be assumed that the significant interaction between soil textural characteristics with their distinct SOC stabilization mechanisms and biochemical quality of organic inputs were decisive for the recorded community alterations of decomposing and particularly proteolytic bacteria. Indications were provided that medium-term accessibility of SOC to the soil microbial decomposers depended on its location and stabilization within the mineral soil matrix. Conversely, we concluded that the abundance of decomposing bacteria (i.e., *npr* gene abundance) was obviously regulated by organic input quality. This had a distinct effect on N availability, which in turn might have contributed to the quantity (stabilization) and quality of SOC (Puttaso et al., 2013).

In the presented paper, the underlying mechanisms for these complex interactions between soil texture determining the SOC background, input quality and microbial decomposer (i.e., proteolytic bacteria) community remained however partially not fully understood. Hence, we propose on basis of the presented report that the integration of spectroscopic (as fast and reliable alternative to commonly used SOC fractionation procedures (e.g., von Lützow et al., 2006)) along with geochemical and molecular data can enhance the insight into organic input quality induced SOC quality changes on bacterial decomposers including particularly proteolytic bacteria. In this context, future research should also consider soil PP oxidizing soil fungi (Kamolmanit et al., 2013; Edwards et al., 2011; Sinsabaugh et al., 2005), which have not been addressed in this study, to get a closer insight into the underestimated dynamics of organic input type dependent soil PP contents.

The presented study was based on only two contrasting soils and a small variety of different organic inputs. It did not consider the acknowledged effects of e.g., soil management, seasonal alternations including accelerating climate variability, as well as species and growth stage of cultivated crops on the dynamics of microbial decomposition processes. Such influence factors need particular attention to get a better insight into the short and long-term dynamics of soil proteolytic bacteria (Vranova et al., 2013). In this regard, it needs to be pointed out that the *npr* gene abundance data shown in this paper represented only the functional potential of neutral metalloprotease in the studied soils. To get a closer insight into the dynamics of active proteolytic bacteria, as regulated by biochemically contrasting organic inputs, we suggest the application of nucleic acid-based stable isotope probing approaches. This technique has approved its potential to demarcate those bacterial community members which were actively involved in the decomposition of contrasting organic inputs (España et

al., 2011). Finally, prospective research should emphasize, apart from the significance of active *npr* genes in response to organic inputs, also other proteolytic genes (e.g., sub gene (encoding serine peptidase), *apr* gene (alkaline metallopeptidase), pepN gene (encoding alanine aminopetidase)) to substantiate the critical contribution of proteolytic bacteria to N mineralization and synchronized crop nutrient supply in agriculturally managed soils (Enowashu et al., 2012; Vanlauwe et al., 2010; Bach et al., 2002).

4.7. Acknowledgements

The authors thank Evonne Oyugi (Tropical Soil Biology and Fertility Institute of the International Centre for Tropical Agriculture (TSBF-CIAT), Nairobi, Kenya) for providing the soil samples from the SOM field experiments in Kenya. The authors thank Lena Rathjen and Elvise Mangoua (Institute of Plant Production and Agroecology in the Tropics and Subtropics, University of Hohenheim) for their laboratory assistance. The first author (F. Rasche) is particularly grateful to the Life Science Center of the University of Hohenheim for the financial support within the program "Project support for young researchers". The contribution of Mary K. Musyoki was partially funded through the PhD program "Global Food Security" of the Food Security Center of the University of Hohenheim. The authors are further grateful to Professor Dr. Ludwig Hölzle (Institute of Environmental and Animal Hygiene and Veterinary Medicine, University of Hohenheim) for access to his ABI 3130 Genetic Analyzer**.**

CHAPTER 5

Response of proteolytic bacteria abundance and their potential enzymatic activity to the fungal biocontrol agent *Fusarium oxysporum* f. sp. *strigae* in contrasting soils

5. Response of proteolytic bacteria abundance and their potential enzymatic activity to the fungal biocontrol agent *Fusarium oxysporum* f. sp. *strigae* in contrasting soils

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5.1. Abstract

In the current study, we have analyzed potential non-target effects of the biocontrol agent *Fusarium oxysporum* sp. f. *strigae* "Foxy-2" on the abundance (i.e. *npr* gene encoding neutral metalloproteases) and potential enzyme activities (i.e. *npr* gene specific Suc-Ala-Ala-Phe-AMC) of protoelytic bacteria colonizing the rhizosphere of maize grown on contrasting soils (i.e., sandy Ferric Alisol versus clayey Humic Nitisol). We hypothesized that "Foxy-2" inoculation induced organic nitrogen (N) competition leading to increased abundance and potential enzymatic activity of proteolytic bacteria as a response mechanism to N competition. To compensate the presumed competition effect, a treatment with N-rich organic inputs (i.e., *Tithonia diversifolia* (TD; CN ratio = 13; lignin content = 8.9%; polyphenol content = 1.7%)) was included. The sandy soil demonstrated significantly higher *npr* gene abundance compared to the clayey soil. The abundance of *npr* genes showed a transient decline when "Foxy-2" was applied, but not when it was combined with TD in the two soils. Similarly, the *npr* gene specific enzyme showed a higher activity when organic inputs were applied in combination with "Foxy-2". It was concluded that soil texture is a key determinant of proteolytic bacteria abundance and enzymatic activities and that sole application of "Foxy-2" may cause initial organic N competition with proteolytic bacteria resulting in a transient reduction in *npr* abundance. This impact was compensated with high quality organic inputs.

Key words: Rhizosphere: Proteolytic bacteria abundance; Proteolytic enzymatic activity; *Fusarium oxysporum* f.sp. *strigae*

5.2. Introduction

Biological control agents (BCAs) for *Striga hermonthica* weed control are currently being examined as potential environmentally friendly components of integrated *Striga* management (Avedi et al., 2014; Zimmermann et al., 2015). This includes the BCA *Fusarium oxysporum* sp. f. *strigae* "Foxy-2", an acknowledged BCA for *Striga* control (Elzein and Kroschel, 2004a; Ndambi et al., 2011; Zarafi et al., 2015). However, considerable uncertainty persists regarding the environmental impacts of such BCAs on non-target organisms which is a key concern prior to their large scale release in the environment (Brimner and Boland 2003). Recent reports on non-target effects of "Foxy-2" on natural rhizosphere nitrogen (N) cycling microorganisms have mainly focused on the abundance and structural composition of functional genes responsible for prokaryotic nitrification (Musyoki et al., 2015).

On the other hand, the ecological impacts of "Foxy-2" on bacterial proteolysis have been overlooked so far. Bacterial proteolysis is a crucial process in soils, where it mobilizes nitrogen (N) organically bound in proteins (Lipson and Näsholm, 2001) through synthesis of proteases including predominantly neutral metalloproteases (*npr*) (Bach and Munch, 2000; Kamimura and Hayano, 2000; Sakurai et al., 2007; Rasche et al., 2014). It has been reported that proteolytic enzymes control the dynamics of the organic N pool which in turn regulates soil N release and hence plant growth (Lipson and Näsholm, 2001; Vranova et al., 2013). It was earlier shown that the community composition of bacterial communities encoding for metalloproteases (*npr*) was mainly influenced by soil texture and nutrient contents particularly organic matter quantity and quality (Mrkonjic Fuka et al., 2007; Sakurai et al., 2007; Rasche et al., 2014). Proteolytic activity was reported to increase in high organic matter and clayey soils (Mrkonjic Fuka et al., 2007; Sakurai et al., 2007).

To explore the potential impacts of "Foxy-2" on the proteolysis processes, as it was not yet considered in risk assessment studies, we investigated the abundance of bacterial *npr* genes and their potential activities (Suc-Ala-Ala-Phe-AMC) in "Foxy-2" treated soils. The effect of soil texture and organic input was considered by evaluating the targeted processes in sandy versus clay textured soils treated with organic N rich residues (*Tithonia diversifolia*) used for compensating any resource competition between "Foxy-2" and indigenous rhizosphere

bacteria with proteolytic abilities. We hypothesized that soil inoculation with the saprophytic fungal biocontrol agent "Foxy-2" induced organic N competition on abundance and potential enzyme activities of the *npr* gene coding bacteria. It was further hypothesized that these competition effects would vary with soil texture with greater competition effect in a sandy than a clayey textured soil. As such, sandy soils may increase proteolytic abundance/enzymatic activity since proteolytic abundance has been shown to increase under N limitation as a mechanism of N stress compensation (Rasche et al., 2014; Chapter 3).

5.3. Materials and methods

The model Fos isolate "Foxy-2" was obtained from *S. hermonthica* collected from North Ghana (Abbasher et al., 1995). Taxonomic identification of the isolate was confirmed by Julius-Kühn-Institut (JKI), Berlin, Germany, where it is deposited under accession number "BBA-67547-Ghana. Soil samples were obtained from a previous rhizobox experiment which was initiated to determine non-target effects of "Foxy-2" on the abundance of nitrifying prokayotes in contrasting tropical soils (Musyoki et al., 2015). The soils were collected from two sites in the central highlands of Kenya; i.e. Embu site: Humic Nitisol (sand: 17%; silt: 18%; clay: 65%) and Machanga site: Ferric Alisol (sand: 66%; silt: 11%; clay: 22%) (FAO, 1998). Maize (*Zea mays* L. variety 'WH507' (provided by Western Seed Company Ltd., Kitale, Kenya) was used as test crop. Maize (*Zea mays* L. variety 'WH507' (provided by Western Seed Company Ltd., Kitale, Kenya) was used as test crop. The selected variety is of high preference by smallholder farmers in Western Kenya due to its tolerance to *S. hermonthica*. Maize seeds were coated with "Foxy-2" (1.15 x 105 colony forming units per seed) through a special seed treatment technology (Elzein et al., 2006a; seed coating processed by SUET GmbH, Eschwege, Germany) to provide uniform inoculum coverage. After coating, coated and uncoated (control) maize seeds were allowed to germinate on wet filter paper to ensure that only germinated seeds were used for the rhizobox experiment. Each rhizobox was filled at the bottom with a 1 cm ground layer of vermiculite (grain size 3-8 mm) for drainage improvement. On top of this layer, soil adjusted to 50% water holding capacity was added. Meanwhile, *S. hermonthica* seeds were surface sterilized according to Elzein et al. (2010) and germination ability of seeds (75%) was checked as described by Kroschel (2002). Both soils were infested artificially with disinfected *S. hermonthica* seeds (20 mg seeds 165 g dry soil-1) and pre-conditioned at 28°C in the dark for 7 days (Kroschel, 2002). After this step, germinated maize seedlings were introduced into the rhizoboxes. After

planting of seedlings, a 1 cm layer of vermiculite was placed as the top layer to reduce evaporation and facilitate water drainage. Details on the management of the rhizobox experiment and rhizosphere soil sampling can be retrieved from Musyoki et al., (2015) (Chapter 2). For this study the following three treatments were considered: i) uncoated maize seeds with no *S. hermonthica* (C), ii) coated maize seeds with "Foxy-2" (F), and iii) coated maize seeds with "Foxy-2" and *T. diversifolia* (F + T). Three replicates of each treatment sampled at 14, 28 and 42 days after planting (DAP) were subjected to quantitative polymerase chain reaction (qPCR) of the *npr* gene using the protocol published by Rasche et al. (2014) (Chapter 4).

The potential activity of the *npr* gene specific enzyme on the basis of the fluorogenic substrate Suc-Ala-Ala-Phe-AMC (7-amido-4-methylcoumarin (7-AMC) (Bachem AG, Bubendorf, Switzerland)) was measured according to Marx et al., (2001) and Poll et al., (2006). The substrate was dissolved in 300 ml dimethylsulfoxide (DMSO), filled to a final volume of 10 ml with autoclaved water (10 mM stock solution) and stored at 4°C until further processing. For analysis, a 0.5 mM working solution of the substrate was prepared with autoclaved buffer (0.05 M Trizma® buffer; Sigma-Aldrich). For preparation of standard stock solution, 0.0876 g 7-AMC (5 mM) was suspended in 100 ml methanol and autoclaved water (1:1). The stock solution was diluted with the buffer to a final concentration of 10 µM. Soil suspensions were prepared by adding 0.5 g thawed soil into 50 ml of autoclaved water and dispersed by ultrasonication for 2 min with 50 J s^{-1} sonication energy. Fifty µl of each suspension were dispensed into 96-well microplates (PP F black 96 well; Greiner Bio-one GmbH, Frickenhausen Germany), followed by 50 µl and 100 µl of 1 mM substrate solution and buffer, respectively. Standard wells received 50 µl soil suspension and 150 µl of standard/buffer-mixture in the following concentrations: 0 mM (0 µl dye solution (10 mM) plus 150 µl buffer), 0.5, 1.0, 2.5, 4.0 and 6.0 mM, respectively. Plates were incubated at 30°C and monitored over a period of 3 h. Fluorescence was measured at 360/460 nm wavelength in a microplate fluorescence reader (Bio-Tek instruments Inc., FLX 800, Germany) after 0, 30, 60 120 and 180 min. Enzyme activity corresponded to an increase in fluorescence and was calculated as a linear regression and presented as nmol g^{-1} h^{-1}.

Since each rhizobox was sampled on 3 sequential dates (DAP 14, 28 and 42), a repeated measures analysis with an autoregressive covariance structure using the "nlme" package (Pinheiro et al., 2014) combined with Tukey-B based post hoc pairwise comparisons using the "lsmeans" package (Lenth, 2013) in the statistic software R (R Core Team, 2013) was

performed to determine effects of "Organic input" ("*T. diversifolia*"), "Soil type" (Embu-clayey and Machanga-sandy soil), "Sampling date" (14, 28 and 42 DAP) and "Foxy-2" on the abundance and potential enzymatic activities of proteolytic bacteria. Gene copy numbers were log transformed to meet the assumptions of parametric statistical tests (Piepho, 2009).

5.4. Results and discussion

Overall, the sandy textured soil had higher *npr* gene abundance than the clayey soil ($P<0.001$) (Table 14, Fig. 12). This was contrary to our expectations from our previous work in which the same clay soil revealed a higher abundance of indigenous total and nitrifying prokaryotes (Musyoki et al., 2015). Moreover, Mrkonjic Fuka et al. (2007) reported obvious positive correlations between abundance of proteolytic communities and soil clay fractions. On the other hand, adsorption and binding of proteinaceos materials by clay minerals was shown to have inhibitory effects on proteolytic activities (Tapp et al. 1994; Shahriari et al., 2010). The lower *npr* abundance in the clayey soil was an indication of possible protein adsorption to clay minerals that may have reduced the availability of proteins to proteolytic bacteria (i.e. *npr* genes). Recent studies in the same soils also indicated that the clayey soil with relatively high polyphenol content reduced the N availability for microbial metabolization, a reason that may further explain the lower *npr* abundance in the clayey soil (Rasche et al., 2014). Contrastingly, the higher *npr* abundance in the sandy soil was explained by the low N content in the sandy soil compared to the clayey soil (Table 15). It was argued that this N limitation is generally balanced by advanced synthesis of proteolytic genes as a responsive mechanism to N stress compensation (Rasche et al., 2014).

In the clayey soil, "Foxy-2" suppressed *npr* gene abundance at 42 DAP in comparison to the untreated soil (control) ($P<0.05$, Fig. 12a). A similar trend was observed in the sandy soil at 28 DAP with lower *npr* abundnce in treatment "Foxy-2" (F) compared to control (C) and "Foxy-2" + "*Tithonia diversifolia*" (F+T) (Fig. 12b). However, the decrease in this soil was only transient as a higher *npr* gene abundance was noted 42 DAP in treatment "Foxy-2" (F) and Foxy-2" + "Tithonia diversifolia" (F+T) compared to the control (Fig.12b). Overall, the reduction in *npr* gene abundance indicated that "Foxy-2" induced a certain competition effect which has been suggested as one of the key interactions between fungal biocontrol agents (e.g., "Foxy-2") and natural soil microbial communities (Brimner and Boland 2003). On the other hand, the varying response of *npr* gene abundance to sampling time in the two

contrasting soils was linked to the differences in the native soil organic matter background as was evidenced by higher extractable organic C and N in the clayey soil (Table 15). Consequently, the clayey soil provided more resources for microbial proliferation and this may have been responsible for the lagged "Foxy-2" induced *npr* reduction in the clayey soil compared to the sandy soil.

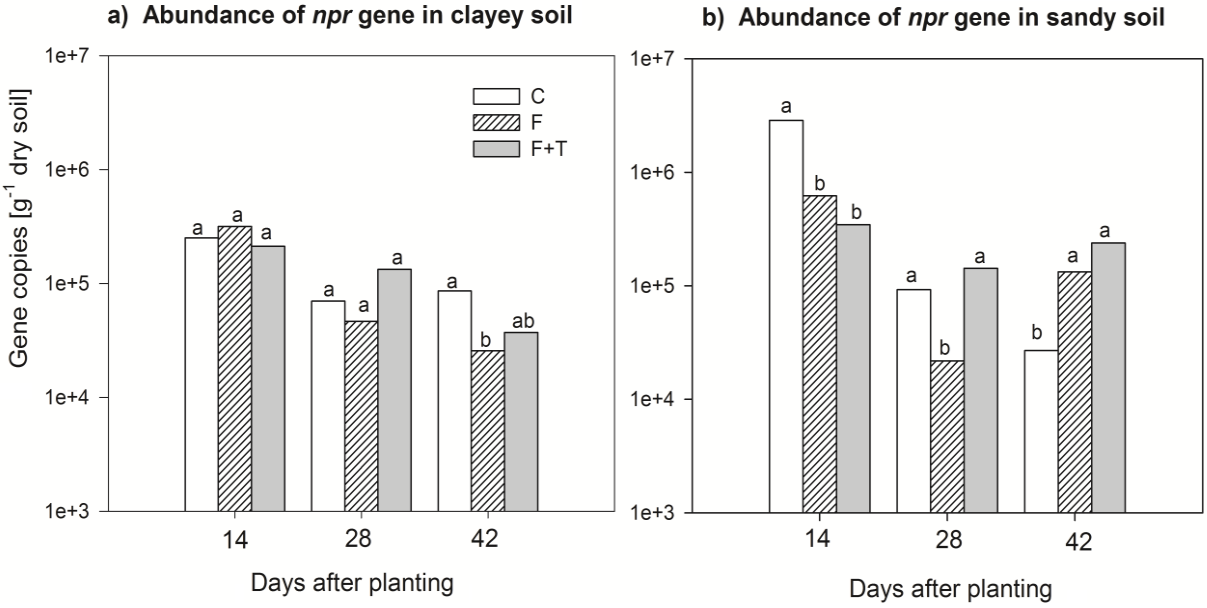

Figure 12. Response of *npr* gene abundance to "Foxy-2" inoculation and *T.diversifolia* in contrasting soils

[a]Data was backtransformed hence standard errors are not shown in the graphical plot

Combination of "Foxy-2" with organic inputs i.e. *Tithonia diversifolia* resulted in higher *npr* abundance in the sandy soil (Fig. 12), a finding which is in agreement with earlier studies (Mrkonjic Fuka et al., 2007; Sakurai et al., 2007). Similarly, potential *npr* enzymatic activity was increased by organic input with higher levels at 14 and 28 DAP in the "Foxy-2" and *Tithonia diversifolia* treatment (F+T) compared to the control (C) and "Foxy-2" (F) ($P<0.05$) in the sandy soil (Table 14, Fig. 12). This agreed with earlier findings (Mrkonjic Fuka et al., 2007; Sakurai et al., 2007; Weintraub et al., 2007) and was further explained by the fact that *Tithonia diversifolia* with its high N content (>2.5%) and low content of polyphenols (<2.0%) (Chivenge et al., 2009; Gentile et al., 2009) is generally largely decomposed already at early stages after application.

No significant differences were determined on the *npr* gene specific enzyme activity (i.e Suc-Ala-Ala-Phe-AMC) between "Foxy-2" inoculated soil and the uninoculated control soil (C)

implying that “Foxy-2” was compatible with the gene specific enzyme Suc-Ala-Ala-Phe-AMC (Fig. 13) ($P<0.001$). Since enzymatic analysis was only performed in the sandy soil, future studies should focus on the effect of “Foxy-2” on potential proteolytic enzymatic activity in clay soils as well as on other proteolytic genes to better understand the soil texture effects on the interactions of “Foxy-2” and proteolytic bacteria (Galantini and Rosell, 2006; Mrkonjic et al., 2007).

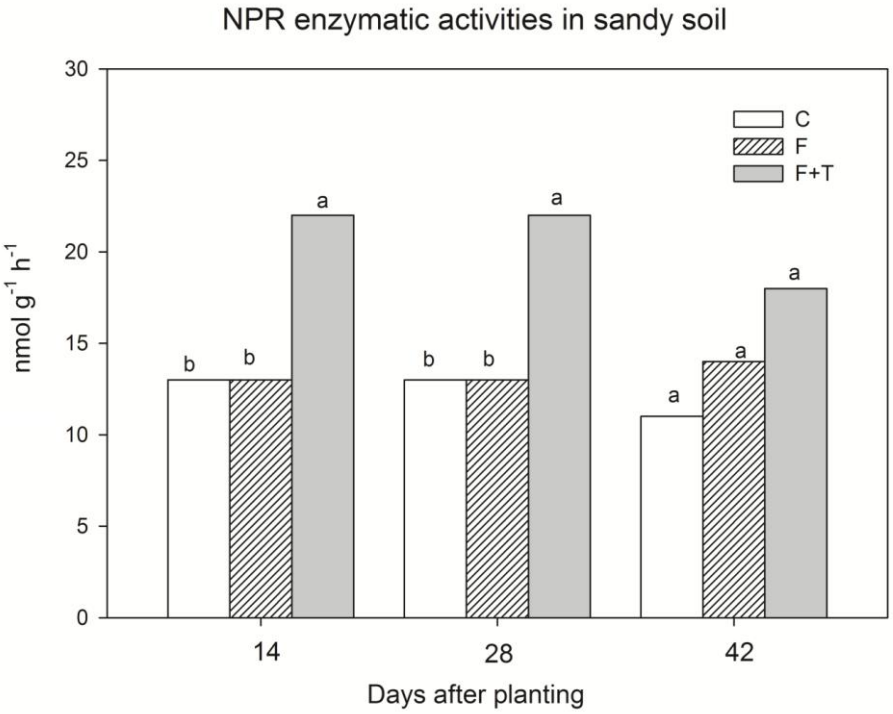

Figure 13. Response of *npr* gene specific enzyeme (suc-Ala-Ala.Phe-AMC) to “Foxy-2” and *T.diversifolia* in a sandy soil

Table 14. Analysis of variance (ANOVA) of proteolytic bacteria abundance, enzymatic activities and soil properties as influenced by soil type (S), sampling time (ST), Treatment (TR) and their interactions

Target group and soil properties	Factor/ source of variation							
	Units	S	ST	TR	S x ST	S x TR	ST x TR	S x ST x TR
qPCR								
npr gene	(Copies g^{-1} dry soil)	**	***	**	*	n.s.	**	***
Enzymatic analysis								
suc-Ala-Ala.Phe-AMC	(nmol g^{-1} h^{-1})		n.s.	*			n.s.	
Soil properties								
TC	(g kg-1)	***		***		n.s.		
N_t	(g kg-1)	***		***		***		
EOC	(mg kg-1)	***		***		***		
EON	(mg kg-1)	***		***		***		
NH_4^+	(mg kg-1)	n.s.		*		n.s.		
NO_3^-	(mg kg-1)	***		n.s.		n.s.		
pH		***		***		***		

[a]Significance levels: ***: P<0.001;**: P<0.01;*: P<0.05; n.s.: not significant (P>0.05).
[b]Potential *npr* enzyme activity was only performed in the sandy soil thus significance levels for soil type effects and their interaction are not shown.
[c]Soil physical-chemical analysis was done 42 days after planting hence no sampling time effect and its interaction with treatment and soil type is shown for soil properties.

Table 15. Soil chemical parameters as influenced by "Foxy-2" and "organic input" 42 days after planting

Soil	Treatment code	TC ($g\ kg^{-1}$)	N_t ($g\ kg^{-1}$)	EOC ($mg\ kg^{-1}$)	EON ($mg\ kg^{-1}$)	NH_4^+ ($mg\ kg^{-1}$)	NO_3^- ($mg\ kg^{-1}$)	pH
Clayey	C	16.9b	1.51a	572a	60ab	3.9a	23.4a	4.5b
(Embu)	F	16.9b	1.47a	589a	55b	6.8a	22.0a	4.6b
	F + T	18.0a	1.57a	619a	65ab	6.4a	3.6b	4.9a
SE		0.02						0.04
Sandy	C	3.69b	0.35b	103b	15.3b	4.5ab	1.99a	4.7b
(Machanga)	F	3.67b	0.36b	100b	20.3b	3.5b	2.07a	4.7b
	F + T	5.64a	0.57a	207a	28.0a	10.0a	3.80a	5.6a
SE		0.02						

[a]Different letters within columns indicate significant differences per soil.

[b]SE= standard error.

[c]Abbreviations: TC: Total carbon, N_t: Total nitrogen, EOC: extractable organic carbon, EON: Extractable organic nitrogen, NH_4^+: ammonia, NO_3^-: nitrate, pH: soil pH.

[d]No standard error is given for N_t, EOC, EON NH_4^+ and NO_3^- since natural log transformation was performed on the data.

5.5. Conclusion

Overall, our results support the theories published about the influence of organic substrate availability and soil texture on proteolytic bacteria. This study also provides evidence that the application of "Foxy-2" as a biological control agent induces transient inhibitory effects on the abundance of the *npr* gene. This reinforces the suggestion for involvement of proteolytic bacteria and their potential enzymatic activity for better understanding of the non-target effects of "Foxy-2" particularly on organic-protein derived N. The *npr* gene was sensitive to "Foxy-2" inoculation in contrasting soils types, however, the effect appears to be transitory and not long-term particularly in the sandy soil. Hence, further studies need to be done under field conditions to validate the rhizobox study and overcome some limitations involved with such studies such as acclimation period and indigenous population density which is often lower in controlled conditions especially in clayey soil where we could not determine if the effects were transitory in this study (Teuben and Verhoef, 1992). Moreover, it is critical to determine the effect of "Foxy-2" on proteolytic enzymatic activities in soils with high clay contents since clay minerals have been shown to have a stabilizing effect on free enzymes consequently leading to increased proteolytic enzyme activities (Mrkonjic et al., 2007). It should also be noted that the *npr* gene used in this study represents only the functional potential of neutral metalloproteases. To get a better understanding of the dynamics of proteolytic bacteria as influenced by "Foxy-2" inoculation, we suggest more research on other proteolytic genes (e.g. *sub* gene (encoding serine peptidase), *apr* gene (encoding alkaline metallopeptidase) and *pep*N gene (encoding alanine aminopeptidase) to substantiate the non-target effects of "Foxy-2" on N mineralization as a critical precursor step to nitrification in the N cycle (Enowashu et al., 2012; Rasche et al., 2014).

CHAPTER 6

General discussion

6. General discussion

Striga hermonthica (Del.) Benth. continues to be a major biotic threat to cereal production and food security in SSA (Parker, 2014). Integrated *Striga* management (ISM), using a combination of control measures has been widely accepted as a lasting solution to the *Striga* problem (Ejeta, 2007; Hearne, 2009). The use of fungal biocontrol agents such as *Fusarium oxysporum* f.sp. *strigae* strains along with tolerant varieties have been proposed as environmentally safe components of ISM (Marley et al., 2004). Since the fungus attacks *Striga* at all development stages, the mycoherbicide strategy has an added advantage in that it contributes to seed bank reduction and prevents reproduction in each season particularly in the absence of the host crop for *Striga* (Schaub et al., 2006). Although results from the use of fungal BCAs from controlled and field conditions have led to the successful control of *S. hermonthica*, its prospects for integration in ISM strategies are questionable (Avedi et al., 2014). The main reason for this is lack of risk assessment studies which are a prerequisite for field application and commercialization of any BCA (Avedi et al., 2014; Hearne, 2009). Therefore, there is an urgent need for environmental risk assessment of *Fusarium oxysporum* f.sp. *strigae* strains particularly on beneficial non-target microorganisms as many studies have mainly focused on host range, yet little is known on the fate of key microorganisms that drive nutrient cycling processes in the soil. There has also been rising concern over such BCAs to displace indigenous microorganisms especially those involved in key nutrient cycling processes i.e. N and C cycling (Mezzalama et al., 1997). On the other hand, high variability in field efficacy of *Striga* biological control agents under certain conditions has often been linked to better rhizosphere competence of indigenous microorganisms compared to the introduced biocontrol microorganism which has to adapt to the new environment (Avedi et al., 2014). However, most of these concerns and explanations are speculative and little is known on the non-target effects of *Fusarium oxysporum* f.sp. *strigae* strains on indigenous microorganisms. Consequently, the main goal of this study was to investigate how fungal biocontrol agents (i.e. *Fusarium oxysporum* f.sp. *strigae* strains) interact with native beneficial microorganisms (nitrifying and proteolytic prokaryotes) in contrasting maize rhizosphere soils by using "Foxy-2" as a model strain.

6.1. Is Foxy-2 compatible with nitrifying prokaryote communities in maize rhizospheres in contrasting soil types?

In depth studies (Chapter 2), were carried out to investigate if "Foxy-2" as a biocontrol agent has non-target competition effects on nitrifying prokaryotes in two contrasting soils by using bacterial and archaeal *amoA* genes (ammonia monooxigenase) (AOB and AOA) as well as the bacterial and archaeal 16S rRNA genes as markers. To compensate presumed potential competition effects by Foxy-2 on nitrifying prokaryotes, high quality organic input (i.e., *Tithonia diversifolia* with C/N ratio = 13, lignin content = 8.9%, polyphenol content = 1.7%) was included as an additional treatment.

Our results from the rhizobox experiment revealed that "Foxy-2" did not negatively affect the abundance of nitrifying prokaryotes but rather promoted total archaeal communities and their AOA counterparts (Chapter 2). The increase in the abundance of archaeal prokaryotes following "Foxy-2" inoculation was, however, dependent on soil texture because it occurred in the sandy soil but not in the clayey soil (Chapter 2). On the other hand, AOB abundance remained unaffected by "Foxy-2" inoculation irrespective of the soil texture. These results suggest a soil type-dependent interaction between "Foxy-2" and archaeal nitrifying prokaryotes and their bacterial counter parts. The soil type-dependent effects were mainly linked to differences in nutrient resource availability, with the clayey soil having larger C and N resources than the sandy soil (Chapter 2). Moreover, the differential increase in archaeal nitrifiers in the sandy but not in the clayey soil following "Foxy-2" inoculation clearly suggests a potential "Foxy-2" driven niche differentiation between archaeal (AOA) and bacterial nitrifiers (AOB). It has been widely acknowledged that AOA communities tend to be prevalent under low nutrient and energy shortage conditions while higher AOB abundance is associated with increasing nitrogen and carbon sources in soils (Lieiniger et al., 2006; Valentine, 2007; Erguder et al., 2009). This was also evident from this study where bacterial prokaryotes showed increased abundance when a high quality organic input was added whereas archaeal prokaryotes increased when "Foxy-2" was inoculated in the soil (Chapter 2). Consequently, the overall assumption was that "Foxy-2" increased the demand for energy resources (i.e. C and N) further increasing nutrient limitation. Although the speculated nutrient limitation was not reflected in the soil chemical data as there were no significant differences between the control (uninoculated) and "Foxy-2" inoculated soils; other mechanisms may have been responsible for the observed "Foxy-2" promotive effect on archaeal abundance such as regulation of plant hormones to increase nutrient uptake which is

one of the mechanisms used by fungal BCAs (Strange, 2005). From this study the mechanism underlying the "Foxy-2" promoting effect on archaeal community abundance was not fully understood and so further research would be required to further elucidate the mechanisms behind the promoting effect observed. It was however, clear that "Foxy-2" did not cause reduction in C and N resources as expected and thus no reduction in the abundance of nitrifying prokaryotes was established. The addition of high quality organic input *Tithonia diversifolia* was beneficial to nitrifying prokaryotes as it further boosted AOA and total bacterial abundance.

Overall, the study revealed that "Foxy-2" as a biological control agent is compatible with nitrifying prokaryotes. The increased abundance of archaeal nitrifiers by "Foxy-2" inoculation in the sandy but not in the clay soil implied that soil texture plays a key role in modulating the interaction between "Foxy-2" and nitrifying prokaryotes. Hence, future BCAs risk assessment studies need to take into account variation in soil texture among sites where the BCA is introduced and this is likely to influence the outcome of the interaction between the BCA and other non-target microorganisms.

6.2. Natural factors are strong determinants of nitrifying prokaryotes than *Fusarium oxysporum* f.sp. s*trigae* (Foxy-2) in maize rhizospheres

Basic environmental conditions, such as temperature, soil physical and chemical characteristics, crop cultivar and growth stages can greatly affect the efficacy of BCAs as well as alter the interactions between the host plant, the BCA and other soil microbes (Lakin and Fravel, 2002: Fravel et al., 2005). Consequently, a major criticism of *Striga* BCAs is that the extent of control may vary with multiple environmental parameters (Fravel et al., 2005). For example, Avedi et al. (2014) attributed variable performance of "Foxy-2" biocontrol against *Striga* in western Kenya to environmental differences such as soil type, pH, climatic conditions and biotic factors. In the same vein, it may be argued that the non-target effects of the fungal biocontrol agent "Foxy-2" on nitrifying prokaryotes may vary with environmental conditions. Hence, we carried out non-target risk assessment analysis in different soil types during two cropping seasons at different crop growth stages to better understand the mechanisms/factors driving the interactions between "Foxy-2" and nitrifying prokaryotes abundance and community structure (Chapter 3) as well as corroborate the findings from our rhizobox experiment (Chapter 2).

In this study, soil type, seasonality and crop growth stages had stronger influence on the abundance and community structure of nitrifying prokaryotes than "Foxy-2" inoculation effects (Chapter 3). Similar to our previous findings from the controlled experiment (Chapter 2), "Foxy-2" inoculation did not have a negative effect on the abundance and community structure of nitrifying prokaryotes. However, no promoting effect of "Foxy-2" on any of the target microorganisms (AOA, AOB, total bacteria and total archaea communities) was determined in the field study. The differences in the results between the rhizobox study and the field study were linked to the strong influence of soil type, seasonality and crop growth stages. For example, in the field study soil type influenced nitrifying prokaryotes abundance and community structure to a greater extent whereas "Foxy-2" inoculation had no effect on the abundance and community structure of nitrifying prokaryotes (Chapter 3). With or without "Foxy-2" inoculation, archaeal abundance and community structure showed distinct effects of soil type (Chapter 3). The Homabay soil which had higher clay content (49%) compared to that at the Busia site (33%) showed higher archaeal abundance and clear community differences for ammonia oxidizing bacteria (AOB), total bacterial and archaeal community structures compared to the Busia site (Chapter 3). The results reaffirmed our previous rhizobox based study that soil texture is one of the main drivers of the interaction between "Foxy-2" and indigenous nitrifying prokaryotes. The clay content in addition to the higher soil pH mainly defined the differences between the two sites in this study. In particular, the archaeal domination observed in the Homabay clay soil was attributed to the higher soil pH in this soil (Homabay = 6.1 to 7.6 versus Busia = 4.9 to 5.4); a finding in agreement with other studies that have shown archaeal community to withstand high soil pH than their bacterial counterparts (Shen et al., 2008). Although the optimal soil pH conditions for "Foxy-2" proliferation remain unexplored it has been reported that clay texture hinders the proliferation of *Fusarium* species including "Foxy-2" either by the often compacted nature of the soil structure or by natural suppression mainly associated with higher microbial diversity in clayey soils (Toyota et al., 1996; Zimmermann et al., 2015). Based on these findings, it could be deduced that the archaeal community had greater rhizosphere competence in clayey soils with high soil pH than the fungal biocontrol agent "Foxy-2".

Seasonality effects also shaped the abundance of nitrifying prokaryotes. Total archaea showed higher abundance during the short rain season (SR) in both soils compared to the long rain season (LR) (Chapter 2). The higher archaeal abundance during the SR season was attributed to high organic C turn over from the previous fallow period as evidenced by the

high values in extractable organic C during this season. This finding supports those from other studies that have shown that archaeal communities preferentially utilize organic derived N resources unlike their bacterial counterparts which are increased by inorganic derived nitrogen (Höfferle et al., 2010). On the contrary, in the long rainy season "Foxy-2" inoculation was associated with a tendency of all genes to increase, especially at the early stages of maize plant growth, an increase which was further promoted by the application of *Tithonia diversifolia* (TD) residues (Chapter 3). These findings clearly indicate that our field sites were nutrient resource limited. This limitation was compensated for by organic N application in the long rainy season but by the organic decomposition from the fallow period prior to setting up the field experiments in the short rainy season. The mechanisms underlying the tendency of "Foxy-2" to promote all genes during the long rainy season remain unclear and need further investigation.

Growth stage showed differences in the abundance and community structure of nitrifying prokaryotes. Overall, total bacterial abundance was higher at early leaf development stage whereas archaeal abundance was higher at later stages (i.e. flowering and the senescence stage) (Chapter 3). Structural community differences were also greater between the early leaf development stage and senescence stage and more so in the Homabay soil than in the Busia soil (Chapter 3). The differences by growth stage were attributed to; (i) potential niche differentiation and specialization between archaeal and bacterial prokaryotes (Prosser and Nicol (2012)) (ii) the quantity (availability) and (iii) quality (degradability) of the organic resources at the three crop growth stages (Hai et al., 2009). In addition, the early growth stages of maize, for example, have been associated with fast growing bacteria (r-strategists) due to the rapidly changing environment (i.e. pH) due to high turnover of acids produced by young roots; while the flowering and subsequent stages were associated with slow growing microorganisms (K-strategists) (Chiarini et al., 1998; Cavaglieri et al., 2009). Building on these concepts, it could be assumed that the higher total bacterial abundance at the early leaf stage was due to (i) tolerance to sudden changes at early growth stages or (ii) their ability to easily degrade nutrients released by young roots whereas the high archaeal abundance at the later stages could be linked to their preference for organic derived N as energy resources (Nihorimbere et al., 2011; Prosser and Nicol 2012). However, future studies need to investigate if the promoted bacteria at early leaf stage are r strategists.

Overall, this study revealed that soil type, seasonality and maize crop growth stage exert stronger influences on nitrifying prokaryotes than the fungal biocontrol agent "Foxy-2".

Moreover, the results showed that "Foxy-2" did not have a negative effect on nitrifying prokaryotes under field conditions. Hence, it is safe to conclude that "Foxy-2" is compatible with indigenous nitrifying prokaryotes in Western Kenya and is a safe *S. hermonthica* biocontrol agent.

6.3. Can proteolytic bacteria abundance and potential enzyme activity be used as bioindicators for soil monitoring under Foxy-2 inoculation in contrasting soils?

The possibility of using ammonia oxidizing bacteria (AOB) and archaea (AOA) as model organisms for soil monitoring has been recently suggested by Wessén and Hallin (2011) and these organisms have also widely been used to study different kinds of soil perturbations in the N cycle (Chang et al., 2001; Nyberg et al., 2006). This is justified because ammonia oxidation is the first rate limiting step in the nitrification process and the quantification of AOB and AOA abundance using real-time polymerase chain reaction (PCR) is among the top ranked, reproducible and sensitive contemporary techniques (Ritz et al., 2009; Wessén and Hallin (2011)). However, nitrification is preceded by proteolysis which is involved in the degradation of protein-nitrogen bond (i.e cleavage of amino acid bonds of organic-derived proteins) paving way for further mineralization, including nitrification (Rasche et al., 2014). Therefore, consideration of proteolytic bacteria which are dominant players in this process may contribute to a better understanding of "Foxy-2" non-target effects on the N cycling process, particularly on organic derived N. As such, quantification of bacterial protease genes (i.e. *npr* (neutral metalloproteases)) and measurement of their potential enzyme activity (Suc-Ala-Ala-Phe-AMC) may be more informative in studying the interactions of the biocontrol agent "Foxy-2" and organic N cycling bacteria.

The study presented in Chapter 3 clearly show that not only organic input application but also the quality of input shaped proteolytic bacterial abundance. The results from this study also indicated that lignin and polyphenol contents of organic inputs were the main determinants of proteolytic abundance, with the abundance decreasing when lignin and polyphenols were lower in the organic input (Chapter 3). These findings clearly indicate that consideration of proteolytic communities in agricultural soils where organic N is the main source of N is important. Building on this study, further investigations on the non-target effects of "Foxy-2" on proteolytic bacteria by using qPCR (abundance) and gene-specific enzyme activity (Suc-Ala-Ala-Phe-AMC) were carried out in clayey and sandy soils (Chapter 5).

Similar to our previous studies on nitrifying prokaryotes, soil texture played a significant role in the abundance of proteolytic bacteria. Overall, the sandy soil had higher *npr* abundance than the clay soil; a finding which was explained by the lower N content in the sandy than in the clayey soil further supporting the hypothesized increase of *npr* gene to limited N availability as a compensation mechanism for N stress (Chapter 4). It was also evident that "Foxy-2" application caused a transient decline in *npr* abundance in both soils. However, the timing of the reduction in *npr* abundance due to "Foxy-2" inoculation differed between soil types with the reduction detected 14 days earlier in the sandy than in the clayey soil. These differences in response to "Foxy-2" inoculation were further linked to the background organic matter in the native soils. The clay soil had a higher background organic matter than the sandy soil; a difference that may explain the observed 14 days lag period of "Foxy-2" reduction effect on *npr* gene abundance in the clay relative to the sandy soil. Overall, the reduction in *npr* abundance in both soils following "Foxy-2" inoculation suggest that "Foxy-2" induced competition with proteolytic bacteria. It is however noteworthy that the suggested "Foxy-2" competition effect was transitory as it could not be traced consistently during the study period (Chapter 5). Moreover, this reduction effect was not detectable in the measured *npr* enzyme activity, implying that the functionality of *npr* protoelytic bacteria was not affected by "Foxy-2" inoculation.

As expected, the application of organic input boosted both the abundance and enzyme activity (i.e *npr* gene specific Suc-Ala-Ala-Phe-AMC) of proteolytic bacteria and further compensated for the transitory "Foxy-2" reduction effect on *npr* abundance. It was concluded that "Foxy-2" inoculation has minor effects on proteolytic abundance which is compensated for by the application of high quality organic inputs such as *Tithonia diversifolia*. The study further showed that consideration of proteolytic bacteria communities would help illuminate our understanding of the N cycling process and that coupling ammonia oxidizing microorganisms with proteolytic bacteria is likely to lead to fruitful future risk assessment studies involving *Fusarium* isolates and N cycling microorganisms.

6.4. Concluding remarks and recommendations for future work

The integration of a biological control component in integrated *Striga* management strategies is faced by limitations such as (i) lack of risk assessment analysis which restrict its registration and use in *Striga* affected countries such Kenya, Ghana, Nigeria and Ethiopia and

(ii) varied performance in *Striga* control according to Avedi et al., 2014. This thesis addresses some of the challenges facing the slow integration of the fungal BCAs into integrated *Striga* management strategies by showing that "Foxy-2" is compatible with nitrifying prokaryotes and proteolytic bacteria communities and hence is environmentally safe for non-target microorganisms. The finding that soil type, seasonality and crop growth stage exert strong influences on nitrifying the abundance and community structure of prokaryotes than "Foxy-2" implies that these factors play a significant role in shaping the interactions between "Foxy-2" and nitrifying prokaryotes and should be carefully considered in future risk assessment studies. The integration of nitrifying prokaryotes and proteolytic bacteria as bio-indicators for soil health monitoring in "Foxy-2" inoculated soils also proved to be a promising way to study "Foxy-2" non-target effects on the N cycle as it revealed "Foxy-2" transient effects on proteolytic bacteria abundance which was not detected by using nitrifying prokaryotes as gene markers. Given that the promoting effect of archaeal abundance by "Foxy-2" was demonstrated under controlled conditions (rhizobox experiment) but not under field conditions, it is clear that more research still needs to be done. Below are a set of recommendations and remarks regarding issues that future studies may address based on the findings reported in this thesis:

i. The promoting effect of archaeal abundance by "Foxy-2" inoculation in a sandy soil could not be fully explained by competition for N and C resources in the soil as there were no significant differences between the control soil and "Foxy-2" inoculated soil in the soil N and C content. Future research needs to focus on exploring the influence of "Foxy-2" on the quantity and quality of root exudates which may have played a key role in the promoting effect of archaeal abundance by "Foxy-2".

ii. The influence of environmental factors such as, soil type, seasonality and crop growth stage on the efficacy of "Foxy-2" should be investigated as these factors may be contributing to the often observed varied performance of *Striga* control by "Foxy-2" as was the case for the interactions between "Foxy-2" and nitrifying prokaryotes.

iii. There is current debate that microbial abundance (qPCR) and diversity (TRFLP) does not necessarily represent all the active microorganisms. Although this study did some enzymatic analysis, which covered a narrow range of activity, it is highly recommended that future studies apply a combination of advanced techniques that

target the active microorganisms such as stable isotope analysis and includes a wide range of beneficial microorganisms such as fungi, mycorrrhizea and plant growth promoting bacteria.

iv. It would be important for future studies to investigate the optimal conditions (i.e soil pH, soil temperature and soil moisture) under which "Foxy-2" is effective in controlling *Striga* as this may contribute to better recommendations on where to use "Foxy-2" as a BCA for best control of *Striga*.

v. Studies by Bozkurt et al. (2015) have revealed that the *Striga* species in East Africa are more virulent than the *Striga* species in Western Africa. Based on this it is recommended that future research should investigate the effectiveness of "Foxy-2" on different *Striga* species.

This thesis examined the non-target effects of Foxy-2 on nitrifying prokaryotes and proteolytic bacteria in contrasting soils in central and Western Kenya. Given that *Striga* is widely spread in several countries in SSA, the presented case studies could not cover the whole range of agroecologies in SSA. Hence, the outcome discussed in the thesis cannot be used to draw general conclusions regarding the entire Sub-Saharan Africa regions affected by *Striga.* Nevertheless, it can be extrapolated to regions with similar climatic conditions and soil types as well.

The findings are scientifically relevant because they demonstrated that "Foxy-2" is compatible with beneficial nitrifying and proteolytic communities and is thus environmentally safe as a BCA in the conditions under which it was tested. Consequently, the study's findings are relevant for the registration process of "Foxy-2" as a BCA especially in Kenya where the study was carried out by showing that no risks on non-target microorganisms are likely to be detected when "Foxy-2" is used as a *Striga* BCA.

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Summary

Striga hermonthica is a major threat to cereal production and food security in Sub-Saharan Africa. Integrated *Striga* management (ISM) has been proposed as one of the best options to control *Striga* weeds. Consistent with this proposal, the integration of resistant crop varieties and biological control agents (BCAs) including *Fusarium oxysporum* f.sp. *strigae* ("Foxy-2") has been proven as an effective and environmental friendly management strategy. Despite being promoted as a critical component of ISM, the use of "Foxy-2" as a *striga* BCA has thus far not been subjected to risk assessment analysis on non-target microorganisms, yet such an assessment is a prerequisite for its large-scale field application. Moreover, the use of *Striga* BCAs such as "Foxy-2" has been criticized for wide differences in their performances in *Striga* control between laboratory/greenhouse and field conditions; differences which have been attributed to variation in biotic and abiotic factors prevailing where the BCAs have been introduced. Hence, this thesis focused on determining the non-target effects of "Foxy-2" on key microorganisms involved in soil nitrogen (N) cycling (i.e., nitrification, proteolysis) in the maize rhizosphere. Specific objectives were to; 1) investigate the non-target effects of "Foxy-2" on the abundance of ammonia-oxidizing archaea (AOA) and bacteria (AOB) as well total archaea and bacteria. This was performed in a rhizobox experiment using two contrasting soils (i.e., sandy Ferric Alisol versus clayey Humic Nitisol) derived from non *Striga* infested field sites in the central highlands of Kenya (Embu and Machanga), 2) determine the extent to which soil type, crop growth (i.e., early leaf development stage (EC30), flowering (EC60) and senescence stage (EC90)) stage and seasonality (i.e. short and long rain seasons) contribute to the dynamics of abundance and structure of nitrifying prokaryotes relative to the effects of "Foxy-2" inoculation on nitrifying prokaryotes in two contrasting agro-ecological sites (Busia and Homabay) in Western Kenya, 3) develop quantitative polymerase chain reaction (qPCR) protocols for quantifying proteolytic bacteria (i.e. *npr* gene encoding neutral metalloproteases) in tropical soils, and 4) use the developed protocols to determine if "Foxy-2" has non-target effects on proteolytic bacteria abundance (qPCR) and potential enzyme activity (i.e. *npr* gene specific Suc-Ala-Ala-Phe-AMC). The methodological approach entailed a combination of rhizobox and field experiments planted with and without *Striga* seeds in combination with maize seeds coated with and without "Foxy-2". Since the study hypothesized that "Foxy-2" induces N resource competition,

consequently threatening N cycling microorganisms, an additional treatment with high quality organic input (*Tithonia diversifolia*) was added. Rhizosphere soils obtained from the experimental maize plants were subjected to molecular (i.e. qPCR) and terminal restriction fragment length polymorphism (TRFLP), biochemical (enzymatic and soil physico-chemical analysis) and statistical analysis.

The results from a rhizobox study showed that "Foxy-2" inoculation leads to an increase in the abundance of total archaea and AOA in the sandy soil but has no effect on the abundance of both micro-organisms in the clayey soil. This suggests that the effect of "Foxy-2" inoculation on nitrifying prokaryotes, particularly the archaeal community, is interactive with soil type. Moreover, AOA abundance remained stable and higher in the control treatments in the clayey soil where *Striga hermonthica* seeds had been applied without "Foxy-2" inoculation further suggesting that AOA are able to surmount nutritional resource limitations caused by the *Striga* weed.

A subsequent field based study demonstrated strong influences of soil type, crop growth stage and seasonality on the abundance and community composition of total and nitrifying prokaryotes but no major effect of "Foxy-2". This implies that these "natural" factors masked the promotive effect of "Foxy-2" on the archaeal community abundance observed in the rhizobox experiment; a phenomenon that was linked to variation in soil organic carbon content mediated by seasonality and crop growth stages.

The third part of the study, which focused on the influence of the quality of organic inputs on the abundance and community composition of proteolytic bacteria, showed that high quality organic inputs (i.e. *Tithonia diversifolia*) caused a reduction in the abundance of proteolytic bacteria. The suppressive effect of *Tithonia diversifolia* on proteolytic bacteria abundance was linked to low lignin and polyphenol composition. Based on the major finding that *Tithonia diversifolia* reduced the abundance of protoeolytic bacteria, additional investigations were carried out using the rhizobox soils from the control, "Foxy-2" and "Foxy-2" + *Tithonia diversifolia* treatments to determine if the application of "Foxy-2" and its combination with high quality inputs further depressed the abundance and potential activity of enzymes of proteolytic bacteria. "Foxy-2" showed a minor reduction effect (i.e. transitory) on the abundance of proteolytic bacteria in the sandy and clayey soils. The transient reduction in abundance *of npr* genes signified a "Foxy-2" induced competition effect on the abundance of proteolytic bacteria which was compensated for by the application of high quality organic input *Tithonia diversifolia*. The "Foxy-2" reduction effect was not reflected in the measured

potential proteolytic enzyme activity, indicating that the functionality of proteolytic bacteria was not affected by "Foxy-2" inoculation.

Overall, the PhD study showed that, "Foxy-2" is compatible with nitrifying and proteolytic prokaryotes and has no adverse non-target risks on the abundance and community composition of key genes involved in N cycling. To mask the transient reduction effects of "Foxy-2" on proteolytic bacteria abundance, combining high quality organic inputs with "Foxy-2" inoculation is recommended. The field study indicated that crop growth stage, seasonality and soil type were stronger determinants of the abundance and community structure of nitrifying prokaryotes than "Foxy-2", reinforcing the importance of considering site-specific factors in risk assessment studies. It could also be assumed that similar factors play significant roles in determining the efficacy of "Foxy-2" in *Striga* control; hence future efficacy studies should evaluate the influence of such factors. Careful examination of the interaction between BCAs and specific sites would be especially useful for improving the efficacy of *Fusarium oxysporum* f.sp. *Strigae* isolates through more research on local isolates which may be better adapted to local soil and climatic conditions than the foreign strain (Ghana isolate) used in this study. Finally, future studies should consider a wide range of microorganisms such as plant growth promoting bacteria with *Striga* suppression potential. The compatibility of such bacteria with biotic suppression potential in combination with "Foxy-2" or other strains may be helpful as they may be harnessed in the future to enhance the performance of *Striga* BCAs.

Zusammenfassung

Striga hermonthica ist ein wichtiger limitierender Faktor der Getreideproduktion und Ernährungssicherheit im subsaharischen Afrika. Die integrierte Kontrolle wird als eine der aussichtsreichsten Möglichkeiten zur Bekämpfung parasitischer Striga-Arten angesehen. Dabei hat sich die Kombination resistenter Getreidesorten und mikrobiologischer Nutzorganismen wie *Fusarium oxysporum* f. sp. *strigae* („Foxy-2“) als effiziente und umweltfreundliche Bekämpfungsstrategie bewährt. Obwohl die Anwendung von „Foxy-2“ als wichtige Komponente in der integrierten *Striga*-Kontrolle propagiert wird, wurde bisher keine Risikoanalyse hinsichtlich ihrer Auswirkungen auf Nicht-Ziel-Mikroorganismen durchgeführt. Eine solche Risikoanalyse ist jedoch unabdingbar für Feldapplikationen im größeren Maßstab. Außerdem wurde ein stark variierender Wirkungsgrad von „Foxy-2“ auf Striga unter Labor, Gewächshaus-und Feldbedingungen beobachtet, was auf unterschiedliche biotische und abiotische Standortfaktoren zurückzuführen ist.

Deshalb zielte die vorliegende Arbeit darauf ab mögliche Nebenwirkungen von „Foxy-2“ auf Schlüsselmikroorganismen des Stickstoffkreislaufes (z.B. Nitrifikation und Proteolyse) in der Maisrhizosphäre zu bestimmen. Spezifische Ziele waren (1) mögliche Nebenwirkungen von „Foxy-2“ auf die Abundanz von Ammonium-oxidierenden Archaeen (AOA) und Bakterien (AOB) sowie auf Gesamtarchaeen und -bakterien zu untersuchen. Dazu wurde ein Rhizobox-Versuch mit zwei unterschiedlichen Böden (sandiger Ferric Alisol bzw. toniger Humic Nitisol) von nicht *Striga*-infizierten Standorten im zentralen Hochland von Kenia (Embu und Machanga) angelegt; (2) Effekte von Bodeneigenschaften, Pflanzenwachstum (frühes Blattentwicklungsstadium (EC 30), Blüte (EC 60) und Seneszenz (EC 90)) und Saisonalität (kurze und lange Regenzeit) auf die Dynamik der Abundanz und Struktur von Gesellschaften nitrifizierender Prokaryoten relativ zum Effekt der Inokulation mit „Foxy-2“ an zwei unterschiedlichen agrarökologischen Standorten (Busia und Homa Bay) in West-Kenia zu quantifizieren; (3) die methodische Entwicklung spezifischer Polymerasekettenreaktionen (qPCR) zur Quantifizierung proteolytischer Bakterien (z.B. neutrale Metalloproteasen kodierende *npr* Gene) in tropischen Böden; (4) Identifizierung von Nebenwirkungen von „Foxy-2“ auf die Abundanz proteolytischer Bakterien (qPCR) und deren Enzymaktivität (z.B. *npr* Gen spezifische Suc-ala-Ala-Phe-AMC) mittels der neu entwickelten Methoden.

Der Versuchsansatz basierte auf einer Kombination von Rhizobox- und Feldversuchen, in denen Behandlungen mit und ohne „Foxy-2“ beschichtetes Maissaatgut sowie mit und ohne *Striga*-Saatgut getestet wurden. Unter der Annahme, dass „Foxy-2“ N-Konkurrenz induziert und die Entwicklung N-umsetzender Mikroorganismen hemmt, wurde eine zusätzliche Behandlung mit einer leicht verfügbaren organischen N-Quelle (*Tithonia diversifolia* Pflanzenmaterial) angesetzt. Rhizosphären-Boden der Maisversuche wurde anhand molekularer (qPCR und terminaler Restriktionsfragment-Längenpolymorphismus (TRFLP)), biochemischer (enzym-und bodenchemische Analysen) und statistischer Verfahren analysiert.

Der Rhizoboxversuch zeigte, dass „Foxy-2“ Inokulation zu einem Anstieg der Abundanzen von Gesamtarchaeen und AOA im sandigen Boden, nicht jedoch im tonigen Boden führte. Demnach bestehen Wechselwirkungen zwischen Effekten von „Foxy-2“ auf nitrifizierende Prokaryoten, insbesondere der Archaeengemeinschaft, und Bodentyp. Außerdem blieb die AOA Abundanz stabil und höher in den Kontrollbehandlungen des tonigen Bodens mit *Striga* ohne „Foxy-2“-Inokulation der Maissaat. Dies lässt darauf schließen, dass AOA in der Lage sind Striga-induzierten Ressourcenmangel zu überstehen.

Die anschließenden Feldversuche zeigten starken Einfluss von Bodentyp, Pflanzenentwicklungsstadium und Saisonalität, jedoch keine nennenswerten Effekte von „Foxy-2“ auf die Abundanz und Struktur der Gesamt- und nitrifizierenden Prokaryotenpopulationen. Demnach überdeckten diese „natürlichen“ Faktoren den im Rhizoboxversuch beobachteten stimulierenden Effekt von „Foxy-2“ auf die Abundanz der Archaeengemeinschaft. Dies lässt sich durch Variabilität im organischen Bodenkohlenstoffgehalt unter Einfluss von Saisonalität und Pflanzenentwicklungsstadium erklären.

Der dritte Teil der Studie befasste sich mit dem Einfluss pflanzlicher N-Inputs auf die Abundanz und Artenzusammensetzung proteolytischer Bakterien. Leicht abbaubares Pflanzenmaterial (*Tithonia diversifolia* mit niedrigem Lignin- und Polyphenolgehalt) induzierte einen Rückgang der Abundanz proteolytischer Bakterien, was eine unterdrückende Wirkung von *Tithonia diversifolia* nahelegt. Auf diesen Ergebnissen basierend wurden weitere Rhizobox-Versuche mit Böden aus den Kontroll-, „“Foxy-2“- und „Foxy-2“ + *Tithonia*-Behandlungen angelegt, um festzustellen ob „Foxy-2“ in Kombination mit *Tithonia* die Abundanz und Enzymaktivität proteolytischer Bakterien zusätzlich unterdrückt. „Foxy-2“ führte zu leichter (d.h. vorübergehender) Unterdrückung der Abundanz proteolytischer Bakterien im sandigen und tonigen Boden. Die vorübergehende Reduzierung der Abundanz

der npr Gene durch „Foxy-2“ deutet auf Ressourcenkonkurrenz, die durch die Applikation von hochqualitativem Pflanzenmaterial (*Tithonia diversifolia*) kompensiert werden kann. Dieser suppressive Effekt von „Foxy-2“ spiegelte sich nicht in den gemessenen proteolytischen Enzymaktivitäten wider, was darauf hinweist, dass die Funktionalität der proteolytischen Bakterien durch „Foxy-2“ nicht beeinflusst wurde.

Zusammenfassend zeigt diese Dissertation, dass die Anwendung von „Foxy-2“ mit nitrifizierenden und proteolytischen Prokaryoten vereinbar ist und keine nachteiligen Wirkungen auf die Abundanz und Struktur wichtiger Gene des N-Kreislaufs der untersuchten Bodenmikroorganismen hat. Um den vorübergehenden suppressiven Effekt von „Foxy-2“ auf die Abundanz proteolytischer Bakterien zu kompensieren, ist die Kombination von „Foxy-2“ Inokulation mit leicht abbaubarem Pflanzenmaterial empfehlenswert. Im Feldversuch beeinflussten Pflanzenentwicklungsstadium, Saisonalität und Bodentyp die Abundanz und Struktur der nitrifizierenden Prokaryoten stärker als „Foxy-2“. Dies unterstreicht die Notwendigkeit der Berücksichtigung standortspezifischer Faktoren in Risikoanalysen zum biologischen Pflanzenschutz. Es kann außerdem angenommen werden, dass vergleichbare Faktoren einen signifikanten Einfluss auf die Effektivität von „Foxy-2“ bei der Kontrolle von Striga haben, was in zukünftigen Studien berücksichtigt werden sollte. Eine gründliche Untersuchung von Interaktionen zwischen „Foxy-2“ und Standortfaktoren ist insbesondere notwendig, um die Wirksamkeit von *Fusarium oxysporum* f. sp. *strigae* durch weitere Forschung zu lokalen Isolaten, die möglicherweise besser an lokale Boden- und Klimabedingungen angepasst sind als das hier verwendete ghanaische Isolat, zu verbessern. Außerdem sollten zukünftige Studien eine große Bandbreite an Mikroorganismen, z.B. pflanzenwachstumsfördernde Bakterien, mit Kontrollpotential gegen *Striga* untersuchen. Eine Kompatibilität solcher Striga unterdrückender Bakterien in Kombination mit „Foxy-2“ oder anderen Isolaten könnte die Wirksamkeit mikrobieller Kontrollstrategien gegen *Striga* weiter verbessern.

Appendices

Appendix A. Additional publications

(related to this thesis and published during the same time frame of doctoral studies)

Zimmermann, J., Madelein, D. K., **Musyoki, M.K**, Altus, V., Watson, A.K., Beed, F., Gorfer, M., Cadisch, G., Rasche, F. (2015). An explicit AFLP-based marker for monitoring *Fusarium oxysporum* f.sp. *strigae* in tropical soils. Biological control, 89: 42-52.

Zimmermann, J., **Musyoki, M. K.,** Cadisch, G., Rasche, F. (2016). Biocontrol agent Fusarium oxysporum f. sp. strigae has no adverse effect on indigenous total fungal communities and specific AMF taxa in contrasting maize rhizospheres. *Fungal Ecology*, *23*, 1-10.

(not related to this thesis)

Kamaa, M. M., Mburu, H. N., Blanchart, E., Chibole, L., Chotte, J. L., Kibunja, C. N., Lesueur, D. (2012). Effects of organic and inorganic applications on soil bacterial and fungal microbial communities' diversity and impacts of earthworms on microbial diversity in the Kabete long-term trial, Kenya. In Bationo et al., (Eds), Lessons learned from long-term soil fertility management experiments in Afric*a* (pp. 121-136), Springer Netherlands.

Kamaa, M., Mburu, H., Blanchart, E., Chibole, L., Chotte, J. L., Kibunja, C., Lesueur, D. (2011). Effects of organic and inorganic fertilization on soil bacterial and fungal microbial diversity in the Kabete long-term trial, Kenya. Biology and Fertility of Soils, 47(3), 315-321.

Conference contributions (oral presentations related to this thesis)

Musyoki, M., Cadisch, G., Enowashu, E., Zimmermann, J., Muema, E. K., Wainwright, H., Rasche, F. (2014). Response of the abundance of nitrifying prokaryotes to *Fusarium* f.sp. *Strigae* in a maize rhizosphere. Tropentag conference 2014. Czech University of life sciences Prague, Czech Republic, 17th-19th September 2014**.**

Musyoki, M., Enowashu, E., Zimmermann, J., Muema, E., Wainright, H., Vanlauwe, B., Rasche, F. (2014). Stimulative effects of the fungal biocontrol agent *Fusarium*

oxypsorum f.sp. *strigae* on nitrifying prokaryotes in a maize rhizosphere European Geosciences Union Assembly Vienna, Austria, 27th April-02nd May 2014.

(oral presentations not related to this thesis)

Musyoki, M.K., Majengo, C., Mutegi, E., Thonar, C., Faye, A., Okalebo, R., Thuita, M., Lesueur, D., Pypers, P. (2011). Increased soybean production through rhizobial inoculation under smallholder conditions in distinct agro-ecological zones of Kenya. Nitrogen & Global change key findings-future challenges conference, 11th-15th April 2011, Edinburgh, Scotland.

Poster presentations (related to this thesis)

Musyoki, M.K., Zimmermann, J., Wainwright, H., Cadisch, G. Rasche, F (2015). Soil type, season and crop growth stage exert a stronger effect on rhizosphere microbial dynamics than the fungal biocontrol agent *Fusarium oxysporum* f.sp. *strigae*. Ecology of soil microorganisms, 29th November-3rd December 2015, Prague, Czech Republic.

Zimmermann, J., **Musyoki, M.K.,** Cadisch, G., Rasche, F. (2015). The biocontrol agent *Fusarium oxyporum* f.sp. *strigae*-Its detection and effects on beneficial indigenous microorganisms in a maize rhizosphere. Ecology of soil Microorganisms, 29th November-3rd December 2015, Prague, Czech Republic.

Rasche, F., **Musyoki, M.K.,** Muema, E.K., Vanlauwe, B., Cadisch, G, (2015). Lasting influence of biochemically contrasting organic inputs on abundance and community structure of total and proteolytic bacteria in tropical soils. Ecology of soil Microorganisms, 29th November-3rd December 2015, Prague, Czech Republic.

Poster presentations (not related to this thesis)

Muema, E., **Kamaa, M.M**., Mugadi D., Jena-Luc Chotte, Mburu, H., Vanlauwe, B., Kibunja, C.N., Lesueur, D. (2008). Impact of soil organic matter applications combined with mineral fertilization on both microbial and fungal soil communities in Kenya. 13th African Association for Biological Nitrogen Fixation (AABNF) congress, 15th-18th December 2008, Hammamet, Tunisia.

Kamaa M.M., Shepherd, K., Verchout, L., Mugadi, D., Mburu, H. Lesueur, D. (2007). Soil degradation Assessment: A comparative analysis of Denaturing Gradient Gel Electrophoresis results and Enzymatic approaches. 2nd International Rhizosphere congress, 26th-31st 2007, Montpellier, France.

Appendix B. Curriculum vitae of Mary Kamaa Musyoki

Education

Cumulative PhD studies for the degree of Dr. Agr. 10/2011-2016

Faculty of Agriculture, University of Hohenheim, Stuttgart

Thesis title: Impacts of the fungal bio-control agent *Fusarium oxysporum* f.sp. *strigae* on plant beneficial microbial communities in the maize rhizosphere

Master Degree (M.sc.) in Environmental Science 10/2008-07/2011

Kenyatta University, Nairobi-Kenya

Thesis title: The effect of organic and mineral fertilizers on soil microbial communities in Kabete Long-Term experiment, Kenya (final grade: no grading)

Bachelor of Science (B.Sc.) in Environmental Science 10/1999-10/2003

Kenyatta University, Nairobi-Kenya

Dissertation title: A comparative analysis of plant and animal biomass: A case study of Konza co-operative Ranch and Farming society in Machakos district (Final grading: First class honours)

Professional Experience

Research Assistant 2009-2011

Compro I Project, International Center for Tropical Agriculture (CIAT),

Nairobi, Kenya

Key responsibilities:

- Base line studies to characterize the Mandate areas of the project in Kenya
- Development of field technicians and farmers' data collection protocols
- Training of farmers on the use and application of successful commercial products

Internship 2005–2006

Tropical Soil Biology and Fertility institute of CIAT (Nairobi, Kenya)

Key responsibilities:

- Developed comprehensive molecular techniques protocols laboratory
- Training of new internship students on molecular techniques
- Preparing of reports for projects evaluation as well as posters for conference presentations

Administrative Assistant 2004-2005

Kamongo Waste Paper and Recycling Company (Nairobi, Kenya)

Key responsibilities:

- Supporting day- to-day administrative management in the accounts department

Research assistant 2003

African Institute for economic and social development (INADES)-Formation international-Machakos, Kenya

Key responsibilities:

- Assisted in data collection and analysis

Publications

Musyoki, M.K., Cadisch, G., Zimmermann, J., Wainwright, H., Beed, F., Rasche, F. (2016) Soil properties, seasonality and crop growth stage exert a stronger effect on rhizosphere prokaryotes than the fungal biocontrol agent *Fusarium oxysporum* f.sp. *strigae*. *Applied Soil Ecology*, 105:126-136.

Zimmermann, J., **Musyoki, M. K.,** Cadisch, G., Rasche, F. (2016). Biocontrol agent *Fusarium oxysporum* f. sp. *strigae* has no adverse effect on indigenous total fungal communities and specific AMF taxa in contrasting maize rhizospheres. *Fungal Ecology*, 23*:* 1-10.

Musyoki, M.K., Cadisch, G., Enowashu, E., Zimmermann, J., Muema, E., Beed, F., Rasche, F. (2015). Promoting effect of *Fusarium oxysporum* [f.sp. *strigae*] on abundance of

nitrifying prokaryotes in a maize rhizosphere across soil types. *Biological Control, 83,* 37-45.

Zimmermann, J., Madelein, D.K., **Musyoki, M.K**, Altus, V., Watson, A.K., Beed, F., Gorfer, M., Cadisch, G., Rasche, F. (2015). An explicit AFLP-based marker for monitoring *Fusarium oxysporum* f.sp. *strigae* in tropical soils. *Biological Control, 89*, 42-52.

Frank, R., **Musyoki, M.K.,** Röhl, C., Muema, E.K., Vanlauwe, B., Cadisch, G. (2014). Lasting influence of biochemically contrasting organic inputs on abundance and community structure of total and proteolytic bacteria in tropical soils. *Soil Biology and Biochemistry, 74,* 204-213.

Kamaa, M.M., Mburu, H.N., Blanchart, E., Livingstone, C., Chotte, J.L., Kibunja, C.N., Lesueur, D. (2012). Effects of organic and inorganic applications on soil micro and macro faunal diversity in Kabete Long-term trial, Kenya in Bationo et al. (eds), Lessons learned from long-term soil fertility management experiments in Africa (pp. 121-136), Spinger, Netherlands.

Kamaa, M.M., Mburu, H., Blanchart, E., Livingstone, C., Chotte, J.C., Kibunja, C., Didier, L. (2011). Effects of organic and inorganic fertilization on soil bacterial and fungal microbial diversity in the Kabete Long-term trial, Kenya. *Biology and Fertility of soils, 47 (3)* 315-321.

Awards

Full PhD scholarship from Food Security Center 2012

Universität Hohenheim, Germany

Hohenheim / September, 2016

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Mary Kamaa Musyoki

Appendix C. Author's declaration

I hereby declare that this doctoral thesis is a result of my own work and that no other than the indicated aids have been used for its completion. All quotations and statements that have been used are indicated. I did not accept the assistance from any commercial agency or consulting firm. Furthermore, I assure that the work has not been used, neither completely nor in parts, for achieving any other academic degree.

Hohenheim / September, 2016

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Mary Kamaa Musyoki