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The biocontrol agent *Fusarium oxysporum* f. sp. *strigae* - Monitoring its environmental fate and impact on indigenous fungal communities in the rhizosphere of maize

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List of Abbreviations

AFLP: amplified fragment length polymorphism

ALS: acetolactate synthase

AMF: arbuscular mycorrhizal fungi

ANOSIM: analysis of similarity

ANOVA: analysis of variance

BCA: biological control agent

Bp: base pair

CAP: canonical analysis of principal coordinates

DAP: days after planting

DGGE: denaturing gradient gel electrophoresis

DNA: desoxyribonucleic acids

ELISA: enzyme-linked immunosorbent assay

EOC: extractable organic carbon

EON: extractable organic nitrogen

EU: European Union

FAO: Food and Agriculture Organization of the United Nations

f. sp.: forma specialis

Fos: *Fusarium oxysporum* f. sp. *strigae*

IITA: International Institute of Tropical Agriculture

IPM: integrated pest management

IPPC: International Plant Protection Convention

IR: imazapyr resistant

ITS: internal transcribed spacer

LR: long rains (cropping season in western Kenya)

MtSSU: mitochondrial small subunit

nMDS: non-metric multidimensional scaling

N_t: total nitrogen

OECD: Organisation for Economic Co-operation and Development

P_{av}: available phosphorus

PCR: polymerase chain reaction

PLFA: phospholipid-derived fatty acids

QPCR: quantitative polymerase chain reaction

RAPD: randomly amplified polymorphic desoxyribonucleic acids

rDNA: ribosomal desoxyribonucleic acids

RNA: ribonucleic acids

SCAR: sequence characterized amplified region

SIX: secreted in xylem

SNP: single nucleotide polymorphism

spp.: species pluralis

SR: short rains (cropping season in western Kenya)

TC: total carbon

TE: transposable element

TEF: translation elongation factor

T-RF: terminal restriction fragment

TRFLP: terminal restriction fragment length polymorphism

VCG: vegetative compatibility grouping

1. General Introduction

1.1 Background and Research Justification

Sub-Saharan Africa represents the region with the highest percentage of undernourished people in the world with 220 million people suffering from hunger (FAO, IFAD and WFP, 2015). Moreover, yield of staples (i.e. cereals) in Sub-Sahara Africa is the lowest in the world (USDA-ERS, 2012), which is in contrast to the high food demand respecting the current and predicted population increase in this region (United Nations, 2015).

This underlines the urgent need to overcome current limitations to cereal production in Sub-Saharan Africa to satisfy the increasing food demand of its growing population. Prominent constraints to cereal production in Sub-Saharan Africa are low soil fertility and drought periods followed by biotic impacts (Fig. 1.1, Reynolds et al., 2015).

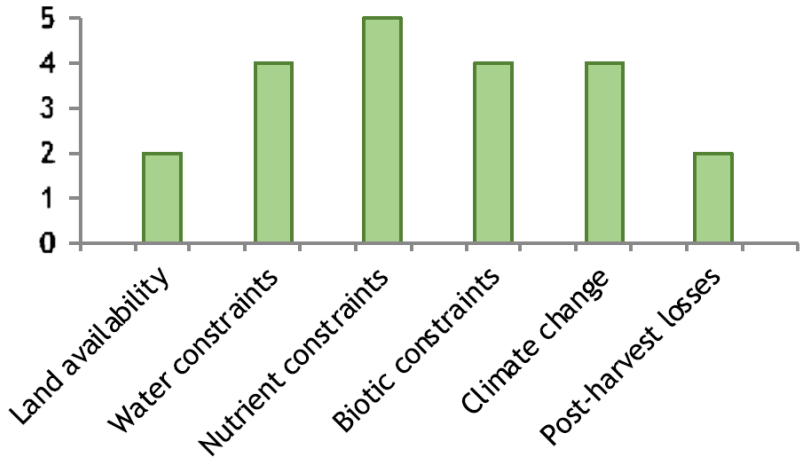

Figure 1.1: Relative severity of constraints to maize production in Sub-Sahara Africa. Source: Reynolds et al. (2015).

Among the biotic constraints to cereal production, infestation with parasitic weeds of the genus *Striga* spp. is considered as the major threat to cereal production and food security in Sub-Saharan Africa (Ejeta, 2007a; Parker, 2014, 2009; Spallek et al., 2013). *Striga* spp. parasitizes cereals like maize, sorghum and millet and infests more than 50 million hectare farmland with intensifying dissemination causing annual yield losses worth of US$ 7 billion (Ejeta, 2007a; Gibbon et al., 2007). Out of the three agriculturally important *Striga* spp. species in the world

(*S. hermonthica*, *S. asiatica* and *S. gesnerioides* (Parker, 2009)), *S. hermonthica* is considered as the most damaging species in Sub-Saharan Africa (Ejeta, 2007a; Parker, 2012).

Although efforts to develop control options have intensified in the last 20 years, recent reviews report increasing severity of the *S. hermonthica* problem in Africa, probably related to the continuing mono-cropping and limited use of fertilisers which all contributes to soil nutrient depletion and, hence, an alteration of the habitat in favour of *S. hermonthica* (Parker, 2012; Spallek et al., 2013). Furthermore, currently deployed *S. hermonthica* control technologies are often laborious, expensive and show only incomplete or transient control ability (reviewed in Hearne, 2009). Several researchers state that no single control technology will be sufficient in controlling *S. hermonthica* and, hence, an integrated control approach should be implemented (Atera et al., 2012; Hearne, 2009; Menkir and Kling, 2007). In this context, intensive research was conducted in the last years on the implementation of biological control agents (BCAs) in combination with resistant crop varieties as integrated control approach against *S. hermonthica*. Particular attention was paid to strains of the soil-borne fungal species *Fusarium oxysporum* f. sp. *strigae* (Fos), which were proven to be effective in the suppression of *S. hermonthica* and non-pathogenic to crops, thereby representing a promising BCA candidate (Elzein and Kroschel, 2006a, 2004; Ndambi et al., 2011). Advantages of the fungal BCA Fos are its straightforward application via seed coating of the crop variety and its ability to multiply and persist in soils, thereby offering potential long-term protection against *S. hermonthica* with one inoculation (Elzein and Kroschel, 2006; Schwarzenbach, K. A., 2008). This potential long-term control ability of Fos would represent an important benefit to smallholder farmers in Sub-Saharan Africa who often suffer cash constraints and may not purchase coated seeds on an annual basis.

Prerequisite for widespread implementation of the biocontrol technology is the official registration of the BCA Fos by country authorities in Sub-Saharan Africa. The FAO and OECD institutions established international registration regulations to ensure the environmental safety of microbial BCAs prior to their widespread release into the environment (FAO, 2006; OECD, 2014). First, a specific tool is required to identify the BCA at the species or strain level, which allows following its population kinetics in inoculated environments, e.g. soils (FAO, 2006; OECD, 2012). Moreover, requirement for successful registration of microbial BCAs is a profound risk assessment to exclude adverse effects on non-target organisms (FAO, 2006; OECD, 2012). Therefore, the present thesis was based on two major objectives:

(1) To develop a specific monitoring tool for the BCA Fos which allows its identification and quantification in inoculated environments and
(2) to assess the potential impact of Fos inoculation on non-target organisms.

Population kinetics of microbial BCA strains in soils vary depending on environmental conditions, such as soil type, resource availability and pH, which can result in inconsistent efficacy rates of the BCA in controlling the target pest (Whipps, 2001). Hence, the envisaged monitoring tool was further implemented in identifying favoured environmental conditions of the BCA Fos, which will facilitate farmer´s decision making about necessary re-inoculation periods and the improvement of soil conditions in favour of the BCA for sustained *S. hermonthica* control.

Cook et al., (1996) reviewed the potential non-target effects of microbial BCAs, including pathogenicity, toxicity and competitive displacement. In this context, host range studies of the BCA Fos confirmed its pathogenicity exclusively to target pests, i.e. *S. hermonthica* and *S. asiatica* (Elzein and Kroschel, 2006). Furthermore, Ndambi B. (2011) has proven that none of the typical *Fusarium* spp. mycotoxins (i.e., beauvericin, fumonisins and moniliformin) were detected in grains of sorghum when treated with the BCA Fos, thus, indicating no risk to humans and animals through nourishment with Fos-treated cereals. The crucial knowledge gap remaining in the risk assessment of the BCA Fos is the potential competitive displacement of indigenous organisms through contention for food webs and colonization area. Fos is applied via seed coating of the crop variety and establishes in the rhizosphere of the crop where it propagates saprophytically waiting for its host *S. hermonthica* (Elzein et al., 2010; Ndambi et al., 2012). The rhizosphere is well known as the microbial hotspot in the soil, also named "rhizosphere effect", due to rhizodeposition but also symbiotic interaction between the plant and certain microbes, e.g. arbuscular mycorrhizal fungi (AMF) (Huang et al., 2014; Smith and Smith, 2012). These rhizosphere microbial communities maintain plant-beneficial functions including nutrient provision, suppression of pathogens and promotion of plant growth (Compant et al., 2005; Liu et al., 2007; van der Heijden et al., 2008). Hence, alteration in abundance and composition of indigenous plant-beneficial rhizosphere microbial communities induced by Fos inoculation might result in adverse effects on crop yield and health. Therefore, the risk assessment within the frame of this thesis emphasized on rhizosphere microbial community dynamics with focus on rhizosphere fungi as these may colonize similar niches as

the fungal BCA Fos and thus compete for similar resources in the rhizosphere (Winding et al., 2004). Specific emphasize was put on AMF community dynamics since this fungal group is well known for their symbiotic association with the majority of plant species and, hence, can serve as valuable risk indicator in the evaluation of the BCA Fos. The impact of Fos on indigenous fungal communities was traded off against natural environmental impacts, such as soil type, crop growth stage and seasonality, which are acknowledged to highly influence soil microbial community abundance and composition. Furthermore, an organic fertilization treatment with nitrogen-rich organic residues (i.e. *Tithonia diversifolia*; Chivenge et al., 2009) was included in the experimental set up to compensate for the hypothesized resource competition between Fos and indigenous soil fungi.

Overall, the gained output of this PhD thesis aims to facilitate the required registration of the BCA Fos by country authorities in Sub-Saharan Africa but also tends to improve the consistency and durability of the biocontrol efficacy in soils.

1.2 Striga hermonthica

1.2.1 Mode of action

S. hermonthica is classified as an obligate hemiparasite with a life cycle closely linked to the host plant as described in Figure 1.2 (Scholes and Press, 2008).

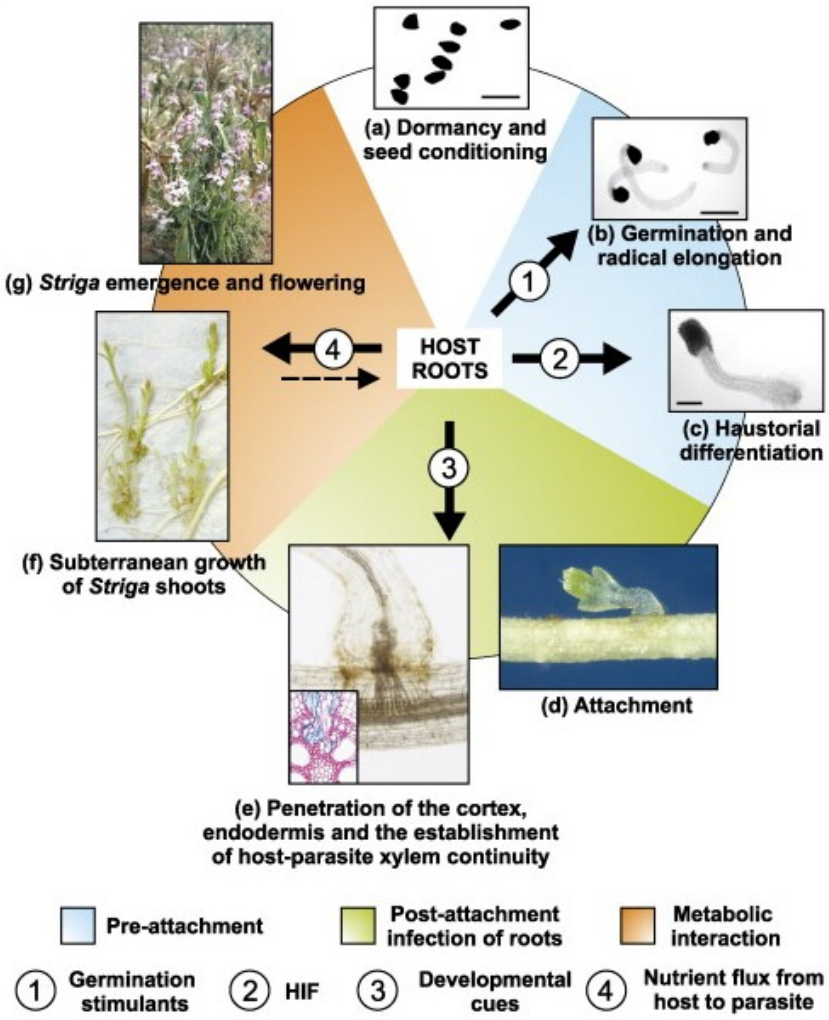

Figure 1.2: Life cycle of *Striga* spp.. HIF = haustorial initiation factors. Source: Scholes & Press (2008).

The germination of *S. hermonthica* seeds rely on the perception of specific root exudates released by host roots, so called germination stimulants with strigolactones being the most studied and extremely capable inducers of *S. hermonthica* germination (Cardoso et al., 2011; Toh et al., 2015; Tsuchiya et al., 2015). *S. hermonthica* seeds fall into a secondary dormancy if no germination stimulants are received during the conditioning time, representing a period of 7-14 days with high moisture and temperature levels (Cardoso et al., 2011). After germination, an initial root is formed to explore the soil for the host, which develops into a haustorium if the host is in close proximity (2-3 mm). The haustorium uses mechanical force and oxidizing enzymes to penetrate the host root and reaches the host vascular system within five days (Press, M. C. and Graves, J. D., 1995). Once connected to the vascular system of the host, the parasite withdraws carbohydrates, aminoacids, nutrients and water, thereby causing leaf chlorosis, stunted shoot growth and reduced photosynthesis in the host plant (Ejeta, 2007). *Striga*

cotyledons emerge from the seed around 24 hours after connecting to the vascular system of the host (Hood et al., 1998). After emergence from the soil, *S. hermonthica* plants start to photosynthesize. However, the parasite remains dependent on its host due to low CO_2 fixation and high dark respiration rates, which result in a negative carbon gain over the 24-h period (Press et al., 1987). *S. hermonthica* flowers about 4 weeks after emergence (Fig. 1.3). Seeds pods are developed after pollination, each containing around 500 tiny seeds resulting in up to 500 000 seeds per plant which are spread into the soil and stay viable for more than 10 years (Berner et al., 1995).

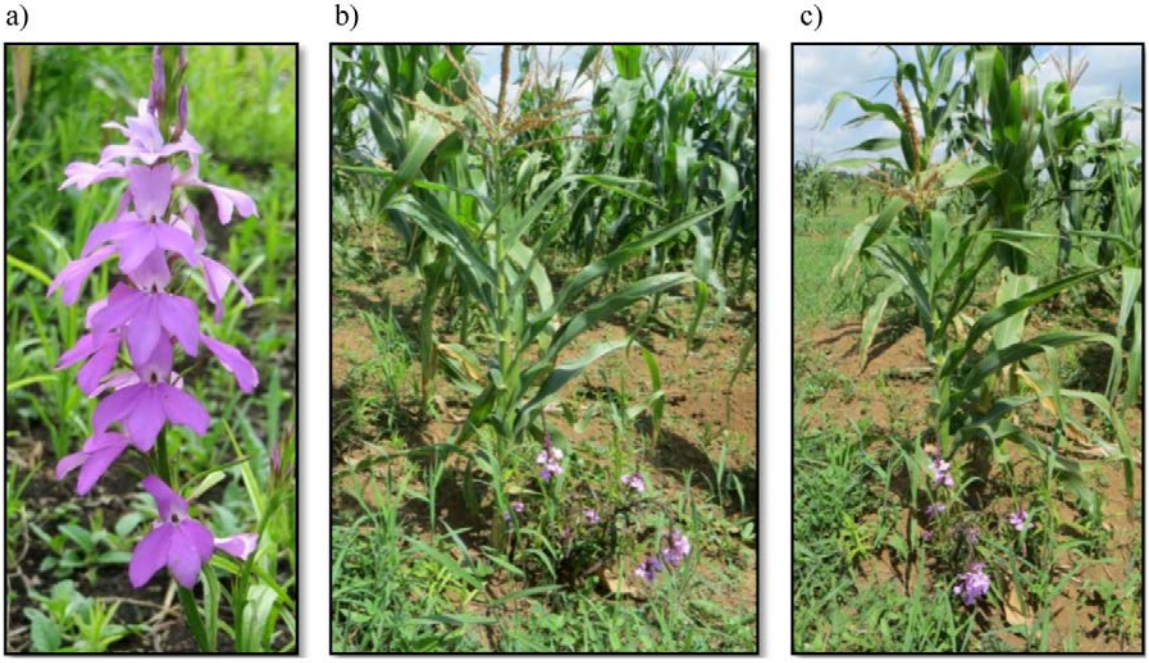

Figure 1.3: a) *Striga hermonthica* at flowering stage. b) and c) *Striga hermonthica* parasitizing on maize plants. Source: Judith Zimmermann.

1.2.2 Control options

Control options against *S. hermonthica* fit into three categories: (1) cultural control, (2) seed-based technologies and (3) biological control (Hearne, 2009). This thesis highlights some of the most promising and frequently implemented control approaches but also supplements each control option with its specific farmer adoption potential and drawbacks. Detailed information on all currently available *Striga* control options can be obtained in reviews from Hearne S.J.

(2009) and Parker C. (2009, 2012, 2014) while Atera et al. (2012) emphasized on the adoption potential of *S. hermonthica* control approaches by Sub-Saharan African farmers.

Cultural control options

Cultural control options include crop rotation, intercropping and management of soil fertility. Most attention in the last years was paid to the push-pull technology which is based on intercropping maize with a forage legume, i.e. *Desmodium uncinatum* Jacq., and planting Napier grass (*Pennisetum purpureum* Schumach) as a border crop (Khan et al., 2014; Pickett et al., 2014). The push-pull technology controls stemborer and *Striga* spp. infestation simultaneously while improving soil fertility and hence, is part of an integrated pest management (IPM) strategy. *Desmodium* repels stem borer moths (push) while Napier grass attracts them (pull). Furthermore, *Desmodium* leads to suicidal *Striga* spp. seed germination through production of germination stimulants (Khan et al., 2002). The technology can be further improved to "climate adapted" push-pull using drought-tolerant crops like *Brachiaria* cv mulato as border crop and greenleaf *Desmodium intortum* as intercrop (Midega et al., 2015). The push-pull technology shows good adoption potential in farms with dairy or fodder production since both companion plants provide high quality animal fodder and facilitate milk production. However, in farms without dairy production, the adoption potential of the push-pull technology is low since farmers have to buy *Desmodium/Brachiaria* seeds, adopt low-till practices and wait several seasons for good establishment in the field (Hearne, 2009).

Improving soil fertility is one of the most important parts of an integrated *Striga* control approach since the degree of *Striga* spp. infestation is strongly correlated with soil nitrogen and phosphorus content. Jamil et al. (2014) and Gebremariam and Assefa (2015) confirmed that the use of organic and inorganic fertilizer can reduce the impact of *S. hermonthica* on cereal hosts. Especially, increasing nitrogen and phosphorus availability have been shown in pot experiments to lower germination stimulant production (i.e. strigolactones) of host roots and, hence, *S. hermonthica* germination and subsequent attachment (Jamil et al., 2011). However, in on-farm field trials results of fertilization were less consistent than in the greenhouse (Jamil et al., 2012). The authors explained this discrepancy with the less predictable availability of mineral nutrients under field conditions due to a several factors such as drought, leaching and runoff (Jamil et al., 2012). This variable efficiency of fertilizer application in conjunction with cash constrains and limited supplies of fertilizer to African farmers clearly reduce the adoption potential of this technology (Atera et al., 2012).

Seed-based control options

Seed-based *S. hermonthica* control technologies mainly rely on resistance breeding in cereals. Cereals like sorghum, which have coevolved with S. hermonthica, show a greater range of tolerance and resistance than introduced cereals, such as maize (Hearne, 2009). Several sorghum varieties have been bred with lower susceptibility towards *Striga* spp. such as “Framida”, “Tiemarifing” and “N13” (Rodenburg et al., 2006, 2005; Ejeta, 2007b). These varieties possess traits such as attachment stage resistance und reduced germination stimulant production (i.e. strigolactones), which improve cereal yields compared to susceptible varieties (Rodenburg et al., 2005). In maize, most promising varieties have been bred from the maize wild progenitor teosinte (*Zea diploperennis*) showing 31% lower yield caused by *S. hermonthica* infection in contrast to 72% yield loss in susceptible controls (Rich and Ejeta, 2008). However, the level of protection by resistant crop varieties achieved to date is either incomplete or transient and insufficient under high *S. hermonthica* infestation levels (Ejeta, 2007b; Hearne, 2009). Moreover, farmers often rate the effectiveness of control by resistant crop varieties lower than their own traditional control methods, for example hand weeding (Hearne, 2009; Atera et al., 2012). Another seed-based *Striga* spp. control technology is the use of herbicide coated maize seed (Makumbi et al., 2015; Ransom et al., 2012). In this case, breeding focused on resistance to the imidazolinone group of the acetolactate synthase (ALS) inhibiting family of herbicides. Seeds of this IR (imazapyr resistant) maize variety are coated with the imidazolinone herbicide “imazapyr” and sold under the name StrigAway® in Kenya. The major disadvantage of the IR based control technology is that farmers need to purchase coated seed on an annual basis since the IR maize is susceptible against *Striga* spp. without herbicide treatment. This potentially limits the adoption of the IR technology. Furthermore, Ahonsi et al. (2004) showed that application of ALS inhibitory herbicides lowered the natural biotic soil suppressiveness against *Striga* spp., which is potentially related to the fungicidal function of these herbicides (Bélai and Oros, 1996). Hence, the use of ALS inhibitory herbicides, i.e. imazapyr, for sustainable *Striga* spp. control remains questionable due to its potential non-target side effects on beneficial soil fungi.

Biological control options

One biological control option against *S. hermonthica* is the use of antagonistic AMF species (i.e. *Glomus clarum, Gigaspora margarita*) which can limit *S. hermonthica* seed germination, growth and attachment (Isah et al., 2013; Lendzemo et al., 2007; Othira et al., 2012). The mechanism behind can be potentially attributed to a reduced excretion of *S. hermonthica* germination stimulants by the host plant when colonized by AMF (Lendzemo et al., 2009). Mycorrhizal fungi offer two advantages as BCAs: (1) they are non-pathogenic, and (2) offer additional plant-beneficial functions such as nutrient provision (Louarn et al., 2012). The non-pathogenicity of AMF facilitates the official registration as biocontrol product, which is further underlined by the fact that several AMF strains are already permitted and commercially used in some countries as plant growth promotion products (i.e., The Landscaper's BioNutrition™, MYKE® PRO). Another potential biocontrol option is the use of certain rhizobacteria, mainly of the genus *Azospirillum* spp., *Pseudomonas* spp., *Bacillus* spp., and *Burkholderia* spp., which showed inhibitory effects on *S. hermonthica* germination and haustorial development under laboratory conditions (Hassan et al., 2011, 2009; Mounde et al., 2015). However, the potential of these bacterial strains as BCAs against *S. hermonthica* is still in the laboratory/greenhouse evaluation stage and needs yet to be validated under field conditions. Finally, chapter 1.3 of this thesis is emphasizing on the use of *Striga*-pathogenic *Fusarium oxysporum* isolates as biological control agents.

1.3 The biocontrol agent *Fusarium oxysporum* f. sp. *strigae*

Several field surveys in Nigeria, Benin, Niger and Burkina Faso resulted in the conclusion that *Fusarium oxysporum* is the most virulent pathogen causing disease in *Striga* spp. (Abbasher et al., 1995; Ciotola et al., 2000, 1995; Elzein and Kroschel, 2004). Most attention was paid to three *Fusarium oxysporum* isolates, i.e. “Foxy-2”, “PSM 197” and “M12-4A”, which were proven to be highly virulent and host-specific to the *Striga* spp. genus and, hence, classified into a new forma specialis (f. sp.) named f. sp. *strigae* (Ciotola et al., 1995; Elzein and Kroschel, 2006; Marley et al., 2004). These *Fusarium oxysporum* f. sp. *strigae* (Fos) strains have shown to saprophytically colonize the roots of the cereal host und subsequently infect *Striga* spp. at the seed or later stages of growth by clogging vessels and causing wilting symptoms (Elzein et al., 2010; Ndambi et al., 2011). Potential application technologies of the Fos strains are soil inoculation with granular (Elzein and Kroschel, 2006b) or coating of crop seeds (Elzein et al., 2006). The seed coating technology contributes to farmer´s adoption of the biocontrol approach

since no additional working steps are required for soil inoculation with the BCA. Furthermore, seed coating allows the convenient combination of the biocontrol technology with resistant crop varieties, which results in an integrated *S. hermonthica* control approach (Beed et al., 2013). The Fos strain “Foxy-2” was collected from diseased *S. hermonthica* plants in North Ghana, Africa, by Abbasher et al. (1995) and represents the best studied Fos isolate by now. Pot experiments under greenhouse conditions have shown that “Foxy-2” can reduce *S. hermonthica* emergence by 98 % (Elzein and Kroschel, 2004; Kroschel et al., 1996; Ndambi et al., 2011). Host range studies were conducted using three *Striga* spp. species and 25 non-target plant species belonging to nine families as described in Elzein et al. (2006a). “Foxy-2” was found to be highly virulent against *S. hermonthica* and *S. asiatica* while *S. gesnerioides* and non-target plant species were not susceptible. Zarafi et al. (2015) recently published contradictory results concerning the host specificity of “Foxy-2” with detecting disease symptoms in solanaceous crops. However, the experimental set up (i.e., use of non-sterilized soils and no verification if detected disease symptoms were caused by “Foxy-2”) raise concern about the validity of their results, as discussed in personal communication with Dr. Fenton Beed (Regional Director of the World Vegetable Center, Bangkok, Thailand) and Prof. Dr. Alan K. Watson (Department of Plant Science, McGill University, Sainte-Anne-de-Bellevue, Canada). Nonetheless, based on these contradictory publications, it seems advisable to repeat the host range studies to corroborate the host specificity of the BCA "Foxy-2".

Efficacy of “Foxy-2” was further evaluated under field conditions in Benin, Burkina Faso Nigeria and Kenya (Avedi et al., 2014; Schaub et al., 2006; Venne et al., 2009; Yonli et al., 2005). Venne et al. (2009) compared the efficacy of two “Foxy-2” application technologies (granular and seed coating) in combination with *Striga*-resistant and *Striga*-susceptible varieties of sorghum and maize. The authors detected distinct efficacy levels of “Foxy-2” depending on application method, crop variety, field site and agro-ecological zone. Granular application clearly enhanced "Foxy-2" efficacy, probably caused by higher nutrient resources included in the granular formulation compared to seed coating. Overall, higher efficacy of “Foxy-2” was observed in combination with maize compared to sorghum and enhanced "Foxy-2" efficacy was detected in areas with higher precipitation rates. Field trials in west Africa clearly corroborated the efficacy of the BCA "Foxy-2" against *S. hermonthica*, while the field trial in east Africa (i.e. Kenya; Avedi et al., 2014) failed in showing any suppressive effect of "Foxy-2". The lacking efficacy of “Foxy-2” in Kenya was attributed to differing environmental conditions across agro-ecological zones with different soil types, as well as seasonal differences

such as precipitation rates and temperature patterns (Gerbore et al., 2013; Velivelli et al., 2014). Hence, proliferation of the BCA "Foxy-2" as influenced by contrasting environmental conditions requires thorough investigation to identify favored conditions for successful establishment and consistent efficacy of the BCA in soils.

Moreover, the pathogenicity of “Foxy-2”, originating from west Africa (Abbasher et al., 1995), towards *S. hermonthica* populations in east Africa needs yet to be validated. In this context, Bozkurt et al. (2015) confimed the high genetic diversity within *S. hermonthica* populations and detected significantly different virulence towards sorghum in east African *S. hermonthica* populations compared to west African *S. hermonthica* populations. This potentially indicates the need to implement native Fos strains as BCAs against *S. hermonthica* to account for different resistance gene pools within *S. hermonthica* populations at distinct regions.

1.3.1 Risk assessment of biological control agents

International legal frameworks for the registration and release of BCAs were established by the International Plant Protection Convention (IPPC) of the FAO (2006) with the „Guidelines for the Export, Shipment, Import and Release of Biological Control Agents and other Beneficial Organisms“ (ISPM-3 2005) and by the Organization for Economic Co-operation and Development (OECD) with the Environment, Health and Safety Publications, i.e. Series on Pesticides No. 67 „OECD Guidance to the Environmental Safety Evaluation of Microbial Biocontrol Agents“ (OECD, 2014).

Some countries or groups of countries like the European Union (EU) have established specific regulations (European Union, 2011) which are fundamentally in line with the IPPC and OECD procedures. In contrast to the EU, congruent regulations are so far not in place in Africa. With an increase in the application of BCAs for pest and disease control, many African countries are now adopting regulations for the registration and release of BCAs (Hoeschle-Zeledon et al., 2013). In 2013, „A Guide to the Development of Regulatory Frameworks for Microbial Biopesticides in Sub-Saharan Africa“ was released by the African Agricultural Technology Foundation (AATF) with the intention to “harmonize country regulations resulting in mutually comparable standards, norms and protocols for the registration of microbial BCAs” (AATF, 2013, p. 10). This guide provided by AATF (2013) is emphasizing on specific features relevant to microbial BCAs but is not dealing with “detailed methodology for compiling and evaluating

data dossiers” (AATF, 2013, p.11) with referring in this context to the existing guidelines provided by the FAO (2006) and OECD (2014).

The guidelines of the FAO (2006) require the development of a precise tool for the specific identification and quantification of the microbial BCA in the pest protection product and in the inoculated environments. Furthermore, the regulations encourage monitoring of the released microbial BCA in target environments in order to assess impact on non-target organisms (FAO, 2006). The specific EU regulations (EU No 546/2011, p. 168) state in more detail "authorities shall evaluate the possibility of exposure of and effects on soil microorganisms and subsequent effects on nitrogen and carbon mineralization in the soil" due to inoculation with the microbial BCA. The OECD guideline suggest that impacts on microbial community structures or on symbiotic activity of arbuscular mycorrhizal fungi due to soil inoculation with the microbial BCA should be assessed (OECD, 2014), i.e. corresponding studies were submitted in the EU dossiers for the registration of *Trichoderma atroviride* I-1237 and *Trichoderma asperellum* strain T34 as BCAs (OECD, 2014).

The fungal BCA Fos is envisaged for commercial introduction in all *S. hermonthica* infested areas, which includes several countries in Sub-Saharan Africa (e.g., Nigeria, Benin, Burkina Faso and Kenya). Risk assessment studies of the BCA Fos, which fulfill the requirements of the above-mentioned FAO and OECD guidelines will facilitate its registration by country authorities in Sub-Saharan Africa since most country regulations rely on this international framework.

1.4 Research Objectives and Hypotheses

The main objectives of this PhD study were:

1) To develop a monitoring tool which enables specific identification and quantification of the BCA Fos in soils
2) To monitor the proliferation and persistence of the BCA Fos in the rhizosphere of maize in contrasting soils
3) To identify biotic and abiotic environmental factors which influence the abundance and proliferation of Fos in soils

4) To assess the potential impact of Fos exposure on the abundance and community composition of non-target indigenous soil fungi in contrasting soils.
5) To assess the impact of the BCA Fos on indigenous fungal communities relative to natural environmental impacts such as soil type, crop growth stage and seasonality.

In this context, the presented research was based on the following hypotheses:

1) Fos abundance in soils is determined by environmental factors, such as soil texture, crop growth stage and seasonality.

    1.1) Sandier soil textures will offer favourable growth conditions to the fungal BCA Fos compared to clayey soil textures, due to greater pore sizes which favour growth of fungal hyphae.

    1.2) Fos abundance will decrease with increasing plant age due to elevated demand of the crop plant for symbiotic AMF at later crop growth stages. AMF are acknowledged to possess antagonistic function against *Fusarium* spp..

    1.3) Fos abundance will increase with enhanced precipitation rates since water improves the dispersal and germination of fungal spores in soils.

2) Fos exposure will result in resource competition between the BCA and indigenous soil fungi and, hence, change the abundance and/or composition of the indigenous soil fungal community.

    2.1) Resource competition between Fos and indigenous soil fungi will be pronounced in resource-poor sandier soils compared to resource-rich clayey soils.

3) Increasing soil nutrient resources (i.e., soil amendment with nitrogen-rich organic residues) will compensate the competition effect between Fos and indigenous soil fungi.

    3.1) This compensation effect will be enhanced in resource-poor sandier soils compared to resource-rich clayey soils.

4) Natural environmental factors, such as soil type, crop growth stage and seasonality, superimpose the impact of the BCA Fos on indigenous fungal communities.

1.5 Review on molecular techniques implemented in this study

This review on molecular techniques is not aiming on detailed explanation and comparison of existing molecular methods for studying soil microbial community dynamics, referring in this context to extensive reviews provided by Hirsch et al. (2013, 2010), Rincon-Florez et al. (2013) and van Elsas and Boersma (2011), but tends to serve as a justification for specific methodical choices in this PhD thesis.

1.5.1 Molecular techniques applied in the development of the monitoring tool for Fos

Fusarium oxysporum are ubiquitous in soils worldwide (Gordon and Martyn, 1997) with agricultural soils usually containing a broad variety of *F. oxysporum* species of differing *forma speciales* (Appel and Gordon, 1994; Edel-Hermann et al., 2015). The envisaged Fos monitoring tool is required to discriminate on the *forma speciales* level between Fos and other close related *F. oxysporum* in soils. Culture-dependent monitoring techniques that rely on visual distinction (i.e. agar plating technique) cannot achieve the required discrimination power of the envisaged monitoring tool because of the high morphological similarity within the species complex *F. oxysporum*. This justified the methodological choice of using molecular DNA-based techniques in the development of the monitoring tool for the BCA Fos. DNA-based techniques show the highest possible resolution power with detecting even single nucleotide polymorphisms (SNPs) (Papp et al., 2003) and are acknowledged as straightforward, more sensitive and specific alternative to culture-based morphological approaches (Hirsch et al., 2010; Lievens, 2006; van Elsas and Boersma, 2011). Requirement for the development of a molecular Fos monitoring tool was the identification of a unique DNA sequence which enables to distinguish Fos from other close related *F. oxysporum* isolates in soils. The unique Fos DNA sequence was identified with the molecular fingerprinting technique “Amplified Fragment Length Polymorphism (AFLP)” which enables genetic comparison screening between close related organisms (Vos et al., 1995). The decision to work with the AFLP approach was justified by its advantages compared to other fingerprinting technologies including microsatellites, randomly amplified polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP). AFLP is confirmed to have higher resolution, reproducibility and sensitivity compared to other techniques without the need of prior sequence information (Abd-Elsalam et al., 2004; Kiprop et al., 2002; Mueller and Wolfenbarger, 1999). In AFLP, a subset of restriction fragments is produced from an enzymatic digest of genomic DNA with a subsequent selective polymerase chain reaction (PCR) amplification. The AFLP approach is similar to RFLP analysis with the

exception that only a subset of fragments is displayed and the number of fragments generated can be controlled by selective primer extensions. The advantages of the AFLP technique have resulted in an enhanced application in the genetic discrimination of the genus *Fusarium* spp. and within the species complex *F. oxysporum* (i.e. Baayen et al., 2000; Bogale et al., 2006; Groenewald et al., 2006; Silva et al., 2013). Cipriani et al. (2009) developed an AFLP based marker to identify the *F. oxysporum* strain “FT2” using PCR in soils.

The unique Fos DNA sequence identified with AFLP fingerprinting forms the baseline for the envisage monitoring tool which should be capable of specifically identifying but also quantifying Fos in soils. Several studies have confirmed the effectiveness of quantitative PCR (qPCR) in the detection and quantification of soil fungal populations (Huang et al., 2016; Jiménez-Fernández et al., 2010; Lievens, 2006) with a number of qPCR assays developed for *F. oxysporum* in soils (e.g. Bluhm et al., 2004; Jiménez-Fernández et al., 2010; Scarlett et al., 2013). Edel-Hermann et al. (2009) employed a qPCR based monitoring tool to follow the population dynamics of the BCA strain *F. oxysporum* "Fo47" in soils. In qPCR, two distinct reporter systems are commonly used: (1) SYBR Green and (2) *Taq*Man probe assays (Smith and Osborn, 2009). SYBR green intercalates with double stranded DNA followed by a fluorescence signal, which increases corresponding to the accumulation of amplicon numbers in every PCR cycle. Requirement for SYBR Green assays is a highly specific PCR reaction, which must be verified by post-PCR melting curve analysis (Smith and Osborn, 2009). The *Taq*Man probe assay relies on a fluorescence labeled probe that hybridizes to a conserved region within the amplicon sequence. The *Taq* Polymerase cleaves the fluorophore from the *Taq*Man probe during the qPCR extension step resulting in a fluorescence signal. The required hybridization of the *Taq*Man probe with the target DNA sequence ensures additional specificity compared to SYBR Green assays (Smith and Osborn, 2009). The *Taq*Man approach is imperative in multiplex-qPCR assays where different targets are coamplified by using different fluorophores. However, it is less frequently used in single qPCR assays due to much higher costs compared to SYBR Green and the required identification of three conserved regions (two primer regions and one probe region) within the short DNA sequence essential for qPCR (typically ~100 base pairs (bp)) which is often not feasible. The Fos monitoring tool relies therefore on a SYBR Green based qPCR assay with post-hoc melting curve analysis.

1.5.2 Molecular tools implemented in the risk assessment of the BCA Fos

1.5.2.1 Analysis of total indigenous soil fungal community abundance and composition

Molecular DNA-based fingerprinting methods have widespread use in the investigation of the abundance and composition of soil microbial communities (Prévost-Bouré et al., 2011; Liu et al., 1997) and the specificity and high resolution quality of these molecular tools is a great advantage compared to conventional methods, like plating-, bioassays and also phospholipid-derived fatty acids (PLFA) analysis (Hirsch et al., 2010). PCR can be applied to amplify marker genes directly from environmental samples. Thus, fungal community profiling does not require cultivation of the fungal targets (Vainio and Hantula, 2000) and molecular fingerprinting methods are therefore not biased by differences in culturability of different fungal taxa within a community (Kennedy and Clipson, 2003; Varma and Oelmüller, 2007). The small ribosomal subunit sequence 18S and the internal transcribed spacer (ITS) region between the mall and large ribosomal subunit are so called marker sequences for fungal communities since they harbour both highly conserved and highly variable sequences (Elsas et al., 2011; Hirsch et al., 2010). The ITS region shows high sequence length variability and is commonly used for high throughput sequencing approaches (Lindahl et al., 2013). Several researchers designed universal primers on highly conserved regions in the 18S rDNA gene sequence, which enable assessing the total fungal abundance in soil samples via qPCR and can be further implemented in molecular fingerprinting methods, i.e. PCR - Denaturing Gradient Gel Electrophoresis (PCR-DGGE) and Terminal Restriction Fragment Length Polymorphism (TRFLP) (e.g. Elsas et al., 2011; Hirsch et al., 2010). Prévost-Bouré et al. (2011) compared 33 different 18S rDNA primer sets to assess their applicability in qPCR but also their soil fungal coverage and specificity. The authors concluded that the best consensus between specificity, coverage and amplicon length was the primer set FF390::FR1 developed by Vanio and Hantula (2000). This justified the use of the primer set FF390::FR1, targeting a conserved region in the 18S rDNA gene, to monitor dynamics in total fungal abundance and community composition using qPCR and TRFLP, respectively, in the present study. The use of internal size standards in TRFLP provides an improved reproducibility of fingerprinting patterns than DGGE (Marsh, 1999).

1.5.2.2 Analysis of indigenous arbuscular mycorrhizal fungal abundance and composition

Detection and quantification of AMF in soils is an important but challenging task in mycorrhiza research. The high genetic variability within AMF hampers primer development with high coverage and specificity to the entire *Glomeromycota* phylum. No single oligonucleotide pair designed until now is able to detect total AMF abundance in soils without serious flaws in specificity with up to 52% non-AMF DNA being coamplified (Xiang et al., 2016; Kohout et al., 2014; Lee et al., 2008, Krüger et al., 2009). Moreover, most oligonucleotides targeting total AMF were specifically designed for high throughput sequencing approaches with amplicon lengths ≥ 800 bp, thereby violating the requirements of proper qPCR assays with preferably short amplicon lengths of ≤ 200 bp (Thonar et al., 2012). Consequently, researchers focused on AMF taxon- or species-specific primer development for subsequent use in qPCR (Boyer et al., 2015; König et al., 2010; Thonar et al., 2012). Thonar et al. (2012) developed and validated qPCR oligoculeotides targeting taxon-specific motifs in the nuclear large ribosomal subunit RNA genes of AMF covering five major AMF taxa: *Cetraspora pellucida*, *Gigaspora margarita*, *Funneliformis mosseae*, *Claroideoglomus claroideum* and *Rhizophagus intraradices*. Field validation of these oligonucleotides confirmed their ability to assess abundance of AMF taxa due to environmental impacts such as soil tillage (Thonar et al., 2012). The high specificity of these taxon-specific primers combined with their applicability in qPCR resulted in enhanced implementation in AMF field research (Janoušková et al., 2015; Jemo et al., 2014; Köhl et al., 2015; Schneider et al., 2015). Until now, the AMF taxon-specific primer sets from Thonar et al. (2012) are the only qPCR oligonucleotides validated for mycorrhiza abundance analysis in tropical soil ecosystems (Jemo et al., 2014), which justified their utilization in AMF abundance analysis in the present thesis.

Similar as for the quantification of AMF, suitable primer sets for monitoring the AMF community composition using TRFLP analysis are limited, since many frequently used AMF oligonucleotides reveal flaws in specificity with coamplifying non-target fungal DNA or even plant DNA (i.e. primer sets AM1::NS31 and AML1::AML2, Kohout et al., 2014). This flaw in specificity can result in serious errors when derived PCR products are used for peak-profile fingerprinting approaches such as TRFLP (Dickie and FitzJohn, 2007). Liu et al. (2011) proposed a new primer combination NS31/AML2, however, results by Kohourt et al. (2014) revealed that even this system co-amplified up to 20% non-AMF fungi. Moreover, about 52%

non-specific amplification was observed using the primer set developed by Redecker (2000) (Kohout et al., 2014). Currently, TRFLP analysis in mycorrhizal communities is strongly dominated by the nested PCR approach using oligonucleotides LR1::FLR2 and FLR3::FLR4 developed by Gollotte et al. (2004) and Trouvelot et al. (1999), respectively, which targets partial sequences in the large subunit ribosomal rDNA gene (Mummey and Rillig, 2007). This nested PCR approach shows reasonable specificity towards Glomeromycota (Mummey and Rillig, 2007) but is known to bias towards *Glomeraceae* (Gamper et al., 2010). A potential alternative was developed by Krüger et al. (2009), who designed a mixed primer set, which amplifies an rDNA gene part of approximately 1500 bp with supposedly improved specificity and coverage. However, this primer set has been tested only in a very limited number of field studies (Fahey et al., 2012; Wang et al., 2011) and has not been implemented so far in subsequent TRFLP analysis. Hence, the best consensus in specificity, field validation and applicability in TRFLP justified the use of the nested PCR approach with primer sets LR1::FLR2 and FLR3::FLR4 (Mummey et al., 2007) for total AMF community composition analysis in the present PhD study.

1.6 Outline of the thesis

This PhD thesis has been written as a cumulative thesis consisting of four research papers, one published (Chapter 2), one accepted for publication (Chapter 3), one submitted (Chapter 4) and one to be submitted (Chapter 5). The thesis begins with a general introduction (Chapter 1) concerning the relevance of the *S. hermonthica* problem to food security in Sub-Saharan Africa, *S. hermonthica* biology and currently deployed and potential *S. hermonthica* control options. Furthermore, it summarizes previous research conducted with the BCA Fos and provides the necessary background about registration regulations concerning microbial BCAs, with this leading over to the objectives and hypotheses. Chapter 2 focuses on the development and validation of a molecular monitoring tool specific for the BCA Fos, including the Fos strain “Foxy-2”. It further emphasizes on monitoring “Foxy-2” abundance as driven by contrasting soil physico-chemical characteristics and availability of organic resources under controlled conditions in a rhizobox experiment. In Chapter 3, the impact of “Foxy-2” exposure on the total indigenous fungal community is assessed under controlled conditions with specific emphasize on plant-beneficial fungal community members such as AMF. Chapter 4 aims on assessing “Foxy-2” proliferation and its impact on total indigenous fungal communities under contrasting

field conditions in western Kenya while Chapter 5 investigates specifically the potential of AMF taxa abundance and total AMF community structure to serve as reliable risk indicators in environmental safety studies. The thesis closes with a general discussion (Chapter 6) highlighting outcomes and limitations of this PhD thesis and finalizing with future recommendations.

2. An explicit AFLP-based marker for monitoring *Fusarium oxysporum* f.sp. *strigae* in tropical soils*

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2.1 Abstract

Our objective was to develop an explicit AFLP-marker to quantify the abundance of *Fusarium oxysporum* f.sp. *strigae* (Fos), an effective soil-borne biocontrol agent (BCA) against the parasitic weed *Striga hermonthica*, in tropical soils. The specificity of the AFLP-marker to Fos was confirmed on basis of comparison of *Fusarium* isolates of differing relatedness to Fos. These consisted of 40 Fos, 17 *Fusarium* spp. and 68 *F. oxysporum* isolates retrieved from tropical and temperate ecosystems. The robustness of the AFLP-marker for monitoring Fos was validated in a controlled incubation experiment. In this experiment, we inoculated the known Fos-BCA "Foxy-2" as model organism via seed coating ($1.15*10^5$ colony forming units per seed) of a tropical maize (*Zea mays* L.) variety to a tropical clayey (Humic Nitisol) and a sandy (Ferric Alisol) soil, both with no indigenous Fos infestation. The proliferation of "Foxy-2" was followed at 14, 28 and 42 days after start of experiment by Fos-specific quantitative PCR using the AFLP-marker. Moreover, the experimental set-up considered two additional factors: (1) presence of *S. hermonthica*, and (2) application of *Tithonia diversifolia* residues as nitrogen-rich resource for supporting "Foxy-2" proliferation. The explicit AFLP-marker was appropriate to reveal that soil type and organic resource availability exhibited distinct effects on abundance of inoculated "Foxy-2". Negative PCR signals, which confirmed the Fos-specificity of the developed AFLP-marker, were retrieved from control soils not inoculated with "Foxy-2". Hence, we verified the applicability of the explicit AFLP-marker for Fos in two soils, but recommend additional broad scale field studies to approve its suitability as Fos monitoring tool for differently managed soils in contrasting tropical agro-ecological zones.

2.2 Introduction

The parasitic weed *Striga hermonthica* is a major constraint to cereal production in Sub-Saharan Africa affecting the livelihood of about 100 million people (Ejeta, 2007a). *S. hermonthica* lives parasitically on cereal crops such as millet (*Pennisetum americanum*), sorghum (*Sorghum bicolor*), maize (*Zea mays*), and rice (*Oryza sativa*) (Elzein and Kroschel, 2004; Marley et al., 2004). It causes annual crop damage ranging from 30 to 90% deemed equivalent to approximately 9 billion US dollars financial loss (Parker and Riches, 1993; van Mourik, 2007; Watson et al., 2007). Control of *S. hermonthica* remains challenging due to its extremely high seed production per plant with seed survival rates in soils of more than 10 years (Parker and Riches, 1993; van Mourik, 2007).

Current *S. hermonthica* control strategies include hand pulling, catch crops (e.g., Sudan grass (*Sorghum sudanense*)) and trap crops (e.g., soybean (*Glycine max*), lucerne (*Medicago sativa*), cotton (*Gossypium* spp.)), as well as the use of tolerant crop varieties. But these approaches were shown to be ineffective when applied individually. Hence, integrated approaches are postulated as control strategies against *S. hermonthica* (Atera et al., 2012; Hearne, 2009; Menkir and Kling, 2007).

Combination of biological control agents (BCAs) such as *Fusarium oxysporum* f.sp. *strigae* (Fos) strains along with tolerant crop varieties has shown respectable control success in field experiments conducted in Burkina Faso, Benin and Nigeria (Schaub et al., 2006; Venne et al., 2009). In this respect, the Fos strain “Foxy-2” was approved to be superior in suppressing all development stages of *S. hermonthica* ranging from germination to flowering (Elzein and Kroschel, 2004; Ndambi et al., 2011). In addition, Ndambi et al. (2011) reported that “Foxy-2” colonized saprophytically the roots of cereals, where the biocontrol activity of “Foxy-2” was initialized after *S. hermonthica* attacked the root system. Hence, integration of Fos with tolerant crop varieties is, in comparison to the use of agrochemicals, an environmentally friendly and durable strategy to combat *S. hermonthica*.

Schaub et al. (2006) and Venne et al. (2009) showed, however, that the success of “Foxy-2” in controlling *S. hermonthica* under field conditions is variable, presumably as a consequence of differing environmental conditions across agro-ecological zones with different soil types, as well as rainfall and temperature patterns. Thus, if a prospective broad scale application of BCA Fos in agricultural fields infested by *S. hermonthica* is anticipated, its proliferation and survival

potential in differently managed soil ecosystems requires a thorough evaluation. However, a specific monitoring tool is still lacking allowing the robust quantification of Fos abundance in target environments and also quality assessment during inoculum production.

Conventional techniques for monitoring fungi in soils include the estimation of colony forming units (cfu) on selective media and immunological assays (e.g., ELISA) (Lievens and Thomma, 2005). These methods are generally time-consuming, require taxonomical expertise, and often lack in specificity for the organism of interest (Ward et al., 2004). Molecular, DNA-based techniques compensate many of known limitations of such conventional analyses. Recently, application of quantitative polymerase chain reaction (qPCR) emerged as a suitable method for specific monitoring of microorganisms in soils (Edel-Hermann et al., 2011; Jiménez-Fernández et al., 2010; Providenti et al., 2009). A number of qPCR assays were developed previously for *Fusarium* spp. (Bluhm et al., 2004; Pasquali et al., 2006; Waalwijk et al., 2004). Edel-Hermann et al. (2011) developed a strain-specific qPCR assay based on a sequence-characterized amplified region marker (SCAR) for quantification of BCA “Fo47”, a soil-borne *F. oxysporum* strain.

Amplified fragment length polymorphisms (AFLP; Vos et al., 1995) was developed as one of the most recognized DNA fingerprinting techniques to analyze fungal populations including *Fusarium* spp. in soils (e.g., Abd-Elsalam et al., 2004; Kiprop et al., 2002; Leslie et al., 2005; Silva et al., 2013). The reproducibility and resolution of AFLPs are superior to those of other markers (e.g., random amplified polymorphic DNAs, restriction fragment length polymorphisms, microsatellites) (Mueller and Wolfenbarger, 1999). AFLP-markers for Fos as necessary prerequisite for monitoring its proliferation in soils are yet to be developed.

Our objective was thus to develop an explicit AFLP-marker for monitoring the abundance of Fos in soils via qPCR. The developed AFLP derived marker was tested for its specificity against 40 Fos, 17 *Fusarium* spp. and 68 *F. oxysporum*

isolates and validated in a controlled incubation experiment. This experiment included two contrasting tropical soils (sandy Ferric Alisol versus clayey Humic Nitisol), with and without *S. hermonthica* infestation, to which "Foxy-2" was inoculated and its proliferation monitored during 42 days. Soils were not sterilized to validate the specificity of the monitoring tool against the indigenous soil fungal population and to monitor the "Foxy-2" proliferation under natural conditions. Additionally, an organic fertilization treatment with *Tithonia diversifolia* residues, a widely used green manure in Sub-Saharan Africa (Gachengo et al., 1998; Jama et al., 2000), was included to investigate its stimulating effect on the saprophytic survival of "Foxy-2" in studied soils.

2.3 Material and methods

2.3.1 AFLP marker development

2.3.1.1 Fungal strains, growth conditions and DNA extraction

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". Details on the origin of *Fusarium* spp. and *F. oxysporum* isolates involved in this study are given in Table 2.1. Fos isolates CAV 6021 – CAV 6236 (Table 2.1) were recently collected from diseased *S. hermonthica* plants in Kenya and Nigeria, and pathogenicity tests were conducted to confirm their pathogenicity to *S. hermonthica* plants (H. Wainwright and A. Elzein, unpublished data). Fos isolates were further characterized by vegetative compatibility group (VCG) testing and phylogenetic analysis of Tef-1α and MtSSU DNA sequences (M. de Klerk, unpublished data). Strains were propagated in potato dextrose broth at 28°C for 3 days, followed by DNA extraction (UltraClean Microbial DNA Isolation Kit, MO BIO Laboratories Inc., Carlsbad, CA). Concentration and quality of isolated DNA were determined on a Nanodrop ND-1000 (Nanodrop Technologies, Wilmington, DE, USA).

Table 2.1: List of *Fusarium* isolates used in this study.

Code	Species	Origin	Isolated from	Reference
Foxy-2	*F. oxysporum* f.sp. *strigae*	Ghana	*Striga hermonthica*	Elzein et al. (2004)
M6-1A	*F. oxysporum* f.sp. *strigae*	Mali	*Striga hermonthica*	Ciotola et al. (1995), Venne et al. (2008)
PSM 197	*F. oxysporum* f.sp. *strigae*	Nigeria	*Striga hermonthica*	Marley et al. (2004)
N2-5A	*F. oxysporum* f.sp. *strigae*	Niger	*Striga hermonthica*	Ciotola et al. (1995), Venne et al. (2008)
M5-1B	*F. oxysporum* f.sp. *strigae*	Mali	*Striga hermonthica*	Ciotola et al. (1995), Venne et al. (2008)
N1-2A	*F. oxysporum* f.sp. *strigae*	Niger	*Striga hermonthica*	Ciotola et al. (1995), Venne et al. (2008)
M8-5A	*F. oxysporum* f.sp. *strigae*	Mali	*Striga hermonthica*	Ciotola et al. (1995), Venne et al. (2008)
K7-5A	*F. oxysporum* f.sp. *strigae*	Kenya	*Striga hermonthica*	Ciotola et al. (1995), Venne et al. (2008)
N5-1A	*F. oxysporum* f.sp. *strigae*	Niger	*Striga hermonthica*	Ciotola et al. (1995), Venne et al. (2008)
B7-5A	*F. oxysporum* f.sp. *strigae*	Burkina Faso	*Striga hermonthica*	Ciotola et al. (1995), Venne et al. (2008)
M12-4A	*F. oxysporum* f.sp. *strigae*	Mali	*Striga hermonthica*	Ciotola et al. (1995), Venne et al. (2008)
FK1	*F. oxysporum* f.sp. *strigae*	Kenya	*Striga hermonthica*	Wainwright H. (2014, unpublished data)
FK2	*F. oxysporum* f.sp. *strigae*	Kenya	*Striga hermonthica*	Wainwright H. (2014, unpublished data)
FK3	*F. oxysporum* f.sp. *strigae*	Kenya	*Striga hermonthica*	Beed et al. (2013)
FK4	*F. oxysporum* f.sp. *strigae*	Kenya	*Striga hermonthica*	Wainwright H. (2014, unpublished data)
FK5	*F. oxysporum* f.sp. *strigae*	Kenya	*Striga hermonthica*	Wainwright H. (2014, unpublished data)
CAV 6021	*F. oxysporum* f.sp. *strigae*	Kenya	*Striga hermonthica*	de Klerk M. (2014, unpublished data)
CAV 6023	*F. oxysporum* f.sp. *strigae*	Kenya	*Striga hermonthica*	de Klerk M. (2014, unpublished data)
CAV 6039	*F. oxysporum* f.sp. *strigae*	Kenya	*Striga hermonthica*	de Klerk M. (2014, unpublished data)
CAV 6040	*F. oxysporum* f.sp. *strigae*	Kenya	*Striga hermonthica*	de Klerk M. (2014, unpublished data)
CAV 6044	*F. oxysporum* f.sp. *strigae*	Kenya	*Striga hermonthica*	de Klerk M. (2014, unpublished data)
CAV 6045	*F. oxysporum* f.sp. *strigae*	Kenya	*Striga hermonthica*	de Klerk M. (2014, unpublished data)
CAV 6067	*F. oxysporum* f.sp. *strigae*	Kenya	*Striga hermonthica*	de Klerk M. (2014, unpublished data)
CAV 6077	*F. oxysporum* f.sp. *strigae*	Kenya	*Striga hermonthica*	de Klerk M. (2014, unpublished data)
CAV 6211	*F. oxysporum* f.sp. *strigae*	Kenya	*Striga hermonthica*	de Klerk M. (2014, unpublished data)

CAV 6090	*F. oxysporum* f.sp. *strigae*	Nigeria	*Striga hermonthica*	Elzein A. (2014, unpublished data)
CAV 6091	*F. oxysporum* f.sp. *strigae*	Nigeria	*Striga hermonthica*	Elzein A. (2014, unpublished data)
CAV 6092	*F. oxysporum* f.sp. *strigae*	Nigeria	*Striga hermonthica*	Elzein A. (2014, unpublished data)
CAV 6116	*F. oxysporum* f.sp. *strigae*	Nigeria	*Striga hermonthica*	Elzein A. (2014, unpublished data)
CAV 6135	*F. oxysporum* f.sp. *strigae*	Nigeria	*Striga hermonthica*	Elzein A. (2014, unpublished data)
CAV 6143	*F. oxysporum* f.sp. *strigae*	Nigeria	*Striga hermonthica*	Elzein A. (2014, unpublished data)
CAV 6166	*F. oxysporum* f.sp. *strigae*	Nigeria	*Striga hermonthica*	Elzein A. (2014, unpublished data)
CAV 6167	*F. oxysporum* f.sp. *strigae*	Nigeria	*Striga hermonthica*	Elzein A. (2014, unpublished data)
CAV 6233	*F. oxysporum* f.sp. *strigae*	Nigeria	*Striga hermonthica*	Elzein A. (2014, unpublished data)
CAV 6236	*F. oxysporum* f.sp. *strigae*	Nigeria	*Striga hermonthica*	Elzein A. (2014, unpublished data)
573	*F. oxysporum* f.sp. *strigae*	Benin	*Striga hermonthica*	Venne et al. (2008)
690	*F. oxysporum* f.sp. *strigae*	Burkina Faso	*Striga hermonthica*	Venne et al. (2008)
782	*F. oxysporum* f.sp. *strigae*	Burkina Faso	*Striga hermonthica*	Venne et al. (2008)
783	*F. oxysporum* f.sp. *strigae*	Burkina Faso	*Striga hermonthica*	Venne et al. (2008)
784	*F. oxysporum* f.sp. *strigae*	Burkina Faso	*Striga hermonthica*	Venne et al. (2008)
773	*Fusarium* spp.	Burkina Faso	*Striga hermonthica*	Yonly et al. (2005)
774	*Fusarium* spp.	Burkina Faso	*Striga hermonthica*	Yonly et al. (2005)
775	*Fusarium* spp.	Burkina Faso	*Striga hermonthica*	Yonly et al. (2005)
777	*Fusarium* spp.	Burkina Faso	*Striga hermonthica*	Yonly et al. (2005)
CAV 6017	*F. incarnatum-equiseti* species complex	Kenya	*Striga hermonthica*	de Klerk M. (2014, unpublished data)
CAV 6028	*F. equiseti*	Kenya	*Striga hermonthica*	de Klerk M. (2014, unpublished data)
CAV 6032	*F. solani*	Kenya	*Striga hermonthica*	de Klerk M. (2014, unpublished data)
CAV 6093	*F. semitectum*	Nigeria	*Striga hermonthica*	de Klerk M. (2014, unpublished data)
CAV 6056	*F. incarnatum*	Kenya	*Striga hermonthica*	de Klerk M. (2014, unpublished data)
CAV 191	*F. oxysporum* f.sp. *cubense*	Indonesia	*Musa* L.	Groenewald et al. (2006)
AV 789	*F. oxysporum* f.sp. *cubense*	Australia	*Musa* L.	Sutherland et al. (2013)
CAV 528	*F. oxysporum* non pathogen	South Africa	*Musa* L.	Belgrove et al. (2011)
CAV 529	*F. oxysporum* non pathogen	South Africa	*Musa L.*	Belgrove et al. (2011)

CAV 095	*F. oxysporum* f.sp. *cubense* STR4	South Africa	*Musa* L.	FABI, University Pretoria, South Africa
CAV 311	*F. oxysporum* f.sp. *cubense* TR4	Indonesia	*Musa* L.	FABI, University Pretoria, South Africa
CAV 183	*F. oxysporum* f.sp. *cubense* race 1	Australia	*Musa* L.	Groenewald et al. (2006)
CAV 556	*F. oxysporum* non pathogen	South Africa	*Musa* L.	Belgrove et al. (2011)
CAV 315	*F. oxysporum* f.sp. *lycopersici*	South Africa	*Solanum lycopersicum*	PPRI 5456
CAV 317	*F. oxysporum* f.sp. *melonis*	South Africa	*Cucurbitaceae*	PPRI 4923
CAV 324	*F. oxysporum* f.sp. *niveum*	-	*Citrullus lanatus*	Viljoen A. (University Stellenbosch, SA)
CAV 326	*F. oxysporum* f.sp. *conglutin*	USA	*Brassica*	CBS 186.53
CAV 327	*F. oxysporum* f.sp. *dianthi*	Netherlands	*Carnation*	CBS 491.97
CAV 328	*F. oxysporum* f.sp. *gladioli*	Netherlands	*Gladiolus*	CBS 137.97
CAV 329	*F. oxysporum* f.sp. *lini*	Canada	*Linum usitatissimum*	CBS 259.51
CAV 331	*F. oxysporum* f.sp. *tulipae*	-	*Tulipa*	CBS 195.65
CAV 333	*F. oxysporum* f.sp. *phaseoli*	USA	*Phaseolus vulgaris*	CBS 935.73
CAV 334	*F. oxysporum* f.sp. *pisi*	UK	*Pisum sativum*	CBS 127.73
CAV 336	*F. oxysporum* f.sp. *raphanni*	Germany	*Raphanus sativus*	CBS 488.76
CAV 338	*F. oxysporum* f.sp. *elaeidis*	Suriname	*Elaeis guineensis*	CBS 783.83
CAV 339	*F. oxysporum* f.sp. *nicotianae*	-	*Nicotiana tabacum*	CBS 179.32
CAV 340	*F. oxysporum* f.sp. *passiflorae*	Brazil	*Passiflora edulis*	CBS 744.79
CAV 341	*F. oxysporum* f.sp. *perniciosum*	Iran	*Mimosa*	CBS 794.70
CAV 345	*F. oxysporum* f.sp. *chrysanthemi*	USA	*Chrysanthemum*	CBS 129.81
MRC 2301	*F. proliferatum*	USA	*Zea mays*	Marasas et al. (1986)
MRC 0115 a	*F. subglutinans*	South Africa	*Zea mays*	Marasas et al. (1986)
RL 31084	*F. graminearum*	USA	*Zea mays*	Cuomo et al. (2007)
C 8267	*F. verticillioides*	South Africa	*Zea mays*	Fandohan et al. (2005)
CAV 337	*F. oxysporum* f.sp. *vasinfectum*	Israel	*Gossypium*	CBS 411.90
KSU 11404	*F. lateritium*	Australia	*Corylus* L.	Viljoen A. (University Stellenbosch, SA)
CAV 343	*F. oxysporum* f.sp. *melonis*	Israel	*Curcurbitaceae*	CBS 424.90
FOG	*F. oxysporum*	Italy	*Orobanche ramosa*	Kohlschmid et al. (2009)

FT2	*F. oxysporum*	Italy	*Orobanche ramosa*	Boari et al. (2004)
AC3	*F. oxysporum*	Italy	*Orobanche cumana*	-
CAV 330	*F. oxysporum* f.sp. *lycopersici*	Israel	*Solanum lycopersicum*	CBS 413.90
FOL	*F. oxysporum*	Italy	*Solanum lycopersicum*	-
rad-lyc	*F. oxysporum* f.sp. radicis-lycopersici	Germany	*Solanum lycopersicum*	-
lycopers	*F. oxysporum* f.sp. *lycopersici*	Netherlands	*Solanum lycopersicum*	-
melonis	*F. oxysporum* f.sp. *melonis*	Frankreich	*Curcurbitaceae*	-
gladioli	*F. oxysporum* f.sp. *gladioli*	Germany	*Gladiolus* L.	-
308	*F. oxysporum*	Germany	*Dianthus caryophyllus*	Vögele R. (University Hohenheim, Germany)
309	*F. oxysporum*	Germany	*Dianthus caryophyllus*	Vögele R. (University Hohenheim, Germany)
311	*F. oxysporum*	Germany	*Dianthus caryophyllus*	Vögele R. (University Hohenheim, Germany)
312	*F. oxysporum*	Germany	*Dianthus caryophyllus*	Vögele R. (University Hohenheim, Germany)
313	*F. oxysporum*	Germany	*Dianthus caryophyllus*	Vögele R. (University Hohenheim, Germany)
314	*F. oxysporum*	Germany	*Dianthus caryophyllus*	Vögele R. (University Hohenheim, Germany)
315	*F. oxysporum*	Germany	*Dianthus caryophyllus*	Vögele R. (University Hohenheim, Germany)
316	*F. oxysporum*	Germany	*Dianthus caryophyllus*	Vögele R. (University Hohenheim, Germany)
317	*F. oxysporum*	Germany	*Dianthus caryophyllus*	Vögele R. (University Hohenheim, Germany)
318	*F. oxysporum*	Germany	*Dianthus caryophyllus*	Vögele R. (University Hohenheim, Germany)
319	*F. oxysporum*	Germany	*Dianthus caryophyllus*	Vögele R. (University Hohenheim, Germany)
320	*F. oxysporum*	Germany	*Dianthus caryophyllus*	Vögele R. (University Hohenheim, Germany)
321	*F. oxysporum*	Germany	*Dianthus caryophyllus*	Vögele R. (University Hohenheim, Germany)
322	*F. oxysporum*	Germany	*Dianthus caryophyllus*	Vögele R. (University Hohenheim, Germany)
323	*F. oxysporum*	Germany	*Dianthus caryophyllus*	Vögele R. (University Hohenheim, Germany)
324	*F. oxysporum*	Germany	*Dianthus caryophyllus*	Vögele R. (University Hohenheim, Germany)
325	*F. oxysporum*	Germany	*Dianthus caryophyllus*	Vögele R. (University Hohenheim, Germany)
326	*F. oxysporum*	Germany	*Dianthus caryophyllus*	Vögele R. (University Hohenheim, Germany)
329	*F. oxysporum*	Germany	*Dianthus caryophyllus*	Vögele R. (University Hohenheim, Germany)
W152	*F. oxysporum*	Kenya	*Warburgia ugandensis*	Mitter B. (AIT GmbH, Austria)

ACC01	*F. oxysporum*	Austria	Metal working fluid	Gorfer M. (BOKU, Austria)
ACC02	*F. oxysporum*	Austria	Metal working fluid	Gorfer M. (BOKU, Austria)
ACC03	*F. oxysporum*	Austria	Metal working fluid	Gorfer M. (BOKU, Austria)
ACC04	*F. oxysporum*	Austria	Metal working fluid	Gorfer M. (BOKU, Austria)
ACC05	*F. oxysporum*	Austria	Metal working fluid	Gorfer M. (BOKU, Austria)
ACC06	*F. oxysporum*	Austria	Metal working fluid	Gorfer M. (BOKU, Austria)
ACC07	*F. oxysporum*	Austria	Metal working fluid	Gorfer M. (BOKU, Austria)
ACC08	*F. oxysporum*	Austria	Metal working fluid	Gorfer M. (BOKU, Austria)
ACC09	*F. oxysporum*	Austria	Metal working fluid	Gorfer M. (BOKU, Austria)
NG_H15	*F. oxysporum*	Austria	Agricultural soil	Gorfer et al. (2011)
NG_H16	*F. oxysporum*	Austria	Agricultural soil	Gorfer et al. (2011)
G-5111	*F. nygamai*	Australia	Agricultural soil	Viljoen A. (University Stellenbosch, SA)
KSU 18979	*F. redolens*	Germany	Agricultural soil	Viljoen A. (University Stellenbosch, SA)
CAV 240	*F. oxysporum*	South Africa	Agricultural soil	Nel et al. (2006)
KSU 11478	*F. babinda*	Australia	Forest soil	Viljoen A. (University Stellenbosch, SA)
SC1103_05	*F. oxysporum*	Austria	Indoor air	Viljoen A. (University Stellenbosch, SA)

2.3.1.2 Amplified fragment length polymorphism (AFLP) procedure

All primers (Biomers GmbH, Ulm, Germany) used are listed in Table 2.2. A selection of nine Fos and six *F. oxysporum* isolates out of 125 isolates (Table 2.1) were included in AFLP fingerprinting to reduce labor and costs.

Table 2.2: List of primers including the developed Fos marker (Kb1::Kb2) used in this study.

Primer	Function	Sequence
*Eco*RI-ad forward	*Eco*RI-Adapter	5'-CTCGTAGACTGCGTACC-3'
*Eco*RI-ad reverse	*Eco*RI-Adapter	3'-CATCTGACGCATGGTTAA-5'
*Mse*I-ad forward	*Mse*I-Adapter	5'-GACGATGAGTCCTGAG-3'
*Mse*I-ad reverse	*Mse*I-Adapter	3'-TACTCAGGACTCAT-5'
*Eco*RI-0	Pre-selective amplification	5'-GAC TGC GTA CC AAT TC-3'
*Mse*I-0	Pre-selective amplification	5'-GAT GAG TCC TGA GTA A-3'
*Eco*RI-ACT	Selective amplification	5'-GAC TGC GTA CC AAT TC ACT-3'
*Mse*I-AA	Selective amplification	5'-GAT GAG TCC TGA GTA AAA-3'
Kb3-forward	Fos marker	5'-GGACGAACTGACAGCCCTAC-3'
Kb3-reverse	Fos marker	5'-GTAACCGTAATATTGTTCAGAGCTC-3'

AFLP fragments were generated as described by Vos et al. (1995) with minor modifications. Digestion was performed at 37°C for 3 h using 300 ng genomic DNA extracted from each fungal isolate, 1x NEBuffer 4 (New England Biolabs Inc. (NEB), Ipswich, MA, USA), 1 U *Mse*I (NEB), 1 U *Eco*RI (NEB), 0.1 mg ml^{-1} bovine serum albumin (NEB), and then adding water to a total volume of 50 µl. Preparation of the *Eco*RI adapter was done with 100 pmol *Eco*RI-ad forward, 100 pmol *Eco*RI-ad reverse and 1x Buffer C (Promega GmbH, Mannheim, Germany). Preparation of the *Mse*I adapter was conducted with 950 pmol *Mse*I-ad forward, 950 pmol *Mse*I-ad reverse and 1x Buffer C. Both adapter reactions were incubated at 96°C for 5 min, and cooled down slowly to room temperature. T4 DNA Ligase buffer (1x, NEB), 1 U T4 DNA Ligase (NEB), 1.5 µl *Eco*RI adapter and 1.5 µl *Mse*I adapter were mixed and filled up to 10 µl with water. Ten µl ligation mix were added to each restriction digestion mix and incubated at 16°C for 12 h. From each ligation, 2 µl were used as template for pre-selective PCR (1x PCR buffer, 1.5 mM $MgCl_2$, 0.25 mM dNTPs, 0.25 µM *Eco*RI pre-selective primer (*Eco*RI-0), 0.25 µM *Mse*I pre-selective primer (*Mse*I-0), and 1 U of *Taq* polymerase (Bioline GmbH, Luckenwalde, Germany) in a total volume of 20 µl). Pre-selective PCR started with 72°C for 2 min and 94°C for 2 min, which was followed by 26 cycles of 94°C for 1 min, 56°C for 1 min,

and 72°C for 1 min. A final extension at 72°C for 5 min completed the reaction. Pre-selective amplicons were diluted 5-fold for subsequent selective PCR. Selective primers were *Eco*RI-ACT and *Mse*I-AA, and the PCR mix was similar to that used for pre-selective PCRs with 2 µl diluted template. Selective PCR included an initial denaturation of 94°C for 2 min, which was followed by 15 cycles of 94°C for 30 s, 65°C for 30 s (with a decrease of 0.7°C in each successive cycle), and 72°C for 1 min; then 25 cycles each of 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min; with a final extension at 72°C for 2 min. Amplicons were visualized with GelRed™ (Biotrend Chemikalien GmbH, Cologne, Germany) staining following electrophoresis (90 V, 3 h) in an agarose-synergel (0.7% agarose and 0.5% Synergel™ (Carl Roth GmbH, Karlsruhe, Germany)).

Target bands in the AFLP fingerprint gel (highlighted in Fig. 2.1) were excised and purified (QIAquick Gel Extraction Kit, Qiagen GmbH, Hilden, Germany). Since DNA quantity after purification from agarose gel was very low, purified DNA was used as template for an additional PCR (2 µl template DNA, 1x PCR buffer, 2 mM $MgCl_2$, 0.25 mM dNTPs, 0.25 µM *Eco*RI-ACT, 0.25 µM *Mse*I-AA, and 1 U of proofreading high fidelity Velocity polymerase (Bioline GmbH)) with the same PCR conditions as used for selective PCR in AFLP (see above). Amplicons were checked for correct band size on a 1.5% agarose gel and purified (Invisorb® DNA CleanUP Kit, Stratec Molecular GmbH, Berlin, Germany). DNA concentration of purified amplicons was determined as described above and was adjusted to the recommended DNA concentration for sequencing. Diluted amplicons were sequenced with the *Mse*I-AA primer at LGC Genomics GmbH (Berlin, Germany).

2.3.1.3 Marker design

Obtained DNA sequences were analyzed using BLAST (Altschul et al., 1990) of the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov) and the *Fusarium* database (*Fusarium* Comparative Sequencing Project, Broad Institute of Harvard and MIT) to determine if any sequence homologues exist in databases. Primers targeting internal sequences of AFLPs were designed using Primer-BLAST (http://www.ncbi.nlm.nih.gov/ tools/primer-blast) in consideration of relatively high annealing temperatures (≥ 60°C) and amplicon sizes smaller than 200 bp to reduce generation of artifacts and to achieve proper reaction efficiencies during qPCR. The designed primer pair (Kb1::Kb2, Table 2.2) generated an amplicon of 165 bp with the following sequence:

5´-
GGACGAACTGACAGCCCTACGAGAATGGCTTGATGAGAACCTACGGAAAGGGTTT
ATCAGGCCAAGCTCCTCCCCCGTGGCATCCCCTGTGCTGTTCGTAAAGAAGCCAG
GAGGAGGACTACGATTCTGCGTAGACTACAGAGCTCTGAACAATATTACGGTTAC -
3´.

2.3.1.4 PCR protocol for the developed marker

The primer pair was first tested for its specificity on all isolates (i.e., 40 Fos, 17 *Fusarium* spp. and 68 *F. oxysporum* isolates (Table 2.1)). Five ng of the *Fusarium* isolates DNA was used as template for PCR (1x PCR buffer, 1.5 mM $MgCl_2$, 0.25 mM dNTPs, 0.25 µM of each primer and 1 U of *Taq* polymerase (Bioline GmbH) in a total volume of 20 µl). PCR started with 94°C for 2 min, followed by 35 cycles of 94°C for 1 min, 65°C for 30 s, and 72°C for 30 s. Reaction was completed with a final extension at 72°C for 2 min. Amplicons were visualized with GelRed™ (Biotrend Chemikalien GmbH) staining following electrophoresis (120 V, 45 min) in a 1.5% agarose gel. In a second step, the developed primer set was validated in DNA extracts obtained from soils of the incubation experiment (section 2.3.2) with the PCR protocol described above using 10 ng soil DNA as template.

2.3.2 Rhizobox experiment

A rhizobox experiment was conducted to validate the developed Fos marker in two contrasting tropical soils. Details on the set-up of the rhizobox experiment can be retrieved from a parallel study conducted by Musyoki et al. (2015). For better comprehensibility, a summary is provided as follows. The rhizobox experiment was arranged as a completely randomized design with 6 treatments with 3 replicates each: i) uncoated maize seeds with no *S. hermonthica* (C), ii) uncoated maize seeds and *S. hermonthica* (C+S), iii) coated maize seeds with “Foxy-2” (F), and iv) coated maize seeds with “Foxy-2” and *S. hermonthica* (F+S), v) coated maize seeds with “Foxy-2” and *T. diversifolia* (F+T), as well as vi) coated maize seeds with “Foxy-2”, *S. hermonthica* and *T. diversifolia* (F+S+T).

Rhizosphere soil samples were taken 14, 28 and 42 days after planting (DAP). For this, the rhizobox was opened and approximately 2 g of root adhered soil was taken carefully from several positions in order not to destruct the rooting system. Rhizosphere soil was gently scraped off with sterile forceps and transferred into sterile sampling bags. Chemical soil

analyses were done using bulk soil samples at 42 DAP. Rhizosphere soil samples for molecular analysis were freeze-dried and stored at -20°C, while bulk soils for chemical analyses were kept at -20°C without freeze-drying.

2.3.3 Molecular and chemical analysis of soil samples

2.3.3.1 DNA extraction from rhizosphere samples

Total genomic DNA from rhizosphere soil samples was extracted using the Fast DNA® Spin Kit for Soil (MP Biomedicals, Solon, OH, USA) following the manufacturer's instructions with slight modifications. Briefly, 0.4 g freeze-dried soil was bead-beated for 30 s with a beating power of 5.5 m s^{-1} using a FastPrep®-24 Instrument (MP Biomedicals). Extracted DNA was quantified as described above. A soil spiking experiment was included to account for soil type depending DNA extraction efficiencies influencing "Foxy-2" gene copy recovery: 400 mg of freeze-dried soil samples obtained from control sets of the rhizobox experiment were transferred into the beat beating tubes of the DNA extraction kit (MP Biomedicals). Soil samples in tubes were spiked with cloned "Foxy-2" amplicons of known concentration (10^3 "Foxy-2" gene copies). Recovery of "Foxy-2" amplicons after DNA extraction was determined using the qPCR protocol described in section 2.3.2.

2.3.3.2 Quantitative PCR protocol for the developed AFLP marker

Each reaction (20 µl) contained 10 ng rhizosphere soil DNA template, 10 µl of Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 0.2 µM of each primer Kb1 and Kb2, as well as 0.2 µl T4 gene 32 protein (500 ng μl^{-1}, MP Biomedicals). A cloned amplicon was used as standard in 10-fold serial dilutions of known DNA concentration. PCR runs were performed on a StepOnePlus™ Real-Time PCR System (Applied Biosystems). Reactions started with initial denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 94°C for 30 s, annealing at 65°C for 30 s, and polymerization at 72°C for 1 min as well as one additional step at 77°C for 30 s for signal detection. Occasionally, small peaks occurred in the melting curve between 72 and 76°C due to primer dimers not detected by electrophoresis in a 1.5% agarose gel (data not shown). To avoid measurement of fluorescence signal emitted by these primer dimers, fluorescence of target amplicon (melting temperature (Tm) = 81.8°C) was detected at 77°C. Each DNA sample was processed in triplicate reactions,

while standards were run in duplicates. Melting curve analysis of amplicons was conducted to confirm that fluorescence signals originated from specific amplicons and not from primer dimers or other artifacts. Quantification of gene copies was calculated by comparing values of threshold cycles (Ct) to values of crossing points of the linear regression line of the standard curve using StepOne™ software version 2.2 (Applied Biosystems).

2.3.3.3 Measurement of soil chemical parameters

Total carbon (TC), total nitrogen (N_t), extractable organic C (EOC), extractable N (EON), ammonia (NH_{4+}), nitrates (NO_{3-}) and pH were recorded using bulk soils from each rhizobox shortly taken at the last sampling date (DAP 42). Details on analytical procedures can be obtained in Musyoki et al. (2015).

2.3.4 Statistical analysis

Each rhizobox was sampled at three sequential dates (DAP 14, 28 and 42). Hence, a repeated measures analysis with an autoregressive covariance structure using the "nlme" package (Pinheiro et al., 2014) combined with post hoc Tukey-B tests using the "lsmeans" package (Lenth, 2013) in the statistic software R (R Core Team, 2013) was performed to determine effects of "*S. hermonthica*", "*T. diversifolia*", "Soil type" and "Sampling date" on abundance of "Foxy-2". For this, obtained "Foxy-2" gene copy numbers were square root transformed to meet the assumptions of parametric statistical tests. Pearson's correlation coefficients were used to assess significant relations between "Foxy-2" abundance and soil chemical parameters (Musyoki et al., 2015).

2.4 Results

2.4.1 Development of the molecular monitoring tool for BCA Fos

2.4.1.1 AFLP fingerprint

In an initial test, several primer combinations with different selective primer extensions (Vos et al., 1995) were evaluated on their ability to separate the Fos group from other *Fusarium* spp. and *F. oxysporum* strains via AFLP. Selective primer extensions were suited for visual

fingerprint comparison resulting in a three base-pair extension of the selective *Eco*RI-primer and a 2 base-pair extension of selective *Mse*I-primer. Gel electrophoresis with 0.7% agarose + 0.5% synergel combination provided an optimal separation of fragments with 100 to 1000 bp. The fingerprint, from which the explicit marker for Fos finally resulted, was based on 15 out of 125 pre-selected isolates (Fig. 2.1). AFLP fingerprinting with primers *Eco*RI-ACT/*Mse*I-AA separated clearly the Fos group from the other 6 *F. oxysporum* isolates tested. The highlighted bands were excised, purified, sequenced and used for AFLP marker development. The suitability of the *Eco*RI-ACT/*Mse*I-AA fingerprint for marker development was substantiated against other primer combinations (e.g., *Eco*RI-TTG/*Mse*I-CA) from which no Fos group separating bands were identified (Fig. 2.2).

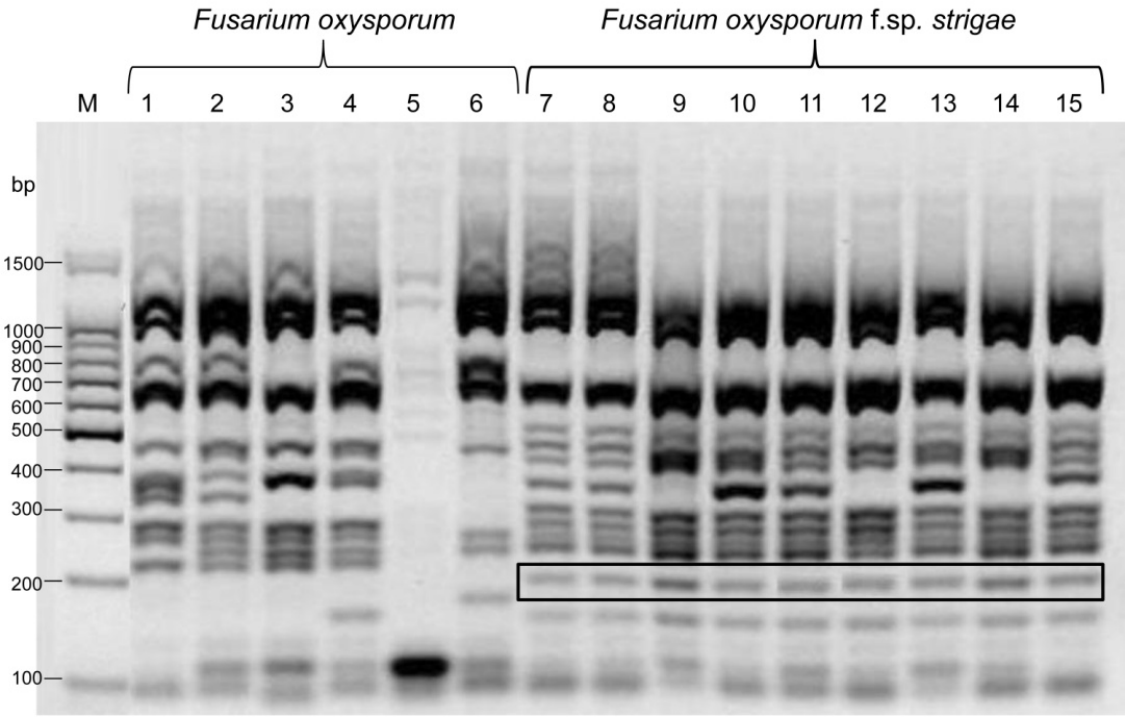

Figure 2.1: AFLP fingerprint obtained for a selection of *Fusarium oxysporum* f.sp. *strigae* (Fos) and *Fusarium oxysporum* isolates with the primer pair *Eco*RI-ACT and *Mse*I-AA. Lane M shows the 100 bp ladder (Promega GmbH, Mannheim, Germany). Bands which were excised from gel and used for marker development are given in the black box. Numbers above lanes represent the following *Fusarium* isolates (codes described in Table 2.1): 1 = AC3, 2 = FOG, 3 = *melonis*, 4 = *lycopers*, 5 = ACC01, 6 = 308, 7 = "Foxy-2", 8 = PSM197, 9 = M6-1A, 10 = M12-4A, 11 = N2-5A, 12 = K7-5A, 13 = B7-5A, 14 = M5-1B, 15 = N5-1A.

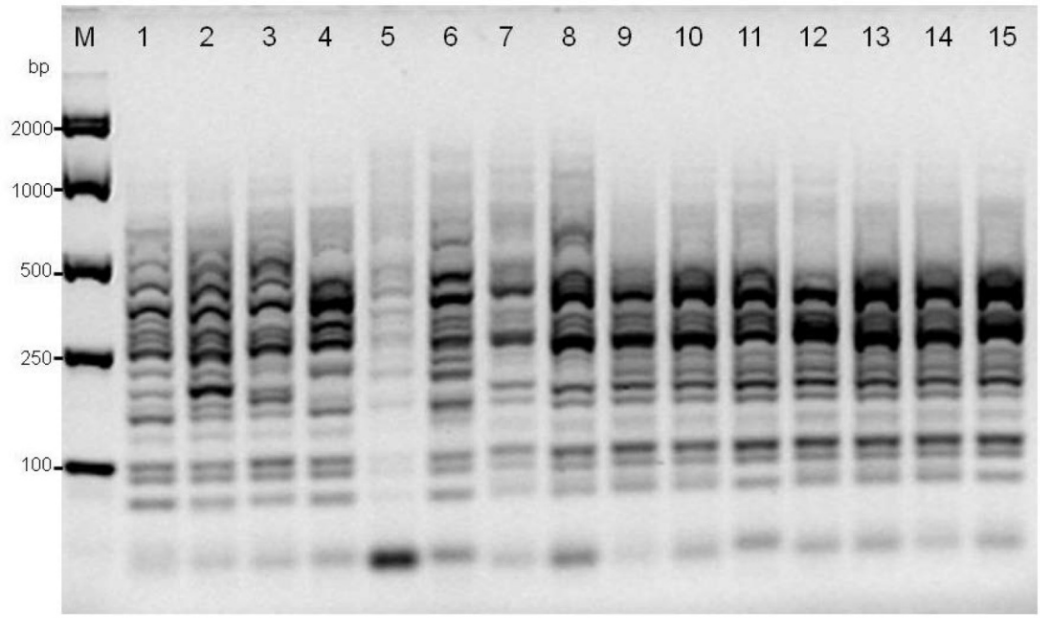

Figure 2.2: AFLP fingerprint obtained for a selection of *Fusarium oxysporum* f.sp. *strigae* (Fos) and *Fusarium oxysporum* isolates with the primer pair *Eco*RI-TTG and *Mse*I-CA. Lane M shows the EasyLadder I (Bioline USA Inc., Taunton, USA). Numbers above lanes represent the following *Fusarium* isolates (codes described in Table 2.1): 1 = AC3, 2 = FOG, 3 = melonis, 4 = lycopers, 5 = ACC01, 6 = 308, 7 = "Foxy-2", 8 = PSM197, 9 = M6-1A, 10 = M12-4A, 11 = N2-5A, 12 = K7-5A, 13 = B7-5A, 14 = M5-1B, 15 = N5-1A.

2.4.1.2 Primer specificity testing

The AFLP derived primer pair Kb1::Kb2 (Table 2.2) was checked for its specificity to Fos via PCR including 40 Fos, 17 *Fusarium* spp. and 68 *F. oxysporum* isolates belonging to 15 different species and 20 forma speciales (f.sp.) (Table 2.1; Fig. 2.3). The only non-Fos isolate amplifying with the developed primers was *F. oxysporum* f.sp. *melonis* (CAV 343, Table 2.1) originating from Israel. Its amplicon showed the same fragment length (165 bp) and DNA sequence as the Fos amplicon. All other non-Fos *Fusarium* spp. and *F. oxysporum* isolates, including the f.sp. *melonis* of African (CAV 317, Table 2.1) and European (*melonis*, Table 2.1) origin, were not detectable with the developed PCR protocol used for specificity testing.

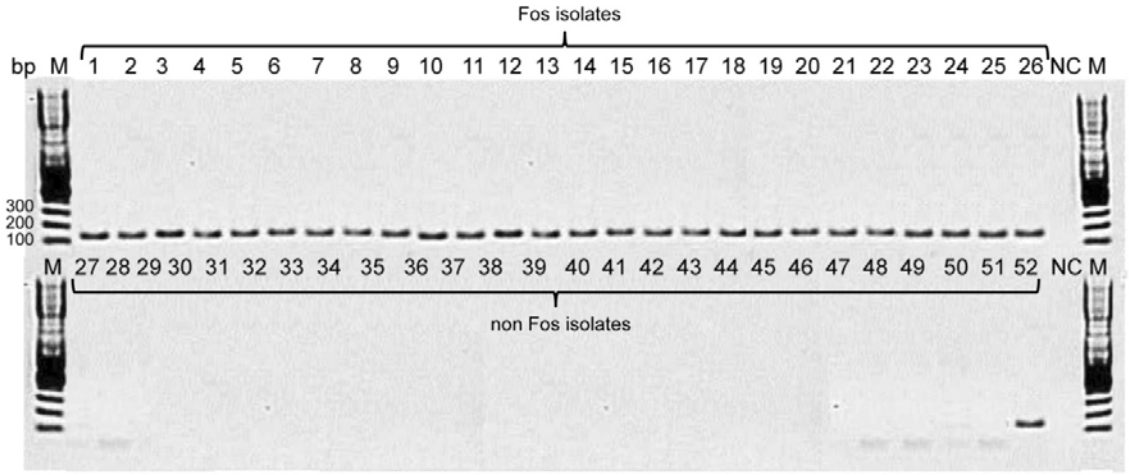

Figure 2.3: Agarose gel picture shows the specificity testing of the developed Fos marker Kb1:Kb2 in PCR including 52 representative *Fusarium* isolates selected from table 2.1. Lane M indicates the 100 bp ladder (Promega GmbH, Mannheim, Germany). Numbers 1-26 represent Fos isolates with 1 = "Foxy-2". Numbers 27-52 represent non-Fos isolates with 52 = CAV 343. NC = negative control.

Alignment of the 165 bp amplicon sequence in BLAST using specifically MEGABLAST for highly similar sequences resulted in no match. With using discontiguous MEGABLAST for comparing diverged sequences, the highest match (76% identity) was found for *Magnaporthe oryzae* covering 70% of the amplicon sequence. Further alignment in the *Fusarium* database showed the highest match (84% identity) with "*F. oxysporum* II5: Supercontig 200: 2407-2554" with sequence coverage of 90%. Alignment of primer sequences Kb1 and Kb2 in Primer-BLAST resulted in one match with *Cryptococcus neoformans* (accession number: NC006685.1) showing an identity score of 80% to both primers Kb1 and Kb2 and generating an amplicon of 4113 bp length. This could be clearly distinguished from the desired Fos amplicon of 165 bp. Additionally, due to the fact that *C. neoformans* is not soil borne, it is considered not to interfere with the Fos monitoring tool for envisaged soil application. Further alignment of primer sequences Kb1::Kb2 in the *Fusarium* database, which includes among others the full genome sequence of one f.sp. *melonis* isolate (NRRL 26406, Ma et al., 2014), resulted in no match. This substantiated the specificity of the developed marker Kb1::Kb2 to Fos in target African soils.

2.4.1.3 Quantitative PCR protocol

Accurate standard curves were generated in the range of $4.66*10^5$-10^1 "Foxy-2" gene copy numbers (Fig. 2.4), thus the detection limit of the qPCR assay was approximately 10 gene

copies of “Foxy-2” per reaction. Standard regression lines approved, according to Smith and Osborn (2009), high quality performance (i.e., reaction efficiencies ≥ 94 %, coefficients of determination (R^2) ≥ 0.997). Specificity of amplifications was verified by melting curve analysis (Fig. 2.4). Control sets without “Foxy-2” inoculation showed no amplification in qPCR confirming the specificity of the AFLP marker to Fos in the two model soils from Embu (clayey Humic Nitisol) and Machanga (sandy Ferric Alisol). Moreover, the soil spiking experiment confirmed the soil type independent “Foxy-2” DNA recovery after DNA extraction since no significant difference in “Foxy-2” gene copy numbers between the clayey and sandy soils was detected ($P > 0.05$).

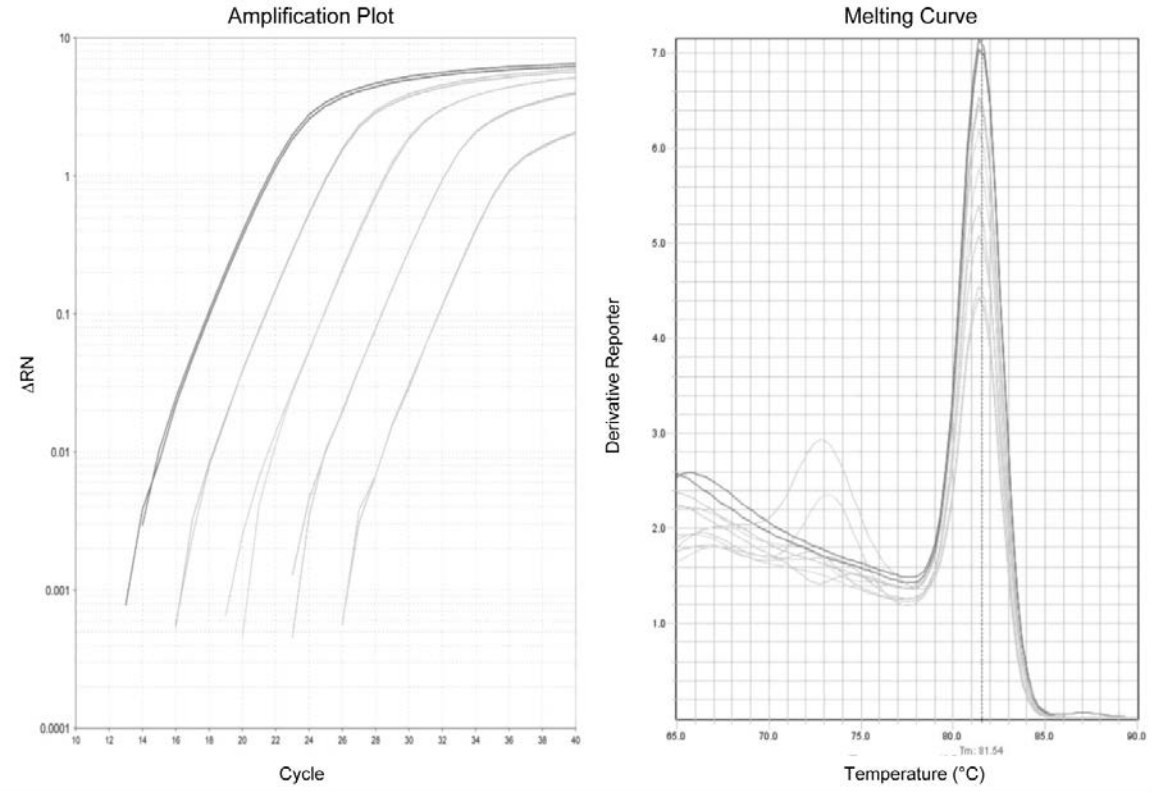

Figure 2.4: Amplification plot of qPCR standards obtained from the developed Fos marker Kb1::Kb2 ranging from $4.66*10^5$ to 10^1 Fos gene copies. Melting curve of qPCR standards shows a Tm = 81.5°C. As a small peak occurred at Tm = 71°C to 76°C due to primer dimers, the fluorescence signal for quantification of Fos was recorded at 77°C.

2.4.2 Validation of the monitoring tool

Figure 2.5 displays the proliferation of “Foxy-2” in the rhizosphere soils of the four “Foxy-2” amended treatments at each sampling date (i.e., DAP 14, 28 and 42). In the non-inoculated control sets, Fos was not detected and these samples were then excluded from statistical

analysis. Clear interactions were found between treatments “Soil type” and “*T. diversifolia*” ($P < 0.001$) as well as “Soil type” and “Sampling date” ($P < 0.01$). No *S. hermonthica* root attachment and emergence was detected in the rhizobox experiment over all treatments within the period of 42 days. Accordingly, the “*S. hermonthica*” treatment (F+S) did not induce any effect on “Foxy-2” gene copy numbers ($P > 0.05$) compared to the “Foxy-2” treatment (F). Treatments with *T. diversifolia* residues (F+T, F+S+T) promoted “Foxy-2” abundance in both soils ($P < 0.001$). In general, *T. diversifolia* treated sandy soils revealed a higher “Foxy-2” abundance than their clayey counterparts ($P < 0.001$). From day 28 onwards, treatment *T. diversifolia* in the clayey soils showed, in comparison to the non-treated soils, an increase of “Foxy-2” gene copy numbers ($P < 0.001$). In the sandy soils, a similar effect was detected from day 14 onwards ($P < 0.001$).

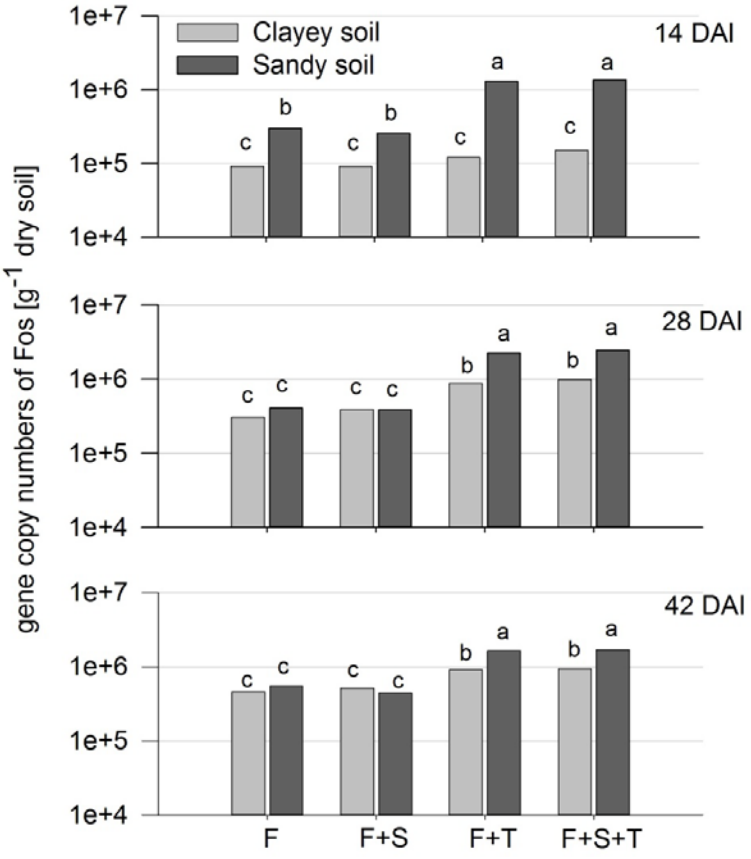

Figure 2.5: Abundance of Fos at 14 (A), 28 (B) and 42 (C) days after inoculation into the clayey Humic Nitisol (Embu) and sandy Ferric Alisol (Machanga) with the four treatments: coated maize with “Foxy-2” (F), coated maize with “Foxy-2” and *S. hermonthica* (F+S), as well as coated maize with “Foxy-2” and *T. diversifolia* (F+T) and coated maize with “Foxy-2”, *S. hermonthica* and *T. diversifolia* (F+S+T). Different letters indicate significant differences in Fos gene copy number at $P < 0.05$.

2.4.2.1 Correlation between “Foxy-2” gene copy numbers and soil chemical data

Pearson’s correlations were assessed between “Foxy-2” gene copy numbers and soil chemical data (Musyoki et al., 2015, Appendix A.1) at DAP 42. Positive correlations were calculated between “Foxy-2” gene copy numbers and pH (clayey soils: $r = 0.706$, $P < 0.01$; sandy soils: $r = 0.931$, $P < 0.0001$) and total carbon (TC) (clayey soils: $r = 0.751$, $P < 0.001$; sandy soils: $r = 0.726$, $P < 0.001$) in both soils. In the sandy soils, “Foxy-2” gene copy numbers showed a positive correlation with extractable organic carbon (EOC) ($r = 0.894$, $P < 0.001$), total nitrogen (N_t) ($r = 0.747$, $P < 0.001$) and ammonium (NH_4^+) ($r = 0.823$, $P < 0.05$). Nitrate (NO_3^-) showed a negative correlation with “Foxy-2” gene copy numbers in the clayey soils ($r = -0.870$, $P < 0.01$).

2.5 Discussion

2.5.1 Development of the molecular monitoring tool for Fos

In the present study, we developed a qPCR assay that allows DNA-based quantification of Fos in soils. For this purpose, a Fos-specific DNA fragment was identified using PCR-based AFLP fingerprinting. Specificity of the generated AFLP marker was validated against 40 Fos and 85 non-Fos isolates representing 15 different species and 20 formae speciales (f.sp.). This was, compared to other studies (e.g., Cipriani et al., 2009; Dubey et al., 2013), an extensive collection of isolates of varied relatedness and was hence an essential fundament for obligatory marker specificity testing. Additional alignment of the AFLP marker sequence in BLAST and in the *Fusarium* database substantiated the specificity of the AFLP marker to Fos. However, one *F. oxysporum* f.sp. *melonis* (Fom) isolate originating from Israel (i.e., CAV 343) gave a positive PCR signal when using the newly developed Fos marker. This co-amplifying isolate was characterized previously to be a member of race 1.2 (Risser, 1976) belonging to the vegetative compatibility group (VCG) 0138 with its geographical restriction to Israel (Katan et al., 1994). Due to this geographical divergence, no interference of the Fom isolate with the Fos marker is expected in African soils for which our marker was specifically developed. Additional Fom isolates originating from Africa (CAV 317, Table 2.1) and Europe (*melonis*, Table 2.1) were tested and these did not show a PCR signal on basis of the developed Fos-marker. Furthermore, the *Fusarium* database includes the full DNA sequence of a Fom isolate, which did also provide

no match with the Fos-marker. This clearly strengthened the Fos-marker specificity for envisaged application in target African soils.

2.5.2 Validation of the monitoring tool in soils

The negative signals retrieved in qPCR from control sets in the rhizobox experiment confirmed the specificity of the developed Fos-marker to the inoculated Fos-strain "Foxy-2" in the two tropical soils from Embu (clayey Humic Nitisol) and Machanga (sandy Ferric Alisol). We showed that "Foxy-2" proliferated in the maize rhizosphere. Further, it was obvious that distinct soil chemical characteristics and particularly the application of *T. diversifolia* residues stimulated "Foxy-2" abundance. The *S. hermonthica* treatment did not pose a significant effect on "Foxy-2" proliferation, which was related to the non-existent *S. hermonthica* root infection. This finding contrasted our hypothesis that the presence of the host *S. hermonthica* would stimulate the proliferation of "Foxy-2". Hence, we argued that the duration of the rhizobox experiment (42 days) was probably not sufficient to observe the expected *S. hermonthica* effect on "Foxy-2" abundance. Therefore, we recommend further experiments under an extended period to investigate the presumed interaction between *S. hermonthica* and "Foxy-2".

The general assumption that sandy soils compared to clayey soils offer favorable growth conditions to fungi (Dominguez et al., 2001; Stotzky and Martin, 1963) was not observed in this study since no distinction in "Foxy-2" abundance was detected between the two soils throughout the experiment. Nevertheless, addition of *T. diversifolia* residues posed a stronger effect on propagation of "Foxy-2" in the sandy soils. Application of *T. diversifolia* residues was accompanied with pronounced increases in ammonium (NH_4^+) levels in the sandy than clayey soils. Accordingly, a positive correlation was detected between NH_4^+ content and "Foxy-2" gene copy number in the sandy soils. Nishio and Furusaka (1970) as well as Ranjard and Richaume (2001) have shown higher NH_4^+ levels in sandy soils as a result of lower colonization of nitrifying microorganisms. Analysis of nitrifying prokaryotes abundance in the same rhizobox experiment confirmed the lower abundance of nitrifying prokaryotes in the sandy soils as opposed to the clayey soils (parallel study of Musyoki et al., 2015). This fact induced obviously an advantageous resource availability for "Foxy-2" utilizing the released NH_4^+ from *T. diversifolia* residue decomposition (Rasche et al., 2014). Hence, supplementation of *T. diversifolia* residues offered a favourable environment for saprophytic proliferation of "Foxy-2" which was pronounced in resource poor sandy soils with low abundance of nitrifying prokaryotes.

2.6 Conclusions

Our results manifested that AFLP derived markers provide a robust strategy to selectively and sensitively detect and enumerate microorganisms such as Fos in African soils via qPCR. We confirmed the presence and accuracy of the predicted PCR fragment via melting curve analysis and gel electrophoresis. Both approaches approved the specific amplification of the desired fragment as generated by the AFLP derived marker. With respect to this sensitivity, we found clear indications that propagation of Fos (i.e., “Foxy-2”) was driven by distinct physico-chemical soil characteristics and organic fertilization (saprophytic survival).

To further approve the specificity and application robustness of the Fos explicit AFLP marker in contrasting environments, in which Fos may proliferate differently in the long-term including its success in controlling *S. hermonthica*, we recommend specific validations of the qPCR-based monitoring tool in a broad variety of contrasting tropical soils of Africa and cropping systems including site-specific organic matter management strategies (e.g., use of organic residues such as *T. diversifolia*) (Rasche and Cadisch, 2013).

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3. Biocontrol agent *Fusarium oxysporum* f.sp. *strigae* has no adverse effect on indigenous total fungal communities and specific AMF taxa in contrasting maize rhizospheres*

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3.1 Abstract

We studied the effects of *Fusarium oxysporum* f.sp. *strigae* (Fos), a soil-borne biocontrol agent (BCA) against *Striga hermonthica*, on total fungal and arbuscular mycorrhizal fungal (AMF) taxa in rhizospheres of maize in a clayey and sandy soil. Effects of Fos-BCA "Foxy-2" were evaluated against (1) *S. hermonthica* presence, and (2) organic fertilization with *Tithonia diversifolia* residues at 14, 28 and 42 days after "Foxy-2" inoculation via DNA-based quantitative PCR and TRFLP fingerprinting. In both soils, "Foxy-2" occasionally promoted total fungal abundance, while the community composition was mainly altered by *T. diversifolia* and *S. hermonthica*. Notably, "Foxy-2" stimulated AMF *Gigaspora margarita* abundance, while *G. margarita* was suppressed by *S. hermonthica*. Total fungal and AMF abundance were promoted by *T. diversifolia* residues. In conclusion, "Foxy-2" exposed no adverse effects on indigenous rhizosphere fungal communities substantiating its environmental safety as BCA against *S. hermonthica*.

3.2 Introduction

The parasitic weed *Striga hermonthica* is a major constraint to cereal production in Sub-Saharan Africa causing yield losses worth of US$ 9 billion (Gibbon et al., 2007; Ejeta, 2007). *S. hermonthica* parasitizes on staples such as millet (*Pennisetum americanum*), sorghum (*Sorghum bicolor*), maize (*Zea mays*), and rice (*Oryza sativa*) (Elzein and Kroschel, 2004; Marley et al., 2004). It infests more than 50 million hectare farmland with intensifying dissemination in Sub-Saharan Africa, which makes it to one of the gravest threads to food security in this region (Parker, 2012). Control of *S. hermonthica* remains challenging due to its high seed production per plant with seed survival rates in soils of more than ten years (Parker and Riches, 1993; van Mourik, 2007). It has been widely accepted that a single control method is not effective against *S. hermonthica*, hence, integrated approaches are postulated as control strategies (Atera et al., 2012; Hearne, 2009; Menkir and Kling, 2007).

The combination of biological control agents (BCAs) such as *Fusarium oxysporum* f.sp. *strigae* (Fos) along with tolerant crop varieties revealed respectable control abilities against *S. hermonthica* under field conditions in Burkina Faso, Benin and Nigeria (Schaub et al., 2006; Venne et al., 2009). In particular, the Fos strain “Foxy-2” was shown to be effective in suppressing all development stages of *S. hermonthica* ranging from germination to flowering (Elzein and Kroschel, 2004; Ndambi et al., 2011). In addition, Ndambi et al. (2011) reported that “Foxy-2” colonized endophytically the roots of the host crop (e.g., sorghum), where the biocontrol activity of “Foxy-2” was initialized after *S. hermonthica* attacked the root system. In contrast to previous studies performed in West Africa (e.g., Schaub et al., 2006; Venne et al., 2009), recent efficacy studies of “Foxy-2” in Kenya showed no effective biocontrol ability of “Foxy-2” against *S. hermonthica* (Avedi et al., 2014). These contradictory results were explained by potential genetic distinctions between Eastern and Western African *S. hermonthica* varieties, but also by abiotic and biotic environmental factors influencing the proliferation and hence efficacy of “Foxy-2” in foreign ecosystems (Avedi et al., 2014). The latter knowledge gap was issued by Zimmermann et al. (2015) who, using a Fos specific and quantitative monitoring tool, followed the fate of BCA Fos after inoculation into foreign soil ecosystems. In their study, it was evidenced that Fos proliferation was indeed controlled by physico-chemical soil characteristics and by the availability of organic resources for which Fos stands in competition with indigenous microorganisms in the rhizosphere of the host crop. The latter fact

requires particular attention as Fos is a soil borne fungus and proliferates saprophytically and endophytically in crop rhizospheres and roots, respectively (Ndambi et al., 2011).

Soil microorganisms maintain critical soil functions including nutrient cycling as well as turnover and stabilization of soil organic matter (Kunlanit et al., 2014; van der Heijden et al., 2008). A range of soil microorganisms was shown to suppress soil-borne plant diseases and to promote plant growth (Compant et al., 2005; Rasche et al., 2006; Liu et al., 2007). With respect to resource acquisition in soils, it was recently speculated that there might exist a potential resource competition between Fos and indigenous soil microorganisms (Zimmermann et al., 2015). Hence, it could be hypothesized that the release of Fos in soils may expose a considerable effect on the abundance and community composition of functionally relevant indigenous soil microorganisms which may in turn influence crop health and yield. The impact of the Fos strain "Foxy-2" on the abundance of total indigenous bacterial communities and plant-beneficial prokaryotic nitrifiers in a maize rhizosphere was emphasized by Musyoki et al. (2015) who detected no negative side effects of "Foxy-2" on root-associated bacteria.

In the study we present here, we focused on community dynamics of rhizosphere fungi as these may colonize similar niches as Fos and thus compete for similar resources in the rhizosphere (Winding et al., 2004). We put major emphasis on functionally relevant members of the fungal community focusing primarily on arbuscular mycorrhizal fungi (AMF) colonizing crop roots. The focus on AMF is justified due to their beneficial effects on crop growth and crop stress compensation (Smith and Smith, 2012). We studied the response of fungal communities to Fos inoculation in two contrasting (clayey Humic Nitisol versus sandy Ferric Alisol) soils from Kenya which were not naturally infested with Fos. A rhizobox experiment was conducted in which the selected soils were treated with the Fos strain "Foxy-2" via seed coating of a tropical maize variety used as test crop. Two additional factors were considered: (1) presence of *S. hermonthica*, and (2) application of *Tithonia diversifolia* residues, a widely used green manure in Sub-Saharan Africa (Gachengo et al., 1998; Jama et al., 2000; Opala et al., 2015), to cover the hypothesized resource competition effects. *T. diversifolia* is classified as high quality organic fertilizer with low C/N ratio (Chivenge et al., 2009) and provides easy accessible C sources and high N availability to stimulate indigenous fungal communities (Zimmermann et al., 2015). The response of the total fungal abundance was monitored at 14, 28 and 42 days after inoculation (DAI) using DNA-based quantitative polymerase chain reaction (qPCR), while

fungal community composition (terminal restriction fragment length polymorphism (TRFLP) fingerprinting) and AMF taxa abundance (qPCR) were monitored at 42 DAI.

3.3 Material and methods

3.3.1 Rhizobox experiment

3.3.1.1 Preparatory work

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. Maize (*Zea mays* 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 dried "Foxy-2" chlamydospore inoculum (1.15×10^5 colony forming units per seed) homogenized into 20% arabic gum used as adhesive through a special seed treatment technology (Elzein et al., 2006; seed coating processed by SUET GmbH, Eschwege, Germany) to provide uniform inoculum coverage. *S. hermonthica* seeds (originating from Sudan) were surface sterilized according to Elzein et al. (2010) and germination ability of seeds (75%) was checked as described by Kroschel (2002).

3.3.1.2 Rhizobox set-up

Details on the experimental set up can be retrieved from a parallel study conducted by Musyoki et al. (2015).

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

3.3.1.3 Rhizosphere and bulk soil samplings

Rhizosphere samples for molecular analyses were taken 14, 28 and 42 days after inoculation (DAI). For this step, the rhizobox was opened and approximately 2 g of root adhered soil was taken carefully from several positions in order not to destruct the root system. Rhizosphere soil was gently scraped off with sterile forceps and transferred into sterile sampling bags. Soil samples (bulk soil) for chemical analyses were obtained at 42 DAI. 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. One proportion of the obtained rhizosphere soil samples was used in parallel studies to investigate the proliferation of the BCA "Foxy-2" (Zimmermann et al., 2015) and its impact on indigenous prokaryotic communities (Musyoki et al., 2015), while another proportion was used in the present study to assess the impact of "Foxy-2" on indigenous fungal communities.

3.3.2 Analysis of fungal communities

3.3.2.1 DNA extraction from rhizosphere samples

Total genomic DNA from rhizosphere samples was extracted using the Fast DNA® Spin Kit for Soil (MP Biomedicals, Solon, OH, USA) following the manufacturer's instructions with slight modifications. Briefly, 0.4 g freeze-dried soil was bead-beated for 30 s with a beating power of 5.5 m s^{-1} using a FastPrep®-24 Instrument (MP Biomedicals). Concentration and quality of DNA were determined on a Nanodrop ND-1000 (Nanodrop Technologies, Wilmington, DE, USA) and DNA was stored at -20°C.

A soil spiking experiment was conducted including the two soils used in the rhizobox experiment to account for soil type depending DNA extraction efficiencies influencing fungal gene copy recovery (details described in Zimmermann et al., 2015). Results of the soil spiking experiment verified that DNA extraction efficiency was soil type independent (Zimmermann et al., 2015).

3.3.2.2 Total fungal abundance

Quantification of 18S rDNA gene copy numbers in soils was performed using oligonucleotides FF390 (5′-CGATAACGAACGAGACCT-3′) and FR1 (5′-AICCATTCAATCGGT

AITCATTCA-3′) (Vainio and Hantula, 2000). Each reaction (20 µl) contained 5 ng DNA template, 10 µl of Power SYBR® Green Master Mix (Applied Biosystems, Foster City, CA, USA), 0.2 µl T4 gene 32 protein (500 ng μl^{-1}, MP Biomedicals), and 0.4 µM of each oligonucleotide. A cloned amplicon was used as standard in 10-fold serial dilutions of known DNA concentration (Kamolmanit et al., 2013). PCR runs were performed on a StepOnePlus™ Real-Time PCR System (Applied Biosystems). Cycling started with initial denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s and polymerization at 70°C for 1 min. Each DNA sample was processed in triplicate reactions, while standards were run in duplicates. Melting curve analysis of amplicons was conducted to confirm that fluorescence signals originated from specific amplicons and not from oligonucleotide dimers or other artifacts. An average reaction efficiency of 86% was achieved with R^2 values consistently > 0.98. Quantification of gene copies was calculated by comparing the values of threshold cycles (Ct) to the values of the crossing points of the linear regression line of the standard curve using StepOne™ software version 2.2 (Applied Biosystems).

It needs to be considered that the inoculated Fos strain “Foxy-2” is part of the total fungal abundance. Both were quantified with the used approach and hence, it was likely that the abundance of “Foxy-2” has superimposed the abundance of the indigenous fungal population. We have subtracted “Foxy-2” abundance from the total fungal abundance using the following procedure. “Foxy-2” was propagated in 5 ml potato dextrose broth at 28°C for 3 days, followed by DNA extraction (UltraClean Microbial DNA Isolation Kit, MO BIO Laboratories Inc., Carlsbad, CA). Concentration and quality of “Foxy-2” DNA were determined as described above. Five ng of “Foxy-2” DNA was used as template #1 for Fos-specific qPCR (using oligonucleotides Kb1::Kb2 with the protocol published in Zimmermann et al. (2015) and template #2 for 18S rDNA qPCR (see above). The 5 ng “Foxy-2” DNA template used for both qPCR assays corresponded to $2.3*10^5$ “Foxy-2” gene copies and $4.6*10^5$ 18S rDNA gene copies resulting in a ratio of 1:2 between “Foxy-2” and 18S rDNA gene copies. Accordingly, the previously measured “Foxy-2” gene copy numbers in the soils of the identical rhizobox experiment (Zimmermann et al., 2015) were first multiplied with factor 2 and then subtracted from total 18S rDNA gene copy numbers. This calculation resulted in the adjusted 18S rDNA gene copy numbers reflecting the abundance of the total indigenous fungal population.

3.3.2.3 Fungal community composition

The fungal community composition was studied by terminal restriction fragment length polymorphism (TRFLP) analysis using the same oligonucleotide set as applied for 18S rDNA qPCR (Vainio and Hantula, 2000; Kamolmanit et al., 2013). The 18S rDNA gene was amplified in 25-µl reactions containing 5 ng DNA template, 1× PCR buffer, 2 U Taq DNA polymerase (Bioline GmbH, Luckenwalde, Germany), 0.2 mM of each deoxynucleoside triphosphate (dNTP), 0.4 µM of each oligonucleotide (FF390::FR1), and 1.5 mM $MgCl_2$. The forward oligonucleotide FF390 was labelled with the fluorescent dye FAM-6. PCRs were started with initial denaturation at 95°C for 1 min, followed by 30 cycles consisting of a denaturation at 95°C for 30 s, an annealing step at 52°C for 45 s, and elongation at 72°C for 2 min. Reactions were completed with a final elongation step at 72°C for 10 min. Amplicons were purified using the Invisorb Fragment CleanUp Kit (Stratec Biomedical AG, Birkenfeld, Germany) following the manufacturer's instructions. For digestion, 200 ng of amplicons were incubated with 5 U *Msp*I restriction endonuclease (Promega GmbH, Mannheim, Germany) at 37°C for 4 h followed by 65°C for 20 min enzyme inactivation. Digested products were desalted with Sephadex™ G-50 (GE Healthcare) (Frank Rasche et al., 2006) and amended with 7.75 µl Hi-Di formamide (Applied Biosystems) and 0.25 µl internal size standard GeneScan™-500 ROX™ (Applied Biosystems). Mixtures were denaturated at 95°C for 2 min, followed by immediate chilling on ice. TRFLP profiles were recorded on an ABI Genetic Analyzer 3130 (Applied Biosystems). Peak Scanner software (version 1.0, Applied Biosystems) was used to compare relative lengths of terminal-restriction fragments (T-RFs) with the internal size standard and to compile electropherograms into numeric data sets, in which T-RF length and height >100 fluorescence units (Fredriksson et al., 2014) were used for statistical profile comparison. TRFLP profiles used for statistical analyses were normalized according to Dunbar et al. (2000). A requirement for analyzing "Foxy-2" induced alterations in indigenous fungal community composition was the deletion of "Foxy-2" T-RF from TRFLP profiles. To account for this, we used the following procedure: Fos strain "Foxy-2" was propagated in 5 ml potato dextrose broth at 28°C for 3 days, followed by DNA extraction (UltraClean Microbial DNA Isolation Kit, MO BIO Laboratories Inc., Carlsbad, CA). Concentration and quality of "Foxy-2" DNA were determined as described above. Five ng of "Foxy-2" DNA was used as template for 18S rDNA PCR with oligonucleotides described above in triplicate reactions. PCR amplicons were purified using the Invisorb® Fragment CleanUp (Stratec Molecular GmbH, Berlin, Germany), quantified as described above and adjusted to the recommended DNA concentration for sequencing.

Sequencing was done with the 18S rDNA primer FR1 (LGC Genomics GmbH, Berlin, Germany) and 18S rDNA sequences of “Foxy-2” were submitted to http://www.restrictionmapper.org/ to identify restriction cutting sites with the enzyme *Msp*I used for TRFLP analysis. The resulting T-RF of “Foxy-2” with 168 base pair length was deleted from TRFLP profiles.

3.3.2.4 AMF taxa abundance

AMF taxon-specific oligonucleotides specifically developed for qPCR assays (Thonar et al., 2012) were used in this study since universal AMF primers (i.e., NS31::AM1, AML1::AML2 and NS31::AML2; Helgason et al., 1998; Lee et al., 2008; Simon et al., 1992) lack in specificity for the intended Sybr Green qPCR approach (Kohout et al., 2014). Moreover, it is worthwhile noting that these universal AMF oligonucleotides were not appropriate for qPCR assays as they produce amplicon lengths up to 1800 base pairs. Hence, monitored AMF taxa in this study served as model organisms to investigate potential non-target effects of the BCA “Foxy-2” on indigenous AMF. Thonar et al. (2012) developed qPCR oligonucleotides for five major AMF taxa (i.e., *Rhizoglomus irregulare, Funneliformis mosseae, Gigaspora margarita, Cetraspora pellucida, Claroideoglomus claroideum)*. In a first step, the two soils used in the rhizobox experiment (i.e., sandy and clayey soil) were checked for any occurrence of the five AMF taxa. For this, we have used a nested PCR approach to obtain a higher sensitivity. PCR #1 with oligonucleotides LR1::FLR2 (Jansa et al., 2002) was amplified in 25-µl reactions containing 10 ng soil DNA template, 1× PCR buffer, 2 U Accuzyme DNA polymerase (Bioline GmbH, Luckenwalde, Germany), 0.2 mM of each dNTP, 0.4 µM of each oligonucleotide (LR1::FLR2), and 1.5 mM $MgCl_2$ with cycling conditions described in Jansa et al. (2002). Amplicons of PCR #1 were diluted 1:200 and used as template (2 µl) for PCR #2 with taxon-specific AMF oligonucleotides developed by Thonar et al. (2012) using 25 µl reactions containing 1× PCR buffer, 2 U Accuzyme DNA polymerase (Bioline GmbH, Luckenwalde, Germany), 0.2 mM of each dNTP, 0.4 µM of each oligonucleotide, and 2.0 mM $MgCl_2$. PCR #2 was started with initial denaturation at 95°C for 5 min, followed by 35 cycles consisting of a denaturation at 95°C for 1 min, an annealing step (Table 3.1) for 45 s, and elongation at 72°C for 2 min. Reactions were completed with a final elongation step at 72°C for 10 min. PCR #2 amplicons were visualized with GelRed™ (Biotrend Chemikalien GmbH, Cologne, Germany**)** staining in an agarose gel (1% agarose (Carl Roth GmbH, Karlsruhe, Germany)) following electrophoresis (120 V, 45 min). The nested PCR approach identified two AMF taxa naturally occurring in each soil used

in our rhizobox experiment (i.e., *Cetraspora pellucida* and *Gigaspora margarita* in the clayey soil and *Cetraspora pellucida* and *Claroideoglomus claroideum* in the sandy soil).

The qPCR assays were conducted for the identified AMF taxa as follows: Each qPCR (20 µl) contained 50 ng DNA template, 10 µl of Brilliant III Ultra-Fast SYBR®Green QPCR Master Mix (Agilent Technologies, Santa Clara, USA), 0.3 µl of 1:50 diluted passive reference dye (Agilent Technologies), 0.2 µl T4 gene 32 protein (500 ng μl^{-1}, MP Biomedicals) and 0.5 µM of each oligonucleotide corresponding to the assayed AMF taxa. A cloned amplicon was used for each AMF taxa as standard in 10-fold serial dilutions of known DNA concentration. PCR runs were performed on a StepOnePlus™ Real-Time PCR System (Applied Biosystems). Cycling started with initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 94°C for 15 s, individual annealing temperature (Table 3.1) for 30 s and polymerization at 72°C for 1 min. An additional step at 76°C for 30 s was included for signal detection. Occasionally, small peaks occurred in the melting curve between 72 and 75°C due to oligonucleotide dimers not detected by electrophoresis in a 1.5% agarose gel (data not shown). To avoid measurement of fluorescence signal emitted by these oligonucleotide dimers, fluorescence of target amplicon (*C. pellucida* amplicon T_m=81°C, *G. margarita* amplicon T_m=82°C, *C. claroideum* amplicon T_m=80°C) was detected at 76°C. Each DNA sample was processed in triplicate reactions, while standards were run in duplicates. Melting curve analysis of amplicons was conducted to confirm reaction quality as described above. Quantification of gene copies was processed as described above and average qPCR reaction efficiencies were 89% for *C. pellucida*, 86% for *G. margarita*, and 84% for *C. claroideum* with R^2 values consistently > 0.98.

Table 3.1: Sequences of oligonucleotides (Thonar et al., 2012) and corresponding annealing temperatures used for quantitative PCR of the different AMF taxa.

Target AMF species	Oligonucleotide sequences (5'-3')	Annealing temperatures [°C]
Cetraspora pellucida	AGAAACGTTTTTTACGTTCCGGGTTG CCAAACAACTCGACTCTTAGAAATCG	54
Gigaspora margarita	CTTTGAAAAGAGAGTTAAATAG GTCCATAACCCAACACC	48
Claroideoglomus claroideum	GCGAGTGAAGAGGGAAGAG TTGAAAGCGTATCGTAGATGAAC	52

3.3.3 Measurement of soil chemical parameters

For statistical purpose, data on total carbon (TC), total nitrogen (N_t), extractable organic C (EOC), extractable organic N (EON), ammonia (NH_{4+}), nitrates (NO_{3-}) and pH of soils were retrieved from Musyoki et al. (2015). Plant-available phosphorus (P_{av}) was extracted with the Bray-Kurtz P1 test (Bray and Kurtz, 1945) and content of P_{av} in extracts was quantified at 882 nm on a spectrophotometer (SPECORD 50, Analytik Jena AG).

3.3.4 Statistical analyses

Each rhizobox was sampled at three sequential dates (DAI 14, 28 and 42). Therefore, a repeated measurements analysis with an autoregressive covariance structure using the "nlme" package (Pinheiro et al., 2014) combined with post hoc Tukey-B tests using the "lsmeans" package (Lenth, 2013) in R (R Core Team, 2013) was performed to determine effects of the 5 factors "Foxy-2", "*S. hermonthica*", "*T. diversifolia*", "Soil type" and "Sampling date" on 18S rDNA abundance. AMF species abundance was monitored only at 42 DAI. Hence, for analysis of AMF species abundance, a multifactorial ANOVA was applied in R combined with post hoc Tukey-B tests with the factors mentioned above, but excluding factor "sampling date". Pearson's correlation coefficients were used to assess significant relations between total fungal and AMF species abundance and soil chemical parameters (Musyoki et al., 2015) across all treatments in each soil at 42 DAI (6 observations).

TRFLP data sets were analysed on basis of Bray-Curtis similarity coefficients (Legendre and Legendre, 1998). A similarity matrix was generated for all possible pairs of samples for each target gene. This similarity matrix was used for analysis of similarity (ANOSIM) statistics (Clarke, 1993) to test if the composition of target fungal communities was altered by factors "Foxy-2", "*S. hermonthica*", "*T. diversifolia*" and "Soil type". 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 pair wise 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). Treatment separation was visualized by non-metric multidimensional scaling (nMDS). nMDS calculates a stress value indicating the fitness of similarity ranking, where a stress value below 0.2 warrants a justified treatment separation (Clarke and Warwick, 2001). Calculation of similarity

coefficients, ANOSIM and nMDS were carried out using Primer for Windows version 6 (Primer-E Ltd., Plymouth, UK). To verify if considered soil chemical parameters (Musyoki et al., 2015) were decisive for the observed treatment-driven community composition shifts of the total fungal population, the DistLM procedure of PERMANOVA+ in Primer v6 (Primer-E Ltd.) was used (Clarke and Gorley, 2006). This procedure calculates a linear regression between the diversity of fungal communities using the Shannon diversity index and log transformed soil chemical data (Legendre and Anderson, 1999).

3.4 Results

3.4.1 Total fungal abundance

Figure 3.1 displays the total fungal abundance (18S rDNA gene copies) of the 6 rhizobox treatments at each sampling date (i.e., DAI 14, 28 and 42). Treatment “Foxy-2” (F) promoted the total fungal abundance (18S rDNA gene copies) at 28 DAI in the sandy soil ($P < 0.01$) and at 42 DAI in the clayey soil ($P < 0.001$) compared to the control treatment (C). No *S. hermonthica* root attachment and emergence was detected overall treatments within the incubation period of 42 days. Nonetheless, treatment “*S. hermonthica*” (C+S) induced a stimulating effect on total fungal abundance throughout all sampling dates compared to treatment C ($P < 0.001$). The stimulating effect of *S. hermonthica* on total fungal abundance was less pronounced when inoculated together with “Foxy-2” (F+S) ($P < 0.01$). Addition of *T. diversifolia* residues (F+T, F+S+T) promoted total fungal abundance in both soils at all sampling dates compared to treatment F ($P < 0.001$).

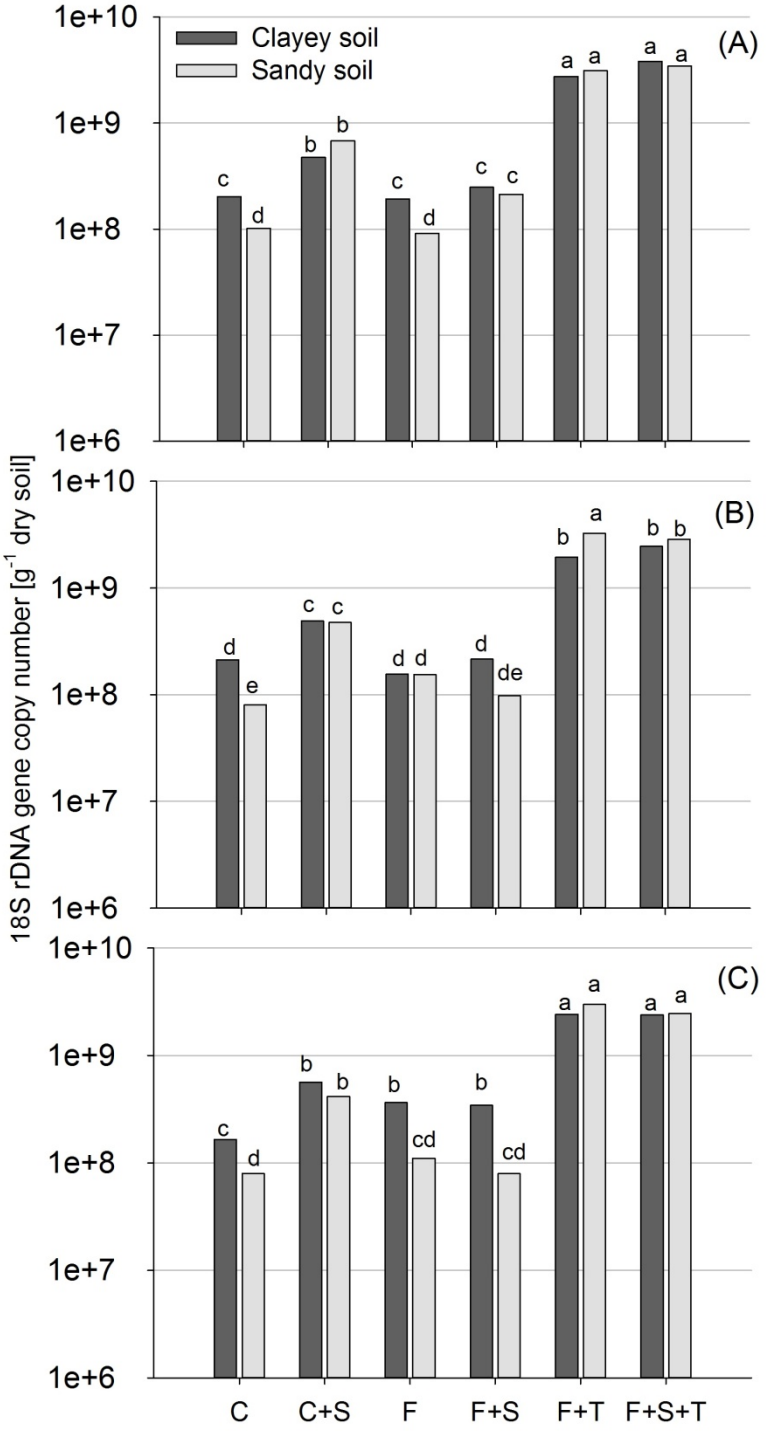

Figure 3.1: Adjusted total fungal abundance on basis of 18S rDNA gene copy numbers determined at 14 (A), 28 (B) and 42 (C) after "Foxy-2" inoculation in the two soils ("clayey" (Humic Nitisol), "sandy" (Ferric Alisol)). Different letters indicate significant differences at $P < 0.05$. Treatment codes are: uncoated maize (C), uncoated maize with *S. hermonthica* (C+S), coated maize with "Foxy-2" (F), coated maize with "Foxy-2" and *S. hermonthica* (F+S), coated maize with "Foxy-2" and *T. diversifolia* (F+T), and coated maize with "Foxy-2", *S. hermonthica* and *T. diversifolia* (F+S+T).

3.4.2 Fungal community composition

Table 3.2 displays the results of analysis of similarity (ANOSIM) of TRFLP profiles. In the clayey soil ANOSIM resulted in the strongest community separation between control treatments (C, C+S) and *T. diversifolia* amended treatments (F+T, F+S+T) with R=1. Control treatment (C) versus "Foxy-2" treatment (F) resulted in R=0.333. In the same soil, treatment C+S induced a community composition distinction with treatment C (R=0.556). Moreover, treatment F was different from *T. diversifolia* amended treatment (F+T) (R=0.915) and F+S+T (R=0.989).

In the sandy soil, a clear community difference was detected between control treatment C versus *T. diversifolia* amended treatments (F+T, F+S+T) with R=1. Treatment F showed a community distinction to the *T. diversifolia* amended treatments (F+T, F+S+T) with R=1. Treatment C was only slightly different from treatment F (R=0.259), while treatments C and C+S showed a community difference of R=0.364.

Effects of factors "Foxy-2", "*S. hermonthica*" and "*T. diversifolia*" on the community composition of the total fungal population were confirmed by nMDS showing clear separations between treatments with stress values of 0.14 in the clayey (Fig. 3.2a) and 0.09 in the sandy (Fig. 3.2b) soils.

Table 3.2: Analysis of similarity (ANOSIM) of TRFLP datasets based on pairwise comparison of treatments. The magnitude of *R* indicates the degree of separation between two tested communities. An *R* score of 1 indicates a complete separation, while 0 indicates no separation.

Soil	Treatment (pairwise comparison)	*R* statistic	Significance level
Clayey soil (Embu)	C, C+S	0.556	0.01*
	C, F	0.333	0.04*
	C, F+S	0.593	0.01*
	C, F+T	1	0.01*
	C, F+S+T	1	0.01*
	C+S, F	0.333	0.04*
	C+S, F+S	0.111	0.06^{ns}
	C+S, F+T	1	0.01*
	C+S, F+S+T	1	0.01*
	F, F+S	0.667	0.01*
	F, F+T	0.915	0.01*
	F, F+S+T	0.989	0.01*
	F+S, F+T	1	0.01*
	F+S, F+S+T	0.667	0.01*
	F+T, F+S+T	0.407	0.01*
Sandy soil (Machanga)	C, C+S	0.364	0.06^{ns}
	C, F	0.259	0.06^{ns}
	C, F+S	0.630	0.01*
	C, F+T	1	0.01*
	C, F+S+T	1	0.01*
	C+S, F	0.259	0.08^{ns}
	C+S, F+S	0.222	0.06^{ns}
	C+S, F+T	0.667	0.01*
	C+S, F+S+T	0.444	0.02*
	F, F+S	0.37	0.02*
	F, F+T	1	0.01*
	F, F+S+T	1	0.01*
	F+S, F+T	0.926	0.01*
	F+S, F+S+T	0.926	0.01*
	F+T, F+S+T	0.333	0.06^{ns}

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

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).

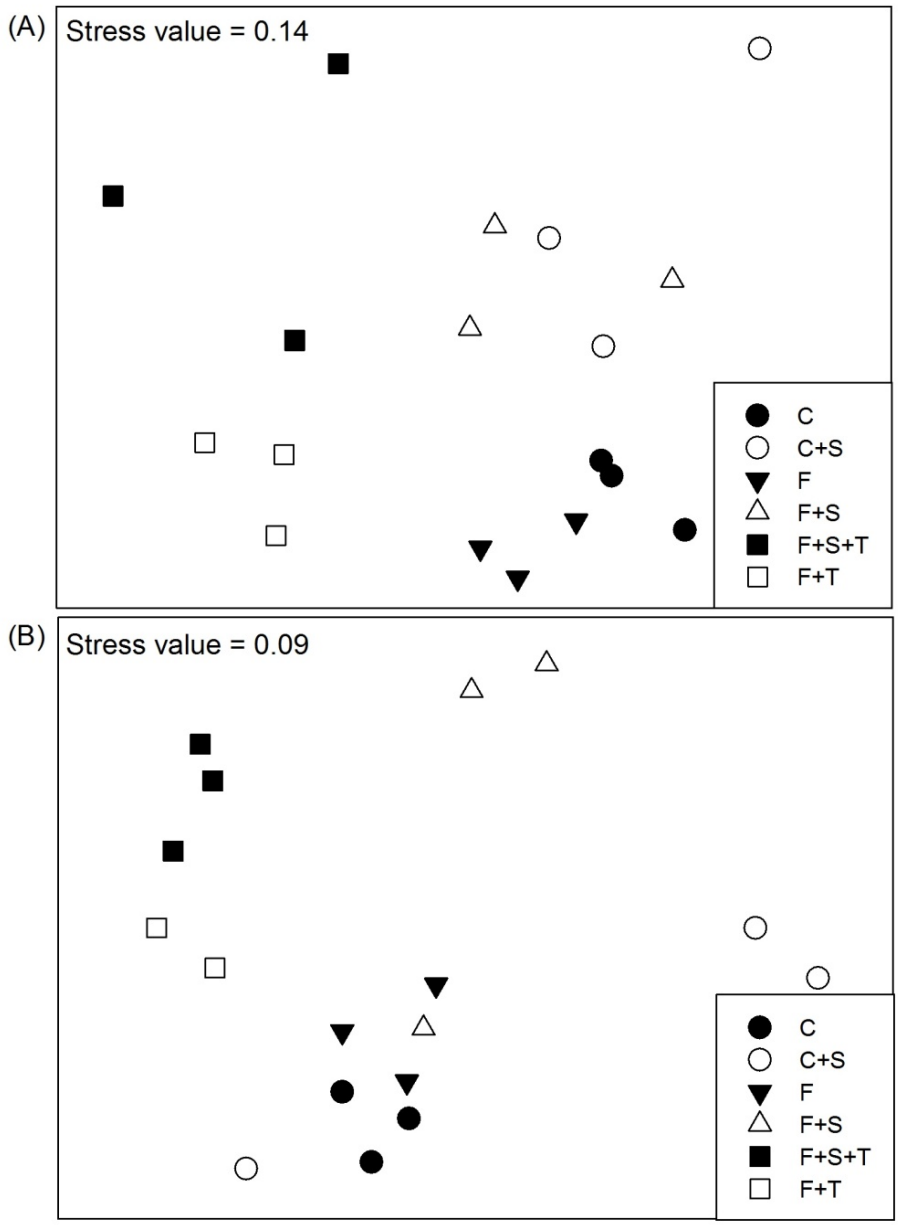

Figure 3.2: Bray-Curtis similarity-based non-metric multidimensional scaling plot (nMDS) of normalized T-RFLP data obtained from *Msp*I-digested 18S rDNA amplicons visualizing the differences in fungal community composition in the clayey (A) and sandy (B) soil according to the following treatments: uncoated maize (C), uncoated maize with *S. hermonthica* (C+S), coated maize with "Foxy-2" (F), coated maize with "Foxy-2" and *S. hermonthica* (F+S), coated maize with "Foxy-2" and *T. diversifolia* (F+T) and coated maize with "Foxy-2", *S. hermonthica* and *T. diversifolia* (F+S+T).

3.4.3 AMF taxa abundance

AMF *Cetraspora pellucida* was detected in both soil types, while *Gigaspora margarita* and *Claroideoglomus claroideum* were detected only in the clayey and sandy soils, respectively (Fig. 3.3). Abundance of *C. pellucida* was higher in the clayey than the sandy soil when not treated with *T. diversifolia* residues ($P < 0.001$). An opposite effect was detected for *T. diversifolia* residue treatments ($P < 0.001$). The highest *G. margarita* abundance was detected in the *T. diversifolia* treatments (F+T, F+S+T) ($P < 0.001$). Additionally, its abundance was promoted by "Foxy-2" (F) compared to the control (C) ($P < 0.001$). Conversely, a suppressive effect on *G. margarita* abundance was detected under *S. hermonthica* treatments (C+S, F+S) compared to the respective controls (C, F). *C. claroideum* abundance was promoted in *T. diversifolia* treatments compared to all other treatments ($P < 0.001$).

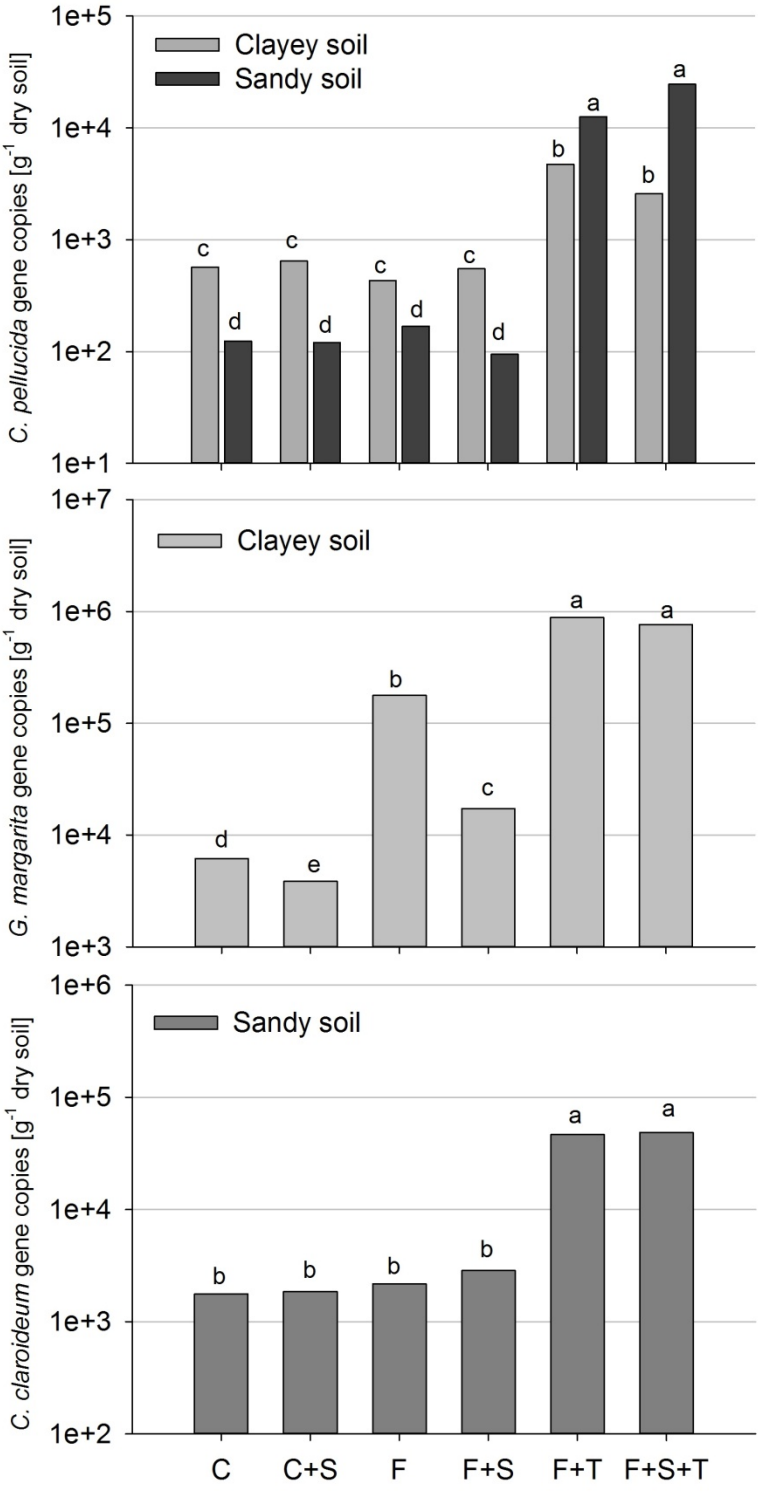

Figure 3.3: AMF taxa abundance at 42 DAI in the two soils ("clayey" (Humic Nitisol), "sandy" (Ferric Alisol)). Different letters indicate significant differences at $P < 0.05$. Treatment codes are: uncoated maize (C), uncoated maize with *S. hermonthica* (C+S), coated maize with "Foxy-2" (F), coated maize with "Foxy-2" and *S. hermonthica* (F+S), coated maize with "Foxy-2" and *T. diversifolia* (F+T), and coated maize with "Foxy-2", *S. hermonthica* and *T. diversifolia* (F+S+T).

3.4.4 Correlation of community abundance and composition with soil chemical data

Pearson's correlation coefficients (Table 3.3) between 18S rDNA gene copy numbers, AMF species abundance and soil chemical data (Musyoki et al., 2015, Appendix A.1) were determined across all treatments in each soil at 42 DAI. For total fungal abundance, 18S rDNA gene copy numbers showed in the clayey soil a negative correlation with plant-available P (P_{av}) (r = -0.649, $P < 0.05$, Fig. 3.4B). *C. pellucida* abundance revealed in the sandy soil positive correlations with extractable organic nitrogen (EON) (r = 0.563, $P < 0.05$), while *G. margarita* abundance in the clayey soil was negatively correlated with P_{av} (r = -0.634, $P < 0.05$, Fig. 3.4A). Moreover, a positive correlation was calculated in the sandy soil between Fos gene copy numbers (Zimmermann et al., 2015) and adjusted 18S rDNA gene copy numbers (r = 0.741, $P < 0.01$).

Shannon diversity indexes calculated from the TRFLP data of total fungal communities and log transformed soil chemical data revealed in the clayey soil positive correlations for soil pH (r = 0.775, $P < 0.001$), EOC (r = 0.748, $P < 0.001$), NH_4^+ (r = 0.606, $P < 0.01$), TC (r = 0.602, $P < 0.01$) and P_{av} (r = 0.551, $P < 0.05$).
In the sandy soil, positive correlations were detected for soil pH (r = 0.669, $P < 0.01$), N_t (r = 0.645, $P < 0.01$), EON (r = 0.640, $P < 0.01$), EOC (r = 0.621, $P < 0.01$), TC (r = 0.599, $P < 0.01$) and NH_4^+ (r = 0.497, $P < 0.05$).

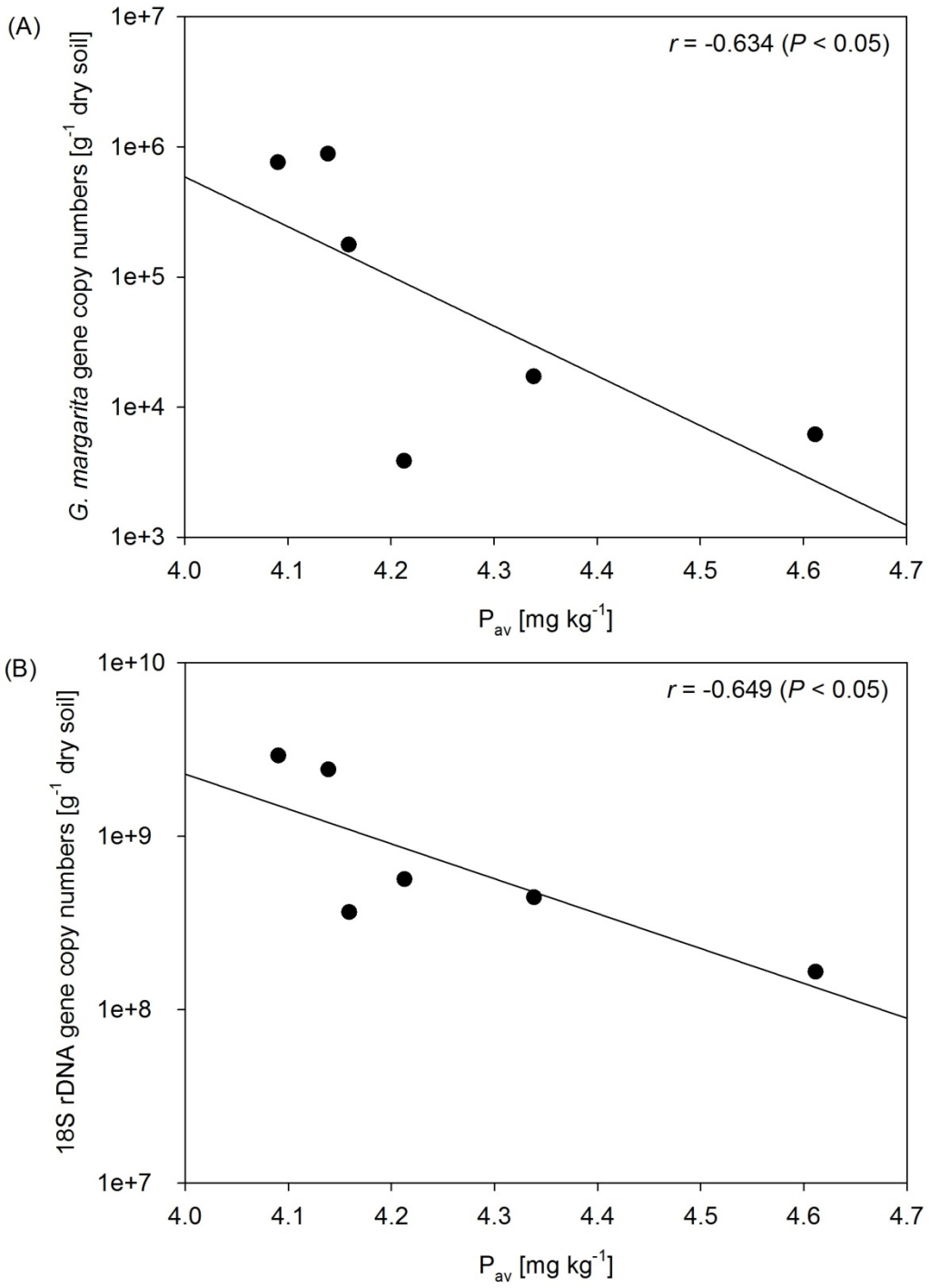

Figure 3.4: Scatterplots between (A) *G. margarita* gene copy numbers and plant-available phosphorus (P_{av}) and (B) 18S rDNA gene copy numbers and P_{av} content. Pearson correlation coefficient (*r*) and *P* values are indicated in each plot.

Table 3.3: Pearson's linear correlation coefficients between target gene abundance (18S rDNA, *C. pellucida*, *C. claroideum* and *G. margarita*) and soil chemical data at 42 DAI.

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}]	P_{av} [mg kg^{-1}]	Soil pH
Clayey soil (Embu)	18S rDNA	0.028^{ns}	0.171^{ns}	0.463^{ns}	0.109^{ns}	0.398^{ns}	0.062^{ns}	**-0.649***	0.196^{ns}
	C. pellucida	-0.187^{ns}	0.065^{ns}	0.268^{ns}	-0.040^{ns}	0.392^{ns}	0.223^{ns}	-0.409^{ns}	0.080^{ns}
	G. margarita	-0.089^{ns}	0.127^{ns}	0.252^{ns}	0.031^{ns}	0.420^{ns}	0.180^{ns}	**-0.634***	0.093^{ns}
Sandy soil (Machanga)	18S rDNA	0.121^{ns}	0.154^{ns}	0.199^{ns}	0.223^{ns}	0.121^{ns}	0.066^{ns}	-0.311^{ns}	0.121^{ns}
	C. pellucida	0.459^{ns}	0.505^{ns}	0.464^{ns}	**0.563***	0.257^{ns}	0.043^{ns}	0.069^{ns}	0.404^{ns}
	C. claroideum	0.215^{ns}	0.283^{ns}	0.243^{ns}	0.307^{ns}	0.159^{ns}	-0.016^{ns}	-0.103^{ns}	0.198^{ns}

Abbreviations: TC: Total carbon, N_t: Total nitrogen, EOC: Extractable organic carbon, EON: Extractable organic nitrogen, NH_4^+: ammonia, NO_3^-: nitrate, P_{av}: Plant-available phosphorus.

Significance levels: ns: $P>0.05$; *$P<0.05$; **$P<0.01$; ***$P<0.001$.

3.5 Discussion

3.5.1 Impact of “Foxy-2” on indigenous AMF

In the present study, we assayed the potential impacts of the BCA “Foxy-2” on the total indigenous soil fungal community, as well as fungal community parts with proven beneficial functions (i.e., AMF), colonizing the rhizosphere of maize. It was one major finding that “Foxy-2” had a promoting effect on abundance of AMF *G. margarita*, while the other two monitored AMF taxa remained unaffected by the BCA. Similarly, *G. margarita* abundance was suppressed by *S. hermonthica* which was compensated when “Foxy-2” was inoculated. Hence, our findings implied a tripartite interaction between „Foxy-2“, AMF *G. margarita* and *S. hermonthica*. A mechanistic linkage between *S. hermonthica* and AMF is the root exudate „strigolactone“, a known stimulating agent of *S. hermonthica* germination (Yoneyama et al., 2010) and also AMF root colonization (Bouwmeester et al., 2007; Besserer et al., 2006). Exudation of strigolactones is specifically increased when crops are cultivated under phosphorus (P) limitation. Under such circumstances, symbiotic AMF are attracted by the crop (Jamil et al., 2014; Czarnecki et al., 2013; Yoneyama et al., 2012). Consequently, we determined a negative correlation between plant available P and abundance of AMF *G. margarita* along with the total fungal community, a finding in line with earlier reports (Ryan et al., 2000; Smith and Read, 2008). Likewise, it was reported that root colonization by AMF (i.e., *Glomus clarum*, *G. margarita*; Othira et al., 2012) reduced the infection of crops (e.g., sorghum, maize) by *S. hermonthica* (Gworgwor and Weber, 2003; Lendzemo et al., 2007, 2005; Othira et al., 2012) as caused by down-regulated strigolactone formation following mycorrhizal colonization of crop roots (Aroca et al., 2013; López-Ráez et al., 2011; Lendzemo et al., 2009).

Interestingly and contradictory to earlier studies (Gworgwor and Weber, 2003; Lendzemo et al., 2007, 2005; Othira et al., 2012) where AMF presence suppressed *S. hermonthica*, our results showed for the first time a suppressive effect of *S. hermonthica* on AMF *G. margarita.* Hence, it remained speculative which actual mechanism underlay this observed interaction between *S. hermonthica* and AMF *G. margarita*, especially under the given short experimental period during which no *S. hermonthica* root attachment or emergence was visually detected. It could be however assumed that germination of *S. hermonthica* seeds started at the end of the experiment. Thereby, competition for infection sites on the crop roots may have been the main driver of the observed interaction. In this context, the role of cytotoxic and antifungal

compounds (i.e., iridoids; Céspedes et al., 2014; Silva et al., 2007) potentially excreted by *S. hermonthica* under these particular situations (Rank et al., 2004) may have played a regulating role.

Our results indicated that the promoting effect of „Foxy-2“ compensated the suppressive effect of *S. hermonthica* on AMF *G. margarita* which would obviously represent an additional benefit when implementing „Foxy-2“ as BCA. According to our findings, several studies reported enhanced mycorrhization of crop roots when AMF and saprophytic *Fusarium oxysporum* were applied simultaneously (Diedhiou et al., 2003; Fracchia et al., 2000; Garcia-Romera et al., 1998). The mechanism behind this obvious interaction is however yet to be understood.

Organic N fertilization (i.e., *T. diversifolia* residues) promoted the abundance of all studied AMF taxa which was underlined by positive correlations with extractable organic nitrogen (EON) contents in soils as well as by earlier findings by Gryndler et al. (2005). Hodge and Fitter (2010) showed that AMF scavenged substantial amounts of nitrogen (N) from decomposing organic materials not only for the transfer to the plant during symbiosis but also as an important N source for maintenance of their own metabolism. Furthermore, Aleklett and Wallander (2012) confirmed the ability of high quality fertilizers with low C/N ratio (i.e., *T. diversifolia*, Chivenge et al., 2009) to stimulate AMF abundance in contrast to low quality fertilizers with high C/N ratio which were shown to be ineffective. Accordingly, this justified the consideration of *T. diversifolia* as organic fertilization treatment to compensate potential suppressive effects of “Foxy-2” on indigenous soil fungal communities through providing additional N and C resources to the rhizosphere microbial community. Our results indicated, however, that *T. diversifolia* in conjunction with “Foxy-2” was not essentially required since no suppressive effects of “Foxy-2” on AMF were detected. On the other hand, as we have shown the promoting effect of *T. diversifolia* on AMF abundance, it may be further considered for general soil fertility improvement by Sub-Saharan farmers.

3.5.2 Impact of “Foxy-2” relative to other factors on total fungal community

We found a promoting effect of “Foxy-2” on total fungal abundance in both soil types which was not due to the presence of Foxy-2 itself since we accounted for this. However, effects of “Foxy-2” on total fungal abundance were detected only temporary in the sandy soil at DAI 28 which ceased at DAI 42. In the clayey soil, the promoting effect of “Foxy-2” on total fungal abundance was delayed and only visible at DAI 42. This was in accordance with other studies

reporting on only transient effects of inoculated endophytic and saprophytic *Fusarium* strains on indigenous soil microbial communities (Ghini et al., 2000; Gullino et al., 1995). For example, Karpouzas et al. (2011) detected only minor temporary effects of an endophytic *Fusarium* strain on the fungal community in the tomato rhizosphere of a sandy loam soil, while a tomato-pathogenic *Fusarium* strain caused long lasting effects on the respective fungal community. However, stronger effects of pathogen on the abundance and community structure of indigenous soil microbial communities were also reported (Berendsen et al., 2012; Ding et al., 2014; Karpouzas et al., 2011).

Further attention needs to be put on our findings that *T. diversifolia* exhibited stronger effects on total fungal abundance than "Foxy-2". This was in accordance to recent findings by Kamolmanit et al. (2013) and Lee et al. (2013) who showed that higher N availability in organic residues increased total fungal abundance in soils in contrast to organic residues with low N availability or mineral fertilizer. Our results were further substantiated by España et al. (2011) and Poll et al. (2010) who confirmed that fast-growing opportunistic fungi were stimulated by easy accessible C sources and high N availability which corresponded to the *T. diversifolia* effect observed in our study. According to the organic input induced alterations of the fungal abundance, we found similar responses of the fungal community composition as supported by positive correlations between Shannon diversity indexes with chemical soil properties (e.g., ammonia, N_t, EON). These findings matched those of Yu et al. (2015) who suggested that the soil microbial community composition is mainly structured by physico-chemical soil characteristics including nutrient status.

3.6 Conclusions

The exclusion of non-target effects of introduced microbial BCAs on the indigenous soil microbial community is inevitable for registration and commercialization of a BCA such as "Foxy-2". In the current study, we evaluated the effects of the fungal BCA "Foxy-2" on the total indigenous soil fungal abundance and composition, as well as fungal community members with proven beneficial functions (i.e., AMF). A major highlight of this study was the promoting effect of "Foxy-2" on the AMF *G. margarita*, while the other two monitored AMF taxa (i.e., *C. pellucida, C. claroideum*) remained unaffected by the BCA. Hence, we concluded that no suppressive effects of "Foxy-2" on AMF are to be expected in the future when implementing "Foxy-2" as an environmentally safe BCA.

Additionally, further research should be addressed to evaluate the promoting effect of “Foxy-2” on AMF *G. margarita* under long-term conditions and emphasize on a broader variety of potential crops (i.e., sorghum) since several AMF taxa show specialization to a particular host (Martínez-García and Pugnaire, 2011). In this context, additional AMF taxa should be tested on their potential compatibility with “Foxy-2” with emphasize those AMF taxa with proven suppression of *S. hermonthica* (i.e., *Glomus clarum*; Othira et al., 2012).

The present study was based on a short-term, controlled rhizobox experiment and, hence, similar experiments should be conducted under natural field conditions with extended time periods to fully understand the ecological effects of “Foxy-2”. These future experiments should account for relevant factors such as crop variety and development, a broader range of fertilization regimes and soil types, as well as seasonal characteristics including rainfall and temperature patterns.

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4. Proliferation of the biocontrol agent *Fusarium oxysporum* f. sp. *strigae* and its impact on indigenous rhizosphere fungal communities under different agro-ecologies*

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4.1 Abstract

Our objectives were to (1) monitor the proliferation of the biocontrol agent (BCA) *Fusarium oxysporum* f. sp. *strigae* strain "Foxy-2", an effective soil-borne BCA against the parasitic weed *Striga hermonthica*, in the rhizosphere of maize under different agro-ecologies, and (2) investigate its impact on indigenous rhizosphere fungal community abundance and composition. Field experiments were conducted in Busia and Homa Bay districts in western Kenya during two cropping seasons to account for effects of soil type, climate, growth stage and seasonality. Maize seeds were coated with or without "Foxy-2" and soils were artificially infested with *S. hermonthica* seeds. One treatment with nitrogen rich organic residues (*Tithonia diversifolia*) was established to compensate hypothesized resource competition between "Foxy-2" and the indigenous fungal community. Rhizosphere soil samples collected at three growth stages (i.e., EC30, EC60, EC90) of maize were subjected to abundance measurement of "Foxy-2" and total indigenous fungi using quantitative polymerase chain reaction (qPCR) analysis. Terminal restriction fragment length polymorphism (TRFLP) analysis was used to assess potential alterations in the fungal community composition in response to "Foxy-2" presence. "Foxy-2" proliferated stronger in the soils with a sandy clay texture (Busia) than in those with a loamy sand texture (Homa Bay) and revealed slightly higher abundance in the second season. "Foxy-2" had, however, only a transient suppressive effect on total indigenous fungal abundance, which ceased in the second season and was further markedly compensated after addition of *T. diversifolia* residues. Likewise, community structure of the indigenous fungal community was mainly altered by maize growth stages, but not by "Foxy-2". In conclusion, no adverse effects of "Foxy-2" inoculation on indigenous fungal rhizosphere communities were observed corroborating the safety of this BCA under the given agro-ecological conditions.

4.2 Introduction

Striga hermonthica is an endemic parasitic weed of maize (*Zea mays*) and other cereal crops including sorghum (*Sorghum bicolor*), millet (*Pennisetum americanum*) and rice (*Oryza sativa*) which are main staple crops in Sub-Saharan Africa (Ejeta, 2007a; Elzein and Kroschel, 2004; Marley et al., 2004). In western Kenya, *S. hermonthica* infests about 76% of total area under maize and sorghum causing annual crop loss equivalent to 41 million US$ (Kanampiu et al., 2002; Vanlauwe et al., 2008).

The *Fusarium oxysporum* f. sp. *strigae* (Fos) strain "Foxy-2" was isolated from diseased *S. hermonthica* plants (Abbasher et al., 1995). This strain was proven to be effective in the suppression of all development stages of *S. hermonthica* ranging from germination to flowering (Elzein and Kroschel, 2004; Ndambi et al., 2011). Field experiments in Burkina Faso, Benin and Nigeria confirmed the combination of "Foxy-2" along with tolerant crop varieties as an effective integrated control approach against *S. hermonthica* (Schaub et al., 2006; Venne et al., 2009). Avedi et al. (2014) and Venne et al. (2009) showed, however, lack in control consistency of the biocontrol approach under field conditions, presumably as a consequence of differing environmental conditions across agro-ecological zones with different soil types, as well as rainfall and temperature patterns (Gerbore et al., 2013; Velivelli et al., 2014). Thus, a thorough understanding of environmental conditions, which promote the proliferation and persistence of the BCA "Foxy-2" is required to ensure consistent and sustained *S. hermonthica* control. In this context, the monitoring of "Foxy-2" proliferation under contrasting soil conditions was issued by Zimmermann et al. (2015) who, using a Fos specific and quantitative monitoring tool, confirmed under controlled conditions that "Foxy-2" proliferation was determined by soil texture and promoted by the amendment of nitrogen (N)-rich organic resources. The latter fact requires particular attention since "Foxy-2" is a soil borne fungus and proliferates saprophytically and endophytically in crop rhizospheres and roots, respectively, where it has to compete with indigenous microorganisms for organic resources (Ndambi et al., 2011).

Potential competition between "Foxy-2" and indigenous microorganisms can determine the proliferation of the BCA and, in turn, may alter the abundance and composition of indigenous microbial communities. Soil microorganisms maintain important soil functions including nutrient cycling, suppression of soil-borne plant pathogens as well as promotion of plant growth (Compant et al., 2005; Liu et al., 2007; van der Heijden et al., 2008). Hence, it needs to be

confirmed that the release and successful proliferation of “Foxy-2” in soils does not alter negatively the abundance and community composition of functionally relevant indigenous soil microorganisms. The exclusion of adverse effects of “Foxy-2” on non-target organisms is mandatory for the official registration of the BCA by regulatory authorities of Sub-Saharan African countries which generally oblige, according to international registration regulations, a profound risk assessment of BCAs (FAO, 2006; OECD, 2014). In this context, recent studies by Musyoki et al. (2015) and Zimmermann et al. (2016) emphasized that the BCA “Foxy-2” exposed no negative effects on the abundance and community composition of indigenous soil prokaryotic and fungal populations. However, as these studies were conducted under short-term and controlled laboratory incubation conditions, progressive field studies are required over extended time periods considering additional factors such as seasonality and crop growth stages that determine the dynamics of rhizosphere microbial communities (Hai et al., 2009; Rasche et al., 2006; Marschner et al., 2003; Rasche et al., 2014).

We hypothesized that “Foxy-2” proliferation in soils is strongly controlled by agro-ecological conditions including soil type and climate, as well as organic residue inputs, seasonality and crop growth stage. Secondly, it was hypothesized that “Foxy-2” presence induces a considerable resource competition for indigenous fungi in the maize rhizosphere inducing alterations of their abundance and community composition. To account for this, we further hypothesized that this resource competition could be compensated by the application of N-rich organic residues (e.g., *T. diversifolia*, Chivenge et al., 2009). To test these hypotheses, the presented research considered two main objectives: (1) Monitor the proliferation of the BCA “Foxy-2” and (2) investigate potential alterations in total indigenous fungal abundance and composition due to “Foxy-2” exposure under field conditions in western Kenya. Both objectives were assayed at three distinct growth stages of maize cultivated in two contrasting field sites during two cropping seasons.

4.3 Material and methods

4.3.1 Fungal biocontrol agent

The 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 the Julius-Kühn-Institut (JKI), Berlin, Germany, where it is deposited under accession number “BBA-

67547-Ghana". Since then, the isolate is preserved at -80ºC at the Institute of Agricultural Sciences in the Tropics, University of Hohenheim, Stuttgart, Germany.

4.3.2 Field experiments

4.3.2.1 Study site description

The studied soil samples originated from field experiments conducted in post-entry quarantine facilities at the Busia Agricultural Training Centre (0° 26'S-34° 15' E) and the Homabay Agricultural Training Centre (0° 40'-0°S and 0° 34° 50'E), both located in Western Kenya (Avedi et al., 2014). The two sites were selected based on their previously described high *S. hermonthica* infestation (De Groote et al., 2008). The study areas have bimodal rainfall patterns with two growing seasons, the first rainy season with long rains (LR) from March to August and second rainy season with short rains (SR) from September to January. Precipitation rates and temperature patterns for the years 2012 and 2013 in Busia and Homa Bay district are presented in Figure 4.1. Busia district received 1157 mm precipitation in the SR of 2012/2013 and 606 mm in the LR of 2013, while the mean temperature was 27.4°C and 26.8°C in the SR and LR of 2012/2013, respectively. Homa Bay district received 383 mm precipitation in SR of 2012/2013 and 481 mm in the LR of 2013, while the mean temperature was 29.5°C and 29.0°C in the SR and LR of 2012/2013, respectively. The soils at Homa Bay were classified as vertic Phaeozems with a loamy clay texture (49% clay, 19% silt, 32% sand), while Busia has orthic Acrisols with a sandy clay texture (33% clay, 22% silt, 45% sand) (IUSS Working Group WRB, 2015).

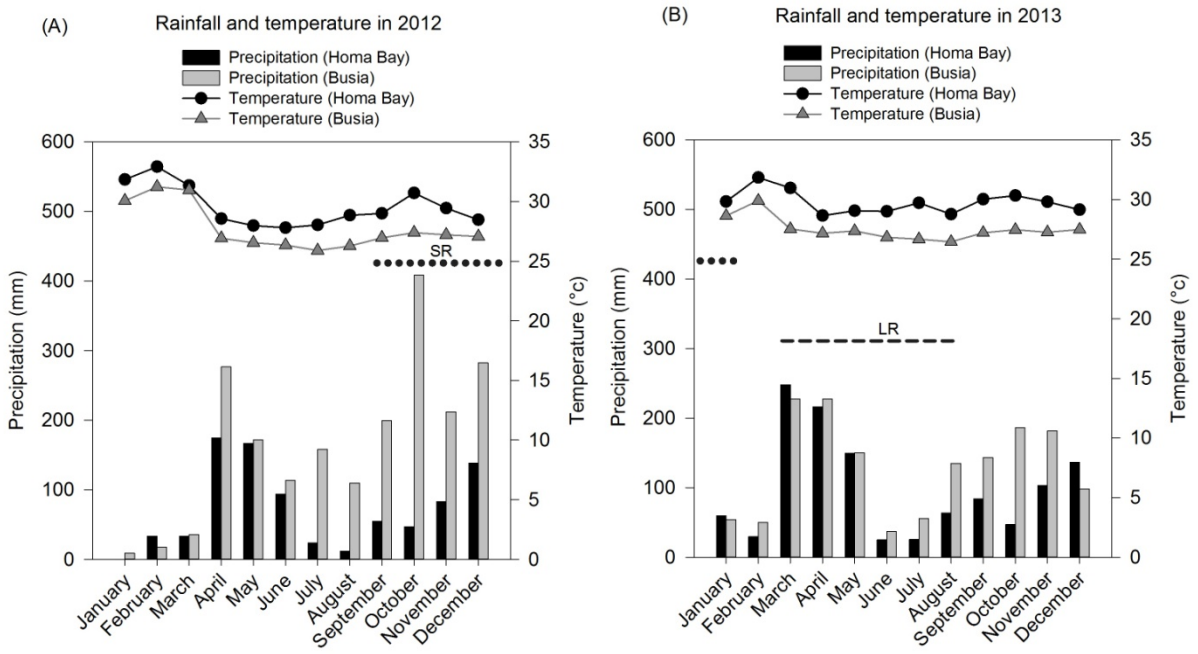

Figure 4.1: Precipitation rate and air temperature per month of the years 2012 (A) and 2013 (B) in Homa Bay and Busia district. Dotted and dashed lines represent the time period of the short rain season (SR) and long rain season (LR), respectively. Data were retrieved with slight modifications from Musyoki et al. (2016).

4.3.2.2 Field experiment set up and rhizosphere sampling

Details on the field experimental set up can be retrieved from a parallel study conducted by Musyoki et al. (2016). For better comprehensibility, a summary is provided as follows. The study covered two seasons (SR; September 2012 to January 2013; LR; April 2013 to August 2013). *Zea mays* L. variety 'WH507' (provided by Western Seed Company Ltd., Kitale, Kenya), which is tolerant to *S. hermonthica* and of high preference by smallholder farmers in western Kenya, was planted in 3 m x 2.7 m plots with a row spacing of 70 cm x 30 cm. 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+S), ii) coated maize with "Foxy-2" and *S. hermonthica* (F+S), and iii) coated maize with "Foxy-2", *S. hermonthica* and *Tithonia diversofolia* residues (F+S+T).

All treatments received diammonium phosphate (23.5 kg N ha^{-1}, 60 kg P_2O_5 ha^{-1}) at sowing. For treatments C+S and F+S, additional N was split applied in the form of calcium ammonium nitrate ($CaNH_4NO_3$) at a rate of 120 kg N ha^{-1} with 1/3 and 2/3 added 3 and 8 weeks after sowing, respectively. 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 (Gacheru and Rao, 2001)). The organic residue was hand-incorporated to a soil depth of 0-15 cm at the onset of each rainy season.

Rhizosphere samples (approximately 50 g) were collected according to standard procedures (Milling et al., 2005) at EC 30 (early leaf development stage), EC 60 (flowering stage), and EC 90 (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 and rhizosphere soil samples of three representative plants per plot were then mixed to one composite sample. Soils were freeze-dried to avoid further microbial activity and stored in a dark and dry place. One proportion of the obtained rhizosphere soil samples was used to study the impact of "Foxy-2" on indigenous prokaryotic communities (Musyoki et al., 2016) while the other proportion was used in the present study to assess the "Foxy-2" abundance and its impact on indigenous fungal communities.

4.3.3 Analysis of fungal communities

4.3.3.1 DNA extraction from rhizosphere samples

Total genomic DNA from rhizosphere samples was extracted using the Fast DNA® Spin Kit for Soil (MP Biomedicals, Solon, OH, USA) following the manufacturer's instructions with slight modifications. Briefly, 0.4 g freeze-dried soil was bead-beated for 30 s with a beating power of 5.5 m s^{-1} using a FastPrep®-24 Instrument (MP Biomedicals). Concentration and quality of DNA were determined on a Nanodrop ND-1000 (Nanodrop Technologies, Wilmington, DE, USA) and DNA was stored at -20°C.

A soil spiking experiment was conducted including the two soils of the field experiments (i.e., Busia and Homa Bay) to account for soil type depending DNA extraction efficiencies influencing fungal gene copy recovery. Briefly, 400 mg of freeze dried soil samples obtained from control sets of the field experiment were transferred into the beat beating tubes of the DNA extraction kit (MP Biomedicals). Soil samples in tubes were spiked with cloned "Foxy-2" amplicons of known concentration (10^3 "Foxy-2" gene copies). Recovery of "Foxy-2" amplicons after DNA extraction was determined using the qPCR protocol with "Foxy-2" specific oligonucleotides Kb1::Kb2 as described in section 2.3.2. Results of the soil spiking experiment verified that DNA extraction efficiency was soil type independent.

4.3.3.2 “Foxy-2” abundance

Quantification of “Foxy-2” gene copy numbers in soils was performed using Fos-specific oligonucleotides Kb1 (5’-GGACGAACTGACAGCCCTAC-3’) and Kb2 (5’-GTAACCGTAATATTGTTCAGAGCTC-3’) (Zimmermann et al., 2015). Each reaction (20 µl) contained 10 ng rhizosphere soil DNA template, 10 µl of Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 0.2 µM of each oligonucleotide Kb1 and Kb2, as well as 0.2 µl T4 gene 32 protein (500 ng μl^{-1}, MP Biomedicals). A cloned amplicon was used as standard in 10-fold serial dilutions of known DNA concentration. PCR runs were performed on a StepOnePlus™ Real-Time PCR System (Applied Biosystems). Reactions started with initial denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 94°C for 30 s, annealing at 62°C for 30 s, and polymerization at 72°C for 1 min as well as one additional step at 77°C for 30 s for signal detection. Occasionally, small peaks occurred in the melting curve between 72 and 76°C due to primer dimers not detected by electrophoresis in a 1.5% agarose gel (data not shown). To avoid measurement of fluorescence signal emitted by these primer dimers, fluorescence of target amplicons (melting temperature (Tm) = 81.8°C) was detected at 77°C. Each DNA sample was processed in triplicate reactions, while standards were run in duplicates. Melting curve analysis of amplicons was conducted to confirm that fluorescence signals originated from specific amplicons and not from primer dimers or other artefacts. An average reaction efficiency of 96.8% was achieved. Quantification of gene copies was calculated by comparing values of threshold cycles (Ct) to values of crossing points of the linear regression line of the standard curve using StepOne™ software version 2.2 (Applied Biosystems).

4.3.3.3 Total fungal abundance

Quantification of 18S rDNA gene copy numbers in soils was performed using oligonucleotides FF390 (5′-CGATAACGAACGAGACCT-3′) and FR1 (5′-AICCATTCAATCGGTAITCATTCA-3′) (Vainio and Hantula, 2000) and a cloned amplicon as standard (Kamolmanit et al., 2013). Each reaction (20 µl) contained 5 ng DNA template, 10 µl of Power SYBR® Green Master Mix (Applied Biosystems), 0.2 µl T4 gene 32 protein (500 ng μl^{-1}, MP Biomedicals), and 0.4 µM of each oligonucleotide. Cycling started with initial denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 94°C for 30 s,

annealing at 50°C for 30 s and polymerization at 70°C for 1 min. Average reaction efficiency was 92.5% and quantification of gene copies was done as described above.

It needs to be considered that the inoculated Fos strain “Foxy-2” is part of the total fungal abundance. Hence, it was likely that the abundance of “Foxy-2” was superimposed on the abundance of the indigenous fungal population. To account for this, “Foxy-2” abundance was subtracted from total fungal abundance as described in the following procedure. “Foxy-2” was propagated in 5 ml potato dextrose broth at 28°C for 3 days, followed by DNA extraction (UltraClean Microbial DNA Isolation Kit, MO BIO Laboratories Inc., Carlsbad, CA). Concentration and quality of “Foxy-2” DNA were determined as described above. Five ng of “Foxy-2” DNA was used as template #1 for Fos-specific qPCR (using oligonucleotides Kb1::Kb2 with the protocol published in Zimmermann et al. (2015)) and template #2 for 18S rDNA qPCR (see above). The 5 ng “Foxy-2” DNA template used for both qPCR assays corresponded to $2.3*10^5$ “Foxy-2” gene copies and $4.6*10^5$ 18S rDNA gene copies resulting in a ratio of 1:2 between “Foxy-2” and 18S rDNA gene copies. Accordingly, the previously measured “Foxy-2” gene copy numbers in the soils from Busia and Homa Bay were first multiplied with factor 2 and then subtracted from total 18S rDNA gene copy numbers. This calculation resulted in the adjusted 18S rDNA gene copy numbers reflecting the abundance of the total indigenous fungal population.

4.3.3.4 Fungal community composition

The fungal community composition was studied by terminal restriction fragment length polymorphism (TRFLP) analysis using the same oligonucleotide set as applied for 18S rDNA qPCR (Vainio and Hantula, 2000; Kamolmanit et al., 2013). As no rapid response of the community composition to the respective treatments was expected in the first study season (SR), T-RFLP analysis was only performed on rhizosphere soil samples obtained during the second study season (LR). The 18S rDNA gene was amplified in 25-µl reactions containing 5 ng DNA template, 1× PCR buffer, 2 U Taq DNA polymerase (Bioline GmbH, Luckenwalde, Germany), 0.2 mM of each deoxynucleoside triphosphate, 0.4 µM of each oligonucleotide (FF390::FR1), and 1 mM $MgCl_2$. The forward oligonucleotide FF390 was labelled with the fluorescent dye FAM-6. PCRs were started with initial denaturation at 95°C for 1 min, followed by 30 cycles consisting of a denaturation at 95°C for 30 s, an annealing step at 52°C for 45 s, and elongation at 72°C for 2 min. Reactions were completed with a final elongation step at

72°C for 10 min. Amplicons were purified using the Invisorb Fragment CleanUp Kit (Stratec Biomedical AG, Birkenfeld, Germany) following the manufacturer's instructions. For digestion, 200 ng of amplicons were incubated with 5 U *Msp*I restriction endonuclease (Promega GmbH, Mannheim, Germany) at 37°C for 4 h followed by 65°C for 20 min enzyme inactivation. Digested products were desalted with Sephadex™ G-50 (GE Healthcare) (Frank Rasche et al., 2006a) and amended with 7.75 µl Hi-Di formamide (Applied Biosystems) and 0.25 µl internal size standard GeneScan™-500 ROX™ (Applied Biosystems). Mixtures were denaturated at 95°C for 2 min, followed by immediate chilling on ice. TRFLP profiles were recorded on an ABI Genetic Analyzer 3130 (Applied Biosystems). Peak Scanner software (version 1.0, Applied Biosystems) was used to compare relative lengths of terminal-restriction fragments (T-RFs) with the internal size standard and to compile electropherograms into numeric data sets, in which T-RF length and height >100 fluorescence units (Fredriksson et al., 2014) were used for statistical profile comparison. TRFLP profiles used for statistical analyses were normalized according to (Dunbar et al., 2000a).

A requirement for analyzing "Foxy-2" induced alterations in indigenous fungal community composition was the deletion of "Foxy-2" T-RF from TRFLP profiles. The explicit "Foxy-2" T-RF was deleted from fungal TRFLP profiles using the following procedure: "Foxy-2" was propagated in 5 ml potato dextrose broth at 28°C for 3 days. DNA was extracted (UltraClean Microbial DNA Isolation Kit, MO BIO Laboratories Inc., Carlsbad, CA), and quantified as described above. Five ng DNA was amplified in triplicate reactions using oligonucleotides FF390::FR1. Amplicons were purified (Invisorb® Fragment CleanUp kit (Stratec Molecular GmbH), quantified and sequenced with oligonucleotide FR1 (LGC Genomics GmbH, Berlin, Germany). 18S rDNA sequences of "Foxy-2" were submitted to http://www.restrictionmapper.org/ to identify the restriction cutting site with the enzyme Msp*I* used for TRFLP. The resulting T-RF of "Foxy-2" with 168 base pair length was deleted from all TRFLP profiles.

4.3.4 Statistical analysis

Statistical analyses on obtained qPCR data sets ("Foxy-2" and 18S rDNA gene copy numbers) were performed using R software (Software R 3.0.1, R foundation for Statistical Computing, Vienna, Austria, http://www.R-project.org). QPCR data was log transformed to meet the assumptions of parametric statistical tests. Effects of factors "Treatment" (Control, "Foxy-2", *T. diversifolia*), "Maize growth stage", "Season" and "Field site" on abundance of both studied

genes in qPCR were assessed using linear mixed-effects models in R with the “nlme” package (Pinheiro et al., 2016). Since repeated measures (three maize growth stages) were taken within each season, a random effect was used in the model to account for serial autocorrelation at each plot. Furthermore, the factor “Maize growth stage” was nested in “Season”. An autoregressive variance-covariance structure was fitted to compensate for the proximity of the observations (Piepho, 2009). Least squares means comparison between factors was done using the Tukey´s range test (P<0.05). Bar charts displaying gene copy numbers were shown without standard errors due to back transformation of the least square means. For statistical purpose, soil chemical data were retrieved from a parallel study by Musyoki et al. (2016, Appendix B.1), where analytical procedures were described. The impact of soil chemical properties (Musyoki et al., 2016, Appendix B.1) on “Foxy-2” and total fungal abundance was assessed by adding the soil chemical parameters to the above-described linear mixed-effects model as co-variables. The resulting model was stepwise reduced based on the Akaike information criterion (AIC) by using the “stepAIC” function of the R package “MASS” (Venables and Ripley, 2002).

TRFLP data sets generated for 18S rDNA were assayed based on Bray-Curtis similarity coefficients (Rees et al., 2005; Kamolmanit et al., 2013). The similarity matrix was used for analysis of similarity (ANOSIM) to test the hypothesis that total fungal composition was altered by factors ”Treatment”, “Field site”, and “Maize 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). Treatment separation was visualized by canonical analysis of principal coordinates (CAP) on the basis of Bray-Curtis similarity indices (Anderson and Willis, 2003). Calculation of similarity coefficients, ANOSIM and CAP were carried out using Primer for Windows version 6 (Primer-E Ltd., Plymouth, UK). To verify if considered soil chemical properties (Musyoki et al., 2016) were decisive for the observed treatment-driven community composition shifts of the total fungal population, the DistLM procedure of PERMANOVA+ in Primer v6 (Primer-E Ltd.) was used (Clarke and Gorley, 2006). This procedure calculates a linear regression between the diversity of fungal communities using the Shannon diversity index and log transformed soil chemical data (Legendre and Anderson, 1999).

4.4 Results

4.4.1 “Foxy-2” abundance

No “Foxy-2” was detected in the control treatments (C+S) which were consequently excluded from statistical analysis. Abundance of “Foxy-2” was higher in soils with a sandy clay texture (Busia) than in those with a loamy clay texture (Homa Bay) ($P<0.001$) (Fig. 4.2). A significantly higher abundance of “Foxy-2” was observed in the second season ($P<0.05$) at both field sites. *T. diversifolia* amendment stimulated “Foxy-2” abundance at Busia ($P<0.001$) but not at Homa Bay ($P>0.05$). A significant decrease in “Foxy-2” gene copies was detected from the first two maize growth stages (EC 30 and EC 60) to senescence (EC 90) in season 1 at both field sites ($P<0.01$). The impact of fixed factors (“Field site”, “Season”, “Maize growth stage” and “Treatment”) and their interactions on gene abundance (i.e., “Foxy-2”, total fungi) and soil chemical properties is summarized in Table 4.1, while the impact of soil chemical properties on gene abundance was assessed across field sites and within each field site (Table 4.2). The pH ($P<0.01$) and ammonia ($P<0.05$) values showed a negative relationship with “Foxy-2” gene copy numbers across field sites. In Busia, a negative relationship was detected between “Foxy-2” abundance and pH ($P>0.01$), while EON ($P<0.05$) was positively related with “Foxy-2” gene copy numbers. In Homa Bay, “Foxy-2” abundance showed a negative relationship with pH ($P<0.01$), TC ($P<0.05$) and EOC ($P<0.05$).

Table 4.1: Effects of factors "Field Site", "Season", "Treatment" and "Growth stage" and their interactions on gene abundance ("Foxy-2", Total fungi) and soil chemical properties. Significant values at $P < 0.05$ are highlighted in bold.

		Factor	"Foxy-2" [gene copies g^{-1} dry soil]	Total fungi [gene copies g^{-1} dry soil]	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
		Field Site	**0.000**	**0.000**	**0.000**	**0.000**	0.647	0.599	**0.003**	**0.009**	**0.000**
Busia		Season (SS)	**0.011**	0.062	**0.000**	**0.000**	0.540	**0.001**	0.081	0.731	**0.000**
	Season 1	Growth stage (EC)	**0.001**	0.142	**0.000**	**0.000**	0.068	0.190	**0.004**	0.119	**0.000**
		Treatment F (F)	-	**0.000**	0.154	0.444	0.746	0.176	0.387	0.644	0.288
		Treatment F+T (FT)	**0.000**	**0.000**	0.214	**0.020**	0.080	0.201	0.723	0.336	**0.000**
		EC x F	-	0.088	**0.006**	0.835	0.977	0.523	0.446	0.992	**0.010**
		EC x FT	**0.000**	0.161	**0.001**	**0.025**	0.062	0.183	0.695	0.624	**0.000**
	Season 2	Growth stage (EC)	0.230	0.285	**0.000**	**0.006**	**0.002**	**0.008**	0.008	0.787	**0.000**
		Treatment F (F)	-	0.072	**0.000**	0.061	0.421	0.076	**0.046**	**0.023**	**0.001**
		Treatment F+T (FT)	**0.000**	**0.000**	**0.009**	0.410	0.549	0.980	0.217	0.521	**0.001**
		EC x F	-	0.118	**0.001**	**0.017**	**0.005**	0.145	0.299	**0.049**	**0.000**
		EC x FT	0.238	0.325	**0.038**	0.292	**0.048**	0.849	0.631	0.296	**0.000**
Homa Bay		Season (SS)	**0.020**	0.630	**0.001**	**0.000**	0.787	0.550	0.111	**0.003**	**0.000**
	Season 1	Growth stage (EC)	**0.000**	0.093	0.792	**0.001**	0.523	**0.002**	**0.008**	**0.001**	**0.000**
		Treatment F (F)	-	**0.000**	0.344	0.815	0.523	0.331	0.874	0.212	**0.000**
		Treatment F+T (FT)	0.091	**0.000**	0.537	0.078	0.208	0.072	0.663	**0.003**	**0.000**
		EC x F	-	0.096	0.802	0.954	0.406	0.974	0.893	0.073	**0.000**
		EC x FT	0.107	0.130	0.071	**0.011**	0.951	0.176	0.096	0.341	**0.000**
	Season 2	Growth stage (EC)	0.057	0.149	**0.000**	**0.000**	**0.001**	**0.002**	**0.000**	**0.003**	**0.000**
		Treatment F (F)	-	0.084	0.634	**0.022**	**0.037**	0.730	0.406	0.076	**0.000**
		Treatment F+T (FT)	0.120	**0.000**	**0.002**	**0.000**	0.943	0.217	0.601	0.086	**0.000**
		EC x F	-	0.204	0.661	**0.004**	**0.049**	0.464	0.086	0.372	**0.000**
		EC x FT	0.244	0.189	**0.019**	**0.000**	0.597	0.400	0.518	0.297	**0.000**

Abbreviations: F = "Foxy-2"; F+T = "Foxy-2" + *T. diversifolia*. TC: Total carbon, N_t: Total nitrogen, EOC: Extractable organic carbon, EON: Extractable organic nitrogen, NH_4^+: ammonia, NO_3^-: nitrate, pH: soil pH.

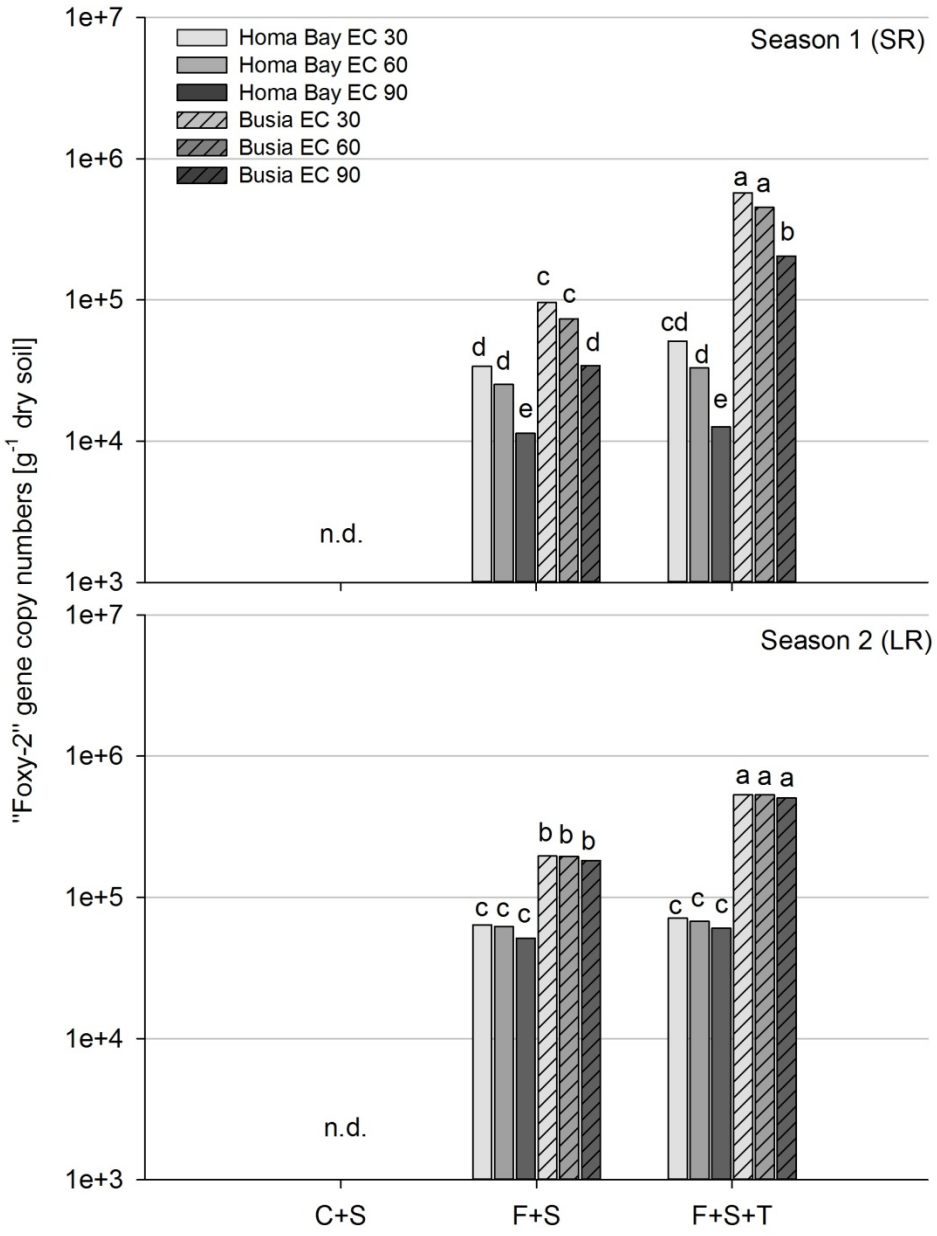

Figure 4.2: "Foxy-2" abundance during three maize growth stages (EC 30 (early leaf development stage), EC 60 (flowering stage), EC 90 (senescence stage)) in Season 1 (SR, short rains) and Season 2 (LR, long rains) at the two field sites Homa Bay and Busia. Treatments: uncoated maize and *S. hermonthica* (C+S), coated maize with "Foxy-2" and *S. hermonthica* (F+S), coated maize with "Foxy-2", *S. hermonthica* and *Tithonia diversofolia* residues (F+S+T). Different letters indicate significant differences at $P < 0.05$. Abbreviation n.d. = not detected.

Table 4.2: Impact of soil chemical properties on “Foxy-2” and 18S rDNA gene copy numbers (Total fungi). Significant values at $P < 0.05$ are highlighted in bold.

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}]	Soil pH
Across field sites	"Foxy-2"	0.076^{ns}	0.121^{ns}	0.251^{ns}	0.389^{ns}	**0.032***	0.143^{ns}	**0.004****
	Total fungi	**0.013***	0.183^{ns}	0.219^{ns}	0.223^{ns}	0.129^{ns}	0.203^{ns}	**0.003****
Busia	"Foxy-2"	0.181^{ns}	0.143^{ns}	0.201^{ns}	**0.028***	0.086^{ns}	0.308^{ns}	**0.006****
	Total fungi	0.092^{ns}	**0.037***	**0.042***	0.182^{ns}	0.159^{ns}	0.231^{ns}	**0.005****
Homa Bay	"Foxy-2"	**0.026***	0.078^{ns}	**0.041***	0.146^{ns}	0.195^{ns}	0.127^{ns}	**0.004****
	Total fungi	**0.035***	0.101^{ns}	0.082^{ns}	0.123^{ns}	0.091^{ns}	0.216^{ns}	**0.019****

Significance levels: ns: $P>0.05$; *$P<0.05$; **$P<0.01$; ***$P<0.001$.

Abbreviations: “Foxy-2”: “Foxy-2” gene copy numbers; Total Fungi: 18S rDNA gene copy numbers; TC: Total carbon, N_t: Total nitrogen, EOC: Extractable organic carbon, EON: Extractable organic nitrogen, NH_4^+: ammonia, NO_3^-: nitrate.

4.4.2 Total fungal abundance

Total fungal abundance was higher in Busia compared to Homa Bay throughout all treatments (P<0.001, Fig. 4.3). "Foxy-2" inoculation induced a decrease in total fungal abundance only in season 1 at both field sites (P<0.001), while *T. diversifolia* amendment induced an increase in total fungal abundance at both field sites throughout both seasons (P<0.001). Maize growth stage revealed no effect on total fungal abundance (P>0.05). For the total fungal community, gene copy numbers across field sites were negatively related to pH (P<0.01) and TC (P<0.05). In Busia, a negative relation between pH (P<0.01) and total fungal abundance was observed while N_t (P<0.05) and EOC (P<0.05) showed positive relations with total fungal gene copies. In Homa Bay, total fungal abundance was negatively related to TC (P<0.05) and pH (P<0.05).

4.4.3 Fungal community composition

Analysis of similarity (ANOSIM) of TRFLP profiles revealed distinct fungal community compositions between Busia and Homa Bay (R=1, P<0.001). Within each field site, factor "Treatment" showed no significant impact on fungal community composition (Homa Bay: global R=0.119, P>0.05; Busia: global R=0.073, P>0.05), while "Maize growth stage" induced strong alterations in fungal composition (Homa Bay: global R=0.410, P<0.01; Busia: global R=0.627, P<0.01) (Table 4.3, Fig. 4.4). At Homa Bay, EC 60 versus EC 90 resulted in the strongest fungal community alteration (R=0.580, P<0.01), while at Busia, fungal community separation was most pronounced between EC 30 and EC 90 (R=0.840, P<0.01). Shannon diversity indexes calculated from TRFLP data and log transformed soil chemical data revealed no significant correlations at both field sites.

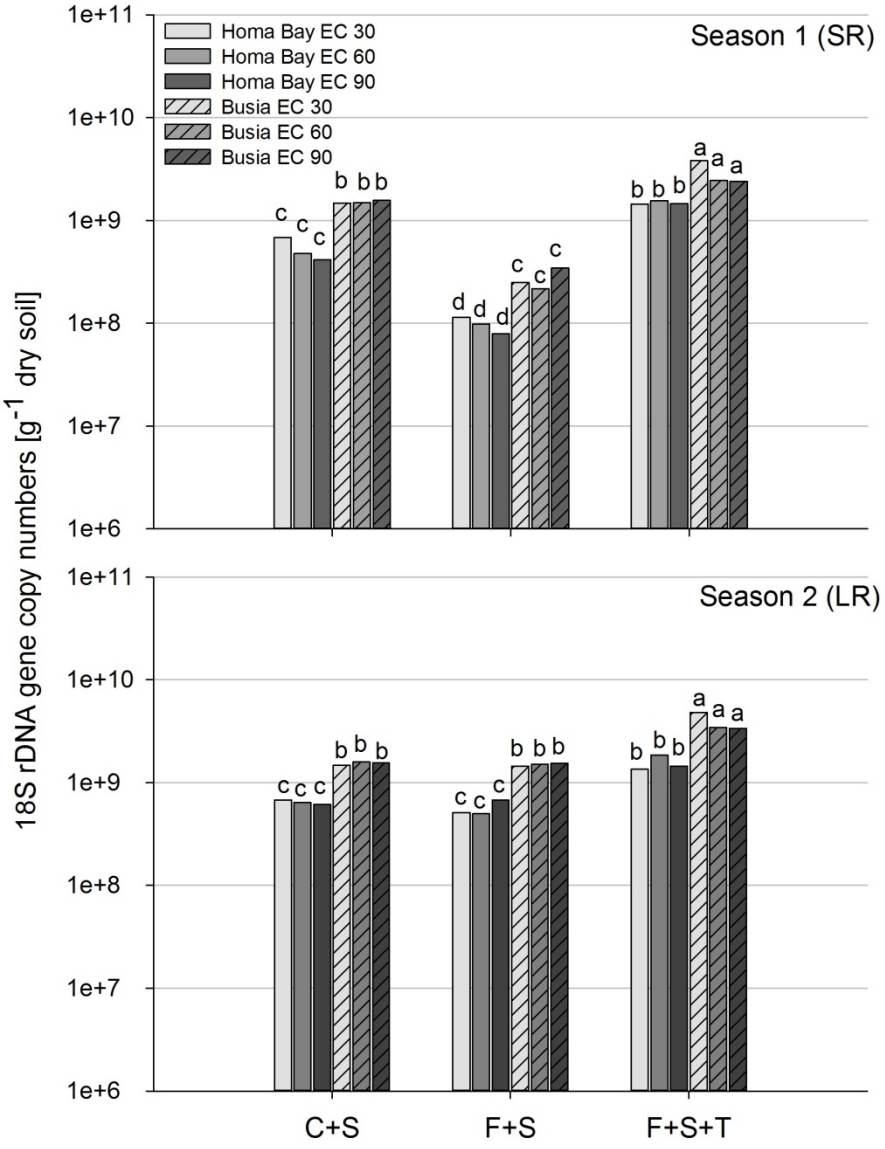

Figure 4.3: Total fungal abundance during three maize growth stages (EC 30 (early leaf development stage), EC 60 (flowering stage), EC 90 (senescence stage)) in Season 1 (SR, short rains) and Season 2 (LR, long rains) at the two field sites Homa Bay and Busia. Treatments: uncoated maize and *S. hermonthica* (C+S), coated maize with "Foxy-2" and *S. hermonthica* (F+S), coated maize with "Foxy-2", *S. hermonthica* and *Tithonia diversofolia* residues (F+S+T). Different letters indicate significant differences at $P < 0.05$.

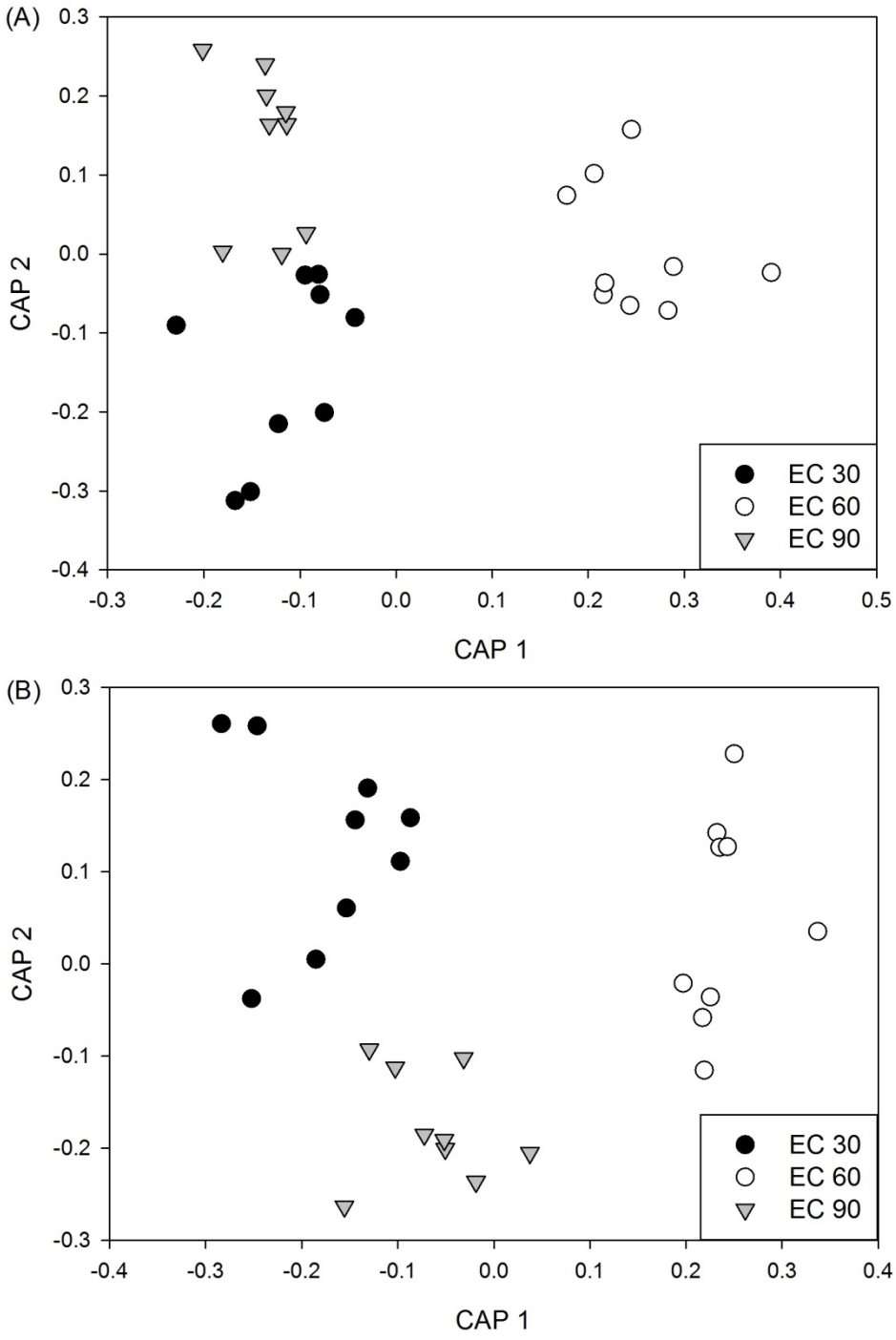

Figure 4.4: Canonical analysis of principal coordinates (CAP) ordination on the basis of Bray-Curtis similarity indices of normalized TRFLP data obtained from Msp*I*-digested 18S rDNA amplicons to visualize the differences in fungal community composition in Homa Bay (A) and Busia (B) according to the three maize growth stages (EC 30 (early leaf development stage), EC 60 (flowering stage), EC 90 (senescence stage)).

Table 4.3: Analysis of similarity (ANOSIM) of total fungal TRFLP datasets based on global *R* values for the factors treatment and maize growth stage and single *R* values for pairwise comparison within treatments and maize growth stages. The magnitude of *R* indicates the degree of separation between two tested communities. An *R* score of 1 indicates a complete separation, while 0 indicates no separation.

Soil	Factor	Global *R*
Busia	Treatment	0.073^{ns}
	Maize growth stage	0.627**
Homa Bay	Treatment	0.119^{ns}
	Maize growth stage	0.410**

Soil	Treatment (pairwise comparison)	*R* statistic
Busia	C+S vs. F+S	0.074^{ns}
	C+S vs. F+S+T	0.160^{ns}
	F+S vs. F+S+T	0.173^{ns}
Homa Bay	C+S vs. F+S	0.086^{ns}
	C+S vs. F+S+T	0.049^{ns}
	F+S vs. F+S+T	0.184^{ns}

Soil	Maize growth stage (pairwise comparison)	*R* statistic
Busia	EC30 vs. EC60	0.753**
	EC30 vs. EC90	0.840**
	EC60 vs. EC90	0.420*
Homa Bay	EC30 vs. EC60	0.395*
	EC30 vs. EC90	0.333*
	EC60 vs. EC90	0.580**

Significance levels: ns: $P>0.05$; *$P<0.05$; **$P<0.01$; ***$P<0.001$.

Treatment codes: C+S = uncoated maize + *S. hermonthica*, F+S = coated maize (with „Foxy-2“) + *S. hermonthica* and F+S+T = coated maize + *S. hermonthica* + *T. diversofolia*. EC30 = early leaf development stage, EC60 = flowering stage, EC90 = senescence stage.

4.5 Discussion

Identification of favoured agro-ecological conditions for ideal proliferation of the fungal BCA "Foxy-2" in the rhizosphere of crops is inevitable to optimally control the weed *S. hermonthica*. It was recently suggested that Foxy-2" abundance is generally determined by physico-chemical soil properties and availability of organic N resources (Zimmermann et al., 2015), but effects of other relevant impact factors like crop growth stage and seasonality under natural field conditions are yet to be understood. It was our primary objective to overcome these constraints through monitoring the proliferation of "Foxy-2" in the rhizosphere of crops cultivated at two agro-ecologically contrasting field sites in western Kenya considering three plant growth stages throughout two cropping seasons. Secondly, we investigated the potential impact of "Foxy-2" on indigenous rhizosphere fungal community abundance and composition considering N-rich organic residues to compensate for any resource competition.

4.5.1 "Foxy-2" prefers sandier soil textures with low pH and carbon background

Our study identified agro-ecological distinctions as substantial drivers of "Foxy-2" proliferation in assayed maize rhizospheres which was mainly attributed to contrasting soil textures, soil carbon background and pH, but also different climatic conditions (i.e., rainfall, temperature). "Foxy-2" exhibited a major preference towards the sandier soil type (i.e., Busia; orthic Acrisol) with low total carbon (TC) content and lower pH (~5) which corroborated recent observations on "Foxy-2" by Zimmermann et al. (2015) and *Fusarium* spp. in general (Fang et al., 2012; Höper et al., 1995; Senechkin et al., 2014). Higher precipitation rates and lower mean temperatures at the field site Busia reinforced the influence of soil type on "Foxy-2" proliferation as was also revealed by Venne et al. (2009) showing increased "Foxy-2" efficacy in areas with high rainfall amounts.

We found clear indications that the abundance of "Foxy-2" was limited by the higher level of suppressiveness towards *Fusarium* spp. in the clayey soil (Homa Bay) with its strong soil organic carbon (SOC) background and higher pH (~7). This finding is supported by earlier studies correlating soil suppressiveness to *Fusarium* spp. to abiotic soil characteristics such as clay content and pH (Höper et al., 1995; Yergeau et al., 2010). Furthermore, certain prokaryotes (i.e., *Bacillus* spp., *Pseudomonas* spp.) are acknowledged to act as antagonists of *Fusarium* spp. (Farooq and Bano, 2013; Köhl et al., 2015), thereby substantially contributing to soil suppressiveness. In this context, we found in a parallel study substantially higher abundance of

archaea in the rhizosphere of maize grown on a clayey soil (Homa Bay) compared to a sandy soil (Busia) (Musyoki et al., 2016). A promoting effect of high soil pH (~7) and strong SOC background on archaea abundance was earlier acknowledged (Bengtson et al., 2012; Pereira e Silva et al., 2012). Moreover, our assumption on biotic factors (i.e., archaeal community) determining proliferation of "Foxy-2" is further supported by our finding that advanced maize growth development hampered "Foxy-2" abundance while stimulating archaea abundance in maize rhizospheres (Musyoki et al., 2016) and also being acknowledged to stimulate mycorrhiza abundance (Grigera et al., 2007), a microbial group known to act as antagonists of *Fusarium* spp. (Hu et al., 2010; Shukla et al., 2015).

Interestingly, re-inoculation of "Foxy-2" in the second season resulted not only in higher abundance levels, but also in increased resilience of the BCA towards biotic factors (i.e., maize growth stage) which was attributed to the rapid adaptation of "Foxy-2" after repeated exposure to specific agro-ecological conditions (Griffiths and Philippot, 2013).

4.5.2 "Foxy-2" had only a transient suppressive effect on indigenous fungal abundance

"Foxy-2" induced only a transient suppressive effect on abundance of total indigenous fungi in the studied crop rhizospheres, while their community structure remained unaffected. According to Griffiths and Philippot (2013), our results suggested a strong tolerance and resilience potential of the native fungal community towards invading microorganisms (i.e., "Foxy-2"). Similarly, Edel-Hermann et al. (2009) and Savazzini et al. (2009) observed only transient community shifts in indigenous microbial populations in response to inoculation with fungal BCAs (i.e., *Fusarium oxysporum*, *Trichoderma atroviride).*

The suggested suppressive effect of "Foxy-2" on indigenous fungal abundance was superimposed by organic resource availability, but also TC and pH constituting main drivers of total rhizospheric fungal abundance assayed in this study as corroborated by Liu et al. (2015) and Rousk et al. (2009). Furthermore, fungal community structure was mainly determined by maize growth stage, while "Foxy-2" presence did not induce any effect. This finding was verified by Cavaglieri et al. (2009) and Chiarini et al. (1998) confirming the impact of plant development stage on rhizosphere microbial community structures.

4.5.3 Nitrogen-rich organic residues compensate suppressive effects of “Foxy-2”

Interestingly, organic amendments with *T. diversifolia* compensated the transient suppressive effect of “Foxy-2” on indigenous rhizosphere fungal abundance. This implied a competitive situation for resources between “Foxy-2” and indigenous rhizosphere fungi which was supposedly equalized by the provision and accessibility of additional organic N resources (i.e., *T. diversifolia*) promoting the abundance of “Foxy-2” and total fungal community. This interpretation corresponded to previous findings confirming that higher N availability in organic residues (i.e., *T. diversifolia* with low a C/N ratio (Chivenge et al., 2009)) increased total soil fungal abundance in contrast to organic residues with low N availability (high C/N ratio) or mineral fertilizers (España et al., 2011; Kamolmanit et al., 2013; Lee et al., 2013; Zimmermann et al., 2016).

4.6 Conclusions

An important intervention of prospective rhizosphere engineering is the use of plant-beneficial microbial inoculants to improve crop yield and health (Dessaux et al., 2016; Quiza et al., 2015; Zhang et al., 2015). The consistent efficacy and environmental safety of these microbial inoculants need to be thoroughly assessed prior to their large scale implementation in contrasting agro-ecosystems (Quiza et al., 2015). The present study successfully identified favoured environmental growth conditions of the rhizosphere-acting BCA “Foxy-2” which will contribute to its proliferation in soils increasing its potential to act effectively against the parasite *S. hermonthica*. Based on our results, persistence and establishment of “Foxy-2” in crop rhizospheres could be appraised if considering site-specific factors such as soil texture, soil carbon background, soil pH and climatic conditions (e.g., rainfall and temperature patterns).

Site-specific conditions could be adjusted in favour of the BCA with increased availability of additional organic N materials if soil resource limitation is prevalent. Moreover, our results indicated that N-rich residues are applicable to compensate a possible resource competition between the BCA and indigenous rhizosphere microorganisms. However, the observed resource competition in this study was only of transient nature and indigenous rhizosphere fungal communities exhibited a strong resilience against “Foxy-2” exposure substantiating the environmental safety of the BCA.

Nonetheless, it needs to be emphasized that measured total fungal abundance was not linked directly with soil fungal activity and functionality (Brankatschk et al., 2011). Hence, prospective studies should focus on the impact of “Foxy-2” on rhizosphere functions mediated by microorganisms (i.e., organic matter decomposition, nutrient cycling) (Musyoki et al., 2015) or specific microbial groups known for their beneficial functions in rhizospheres (e.g., arbuscular mycorrhizal fungi). Moreover, hypothesized biotic factors contributing to soil suppressiveness towards ”Foxy-2” such as archaeal and arbuscular mycorrhizal communities need further investigation to decipher the underlying mechanisms of this microbial interaction in crop rhizospheres.

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5. AMF taxon-specific responses to inoculated microbial biocontrol agents in the rhizosphere of maize – implications for environmental safety studies*

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* Contribution of Authors: Jana Bergmann conducted the molecular work (qPCR and TRFLP), analysed the derived data within the frame of her master thesis and has written the material & methods part of this manuscript. Judith Zimmermann has written the abstract, introduction, results and discussion chapter, conducted the field trials in western Kenya and supervised Jana Bergmann during her master thesis. Mary K. Musyoki was involved in the conduction of the field trials and soil sample collection. Frank Rasche supervised the entire experimental set up and reviewed the manuscript.

5.1 Abstract

The intentional introduction of microbial biocontrol agents (BCAs) into soil ecosystems might induce alterations in indigenous plant-beneficial soil microbial communities with subsequent adverse effects on crop yield and health. Arbuscular mycorrhizal fungi (AMF) are acknowledged to maintain plant-beneficial functions and a critical knowledge gap remains if AMF abundance and community composition could serve as reliable indicators for evaluating the environmental safety of microbial BCAs. The present study aimed on assessing the potential role of AMF as suitable risk indicator by using the well investigated fungal BCA *Fusarium oxysporum* f. sp. *strigae* strain "Foxy-2" as model organism to study its effect on indigenous AMF communities in the rhizosphere of maize. A two-season field experiment was conducted in Busia and Homa Bay districts in western Kenya to account for effects of soil type, climate and seasonality. One treatment with nitrogen rich organic residues (*Tithonia diversifolia*) was included to compensate potential resource competition between "Foxy-2" and indigenous AMF. The prevalent AMF taxa *Rhizophagus intraradices* and *Funneliformis mosseae* were considered as model indicators to study the impact of the BCA "Foxy-2" on AMF taxa abundance by using quantitative polymerase chain reaction (qPCR) analysis while terminal restriction fragment length polymorphism (TRFLP) analysis was used to assess potential alterations in total AMF community composition. Rhizosphere soil samples were collected at three growth stages of maize (i.e. EC 30, EC 60 and EC 90) and subjected to qPCR and TRFLP analysis. Our results revealed taxon- and site-specific responses of AMF abundance to "Foxy-2" inoculation with *R. intraradices* being transiently suppressed by "Foxy-2" solely at the field site Busia. This suppressive effect was compensated by the organic residue amendment. No "Foxy-2" induced effects on abundance of AMF taxon *F. mosseae* and composition of total AMF community were detected. Natural environmental factors such as seasonality and plant growth stage clearly superimposed the effect of "Foxy-2" on AMF. However, the detected AMF taxon-specific responses to "Foxy-2" inoculation but also natural environmental impacts imply that AMF taxa diverge in their functional traits, thereby varying in their interference potential with microbial BCAs. Consequently, AMF taxa abundance cannot be used as model risk indicator representative for the entire AMF community. Conclusively, risk assessment studies should not solely rely on monitoring AMF taxa abundance in response to BCA inoculation but further include analysis on total AMF community composition and other plant-beneficial rhizosphere microbes, such as nitrifying prokraryotes, to draw conclusions on the environmental safety of a microbial BCA.

5.2 Introduction

International guidelines for the registration and release of microbial biocontrol agents (BCAs) require a thorough risk assessment of the BCA prior to its large scale application in agro-ecosystems (FAO, 2006; OECD, 2014). The intentional introduction of microbial BCAs into soils might result in resource competition between the BCA and indigenous soil microbes, thereby altering the native soil microbial community (Edel-Hermann et al., 2009; Karpouzas et al., 2011). Plant-beneficial soil microbes such as arbuscular mycorrhizal fungi (AMF) are acknowledged to substantially contribute to enhanced crop yield, health and stress tolerance (Bhardwaj et al., 2014; Nadeem et al., 2014; van der Heijden et al., 2015). Hence, BCA induced alterations in indigenous plant-beneficial microbial communities have to be classified as a major risk with adverse consequences on the productivity in agro-ecosystems. Although several studies assessed the impact of microbial BCAs on total indigenous bacteria and fungi (e.g. Edel-Hermann et al., 2009; Karpouzas et al., 2011; Savazzini et al., 2009; Zhang et al., 2008), only very limited data is available on dynamics of functionally relevant soil microbial community members such as AMF (Timms-Wilson et al., 2005; Zimmermann et al., 2016). This knowledge gap is potentially reinforced by the high genetic variability within AMF communities, which hampers oligonucleotide development with high coverage and specificity to the entire *Glomeromycota* phylum, thereby complicating the use of DNA based molecular approaches for the monitoring of total AMF abundance. One problem-solving approach could be the monitoring of specific AMF taxa representative as model indicators for the entire AMF community. Our recent publication (Zimmermann et al., 2016) was to our knowledge the first attempt of using AMF taxa abundance as risk indicators for environmental safety studies of microbial BCAs. However, this study was conducted under short-term controlled conditions and progressive studies under natural field conditions are lacking until now. Therefore, the primarily objective of the present study was to further assess the potential of specific AMF taxa to serve as valuable risk indicators in environmental safety studies for microbial BCAs under contrasting field conditions in western Kenya. Moreover, we complemented AMF taxa abundance analysis with total AMF community composition analysis to enable the detection of BCA induced shifts in total AMF community structure. We implemented the fungal BCA *Fusarium oxysporum* f. sp. *strigae* strain “Foxy 2” as model BCA to investigate its impact on indigenous AMF communities. “Foxy-2” is applied to the soil by seed coating of the crop plant with dried chlamydospores of the fungus (Elzein et al., 2006). These dormant spores are able to proliferate and fungal hyphae colonize the root of the crop plant as well as the rhizosphere,

hereby protecting the plant against attacks of the parasite *Striga hermonthica* (Elzein et al., 2010; Elzein and Kroschel, 2004; Ndambi et al., 2011). Consequently, the fungal BCA "Foxy-2" potentially occupies the same colonization sites (i.e. plant roots and rhizosphere) in the soil as plant-beneficial AMF, thereby increasing the potential of resource competition between these two fungal groups (Winding et al., 2004). Therefore, AMF communities could serve as tool to study potential non-target side effects of "Foxy-2" on plant-beneficial rhizosphere fungi. However, studies should further consider natural environmental impacts acknowledged to determine AMF communities (i.e. plant growth stage and seasonality; Grigera et al., 2007; Guadarrama et al., 2014; Lingfei et al., 2005) to trade-off potentially observed BCA induced effects. Therefore, we included the effects of soil type, plant growth stage and seasonality in our experimental set up to trade-off observed "Foxy-2" induced effects on native AMF communities against these natural environmental impacts. Moreover, a treatment with nitrogen-rich organic residues (*Tithonia diversifolia,* (Chivenge et al., 2009) was included to compensate any resource competition between "Foxy-2" and indigenous AMF. *T. diversifolia* is a widely used green manure in Sub-Saharan Africa which supplies easy accessible C and N resources to the soil microbial community (Gachengo et al., 1998; Jama et al., 2000; Opala et al., 2015). We used the two prevalent AMF taxa *Rhizophagus intraradices* and *Funneliformis mosseae* as model AMF to study the impact of "Foxy-2" and natural environmental factors on AMF abundance by quantitative polymerase chain reaction (qPCR). Potential shifts in total AMF community composition in response to monitored factors were detected by terminal restriction fragment length polymorphism (TRFLP). Our objectives were assayed at three distinct plant growth stages of maize (EC 30, EC 60 and EC 90) during two cropping seasons in Busia and Homa Bay districts in western Kenya.

5.3 Material & Methods

5.3.1 Field experimental set up

Details on the experimental set up can be retrieved from a parallel study conducted by Musyoki et al. (2016). For better comprehensibility, a summary is provided in Chapter 4 section 4.3.2.2 of the present thesis.

The experimental design included three different treatments: i) uncoated maize and *S. hermonthica* (C+S), ii) coated maize with 'Foxy-2' and *S. hermonthica* (F+S), and iii) coated maize with 'Foxy-2', *S. hermonthica* and *Tithonia diversifolia* residues (F+S+T).

5.3.2 Analysis of AMF communities

5.3.2.1 DNA extraction from soil samples

Total genomic DNA from rhizosphere samples was extracted using the Fast DNA® Spin Kit for Soil (MP Biomedicals, Solon, OH, USA) following the manufacturer's instructions with slight modifications. Briefly, 0.4 g freeze-dried soil was bead-beated for 30 s with a beating power of 5.5 m s^{-1} using a FastPrep®-24 Instrument (MP Biomedicals). Concentration and quality of DNA were determined on a Nanodrop ND-1000 (Nanodrop Technologies, Wilmington, DE, USA) and DNA was stored at -20°C.

A soil spiking experiment was conducted as described in Chapter 4 section 4.3.3.1 of the present thesis. Results of the soil spiking experiment verified that DNA extraction efficiency was soil type independent.

5.3.2.2 AMF taxa abundance

5.3.2.2.1 Nested PCR

AMF taxon-specific oligonucleotides described by Thonar et al. (2012), developed specifically for the application in quantitative real-time PCR, were used to quantify representative AMF taxa in the soil. Primers developed by Thonar et al. (2012) cover the 5 major AMF taxa *Cetraspora pellucida*, *Gigaspora margarita*, *Funneliformis mosseae*, *Claroideoglomus claroideum* and *Rhizophagus intraradices*. To test for the presence of these taxa in the studied soils from Homabay and Busia, a nested PCR approach was chosen as it provides a higher sensitivity than direct amplification from soil samples. First, a PCR with the oligonucleotides LR1::FLR2 (Trouvelot et al., 1999) was conducted with the following conditions: initial denaturation at 95 °C for 5 min, then 35 cycles with a denaturation step at 95 °C for 15 s, an annealing step at 59 °C for 30 s and an elongation step at 72 °C for 1 min. Final elongation was at 72 °C for 10 min. The reactions (25 µl) contained 5 ng DNA template, 1x PCR buffer, 1.25

U Accuzyme DNA polymerase (Bioline GmbH, 271 Luckenwalde, Germany), 0.2 mM of each deoxynucleoside triphosphate (dNTP), 1.5 mM $MgCl_2$, 0.1 µl T4 gene 32 protein (500 ng μl^{-1}, MP Biomedicals) and 0.3 µM of both oligonucleotides. Amplicons of this PCR were used undiluted as template for a subsequent qPCR with the AMF taxon-specific primers (Thonar et al., 2012). 20 µl reactions included 10 µl Power SYBR® Green Master Mix (Applied Biosystems, Foster City, CA, USA), 0.2 µl T4 gene 32 protein (500 ng μl^{-1}, MP Biomedicals) and 0.35 µM of each oligonucleotide. DNA template quantities of 5 ng, 10 ng, 20 ng and 30 ng were tested for each primer pair. Cycling was performed as described by Thonar et al. (2012): initial DNA denaturation and polymerase activation at 95 °C for 15 min, 45 cycles of denaturation at 95 °C for 10 s, annealing at the optimized temperature for each oligonucleotide pair (Table 5.1) for 30s and polymerization at 72 °C for 1 min. *R. intraradices* was detected in both soils, while *F. mosseae* was only present in the clayey soil from Homabay.

Table 5.1: AMF taxon-specific oligonucleotides used for qPCR ((Thonar et al., 2012), modified).

Target AMF taxon	Primer name	Sequences (5' --> 3')	Amplicon size [bp]	Annealing temperature [°C]
Rhizophagus intraradices	*intra-f*	TTCGGGTAATCAGCCTTTCG	250	52
	intra-r	TCAGAGATCAGACAGGTAGCC		
Funneliformis mosseae	*mos-f*	GGAAACGATTGAAGTCAGTCATACCAA	122	54
	moss-r	CGAAAAAGTACACCAAGAGATCCCAAT		

5.3.2.2.2 AMF- taxon specific qPCRs

For the taxon *F. mosseae*, 20 ng DNA template were used in a qPCR reaction of 20 µl with 10 µl Power SYBR® Green Master Mix (Applied Biosystems, Foster City, CA, USA), 0.2 µl T4 gene 32 protein (500 ng μl^{-1}, MP Biomedicals) and 0.5 µM of each oligonucleotide (see Table XX). Cycling started with an initial denaturation step at 95 °C for 10 min, which was followed by 45 cycles with denaturation at 95 °C for 15 s, annealing for 30 s (Table 5.1), elongation at 72 °C for 1 min and an additional step for signal detection at 75 °C for 30 s. As the melting curve showed small peaks between 70 °C and 74 °C, which was assigned to oligonucleotide dimers, fluorescence was detected at 75 °C (*F. mosseae* amplicon T_m= 77 °C) to avoid acquisition of these non-target signals. A 10-fold serial dilution of a cloned amplicon with known DNA concentration served as a standard for the quantification of *F. mosseae.* For the standard, duplicates of the reaction were processed, while the DNA samples were run in triplicates. Two non-template controls (NTC) were included in every run, to assure the specificity of the reaction. An average efficiency of 85.5 % with R^2 values consistently > 0.99 were achieved for the reactions. The number of gene copies was calculated by measuring the values of threshold cycles against their crossing points on the regression line of the standard curve using StepOne™ software version 2.2 (Applied Biosystems).

The qPCR assays for *R. intraradices* were performed under the same cycling conditions as *F. mosseae* with its respective annealing temperature (Table 5.1). Fluorescence was also detected at 75 °C (*R. intraradices* amplicon T_m= 83 °C). The 20 µl reaction mix contained 10 ng DNA template, 10 µl Power SYBR® Green Master Mix (Applied Biosystems, Foster City, CA, USA), 0.1 µl T4 gene 32 protein (500 ng μl^{-1}, MP Biomedicals) and 0.5 µM of each of the *R. intraradices* oligonucleotides. The qPCR assays showed an average efficiency of 93.4 % with

R^2 values > 0.99. The gene copy numbers for *R. intraradices* were calculated as described above.

5.3.2.3 AMF community composition

The community structure of AMF was studied by terminal restriction fragment length polymorphism (TRFLP) analysis, based on a nested PCR approach with the primer pairs LR1-FLR2 (Trouvelot et al., 1999) and FLR3-FLR4 (Gollotte et al., 2004; Verbruggen et al., 2012). The LR1-FLR2 oligonucleotides target the large-subunit (LSU) rDNA of all fungi. Amplification of this DNA-section was performed in 25 µl reactions containing 1x PCR buffer, 0.5 U Accuzyme DNA polymerase (Bioline GmbH, Luckenwalde, Germany), 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 0.1 µl T4 gene 32 protein (500 ng μl^{-1}, MP Biomedicals) and 0.3 µM of each oligonucleotide (LR1-FLR2). DNA template concentrations of 10 ng and 2.5 ng for Homa Bay and Busia samples, respectively, were added to the reactions. Cycling started with an initial denaturation step at 95 °C for 5 min, followed by 35 cycles consisting of denaturation at 95 °C for 15 s, annealing at 58 °C for 30 s and elongation at 72 °C for 1 min. A final elongation step at 72 °C for 10 min completed the reaction. The strength of amplification showed differences for the two soils (Busia and Homa Bay) and the three sampling dates (EC 30, EC 60 and EC 90), therefore the dilution step for the subsequent second PCR was optimized for each soil and sampling date (Table 5.2). The second PCR consisted of 50 µl reactions with 2 µl template, 1 x PCR buffer, 0.2 mM of each dNTP, 3 mM $MgCl_2$, 0.2 µl T4 gene 32 protein (500 ng μl^{-1}, MP Biomedicals) and 0.6 µM of both oligonucleotides (FLR3-FLR4). The forward oligonucleotide FLR3 was labelled with the fluorescent dye FAM-6. Cycling started at 95 °C for 5 min, followed by 30 cycles each of 95 °C for 15 s, 54 °C for 30 s and 72 °C for 1 min, concluding with 72 °C for 10 min. All amplicons were purified with the Invisorb® Fragment CleanUp Kit (Stratec Biomedical AG, Birkenfeld, Germany) according to the manufacturer's instructions. Elution was performed with 20 µl ddH_2O and amplicon concentrations were measured on a NanoDrop as described above. 200 ng of amplicons were digested in a 40 µl reaction with 5 U *AluI* restriction endoncuclease (New England Biolabs, Ipswich, MA, USA) and 1x CutSmart® buffer (New England Biolabs). Incubation was performed at 37 °C for 2 h with a subsequent heat inactivation step at 80 °C for 20 min. The digested products were desalted on Sephadex™ G-50 (GE Healthcare) columns (Frank Rasche et al., 2006b). 2 µl of each desalted sample was mixed with 7.75 µl Hi-Di formamide (Applied Biosystems) and 0.25 µl internal size standard GeneScan™-500 ROX™ (Applied Biosystems). The samples were denaturated at 95 °C for 3

min and immediately chilled on ice for 5 min. T-RFLP profiles were recorded on an ABI Genetic Analyzer 3130 (Applied Biosystems). Analysis of T-RFLP peak profiles was conducted with the Peak Scanner Software 2 (Version 2.0, Applied Biosystems). The relative lengths of terminal restriction fragments (T-RFs) were compared to the internal standard and numeric data sets were compiled from electropherograms, using the T-RF lengths and peak heights > 100 fluorescence units for statistical analysis. T-RFLP profiles were normalized as described by (Dunbar et al., 2000b).

Table 5.2: PCR amplicon dilutions used for second PCR with primers FLR3::FLR4.

Soil	Sampling date	Dilution
Busia	EC 30	no dilution
	EC 60	no dilution
	EC 90	1/20
Homa Bay	EC 30	no dilution
	EC 60	1/20
	EC 90	1/20

5.3.3 Statistical analysis

AMF abundance was analyzed in R (RStudio Team, 2015) using the 'nlme' package (Pinheiro et al., 2007) for a repeated measurement analysis with an autogressive covariance structure. The effects of the factors "treatment", "season" and "sampling date" were determined with subsequent post hoc Tukey-B test using the 'lsmeans' package (Lenth, 2013). Logarithmic transformation was performed on qPCR data to meet the assumptions of parametric statistical tests. For the graphical depiction, gene copy numbers were shown without standard errors based on the back transformation of least square means. For statistical purpose, soil chemical data were retrieved from a parallel study by Musyoki et al. (2016, Appendix B.1), where analytical procedures were described. The impact of soil chemical properties (Musyoki et al., 2016, Appendix B.1) on AMF taxa abundance was assessed by adding the soil chemical parameters to the above described linear mixed-effects model as co-variables. The resulting model was

stepwise reduced based on the Akaike information criterion (AIC) by using the “stepAIC” function of the R package “MASS” (Venables and Ripley, 2002).

Datasets obtained by TRFLP analysis were studied on the basis of Bray-Curtis similarity coefficients (Legendre and Legendre, 2012). A resemblance matrix for all possible pairs of samples was created and subsequently used for analysis of similarity (ANOSIM) to determine effects of the factors “site”, “treatment”, “season” and “sampling date” on the community composition of AMF. This analysis calculates a test statistic ‘*R*’ based on rank similarities between the sample matrix. The significance of a studied factor in the data set is first assessed in ANOSIM by calculation of a ‘global’ *R*, followed by pair wise comparison of communities. Hereby a value of *R* between -1 and +1 is generated, with an *R* value of +1 indicating complete separation of communities while an *R* score of 0 indicates no separation (Rees et al., 2004). Negative *R* values between 0 and -1 are reportedly unlikely to occur as they would suggest greater dissimilarities among the replicates of one sample than between replicates of different samples (Clarke, 1993). However, it has been shown that negative *R* values can occur quite frequently for some habitats and might result for various reasons, including outliers, patchiness of the habitat or problems with the sampling design (Chapman and Underwood, 1999). Treatment separation was visualized by canonical analysis of principal coordinates (CAP) on the basis of Bray-Curtis similarity indices (Anderson and Willis, 2003). Computation of similarity coefficients, ANOSIM and CAP was performed in Primer for Windows version 6 (Primer-E Ltd., Plymouth, UK, (Clarke, 1993)). To verify if considered soil chemical properties (Musyoki et al., 2016) were decisive for the observed treatment-driven community composition shifts of the total fungal population, the DistLM procedure of PERMANOVA+ in Primer v6 (Primer-E Ltd.) was used (Clarke and Gorley, 2006). This procedure calculates a linear regression between the diversity of fungal communities using the Shannon diversity index and log transformed soil chemical data (Legendre and Anderson, 1999).

5.4 Results

5.4.1 AMF taxa abundance

5.4.1.1 *R. intraradices* abundance

The AMF taxon *R. intraradices* was detected in both soils used in this study (i.e. Busia and Homa Bay). In Homa Bay, abundance of *R. intraradices* was higher in the second season (LR) compared to the first season (SR) ($P < 0.001$, Fig. 5.1). Gene copy numbers of *R. intraradices* showed no significant differences between sampling dates as well as treatments in both seasons at Homa Bay ($P > 0.05$).

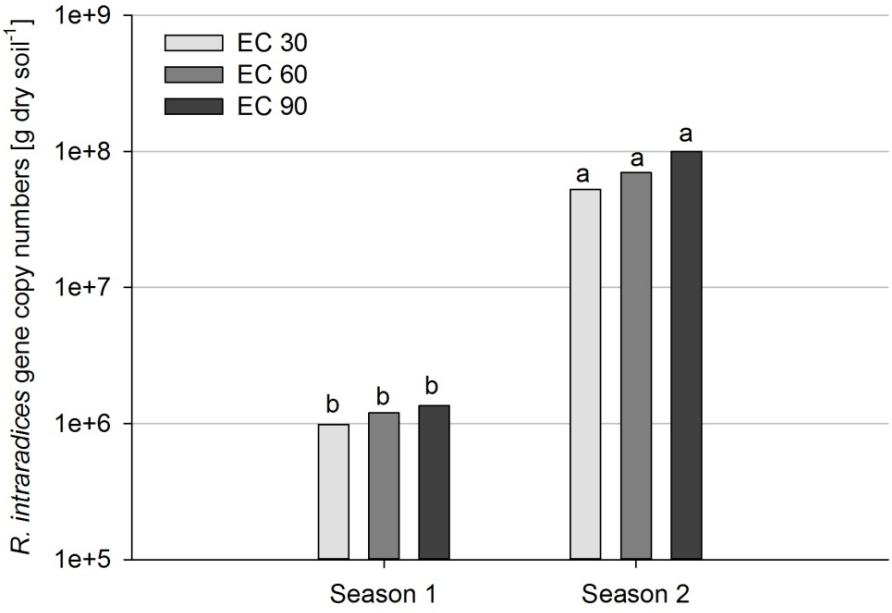

Figure 5.1: *R. intraradices* gene copy numbers during three plant growth stages in two cropping seasons at field site Homa Bay. EC 30 = early leaf development stage, EC 60 = flowering stage, EC 90 = senescence stage. Different letters indicate significant differences at $P < 0.05$.

In Busia, *R. intraradices* abundance significantly increased from the first to the second season ($P < 0.001$). However, as gene copy numbers of *R. intraradices* in the first season were in general very low and thus sometimes below the detection level, not enough replicates were acquired for all sampling dates to make more detailed statements about differences within Season 1. Therefore, analyses on effects of "Sampling date" and "Treatment" on *R. intraradices* abundance were only obtained for Season 2. In the second season, differences in *R. intraradices* gene copy numbers between treatments were observed (Fig. 5.2). In the early leaf development

stage of maize (EC 30), abundance was significantly lower in the 'Foxy 2' treatment (F) compared to the control (C) and *T. diversifolia* amended treatment (F+T) ($P < 0.05$). However, in EC 60 and EC 90, no significant treatment effect was detected anymore ($P > 0.05$). No significant relation between *R. intraradices* gene copy numbers and soil chemical properties was detected.

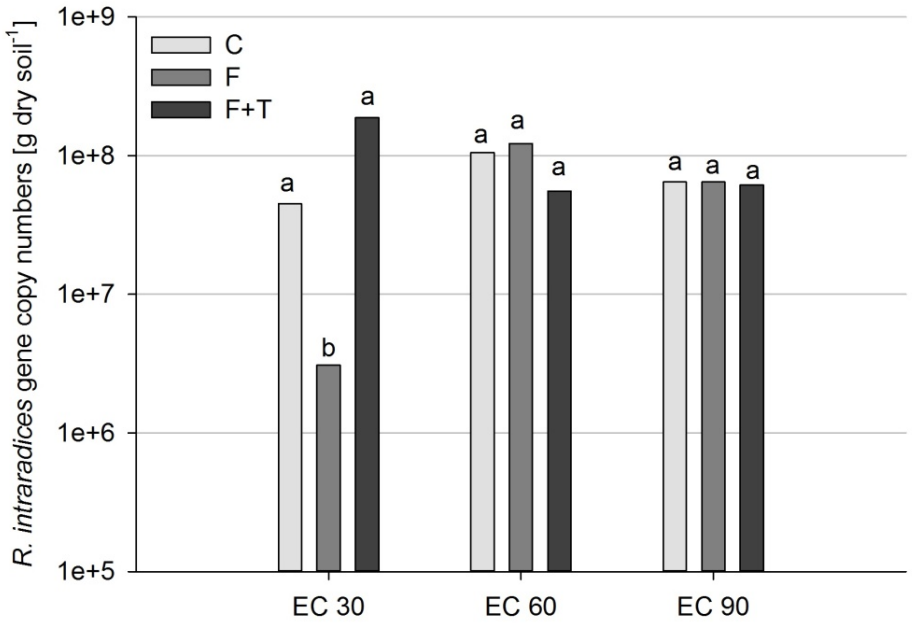

Figure 5.2: *R. intraradices* gene copy numbers for the three treatments (C = control, F = "Foxy-2", F+T = "Foxy-2" + *T. diversifolia*) during each plant growth stage in season 2 at the field site Busia. EC 30 = early leaf development stage, EC 60 = flowering stage, EC 90 = senescence stage. Different letters indicate significant differences at $P < 0.05$.

5.4.1.2 *F. mossae* abundance

The AMF taxon *F. mossae* was detected solely at Homa Bay site. A significant increase in *F. mossae* gene copy numbers from the first to the second season was observed ($P < 0.001$, Fig. 5.3). In season 1, gene copy numbers of *F. mosseae* were significantly higher during the flowering stage of maize (EC 60) compared to the early leaf development stage (EC 30) ($P < 0.01$). At the senescence stage of the crop plant (EC 90), abundance of *F. mosseae* was highest ($P < 0.01$). In the second season, no significant differences in *F. mossae* abundance between sampling dates were detected anymore ($P > 0.05$). No significant treatment effect was observed for any of the three maize growth stages in both seasons. No significant relation between *F. mossae* gene copy numbers and soil chemical properties was detected.

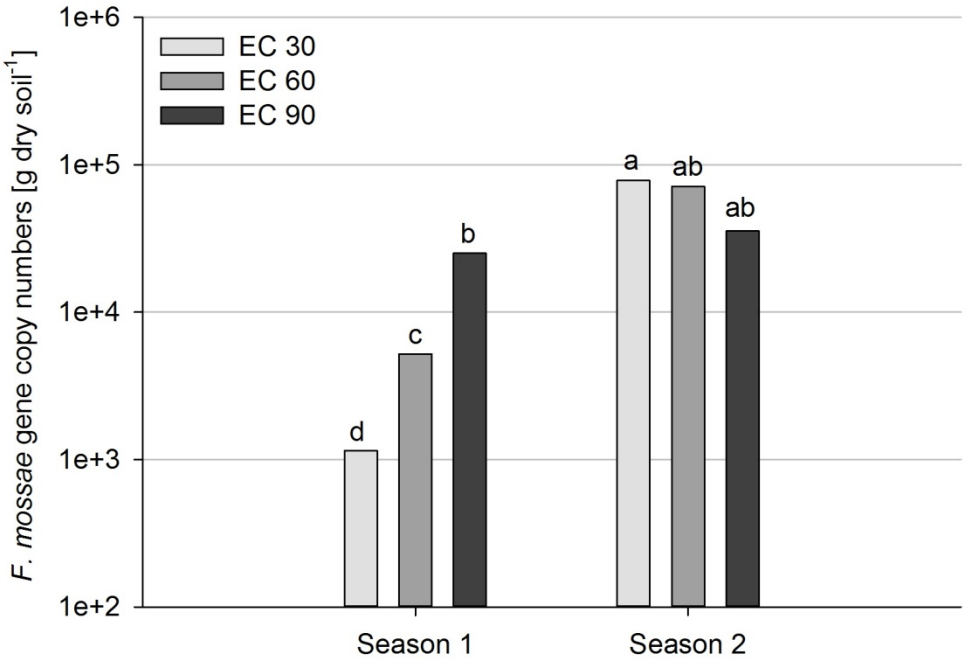

Figure 5.3: *F. mossae* gene copy numbers during three plant growth stages in two cropping seasons at field site Homa Bay. EC 30 = early leaf development stage, EC 60 = flowering stage, EC 90 = senescence stage. Different letters indicate significant differences at $P < 0.05$.

5.4.2 AMF community composition

Analysis of similarity (ANOSIM) of TRFLP profiles showed a minor but significant separation of communities for the two soils from Busia (orthic Acrisol) and Homa Bay (vertic Phaeozem) with R = 0.194 ($P < 0.001$).

5.4.2.1 Busia site

The results of ANOSIM for TRFLP profiles acquired from Busia are displayed in Table 5.3. The AMF community composition was highly similar between the two studied seasons (SR and LR season) with R = 0.009. In the first season, the strongest community separation was observed between the third sampling date (EC 90) and the first two sampling dates (EC 30, EC 60) with R = 0.979 and R = 0.987, respectively (Fig. 5.4a). While strong separation was observed in the SR season, in the second season (LR) all three sampling dates showed intermediate separation between AMF communities, with the first and third sampling date (EC 30, EC 90) showing the highest separation with R = 0.402 (Fig. 5.4b). The treatments (C, F,

F+T) induced no significant community separation for all three sampling dates in both season. The observed negative R values are likely due to higher differences within replicates in each treatment than between treatments as described in section 5.3.3 of the manuscript. No significant relation between soil chemical properties and AMF community composition were observed.

Table 5.3: Results of the analysis of similarity (ANOSIM) of total AMF TRFLP data sets for the first and second season (SR = short rains, LR = long rains) from Busia site. For both seasons, sampling dates EC 30 (early leaf development stage), EC 60 (flowering stage) and EC 90 (senescence stage) are compared. Within sampling dates, three treatments are compared: C = control, F = "Foxy-2", F+T = "Foxy-2"+ *T. diversifolia*. The magnitude of *R* indicates the degree of separation between two tested communities. An *R* score of 1 indicates a complete separation, while ≤ 0 indicates no separation.

	Pairwise comparison	***R* value**	**Significance level**		**Pairwise comparison**	***R* value**	**Significance level**
Busia				**Busia**			
	SR, LR	0.009	ns				
Season 1				**Season 2**			
	EC 30, EC 60	0.071	ns		EC 30, EC 60	0.257	**
	EC 30, EC 90	0.979	***		EC 30, EC 90	0.402	***
	EC 60, EC 90	0.987	***		EC 60, EC 90	0.347	***
EC 30				**EC 30**			
	F, F+T	-0.185	ns		F, F+T	0.056	ns
	F, C	-0.167	ns		F, C	-0.222	ns
	F+T, C	0.037	ns		F+T, C	-0.111	ns
EC 60				**EC 60**			
	F, F+T	-0.259	ns		F, F+T	-0.333	ns
	F, C	-0.148	ns		F, C	-0.278	ns
	F+T, C	-0.148	ns		F+T, C	-0.407	ns
EC 90				**EC 90**			
	F, F+T	0.259	ns		F, F+T	0.330	ns
	F, C	0.333	ns		F, C	-0.417	ns
	F+T, C	-0.148	ns		F+T, C	-0.250	ns

Significance levels: ns $P > 0.05$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

5.4.2.2 Homa Bay site

ANOSIM results of TRFLP profiles for the soil samples from Homa Bay are summarized in Table 5.4. There was no significant separation of communities between the SR and the LR season (R = 0.013).

In the first season, the strongest community separation was observed between the first and the second sampling date (EC 30, EC 60) with R = 0.359. Contrary to findings for Busia, communities obtained during the flowering stage and the senescence stage of maize showed the highest similarity (R = 0.113), which was confirmed by CAP analysis (Fig. 5.4c). In the second

season, the strongest community separation was detected between the first and third sampling date (EC 30, EC 90) with R = 0.413 (Table 5.4, Fig. 5.4d). No distinct effects of the treatments (C, F, F+T) were observed throughout all sampling dates in both seasons. However, for the third sampling date (EC 90) of the second season (LR), the number of replicates for which a TRFLP data set could successfully be acquired was too low to make significant assertions about treatment effects. The observed negative R values are likely due to higher differences within replicates in each treatment than between treatments as described in section 5.3.3 of the manuscript. No significant relation between soil chemical properties and AMF community composition were detected.

Table 5.4: Results of the analysis of similarity (ANOSIM) of total AMF TRFLP data sets for the first and second season (SR = short rains, LR = long rains) from Homa Bay site. For both seasons, sampling dates EC 30 (early leaf development stage), EC 60 (flowering stage) and EC 90 (senescence stage) are compared. Within sampling dates, three treatments are compared: C = control, F = "Foxy-2", F+T = "Foxy-2"+ *T. diversifolia*. The magnitude of *R* indicates the degree of separation between two tested communities. An *R* score of 1 indicates a complete separation, while ≤ 0 indicates no separation.

	Pairwise comparison	***R value***	**Significance level**		**Pairwise comparison**	***R value***	**Significance level**
Homa Bay				**Homa Bay**			
	SR, LR	0.031	ns				
Season 1 (SR)				**Season 2 (LR)**			
	EC 30, EC 60	0.359	**		EC 30, EC 60	0.165	*
	EC 30, EC 90	0.273	**		EC 30, EC 90	0.413	**
	EC 60, EC 90	0.113	ns		EC 60, EC 90	0.178	ns
EC 30				**EC 30**			
	F, F+T	-0.333	ns		F, F+T	-0.250	ns
	F, C	-0.241	ns		F, C	0.000	ns
	F+T, C	-0.315	ns		F+T, C	-0.074	ns
EC 60				**EC 60**			
	F, F+T	-0.185	ns		F, F+T	-0.519	ns
	F, C	-0.547	ns		F, C	-0.519	ns
	F+T, C	0.000	ns		F+T, C	-0.352	ns
EC 90				**EC 90**			
	F, F+T	-0.333	ns		F, F+T	n.d.	n.d.
	F, C	-0.278	ns		F, C	n.d.	n.d.
	F+T, C	-0.037	ns		F+T, C	n.d.	n.d.

Significance levels: ns $P > 0.05$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. n.d. = no data available.

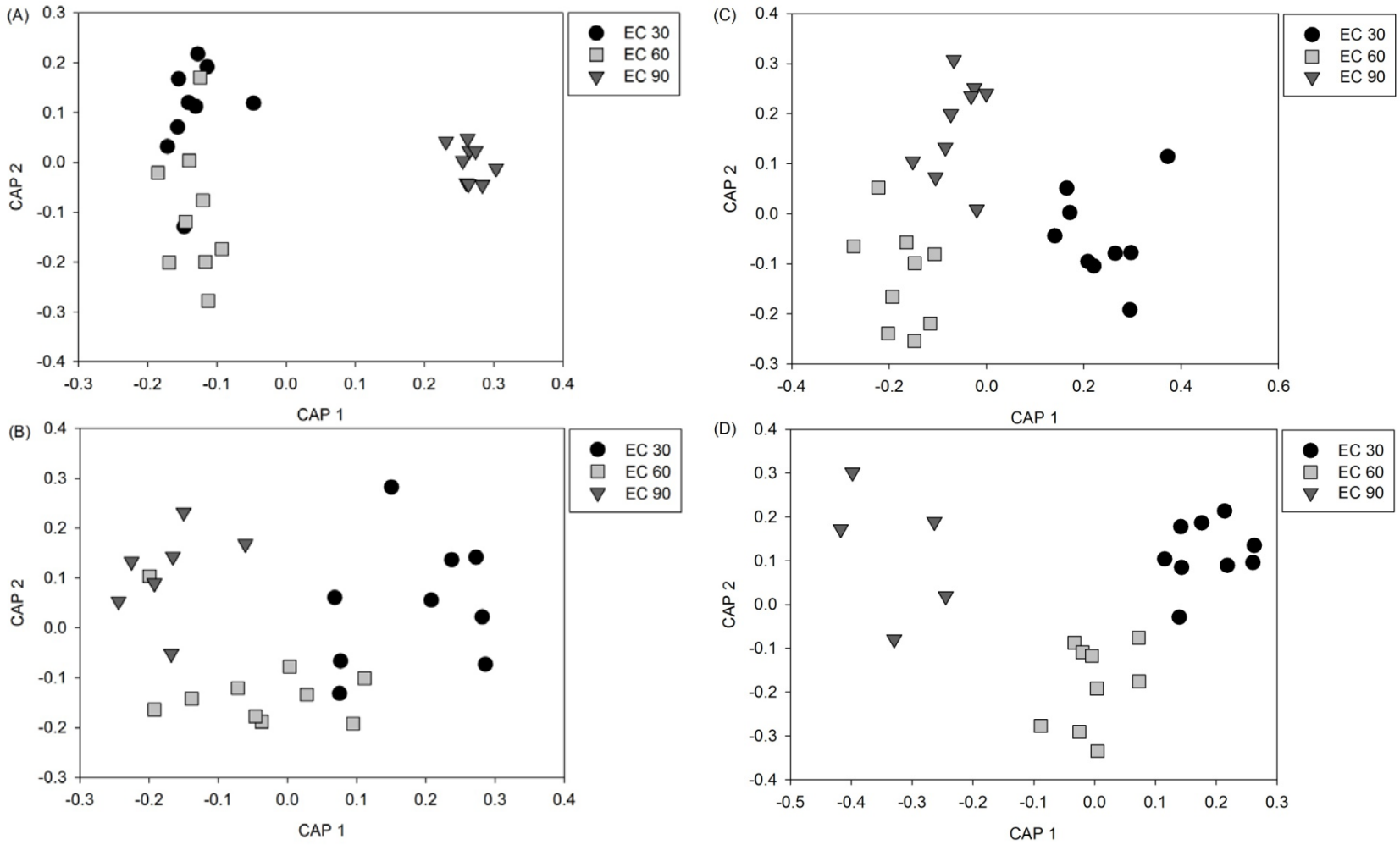

Figure 5.4:

Canonical analysis of principal coordinates (CAP) ordination on the basis of Bray-Curtis similarity indices of normalized TRFLP data obtained from Alu*I*-digested DNA amplicons to visualize the differences in total AMF community composition at Busia site in Season 1 (A) and Season 2 (B) and Homa Bay site in Season 1 (C) and Season 2 (D) according to the three maize growth stages (EC 30 = early leaf development stage, EC 60 = flowering stage, EC 90 = senescence stage).

5.5 Discussion

AMF maintain crucial plant-beneficial functions such as plant nutrient provision, suppression of plant pathogens and enhanced stress tolerance (Smith and Read, 2010; van der Heijden et al., 2008). The intentional introduction of microbial BCAs into the rhizosphere might result in competitive displacement of indigenous plant-beneficial rhizsophere microbes (i.e. AMF) with subsequent adverse effects on crop yield and health. Therefore, AMF might serve as valuable risk indicators in environmental safety studies of microbial BCAs. The present study aimed on assessing the impact of the BCA "Foxy-2" on indigenous AMF taxa abundance and AMF community composition at two distinct field sites in western Kenya (i.e. Busia and Homa Bay) during two cropping seasons. Since it is acknowledged that AMF communities are substantially driven by natural environmental factors such as plant growth stage and seasonality, those factors were included in the experimental set up to trade off potential "Foxy-2" induced effects.

The present study revealed taxon- and site-specific responses of AMF abundance to "Foxy-2" inoculation with *R. intraradices* abundances being transiently suppressed at the field site Busia but not at Homa Bay. In contrast, AMF taxon *F. mossae* was not affected by "Foxy-2" inoculation. The observed transient suppressive effect of "Foxy-2" on AMF taxa *R. intraradices* in the nutrient-poor sandy soil type (i.e. Busia) was compensated by the N-rich organic amendment *T. diversifolia*. The compensatory effect of *T. diversifolia* in the nutrient-poor soil of Busia implies a resource competition between the BCA and *R. intraradices* which was equalized by the provision of additional organic resources (i.e. *T. diversifolia* residues). Our finding is corroborated by other studies which observed stimulating effects of N-rich organic residues on soil fungal communities (España et al., 2011; Kamolmanit et al., 2013; Lee et al., 2013; Zimmermann et al., 2016). In contrast to the minor and transient effect of "Foxy-2" on indigenous AMF communities, we detected substantial effects of plant growth stage and seasonality on AMF community dynamics. Overall, AMF taxa abundance was mainly shaped by seasonal differences while AMF community composition was substantially driven by plant growth stage. However, we detected taxon-specific responses to the monitored environmental factors with *R. intraradices* abundance being solely affected by seasonal changes and soil type dependent by "Foxy-2" while *F. mossae* abundance revealed a close relation with the maize plant, with increasing abundance over the course of the crop plant in season 1 at Homa Bay. Similarly, AMF taxon-specific responses was shown by Zimmermann et al. (2016), observing

an interaction between “Foxy-2” and AMF taxon *Gigaspora margarita* while two other monitored AMF taxa remained unaffected by “Foxy-2” inoculation. Hence, our findings contribute to the emerging research opinion that different AMF species fulfil distinct functional traits in the rhizosphere, resulting in niche differentiation within the AMF community (de Novais et al., 2014; Feddermann et al., 2010; Verbruggen and Toby Kiers, 2010). In this respect, Boyer et al. (2015) found evidence that *F. mossae* plays a significant role in enhancing the drought tolerance of the plant which matches well with the low precipitation rates and high temperatures detected specifically in season 1 at Homa Bay.

The nutrient depleted soil in Busia substantially reinforced the effect of plant growth stage on total AMF community composition suggesting a stronger demand and selection for specific AMF community members during the course of the plant under nutrient limited soil conditions. It can be assumed that the maize plant selects for AMF species with certain functional traits matching its alternating nutrient requirements across developmental stages. While the general ability of plants to shape the rhizosphere microbial community by distinct root exudation patterns is well investigated (Berg and Smalla, 2009; Broeckling et al., 2008; Chaparro et al., 2014; Huang et al., 2014), only a limited number of researchers investigated the impact of plant growth stage on AMF communities so far (Grigera et al., 2007; Husband et al., 2002). Thereby, the underlying plant-microbe interaction observed in our study still needs to be deciphered in more detail.

5.6 Conclusions

The intentional introduction of microbial BCAs into soil ecosystems requires thorough evaluation of potential associated risks on non-target soil microorganisms, especially on functional relevant soil microbes such as AMF. The present study corroborated the environmental safety of the fungal BCA “Foxy-2” since no sustained “Foxy-2” induced effects on indigenous plant-beneficial AMF communities were detected. However, taxon- and site-specific responses of AMF to monitored effects (i.e., “Foxy-2”, seasonality and plant growth stage) substantiated the divergence of functional traits within the AMF community. Our study implies that this divergence in functional traits within the AMF community results in niche differentiation between AMF taxa with varying interference potential of distinct AMF taxa to BCA inoculation. Consequently, the outcome of risk assessment studies using AMF taxa abundance is strongly determined by the monitored AMF taxa and prevailing environmental

conditions. In this context, environmental safety studies should not solely rely on AMF taxa abundance as risk indicator but further include analyses on AMF community composition and other plant-beneficial microbial communities such as nitrifying prokaryotes (i.e. Musyoki et al., 2015; 2016) to draw final conclusions on the safety of a microbial BCA. Progressive studies should further investigate the functional traits of distinct AMF taxa under contrasting environmental conditions to elucidate the interference potential of indigenous AMF with introduced microbial BCAs.

6. General Discussion & Outlook

6.1 Overview

Within this thesis frame, the development of a DNA-based monitoring tool for the fungal BCA Fos was achieved which allows following its population kinetics in soils as driven by contrasting environmental impacts, such as soil type, plant growth stage and seasonality (Chapter 2 and Chapter 4). The developed monitoring tool is essential for official registration of the BCA by country authorities in Sub-Sahara Africa and furthermore enables to identify favoured environmental conditions of the BCA, by this contributing to consistent and sustained efficacy of the biocontrol approach against the parasitic weed *S. hermonthica*. Furthermore, indigenous rhizosphere fungal population dynamics as driven by Fos inoculation and natural environmental impacts were assessed (Chapter 3-5) (1) to evaluate potential effects of Fos inoculation on abundance and composition of indigenous rhizosphere fungi and (2) to trade-off observed Fos-induced effects against natural impact factors such as plant growth stage or seasonality. Within this risk assessment studies, emphasize was put on community dynamics of arbuscular mycorrhizal fungi (AMF) since this fungal group is acknowledged to provide important ecosystem services, such as plant nutrient provision, which are essential for sustained productivity in agro-ecosystems. The potential of AMF taxa abundance to serve as suitable risk indicator in environmental safety studies of microbial BCAs was critically assessed, thereby reinforcing the need of deciphering the diverging functional traits within AMF communities. The gathered data from the risk assessment studies corroborated the environmental safety of the BCA Fos against the investigated microbial communities and highlighted the driving factors of rhizosphere fungal community dynamics, with this contributing to ongoing and future research concerning the resistance and resilience of soil microbial communities to disturbance.

In this discussion, methodical limitations in the development of DNA-based monitoring tools for soil microbes are discussed, including specific obstacles in the development of the monitoring tool for the BCA Fos. Derived results of this PhD thesis on rhizosphere fungal community dynamics are set into a broader context in terms of resistance and resilience of soil microbial communities to disturbance but also prospective rhizosphere engineering. Major concerns of country authorities and societies about evolutionary adaptation and gene transfer in microbes and its implication for host specificity of microbial BCAs are addressed with

emphasize on *Fusarium* spp.. Finally, future research areas concerning the BCA Fos are highlighted.

6.2 Limitations in the development of specific molecular monitoring tools for soil microbes, case example Fos

In Chapter 2, the development of a specific monitoring tool for the BCA Fos was achieved based on AFLP derived molecular markers. The developed tool is capable to monitor population dynamics of Fos strains in soils as influenced by contrasting environmental conditions. The derived information can be implemented to predict the stability and persistence of the BCA under given environmental circumstances, which contributes to sustained and consistent control of the weed *S. hermonthica*. The unique feature of the evolved Fos monitoring tool is its capability to cover the entire taxonomic group *forma specialis strigae*. Hence, we identified genetic markers that are associated with the ability to cause disease on a specific host, i.e. *Striga* spp. (Lievens et al., 2007). This enables country authorities to implement native Fos strains without the need of developing additional monitoring tools. Within the *F. oxysporum* species complex, many *forma speciales* comprise strains that belong to multiple vegetative compatibility groups (VCGs) suggesting a polyphyletic origin (Correll, 1991; Lievens et al., 2008). This also applies to the *forma specialis strigae* with phylogenetic analysis revealing two distinct clades for west- and east African Fos strains (de Klerk M. & Viljoen A., unpublished data). The development of specific molecular monitoring tools covering an entire polyphyletic *forma speciales* is an intricate task due to the high genetic variability within this taxonomic group. In contrast to the present study, most monitoring tools developed for a particular *forma specialis* were only tested on specificity using a relatively small collection of strains not representative for the entire group of *forma specialis* (e.g. Dubey et al., 2014; Lin et al., 2010; Scarlett et al., 2013). Furthermore, attempts to develop molecular markers covering polypyletic *forma speciales* resulted often in substantial flaws in specificity as described well in Lievens et al. (2007). In this context, the developed monitoring tool targeting the f. sp. *strigae* revealed very high specificity when tested against 85 non-Fos isolates representing 15 different species and 20 distinct *forma speciales*. Only one f. sp. *melonis* isolate originating from Israel coamplified with the Fos monitoring tool which is not expected to interfere with the monitoring tool envisaged for implementation in Sub-Sahara Africa.

However, results derived from specificity testing of molecular monitoring tools depend on critical circumstances, which are highlighted in the following discussion. The common approach to verify the specificity of a DNA based detection tool for microbes is the alignment of the developed molecular markers in databases (e.g. BLAST), testing their specificity against an extensive isolate library and, finally, in the target environment (e.g. soils). Hence, specificity of a molecular monitoring tool is determined by the database richness, the size and content of the available isolate library and the microbial composition of the involved environmental samples. Although sequence databases continuously increase in their richness, only about 0.36% of approximately 100.000 identified fungal species are completely sequenced (Azvolinsky, A., 2014). This percentage becomes however relative when considering the total number of fungal species amounting up to 5 million (Blackwell, 2011; O'Brien et al., 2005). Fortunately, the situation within the *F. oxysporum* species complex is not as obscure compared to other fungal species. Within the *F. oxysporum* comparative sequence project (partly published in Ma et al. (2010)) whole genome sequences of several *F. oxysporum* strains were included in gene databases (Table 6.1) which clearly improved the extent and reliability of the AFLP derived Fos marker assignment.

Table 6.1: Strains involved in the *F. oxysporum* comparative sequence project funded by the National Research Initiative within the U.S. Department of Agriculture's National Institute for Food and Agriculture. Source: Broad Institute, Cambridge, USA.

NRRL #	Strain	forma specialis	Host
37622	HDV247	*pisi*	*Pisum*
32931	FOSC 3-a	(human)	*Homo*
54002	Fo47	(biocontrol)	Soil
54003	MN25	*lycopersici* race 3	*Lycopersicum*
54008	PHW808	*conglutinans,* race 2	*Brassica/Arabidopsis*
54005	PHW815	*raphani*	*Raphanus/Arabidopsis*
26381	CL57	*radicis-lycopersici*	*Lycopersicum*
54006	II5	*cubense* tropical race 4	*Musa*
26406		*melonis*	*Cucurbita*
25433		*vasinfectum*	*Gossypium*

Another drawback remaining is the low reliability of sequence assignment in gene databases. Several microbial species were and are misidentified with estimated 20% of fungal DNA sequences, which may have incorrect lineage designations in GenBank (Bidartondo, 2008). This hampers the development of species- or strain specific microbial detection tools as described in O'Donnell et al., (2015) who emphasized on *Fusarium* identification and sequence alignment in databases. For instance, Jiménez-Fernández et al., (2010) experienced the impact of misidentified fungal species during their development of a *F. oxysporum* detection tool, concluding that some of the involved fungal isolates were erroneous assigned to the *F. oxysporum* species complex. In this context, all Fos isolates involved in the present PhD study were thoroughly tested on their pathogenicity towards *S. hermonthica* while VCG testing and phylogenetic analysis further confirmed their correct assignment to *F. oxysporum* f. sp. *strigae*. In the second step of specificity testing, designed molecular markers were tested against the available isolate library. The extent of the isolate library which is considered sufficient for specificity testing varies largely in literature with around 21 isolates (Dubey et al., 2014) as the lowest and 102 (Edel-Hermann et al., 2011) as the upper limit in the development of molecular detection tools for *F. oxysporum* strains. Therefore, 125 isolates, including 85 non-Fos isolates, involved in specificity testing of the monitoring tool for the BCA Fos can be regarded as an extensive library collection compared to previous studies.

The most important step in the evaluation of microbial monitoring tools is the specificity testing in target environments, e.g. soils. Soil microbial communities are extremely diverse containing millions of species within one single ecosystem (Bardgett and van der Putten, 2014) with estimated 200-235 fungal operational taxonomic units (OTU) per gram soil (Bardgett and van der Putten, 2014; Fierer et al., 2007). Moreover, the diversity of most fungal groups is acknowledged highest in tropical soil ecosystems (Tedersoo et al., 2014), which are envisaged for implementation of the Fos monitoring tool. Consequently, to confirm the specificity of developed monitoring tools in these diverse soil ecosystems is one of the major challenges which was met with the Fos monitoring tool in four distinct soil ecosystems.

However, although the specificity of the Fos monitoring tool was extensively validated compared to existing molecular monitoring tools for soil microbes, it has to be generally assumed that the specificity of DNA-based monitoring tools envisaged for implementation in contrasting soil ecosystems is never complete and requires perpetual evaluation if implemented in new soil environments.

6.3 Rhizosphere fungal community dynamics – implications derived by this PhD study

One major objective of this thesis was to assess the impact of the BCA Fos on indigenous rhizosphere fungi, traded off against natural environmental impacts, thereby disclosing detailed insights into rhizosphere fungal community dynamics over the period of successive cropping seasons (Chapter 3-5). In recent years, climate change scenarios and the general intensification of human activities in form of land management and cultivation resulted in increasing awareness of the importance to monitor soil microbial community dynamics and their mediated processes in response to environmental impacts (Bradford, 2013; Pold and DeAngelis, 2013; Schuur et al., 2015). Given the crucial importance of maintaining important ecosystem functions mediated by soil microbes (van der Heijden et al., 2008), the resistance and resilience of soil microbial communities to disturbance emerged as a substantial research area (Griffiths and Philippot, 2013). However, past research has led to a significantly greater understanding of the dynamics, structure and function of soil bacterial communities compared to their fungal counterparts (Orgiazzi et al., 2012; Rousk et al., 2010). Rousk and Frey (2015) recently revised the hypothesis of bacterial-to-fungal dominance in mediating crucial soil functions (e.g. carbon mineralization) and demonstrated the need to re-explore our basic understanding for microbial

communities and their associated function in soil. The emphasise of the present thesis on rhizosphere fungal community dynamics fills this knowledge gap by illuminating the response of rhizosphere fungi to specific environmental impacts with advanced focus on AMF community dynamics (Chapter 3-5). AMF are acknowledged to provide crucial ecosystem services such as plant growth promotion and alleviation of plant stress, which are fundamental for sustained productivity of agro-ecosystems (Azcón-Aguilar et al., 2009; Smith and Read, 2010). Hence, monitoring the response of AMF communities towards contrasting environmental impacts might allow predicting the consequences of human-driven land management practices on specific ecosystem services. Thereby, AMF could serve as valuable risk indicators in environmental safety studies. However, monitoring of total AMF population dynamics in response to environmental impacts remains challenging due to lacking molecular monitoring tools with high coverage and specificity towards the entire phylum *Glomeromycota* (Kohout et al., 2014; Xiang et al., 2016). To circumvent this problem, we investigated the potential of prevalent AMF taxa to serve as model risk indicators representative for the whole AMF community. However, our results indicate a critical drawback if risk assessment studies are solely based on AMF taxa dynamics due to strong niche differentiation within AMF communities (Chapter 3 and Chapter 5). Consequently, observed AMF taxon-specific responses towards distinct environmental impacts are not representative for the entire AMF community and cannot be upscaled to the genus or family level. This implication derived by the present PhD study is essential for proper and reliable conduction of future environmental safety studies in soil ecologies. It also needs to be acknowledged that research on rhizosphere fungal ecology has intensively focused on AMF communities while the potential plant-beneficial role of other rhizosphere fungi was neglected (Khidir et al., 2010; Porras-Alfaro et al., 2008). The present PhD study addressed this issue by combining total fungal community analysis with AMF community analysis, which allows differentiating between the responses of AMF against other fungal community members in the rhizosphere. The importance of this combined approach has been highlighted by our finding that non-AMF fungi play a substantial role in maize-rhizobiome interaction as indicated by TRFLP analysis conducted in Chapter 4 and 5. In season 2 at both field sites (i.e. Busia and Homa Bay), *R* values obtained from pairwise comparison of distinct plant growth stages showed substantially stronger total fungal community separation compared to total AMF community separation. Consequently, the observed strong effect of plant growth stage on total fungal community structure cannot be solely attributed to alterations in the AMF community implying that other rhizosphere fungi played a substantial role in this plant-rhizobiome interaction. Hence, progressive research should focus on elucidating the

contribution of non-AMF fungi to important ecosystem services in the rhizosphere of cereals. In the last years, a new group of endophytic non-AMF fungi called dark septate endophytes (DSE), which seem to maintain similar ecosystems services as AMF, has raised the interest of several researchers (Jumpponen, 2001; Li et al., 2011; Porras-Alfaro et al., 2008; Wang et al., 2016). However, the mechanisms how DSE contribute to plant nutrient provision, pathogen suppression and plant growth are still poorly understood (Porras-Alfaro et al., 2011; Li et al., 2011). The symbiotic interaction between DSE and cereals, such as maize, has been so far only investigated under metal-contaminated soil conditions (Li et al., 2011; Wang et al., 2016). The current knowledge gap on the role of plant-beneficial non-AMF fungi in the rhizosphere of cereals is reinforced by results derived from the present PhD thesis, thereby strengthening the basis for progressive research in this area.

Another knowledge gap tackled by the present study is the impact of plant-beneficial fungal inoculants (i.e. BCAs) on indigenous rhizosphere microbial communities. So far, potential non-target side effects of fungal inoculants were predominantly tested in bulk soils (Edel-Hermann et al., 2009; Savazzini et al., 2009; Schwarzenbach, K. A., 2008) although the rhizosphere is acknowledged to comprise significantly higher microbial density and activity (Gomes et al., 2003; Prashar et al., 2014). Thereby, rhizosphere microbial communities are more vulnerable to environmental stress and competitive displacement compared to their bulk soil counterpart (Kuzyakov and Xu, 2013; Moawad et al., 1984). Recently, Rodriguez and Sanders (2015) highlighted this knowledge gap by calling attention for missing environmental impact studies when using AMF inoculants as plant growth promoting products in crop rhizospheres. In this context, findings gained by this PhD study (Chapter 3-5) and Musyoki et al. (2016, 2015) can serve as first indicator for strong resistance and resilience of native rhizosphere microbial communities to newly inoculated fungal strains. This finding gains even more importance considering future prospects on rhizosphere engineering (Fig. 6.1) for improved crop yield, health and resistance to environmental stress (Dessaux et al., 2016b; Haldar and Sengupta, 2015; Quiza et al., 2015; Zhang et al., 2015). According to Quiza et al. (2015), a major part of prospective rhizosphere engineering will take place via application of microbial inoculants with beneficial functions (e.g. AMF, plant growth promoting rhizobacteria or BCAs). However, authors concordantly state potential non-target side effects of these microbial inoculants on native microbial communities as major obstacle (Dessaux et al., 2016; Zhang et al., 2015; Quiza et al., 2015). The present PhD thesis not only investigates this issue but also highlights strategies to compensate potential competition between microbial inoculants and native microbes and by

this enhancing both the efficacy of the inoculants and stress tolerance of the native microbial community (i.e. *T. diversifolia* treatment). Within the frame of climate change scenarios, strategies to bring soil microbial communities from a vulnerable state to a resilient state (Fig. 6.1) are vital for sustained productivity in agro-ecosystems. Hence, the implication derived by this PhD study that high quality organic residue with low C/N ratio might serve as resource buffer to compensate potential resource competition between plant-beneficial inoculants and native microbial communities is worth to be further investigated by implementing a variety of potential inoculants.

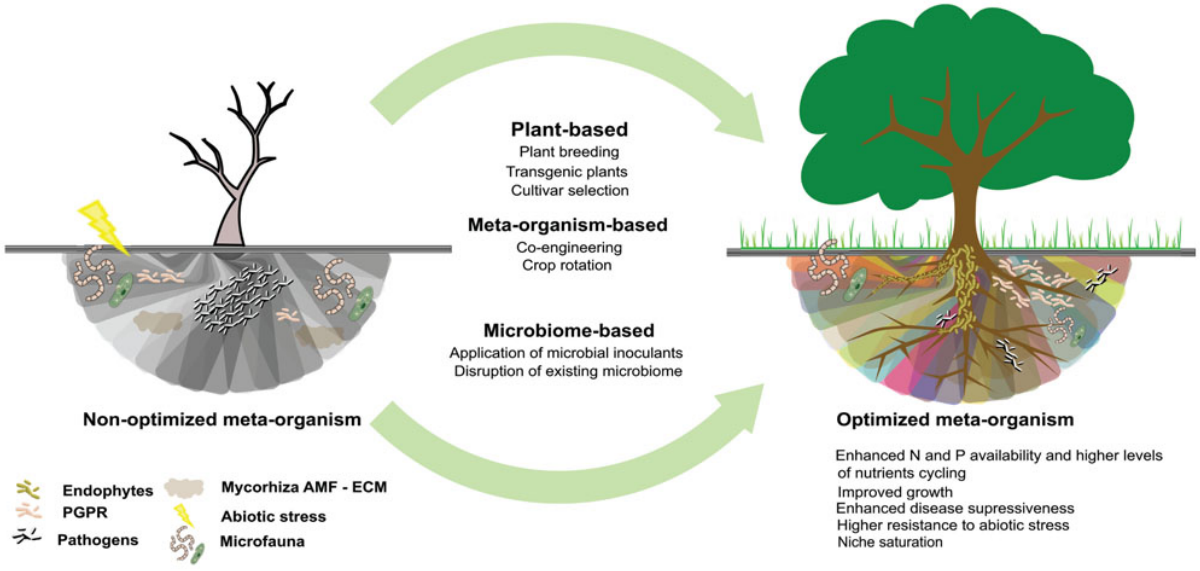

Figure 6.1: Different approaches to rhizosphere microbiome engineering used to bring the microbiome from a low diversity and vulnerable state, with limited functions and productivity, to a diverse and resilient state with high functional redundancy and consistent functioning across variable environments and increased resistance to pathogen invasion**.** Source: Quiza et al. (2015).

6.4 Evolutionary adaptation and gene transfer in microbes – current risk assessment of microbial BCAs only a snapshot?

The risk assessment studies conducted within the frame of the present PhD thesis intended to contribute to successful registration of Fos strains as BCAs against the parasite *S. hermonthica*. Three major risks associated with microbial BCAs (pathogenicity, toxicity and competitive displacement (Cook et al., 1996)) were thoroughly investigated in relation to the BCA Fos (Elzein et al., 2006; Ndambi et al., 2011; Zimmermann et al., 2016; Musyoki et al., 2015, 2016). However, one disregarded risk arising with the intentional introduction of microbial BCAs in

agro-ecosystems includes the possibility of evolutionary adaptation of the BCA to new hosts (Sundh, 2012; Van Klinken and Edwards, 2002). According to Van Klinken and Edwards (2002), two potential consequences might evolve if this risk is being neglected: (1) Release of potentially harmful BCAs or (2) the unnecessary rejection of BCAs because of unfounded fears of post-release evolution. Therefore, the following discussion will resume the current knowledge concerning gene transfer, pathogenetics and host specificity evolvement particular in the genus *Fusarium* spp., with concluding remarks on the BCA Fos.

Generally, comparative analyses have revealed that *Fusarium* spp. genomes are structured into two distinct regions: (1) regions responsible for vital functions (core genome) and (2) regions responsible for pathogenicity, virulence and secondary metabolites (accessory genome) (Ma et al., 2013). Interestingly, supernumerary chromosomes, which are not present in all isolates of the same species, were detected in many pathogenic fungi including *Fusarium* spp.. These supernumerary chromosomes usually comprise a large number of transposable elements (TEs), which distinguishs them from the core genome (Ma et al., 2013; Schmidt and Panstruga, 2011). Transposable elements are DNA sequences that can change their position within a genome; thereby TEs play a profound role in genome evolution because they can contribute to chromosomal rearrangements, gene innovation and gene dublications (Fedoroff, 2012; Grandaubert et al., 2014; Schmidt and Panstruga, 2011). The pathogenetics within *F. oxysporum* are complex by involving a number of effector genes that are required for pathogenicity, with for instance nearly 350 putative effectors in race 1 of *F. oxysporum* f. sp. *cubense* (Guo et al., 2014). Effectors are proteins secreted by the fungus that modulate the interaction between the fungus and its host (Presti et al., 2015). *Fusarium* pathogenicity genes are categorized in two classes (1) basic effector genes, which are shared by different pathogenic fungi and (2) specialized effector genes, which are individual for a specific *Fusarium*-host interaction, for instance *F. oxysporum* f. sp. *lycopersici* established a gene-for-gene interaction with its tomato host (Ma et al., 2013). Best-studied effectors within the *F. oxysporum* species complex are the so-called “secreted in xylem” (*SIX*) effectors, which are produced by *F. oxysporum* f. sp. *lycopersici* during infection of tomato (De Wit et al., 2009). Some effectors, such as *SIX*, play significant roles in determining host specificity. For instance, *SIX4*, *SIX3* and *SIX1* are the avirulence genes that interact with the tomato resistance genes *I-1* (*Immunity-1*), *I-2*, and *I-3*, respectively (Rep et al., 2004). Interestingly, all currently known effector genes of *F. oxysporum* f.sp. *lycoperisici* are located on one supernumerary chromosome as confirmed by Ma et al. (2010). Moreover, Ma et al. (2010) were able to transfer two of the supernumerary

chromosomes of *F. oxysporum* f.sp. *lycoperisici* to a non-pathogenic *F. oxysporum* strain, which resulted in a new pathogenic lineage. Ma et al. (2010) assumed that high TE content and small size of a chromosome substantially improves its transfer propensity. Ma et al. (2010) stated that this novel finding of horizontal chromosome transfer between *F. oxysporum* strains could serve as explanation for the polyphyletic origin of most *forma speciales* and the rapid development of new pathogenic lineages within the *F. oxysporum* species complex. Since then, horizontal chromosome and gene transfer has been demonstrated in *F. oxysporum* f. sp. *canariensis*, f. sp. *phaseoli* and f. sp. *pisi* (Laurence et al., 2015; Milani et al., 2012). Rocha et al. (2015) have shown both vertical and horizontal gene transfer between natural *F. oxysporum* populations and isolates from agro-ecosystems, thereby concluding that horizontal gene transfer of effector genes increases the evolutionary potential of the pathogen with important consequences for long-term disease management. To our current knowledge, non-pathogenic *F. oxysporum* strains can convert into pathogenic strains by receiving a specific chromosome from a donor. The crucial knowledge gap remains if a pathogenic *F. oxysporum* strain can broaden its host range by receiving additional chromosomes harbouring distinct effectors. Ma et al. (2010) included in their study one non-pathogenic *F. oxysporum* strain and two pathogenic strains *(F. oxysporum* f. sp. *melonis* and f. sp. *cubense*). Both pathogenic strains failed in receiving the donor chromosome while the non-pathogenic strain successfully included it. Consequently, it can be assumed that the receptivity of a strain is limited to a certain number of supernumenary chromosomes. This hypothesis is further corroborated by the fact that all currently known *forma speciales* within the *F. oxysporum* species complex show a narrow host range and no significant extension of the host range of a particular *forma specialis* has been reported yet.

Therefore, it can be concluded that an extension of the host range of the BCA Fos due to horizontal chromosome transfer is highly improbable while there is scientific ground that the BCA Fos might serve as donor strain for non-pathogenic *F. oxysporum* strains. However, evidence on supernumenary chromosomes in the *forma specialis strigae* genome is still lacking and progressive research on effector genes responsible for host specificity against *S. hermonthica* is highly recommendable. In this context, it should also be investigated if the developed Fos-specific monitoring tool (Chapter 2) potentially targets an effector gene involved in host specialisation towards *Striga* spp.. This might be an explanation for the coverage of the entire polyphyletic *forma specialis strigae* without substantial flaws in specificity. This assumption is also supported in literature with highly specific monitoring tools developed on basis of the *SIX* effector gene targeting the *forma specialis lycopersici* (Lievens et al., 2009).

Moreover, potential adaptation of *S. hermonthica* accompanied with the development of new resistance genes reinforces the need of a thorough understanding of the pathogenetics of the BCA Fos for sustained *S. hermonthica* control in the future.

6.5 Outlook: Protective *Fusarium oxysporum* strains – double role of the BCA Fos in crop protection in Sub-Sahara Africa?

There is increasing awareness of the potential of *F. oxysporum* strains as BCAs against plant pests due to their easy way of mass production and formulation (Fravel et al., 2003). In general, *F. oxysporum* strains used as BCAs can be classified into two groups: (1) pathogenic and (2) non-pathogenic/protective. Non-pathogenic *F. oxysporum* strains are implemented to control diseases caused by pathogenic strains of the same genus (Alabouvette et al., 2009). The term "non-pathogenic" is often used in this context in literature but may be misleading, since these *F. oxysporum* strains are not proven to lack virulence genes but act non-pathogenic to the crop species tested so far. Fravel et al (2009) suggests the term "protective" as alternative. The mechanisms behind the protective ability of these *F. oxysporum* isolates can be attributed to resource competition (Alabouvette et al., 2009; Fravel et al., 2003) and induced plant resistance (Olivain et al., 2006). It has been shown that these protective *F. oxysporum* strains not only intensively colonize the surface of crop roots but also invade into the inner root layers, such as the epidermis, the hypodermis and cortical cells (e.g. Nahakova et al, 2008; Fravel et al., 2003). The crop plant responded to the fungal invasion with structural barriers such as lignification of cell walls, wall appositions and intercellular plugging (Fravel et al., 2003), by this enhancing protection against subsequent pathogen infection (Benhamou & Garrand, 2001; Olivain et al., 2003; Fravel et al., 2009). Interestingly, similar patterns of crop root colonization and invasion into the inner root layers were detected with the Fos strain "Foxy-2" (Elzein et al., 2010; Beninweck Ndambi et al., 2012). First indication on plant defence reaction to "Foxy-2" colonization in form of lignifications of endodermic root cells exists (Elzein et al., 2010) but studies on the potential protective ability of "Foxy-2" against crop pathogenic *Fusarium* spp. are lacking until now. *Fusarium* diseases in cereals are considered as major threat to food safety all over the world due to critical levels of toxic and carcinogenic fungal metabolites accumulating in grains. In Benin, Burkina Faso and Ghana, 90-100% of maize kernels are contaminated with the *Fusarium* toxin "Fumonisin B_1" with *F. verticillioides* being the causing agent (Dutton, 2009). Although *F. verticillioides* causes symptoms on both the below- and

above-ground parts of plants, it is assumed that maize roots are the major areas that are initially infected by *F. verticillioides* (Oren et al., 2003; Wu et al., 2013). A systemic resistance reaction in the maize roots induced by “Foxy-2” invasion could therefore potentially reduce subsequent infection with *F. verticillioides* and successive accumulation of *Fusarium* toxins in kernels. Combating two major threats to cereal production in Sub-Saharan Africa, *Striga* spp. infestation and *Fusarium* diseases, with a double protective BCA would represent an environmental friendly solution and crucial step towards food security in this region. Moreover, the potential double protective role of Fos strains in cereal production strengthens the importance of the derived results from the present PhD thesis which provides, together with the work of Elzein et al. (2006), Ndambi et al. (2011) and Musyoki et al. (2015. 2016), the necessary basis for successful registration of Fos strains as BCAs in Sub-Saharan Africa.

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Summary

The fungal biocontrol agent (BCA) *Fusarium oxysporum* f. sp. *strigae* strain "Foxy-2" has proven to be effective in the suppression of the parasitic weed *Striga hermonthica*, which causes substantial yield losses in cereals in Sub-Saharan Africa. A prerequisite for widespread implementation of the biocontrol technology is the official registration of the BCA "Foxy-2" by country authorities in Sub-Saharan Africa. The FAO and OECD institutions established international registration regulations to ensure the environmental safety of microbial BCAs. The present thesis aimed on assessing the potential of the BCA "Foxy-2" to meet these registration requirements and was, therefore, based on the following two major objectives: (1) A specific monitoring tool for the BCA "Foxy-2" was developed which allows its identification and quantification in inoculated environments and (2) risk assessment studies were conducted to assess potential side effects of "Foxy-2" on non-target organisms. "Foxy-2" is applied via seed coating and establishes in the rhizosphere of the crop where it propagates saprophytically waiting for its host *S. hermonthica*. The intentional introduction of microbial BCAs, such as "Foxy-2", into the rhizosphere might induce alterations in indigenous plant-beneficial rhizosphere microbial communities (i.e. arbuscular mycorrhizal fungi (AMF)) with subsequent adverse effects on crop yield and health. Therefore, risk assessment studies within the frame of this thesis focused on rhizosphere microbial community dynamics with emphasis on rhizosphere fungi as these may compete for similar resources in the rhizosphere as the fungal BCA "Foxy-2". Specific considerations were given on AMF community dynamics since this fungal group is acknowledged to maintain important ecosystem services and, hence, can serve as valuable risk indicator in the evaluation of the BCA "Foxy-2". The methodological approach comprised a combination of rhizobox and field experiments, including contrasting tropical soils inoculated with and without *S. hermonthica* seeds and planted with maize seeds coated with and without "Foxy-2". Rhizosphere soils obtained from maize plants were subjected to molecular (i.e. quantitative PCR (qPCR) and terminal restriction fragment length polymorphism (TRFLP)), soil physico-chemical and statistical analyses. The impact of "Foxy-2" on indigenous soil fungal communities was traded off against natural environmental impacts, such as soil type (i.e. sandy Ferric Alisol versus clayey Humic Nitisol), crop growth stage (i.e. early leaf development stage, flowering stage and senescence stage) and seasonality (i.e. short and long rain seasons), which are acknowledged to highly influence soil fungal community

abundance and composition. Furthermore, an organic fertilization treatment with nitrogen-rich organic residues (i.e. *Tithonia diversifolia*) was included in the experimental set up to compensate for the hypothesized resource competition between "Foxy-2" and indigenous rhizosphere fungi. The envisaged monitoring tool was further implemented in identifying favoured environmental conditions for the BCA "Foxy-2", which will facilitate the improvement of soil conditions in favour of the BCA for sustained and consistent *S. hermonthica* control under contrasting environmental conditions.

The first research study focused on the development and validation of a specific molecular monitoring tool for the BCA "Foxy-2", which was based on AFLP fingerprinting and subsequent quantitative PCR (qPCR). The specificity of the AFLP-marker to "Foxy-2" was confirmed on basis of comparison of *Fusarium* isolates of differing relatedness to "Foxy-2". The robustness of the AFLP-marker for monitoring "Foxy-2" was validated in a controlled rhizobox experiment revealing that soil type and organic resource availability exhibited distinct effects on abundance of inoculated "Foxy-2". Negative PCR signals, which confirmed the specificity of the developed AFLP-marker, were retrieved from control soils not inoculated with "Foxy-2".

The same rhizobox experiment was implemented in the second research study to investigate the impact of "Foxy-2" on total indigenous rhizosphere fungal community abundance and composition with specific emphasize on AMF taxa abundance, using molecular approaches such as qPCR and TRFLP fingerprinting. In both soils, "Foxy-2" occasionally promoted total fungal abundance, while the community composition was mainly altered by *T. diversifolia* and *S. hermonthica*. Notably, "Foxy-2" stimulated AMF *Gigaspora margarita* abundance, while *G. margarita* was suppressed by *S. hermonthica*. Total fungal and AMF abundance were promoted by *T. diversifolia* residues. In conclusion, "Foxy-2" exposed no adverse effects on indigenous rhizosphere fungal communities substantiating its environmental safety as BCA against *S. hermonthica*.

The third research study emphasized on monitoring "Foxy-2" proliferation and its impact on indigenous soil fungal communities at two contrasting field sites in western Kenya (i.e. Busia and Homa Bay) during two cropping seasons. "Foxy-2" proliferated stronger in the soils from Busia (sandy clay) than Homa Bay (loamy sand) and revealed slightly higher abundance in the second season. "Foxy-2" had only a transient suppressive effect on total indigenous fungal abundance, which ceased in the second season and was further markedly compensated after

addition of *T. diversifolia* residues. Likewise, community structure of the indigenous fungal community was significantly altered by maize growth stages, but not by "Foxy-2". In conclusion, no major adverse effects of "Foxy-2" inoculation on indigenous fungal rhizosphere communities were observed corroborating the safety of this BCA.

The fourth research study emphasized the potential of AMF taxa abundance and total AMF community composition to serve as reliable risk indicators in environmental safety studies of microbial BCAs. Our results revealed taxon- and site-specific responses of AMF abundance to "Foxy-2" inoculation. Natural environmental factors such as seasonality and plant growth stage clearly superimposed the effect of "Foxy-2" on AMF. However, the detected AMF taxon-specific responses to "Foxy-2" inoculation but also natural environmental impacts imply that AMF taxa diverge in their functional traits, thereby varying in their interference potential with microbial BCAs. Conclusively, risk assessment studies should not solely rely on monitoring AMF taxa abundance in response to BCA inoculation but further include analyses on AMF community composition and other plant-beneficial microbial communities such as nitrifying prokaryotes, to draw conclusions on the safety of a microbial BCA.

Within this thesis frame, the development of a DNA-based monitoring tool for the fungal BCA "Foxy-2" was achieved which allows following its population kinetics in soils as driven by contrasting environmental impacts, such as soil type, plant growth stage and seasonality. The developed monitoring tool is essential for official registration of the BCA by country authorities in Sub-Saharan Africa and furthermore enables to identify favoured environmental conditions of the BCA, by this contributing to consistent and sustained efficacy of the biocontrol approach against the parasitic weed *S. hermonthica*. The gathered data from the risk assessment studies corroborated the environmental safety of the BCA "Foxy-2" and highlighted the driving factors of rhizosphere microbial community dynamics, with this contributing to ongoing and future research concerning the resistance and resilience of soil microbial communities to disturbance. Finally, the potential of AMF taxa abundance to serve as suitable risk indicator in environmental safety studies of microbial BCAs was critically assessed, thereby reinforcing the need of deciphering the diverging functional traits within AMF communities, which potentially determine the interference potential of distinct AMF taxa towards microbial BCA strains.

German Summary

Der pilzliche Biokontrollagent (BKA) *Fusarium oxysporum* f. sp. *strigae* Stamm “Foxy-2” stellt eine wirksame Kontrollmöglichkeit gegen das parasitäre Unkraut *Striga hermonthica* dar, welches beachtliche Getreideverluste in Sub-Sahara Afrika verursacht. Voraussetzung für den großflächigen Einsatz dieser biologischen Unkrautkontrolle ist die offizielle Registrierung des BKA “Foxy-2” durch Länderbehörden in Sub-Sahara Afrika. Die FAO und OECD entwickelten internationale Regularien für die Registrierung von mikrobiellen BKA um die Umweltverträglichkeit dieser biologischen Pestizide zu gewährleisten. Die Hauptziele der vorliegenden Dissertation basierten deshalb auf den Anforderungen dieser internationalen Regularien: (1) Eine spezifische Detektionsmethode wurde entwickelt, durch die der BKA in inokulierten Umgebungen identifiziert und quantifiziert werden kann. (2) Risikoanalysen wurden durchgeführt um nachteilige Effekte auf nicht-Ziel Organismen durch die Inokulation mit dem BKA auszuschließen. Der BKA „Foxy-2“ wird durch Saatgutbeschichtung in den Boden ausgebracht, wo er sich in der Rhizosphäre der Getreidepflanze etabliert und saprophytisch überdauern kann. Die Etablierung des inokulierten BKA in der Rhizosphäre könnte sich nachteilig auf einheimische pflanzennützliche Rhizosphärenmikroorganismen auswirken mit entsprechend negativen Folgen für den Pflanzenertrag und die Pflanzengesundheit. Deshalb konzentrierten sich die Risikoanalysen innerhalb dieser Dissertation auf die Dynamiken von Rhizopshärenmikroorganismen mit Fokus auf Rhizosphärenpilze, da diese um die gleichen Ressourcen wie der pilzliche BKA „Foxy-2“ konkurrieren. Ein besonderer Fokus wurde auf die Dynamiken von arbuskulären Mykorrhiza gelegt, da diese für ihren Beitrag zu wichtigen Ökosystemleistungen anerkannt sind und deshalb als wertvolle Risikoindikatoren in der Bewertung des BKA „Foxy-2“ miteinbezogen werden können. Der methodische Ansatz beinhaltete eine Kombination aus Rhizobox – und Feldversuchen unter Einbezug von zwei unterschiedlichen Böden welche jeweils mit oder ohne *S. hermonthica* inokuliert wurden und mit Mais, mit oder ohne „Foxy-2“ Beschichtung, bepflanzt wurden. Bodenproben aus der Rizosphäre der Maispflanzen wurden molekularbiologischen (quantitative PCR (qPCR) und terminalem Restriktionslängenpolymorphismus (TRFLP)), bodenchemischen und statistischen Analysen unterzogen. Der Einfluss von „Foxy-2“ auf einheimische Rhizosphärenpilze wurde gegen natürliche Einflüsse abgewägt (z.B. Bodentyp, Pflanzenwachstumsstadien und Saisonalität) welche nachweislich einen entscheidenden Einfluss auf die bodenpilzliche Abundanz und Gemeinschaftsstrukur haben können. Außerdem wurde eine Behandlung mit organischer

Düngung (stickstoffreiche organische Pflanzenrückstände, z.B. *Tithonia diversifolia*) miteinbezogen um die angenommene Ressourcenkonkurrenz zwischen „Foxy-2“ und einheimischen Rhizosphärenpilzen auszugleichen. Die angestrebte Detektionsmethode wurde desweiteren angewendet um günstige Bodenbedingungen zu identifizieren, welche zu einer erfolgreichen Etablierung und anhaltende Effektivität des BKA „Foxy-2“ gegen das parasitäre Unkraut *S. hermonthica* beitragen.

Die erste wissenschaftliche Studie befasste sich mit der Entwicklung und Validierung eines spezifischen DNA-basierten molekularen Detektionssystems für den BKA „Foxy-2“, welches auf AFLP Fingerprinting und anschließender quantitativer PCR beruhte. Die Spezifität des AFLP-Markers wurde anhand pilzlicher Vergleichsisolate und genetischer Datenbanken bestätigt. Die Eignung des AFLP-Markers „Foxy-2“ spezifisch in Böden zu detektieren und quantifizieren wurde in einem Rhizbox-Versuch unter kontrollierten Bedingungen untersucht. Die Ergebnisse zeigten, dass „Foxy-2“ Abundanz im Boden durch den Bodentyp und die Verfügbarkeit organischer Resourcen beeinflusst wird. Es wurde keine qPCR Amplifikation in den Kontrollböden detektiert, wodurch die Spezifität des AFLP-Markers bestätigt werden konnte.

Derselbe Rhizobox-Versuch wurde in der zweiten wissenschaftlichen Studie verwendet um die Auswirkung der „Foxy-2“ Inokulation auf die einheimische Gesamtpilzabundanz und -struktur zu untersuchen. Ein spezifischer Fokus wurde hierbei auf die Abundanz bestimmter arbuskulärer Mykorrhiza Taxa gelegt. In beiden Böden wurde eine kurzzeitige Stimulation der Gesamtpilzabundanz durch „Foxy-2“ beobachtet, während die pilzliche Gemeinschaftsstruktur vorwiegend durch Zugabe von *T. diversifolia* und *S. hermonthica* beeinflusst wurde. Außerdem wurde das Mykorrhiza Taxon *Gigaspora margarita* durch die Inokulation mit „Foxy-2“ stimuliert und durch die Zugabe von *S. hermonthica* unterdrückt. Die Gesamtpilzabundanz und Mykorrhiza Taxonabundanz wurden durch die Zugabe von *T. diversifolia* stimuliert. Zusammenfassend wurde bestätigt, dass die Inokulation mit „Foxy-2“ keine nachteiligen Effekte auf die einheimische Pilzgemeinschaft in der Rhizosphäre ausübte, wodurch die Umweltverträglichkeit des BKA „Foxy-2“ untermauert werden konnte.

Die dritte wissenschaftliche Studie konzentrierte sich auf die Quantifizierung ds BKAs „Foxy-2“ und seiner Effekte auf einheimische Bodenpilze in zwei verschiedenen Feldstandorten in West-Kenia (Busia und Homa Bay) während zwei aufeinanderfolgender Ernteperioden. Die

Ergebnisse zeigten eine höhere „Foxy-2“ Abundanz am Standort Busia (sandig-toniger Boden) im Vergleich zu als Homa Bay (lehmig-sandiger Boden). Außerdem wurde eine leicht erhöhte Abundanz des BKA in der zweiten Ernteperiode detektiert. Die Inokulation mit „Foxy-2“ zeigte eine kurzzeitig unterdrückende Wirkung auf die einheimische Pilzabundanz, welche allerdings in der zweiten Ernteperiode nicht mehr detektiert werden konnte. Dieser kurzzeitig unterdrückende Effekt wurde außerdem durch die Zugabe von *T. diversifolia* kompensiert. Die Gesamtpilzstruktur wurde hauptsächlich durch das Pflanzenentwicklungsstadium beeinflusst, während „Foxy-2“ keine Wirkung zeigte. Zusammenfassend wurden keine anhaltend nachteiligen Effekte der „Foxy-2“-Inokulation auf einheimische Bodenpilzgemeinschaften festgestellt, wodurch die Umweltsicherheit des BKA weiter bekräftigt werden konnte.

Die vierte wissenschaftliche Studie untersuchte die Eignung von Mykorrhiza Taxaabundanz und Gemeinschaftsstruktur als Risikoindikatoren in Umweltverträglichkeitsstudien von mikrobiellen BKA. Die Ergebnisse zeigten Taxon- und Standort-spezifische Reaktionen der Mykorrhizaabundanz auf die Inokulation mit „Foxy-2“. Natürliche Umweltfaktoren, wie z.B. Saisonalität und Pflanzenentwicklungsstadien, exhibierten deutlich stärkere Effekte auf die Mykorrhizagemeinschaft im Vergleich zu „Foxy-2“. Die Taxon-spezifischen Reaktionen zu den untersuchten Faktoren implizieren, dass sich Mykorrhiza Taxa in ihren Funktionseigenschaften unterscheiden und dadurch auch in ihrem Interferenzpotential mit mikrobiellen BKA. Deshalb sollten Umweltverträglichkeitsstudien nicht ausschließlich auf Taxa Abundanzstudien basieren, sondern zusätzlich die Mykorrhiza Gemeinschaftstruktur oder andere pflanzennützliche mikrobielle Gemeinschaften (z.B. nitrifizierende Prokaryoten), miteinbeziehen.

Innerhalb dieser Dissertation wurde eine spezifische Detektionsmethode für den BKA „Foxy-2“ entwickelt, welche die Quantifizierung des BKA in Böden unter dem Einfluss verschiedenster Umweltfaktoren ermöglicht. Diese Detektionsmethode ist außerdem Voraussetzung für die erfolgreiche Registrierung des BKA durch Länderbehörden in Sub-Sahara Afrika und ermöglicht die Indentifizierung günstiger Umwelbedingungen für eine anhaltende Effektivität des BKAs gegen *S. hermonthica*. Desweiteren wurde der Einfluss des BKA „Foxy-2“ auf die einheimische Bodenpilzgemeinschaft untersucht, welcher gegen natürliche Umwelteinflüsse abgewägt wurde. Innerhalb dieser Umweltverträglichkeitsstudien wurde ein spezifischer Fokus auf arbuskuläre Mykorrhiza gelegt, da diese Pilzgruppe für ihre pflanzennützliche Funktion im Boden anerkannt ist. Die Eignung der Mykorrhizaabundanz als Risikoindikator für Umweltverträglichkeitsstudien wurde kritisch evaluiert und untermauerte

den Bedarf zukünftiger Studien, welche die verschiedenen Funktionseigenschaften innerhalb der Mykorrhizagemeinschaft entschlüsseln. Die erhaltenden Ergebnisse aus den Umweltverträglichkeitsstudien bestätigen die Sicherheit des BKA „Foxy-2“ und veranschaulichen den Einfluss verschiedenster Unweltfaktoren auf die Dynamiken der Bodenpilzgemeinschaft. Dies liefert einen wichtigen Beitrag für zukünftige Forschungsstudien zur Widerstandsfähigkeit und Resilienz bodenmikrobieller Gemeinschaften gegen Störfaktoren.

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Appendices

Appendix A: Chemical properties of soils used in the rhizobox experiment (Chapter 2 and Chapter 3). Source: Musyoki et al. (2015).

Table A.1: Soil chemical properties (mean ± standard deviation) obtained from the rhizobox experiment at DAP 42. Different letters within columns indicate significant differences (P<0.05). Source: Musyoki et al. (2015).

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
Embu	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
	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	71.26 ± 7.6a	5.2 ± 0.2ab	4.3 ± 2.4bc	4.9 ± 0.1a
Statistics		***	**	**	*	**	**	***
Machanga	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
	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.

Appendix B: Chemical properties of soils from the field experiments (Chapter 4 and Chapter 5). Source: Musyoki et al. (2016).

Table B.1: Soil chemical parameters of the differently treated soils from Busia and Homa Bay recorded at EC 30 and EC 90 in two cropping seasons (i.e., SR and LR). Values are given as mean (n=3). Different letters within a column indicate significant differences within growth stages (P<0.05). Standard error (SE) is given only for non back-transformed means. Source: Musyoki et al. (2016).

Site	Growth stage	Treatment	Total N		Total C		EOC		EON		NH_{4+}		NO_{3-}		pH	
			[g kg^{-1}]		[g kg^{-1}]		[mg kg^{-1}]		[mg kg^{-1}]		[mg kg^{-1}]		[mg kg^{-1}]			
			SR	LR	SR	LR	SR	LR	SR	LR	SR	LR	SR	LR	SR	LR
Busia	EC 30	C+S	1.42b	2.17a	14.26a	17.07a	242a	273a	22ab	118a	71a	35b	28a	40b	5.48a	5.33c
		F+S	1.38b	2.07a	15.05a	14.76b	264a	247a	14b	203a	97a	81a	29a	101a	5.49a	5.41b
		F+S+T	1.60a	2.14a	14.92a	16.07a	294a	264a	31a	132a	67a	52ab	37a	55b	5.35b	5.43a
		SE	0.04	0.02	0.17	0.34	-	-	-	-	-	-	-	-	0.02	0.02
	EC 90	C+S	2.01a	2.03a	19.50a	15.00a	281a	197b	37a	49a	13a	13a	0a	36a	5.64c	4.91b
		F+S	1.96a	2.09a	16.94b	14.73a	283a	256a	34a	92a	13a	21a	1a	15b	5.70b	4.86c
		F+S+T	1.97a	2.05a	15.66c	14.87a	301a	224ab	26a	70a	14a	17a	1a	20b	5.81a	4.99a
		SE	0.02	0.02	0.60	0.09	-	-	-	-	-	-	-	-	0.03	0.02
Homa Bay	EC 30	C+S	2.37a	3.23b	27.29b	32.68a	294a	295a	204a	148a	123a	76a	34a	18a	7.04b	6.68a
		F+S	2.40a	3.40a	28.48a	32.98a	263a	226b	142ab	150a	143a	89a	38a	14a	6.91c	6.17b
		F+S+T	2.10b	2.36c	26.52b	28.86b	266a	281ab	92b	198a	99a	82a	16b	14a	7.26a	6.11c
		SE	0.05	0.16	0.32	0.69	-	-	-	-	-	-	-	-	0.05	0.09
	EC 90	C+S	1.65c	1.76a	25.74c	27.51a	273a	156a	48a	25a	4ab	3ab	9a	5a	7.35b	7.59a
		F+S	1.83b	1.71ab	28.38b	28.13a	221ab	172a	33a	22a	4b	2b	5a	5a	7.93a	7.22c
		F+S+T	2.01a	1.63b	30.19a	26.05b	214b	178a	40a	29a	13a	4a	2a	5a	7.27c	7.41b
		SE	0.06	0.02	0.66	0.41	-	-	-	-	-	-	-	-	0.10	0.05

Abbreviations: C+S = uncoated maize + *S. hermonthica*, F+S = coated maize (with „Foxy-2“) + *S. hermonthica* and F+S+T = coated maize + *S. hermonthica* + *T. diversofolia*; SR = short rains, LR = long rains, EC 30 = Early leaf development stage and EC 90 = Senescence stage; TC: Total carbon, N_t: Total nitrogen, EOC: Extractable organic carbon, EON: Extractable organic nitrogen, NH_4^+: ammonia, NO_3^-: nitrate, pH: soil pH.