FACULTY OF AGRICULTURAL SCIENCES

Institute of Plant Production and Agroecology

in the Tropics and Subtropics

Field: Plant Production

University of Hohenheim

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Investigating the mode of action of the mycoherbicide component *Fusarium oxysporum* f.sp. *strigae* on *Striga* parasitizing sorghum and its implication for *Striga* control in Africa

Dissertation
Submitted in fulfillment of the requirements for the degree “Doktor der Agrarwissenschaften”
(Dr.sc.agr. / Ph.D. in Agricultural Sciences)

to the

Faculty of Agricultural Sciences

Presented by

Ndambi Beninweck Endah

Kumbo, Cameroon
2011

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Special thanks to Katholischer Akademischer Ausländer-Dienst (KAAD) for financial support during the study period.

This thesis was accepted as a doctoral dissertation in fulfillment of the requirements for the degree “Doktor der Agrarwissenschaften” (Dr.sc.agr. / Ph.D. in Agricultural Sciences) by the Faculty of Agricultural Sciences at the University of Hohenheim on 23rd August 2011

Date of oral examination: 2nd November 2011

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Dedicated to God Almighty who sent His son Jesus Christ to die on the cross of calvary for our sins

ACKNOWLEDGEMENTS

I would like to first of all give all the glory to God Almighty, who gave me life, the opportunity and the strength to carry out this research. Without Jesus, I would not have been able to achieve all what I achieved. I am very grateful.

I am deeply indebted to Prof. Dr. Georg Cadisch, my academic father, who catered for me as far back as from the days of my masters programme, ensuring that not only am I physically ok, but also looking into my financial well being. I am grateful to him for taking time to go through my manuscripts to correct even missing comas, making very useful suggestions, criticisms and additions without which I would not have achieved what I have today. When I look back, I see lots of moments of happiness and smiles as we discussed my work together; moments which remain in my memory due to his friendliness, readiness, willingness and kind nature of putting forward his ideas, critics, and advice. I deeply believe he was sent to me by God.

I am also deeply indebted to Dr. Heller for all her ceaseless efforts in going through the manuscripts for the chapters in this thesis and also abstracts for the numerous conferences which I attended way back as from my masters programme. I can remember our night meetings which had became a routine just to ensure that I meet up with datelines for submission. Not forgetting all the nice food and fruits from her garden which I used to receive sometimes every week. My gratitude also goes to Dr. Gasim for his constant support and suggestions and for providing seeds used for the experiments. Lots of thanks also go to Prof. Otmar Spring for his constant suggestions and support and to Prof. Petr Karlovsky for freely offering his lab to do the larger part of the work in chapter 5, without any charges and for carefully correcting the manuscript for submission. I am really grateful. Special thanks go to Anyangwe Racheal and Bens for their moral support. Special thanks also go to Frau Rassow Bärbel, the lab technician, who has always been patient and willing to explain and demonstrate the various lab techniques which were all new to me. I would also like to thank Dr. Jens Gopfert, Dr. Jens Pfannstiel, Klaibler Idris who contributed to this work. I am also grateful to Judith Zimmermann for her assistance and support. Also to Stefan Becker for technical support. Many thanks to Prof. Marco Thines, and Dr. Frank Rasche for their constant support and advice. My gratitude also goes to Katharina Doell who put in lots of effort to contribute to the last part of this work.

I am also grateful to KAAD for financial assistance without which I would not have been able to concentrate to do this work. Also to the KAAD family with whom I shared lots of

laughs, fun, dances, singing, church services, dishes, seminars, tours, walks, Dr. Marko Kuhn, Frau Saure, and all the KAAD scholars. And to my God given family of Deeper Life Bible Church Stuttgart to whom I owe lots of gratitude Adaku Ebenezar & Abigail, Amosu, Rose & Lucky Okosodo, Nkadji, Alex & Freda Marful, Olatunjuyeh, Oliver Ngueme, Peace Amoatey, Ibe, Adeniyi, Sandra, Marcus, Adisa. I deeply appreciate the efforts of my dear Adesua Joy who did the German translation and Grace Facinet & Sosthène who did the French translation. I am also grateful to my loving brother Dr. Ndambi Asaah, who always had time to proof read my manuscripts and whose contributions were always useful. Not forgetting his wife Ada and children who are my biological family in Germany, for their moral support. I would also like to thank my family back home in Africa Ndambi Paul, Hannah, Mafor, Ofoui, Apha, Ndaya, for all their support both spiritually, socially and morally.

I also owe lots of gratitude to my friends, colleagues and former colleagues of the Institutes of plant production and Botany with whom I shared lots of time, dishes, tours, seminars and experiences: Dr. Mingrelia España, Dr. Wanwisa Punsak, Dr. Juan Cobo, Dr. Petra Schmitter, Michael Scott Demyan, Juan Carlos Laso, Betha Lusiana, Dr. Teodardo Calles, Por Bharudacha, Aunnop Puttaso, Melvin Lippe, Irene Chukwumah, Christian Brandt, Thanh Nguyen, Silke Will, Aspasia Werner, Dr. Carsten Marohn, Chalermchart Wongleecharoen, Christian Brandt, Erick Towett, Kefyalew Sahle, Khalid Hussain, Lena Rathjen, May, Qicheng Bei, Richard Alabeh, Scott Demyan, Thomas Hilger, Tuan Vu Dinh, Elisabeth Zimmermann, Gabriele Kircher, Buck Ingrid, De Mol Michiel, Dr. Dinter Ina, Dr. Dalitz Helmut, Eisele Gabi, Friedrich Micheal, Dr. Gliniars Roberts, Dr. Gopfert Jens, Prof Haas Klaus, Dr. Hammer Timo, Prof. Manfred Kuppers, Land Alexander, Liner Susanne, Dr. Musila Winnie, Dr. Oesker Mathias, Reichle Daniel, Remmele Sabine, Rucker Erika, Dr. Stika Hans-Peter, Dr. Waltemathe Jorg, Weiss Mirjam, Zipper Reinhard, Sökücü Aylin, Hradecky Marc, Raupp Frank, Boust Helena, Runge Fabian, Asta Chimene, Grasse Wolgang, Aschenbrenner Katharina, Trebing-Niyazi Anne and Klaus Hartmann. Special thanks go to Dr. Pfiz Micheal, Magnus Wachendorf and Anna Krupp for correcting the German translation. I am also very grateful to the family of Dr. Reiner Zimmerman and his wife Horna for kindly hosting me during my stay in Göttingen for part of this work.

Finally, my very special thanks and appreciation to my loving treasure husband Matthew, who even though being so far away in Africa, was constantly encouraging me especially in moments when I felt like giving up, he was always telling me to push ahead. I would like to say without his constant support and encouragement, this work would not have been what it is.

CONTENTS

LIST OF ABBREVIATIONS

BEA : Beauvericin
CP : Cortical Parenchyma
CC : Central Cylinder
Co : Cortex
C : Chloroplast
CTAB: Cetyl Trimethylammonium Bromide
CWDE : Cell Wall Degrading Enzymes
CX : Central Xylem Elements
CFU : Colony Forming Units
ER : Endoplasmic reticulum
E : Endodermis
FB : Fumonisin B
FUM1 : Fumonisin 1
FC : Fumonisin C
FP : Fumonisin P
Foxy SS : Foxy 2 coated Susceptible Sorghum
Foxy ST : Foxy 2 coated Tolerant Sorghum
Foxy 2 : *Fusarium oxysporum* f.sp. *strigae* Elzein et Thines
G : Golgi bodies
HPLC-MS-MS : High Liquid Performance Chromatography Mass Spectroscopy
HI : Haustorial Intrusive cell
HB : Hyaline Body
H : Hyphae
IEW : Inner Endodermal Wall
LR White – London Resin White
LM : Light Microscopy
ML : Middle Lamella
M : Mitochondria
N : Nucleus
OEW : Outer Endodermal Wall
PDB : Potato Dextrose Broth
PDA : Potato Dextrose Agar
PE : Phloem
PCR : Polymerase Chain Reaction
RT : Room Temperature
RPM : Rotations Per Minute
Rh : Root Hairs
R : Rhizodermis
SL : *Striga* seedling
SEM : Scanning Electron Microscopy
SR : Sorghum Root
SNA : Sucrose Nutrient Agar
SCP: Sorghum Cortical Parenchyma
SXP:*Striga* Xylem Parenchyma
S : Sclerenchyma cells
TEM : Transmission Electron Microscopy
Va : Vacuole
VB : Vascular Bundle
V : Vessel
VE : Vessel Element

VL : Vessel Lumen
VW : Vessel Wall

CHAPTER 1

General Introduction

1. General Introduction

1.1. *Striga*, a threat to food security in Sub-Saharan Africa

Parasitic weeds of the genus *Striga* belonging to the family Orobanchaceae (Olmstead et al., 2001) are a threat to crop production and remain of great agronomic importance even after many years of research (Aflakpui et al., 2008). Popularly known as witchweeds, *Striga* spp. obtain water, nutrients and carbohydrates from their hosts (Oswald, 2005). Nearly 50 million hectares of field crops are infested by *Striga* annually (Ejeta, 2007a). The most widespread and damaging species is *S. hermonthica* (Lendzemo et al., 2006) which has been described as the most socio-economically important weed in eastern, western and central Africa (Gressel et al., 2004; Gethi et al., 2005). It parasitizes cereals (Mohamed et al., 2006; Aflakpui et al., 2008), mostly in the savannah regions of Africa (Reda et al., 2005; van Ast et al., 2005), thereby affecting the livelihood of approximately 100 million people (Kanampiu et al., 2002). Attack by members of the genus *Striga*, can completely destroy the yield of cereal crops (Khan et al., 2008; Khan et al., 2010). As at 2008, the annual economic loss in maize croplands due to *Striga* in Sub-Saharan Africa was reported to be US$ 383 million (Woomer et al., 2008).

There is growing evidence that the *Striga* problem is worsening, thus posing a serious problem to small subsistence farmers (Ejeta, 2007a). The problem is aggravated by high human population densities which have forced farmers to adopt permanent cropping systems (van Ast et al., 2005) with intensive land use (Aflakpui et al., 2008) making conditions favourable for *Striga*. Being the only continent where per capita food production has been on the decline in the past two decades (Muchena et al., 2005), Africa is faced with the increasing challenge to feed its growing population. Approximately 80% of the population of Sub-Saharan Africa depends on agriculture for food but the agricultural sector is characterized by low productivity with *Striga* being one of the causes for low yields (Khan et al., 2010). To ensure food security, increase in crop production is required and one way of achieving this could be by putting more efforts in fighting the witch weed. However, past efforts to combat *Striga* like hand weeding, have had very little effects (Lendzemo et al., 2007) and limited success. This is because of the close association of its life cycle to that of its hosts (Oswald, 2005), its prolific seed production (Pieterse and Pesch, 1983) and the fact that the seeds germinate only in the presence of strigolactones (Bouwmeester et al.,

2007; Steinkellner et al., 2007) as germination and haustorial formation are dependent on *Striga* seeds receiving chemical cues from host roots (Stewart and Press, 1990).

1.2. *Striga*, its distribution

Members of the parasitic angiosperm genera *Aeginetia, Alectra, Orobanche* and *Striga* have been classified as the most serious parasitic weeds in the world. These genera which belong to the family Orobanchaceae, are obligate root parasites as they require host plants to complete their life cycle (Olmstead et al., 2001). While the broomrapes (*Orobanche* spp.) and *Aeginetia* are holoparasites (entirely lack chlorophyll), the others including *Striga* are hemiparasites having chlorophyll (can photosynthesize) but still rely on hosts for water and minerals. More than 30 species of the genus *Striga* are recognized worldwide with 22 being endemic in Africa which is the center of distribution and diversity of these witchweeds (Mohamed et al., 2001). *Striga* species occur throughout Africa where they are in greater diversity in the grassland savannas north of the equator. There are invasive *Striga* populations in the Arabian Peninsula, South Asia, Australia, and the United States (Mohamed et al., 2001). The most destructive witchweeds which include *S. hermonthica* (Del.) Benth*, S. gesneriodes* (Willd.) Vatke*, S. asiatica* (L.) Kuntze (sensu stricto) are all native to Africa. *Striga* spp. can be found in most regions south of the Sahara except areas where either precipitation is too high or temperatures are too low for its development (Musselman, 1987; Lagoke et al., 1991). *S. gesneriodes* occurs throughout Africa while *S. hermonthica* is a serious pest to cereal production especially in the Sahel region (Senegal to Ethiopia). *S. asiatica* is common in southern and central Africa and in localized areas of West Africa. *S. hirsutu* Benth and *S. lutea* Lour. are closely related to *S. asiatica* and are confined to natural grasslands (Mohamed et al., 2006). *S. forbesii* is significant only in niche areas in Zimbabwe (Gressel et al., 2004).

1.3. Susceptibility of hosts to *Striga* spp.

Striga spp. attach to the roots of specific host plants on which they depend for survival (Oswald, 2005). Continuous cultivation of the same host plant on the same piece of land increases the chances of *Striga* attack as the seed bank will be continuously built up. Crops which produce high amounts of the corresponding *Striga* germination stimulant (e.g. strigolactones) will also be highly vulnerable to attack by *Striga* spp.

Sorghum (*Sorghum bicolor* (L.) Moench), maize (*Zea mays* L.) and millet (*Pennisetum glaucum* (L.) R. Br.) are highly susceptible to *S. hermonthica* infestation (Kim et al., 1997; Ma et al., 2004). These crops are frequently cultivated in a mono-cropping system in many African countries, thus aggravating the situation (Ma et al., 2004; Reda et al., 2005). In Ghana for example, the most important host crop for *S. hermonthica* is maize (Aflakpui et al., 2008), meanwhile, in a large part of Northern Cameroon, the major staple foods including sorghum and millet suffer seriously due to *S. hermonthica* parasitism (Lendzemo et al., 2005). Furthermore, *S. hermonthica* has been reported to cause sometimes 70 – 100% crop loss in maize, sorghum and pearl millet in Nigeria (Emechebe et al., 2004). Similarly, Aflakpui et al., 2008 reported that *S. asiatica* causes severe damage to maize, sorghum, and millet in West Africa while *S. aspera* causes severe losses in upland rice in Ivory Coast. Damage by this hemiparasite, is often severe because it has a remarkably phytotoxic effect on the host plant it evades (Ejeta, 2007a). To a lesser extent, crops also susceptible to *Striga* spp. attack include bambara groundnut (*Vigna subterranean* (L.) Verdc), rice (*Oryza sativa* L.), sugar cane (*Saccharum officinarum*), and soybean (Mohamed et al., 2006; Aflakpui et al., 2008). *S. gesneriodes* has been reported to cause severe damage to cowpea (*Vigna unguiculata* (L.) Walp.) in West Africa and caused problems on tobacco (*Nicotiana tabacum* L.) in Southern Africa and also sweet potato (*Ipomoea batatas* (L.) Lam.) in East Africa (Parker, 1991).

1.4. *Striga hermonthica* parasitizing sorghum

S. hermonthica produces a prolific number of seeds about 5000 – 10000 per plant (Smith and Webb, 1996) and sometimes even more (Ejeta et al., 1992), which when shed, are dormant and can remain viable for up to 20 years under dry conditions (Doggett, 1988). At the start of the rainy season, the seeds undergo preconditioning by absorbing water for some weeks (one to three weeks) (Musselman, 1987; Verkleij and Kuiper, 2000). The host-parasite relationship is initiated underground when root exudates released by the host plant stimulate the germination of *S. hermonthica* seeds. In the presence of a host in close proximity (2 – 3 mm) to the germinated *Striga* seedling, a haustorium is formed (Fig. 1.1). The structure of sorghum roots (monocots), shows an endodermal layer which separates the outer cortical parenchymal cells from the cells of the central cylinder. *Striga* parasitic cells known as haustorial intrusive cells invade first the host root cortex and reach the host vascular system in about five days (Musselman and Press, 1995; Ba, 1988). Subsequently, a

direct xylem connection is established between the parasite and its host (Stewart and Press, 1990). Active penetration of *S. hermonthica* into mature water conducting elements of its host occurs through pits (Dörr et al., 1994). The structure of the haustorium consists of the hyaline body having characteristic large nucleated cells with dense cytoplasm, xylem elements which traverse it, and a xylem disc (Ba, 1988; Dörr, 1997). When attached, *S. hermonthica* plants grow below ground for 4 to 7 weeks prior to emergence (Pieterse and Pesch, 1983).

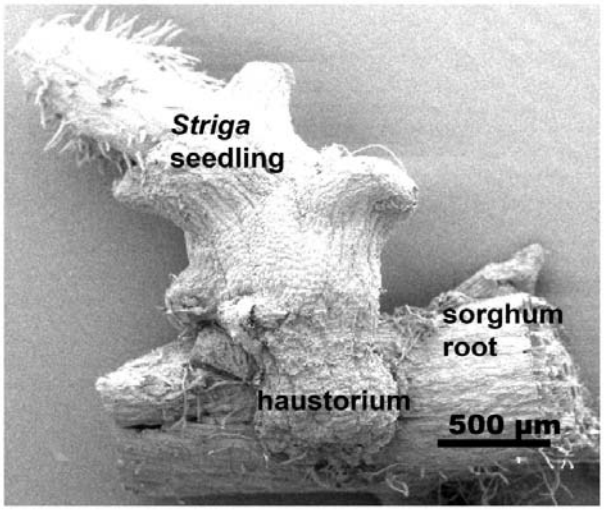

Fig. 1.1. Young underground seedling of *Striga hermonthica* attached to a sorghum root

Sorghum is amongst the most important food and cash crops for millions of rural families in Sub-Saharan Africa (Khan et al., 2010). Sorghum production is greatly hampered by damage due to *S. hermonthica* (Lendzemo et al., 2009). The production of sorghum remained at 19 million tonnes, while the area harvested increased from 18 million ha in 2005 to 20 million ha in 2007 (FAO STAT). In West Africa alone, about 12,000 hectares of land under sorghum cultivation is estimated to be under infestation by *Striga* spp. (Gressel et al., 2004), causing 65 – 70% yield losses in sorghum alone (Ejeta et al., 1992). In African countries, highly susceptible sorghum cultivars are Shanqui Red, P954063 and IS4225, while resistant cultivars include SRN 39, IS 9839, PSL85061, 'Brhan' and El-Mota (Vogler et al., 1996; Ejeta, 2007a).

There are diverse effects of *S. hermonthica* infection on sorghum. For example, *Striga* infection lowers the photosynthetic and transpiration rate of sorghum plants (Frost et al., 1997; Rank et al., 2004; Lendzemo et al., 2006). Stable isotope labeling of *S. hermonthica* in association with sorghum has shown that 35 – 60% of its carbon is

derived from the host (Pageau et al., 1998). When infected by *S. hermonthica*, two tested cultivars of sorghum (tolerant and susceptible to *S. hermonthica*) were significantly shorter than uninfected controls (Frost et al., 1997). The *S. hermonthica* infected sorghum plants had significantly less shoot and root biomass, with significantly smaller leaf areas. The leaf chlorophyll and nitrogen per unit area was greater in *S. hermonthica* infected sorghum plants than in controls (Frost et al., 1997). Analysis of the relationship between the rate of photosynthesis (A) and intercellular CO_2 (C_i), A/C_i as well as ^{13}C isotope discrimination showed a stomatal limitation to photosynthesis in the leaves of *Striga*-infected sorghum plants (Frost et al., 1997) due to the attack by *Striga* or maybe due to nutrient or water competition. Furthermore, the plant growth regulator, abscisic acid, had a double concerntration in the xylem sap of S. hermonthica infected sorghum than in non-infected plants (Frost et al., 1997). Thus the major limitation to productivity is a parasite-induced lowering of host photosynthesis on a canopy scale (Graves et al., 1989). There is however, not a simple relationship between changes in the rate of photosynthesis and alteration in the growth of *Striga* infected plants (Frost et al., 1997).

1.5. Management options for the control of *Striga* in Sub-Saharan cropping systems

Being such a wide spread and severe problem, the control of *Striga* spp. needs an integrated approach (Lendzemo and Kuyper, 2001). There are lots of factors that favour the existence and survival of *Striga* spp. These include: its prolific seed production (5000 – 10000 seeds, Smith and Webb, 1996); the seeds can remain viable for up to 20 years under dry conditions; its intimate life cycle with that of its host and the fact that its seeds germinate only in the presence of a host, furthermore most of its host crops are frequently cultivated in a mono-cropping system with reduced fallow periods (Aflakpui et al., 2008). Additionally, the climatic conditions in the savannas of Africa, with a pronounced dry season and predominantly shallow sandy alfisols are highly suitable for the parasitic plant (Aflakpui et al., 2008). There is no ‘best method’, as no single method effectively reduces *Striga* spp. and improves crop yield in a sustainable way, therefore, a combination of methods needs to be developed (Oswald, 2005).

Breeding for Striga resistance

Ejeta et al. (2000) suggested the following mechanisms of *S. hermonthica* resistance in sorghum: low germination stimulant (LGS) production, low production of the haustorial initiation factor, hypersensitive response, and the incompatible response to parasitic invasion of host genotypes. Amongst these, the best characterized resistance phenotype is low germination stimulant production (Ejeta, 2007a). Breeding involves screening to identify resistant sources and introgression of resistant genes from these sources into elite sorghum lines (Ezeaku and Gupta, 2004; Ejeta, 2007a). This is because the *Striga* resistant varieties (examples are SRN 39 and IS 9839) have undesirable traits such as low grain yield, short stature and small grains which limit acceptance by farmers. By applying pedigree breeding for instance, Ezeaku and Gupta (2004) used SRN 39 and IS 9839 to generate the ICSV 00090 NG variety which combines potential for high yield and resistance to *Striga* spp. However, the mechanisms of resistance are not yet fully understood (Oswald, 2005) eventhough Maiti et al. (1984) showed that resistant sorghum cultivars had marked endodermal and pericyclic thickening with the deposition of silica in their endodermal cells, which were lacking in the susceptible cultivars. Also, there are numerous strains of *S. hermonthica* and *S. asiatica* with different levels of virulence implying that host crops can show resistance in one region but succumb to *Striga* spp in another. For example, Muth et al. (2011) used marker-assisted backcrossing to transfer *Striga* resistance from the low yielding N13 into adapted sorghum genotypes like CSM335 and Tiémarifing but they observed a genotype versus environment effect for some quantitative trait loci classes in that resistance was observed in some regions but not in others.

Cultural control measures

Some practiced cultural control options include crop rotation and soil fertility management by application of N fertilizer as crops produce low germination stimulant in fertile soils (Jamil et al., 2011) and the plant grows faster and stronger thus outgrowing *Striga*. However, De Groote et al. (2010) reported that fertilizer application increased yield but this was not enough to justify the cost of fertilizers. Other control measures include the use of trap and catch crops which make growth conditions for *Striga* less favourable (Lagoke et al., 1991; Oswald, 2005). These methods can reduce the *Striga* seed bank in the soil over time but they need several cropping seasons until an effect can be observed. Trap crops are crops which cause *S.*

hermonthica germination but do not act as hosts to *Striga* such as cotton (Sauerborn et al., 2000; Botanga et al., 2003) or cowpea (Ghehounou and Adango, 2003) which are also important cash crops in savannah Africa. Unfortunately cowpea is susceptible to *S. gesneriodes* (Dube and Olivier, 2001) thus cannot be used in areas of its infestation. The method of using trap crops is highly disadvantageous because it shortens the crop-growing season and requires high labor therefore is not usually adopted by farmers. Hand and hoe weeding of *S. hermonthica* plants which are the most prevalent options are practiced after the parasite emerges above ground and after which it has already inflicted significant damage to the crop (Ejeta, 2007*a;* De Groote et al., 2008). Even though a straight forward approach, *S. hermonthica* densities are usually so high that hand-weeding is extremely time-consuming and *S. hermonthica* usually emerges after the 'normal' weeding of the crop which means the need of coming back to weed the crop several times, therefore not done by most farmers. In pot experiments, van Ast et al. (2005) showed that the use of transplants, deep planting and shallow soil-tillage strongly delayed and reduced *S. hermonthica* infection on both susceptible and tolerant sorghum cultivars. This effect was stronger when all these measures were combined together (van Ast et al., 2005). Although these methods reduced *S. hermonthica* infection in the field, they had no beneficial effect on crop yield (van Ast et al., 2005).

The push-pull is a strategy to control stemborer and *S. hermonthica* in maize farms. The maize crops are intercropped with the forage legume, *Desmodium uncinatum* and napier grass as the border crop. Control of *Striga* is provided by the legume through an allelopathic mechanism in which their root exudates stimulate suicidal germination of *S. hermonthica* seeds. The chemicals exuded inhibit attachment of *S. hermonthica* to maize roots, thereby causing abortive germination of *Striga* seeds that fail to develop and attach to host crops (Khan et al., 2002; Khan et al., 2010). This technology is effective and also enhances productivity of maize farms through suppression and elimination of *Striga* (Khan et al., 2000; Khan et al., 2002). The method is however difficult and slow to establish (Woomer et al., 2008). Adoption by farmers is low because it requires poor farmers to either invest too much money (high initial investment costs in particular for *Desmodium* seeds), labor, or sacrifice land for their important crops to grow crops that have little market value (De Groote et al., 2010).

Intercropping sorghum with other crops also serves as control against *Striga*. For example, the density of emerged *S. hermonthica* plants was reduced when sorghum was intercropped with groundnut (*Arachis hypogaea*) in Gambia (Carson, 1989), dolichos lablab (*Lablab purpureus*) in Sudan (Babiker et al., 1993), cowpea in Cameroon (Carsky et al., 1994), cowpea and haricot in Ethiopia (Reda et al., 1997), and in most cases caused increase in sorghum yield. Intercropping sorghum with sesame (*Sesamum indicum*) reduced *S. hermonthica* and also increased crop vigour (Hudu and Gworgwor, 1998; Reda et al., 2005). The disadvantage of this method is that planting and weeding are more difficult and it requires several accompanying technologies.

Herbicide

Seed dressing of imazapyr-resistant maize with this herbicide has also been used to control *S. hermonthica*. In this technique, relatively small amounts of the herbicide, imazapyr (a systemic herbicide) are applied to maize seeds which provide several weeks' chemical protection from parasite attack (Kanampiu et al., 2002). *Striga* plants die off immediately after attachment to roots of treated plants. The maize remains *Striga* free for the first weeks after planting with significant increase in yields (Kanampiu et al., 2002). This method however has some serious drawbacks because of fast emerging resistance to the herbicide, as the resistance to the herbicide is based on a single recessive gene such that out-crossing of this maize, results in plants which are no longer resistant (Oswald, 2005). This implies that farmers have to buy new seeds every season which is unlikely to happen. Another draw-back is that herbicide resistance is available only for maize thus not used in areas where sorghum is the staple food crop.

Seed treatment with brine NaCl

Treatment of sorghum seeds, be they *Striga* susceptible or resistant varieties, with brine (NaCl) resulted in a significant reduction in *S. hermonthica* shoot emergence in field trials (Gworgwor et al., 2002). Sorghum seeds were soaked overnight in brine solution, drained and air dried before sowing. Brine also had an effect on the plant height and leaf area index which increased as compared to controls. At concentrations of 1.5 M, brine could effectively control *Striga* and increase sorghum grain yield in field experiments. Successful control of *Striga* by seed treatment using brine has also been

reported for maize (Ogunremi and Olaniyan, 1994). But the mechanism by which brine controls *Striga* is not clear and needs to be investigated.

Extracts of traditional Chinese medicinal herbs

Some traditional Chinese herb extracts can increase the rate of *S. hermonthica* seed germination to more than 50% in vitro. These herbs include *Acorus gramineus* Soland., *Agrimonia pilosa* ledeb. var. *japonica* (Miq.) Nakai, *Areca catechu* L., *Citrus tangerine* Hort. et Tanaka, *Cudrania cochinchinensis* (Lour,) Kuda et Masam. among others. The herbs are non host plants which can stimulate *Striga* germination and at the same time provide high value alternative crops used for treating human and livestock diseases. Water extracts from 27 herbs have been shown to inhibit *S. hermonthica* germination. The undiluted extract from *Curcuma longa* L. completely inhibited *S. hermonthica* germination, while extracts from others reduced germination by more than 50% (Ma et al., 2004). However, these experiments were carried out under controlled laboratory conditions and it would be necessary for studies to be done in *Striga* infested fields before considering their potential for use to effectively control *S. hermonthica*.

Biological control options

Biological control has gained considerable attention in recent years (Boss et al., 2007; Sauerborn et al., 2007), as there is public concern regarding environmental problems associated with the use of chemical pesticides (Boss et al., 2007). The basis of biocontrol is the use of beneficial natural enemies to suppress the growth or reduce the population of a target species (Fuchs et al., 1999; Kroschel and Müller-Stöver, 2004). Biological control could involve pathogenicity of host-specific natural enemies (mostly phytopathogenic fungi) to weeds or it could also be due to microbial antagonistic effects which protect the plant, especially roots from attack (Benhamou and Garand, 2001; Benhamou et al., 2002).

a) *Insects*

Smicronyx spp. (Coleoptera: Curculionidae), the gall-forming weevils, are specialized on *Striga* species, as they prevent seed production by developing larvae inside the seed capsules of *S. hermonthica* and thus contribute to reducing the spread and reproductive capacity of *Striga*. Nonetheless, the effectiveness of

preventing *Striga* seed set is limited and not enough to significantly lower the soil seed bank (Smith et al., 1993; Klein et al., 1999).

b) Bacteria

Two strains of *Azospirillum brasilense* L2 and L4 isolated from the rhizosphere of sorghum were able to prevent germination of *S. hermonthica* seeds when stimulated to grow in the presence of *Sorghum vulgare* roots in vitro (Bouillant et al., 1997). L4 has a plant growth promoting effect therefore was beneficial for sorghum growth. When suspended in a synthetic germination stimulant, *Azospirillum* cells blocked radicle elongation forming germ tubes that were shorter and thicker. The radicles manifested an abnormal morphology as seen under light microscopy and had no vacuolated cells in the root elongation zone which could cause the lack of germ tube growth. These bacterial cells were not detected at the surface or inside the *Striga* radicles (Miche et al., 2000). Further tests are however needed in the field so as to investigate the interactions with other soil microbial organisms before their consideration as biocontrol agents.

c) Fungi

The fungi which are presently reported to have potential for use as biocontrol agents against *Striga hermonthica* are arbuscular mycorrhizal fungi and *Fusarium* spp. This does not however limit the potential of other organisms.

Arbuscular mycorrhizal fungi

Arbuscular mycorrhizal fungi (*Glomus clarum* and *Gigaspora margarita*) negatively affect *S. hermonthica* seed germination, reduce the number of *S. hermonthica* seedlings attaching and emerging and also delay the emergence time of *S. hermonthica* (Lendzemo et al., 2006). The fungus also enhances the performance of the sorghum host with a larger beneficial effect on above ground biomass (especially grains) (Lendzemo and Kuyper, 2001). When roots of sorghum were inoculated and colonized by arbuscular mycorrhiza, the number of *S. hermonthica* which attached and emerged was greatly reduced in pot experiments (Lendzemo, 2004). Root exudates from sorghum plants colonized by arbuscular mycorrrhizal fungi caused a reduction in *S. hermonthica* seed germination by 49% after 45 days (Lendzemo, 2004). However, field experiments assessing the role of arbuscular mycorrhizal fungi in *Striga* management are challenging, as studies on how the

dominant cropping systems in savanna Africa affect species composition, abundance and functioning of arbuscular mycorrhizal fungal species in farmers' fields are required. Alternatively, inoculation could be done but practically the cost effectiveness is often not favourable. Constraints also involve the multifuntionality of the symbiosis e.g. the same fungus shows different benefits to the same plant under different environmental conditions or the same fungus shows different benefits to different plants under the same environmental conditions (Lendzemo et al., 2005).

Fusarium

Fusarium spp. are soil-borne fungi and are distributed world wide in every type of soil (Fravel et al., 2003; Kroschel and Müller-Stöver, 2004). The life cycle of *F. oxysporum* has been described to include a saprophytic phase in which they can survive as chlamydospores, followed by germination, growth, and penetration into epidermal and cortical cells of roots, and the phase of parasitic growth into host plants following entry into the xylem elements (Beckman and Roberts, 1995). In the saprophytic phase, these fungi grow and survive for long periods on soil organic matter and in the rhizosphere of many plant species (Garrett, 1970). Many strains are pathogenic infecting roots through a wound or lateral roots causing either root rots or penetrating through the cortex to the xylem and upwards to the stem causing tracheomycosis (Fravel et al., 2003). This affects the host water supply leading to death of the host (Smith et al., 1993; Agrios, 2005). However, non pathogenic *Fusarium* spp. can be used as biocontrol agents against *Fusarium* wilt and has been reported for tomato, watermelon and cucumber amongst others in both greenhouse and field trials (Paulitz et al., 1987; Alabouvette and Couteaudier, 1992; Alabouvette et al., 1993; Larkin et al., 1996; Larkin and Fravel, 1998; Fuchs et al., 1999; Larkin and Fravel, 1999). It is reported that in some cases the biocontrol agent induces resistance to *Fusarium* wilt and in other cases there is competition for nutrients, infection sites and root colonization (Benhamou et al., 2002).

Some isolates proposed for use as biocontrol agents against parasitic weeds include *Fusarium oxysporum* isolates M12-4A, Foxy 2, PSM197 and 4-3-B against *S. hermonthica* (Ciotola et al., 1995; Kroschel et al., 1996; Beed et al., 2007), *F. oxysporum* f.sp. *orthoceras* (FOO) against *Orobanche cumana* (Bedi and Donchev,

1991), *F. arthrosporioides* (FARTH) and *F. oxysporum* (FOXY) against *P. aegyptiaca, P. ramosa and O. cernua* (Amsellem et al., 1999). Widespread use of *Fusarium* spp. as biocontrol agents is however limited because of their ability to produce toxins (Nelson et al., 1992). A wide variety of fungal metabolites are both mycotoxic (toxic to animals and can also affect humans) and phytotoxic (toxic to plants) (Desjardins and Hohn, 1997). Fusaric acid, enniatin, fumonisins, beauvericin, moniliformin, and trichotecenes are amongst the different mycotoxic and phytotoxic compounds produced by *Fusarium* spp (Logrieco et al., 1998; Desjardins, 2006). Phytotoxic effects include necrosis, chlorosis, growth inhibition, wilting and inhibition of seed germination (van Asch et al., 1992; Desjardins and Hohn, 1997; Zonno and Vurro, 1999).

1.6. *Fusarium oxysporum* f.sp. *strigae:* Foxy 2

The fungal pathogen, *Fusarium oxysporum* f.sp. *strigae* Elzein et Thines, Foxy 2 was isolated from diseased *S. hermonthica* plants from Northern Ghana by Abbasher et al. (1995). Foxy 2 is pathogenic to all developmental stages of *Striga* including seeds (Kroschel et al., 1996). Host range studies have shown no negative effect of Foxy 2 on species reported to be highly susceptible to *Fusarium* diseases such as tomatoes, water melon and also on economically important crops cultivated in tropical regions like cotton and sesame (Elzein, 2003). A list of plants tested from 9 different families and proved to have no symptoms of infection when inoculated with Foxy 2 can be seen in Table 1.1. This strain is able to cause a significant reduction in total number of emerged *S. hermonthica* plants by 83%, and *S. asiatica* plants by 91% when pre-inoculated into soil before sowing sorghum (Elzein and Kroschel, 2004a). For its delivery, pesta granules (efficacy of 89% reduction in healthy emerged *Striga* shoots), wheat grain inoculum (efficacy of 97%), as well as sodium alginate (efficacy of 78%), and vermiculite granules (efficacy of 33%), have been studied in the glasshouse (Elzein and Kroschel, 2006a). However, the seed treatment technology which involves film-coating sorghum seeds with Foxy 2 chlamydospores using gum arabic is an attractive option which minimizes the inoculum amount and offers a simple delivery system (Elzein et al., 2006) as seeds are easy to handle. Elzein et al. (2006) reported an efficacy of 81% and 75% reduction of healthy emerged *Striga* shoots in pot and root chamber experiments when sorghum seeds were coated with Foxy 2 using 40% arabic gum. However, the safety of the use of Foxy 2 as biocontrol agent is not yet known and its effects on the desired crop sorghum still need to be investigated.

Table 1: Non-target plants inoculated with Fusarium oxysporum f.sp. strigae, Foxy 2 and tested for host range studies. There was no delay in emergence or decrease in vegetative growth parameters (number of leaves, plant height, root or shoot biomass) in all inoculated plant species compared to their controls.
Source: Elzein and Kroschel (2006b)

Family	*Plant Species*	Common Name	Origin
Chenopodiaecae	*Beta vulgaris* L.	Sugar beet	Germany
Asteraceae	*Helianthus annuus* L.	Sunflower	Hungary
Cucurbitaceae	*Citrullus lanatus (*Thunb.*)* Matsum. et Nakai	Water melon	Germany
	Cucumis sativus L.	Cucumber	Germany
	Cucurbita maxima Duch	Squash	Germany
Fabaceae	*Arachis hypogaea* L.	Groundnut	Mali
	*Cajanus cajan (*L.*)* Huth	Pigeon pea	Ghana
	*Glycine max (*L.*)* Merr.	Soyabean	Ghana
	Lablab purpureus (L.) DC.	Lablab	Sudan
	Pisium sativum L.	Pea	Germany
	Vicia faba L.	Faba bean	Morocco
Malvaceae	*Gossypium barbadense* Mill.	Cotton	Sudan
	Hibiscus esculentus L.	Okra	Ghana
	Hibiscus sabdariffa L.	Roselle	Sudan
Pailionoidae	*Cicer arietinum* L.	Chickpea	Syria
Pedaliaceae	*Sesamum indicum* L.	Sesame	Ethiopia
Poeceae	*Hordeum vulgare* L.	Barley	Germany
	Pennisetum glaucum L.	Millet	Sudan
	Sorghum bicolor (L.) Moench	Sorghum (Dabar)	Sudan
	S. bicolor (L.) Moench	Sorghum (Gadam lhaman)	Sudan
	S. bicolor (L.) Moench	Sorghum (Tabat)	Sudan
	Triticum aestivum L.	Wheat	Germany
	Zea mays L.	Maize	India
Solanaceae	*Lycopersicon esculentum* Mill.	Tomato	Morocco
	Solanum melongena L.	Eggplant	Germany

1.7. Previous attempts to study host-parasite-mycoherbicide interactions

The efficacy and host specificity of *F. oxysporum* (Schlecht.) Synd. & Hans. isolate (FOG) in controlling the parasite *O. ramosa* on its host, tobacco, has been studied by Müller-Stöver et al. (2009). Under controlled conditions, the fungus caused a 55% reduction in underground stages, while in field experiments, FOG reduced number and biomass of *Orobanche* shoots between 50% - 70% in three consecutive years (2006–2008). Tobacco biomass was significantly higher in the fungal treatments (Kohlschmid, 2010).

Very few field studies on host-parasite-mycoherbicide interactions have been reported for *Striga*. Lendzemo et al. (2005) showed that there is a significant interaction effect between *S. hermonthica* and arbuscular mycorhizal fungi on the yield parameters of sorghum in field experiments. They examined stained maize and sorghum roots for mycorhizal colonization using the gridline intersection method (Giovannetti and Mosse, 1980) and showed that 33% of maize and 68% of sorghum plants were colonized by this fungus. In field experiments, inoculation of *S. hermonthica* infested soils with arbuscular mycorhiza and sowing of maize and sorghum caused a significant reduction in the number of *S. hermonthica* shoots due to the fungus (Lendzemo et al., 2005). However, studies disclosing the host-parasite-mycoherbicide interaction at cellular level are rare.

Fig. 1.2. *Striga hermonthica* killed by Foxy 2 (white arrow). Black arrow shows *Striga* which has not yet been killed.

1.8. Justification and research objectives

Control of *Striga* has been so far extremely difficult due to farming practices which result in huge amounts of seeds accumulating in a seed bank, and these seeds remain viable for several years (Lendzemo et al., 2009). Even though the level of *Striga* infestation and damage is increasing; farmers rarely adopt control methods (Oswald, 2005). Reasons being the mismatch between technologies and farmers' socio-economic conditions and the non-availability of economically feasible and effective technologies (Debrah, 1994; Lendzemo and Kuyper, 2001). An appropriated method for *Striga* management adapted for the African farmer is therefore very much needed (Andrianjaka et al., 2007).

The use of fungal biocontrol is now developing as an alternative to the use of chemicals (Boss et al., 2007) because it is a better option considering the intimate life cycle of *Striga* with its hosts. As most of the damage due to *Striga* is done before its emergence, fungal pathogens that can survive in the soil and interfere with the early development stages of *Striga* are thought to be effective. The advantages of such phytopathogenic fungus are that they are usually host-specific and can destroy many stages of the plant including the underground stages as well as seeds thus contributing in reducing the seed-bank (Thomas et al., 1999; Fravel et al., 2003). *Fusarium oxysporum* f.sp. *strigae*, Foxy 2 is thus seen to be suitable against *Striga,* as it is a soil borne fungus. Even though Foxy 2 has been shown to be effective (Fig. 1.2) and specific to *Striga* in the greenhouse (Elzein et al., 2006; Elzein and Kroschel., 2006b), the exact mechanisms of the parasitic process have not been investigated and the events occurring at cellular level during the process from growth/attack to wilting are not known. It is therefore required that mechanisms underlying the mycoparasitic process of such a natural antagonist agent be well understood before its use. It is known that knowledge on aspects such as rate of potential colonization, host specificity, competition and others is necessary for planning the practical application strategy of a biocontrol agent (Deacon and Berry, 1992; Whipps, 1992). Thus, studies on the effectiveness, specificity and timely colonization of this biocontrol agent on *Striga* when delivered as seed coating on sorghum seeds, would be of great interest to contribute to knowledge and substantiate its practical application. Furthermore, to avoid unwanted effects on non-target desired plants (*Striga* hosts), in-depth studies must be carried out to investigate the non-pathogenicity of Foxy 2 to the desired plant sorghum. Its action on *Striga* hosts as well as reactions of hosts towards

the presence of Foxy 2 need to be known as part of the risk assessment to ensure the safe use of such a biocontrol agent. Finally, since some *F. oxysporum* strains are known to produce toxins like fumonisins that have been reported to be of potential risks to human and animal health (Marasas, 1995), it is important that Foxy 2 is evaluated for its toxin production ability so as to verify the safety of the use of this mycoherbicide. On the other hand, the ability of Foxy 2 to produce toxins in *S. hermonthica* shoots which could contribute to the pathogenicity in these plants needs to be investigated. Therefore this study seeks to respond to the research questions: how does Foxy 2 act in its target plant, *Striga hermonthica* and how does the non-target plant sorghum react to Foxy 2?

The main objective of this study was therefore to investigate the mode of action of Foxy 2 in its target weed *Striga hermonthica* and on the roots of *Sorghum bicolor* when delivered as seed treatment on sorghum seeds so as to understand the tripartite interaction between Foxy 2, *S. hermonthica* and sorghum. Adequate investigation of these mechanisms require detailed knowlegde at cell level, thus explaining the use of microscopy in most parts of this study, which provided a high magnification and insight into the process.

The specific objectives were to

- confirm the efficacy of Foxy 2 when coated on two sorghum varieties (*Striga* susceptible and *Striga* tolerant) in reducing healthy *Striga* emergence (Chapter 2)
- examine the action of Foxy 2 in *Striga* seedling haustoria attached to sorghum roots using light and transmission electron microscopy (Chapter 3)
- identify the mechanisms with which Foxy 2 uses to control *S. hermonthica* in compararism to sorghum shoots (Chapter 3)
- study the ability and the extent to which Foxy 2 could colonize and survive on sorghum roots at different stages of growth and compare this to the pathogenic *F. proliferatum* so as to evaluate the defense mechanisms of sorghum and examine the non-pathogenity of Foxy 2 (Chapters 2 and 4)
- investigate the possible production of toxins by Foxy 2 in *Striga* shoots to evaluate pathogenicity and probably in sorghum grains to evaluate its safety (Chapter 5)

1.9. Hypotheses

The hypotheses on which this research was based are that

- Foxy 2 proliferates from the inoculated seed, colonizes the sorghum root and attacks *S. hermonthica* seedlings which attach to the roots,
- Foxy 2 destroys *S. hermonthica* plants by colonizing the vessels and interrupting the flow of water and minerals, and also produces toxins which lead to the death of *S. hermonthica* cells,
- the pathogenic *F. proliferatum* destroys the endodermis and subsequently the sorghum vessels, while Foxy 2 is not able to do so,
- removal of the endodermal barrier could give access of Foxy 2 into the central cylinder which then could colonize the vessels leading to wilting of the sorghum plant.

1.10. Outline of the thesis

This work has been written as a cumulative thesis consisting of 4 research papers, two published (Chapters 2 and 3), one submitted (Chapter 4) and one to be submitted (Chapter 5). In the first chapter, the thesis gives a general introduction which includes an overview of state of the art concerning the *Striga* pandemie and brings out the research already done so far with the *S. hermonthica* biocontrol agent Foxy 2 and others. Chapter 2 reveals the efficacy of Foxy 2 and in addition, light and transmission electron microscopy were used to investigate the ability of Foxy 2 to survive and colonize the surface of sorghum roots. Chapter three gives an in-depth study of the mechanisms of action of Foxy 2 within *S. hermonthica* in the real living complex between the biocontrol agent Foxy 2, the parasite *S. hermonthica,* and its host sorghum. Chapter 4 presents a comparison between the action of Foxy 2 and the pathogenic *F. proliferatum* in sorghum roots. Chapter 5 discloses the role played by the toxin beauvericin in the pathogenicity of Foxy 2 in *S. hermonthica* shoots, and examines the toxin production ability and safety of the use of Foxy 2 by assessing the presence of toxins in sorghum grains and Foxy 2 pure culture. A general discussion is presented in Chapter 6, followed by a section of the references used in the thesis (Chapter 7). The thesis ends with summaries (in English, German and French) and appendices.

CHAPTER 2

Cytological investigations on colonization of sorghum roots by the mycoherbicide *Fusarium oxysporum* f. sp. *strigae* and its implications for *Striga* control using a seed treatment delivery system

2. Cytological investigations on colonization of sorghum roots by the mycoherbicide *Fusarium oxysporum* f. sp. *strigae* and its implications for *Striga* control using a seed treatment delivery system [a]

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2.1. Abstract

The application of the potential *Striga*-mycoherbicide *Fusarium oxysporum* f. sp. *strigae* (Foxy 2) by seed-coating is an appropriate option for delivering and establishing the biocontrol agent in the rhizosphere, the infection zone of the root parasitic weed *Striga hermonthica*. Cytological investigations using light and transmission electron microscopy were performed to assess the pattern and extent of colonization of sorghum roots and shoots by Foxy 2, applied as film-coat on seeds. Germination of Foxy2-treated plants was similar to untreated plants. During sorghum root development, mycelia of Foxy 2 started from seed coat colonizing root surfaces.

[a] **This chapter has been reprinted from:**
Elzein, A., Heller, A., Ndambi, B., De Mol, M., Kroschel, J. & Cadisch, G. (2010). Cytological investigations on colonization of sorghum roots by the mycoherbicide *Fusarium oxysporum* f. sp. *strigae* and its implications for *Striga* control using a seed treatment delivery system. *Biological Control*, Volume 53, Pages 249-257 (DOI:10.1016/j.biocontrol.2010.02.002) with permission from Elsevier (Copyright 2011). The original publication is available at http://www.sciencedirect.com/science/article/pii/S1049964410000332. *Figures 2.1 and 2.4b are not included in the published article.*

The intensity of root colonization increased with time. However, hyphae were not found in the apical zone of the roots. Hyphae growing on the surface of the roots were observed penetrating rhizodermal cells including root hairs, and colonizing the intercellular space and the cells of the cortical parenchyma. Even after four weeks, hyphae were not penetrating the endodermal layer to invade the tissues of the central cylinder. In addition, hyphae of Foxy 2 were also completely absent in the xylem vessels in sorghum shoots even after 11 weeks of sowing, which further proves the non-pathogenicity of the fungus to sorghum. Foxy 2 showed high efficacy in controlling *Striga* in combination with both tolerant and susceptible sorghum varieties in a root chamber experiment, as reflected by the high percentage of diseased *Striga* seedlings (95% and 86%, respectively), recorded after 26 days of sowing. The intensity of fungal root colonization, which coincides with the peak of *Striga* attachments to its host root, will ensure high fungal efficacy and will facilitate the practical use of Foxy 2 against *Striga* using seed treatment delivery. Indeed, this proof of sorghum non-pathogenicity of the fungus will encourage the acceptance of Foxy 2 by authorities and farmers in Africa.

2.2. Keywords: biocontrol efficacy, mycoherbicide, seed coating, root colonization, light microscopy, electron microscopy, *Sorghum bicolor, Fusarium oxysporum* f. sp. *strigae, Striga hermonthica*

2.3. Introduction

The root parasitic weed *Striga hermonthica* (Del.) Benth. is one of the major threats to cereal production in the African savannas, directly marginalising capacity for food production by sometimes causing total crop loss in farmers' fields particularly under drought conditions and infertile soils (Bebawi and Farah, 1981; Sauerborn, 1991; Parker and Riches, 1993; Haussmann et al., 2000; Marley et al., 2002; Gressel et al., 2004; Ejeta, 2007b). The significant yield reductions caused by *Striga* in staple cereals like sorghum (*Sorghum bicolor* L. Moench), maize (*Zea mays* L.), millet (*Pennisetum glaucum* L.) and upland rice (*Oryza sativa* L.) aggravate hunger and poverty for millions of subsistence farmers in Africa. The parasite has a complex life cycle, which is intimately linked to that of its host plants. *Striga* seed germination occurs only when mature seeds are preconditioned by exposure to warm moist conditions for several days followed by germination stimulants derived from host and some non-hosts roots (Worsham, 1987). A germ tube growing close to the host roots elongates towards the

roots, and upon contact develops a haustorium, an organ of attachment to the xylem vessels of the host. The haustorium is the organ of acquisition of nutrients and water from the host (Rich and Ejeta, 2007), causing drought stress and wilting of the host. After several weeks of underground development the parasite emerges above the soil surface and starts flowering and subsequent seed production. Up to 100,000 seeds can be produced by a single plant, which can remain viable for up to 15 years and lead to a re-infestation of the field (Parker and Riches, 1993). Thus, if host plants are frequently cultivated, the *Striga* seed population in the soil increases tremendously and cropping of host plants becomes less and less economically feasible. Hence, a biocontrol agent that is able to attack all developmental stages of *Striga* including seeds is necessary for causing sustainable depletion of the weed seed bank.

In recent years, extensive research efforts and promising results have been made on biological control of *Striga hermonthica* using the soil-borne fungus *Fusarium oxysporum* as a mycoherbicide (Abbasher et al., 1995; Ciotola et al., 1995; Kroschel et al., 1996; Marley et al., 1999). Hence, the potential of *Striga* biocontrol technology as an excellent integrative tool to augment other tricks within integrated approach has gained considerable attention. The *Striga*-mycoherbicidal strain *Fusarium oxysporum* Schlecht f. sp. *strigae* Elzein et Thines abbreviated as “Foxy 2”, is a promising mycoherbicide candidate against *S. hermonthica*. The isolate is highly virulent and host specific to the genus *Striga* (Kroschel et al., 1996; Elzein and Kroschel, 2006b). It possesses a unique DNA sequence enabling it to be considered as a new *forma specialis* (f. sp. *strigae*) (Elzein et al., 2008). Proper formulation, delivery and timely establishment of the potential biocontrol agent “Foxy 2” in the infection zone of *Striga* is necessary for ensuring a high biocontrol efficacy. Hence, progressive research aiming at facilitating and enhancing field application and integration of Foxy 2, resulted in an optimized inoculum production technique of the isolate based on inexpensive agricultural by products (Elzein and Kroschel, 2004b), and in the development of Foxy 2 into Pesta granular formulations or delivery systems using seed treatment technology (Elzein and Kroschel, 2006a; Elzein et al., 2006). The advantage of delivering *Striga*-mycoherbicides by seed treatment procedure over Pesta formulation is that it requires significantly less inoculum amount, establishes the antagonist at the infection sites of *Striga*, and provides a simple, economic, and easy delivery system of biocontrol for subsistence farmers in Sub-Saharan Africa (Ciotola et al., 2000; Elzein et al., 2006).

For controlling root and soil-borne diseases by seed treatment, the antagonists must be able to grow from the seed and colonize the developing roots (Lifshitz et al., 1986; Elad and Chet, 1987; Callan et al., 1990; Jenson and Wolfhechel, 1992). Using a delivery system, where sorghum seeds were coated with Foxy 2 inoculum using gum arabic as an adhesive, a promising control efficacy of *Striga* was achieved in preliminary investigations (Elzein et al., 2006), which suggests the ability of the fungus to colonize the root system of the sorghum plant. Previous microscope observations revealed differences in root colonization intensity of pathogenic and non-pathogenic strains of *Fusarium oxysporum* on different target crops (Alabouvette et al., 2001; Bao and Lazarovits, 2001; Benhamou and Garand, 2001; Olivain et al., 2003). In these studies, root inoculation was done either by direct dipping the root system into a conidial suspension or transplanted in soil heavily infested with conidia. However, in our current research we use a different strain and a novel delivery approach (seed treatment) for microbial agents for root parasitic weeds where the fungal mycoherbicides were film-coated on cereals seeds. Taking into consideration the intimate interaction between host and parasite, time and extent of colonization of the host roots by the fungus is utmost important for ensuring a significant parasite biocontrol and for providing additional evidence supporting the non-pathogenic behaviour of Foxy 2 as a biocontrol agent on non-targets.

The objectives of this study were to perform cytological investigations using transmission electron and light microscopy to study the pattern and intensity of colonization of sorghum roots by Foxy2 as non-pathogenic strain. Efficacy and implication of Foxy2-colonized roots after seed treatment inoculation for successful biocontrol of *Striga* was also evaluated in combination with *Striga*-tolerant and susceptible sorghum cultivars, aiming at providing a potential integrated *Striga* management package.

2.4. Material and Methods

2.4.1. Fungal strain

The fungal strain *F. oxysporum* f.sp. *strigae* “Foxy 2” used in this study was isolated from severely diseased *S. hermonthica* collected in North Ghana by Abbasher et al. (1995). Taxonomic identification of the isolate was confirmed by the Federal Biological Research Center for Agriculture and Forestry, Berlin, Germany, where the isolate was deposited under accession number BBA-67547-Ghana. Since then the isolate was

preserved on Special Nutrient poor Agar (SNA)-medium (Nirenberg, 1976) with 5% (v/v) glycerol at –40oC in the Institute of Plant Production and Agroecology in the Tropics and Subtropics, University of Hohenheim, Stuttgart, Germany.

2.4.2. Inoculum preparation and seed coating procedure

As fungal inoculum, dried chlamydospores were prepared by cultivating actively growing colonies of Foxy 2 in a medium containing 0.5% w/v maize stover (<500 µm) and 20% v/v of wheat (*Triticum aestivum* L.) -based stillage (the spent fermentation broth of ethanol production with poor nutrient constituents) in deionized water. The procedure was previously described (Elzein and Kroschel, 2004b). To prepare dried chlamydospore inoculum, yield of fresh chlamydospores in the pellet was collected after centrifugation and air-dried at room temperature. Thereafter, dried chlamydospores were ground to a fine powder (<100- µm) to facilitate the process of coating and to ensure an even distribution of fungal inoculum on seed films. Viability of the dried material, determined by plating serial dilutions on potato dextrose agar (PDA) plates (Sigma GmbH, Steinheim, Germany), and incubating at room temperature (22±3oC) for three days (Fig. 2.1), was 1.0 x107 CFU g^{-1} (colony forming unit).

The seeds of two sorghum varieties [the *Striga*-susceptible variety “Cowbaula” from Burkina Faso was provided by the International Institute of Tropical Agriculture (IITA-Benin Station) and the *Striga*-tolerant variety “Wad Admed” was provided by the Agricultural Research Corporation (ARC), Sudan] were used in this study. The seeds were first surface sterilised by complete immersion in 1% sodium hypochlorite (NaOCl) for 5 minutes. Then they were rinsed with tap water until water was clear to ensure complete removal of NaOCl, and dried overnight at room temperature. Thereafter, all sorghum seeds were film-coated with a homogenized suspension of gum arabic (AG, 40%) and dried chlamydospores of Foxy 2 (Elzein et al., 2006) using a special seed treatment technology of SUET (SUET Saat-u. Ernte-Technik GmbH, Eschwege, Germany). For coating 1 g of sorghum seeds, 0.048 g of fungal dried chlamydospores with 1.0 x107 CFU g^{-1} were required. The coated seeds were not only uniformly coated, but also abrasion- and dust-free, and with excellent fluidization for assuring high quality coated seeds. All seeds were stored in sealed transparent polyethylene plastic bags (120x90 mm, 50 mµ, Clausen Papier GmbH, Stampe, Germany) at 4 °C before used for further tests.

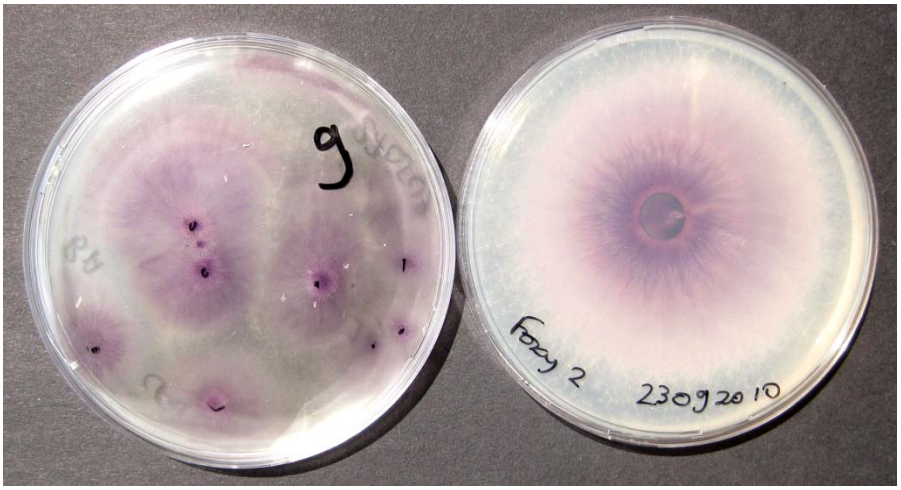

Fig. 2.1. Strain Foxy 2 on agar plate (right) and colonies of Foxy 2 three days to evaluate CFUs (left)

The viability of Foxy 2 on coated sorghum seeds was assayed by dissolving the films of three coated seeds (0.1 g) in 5 ml deionized water in a glass test tube. After serial dilution, 0.1 ml was planted on PDA and CFUs per seed were counted after three days of incubation at 25 ºC. Three replicates per treatment of three replicate plates were used (i.e., nine plates per treatment). The variety "Wad Ahmed" contained $2.4x10^2$ CFUs per seed, while "Cowbaula" had $2.7x10^2$ CFUs per seed.

2.4.3. Cytological investigations

2.4.3.1 Plant material and cultivation

For all cytological investigations the *Sorghum* variety "Wad Admed" were used to study the pattern and extent of colonization of sorghum roots and shoots by Foxy 2 hyphae. Plants were grown *in vitro* between sterile filter paper for 3 weeks, and for longer periods in pots with sterile soil. For *in vitro* cultivation five coated or uncoated (control) sorghum seeds were arranged in rows between two layers of sterile filter paper (Whatman GF/A, 30x20 cm), moistened with sterile water, rolled and wrapped in sterile transparent plastic bags (International Seed Testing Association, 1993) (ISTA). Then the bags were incubated in an upright position at 25 °C in darkness for 48 h and thereafter in 12/12 h light/darkness (Elzein et al., 2006). From each treatment, the roots of 4 fungal-treated sorghum plants and 4 of the corresponding controls were collected at 1, 2, and 3 weeks after germination. Then small root samples (0.5-1.0 cm in length each) were taken from the upper, middle and bottom part of each sampled root for microscopic examinations.

For longer (>3 weeks) periods of cultivation, five Foxy2-coated sorghum seeds were sown in each plastic pot (18×18×18 cm) filled with 4 kg of garden soil (pH 6.7, 5.4% OM), steam-sterilized for 8 h before it was used in pot experiment (Elzein et al., 2006). Pots containing uncoated seeds were set up as controls. Two weeks after sowing, the plants were thinned to three plants per pot, and were watered when necessary. All treatments including controls were replicated four times and arranged as a completely randomized design. The experiment was carried out under glasshouse conditions, where the temperature was adjusted at 34+/-1 ^{o}C / 24+/1.5^{o}C day/night, and relative humidity ranged from 36 to 71%. Lighting was natural except when artificial lights from HQLR-Lamps (1000 W) were used to supplement natural light and to extend day length to 12 hours when necessary. The plants were fertilized with 200 ml of a liquid fertilizer, 0.2% Wuxal® N-P-K (8-8-6) (AGLUKON, GmbH Düsseldorf, Germany), which were split-applied as 100 ml per pot, when the plants were 2 and 4 weeks old. For examining sorghum root colonization, root sections of 0.5 to 1.0 cm in length each, were taken from top, bottom and tip of sampled roots, collected at 4 weeks after planting (WAP). In addition, pieces of sorghum shoots (0.5 to 1.0 cm) were also taken from bottom, middle, and top of the main stems at 5, 6 and 11 WAP for testing sorghum shoot colonization by Foxy 2.

2.4.3.2 Light- and Transmission Electron Microscopy

Light Microscopy (LM): Foxy2-treated and un-treated root samples from sorghum plants, 1, 2 and 3 weeks after germination and grown between sterile filter paper as well as 4-week old root samples from plants grown in sterile soil were collected. Only roots grown in soil were thoroughly washed before fixation. Pieces of sorghum roots (0.5 – 1.0 cm) were taken from the upper (below the seed) and middle part of the root and from the tip region. All root samples were fixed in FAE, a mixture of formaldehyde, acetic acid, and ethanol (70%) (5:5:90 v/v), and stored at 4°C until further processing.

Pieces of sorghum shoots (0.5 – 1.0 cm) from treated and non-treated plants grown in soil were used to collect samples from bottom (1 cm above the base of the root), middle, and top of the shoot after five, six, and 11 weeks of planting. These samples were fixed in 5% buffered glutaraldehyde (0.1 M phosphate buffer, pH 7.2) and also stored at 4°C.

Samples of fixed roots were cut with a razor blade in smaller pieces of about 1 – 5 mm in size and put in fresh fixation solution (FAE). After dehydration in a series of ethanol (75, 90, 100, 100 %, (v/v)) they were infiltrated and embedded in methacrylates Technovit 7100 (Kulzer, Heraeus) or LR-White (Science Services, Munich). Fixed pieces of sorghum shoots cut in sectors of about 5 mm radius and thickness were washed in phosphate buffer, dehydrated in a series of ethanol [30, 50, 75, 90, 95, 100%, (v/v)], and embedded in Technovit 7100. Semithin sections (cross- and longitudinal sections of the roots and shoots) were made by using glass knives for LR-White with the ultratome (Ultracut UCT, Leica) and a metal knife with the rotation microtome HM 340 E (Microm) for Technovit embedded samples. Sections were transferred to Poly-L-Lysin-coated slides, stained with 0.05% aqueous Toluidine blue (Merck) and examined under an Axioplan light microscope (Zeiss). Photographs were taken with a digital camera (Canon Powershot 650). Brightness and contrast of photographs were optimized using Photoshop CS2 (Adobe). *In toto*, 40 root samples were sectioned and investigated by light microscopy.

Transmission Electron Microscopy (TEM): Four-week old roots from treated and un-treated sorghum plants, grown in soil were thoroughly washed under running tap water to remove all adhering particles. Then small samples of the upper region of the roots near the seed, 0.5 x 1-2 mm^2 in size, were cut with a razor blade and immediately fixed in 2.5 % (v/v) buffered glutaraldehyde (0.1 M phosphate buffer, pH 7,2) for one hour and postfixed in 1% (w/v) buffered osmium tetroxide (0.1 M phosphate buffer, pH 7,2) for an other hour. The samples were rinsed three times in distilled water, dehydrated in a series of acetone solutions [30, 50, 70, 100, 100, 100, 100% (v/v)], one hour each step, infiltrated and embedded in Epon 812 substitute (Agar 100 resin, Plano) and polymerized in flat moulds at +60°C. Ultrathin sections were obtained with a diamond knife and the Ultracut UCT (Leica) microtome. They were collected on Pioloform (Science Service, Munich) and carbon coated copper grids, stained with uranyl acetate followed by lead citrate (Reynolds, 1963), and examined with a transmission electron microscope (EM 10, Zeiss) at 60 kV. TEM negatives were scanned (Epson Perfection 2450 Photo) and imported in Photoshop, where brightness and contrast were adjusted.

2.4.4. Bioassay for efficacy evaluation

Following the cytological investigations, the efficacy of 1-year-stored Foxy2-coated sorghum seeds of *Striga*-tolerant Wad Ahmed and susceptible Cowbaula varieties (with 70% fungal viability recovery per seed) in controlling *S. hermonthica* was evaluated using root chamber experiments described by (Linke et al., 2001). *Striga hermonthica* seeds (50 mg) from Sudan (germination 95%), surface sterilized with NaOCl, were evenly distributed on moistened strips of 26×6 cm^2 microfiber glass filter paper (microfiber glass Whatmann GF/A) and placed in the chamber (26 x 6 x 3 cm^3) facing the plexiglass. The chambers were then filled with sterilised sand and wrapped in black plastic supported by rubber bands to prevent light penetration. For preconditioning of the *Striga* seeds, the chambers were moistened and then incubated at room temperature (22±3^oC) for 7 days. Thereafter, the chambers were transferred to the glasshouse, described in section 2.3.1, and three of either Foxy2-coated or uncoated sorghum seeds for each treatment were sown in each chamber between the filter paper and the plexiglass. The treatments tested included: i) Foxy2-coated tolerant sorghum (Foxy ST), ii) Foxy2-coated susceptible sorghum (Foxy SS), iii) uncoated tolerant sorghum (Control ST), and iv) uncoated susceptible sorghum (Control SS). The treatments were arranged as a complete randomized design with 4 replicates. The chambers were placed at an angle (30º) in plastic containers (40×34×12 cm, length, width, depth) to enhance the growth of sorghum roots near the surface of the plexiglass. Two weeks after sowing, the plants in the chambers were fertilized with a solution containing 0.2% liquid fertiliser (Wuxal® N-P-K (8-8-6). Efficacy of Foxy2-coated sorghum seeds in controlling *Striga* was evaluated at 15, 21, and 26 days after sowing. At each evaluation date, root length was measured, and number of healthy and green versus brown, wilted, rotted or with necrotic lesions attached *Striga* seedlings were monitored using a binocular microscope.

For statistical analysis, all data including the arcsine-transformed percentages of diseased *Striga* plants, were tested for normal distribution and variance homogeneity with Shapiro-Wilks-W-Test and the Univariate-Test (Cochrans C, Hartley, Bartlett), respectively. Subsequently, one-way analysis of variance ANOVA was carried out using the STATISTICA software (STATSOFT INC, 2002). In addition, two-way-ANOVA was applied to verify the individual interactions (statistical differences) among the fungal mycoherbicide (as factor I) and sorghum varieties (as factor II). Significant differences between the mean values were determined by Fisher's Least Significant

Difference (LSD) at a significance level of P≤0.05. All results were presented as means of the original data with the standard error of the means, unless otherwise described.

2.5. Results

2.5.1. Colonization of Foxy 2 in sorghum roots

In general, Foxy2-treated plants developed stronger roots than control plants in soil and between filter papers. All sorghum roots exhibited the typical anatomy of monocotyledonous plants with a central cylinder surrounded by an endodermis, cortical parenchyma, and rhizodermis. Within two weeks the innermost cortical layer of the roots differentiated from a characteristic primary endodermis with casparian strips (suberin lamellae) to a tertiary endodermis with a thick inner tangential cell wall. After three to four weeks many stem-borne adventitious roots developed while the first built roots were already in a state of senescence.

Germination of seeds was not negatively influenced by the seed cover containing chlamydospores of Foxy 2. Mycelia of Foxy 2 emerged from chlamydospores during root development and started to colonize root surfaces. Only few hyphae were observed on one week old root samples and there were no hyphae visible on root tips (Fig. 2.2a). In older plants (after two, three, and four weeks), the root surfaces were colonized by Foxy 2; the upper parts of the roots were completely covered with thick mycelia (Fig. 2.2b) that gradually thinned out towards the root tips. Hyphae growing on the surface of the roots were observed penetrating rhizodermal cells. After two weeks, hyphae of Foxy 2 colonized the intercellular space of the cortical parenchyma (Fig. 2.2c) and from there they were also able to penetrate the cortical parenchyma cells. They were found inside many cells of the cortex as well as (inside) rhizodermal cells including root hairs (Fig. 2.2d). There were no reactions of the host cells detectable. In the presence of Foxy 2, the cortex of the roots appeared damaged by hyphae after three to four weeks. Compared to the control only remains of the cortex cell walls or a few collapsed cell layers were left (Figs. 2.2e). Some of the four-week-old control roots, which were already in a state of senescence, showed some collapsed but clearly not destroyed cells (Fig. 2.3a) compared to the roots grown in the presence of Foxy 2 (Fig. 2.3b). Transmission electron micrographs revealed variable stages of degraded cell walls around hyphae as well as condensed and disintegrated cytoplasm where cell organelles were no longer distinguishable (Fig. 2.3c). After four weeks the

central cylinder of the roots still showed intact vessels and xylem parenchyma with functional cytoplasm (Fig. 2.3d) while most of the cortex cells were digested by hyphae (Fig. 2.3b, c). The central cylinders were still enclosed by the thick walled endodermis (Fig. 2.3b, d). Only the thin outer walls of the endodermal cells were collapsed (Fig. 2.3d). Hyphae did not penetrate the thick walled endodermis with its suberin lamellae and were never found in vessels or xylem parenchyma of the central cylinder of the root (Fig. 2.3d). Thus, the colonization of Foxy 2 in the cortex of the roots was not affecting the tissues of the central cylinder. Hyphae of Foxy 2 were growing in the cortex of the roots degrading cell walls and cytoplasm of the parenchymatic tissue step by step (Fig. 2.3c). In addition, during four weeks of colonization Foxy 2 did not develop conidia or chlamydospores in the cortical parenchyma of roots.

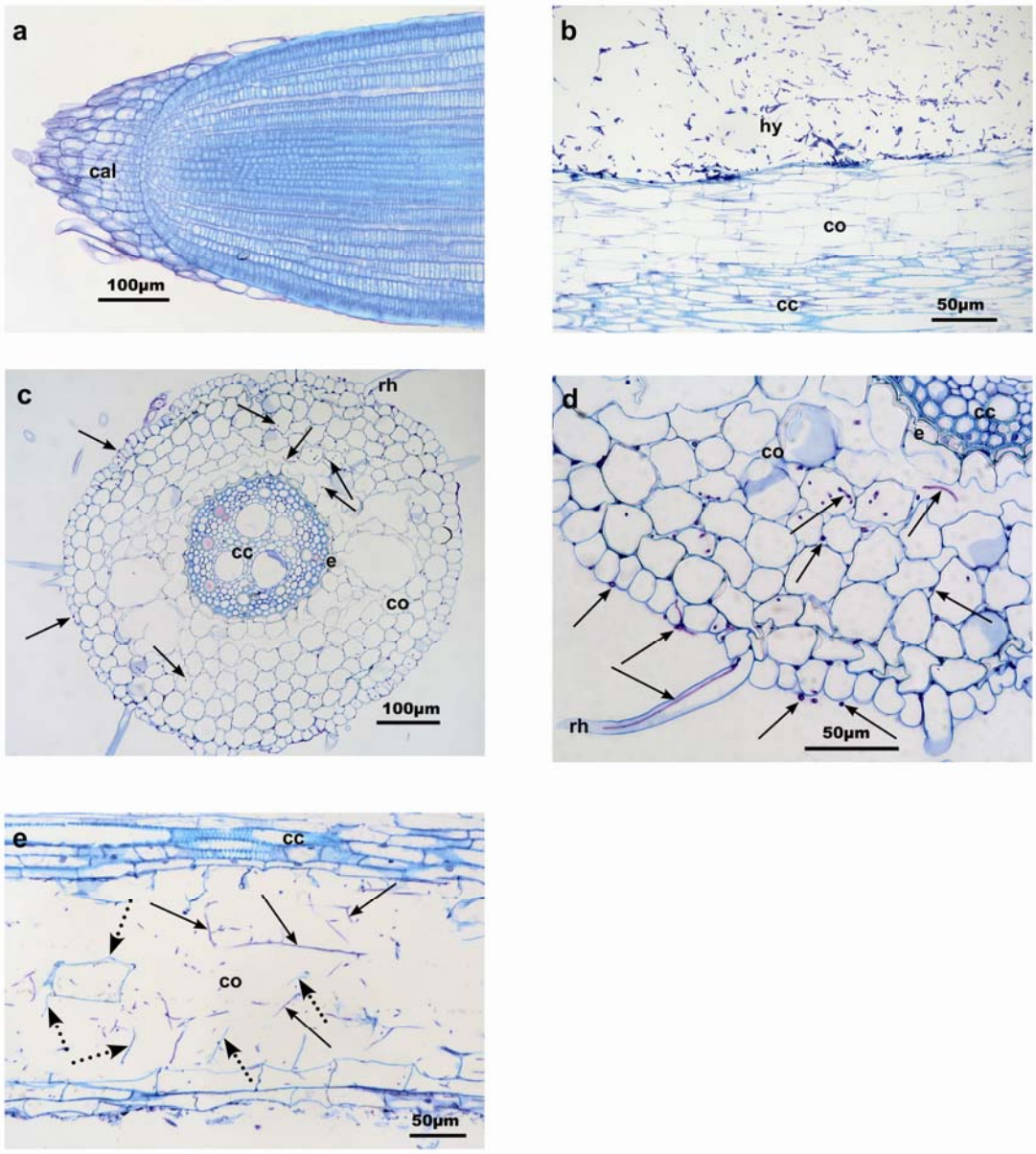

Fig. 2.2. Light micrographs of sorghum roots, grown between filter paper in the presence of Foxy 2, from one to three weeks.

a. Longitudinal section of a one week old root tip. The Calyptra (cal) and all other tissues of the root tip are free of hyphae, also the surface of the root is not colonized by Foxy 2.

b. Longitudinal section of an upper part of a root near the seed of sorghum plant, two weeks old. On the root surface masses of hyphae (hy) are developed. The rhizodermis of the root is attacked by hyphae, but invasion has not followed yet, therefore, cortex (co) and central cylinder (cc) are free of hyphae.

c. Cross section of a middle part of a two week old root of sorghum plant. The root is starting to develop an tertiary endodermis (e) around the central cylinde (cc). The cortex (co) is surrounded by the rhizodermis with root hairs (rh). Hyphae (arrows) are on the surface, in the rhizodermis and inside the cortex (co) of the root. No hyphae are visible in the central cylinder (cc).

d. Detail of Fig. 2.2c: Numerous hyphae (arrows) are growing on the surface of sorghum root, entering the rhizodermis and a root hair (rh) and colonizing the cortical parenchyma (co), where they are visible in the intercellular space and inside the parenchymatic cells. Endodermis (e) and central cylinder (cc) are free of hyphae. Some folded cell walls are due to preparation.

e. Longitudinal section of an upper part of sorghum root after three weeks: Hyphae (arrows) are growing on and in the cortex (co) of the root where they have already destroyed large areas of the tissue. Long strands of hyphae and cell wall remains (dotted arrows) are visible. The central cylinder (cc) is free of hyphae.

2.5.2. Non colonization of Foxy 2 in sorghum shoots

The anatomy of sorghum shoots from seeds treated with Foxy 2 was similar to that of untreated control. The shoots showed the characteristics of most monocotyledonous shoots with vascular bundles scattered within the ground parenchyma and being more closely packed together in the periphery almost forming a ring around the shoot. Shoots of five, six, and eleven weeks old plants did not show any great anatomical difference, only an increase in lignification of vascular bundles and hypodermal tissue occurred with increasing age. There was a complete absence of hyphae in all vessels (Fig. 2.4a) and in all parts of the shoots (Fig. 2.4b) and at all stages of growth.

2.5.3. Efficacy of Foxy 2 in controlling Striga

Efficacy of Foxy 2 in controlling *Striga hermonthica* was evaluated based on the percentage of diseased/dead *Striga* seedlings in the treated root chambers compared to the controls. In general, the fungus Foxy 2 caused severe disease and death of *Striga* seedlings compared to the control treatments, where most of *Striga* seedlings were healthy and vigorous (Fig. 2.5a). *Striga* seedlings (SL) were considered diseased when they turned brown and the colour of the diseased *Striga* changed to dark brown or black (Fig. 2.5b). Browning of the infected seedlings started primarily at the place of connection (haustorium) to the sorghum root, then extended to the growing points, until they wilted and died as a result of the fungal infection.

Throughout the course of the experiment, application of Foxy 2 caused a strong and highly significant increase in the number of diseased *Striga* seedlings on tolerant and susceptible sorghum varieties compared to the controls of both varieties ($p<0.05$) (Table 2.1). The results of the factorial analysis confirmed the efficacy of Foxy 2 by showing highly significant diseases on both varieties ($F = 104.27$, $df = 1, 7$, $p< 0.001$).

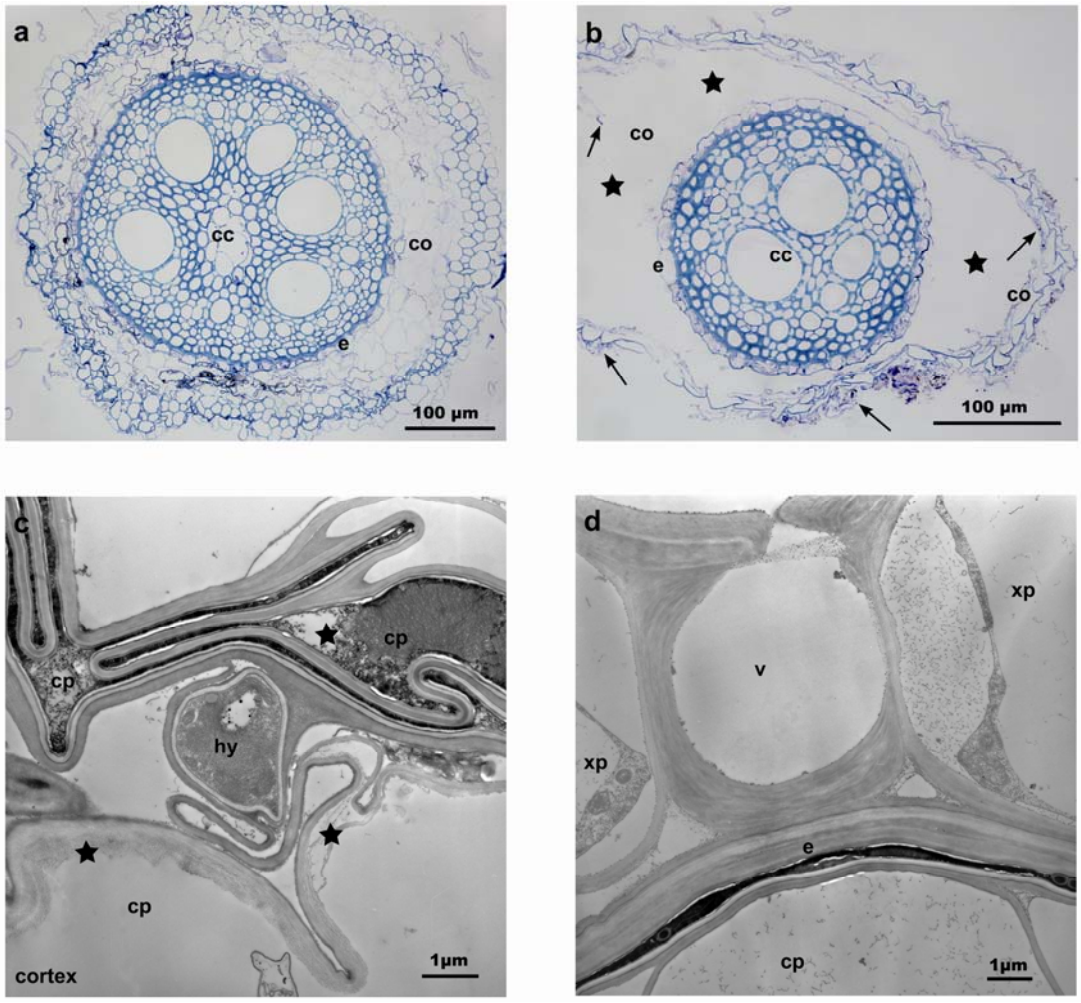

Fig. 2.3. Light- and Transmission elelctron micrographs of roots grown in soil after four weeks.

a. Light micrograph of a cross section of an upper part of sorghum root (control): After four weeks the tertiary endodermis (e) around the central cylinder (cc) is full developed. Rhizodermis and the cortical parenchyma (co) showing already signs of senescence as most of the cells are collapsed.

b. Light micrograph of a cross section of an upper part of sorghum root, grown in the presence of Foxy 2 after four weeks: Vast areas of the cortical parenchyma are disappeared (asterisks). Only few rows of cells and some hyphae (arrows) are left, the other cells of the cortex (co) are disintegrated by Foxy 2 and washed away before preparation. The central cylinder (cc) surrounded by the endodermis (e) is still intact.

c. Transmission electron micrograph of a cross section of an upper part of sorghum root grown in the presence of Foxy 2 after four weeks: Detail of collapsed and destroyed cortical parenchyma cells (cp). In the cortex an active hypha (hy) of Foxy 2 is visible. The cortical parenchyma cells showing variable signs of destruction; partly degraded cell walls and degraded cytoplasm (asterisks).

d. Transmission electron micrograph of a cross section of an upper part of sorghum root, grown in the presence of Foxy 2 after four weeks: Detail of an endodermal cell (e), cortical parenchyma cell (cp), and parts of the central cylinder; xylem parenchyma cells (xp) and a vessel (v). The collapsed endodermal cell (e) is showing the typical thick inner tangential cell wall. While there are no organelles visible in the dark stained cytoplasm of the endodermal cell and the cortical parenchyma cell (cp), the central cylinder is showing an intact vessel (v) and xylem parenchyma cells with functional cytoplasm (xp).

The percentage of diseased/killed *Striga* shoots showed a rapid increase during the course of the experiment, whereas in control treatments the rate of diseased attached *Striga* remained significantly lower (Table 2.1). After 15 days, there was a significant difference in percentage of diseased *Striga* between treated and control treatments in both the tolerant and the susceptible sorghum varieties. At this stage, all *Striga* seedlings were totally healthy i.e., no disease symptoms were recorded, in the control treatments of both varieties, while in Foxy 2-treated tolerant and susceptible varieties 8.5% and 6% diseased seedlings, respectively, were counted.

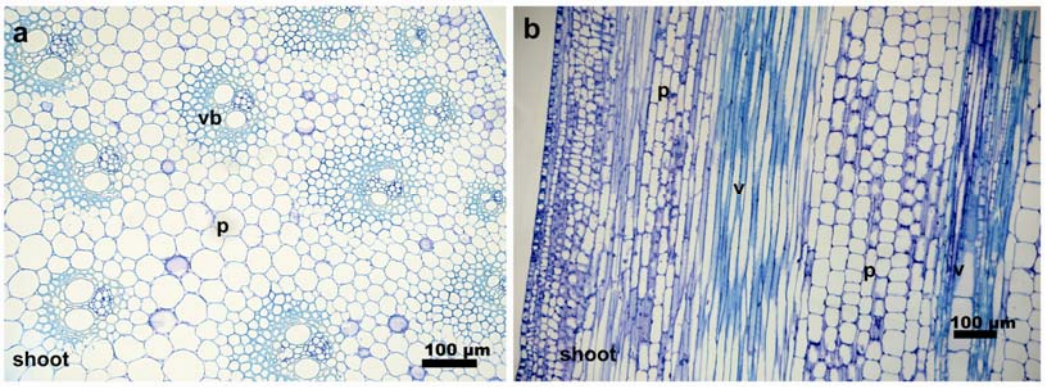

Fig. 2.4. Light micrograph of a cross section of sorghum shoot, grown in soil in the presence of Foxy 2 after five weeks.

a. There are no hyphae in the vascular bundles (vb) or parenchyma (p) and no negative influence of Foxy 2 in the shoots visible. The shoot is showing the typical anatomy of a monocotyledon, vascular bundles (vb) are scattered in parenchymatic tissue (p).

b. Longitudinal section also showing complete absence of hyphae in vessels (v).

Twenty one days after sowing, the combination of Foxy 2 with the tolerant and susceptible varieties caused 56% and 41% disease on *Striga* seedlings, respectively (Table 2.1), whereas, their respective controls have only 14%, and 2%. The results showed a similar tendency after 26 days of sowing i.e. that Foxy 2 treated sorghum of the tolerant variety had 95% diseased *Striga* compared to 86% recorded in the case with the susceptible variety. On the same evaluation date, however, only 27% and 14% seedlings were dead in the control treatments of the tolerant and susceptible varieties, respectively (Table 2.1).

Table 2.1. Effect of Foxy 2, applied as a seed treatment, on sorghum root length and total number of attached and diseased Striga hermonthica seedlings

Treatment[1]	15 days after sowing[2]			21 days after sowing			26 days after sowing		
	Root Length (cm)	No. of SL attached	No. of SL diseased	Root Length (cm)	No. of SL attached	No. of SL diseased	Root Length (cm)	No. of SL attached	No. of SL diseased
Control SS	10.3 (1.6)a	28.3 (7.2)ab	0.0 (0.0)b	11.3 (1.9)a	32.3 (8.4)a	0.8 (0.75)b	11.5 (1.8)a	35.8 (6.9)a	5.0 (4.7)b
Control ST	8.3 (2.2)a	15.0 (5.5)b	0.0 (0.0)b	9.0 (2.4)a	20.8 (4.8)a	3.0 (1.5)b	9.3 (2.5)a	24.0 (5.6)a	6.5 (3.7)b
Foxy SS	12.3 (0.8)a	41.3 (3.4)a	2.5 (1.0)a	13.0 (0.8)a	44.5 (2.9)a	18.3 (3.2)a	13.0 (0.7)a	45.8 (3.7)a	39.5 (4.4)a
Foxy ST	9.0 (1.7)a	35.3 (8.4)a	3.0 (0.4)a	9.8 (1.4)a	38.3 (8.7)a	21.5 (4.4)a	9.8 (1.4)a	40.5 (7.5)a	38.5(6.4)a

[1] Treatments: SS = Sorghum Susceptible; ST = Sorghum Tolerant. [2] SL = Striga seedling. Values in brackets represent standard errors of the means. Mean values followed by different letters within the same column are significant difference after Fischer's LSD-Test at $p<0.05$.

After 26 days of germination, the main effects of type of sorghum variety (tolerant and susceptible) on either sorghum root length or total number of attached *Striga* seedlings were not significant (P>0.05) (F = 2.62, df = 1, 7, p = 0.132, for root length and F = 2.70, df = 1, 7, p = 0.126, for total number of attached *Striga*). Similarly, the interactions between fungal mycoherbicide (factor I) and sorghum variety (factor II) were not significant (P>0.05) for the total number of attached *Striga* (F = 0.083, df = 1, 15, p = 0.777), for the total number of diseased *Striga* (F = 0.127, df = 1, 15, p = 0.728) and for the sorghum root length (F = 0.063, df = 1, 15, p = 0.805). However, both control and Foxy 2-treated susceptible sorghum variety appeared to have higher numbers of attached *Striga* seedlings than the tolerant one (Table 2.1).

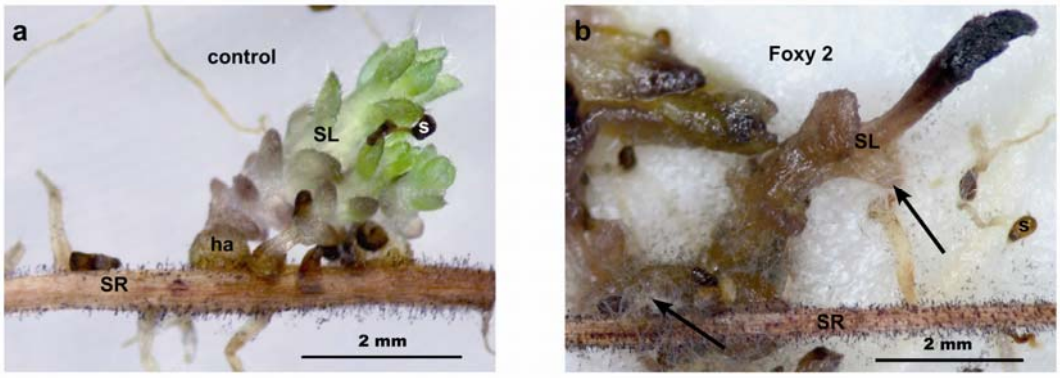

Fig. 2.5. Healthy and diseased/dead *Striga* seedlings as seen under the binocular.
a. Numerous healthy *Striga* seedlings (SL) attached via haustorium (ha) to sorghum roots (SR) in the control treatment three weeks after sowing in root chamber; (*Striga* seed:(s).
b. Diseased/dead *Striga* seedlings (SL) on Foxy 2-treated sorghum root (SR) three weeks after sowing in root chamber.

Although, there was no statistical significant difference (p>0.05) between Foxy 2-treated tolerant and susceptible varieties in causing disease symptoms on the attached *Striga* seedlings (Table 2.1).

2.6. Discussion

Proper delivery and timely establishment of the potential biocontrol agent *F. oxysporum* f. sp. *strigae* “Foxy 2” in the appropriate infection zone of *Striga* is necessary for ensuring a high biocontrol efficacy and facilitating field application and integration. For environmental reasons (minimization of exposure) as well as for the desire to extend the effect of the treatment beyond the germination, there is an increasing interest in biological control of seed- and soil-borne diseases by treating seeds with antagonistic microorganisms (Jenson and Wolfhechel, 1992), for which microbial agents must be able to grow from the seed and colonize the developing root system in order to be effective.

Delivering the potential *Striga*-mycoherbicides by seed treatment (Elzein et al., 2006), which is applicable to all varieties of cereal crops attacked by *Striga*, and compatible with other *Striga*-control methods, could accelerate the adoption and implementation of this biocontrol option by farmers.

2.6.1. Colonization of Foxy 2 in sorghum roots

By coating the sorghum seeds with Foxy 2, the fungus has the opportunity of being the first colonizer of the host roots and then subsequently attacking *Striga* already during its attachment phase. The seed cover of gum arabic with its high sugar content (Grieve, 2007) appears to have provided a suitable nutrient source for Foxy 2 to start the colonization of the root system. Our cytological investigations showed that Foxy 2 was a slow but constant colonizer of roots. While only few hyphae were detectable on roots after one week, there were thick mycelia on the upper parts getting thinner versus the root tips after two to three weeks. Probably, the growth of the roots was faster than the growth of the mycelia. A similar observation was made on roots of tomato (*Lycopersicon lycopersicum*) by Olivain et al. (2006). By investigating the colonization of a pathogenic and a non-pathogenic strain of *F. oxysporum*, they found that both strains were able to colonize the entire root surface, with the exception of the apical zone. This is in contrast to investigations of Turlier et al. (1994) who found that the pathogenic strain *F. oxysporum* f. sp. *lini* Snyder & Hansen was entering the apical zone where the endodermal layer was not yet developed and the xylem tissue became infected when it was an undifferentiated group of cells just formed from subapical initials.

Foxy 2 was not only colonizing the root surface, it was also colonizing the cortical parenchyma of the host roots. In the root cortex hyphae were growing in the intercellular space, but also penetrating parenchyma cells and digesting cell walls and cytoplasm step by step. But Foxy 2 hyphae were not able to pass the endodermal layer to enter the tissues of the central cylinder. Transmission electron microscopy revealed that the central cylinder of the roots showed intact vessels and xylem parenchyma with functional cytoplasm. Hyphae did not penetrate the thickened walls of the endodermal cells and were never found in the central cylinder of the root even after four weeks. This is in contrast to pathogenic *Fusarium* strains which cause typical disease symptoms of tracheomycosis, e.g. wilting, chlorosis, and necrosis, by invading the central cylinder, infecting xylem parenchyma, and colonizing vessels. For example, the pathogen *F. oxysporum* f.sp. *radicis-lycopersici* Jarvis & Shoemaker was penetrating the endodermis

of tomato roots between 96 and 120 h after inoculation, and 24 h later the central cylinder was colonized (Charest et al., 1984).

Foxy 2 acts like other non-pathogenic *Fusarium* strains by colonizing the root surface and the cortex of roots (Olivain et al., 2003; Paparu et al., 2006). Already Olivain and Alabouvette (1997) showed that *Fusarium* strains were able to colonize the cortex of roots to some extend. There are variations in the ability of non-pathogenic *Fusarium* strains to colonize host tissue. While *F. oxysporum* strain Fo47 colonized the root surface and infected only few cortical cells in tomato (Olivain and Alabouvette, 1997), in flax (*Linum usitatissimum* L.) (Olivain et al., 2003) the same strain was able to colonize cortex and central cylinder without disease symptoms in young seedlings of *Eucalyptus viminalis* Labill. (Salerno et al., 2000). There are reports that non-pathogenic fungi cause intense defence reactions such as wall appositions, a general wall thickening, accumulation of osmiophilic material, collapsed cells, or intercellular plugging (Olivain et al., 2003). In the present study, there were no defence reactions in host cells visible.

Although, Foxy 2 was digesting the cortex of roots, there were no negative effects to the growth of the sorghum plants. Instead the Foxy2-colonized plants developed a stronger root system than the control plants. It might be that the reaction of the plant to the destruction of the root cortex is an enhanced growth of the root system and, especially the development of side roots.

The cytological investigations showed that Foxy 2 is present in sorghum roots over a long period of time. The colonization of Foxy 2 started with seed germination, and hyphe were still in the root cortex after four weeks, when roots were already in the state of senescence. Foxy 2 hyphae metabolised step by step the cortical parenchyma cells. They were active and ready to attack *Striga* seedlings over this long period of time being active in and around roots, the site where *Striga* seedlings attach. While it did not cause disease symptoms in the crop sorghum it was highly pathogenic to its specific target weed *Striga* by completely destroying it. Therefore, Foxy 2 applied as seed treatment is a promising biocontrol agent.

2.6.2. Non colonization of Foxy 2 in sorghum shoots

Foxy 2 did not colonize the shoots of sorghum as its hyphae were not detected in the xylem vessels in sorghum shoots even after 11 weeks of sowing. This indicates that hyphae did not enter the vascular tissue of the root to grow through the vessel into the

shoot of sorghum as pathogenic *Fusarium* species would do, and thus further proving evidence of the non-pathogenic behaviour of Foxy 2 to sorghum even at a later stage of growth. Furthermore, our results showed that coating with Foxy 2 did not cause any direct or indirect negative effects on germination and growth of the non-target plant sorghum. Likewise, Elzein and Kroschel (2006b) and Marley et al. (2006) reported immunity of all sorghum cultivars tested in host range studies; none has developed any symptoms of disease infection (e.g. pathogenic *F. oxysporum* strains cause wilting, dieback, necrosis and chlorosis), and no direct or indirect negative effects on their vegetative growth parameters including number of leaves, plant height, photosynthetic rate, and root and shoot biomass, were recorded after heavily inoculation with *F. oxysporum* f. sp. *strigae* (Foxy 2 and PSM 197) inoculum.

2.6.3. Efficacy of Foxy 2-colonized roots in controlling Striga

The presence of the fungus along sorghum roots, on the surface and in the cortex, enables it to act effectively against *Striga* parasitism during the parasite haustorial initiation and establishment of vascular connections with host roots. The application of Foxy 2 by seed treatment on sorghum seeds showed high efficacy against *S. hermonthica* even after one year of storage as it caused disease in >86% of *Striga* seedlings attacking sorghum roots, in both *Striga* susceptible and tolerant varieties (Fig. 2.5b). This high efficacy of Foxy 2 is related to its high pathogenicity to *Striga* and its ability to colonize and spread along roots of sorghum seedlings. Because the life cycle of *Striga* is intimately linked to that of its host, timing and extent of colonization of the host roots by the fungus is of utmost important for ensuring a significant parasite control. Preconditioning, germination, haustorial initiation, development and attachment, and establishment of vascular connections of the parasite take place in the vicinity of host roots and require 18 to 29 days depending on the species and environmental conditions (Rich and Ejeta, 2007). On the other hand, the age of sorghum roots is also a crucial factor in *Striga* parasitism. Maximum *Striga* germination and attachment was reported to take place when sorghum was 3-4 weeks old (Dawoud, 1995). The microscopic investigations confirmed the establishment and long presence of Foxy 2 along the root surface and in the cortex of sorghum during second to fourth week after germination. Thus the peak of number of *Striga* that get attached to host roots coincides with a strong host root colonization by Foxy 2, enabling the fungus to infect nearly all attached *Striga* seedlings. The fact that Foxy 2 is virulent against all developmental stages of *S. hermonthica* including seeds (Kroschel et al., 1996) makes the spreading of fungal mycelia at some distance from the root and in the rhizosphere necessary. An indication

of the spread outside the root surface is that seeds and germ tubes of germinated *Striga* became infected even the development of attachment to the host root (Elzein et al., 2006). Thus, these advantages make the delivery of Foxy 2 by seed treatments appropriate and efficient for causing high and sustainable depletion of the weed seed bank taking into account the high fecundity and longevity of parasite seeds in soils (up to 15 years) (Parker and Riches, 1993).

2.6.4. Integrating Foxy 2 and Striga-tolerant sorghum

Striga resistant (low infestation with *Striga*) and tolerant (high yield in spite of *Striga* infestation) sorghum and maize varieties play an important role in integrated *Striga* management because their application is easy and supposed to be the most cost effective and environment-friendly method in *Striga* control for farmers (Ejeta and Butler, 1993; Gupta and Lagoke, 2000; Ejeta, 2007b). Throughout the course of the experiment, application of Foxy 2 as film-coats on *Striga* tolerant “Wad Ahmed” and susceptible “Cowbaula” sorghum varieties caused highly significant increases in the number of diseased *Striga* seedlings. Foxy caused an increased disease incidence in *Striga* seedlings over time especially, indicating that Foxy 2 and the tolerant variety can make additive effects against early developmental stages of *Striga*. However, the difference between a *Striga* tolerant and the susceptible sorghum variety were small and non significant and need to be tested under field conditions where other parameters such as number of emerged *Striga* plants and yield components are also considered. The sorghum variety “Wad Ahmed” has been identified as tolerant/resistant to *S. hermonthica* based on a low number of emerged *Striga* plants and high grain yields under field conditions of Sudan (Prof. A.G. Babiker, Agricultural Research Corporation, Sudan, pers. com.). The higher percentage of diseased *Striga* seedlings in combination with the tolerant variety in the control treatment could be attributed to its ability to tolerate further *Striga* development by possessing some degree of resistance such as post-attachment hypersensitive reactions with *Striga* or through antibiosis, i.e. reduction of *Striga* development through unfavourable phytohormone supply by the host (Haussmann et al., 2000). This incompatibility may be weakened the *Striga* parasite, which become more susceptible to the fungal infection. The evidence that the sorghum variety “Wad Ahmed” possesses a resistant mechanism was given by Hiraoka and Sugimoto (2008). These and other authors (Mohamed et al., 2003) further described that the roots of the tolerant Wad Ahmed frequently became reddish in response to *Striga* attack, which was also observed in our studies. Furthermore, the premature death of *Striga* under natural conditions is a known phenomenon, which may be in part due to competition for

nutrients, but its exact cause still remains unknown. The recorded dead *Striga* in the control treatments could be in part due to the same causes.

In recent reports, the impact of *Striga* resistant sorghum cultivars showed synergies when used in combination with other agronomic interventions (Mbwaga et al., 2007) or with a mycoherbicidal approach (biocontrol) (Schaub et al., 2006; Elzein et al., 2007) in an integrated *Striga* management program. Therefore, scaling up of Foxy2-seed treatment technology to support and enhance the existing *Striga* control measures could facilitate large-scale application of *Striga*-mycoherbicides in Africa.

In conclusion, the temporal-pattern and extent of colonization and association of *Striga*-mycoherbicide Foxy 2 with sorghum roots supports the efficiency and appropriateness of seed treatment delivery system for ensuring a high biocontrol efficacy. The cytological proof of sorghum non-pathogenicity of the fungus may encourage the acceptance and field application of Foxy 2 by authorities and farmers. The compatibility, synergy and efficacy of *Striga*-mycoherbicide Foxy 2 with *Striga* resistant/tolerant sorghum using seed treatment indicates that effective, adoptable and environmentally friendly integrated control strategies for *Striga* can be successfully realized.

2.7. Acknowledgements

The authors sincerely thank Bärbel Rassow for technical assistance in transmission electron and light microscopy, the seed company SUET (Saat-und Ernte Technik GmbH), Eschwege, Germany, for technical support and coating of seeds, and Prof. A.G. Babiker, Agricultural Research Corporation, Sudan, for supplying of *Striga* seeds. Financial supports by the Alexander von Humboldt Foundation (AvH) and the Eiselen Foundation are gratefully acknowledged.

CHAPTER 3

Colonization and control of *Striga hermonthica* by *Fusarium oxysporum* f. sp. *strigae*, a mycoherbicide component: An anatomical study

3. Colonization and control of *Striga hermonthica* by *Fusarium oxysporum* f. sp. *strigae*, a mycoherbicide component: An anatomical study [b]

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3.1. Abstract

The fungal strain, *Fusarium oxysporum* f. sp. *strigae* (Foxy 2) has proved to be specific and effective in controlling *Striga hermonthica* (Del) "Benth." However, little is known on its mode of action. The objective of this study was to evaluate the time and extent of colonization of the parasite by the fungus which is of critical importance considering the mutual interaction between *S. hermonthica* and its hosts. Anatomical investigations using light and electron microscopy were performed to assess the pattern of colonization and control of *S. hermonthica* seedlings and *Striga* shoots by Foxy 2. The connection between the sorghum root and the *S. hermonthica* seedlings showed that *Striga* haustorial intrusive cells penetrated the central cylinder of sorghum forming a continuous xylem connection but further growth of the *S. hermonthica* plant was prevented by Foxy 2.

[b] **This chapter has been reprinted from:**
Ndambi, B., Cadisch, G., Elzein, A. & Heller, A. (2011). Colonization and control of *Striga hermonthica* by *Fusarium oxysporum* f. sp. *strigae*, a mycoherbicide component: An anatomical study. Biological Control, Volume 58, Issue 2, Pages 149-159, (DOI:10.1016/j.biocontrol.2011.04.015), with permission from Elsevier (Copyright 2011). The original article is available at http://www.sciencedirect.com/science/article/pii/S1049964411000995. *Figure 3.3f is not included in the published article*.

Hyphae invaded and digested all *Striga* seedling tissues belowground including haustorial intrusive cells, hyaline tissue, vessels, central xylem elements and *Striga* cortical parenchyma within 26 days after sowing Foxy 2 coated sorghum seeds. In *S. hermonthica* plants that managed to emerge, hyphae had penetrated and colonized vessels clogging them over long distances, and were identified even in the top of the shoots. In contrast to young seedlings, there was no digestion of vessels in the diseased shoots above-ground. Transmission electron microscopy revealed that some hyphae were embedded in an electron dense wall coating which was formed along the secondary vessel walls in diseased *S. hermonthica* plants. This electron dense wall layer was uneven in thickness and also occurred along xylem parenchyma pit membranes apparently preventing invasion of xylem parenchyma cells. Penetration of intertracheal pit membranes by hyphae was observed from vessel to vessel while attempted penetration of xylem parenchymal pit membranes was foiled by production of wall apposition beneath the pit. Thus, two mechanisms were identified by which Foxy 2 controls *S. hermonthica* i) complete digestion of *S. hermonthica* seedlings inside the host and ii) clogging of vessels of emerged *S. hermonthica* plants by hyphae contributing to wilting and subsequent death.

3.2. Keywords: *Striga hermonthica,* colonization, *Fusarium oxysporum* f.sp*. strigae,* biocontrol, light microscopy, transmission electron microscopy, anatomical

3.3. Introduction

Amongst the root parasitic weeds of the genus *Striga, S. hermonthica* (Del.) Benth. remains the major constraint to cereal production in the tropics and subtropics (Berner et al., 1995; Lagoke et al., 1991; Mboob, 1986; Parker and Riches, 1993) exerting its greatest impact in low-input farming systems. *S. hermonthica* affects several, mainly subsistence cereal crops like sorghum (*Sorghum bicolor* (L.) Moench), maize (*Zea mays,* L.), millet (*Pennisetum glaucum* L.), and rice (*Oryza sativa*, L.). In West African countries (Nigeria, Burkina Faso, Niger, Mali, Ghana, Chad, and Cameroon), yield losses of up to 90 – 100% in sorghum and millet due to *S. hermonthica* were reported (Haussmann et al., 2000). It is estimated that across the African continent annual yield losses caused by *Striga* adversely affect the livelihood of over 300 million people (Ejeta, 2007b).

The haustorium is the organ of parasitism and forms the morphological and physiological bridge between the parasite *Striga* and its hosts through which nutrients and water are obtained by the parasite (Musselman and Press, 1995). Penetration of *Striga* haustoria into the host root takes place after a series of cell differentiation and changes; the centrally located cells of the attached haustorium divide and form a wedge, the cells of this wedge elongate and penetrate the epidermis and cortex of the roots (Rich and Ejeta, 2007). With breaching of the endodermis, intrusions into host vascular elements occur mainly in larger vessels (Dörr, 1997). Using transmission electron microscopy (TEM), Dörr (1997) further showed that the cytoplasm rich haustorial cells penetrate the host vessel member with more than one intrusion which subsequently lose their protoplast. These water and nutrient absorbing structures are called oscula. Ba (1988) described the structure and the ultrastructure of *S. hermonthica* on pearl millet (*Pennisetum typhoides*). Using light microscopy (LM) and TEM he showed that the structure of the haustorium includes a radial vascular system which is surrounded by a hyaline tissue. This hyaline tissue is also embedded within the cortical parenchyma.

For successful control of *Striga*, an integrated approach is required of which biocontrol is an important component. The genus *Fusarium* was identified to be a suitable antagonist against *Striga*. *Fusarium oxysporum* Schecht “Foxy 2”, isolated from diseased *S. hermonthica* plants in Ghana, has been investigated for its use as a biocontrol agent against *S. hermonthica* (Abbasher et al., 1995; Kroschel et al., 1996). It proved to be highly pathogenic (Kroschel et al., 1996) and specific to several *Striga* species leading to its classification as *F. oxysporum* f.sp. *strigae* Elzein et Thines (Elzein et al., 2008). Soil pre-inoculation with Foxy 2 reduced emergence of *S. hermonthica* by 83% and *S. asiatica* by 91%. The infected plants which emerged wilted and died (Elzein and Kroschel, 2006b).

Most studies on the biocontrol of *Striga* have focused on the general aspects such as the suppression or reduction of parasitic weed emergence (efficacy), host range studies and the impact on crop yield. Although some studies have been carried out using light microscopy (LM) and transmission electron microscopy (TEM) to investigate other *Fusarium* sp. as wilt pathogens (Bishop and Cooper, 1983; Ouellette et al., 2006), to our knowledge no detailed investigations have been made to understand the action of the biocontrol agent Foxy 2 in *Striga* plants so as to optimize

conditions for control or for application. To be considered an effective mycoherbicide component, Foxy 2 must be able to timely colonize and infect *S. hermonthica*. By seed coating, the biocontrol agent is brought to the root, the potential infection zone of the plant. Foxy 2 colonizes the cortex of the host sorghum root but is not able to invade the endodermal layer into the tissues of the central cylinder (Chapter 2). Even though Foxy 2 has been shown to be effective and specific to *S. hermonthica* and *S. asiatica* in the greenhouse, the exact mechanisms of the parasitic process have not been investigated and the events occurring at the cellular level during the process, from attack to wilting and death are not known. To date, no anatomical study has been carried out to assess the ability of Foxy 2 to colonize and attack *S. hermonthica* seedlings underground in the real living complex association with its host. It is also not known if Foxy 2 invades the shoots of *S. hermonthica* which grow above-ground or how it affects its functioning. A better understanding of the mode of action of this biocontrol agent on *S. hermonthica* can facilitate the selection of the appropriate and efficient delivery system. Therefore the objective of this research was to study the presence and the pattern of colonization of Foxy 2 at different stages of growth of *S. hermonthica* haustoria (attached to *Striga* susceptible and tolerant sorghum roots to investigate if Foxy 2 acts differently in the two cultivars) when delivered as seed treatment and to trace its resulting infection into the shoots of *S. hermonthica* using LM, scanning electron microscopy (SEM), and TEM.

3.4. Materials and methods

3.4.1. Fungal and plant material

The fungal strain *F. oxysporum* f.sp. *strigae* (Foxy 2) used for the investigations was collected from diseased *S. hermonthica* plants in North Ghana by Abbasher et al. (1995). Identification of the isolate was carried out by the Federal Biological Research Center for Agriculture and Forestry (Berlin, Germany), where the isolate was deposited under accession number BBA-67547-Ghana. A pure culture of the isolate has also been preserved on Special Nutrient poor Agar (SNA)-medium (Nirenberg, 1976) with 5% (v/v) glycerol at –40^{o}C at the Institute of Plant Production and Agroecology in the Tropics and Subtropics, University of Hohenheim, Stuttgart, Germany. The seeds of *S. hermonthica* were collected from Sudan in 2006. Two cultivars of *Sorghum bicolor* (L.) Moench were used: the *Striga*-susceptible cultivar “Cowbaula” from Burkina Faso (provided by the International Institute of Tropical Agriculture (IITA) Benin Station), and the *Striga*-tolerant cultivar “Wad Admed” from

Sudan (provided by the agricultural Research Corporation (ARC), Sudan). Before use, the seeds of both *S. hermonthica* and sorghum were first surface sterilized by complete immersion in 1% sodium hypochlorite (NaOCl) for 5 minutes, and then rinsed with tap water.

3.4.2. Inoculation and plant cultivation

3.4.2.1. Inoculation of Striga-mycoherbicide component: Foxy 2 was inoculated onto sorghum seeds using seed treatment technology provided by SUET (Saat-u. Ernte-Technik GmbH, Eschwege, Germany). The seed coating protocol, developed, and optimized recently by Elzein et al. (2006), involves film-coating of sorghum seeds with a homogenized suspension of dried chlamydospores powder (<100 µm) of Foxy 2 and Gum Arabic as adhesive. The viability (colony forming units, CFUs) of Foxy 2 per coated sorghum seed was determined as described by Elzein et al. (2006). The coated tolerant cultivar "Wad Admed" contained $2.4x10^2$ CFUs per seed, while the susceptible cultivar had $2.7x10^2$ CFUs per seed.

3.4.2.2. Root chamber experiments: Samples of *S. hermonthica* seedlings on sorghum roots for LM and TEM were obtained from root chamber experiments with clear plexiglass as described by Linke et al. (2001). Fifty milligrams of *S. hermonthica* seeds (95% germination rate) were evenly distributed on moistened strips of 26×6 cm filter paper (Whatmann GF/A), and placed in the chamber facing the plexiglass. The vessels were then filled with sterilized sand and wrapped in black plastic inclined to 30º and incubated at room temperature (25ºC) for 7 days. Thereafter, the chambers were transferred to the glasshouse with 12 hours of light having day temperatures of 34 - 39ºC and night temperatures between 23 and 26ºC with a relative humidity of 36 to 71%. In the glasshouse, three Foxy 2-coated sorghum seeds (tolerant or resistant cultivars) were sown in each chamber and for the control, uncoated seeds were sown in other chambers. The chambers were arranged with four replicates for each experiment. Two weeks after sowing, the irrigation water was replaced with 0.2% liquid fertilizer (Wuxal® N-P-K (8-8-6), AGLUKON, GmbH & Co. KG, Düsseldorf, Germany). The experiment was repeated thrice.

3.4.2.3. Pot experiments: Pot experiments were performed in the glasshouse (see above) to obtain *S. hermonthica* shoots (above-ground samples) for microscopic observation. The treatments tested included: i) Foxy 2-coated tolerant sorghum plus *Striga*, ii) Foxy 2-coated susceptible sorghum plus *Striga,* iii) uncoated tolerant

sorghum (Control 1) plus *Striga* and iv) uncoated susceptible sorghum plus *Striga* (Control 2). Four kilograms of sterilized garden soil were filled in 18x18x18 cm black plastic pots and 100 mg *S. hermonthica* seeds were evenly mixed in the top 10 cm of the soil of each pot. Five uncoated or Foxy 2-coated sorghum seeds were sown around the center of each pot for the control and treated pots as treatments, respectively. The pots were replicated four times. After two weeks, sorghum plants were thinned to three in each pot. The plants were fertilized with 200 ml of a liquid fertilizer, 0.2% Wuxal® N-P-K (8-8-6), which was split-applied as 100 ml per pot, when the plants were 2 and 4 weeks old. The plants were watered when necessary.

3.4.3. Microscopy

Samples (0.5 - 1 cm in length) of *S. hermonthica* seedlings and sorghum roots at the points of connections (haustorium) as shown in Fig. 3.1a and b were collected from variable positions along the roots 15, 21, and 26 days after sowing the sorghum seeds in the root chamber experiments. Additionally, samples between 2 and 5 mm long from the top, middle and bottom of each plant, from diseased and healthy *Striga* shoots (of variable length of up to 17 cm) were collected in the pot experiments up to three months after sowing sorghum seeds; i.e. samples were collected 1, 2, 3, 5, and 8 weeks after *Striga* emergence.

3.4.3.1. Light microscopy (LM): All samples were fixed with 5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2) and stored at 4ºC until further preparation steps were performed. Comparable parts of fixed samples of 1-3 mm in size were cut with a razor blade, rinsed in 0.1 M sodium phosphate buffer with pH 7.2 and dehydrated in an ethanol series (30%, 50%, 70%, 90%, 95%, 100% (v/v)) for one hour each. The samples were embedded in methacrylate Technovit 7100 (Kulzer), allowed to polymerize, and harden for at least two hours. Semi thin sections of 5 µm thickness were made using a rotational microtome HM 340E (with a D-shaped metal knife,) and sections were collected on Poly L-lysine coated slides. All sections were stained with Toluidine blue O (MERCK) for one hour and mounted with Roti Histokitt (Carl Roth GmbH). The sections were examined under a Zeiss Axioplan light microscope (Göttingen, Germany) coupled to a digital camera (Canon Powershot A640). Pictures were adjusted for brightness and contrast in Adobe Photoshop CS2 version 9.0.

3.4.3.2. Scanning electron microscopy (SEM): Fresh samples of 3-week-old *S. hermonthica* seedlings attached to sorghum roots from the root chamber experiments were collected using a sharp razor blade. The samples were prepared as described by Honegger (1985) with slight modifications. The samples (0.5 – 1.5 cm in length) were fixed for one hour with 2.5% glutaraldehyde (v/v) in sodium phosphate buffer (pH 7.2). Then the samples were incubated in 5% aqueous detergent solution (washing powder 'Ariel') at 38ºC for three hours to remove cytoplasmic remnants. Thereafter, the samples were washed 10 times in distilled water for 10 minutes each. The samples were then fixed in 1% osmium tetroxide (v/v) in the same buffer for one hour at room temperature. Subsequently, the samples were dehydrated twice in 30% and 50% acetone, each for one hour then left overnight in 70% acetone at room temperature. Dehydration was completed with 90% and 100% acetone (one hour each), after which the samples were critical point dried and sputter coated with gold-palladium. Observations were done using a scanning electron microscope (DSM 940 Zeiss, Germany).

3.4.3.3. Transmission electron microscopy (TEM): For TEM, all samples which were approximately 1 – 3 mm in length were fixed as in SEM and washed three times in the same buffer. Then the samples were post fixed in 1% (v/v) osmium tetroxide in the same buffer for one hour and washed three times in doubled distilled water. The samples then went through a graded ethanol series (30%, 50%, 70%, 90%, 100% (v/v)) as in LM and where embedded in LR White (Science Services, Munich) or dehydrated in the same manner in a graded acetone series (30%, 50%, 70%, 90%, 100% (v/v)) and embedded in Epon 812 (Agar 100 resin, Plano). Embedded samples were allowed to polymerize in flat moulds at 60ºC. The samples were sectioned using an ultratome (Ultracut UCT, Leica), first with glass knives to obtain semi thin sections (1,5 µm thick), then with a diamond knife to obtain ultrathin sections (80 nm thick) which were transferred onto Pioloform (Science Service, Munich) and carbon coated copper (G 200) grids. The Epon embedded sections were stained with lead citrate then uranyl acetate (10 and 30 minutes respectively), while the LR White samples were stained with lead citrate then uranyl acetate and lead citrate again (2, 20 and 2 minutes respectively). Finally the sections were investigated using TEM (EM 10, Zeiss) operating at 60 kV. Negatives were scanned (Epson Perfection 2450 Photo) and the pictures were adjusted for contrast and brightness in Photoshop.

3.5. Results

3.5.1. Infection of S. hermonthica seedlings by Foxy 2

Striga usually attaches underground to its host and depends totally on the host for water and nutrients at this stage. With the naked eye, the point of attachment of *S. hermonthica* to its host could be seen as a globular swelling. This globular basal part of the seedlings, called the haustorium, is the organ that connects the parasite with the sorghum host root (Fig. 3.1). While Fig. 3.1a shows healthy, non-infected *S. hermonthica* seedlings (control), the brown, necrotic *S. hermonthica* seedlings in Fig. 3.1b are infected with Foxy 2. Longitudinal sections of the *S. hermonthica* seedling (SL) and cross sections of sorghum root (SR) revealed the anatomy of the connecting tissues (Fig. 3.2). Numerous haustorial intrusive cells of the parasite (HI) penetrated the cortex of the host root (SCP), cleaved the cortex and pushed the tissue aside. They broke through the endodermis and the central cylinder (CC) of the sorghum root to invade single vessels and to connect them with the xylem of the parasite (Fig. 3.2). *S. hermonthica* oscula, which are the penetration organs, could be identified within sorghum root vessels, which subsequently formed perforated tubes between host and parasite as seen with scanning electron microscopy (Fig. 3.2a and b, arrows). Using light microscopy, a continuous xylem connection was clearly evident (Fig. 3.2c, arrow). Ultrathin sections revealed that these intrusive cells within the haustorium had highly active cytoplasm with numerous large elongated diverse shaped mitochondria, strands of endoplasmic reticulum, golgi bodies, lipid bodies and vacuoles (not shown). In very young *S. hermonthica* seedlings, the central xylem elements (CX) of the parasite ended in the xylem disc which later developed into the xylem system of the shoot (Fig. 3.2c). Using TEM, *S. hermonthica* xylem parenchymal (SXP) cells could be seen among the *S. hermonthica* central xylem elements with large vacuoles (V) and thin layer of cytoplasm (Fig. 3.2d). Phloem elements were not observed. Around the centrally located xylem elements was a cone-shaped, well-developed meristematic tissue, called the hyaline body (HB). Cells of this tissue were thin-walled with dense cytoplasm (Fig. 3.2c and e).

Fig. 3.1. *S. hermonthica* seedlings (SL) attached to susceptible sorghum roots (SR) from the root chamber experiment three weeks after sowing as seen under the binocular. Globular shaped haustoria (circles) at points of attachment. Arrows indicate positions from where samples were collected for microscopy.
a. Healthy, non-infected seedlings (SL).

b. Brown seedlings (SL) infected with Foxy 2.

The hyaline body tissue had a dense deep purple color and was pointed towards the root of sorghum and surrounded by cortical parenchyma (Fig. 3.2c and e). This tissue contained cells having the characteristic large nuclei (N), mitochondria (M), endoplasmic reticulum (ER), and golgi bodies (G) as seen using TEM (Fig. 3.2f). Cells of the *S. hermonthica* cortical parenchyma which formed the outermost layers of the seedlings were less dense with large vacuoles and few mitochondria (not shown).

As sorghum seeds were coated with Foxy 2, the hyphae grew along the root and thus came into contact with attaching *S. hermonthica* seedlings. Fifteen days after sowing a few fungal hyphae were observed to have already penetrated the *S. hermonthica* hyaline body (not shown). After 21 days, the *S. hermonthica* seedling was completely infested by hyphae of Foxy 2 (Fig. 3.3a). The dense cytoplasmic cells of the hyaline body and the *S. hermonthica* cortical parenchyma were no longer identifiable.

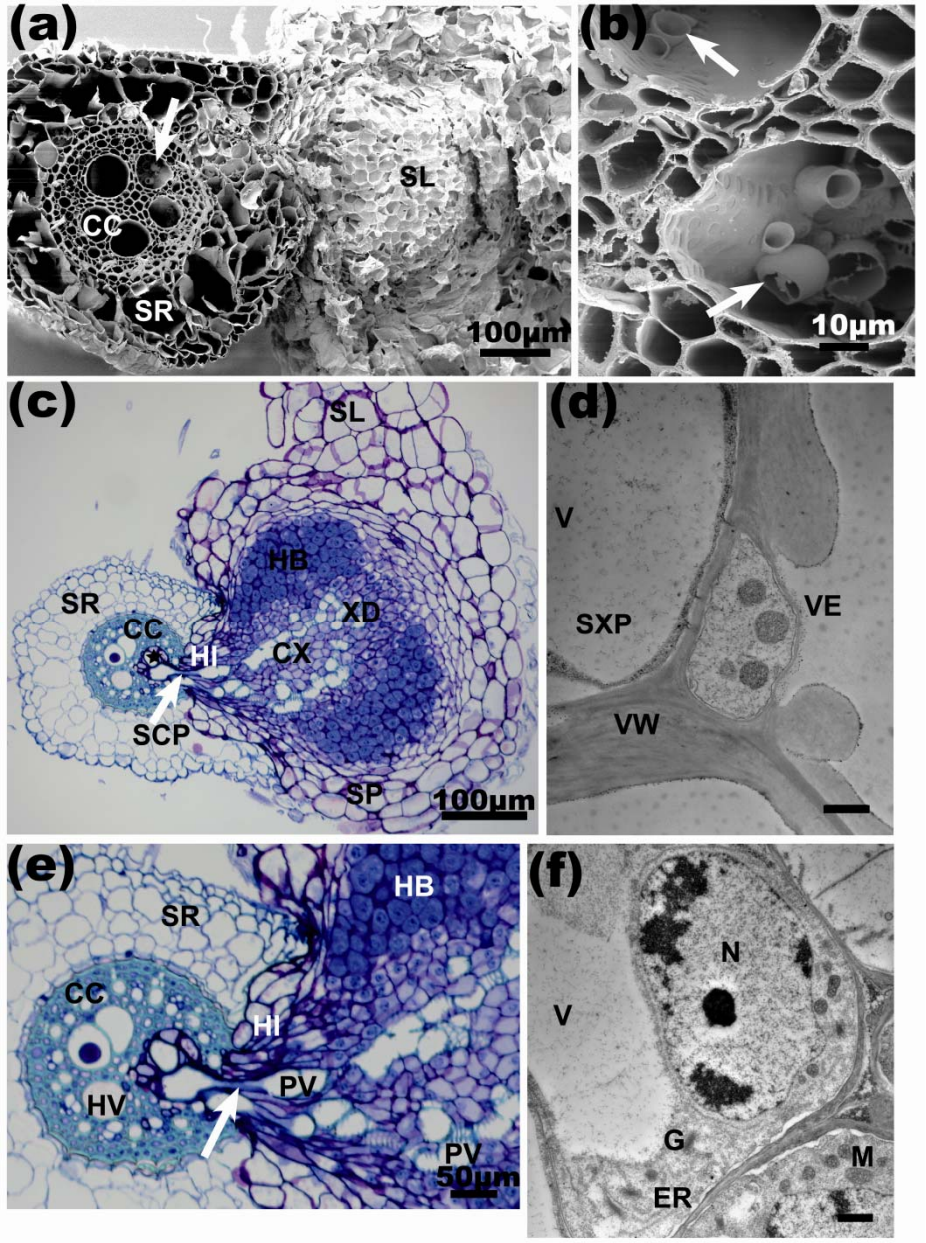

Fig. 3.2. Non-infected *S. hermonthica* seedlings (SL) on tolerant sorghum roots (SR), three weeks after sowing the sorghum seeds. (a) and (b) penetration of seedling (scanning electron micrographs); (c) and (e) light micrographs; (d) and (f) transmission electron micrographs of seedlings embedded in Epon (c) and LR-White (d), *bars = 1 µm*.

a. Overview of a seedling (SL) in longitudinal section parasitizing a sorghum root (SR) in cross section. Seedling has penetrated into the sorghum root central cylinder (CC). Arrow indicates position of oscula.

b. Detail of **(a)** showing oscula (arrows) within the sorghum central cylinder.

c. Light micrograph of seedling as seen in **(a).** The deep purple staining tissues of the parasite (*Striga*) are clearly distinguished from the host (sorghum). Haustorial intrusive (HI) cells protrude the sorghum cortical parenchyma (SCP) and central cylinder (CC). Arrow shows point of connection between *Striga* and sorghum. Haustorial intrusive (HI) cells have completely engulfed one of the central vessels (asterisk) in the central cylinder (CC) of the sorghum root (SR).

d. Part of a vessel element (VE) and *Striga* xylem parenchyma (SXP) with central vacuole (V) and cytoplasm of a seedling.

e. Detail of **(c)** showing a continuous xylem connection (arrow) between host vessel (HV) and parasite vessel (PV).

f. Part of the cell within hyaline tissue has dense cytoplasm with large nuclei (N).

M, mitochondria; ER, endoplasmic reticulum; G, golgi bodies; V, vacuole; CX, Central xylem elements; HB, hyaline body; XD, xylem disc; SP, *Striga* cortical parenchyma; VW, vessel wall.

Observations using TEM showed that organelles in this area could no longer be identified except for the observed electron dense debris (Fig. 3.3b, arrow heads) and some remains of the nucleus (Fig. 3.3b arrows). There was digestion of *S. hermonthica* xylem parenchymal cells by hyphae at this stage (Fig. 3.3c, arrows). Digestion of vessel walls (VW) in the vicinity of hyphae could also be observed (Fig. 3.3c and d). No signs of a resistance reaction of the *S. hermonthica* seedling cells were observed at this stage even though later on during the infection process, the situation was different. Serial sections (close up sections) showed long mycelial strands of Foxy 2 protruding from *S. hermonthica*, within the *S. hermonthica* haustorial intrusive cells into the central cylinder of the sorghum root (Fig. 3.3e, arrows). After perforation of the oscula, hyphae seemed to be blocked at the sorghum vessel wall and did not penetrate nor digest this wall (not shown). However, the sorghum cortical parenchyma (SCP) cells were digested by hyphae such that sometimes gaps appeared between the outer and inner parenchyma layers of the sorghum root (Fig. 3.3e). It is important to note here that the hyphae entered into the sorghum central cylinder within the *S. hermonthica* haustorial cells and not otherwise. Twenty-six days after sowing, most of the *S. hermonthica* tissues were already destroyed including the cortical parenchyma of the seedling (not shown). Hyphae had digested the entire hyaline body and cortical parenchyma cells of *S. hermonthica a* in both the susceptible (Fig. 3.3f) and tolerant sorghum cultivars. Throughout the experiment there was no difference in the extent of intrusion of *S. hermonthica* into susceptible and tolerant sorghum cultivars and there was also no difference in the root anatomy between the two cultivars. *S. hermonthica* seedlings and shoots on both cultivars were equally colonized and severely destroyed by Foxy 2.

3.5.2. Infection of S. hermonthica shoots by Foxy 2

Approximately 5 weeks after sowing, the first *S. hermonthica* shoots emerged. Observation of cross sections of shoots (1, 2, 3, and 5 weeks after emergence) showed the anatomy of most dicotyledons (Fig. 3.4a). The ultrastructure of the vessels showed secondary walls with an electron dense middle lamella running through. Xylem parenchymal cells were also identified with central vacuoles and a thin layer of dense cytoplasm. In untreated *S. hermonthica* shoots (control) no fungal hyphae were detected within cells (Fig. 3.4a and b).

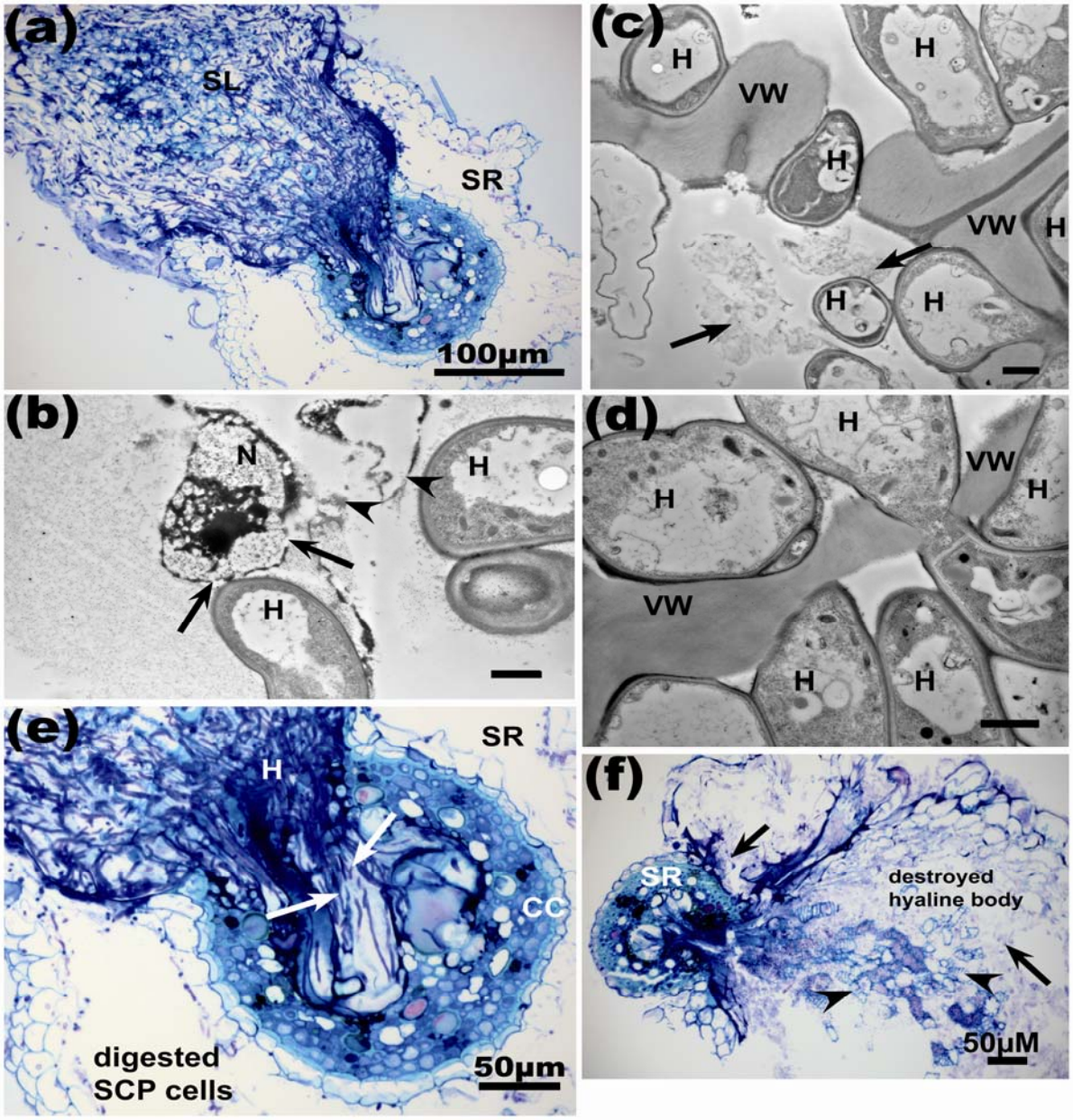

Fig. 3.3. Micrographs of cross sections of Foxy 2 infected *S. hermonthica* seedlings on tolerant sorghum, three weeks after sowing (underground stages) showing the spreading of Foxy 2 hyphae. (a) and (e), light micrographs; (b), (c) and (d), transmission electron micrographs of seedlings embedded in Epon (c) and LR-White (b), (d); *bars = 1 µm*.

a. Sorghum root (SR) of a Foxy 2 treated seed with a lot of hyphae in attached *Striga* seedling (SL).

b. There is extensive destruction of the hyaline tissue such that just cytoplasm debris can be seen (arrow heads) with engraved nucleus (N, arrows).

c. Vessel wall (VW) disintegration in a Foxy 2 infected seedling. Remains of xylem parenchymal cells (arrows) can be seen.

d. Within the area of the central xylem disc of an infected seedling, there is massive digestion of the vessel wall (VW) by hyphae (H).

e. Higher magnification of **(a)** showing Foxy 2 hyphae (H, arrows) invading haustorial intrusive cells and protruding into the central cylinder (CC) of sorghum root. SCP, sorghum cortical parenchyma.

f. Complete destruction of seedling, 26 days after sowing. Hyphae (arrows) can be seen with remains of vessels (arrow heads).

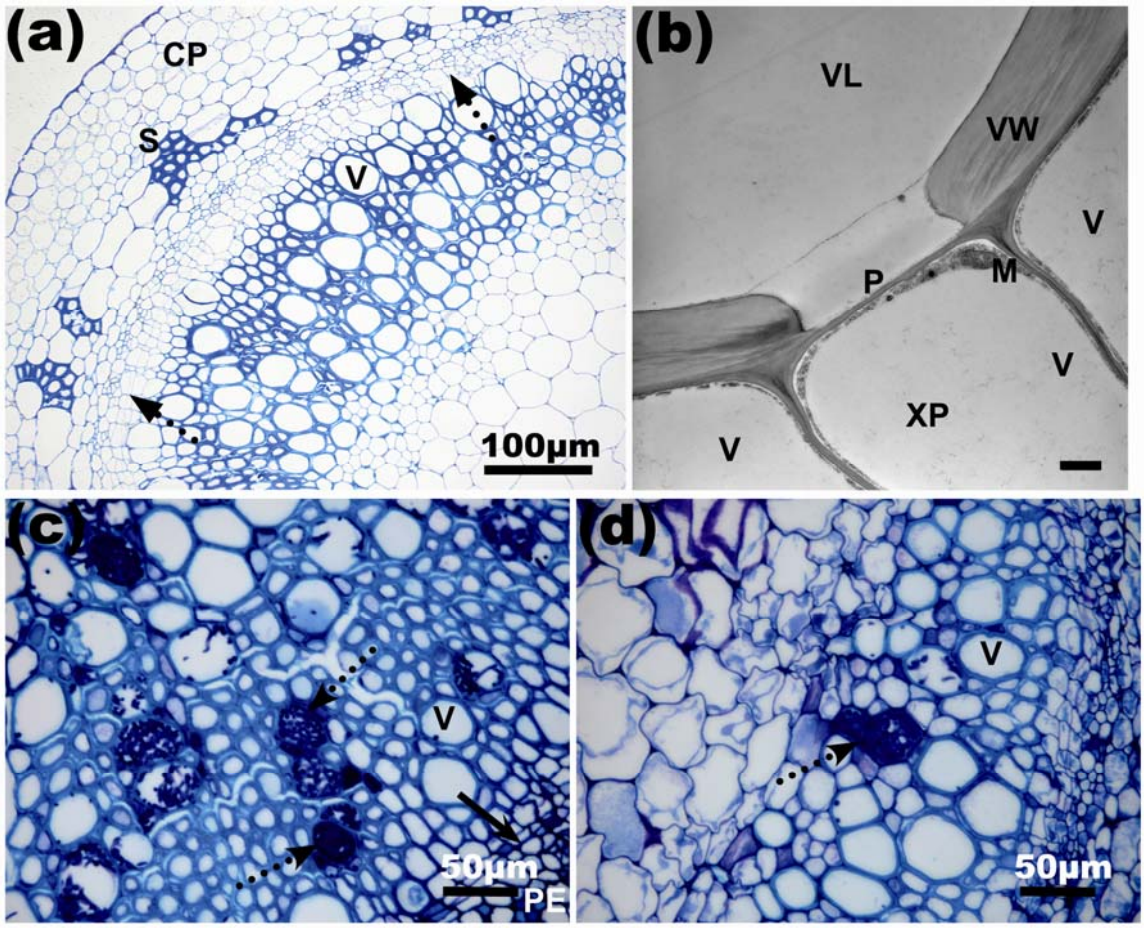

Fig. 3.4. Light and transmission electron micrographs of cross sections of *S. hermonthica* shoots, two weeks after emergence at various heights from the soil showing the anatomy of shoots and the pattern of growth of Foxy 2 hyphae within infected shoots. (a), (c), and (d), light micrographs; (b), transmission electron micrograph, Epon embedding, *bar = 1 µm*.

a. Un-infected shoot at 4 cm above the soil showing absence of hyphae in vessels (V). Within the cortical parenchyma (CP) are sclerenchyma cells (S). Cambial zone is indicated with dotted arrows.

b. Part of a vessel element and xylem parenchymal cells of a non infected shoot showing secondary walls (VW) and clear vessel lumen (VL). A xylem parenchymal pit (P) is also shown with the underlying pit membrane. The xylem parenchymal (XP) cells have a large central vacuole (V) with thin layer of cytoplasm and very few mitochondria (M).

c. Section of a Foxy 2 infected shoot at 1 cm above the soil showing hyphae in many vessels (V), colonizing the vessels (dotted arrows). Cambial zone (arrow) can be seen separating the vessels and the phloem elements (PE).

d. Foxy 2 treated shoot at 17 cm above the soil showing hyphae (dotted arrow) in fewer vessels (V) as compared to the samples close to the soil.

With the naked eye, *S. hermonthica* shoots which emerged from pots with Foxy 2 treated sorghum seeds (1, 2, 3, and 5 weeks after emergence) showed signs of wilting even though still green in color. Shoot samples collected close to the soil (approximately 2 cm above-ground) showed that hyphae had penetrated and were observed in some vessels two weeks after shoot emergence (Fig. 3.4c). The middle (not shown) and top shoot samples (Fig. 3.4d) showed fewer numbers of vessels

penetrated and filled with hyphae as compared to those very close to the soil even though in some sections from the uppermost shoot sample, some vessels were completely blocked by hyphae. Vessels were colonized in a non-uniform manner i.e. colonization had no particular pattern and the relative number of hyphae was variable within the infected vessels (Fig. 3.4c and d). In contrast to control shoots with no hyphae (Fig. 3.5a), longitudinal sections of Foxy 2 infected *S. hermonthica* shoots showed mycelia growing through the vessels (Fig. 3.5b) with some vessels being apparently completely blocked by hyphae (Fig. 3.5c, arrow). The longitudinal sections revealed that hyphae had proliferated and formed compact masses along the vessels over a couple of millimetres (Fig. 3.5b and d), thereby, clogging the vessels. There were no observed anatomical differences between emerged *S. hermonthica* shoots from tolerant and susceptible sorghum cultivars. Nonetheless, the number of *S. hermonthica* plants that emerged from pots with the tolerant cultivar was relatively less than the number of plants from the pots with the susceptible cultivar.

Besides being colonized by Foxy 2, the ultrastructure of *S. hermonthica* shoot vessels invaded by hyphae showed that an electron-dense layer coated the inner secondary cell walls (Fig. 3.6a, arrows). This electron-dense wall layer also occurred along xylem parenchyma pit membranes (Fig. 3.6b, arrow). It was uneven in thickness, increasing sometimes in areas of fungal attachment to the vessel wall and also some hyphal cells in contact or close to the vessel walls were surrounded by a similar electron dense layer (Fig. 3.6a). In contrast to the cells of the *S. hermonthica* haustoria in underground stages, penetration and digestion of secondary walls of vessels by hyphae was never observed in the emerged *S. hermonthica* plants. In the shoots above-ground, hyphae were aligned on the vessel walls (Fig. 3.6c). There was local digestion through the intertracheary pit membranes such that hyphae spread through them from one vessel to another (Fig. 3.6d).

Furthermore, the ultrastructure of *S. hermonthica* shoots revealed that xylem parenchymal cells adjacent to infected vessels had highly active cytoplasm (with lots of mitochondria, endoplasmic reticulum, and lipid bodies; not shown), as compared to xylem parenchymal cells in the control shoots. No penetration of the xylem parenchymal pit membranes was observed although sometimes hyphae fitted closely into this membrane and the underlying xylem parenchyma cells produced a wall apposition just beneath the xylem parenchyma pit membrane (Fig. 3.6c, asterisk).

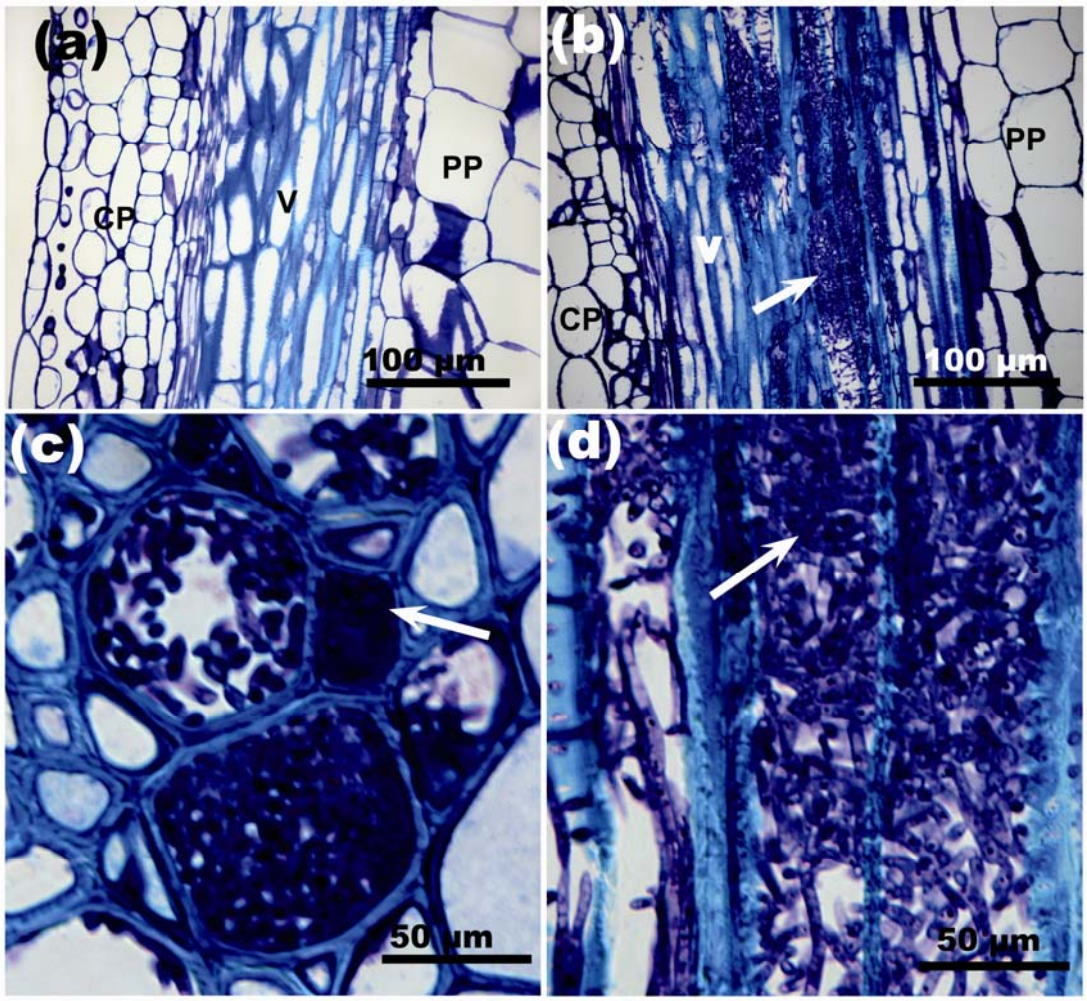

Fig. 3.5. Foxy 2 Infected and non-infected *S. hermonthica* shoots, three weeks after emergence (light micrographs).
a. Longitudinal section of a control shoot showing hyphae free vessels (V).
b. Longitudinal section from a Foxy 2 treated plant with hyphae blocking vessels (arrow) for some distance.
c. Cross section of Foxy 2 infected shoots showing a lot of hyphae in vessels. Some vessels are blocked so that no space can be seen within the vessels (arrow).
d. Enlarged section of **(b)** showing hyphae masses in the vessel (arrow).
PP, pith parenchyma; CP, cortical parenchyma.

3.5.3. Investigation of the dead S. hermonthica shoots

At one week after emergence, *S. hermonthica* plants already manifested symptoms of wilting and at eight weeks after emergence, they were already dead (completely brown) as observed with the naked eye. There was no difference between diseased shoots which were investigated 1, 2, 3, and 5 wks after emergence, in which all samples showed more or less strongly infected vessels with no signs of destruction. However, in the dead shoots (collected 8 wks after emergence), hyphae were found in most of the vessels (Fig. 3.7a, arrows) and hyphae attacked vessel walls and some

digestion of the walls could be observed (Fig. 3.7b, arrows) which was not the case in the shoots of the earlier stages. Dead shoots also had hyphae within dead xylem parenchymal cells that had caused degradation, which was also not the case in the early stages (not shown).

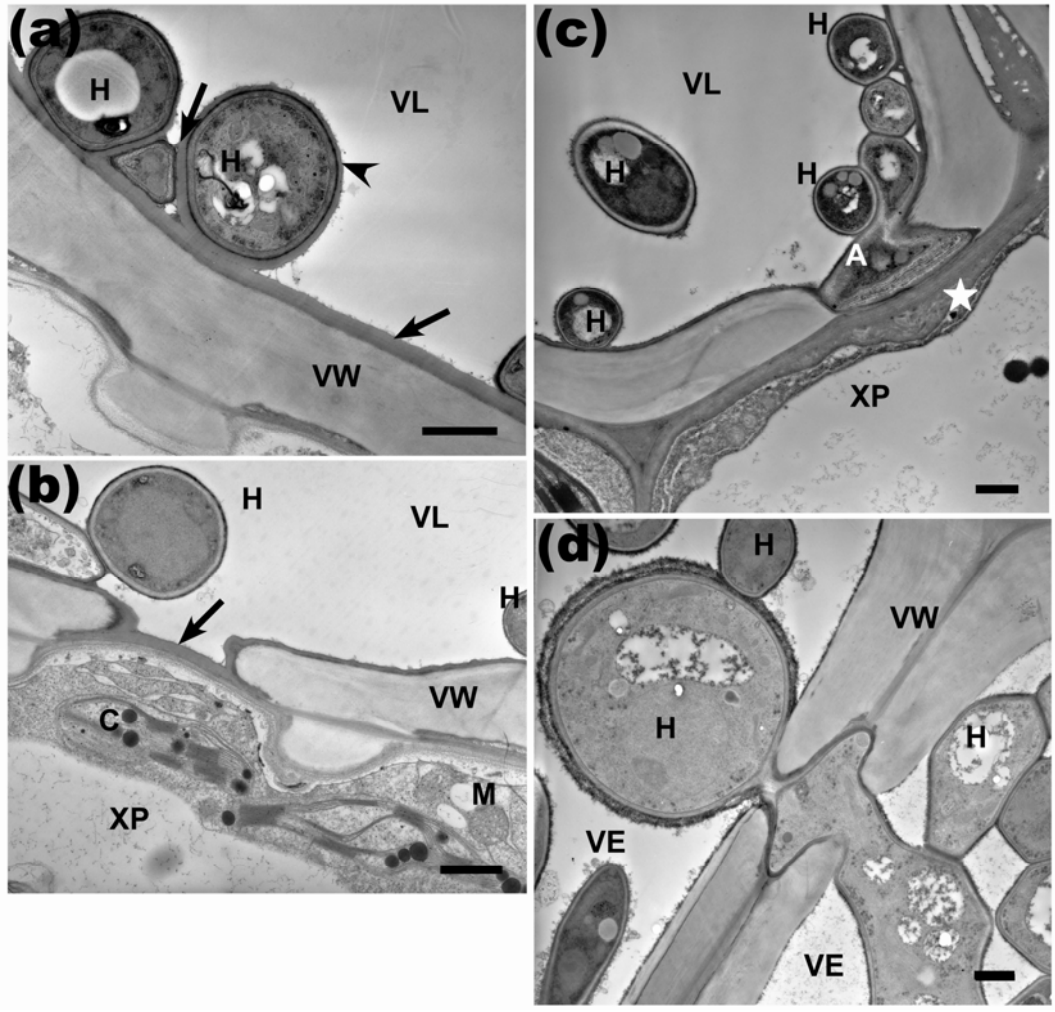

Fig. 3.6. Transmission electron micrographs of infected *S. hermonthica* shoots showing colonization by hyphae in cross sections. Shoots in (c) and (e) were embedded in LR-White and the others in Epon, *bars =1 µm.*

a. Five weeks after emergence, vessel walls (VW) are seen to be coated with an electron dense layer (arrow) which was sometimes found also around the hyphae (arrow head). Hyphae (H) are seen aligned along the vessel wall but not digesting or penetrating the wall.

b. The electron dense layer is observed coating the xylem parenchyma pit membrane (arrow) already two weeks after emergence. The underlying xylem parenchyma (XP) cell shows a dense cytoplasm with chloroplast (C) and mitochondria (M).

c. Two weeks after emergence, a hypha is seen here to spread, completely blocking the pit (A) as it attempts the penetration of a xylem parenchyma pit membrane. The xylem parenchyma (XP) cell produces a wall apposition (asterisk) just beneath the pit.

d. Hypha is seen to constrict as it penetrates through the intertracheary pit in an infected shoot, 5 weeks after emergence.

VE, vessel element; VL, vessel lumen.

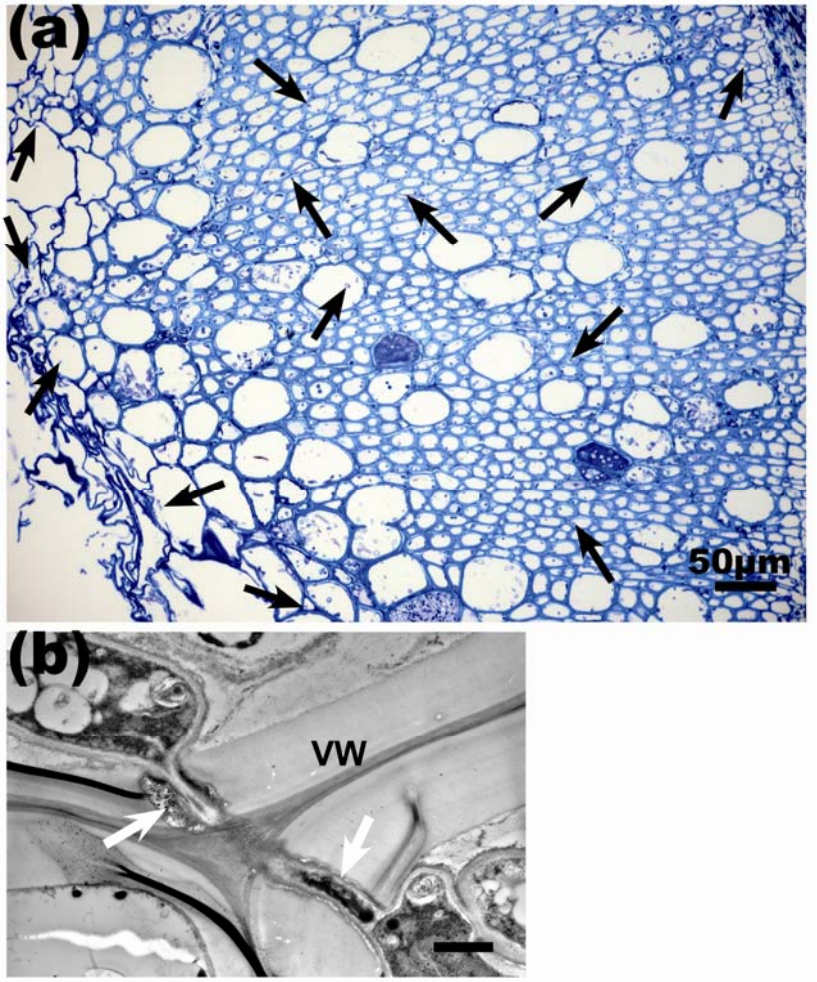

Fig. 3.7. Micrographs of dead *S. hermonthica* shoots collected eight weeks after emergence.
a. In this light micrograph it is observed that hyphae had spread into almost all vessels (arrows) and to surrounding cortical parenchymal cells.
b. Transmission electron micrograph of an LR-White embedded shoot showing digestion of vessel walls (VW) by hyphae (arrows); *bar =1 µm*

3.6. Discussion

3.6.1. S. hermonthica seedling colonization by Foxy 2

Considering the fact that *Striga* produces a prolific number of seeds (5000 – 10000 seeds) per plant (Smith and Webb, 1996), which remain viable in the soil for more than 10 years (Ejeta et al., 1992), destruction of underground stages of *Striga* is of great interest to farmers. In the present study, using scanning electron microscopy, the haustoria of *S. hermonthica* were observed to have penetrated through the cells of the cortical parenchyma of its host root sorghum, into the central cylinder forming a continuous xylem connection as was earlier observed by Musselman and Press (1995). Maass (1999) explains that penetration of the intrusive cells into host tissue is carried out mechanically by pressure on the host endodermal cells and by hydrolytic

enzymes. Upon successful establishment of vascular connections between host and parasite, distinct structures called primary haustoria are formed and later on, hundreds of secondary haustoria may develop and provide additional connections (Parker and Riches, 1993). In earlier studies we showed that by coating the sorghum seeds with the potential biocontrol agent, Foxy 2, the fungus had the opportunity to colonize the roots of sorghum such that the hyphae could then attack *S. hermonthica* as it attaches to the roots (Chapter 2). In this study, when *S. hermonthica* seedlings attached to roots colonized by Foxy 2, the hyphae came in contact and colonized *Striga* seedlings. Most *S. hermonthica* seedlings were destroyed by the fungus within 26 days after sowing. The extent of intrusion of the *S. hermonthica* haustorial intrusive cells into the locally considered susceptible and tolerant sorghum cultivars was not different and the level of destruction in the two cultivars by Foxy 2 was also not different. This further reflected a high pathogenicity of Foxy 2 against *S. hermonthica.* Using TEM, we observed that there was massive digestion of vessel walls in the young *S. hermonthica* seedlings by Foxy 2. The extended digestion of both vessel walls and cell organelles suggested the involvement of cell wall degrading enzymes. This could further be seen in the hyaline tissue where just remains of organelles could be identified. Several enzymes produced by fungal pathogens have been isolated which are responsible for degrading plant cell walls, such as polygalacturonase. Pectinases, pectate lyases, as well as xylanases have been reported to be secreted by other *Fusarium* wilt pathogens (Kang and Buchenauer, 2000; Jorge et al., 2006). The degradation of vessel walls as observed in young *S. hermonthica* seedlings in our experiments has also been observed in other wilt pathogens (Robb et al., 1975; Bishop and Cooper, 1983). Even though enzymes might have caused cell degradation at this early stage, it should however be noted that in the later developmental stages of *S. hermonthica* shoots, the observations were otherwise.

Foxy 2 has been shown to be highly pathogenic against the genus *Striga* but not against other crops including tomato (*Lycopersicon esculentum* Mill.), chickpea (*Cicer arietinum* L.) wheat (*Triticum aestivum* L.) and many others (Elzein and Kroschel, 2006a; Elzein and Kroschel, 2006b; Kroschel et al., 1996). Earlier reports have described *Fusarium* spp. as obligate necrotrophs which are pathogens specialized to their hosts which include cereals and grasses (Isaac, 1992). As with most necrotrophs, Foxy 2 was not restrained as it colonized *S. hermonthica* seedlings. Hyphae invaded all tissues of *S. hermonthica*, from the intrusive cells into the xylem

connection protruding into the host root. While studying the infection of *Arabidopsis thaliana* by the necrotrophic pathogen, *Botrytis cinerea*, (Govrin and Levine, 2000) postulated that the hypersensitive response of the plant facilitated infection by the pathogen. In contrast to their study, there was no hypersensitive reaction of *S. hermonthica* against Foxy 2, and no host defense mechanisms were observed for Foxy 2 at the early underground stages of infection even though later on the plants were seen to react to Foxy 2 invasion. The absence of host reactions at this early stage might have been due to the factor of time to be able to coordinate a defense reaction. This was not the case as reported for *F. oxysporum* f.sp. *lini* on flax seedlings (*Linum usitatissimum*), where attempted penetration of cells through the middle lamella caused the production of appositions close to the penetrating hyphae while invasion of the root cap was somehow retarded (Kroes et al., 1998).

Two weeks after sowing, just a few fungal hyphae were observed in *S. hermonthica* seedlings and most of the tissues were still intact. But a week later, all tissues of the *S. hermonthica* seedlings were destroyed and only few vessel wall remains could be identified within the digested tissues. Hence the colonization process was rather slow as compared to the pathogenic strain, *F. oxysporum* Schlechtend.:Fr. on *Orobanche aegyptiaca*, where colonization was much faster and hyphae were found inside cells and in the intercellular space throughout the tubercles of *Orobanche* within four days of inoculation (Cohen et al., 2002). Additionally, Cohen et al. (2002) observed that the fungus grew through and between infected *Orobanche* cells, resulting in a breakdown of cell walls and the disintegration of cytoplasm in and around the infected cells two days after inoculation. The differences in the progress of the infection between Foxy 2 against *S. hermonthica* and *F. oxysporum* Schlechtend.:Fr. against *Orobanche* may be in part linked to the different fungal strains, but above all, to the inoculation method. In contrast to our experiments, the *Orobanche* tubercles on tomato roots were inoculated by spraying the mycoherbicide as mycelia. Being applied as seed coat, *Striga* got into contact with Foxy 2 only when root surface and cortex were already colonized by the fungus. As Foxy 2 was slowly growing on sorghum roots, there were areas towards the root tips which were not yet colonized by hyphae. *S. hermonthica* seedlings that attached to these areas could have developed without getting in contact immediately with Foxy 2. In such a case the seedlings might have been able to outgrow the fungus and therefore emerged to the surface. It is likely that Foxy 2 was not only able to parasitize attached *S. hermonthica* seedlings, but also *S. hermonthica*

seeds which germinated close to the Foxy 2 colonized roots, therefore killing them before they could attach. However, it is not known how far Foxy 2 hyphae were spreading into the soil around the sorghum root and to what extent *S. hermonthica* seeds in the vicinity could be destroyed. Nonetheless, the investigated seed delivery method is advantageous as the underground stages of *Striga* were attacked, considering that most of the damage to the host is done before its surface emergence.

3.6.2. Mode of action of Foxy 2 in infected S. hermonthica shoots

Despite the introduction of Foxy 2, some *S. hermonthica* plants managed to emerge to the surface. Elzein et al. (2006) reported an efficacy of 81% reduction in healthy emerged *S. hermonthica* shoots compared to the control. In the present study, there were more hyphae in a greater number of vessels at the bottom of the emerged shoots than at the top (1-17 cm above soil), reflecting the fact that Foxy 2 invaded and penetrated first the underground developmental stages of *S. hermonthica*, and then proliferated within the vessels. Since *S. hermonthica* has no roots, Foxy 2 hyphae could get into the seedlings only when *S. hermonthica* seedlings and sorghum roots came into contact or when *S. hermonthica* had already invaded sorghum roots. Depending on the position on the root and the developmental stage of the seedling, hyphae on the surface of the sorghum root could invade *S. hermonthica* intrusive cells or in well developed seedlings hyphae could penetrate the outer layer of the haustorium, then the *Striga* cortical parenchyma and subsequently the xylem, especially vessels. The colonization pattern of Foxy 2 is different from the colonization pattern of non-pathogenic *F. oxysporum* in tissue-cultured Musa plantlets (Musa *spp.* L.) (Salerno et al., 2000). In their investigations, using LM, it was observed that hyphae colonized both roots and rhizomes in the Musa plantlets but did not penetrate the vascular tissues as was the case for Foxy 2 in *S. hermonthica* shoots.

One particular feature of the colonization process of Foxy 2 was that hyphae were mainly located within the vessels in emerged *S. hermonthica* plants. This might be a strategy employed by Foxy 2 in that after the initial colonization, Foxy 2 maintained a localized growth within the vessels so as to be able to colonize the *S. hermonthica* plant to its top. Such a vascular restriction has also been reported for the wilt fungus *F. oxysporum* f.sp. *lycopersici* on tomatoes (Bishop and Cooper, 1984) although some penetration of secondary vessels occurred (Bishop and Cooper, 1983). Unlike in our

observations, *F. oxysporum* f.sp. *lycopersici* was restricted to the lower stem, and tylose formation was also observed which was not the case for Foxy 2.

Diseased shoots (1, 2, 3, and 5 weeks after emergence) showed outward signs of wilting but were still green and not necrotic. In addition to the presence of Foxy 2 hyphae within vessels as observed using LM, ultrastructures disclosed that there was no vessel wall digestion at this stage. Moreover, Foxy 2 was not observed to penetrate the secondary walls of the vessels of diseased *S. hermonthica* shoots. This mode of action could be interpreted in two ways. On one hand, the inability to digest *S. hermonthica* vessel walls by Foxy 2 hyphae could be attributed to the presence of a barrier, like the observed electron dense wall layer which could have prevented vessel wall digestion. On the other hand, non-digestion of vessel walls could also be seen as a strategy used by Foxy 2 to further colonize the plant before killing it. This is in accordance with the explanations of Di Pietro et al. (2001), who stated that fungal plant pathogens have developed strategies to identify appropriate hosts, to overcome defenses, and to optimize growth. As already mentioned, the vessel wall coating in our study could have acted as a barrier taking into consideration the fact that some hyphae approaching the vessel walls were somehow embedded in this coating. The layer might have been a cell wall modification produced by *S. hermonthica* as a defense mechanism against Foxy 2. This was supported by the fact that the adjacent xylem parenchyma cells had active cytoplasm which suggested that they might have been involved in the production of this electron-dense wall layer. The probability that this layer was produced by *S. hermonthica* could be supported by observations of Bishop and Cooper (1983) and Robb and Busch (1982), who reported that vessel coatings were produced by uninfected but physiologically water-stressed plants. Plant defenses induced as responses to pathogens have been reported to include plant cell wall alterations involving phenolics and lignification (Greenberg, 1997). In this study the composition of the coating on *S. hermonthica* vessels was not investigated. Notwithstanding the source of this layer, it is obvious that its presence prevented the digestion of the vessel wall. However, this layer did not stop further tissue colonization by Foxy 2 as hyphae were observed even at the top of the shoots.

One noticeable aspect of the invasion process was the non-penetration of the xylem parenchyma pit membrane. Xylem parenchymal cells were also not colonized. Attempted penetration of xylem parenchymal pit membrane led to the production of a

wall apposition at the site of attempted fungal penetration. This could be seen as a host reaction against penetration and destruction by Foxy 2 hyphae. It has been shown that cell wall modifications, which are induced during plant response, are able to protect the cell wall from digestion by pathogens (Brisson et al., 1994; Greenberg, 1997). The fact that the early *Striga* seedling stages did not react to hyphal infection, in contrast to the later stages, might be explained as due to the extended time needed by the host to be able to respond to the invasion by hyphae of Foxy 2.

3.6.3. Fungal development in dead S. hermonthica shoots

Dead shoots, from their outward appearance were completely brown and drying out. Microscopic observations showed that hyphae were located in almost all vessels in contrast to the initial stages where just few vessels were colonized. This could be seen as a strategy of Foxy 2 in that after colonizing *S. hermonthica* to the top of the plant, i.e. later on during the infection, Foxy 2 rapidly spread and killed the *Striga* plant probably by hyphal clogging of the vessels or production of toxins. Clogging could greatly reduce water passage as their presence interfered with the upward flow of water in the plants, thereby contributing to wilting. Similarly, Agrios (2005) showed using pathogenic *Fusarium* strains that infected vessels in vascular wilts could be filled with pathogens, or substances secreted by the pathogens or by the plant itself in response to attack by the pathogen which could lead to clogging of the vessels. Earlier findings also showed that mechanical plugging as a result of the growth of the pathogenic fungus within the vessels created water deficiency, which was responsible for wilting in tomatoes, watermelon (*Citrullus lanatus* (Thunb.) Matsum & Nakai), cotton (*Gossypium* sp.), and cowpea (Dimond and Waggoner, 1953; Harris, 1940; Massee, 1895; Scheffer and Walker, 1953). For example, a reduction of 88% in vascular water flow was reported in the stem of cabbage plants (*Brassica oleracea* Capitata) infected with *Fusarium* spp. (Beckman, 1987; Melhus et al., 1924). Another possible cause of death of the *S. hermonthica* plant could be the destruction or blocking of the underground continuous xylem connection by Foxy 2 leading to lack of water and nutrients for the further survival of the *Striga* plant. From our TEM investigations, digestion of *S. hermonthica* vessel walls and xylem parenchyma could be noticed at this stage, indicating that Foxy 2 might have acted as a saprophyte at this stage. This is confirmed by Beckman (1987), who explained that the saprophytic phase during the fungal growth initiates when tissues of the infected host start to senesce and die, and the report that *F. oxysporum* (Smith et al., 1993) can survive as

chlamydospores which are dormant in the soil. With the death of the plant, Foxy 2 could invade the parenchymatous tissue.

3.7. Conclusion

Foxy 2 hyphae were able to completely destroy the tissues of the underground stages of young *S. hermonthica* seedlings attached to sorghum roots within 26 days, when applied as film-coat on sorghum seeds. This strongly reduced emergence of *S. hermonthica* and thereby reduced its ability for seed production. The pattern of infection could be summarized in the following sequence of events: i) parasitism of seedlings underground by hyphae growing on sorghum roots, ii) destruction of most seedlings underground, iii) few *S. hermonthica* seedlings escaped, surviving the initial attack, and emerged, iv) vessels of *S. hermonthica* shoots were colonized to the top by hyphae without digesting them, v) finally, blocking of vessels contributed to the death of *S. hermonthica* shoots, and vi) in dead *S. hermonthica* shoots Foxy 2 became saprophytic and hyphae digested vessel walls. Thus, Foxy 2 is fulfilling all necessary requirements for being a promising or potential mycoherbicide for scaling up to support and enhance the existing *S. hermonthica* control measures. In addition to the efficacy of Foxy 2 against *S. hermonthica*, the observed pattern of infection and colonization of the fungal isolate into the tissue of its target weed *S. hermonthica* supports the suitability of seed treatment for delivery of the mycoherbicide.

3.8. Acknowledgements

Many thanks go to Mrs. Bärbel Rassow, the laboratory technician whose efforts greatly contributed to the success of our laboratory work. We also greatly appreciate the efforts of Micheal De Mol for the contributions to the paper. Special thanks also go to the Eiselen foundation and Alexander von Humboldt Foundation (AvH), Germany, for their valuable financial support, which made the realization of our research possible. The technical support of Saat und ErnteTechnik "SUET", Eschwege, Germany, for seed coating is gratefully acknowledged as well. Mrs. Ndambi is also grateful to KAAD Germany for financial support.

CHAPTER 4

Tissue specific reactions of sorghum roots to the mycoherbicide *Fusarium oxysporum* f.sp. *strigae* versus the pathogenic *F. proliferatum*

4. Tissue specific reactions of sorghum roots to the mycoherbicide *Fusarium oxysporum* f.sp. *strigae* versus the pathogenic *F. proliferatum* [c]

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4.1. Abstract

Fusarium oxysporum f. sp. *strigae* (Foxy 2) is a mycoherbicide against *Striga hermonthica.* To ensure the safe use of this biocontrol agent, and as part of the risk assessment, this study was aimed at providing cytological evidence that Foxy 2 does not possess pathogenic behaviour towards the non-target host sorghum. Therefore, we compared the infection processes and sorghum root tissue reactions towards the pathogenic *F. proliferatum* to that of Foxy 2 using light- and transmission electron microscopy. Given that during the growth process, hyphae could get into the central cylinder, tissue specific reactions of sorghum to Foxy 2 were also investigated by wounding the roots (exposing the vascular system), and testing for proliferation of hyphae within the vessels. Results showed that two weeks after sowing, *F. proliferatum* had invaded and destroyed all cell types including the central cylinder while Foxy 2 hyphae were located around the outer endodermal layer and were not

[c] **This chapter has been accepted for publication as:**
Ndambi, B., Cadisch, G., Elzein, A. & Heller, A. (2011). Tissue specific reactions of sorghum roots to the mycoherbicide *Fusarium oxysporum* f.sp. *strigae* versus the pathogenic *F. proliferatum*. Accepted for publication in Biocontrol Science and Technology printed with permission from Taylor and Francis (Copyright 2011).

able to penetrate the latter. There was an increase in blue autoflourescence in the central cylinder and especially the endodermis, probably due to increased phenolics in Foxy 2 infected roots which was not the case for *F. proliferatum*. This might contribute to the inability of Foxy 2 to penetrate the endodermis. Transmission electron microscopy showed extensive degradation of endodermis and vessel walls into thin translucent layers by *F. proliferatum* but not by Foxy 2. In the mechanically wounded and infected roots, Foxy 2 could invade the central cylinder close to the wound but was not identified a few millimetres away from the wound. This implies that it was not able to grow within or destroy the central cylinder even when already present within it; probably due to the observed increased phenolics. Thus, exposure of the vascular system did not serve as a route for the invasion of Foxy 2 which therefore could not further cause tracheomycosis. Therefore, Foxy 2 could be seen as a fungus well suited for biocontrol.

4.2. Keywords: *Fusarium oxysporum* f. sp. *strigae* (Foxy 2), *Striga*, sorghum roots, light- and transmission electron microscopy, *F. proliferatum*, pathogenicity, endodermis

4.3. Introduction

The genus *Fusarium* is widely distributed in plants and soils (Kikot et al., 2009). Many strains of *F. oxysporum* are able to colonize plant roots as non-pathogens or as pathogens causing tracheomycosis, root damage and wilting. Their hyphae directly enter the root, penetrating the rhizodermis and colonizing the cortex to the vascular tissue where they rapidly spread upwards (Di Pietro et al., 2003; Rodriguez-Galvez and Mendgen, 1995). Research has been done on assessing various fungi for their use as biocontrol agents where the mode of action in both the target plant as well as its host is of crucial importance for their safety and effectiveness assessment. However, few investigations have been done to unravel the mechanisms of action of the supposed biocontrol agents on the non-target host plants; making it quite difficult to predict the possibilities of a negative impact on the desired host crops.

Amongst the biocontrol agents under investigation is the potential mycoherbicide *Fusarium oxysporum* f.sp. *strigae* Foxy 2 (Abbasher et al., 1995) used for the control of *Striga hermonthica* which is considered as one of the major challenges to the production of sorghum (de Groote, 2007; Ejeta, 2007b) and therefore, a threat to food

security. Greenhouse experiments have revealed that Foxy 2 is effective and specific to *S. hermonthica* causing a reduction in *Striga* germination of up to 83% (Elzein and Kroschel, 2004a). To ensure the safe use of biocontrol methods, strategies to assess the associated risks need to be developed (Boss et al., 2007). For this, it is necessary first of all to understand the interactions between the biocontrol agent and the parasitic weed together with the host plant which is the non-target plant and the desired crop (Hearne, 2009); before its implementation on large scale can be recommended. We already carried out microscopic investigations on the effect and destruction of *S. hermonthica* by the biocontrol agent Foxy 2 (Chapter 3). Preliminary observations showed that hyphae of Foxy 2 can penetrate the cortex of sorghum roots but provided no further support why the hyphae apparently were not able to cross the endodermis into the central cylinder of sorghum roots (Chapter 2). In contrast, when Foxy 2 attacked *Striga* seedlings attached to sorghum roots, hyphae were able to grow within the *Striga* haustoria into the central cylinder of the sorghum root (Chapter 3). The question; how well and how far Foxy 2 could move and interact within the sorghum central cylinder or if it could ultimately become a potential pathogen was not investigated.

The objectives of the present study were therefore, to investigate and assess the non-pathogenic action and behavior of the mycoherbicide *Fusarium oxysporum f.sp. strigae "*Foxy 2" to sorghum roots in comparison with that of the previously proven pathogenic stain *Fusarium proliferatum* (Souza et al., 2007) so as to provide a comparative evidence that proofs the friendly root colonization of sorghum and ensures the safe use of this biocontrol method. In addition, this study seeks to identify potential defense mechanisms of the host plant, sorghum against the biocontrol agent. In this light, the reaction of sorghum roots to invasion by Foxy 2 was compared to *Fusarium proliferatum*. The hypothesis on which this research was carried out was that Foxy 2 cannot cause major damage to sorghum roots due to the reinforcement of the endodermal barrier making Foxy 2 unable to digest this barrier while *F. proliferatum* can destroy it, therefore, clogging root vessels and thereby causing wilting. Knowing the pattern of colonization of roots by pathogenic and non-pathogenic *Fusarium* could contribute to an understanding of the mechanisms of disease suppression and help to improve the success of biocontrol approaches (Olivain and Alabouvette, 1997; Salerno et al., 2000). Thus the tissue specific reaction of sorghum roots growing from Foxy 2 and *F. proliferatum* coated sorghum seeds was examined

and compared using light- and transmission electron microscopy. It has been shown that the parasitic nematode *Radopholus similis* is prevented from invading the vessels of banana roots due to lignified endodermal cells as identified with fluorescence microscopy (Valette et al., 1998). Therefore, to identify possible defense responses of sorghum roots to invasion by the two *Fusarium* spp*,* fluorescence microscopy was used in the current investigations.

As hyphae of Foxy 2 were able to circumvent the endodermal barrier by growing within *Striga* haustoria into the central cylinder of the sorghum root (Chapter 3), the question arose whether Foxy 2 could afterwards proliferate and cause harm within the central cylinder of sorghum roots. Previous studies have shown that wounds are essential for vascular infection, sometimes enhancing infection and severity of diseases (Doling, 1963; Hepple, 1963; Sequeira et al., 1958; Wardlaw, 1930). Hence, experiments were carried out in this study which involved removing the endodermal barrier (wounding the roots to expose vessels) to allow access of Foxy 2 into the central cylinder so as to test the hypothesis that removal of the endodermal barrier could lead to tracheomycosis resulting in wilting of the sorghum plant as is the case with pathogenic Fusaria. Such studies are essential and valuable to provide direct evidence of the bio-safety of the mycoherbicide towards sorghum plants, a pre-requisite for convincing, facilitating and accelerating the acceptance and application of the *Striga* biocontrol technology by regulatory authorities and farmers in Sub-Saharan Africa.

4.4. Materials and methods

4.4.1. Material preparation

Two *Fusarium* species were used in this study; *Fusarium proliferatum* and *F. oxysporum* f.sp. *strigae* (Foxy 2). *F. proliferatum* was chosen as it has been shown to cause tracheomycosis, root rot, stem and stalk rot and even rot of grains in Zea mays L. (Souza et al., 2007). It was isolated from *Cymbidium* hybrid, leaf spot by H. Nirenberg, IMB 11679 (Schulz) in ‘Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany’ (German collection of microorganisms and cell cultures) and stored under accession number DSMZ 62261. Foxy 2 was isolated in North Ghana by Abbasher et al. (1995) from diseased *Striga hermonthica* plants and taxonomically identified in Berlin, Germany by the Federal Biological Research Center for Agriculture and Forestry, where it is deposited under accession number BBA-67547-Ghana. The

cultures of the two species were maintained/stored on Special Nutrient poor Agar (SNA) (Nirenberg, 1976) with 5% v/v glycerol at -40^{o}C at the institute of Plant Production and Agroecology in the Tropics and Subtropics, University of Hohenheim, Stuttgart, Germany. Before use, the fungi were transferred and cultured on PDA at 25^{o}C for 14 days. Sorghum seeds used were the *Striga*-tolerant variety "Wad Admed" which was provided by the Agricultural Research Corporation, Sudan. The seeds were surface sterilized by immersion in 1% sodium hypochlorite solution for five minutes and rinsed with tap water. Seeds were tested for viability after sterilization.

4.4.1.1. Preparation of conidial suspensions

Microconidia of the two species were cultured by inoculating 250 ml Erlenmeyer flasks containing potato dextrose broth with colonized PDA blocks and placing on a rotary shaker at 200 rpm for 7-14 days at 25^{o}C. Suspensions of microconidia were obtained by blending and centrifuging the media at 4000 rpm for 4 minutes. The concentration of microconidia was evaluated and adjusted to 5 x 10^{8} spores per ml for both species using deionized water.

4.4.1.2. Coating of seeds

Sorghum seeds were coated with microconidia of Foxy 2 and *F. proliferatum*. Three millilitres of microconidial suspensions of both species were put into separate beakers containing 5 ml of 40% (v/v) liquid sterilized (121^{o}C, 20 minutes) arabic gum and stirred. Subsequently, 8 g of surface sterilized sorghum seeds were put into these beakers and allowed to stand for 20 minutes then sieved and dried overnight at 25^{o}C. The viability of the coated seeds and the fungus was evaluated on PDA as described by Elzein et al. (2006). The viability of the fungus in terms of colony forming units (CFU) was determined by plating serial dilutions (in deionized water) on PDA and incubating in a growth chamber at 25^{o}C for three days. The CFUs for Foxy 2 were x 10^{4}/ml while those of *F. proliferatum,* 3 x 10^{4}/ml.

4.4.2. Between paper germination assay

Coated and uncoated (control) sorghum seeds were placed separately between 2 sterilized wet filter papers (Whatman GF/A, 30-20 cm) and rolled. The set up was then sealed in autoclavable plastic bags and incubated in a growth chamber in an upright position at 25^{o}C with a 12 hour fluorescent light day/night alternation. Samples (0.5 cm in length) were collected 7, 14, 21 and 28 days after sowing from two positions:

close to the seed (upper part of the root) and at the root tip and immediately put into freshly prepared fixation solution awaiting further preparation for light microscopy. For transmission electron microscopy, samples were collected three weeks after sowing from the upper part of the root. Eight roots were used for each of the three treatments (uncoated or control, Foxy 2 coated, and *F. proliferatum* coated) and the experiment was repeated thrice.

4.4.3. Wounding and infection of sorghum roots

Uncoated sorghum seeds were allowed to grow on filter paper (as described above) for four days to obtain roots of approximately 6 cm in length. The root tips were then cut off (excised) using a sterilized sharp razor blade, and the remaining wounded root attached to the seed was transferred to a Petri dish with PDA. Subsequently, 1 cm^2 of PDA blocks containing actively growing colonies of the respective fungi (Foxy 2, *F. proliferatum*) were carefully placed 0.5 cm in front of the cut (three roots per Petri dish). Control roots were wounded and transferred to PDA in Petri dishes but were not inoculated. The dishes were sealed with parafilm. Twelve replicates were used for each of the three treatments. All processes were carried out on a clean bench. The set up was incubated in a growth chamber at 25^oC for five days. Root samples were collected using a sharp sterilized razor blade, three and five days after wounding and inoculation, from two positions: just at the wound (0 to 1000 µm, and from 2000 to 3000 µm away from the cut and immediately fixed and prepared for light microscopy. The experiment was repeated four times.

4.4.4. Microscopy

4.4.4.1. Sample preparation for light microscopy (LM)

After collection, the samples (0.1 – 0.3 cm in length) were fixed in 5% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.2 and stored in the refrigerator at -4^oC. For preparation, samples were washed thrice in the same buffer and dehydrated in a graded series of ethanol for one hour each (30, 50, 70, 90 and 100%). Subsequently, they were embedded in Technovit 7100 (Kulzer, Heraeus) and sections of 5 µm thickness were made using a rotational microtome HM 340E (MICROM) with a D-shaped metal knife. The sections were placed on glass slides previously coated with poly-L-lysine, then stained with 0.05% aqueous toluidine blue (Merck) for one hour, washed with distilled water and mounted in Roti®-Histokitt (Carl Roth, GmbH). Observations were made using a Zeiss Axioplan light microscope (Göttingen,

Germany) coupled to a camera (Canon Powershot A640). Photographs were adjusted for contrast and brightness in Adobe Photoshop CS2. Fluorescent microscopic observations were also made from roots collected 2 weeks after sowing. The sections were exposed to the Zeiss filter set 02 (UV) with a 365 nm excitation and 395 nm beam splitter which allows emission at 420 nm. Ammonia (Merck, Germany) was gradually added to the sections and a change from bluish to greenish autoflourescence indicated the presence of ferulic acid (Krishnamurthy, 1999).

4.4.4.2. Sample preparation for Transmission Electron Microscopy (TEM)

For TEM, fresh samples of sorghum roots from the between paper germination assay were cut with a razor blade to sizes of about 0.1 – 0.4 cm in length. The samples were fixed in 0.1 M sodium phosphate (pH 7.2) buffered 5% (v/v) glutaraldehyde (Roth, GmbH) for one hour at room temperature, and washed three times, 10 minutes each in the same buffer. Subsequently, the samples were post fixed in 1% (v/v) osmium tetroxide in the same buffer for one hour at RT. Then, depending on the resin to be used for embedding, the samples were either dehydrated in acetone or ethanol. Samples which were dehydrated in a graded series of ethanol (30, 50, 70, 90, 95, 100%, v/v) for one hour each, and embedded in LR white (Science Services, Munich). Meanwhile those which were dehydrated using acetone in the same manner, were embedded in Epon 812 (Agar 100 resin, Plano). The LR White (in gelatine capsules) and Epon (in mould blocks) samples were both allowed to polymerize at 60^{o}C for 48 hours. Semithin sections (1500 nm or 1,5 µm) were made using an ultratome (Ultracut UCT, Leica) with glass knives, stained with 0.5% aqueous toluidine blue, mounted with Roti®-Histokitt and examined with a Zeiss Axioplan light microscope. Ultrathin sections (about 70 - 80 nm) were made using the same ultratome with diamond knives and collected on carbon and pioloform (Science Service, Munich) coated copper grids (G 200). Epon sections were stained with uranyl acetate for 30 minutes and lead citrate for 10 minutes while LR white embedded sections were stained with uranyl acetate for 10 minutes and lead citrate for two minutes. Sections were then examined with an EM 10 Zeiss transmission electron microscope operating at 60 KV. TEM negatives were scanned with an Epson Perfection 2450 Photo and imported in Photoshop to adjust contrast and brightness.

4.5. Results

4.5.1. Colonization, cell degradation and host reactions of non-wounded sorghum roots infected by Foxy 2 and F. proliferatum

At all stages of growth, Foxy 2 and *F. proliferatum* manifested completely different patterns of colonization. One week after sowing, Foxy 2 hyphae had spread along the upper part of the root surface but had not penetrated the root cells while *F. proliferatum* hyphae had already penetrated the first outer cortical layers (not shown). Two weeks after sowing, *F. proliferatum* hyphae had not only colonized all layers of the cortex but also penetrated the endodermal layer and entered the central cylinder (Fig. 4.1a). At this same stage, Foxy 2 hyphae were observed in the cortex, where digestion of some cells occurred. Even after 3 weeks, Foxy 2 was restricted to the cortex and was not able to penetrate the endodermal layer to colonize the central cylinder (not shown). On the contrary, there was massive digestion of the cells within the central cylinder after 3 weeks in roots infected by *F. proliferatum* (Fig. 4.1b). Also, *F. proliferatum* hyphae colonized root tips (Fig. 4.1c) while the root tips from Foxy 2 coated seeds (not shown) and control roots (Fig. 4.1d) were completely free of hyphae. Subsequently, 4 weeks after sowing, the destruction of root tissues by *F. proliferatum* was severe and in an advanced state where only remnants of the root tissue were left, especially thick walled vessels, endodermal cells, and also few cortical cells (Fig. 4.2a). Longitudinal sections also revealed that the digestion by *F. proliferatum* hyphae created large gaps within the root anatomy (Fig. 4.2b). At the same time, Foxy 2, hyphae had not penetrated endodermal cells; they filled the intercellular spaces at the outer endodermal wall (Fig. 4.2c, circles) and were never observed in the central cylinder (Fig. 4.2c, d).

Three weeks after sowing, transmission electron microscopy of infected tissues revealed that hyphae of both species penetrated the rhizodermis preferably between the anticlinal walls(not shown). Within the cortex, penetration by both species was similar. Invasion by hyphae of both species was always associated with host cell damage involving the degradation of cell walls and cytoplasm resulting in collapse of cells (Fig. 4.3a, 4.3b, 4.3c). Fungal cells grew within host cell walls and this was accompanied by destruction of the middle lamella (Fig. 4.3b white arrows). There was extensive colonization of the intercellular spaces by hyphae of both species.

In roots infected by *F. proliferatum*, cells of the endodermal layer were already dead three weeks after sowing, and the cell walls were degraded to a thin layer (Fig. 4.3c). Parts of the endodermal wall were dissolved around hyphae (Fig. 4.3c). In contrast, no hyphae of Foxy 2 penetrated the thin outer endodermal layer to get into the endodermal cell (Fig. 4.3d).

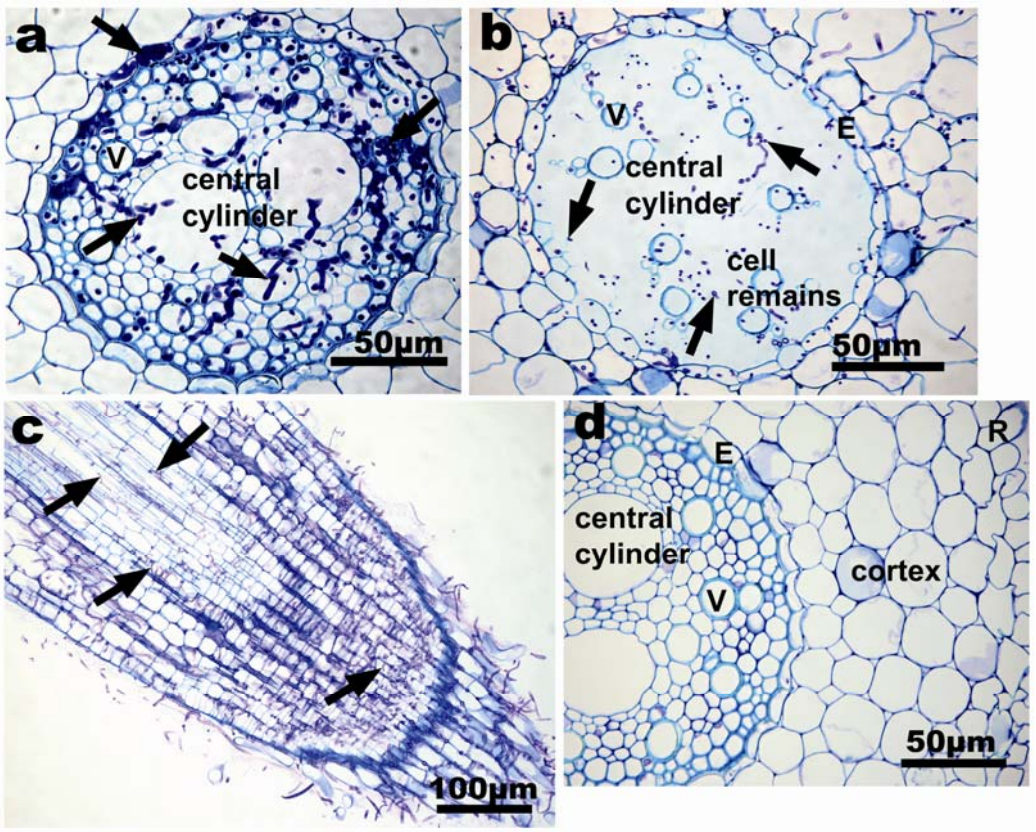

Fig. 4.1. Light micrographs of sections of non-infected and infected sorghum roots showing the invasion by *F. proliferatum* hyphae.

a. Extensive colonization of cells of the endodermis and central cylinder by hyphae (arrows), 2 weeks after sowing.

b. Three weeks after sowing, there is massive digestion of cells within the central cylinder (arrows). Hyphae have digested the whole xylem parenchyma. Also the inner thick wall of the endodermal cells (E) is reduced to a thin layer. Only parts of the thick walled vessels (V) remain.

c. Three weeks after sowing, a longitudinal section of the root tip shows complete colonization by hyphae (arrows).

d. Part of a control root two weeks after sowing. Hyphae are absent and the endodermis (E) with the inner thick wall separates the central cylinder from the cortex. R, rhizodermis; **a, b, d**, cross sections; V, vessels.

Foxy 2 hyphae were limited to the cortex and all cells of the central cylinder were intact as in the controls (not shown). Vessels of the roots from Foxy 2 coated seeds had intact cell walls (Fig. 4.4a, b) and the xylem parenchyma cells also had intact

cytoplasm (Fig. 4.4a). The heavy destructions of the whole central cylinder by *F. proliferatum* were in sharp contrast to the colonization by Foxy 2, for example, some hyphae of *F. proliferatum* grew through the cell walls and were in direct contact with degraded vessel walls (Fig. 4.4c). In most of the *F. proliferatum* samples in a more advanced state of degradation, there was severe dissolution of the cell walls of vessels and parenchyma cells although, hyphae were not in direct contact with all walls.

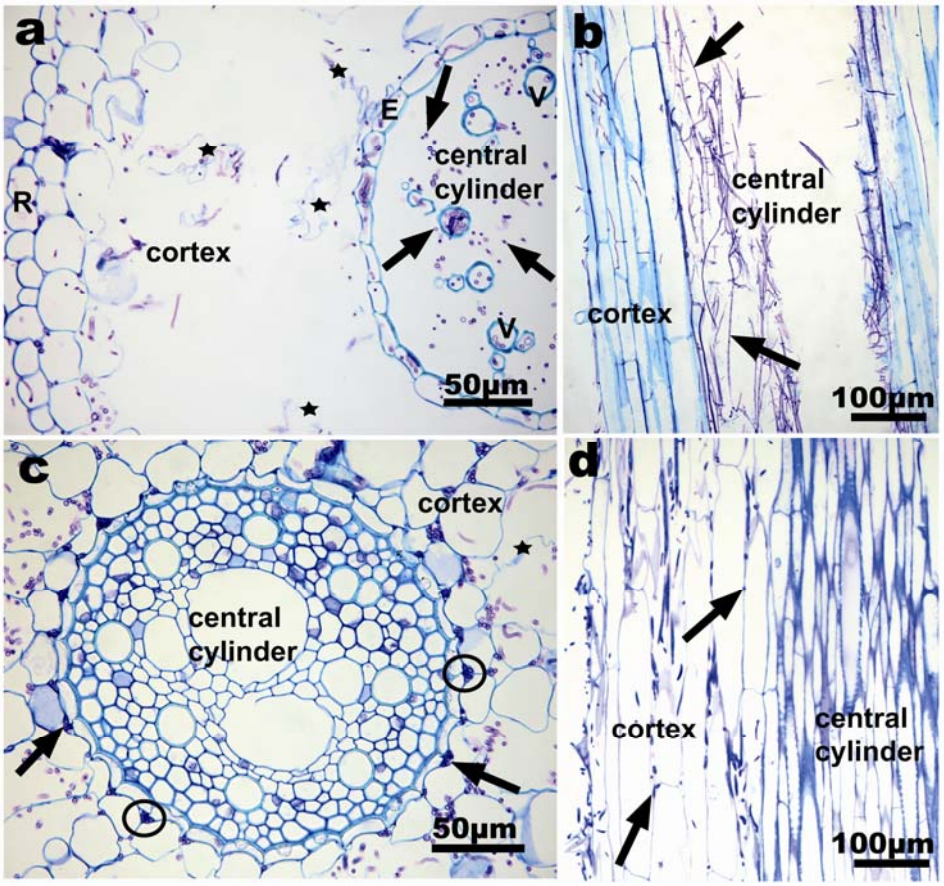

Fig. 4.2. Light micrographs showing the colonization of the upper part of sorghum roots by hyphae.

a. Cross section of part of a root infected by *F. proliferatum,* four weeks after sowing. As a result of digestion, the root cortex has a large gap with cell remnants (asterisks) between the rhizodermis (R) and the endodermis (E). Also, there is massive digestion of cells of the central cylinder by hyphae (arrows) and only some thick walled vessels (V) could be identified. The inner E is reduced to a thin layer.

b. Part of a longitudinal section of a 3 week old root colonized by *F. proliferatum.* Lots of hyphae (arrows) are within the gap created by the digestion of the central cylinder.

c. Cross section of a 4 week old root infected by Foxy 2 with intact central cylinder. Hyphae colonize just the cortex and reach the intercellular spaces of the outer endodermal walls (circles) but don't enter the endodermis and central cylinder. In some areas, hyphae are clogged forming masses on the outer endodermal wall (arrows). There is digestion of some cells within the cortex (asterisk).

d. Part of a longitudinal section of a root infected by Foxy 2. The central cylinder is free of hyphae while they colonize the cortex (arrows).

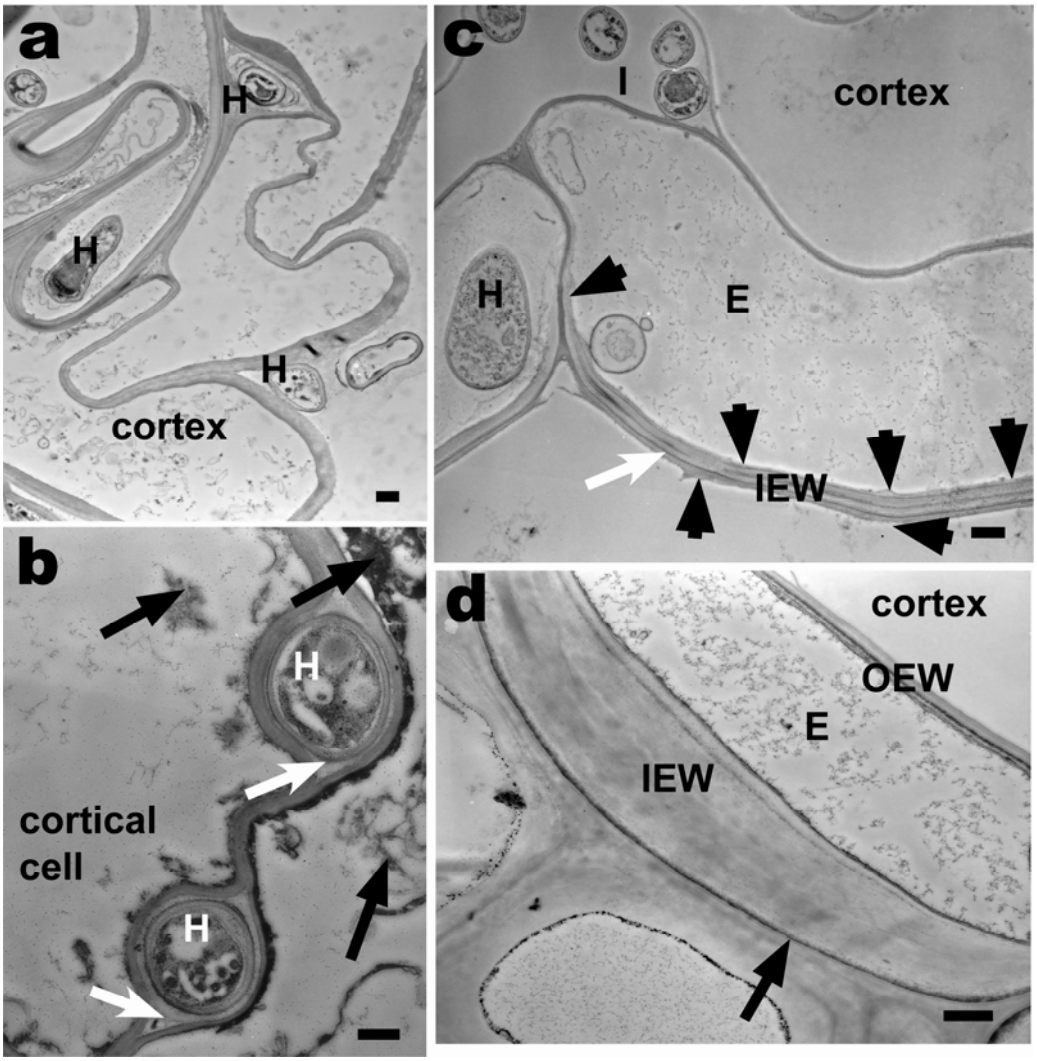

Fig. 4.3. Transmission electron micrographs of cross sections from the upper part of infected sorghum roots, showing details of the cortex and endodermis, three weeks after sowing.

- **a.** *F. proliferatum* hyphae (H) invade cortical cells. Cytoplasm is destroyed leaving remains and cell walls are twisted.
- **b.** Foxy 2 hyphae (H) grow through the cell wall within the cortex with destruction of the cell wall middle lamella (white arrows). Residues of cytoplasm (black arrows) can also be seen in the cortical cells.
- **c.** *F. proliferatum* infected root showing part of the endodermal layer having a very thin inner endodermal wall (IEW). Hyphae (H) are within the endodermis (E) digesting the wall (arrowhead). A large part of the IEW has been digested away (arrowheads) and the position of the casparian strip is seen as the remaining thin layer (arrow). I, Intercellular space.
- **d.** A Foxy 2 infected root showing part of an endodermal cell with a thin outer endodermal wall (OEW) and the thick developed inner (IEW) lignified wall having the casparian strip (arrow). No hyphae can be identified within the endodermal (E) cell and therefore the walls are intact with no signs of digestion.

a, b, d, LR white sections; **c,** Epon section; Bars = 1µm.

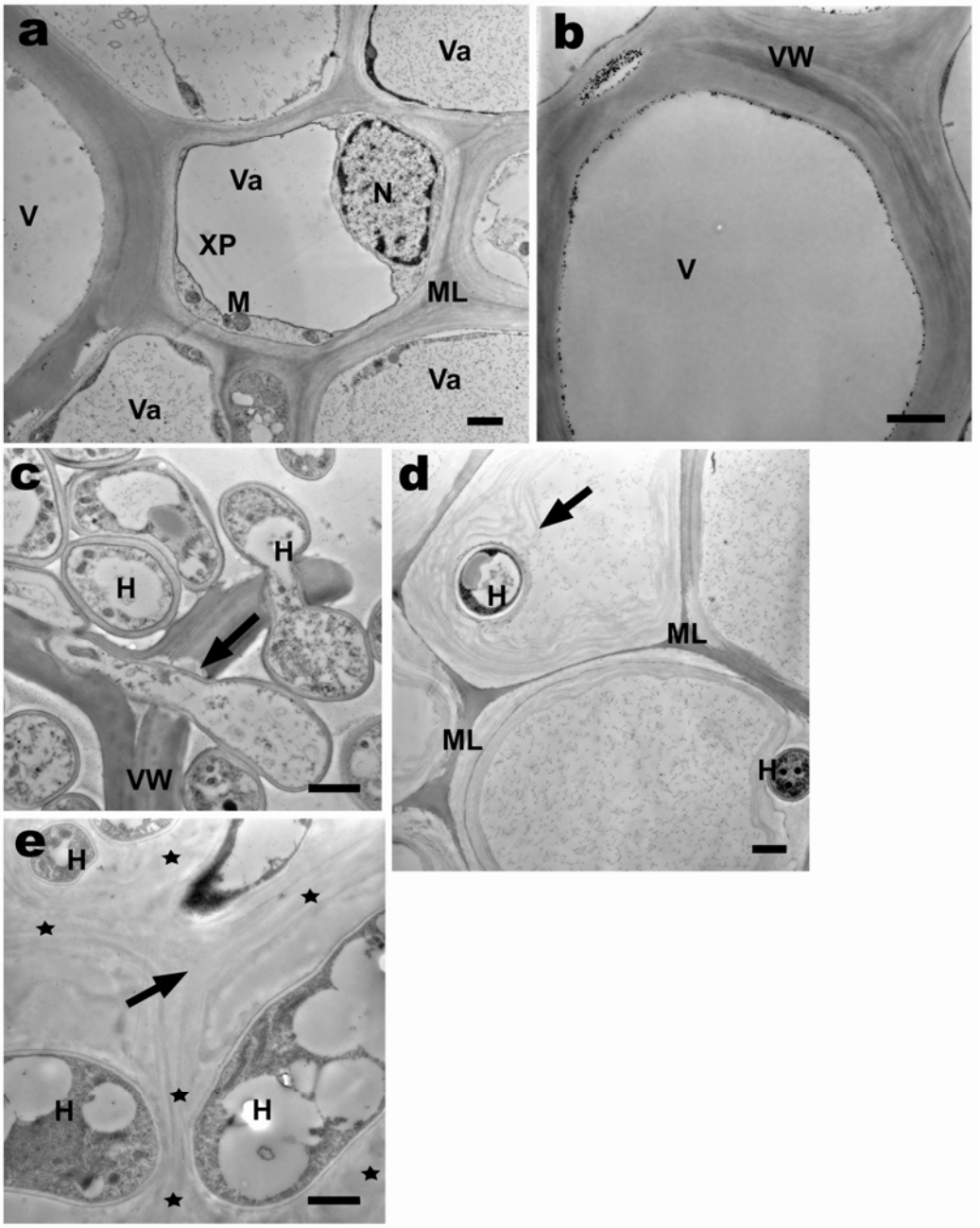

Fig. 4.4. Transmission electron micrographs of cross sections of the upper part of infected sorghum roots showing details of the central cylinder, 3 weeks after sowing.

- **a.** Part of the central cylinder of a Foxy 2 infected root. There is complete absence of hyphae. V, vessel; XP, xylem parenchyma; N, nucleus; M, mitochondrion; Va, vacuole; ML, middle lamella.
- **b.** Foxy 2 infected root revealing part of a hyphae free vessel (V) with lignified intact secondary walls (VW).
- **c.** *F. proliferatum* hyphae (H) in direct contact with vessel walls (VW), and growing through them. There is dissolution of cell wall parts (arrows).
- **d.** *F. proliferatum* hyphae (H) in vessels. The hyphae have started to digest the vessel walls from the inner side towards the middle lamella (ML). Irregular thin disintegrated vessel wall layers can be identified (arrow).
- **e.** Part of *a F. proliferatum* infected root in an advanced state of destruction. Cell walls of vessels and xylem parenchyma have deteriorated (asterisks). Cell organelles within the xylem parenchyma have been digested away. Arrow indicates position of middle lamella.

c, Epon section; **a, b**, **d,** LR white sections; Bars = 1µm

The dissolution of cell walls started from the inner side of the secondary walls and proceeded towards the middle lamellae. Irregular wall layers became visible (Fig. 4.4d). In the end, there was a complete dissolution of all types of cells in the central cylinder (Fig. 4.4e).

Blue autoflourescence in plant cell walls indicates the presence of lignin and other phenolics which might reflect host cell reactions. The upper part of 2 weeks old non infected roots exhibited an intense blue fluorescence of the lignified cell walls of the central cylinder in contrast to the non lignified cells of the cortex (Fig. 4.5a). In roots of the same age infected by Foxy 2, the blue fluorescence of the cell walls of the central cylinder increased (Fig. 4.5b). This increase was more conspicuous on the inner endodermal layer (Fig. 4.5b, arrows). There was also a slight increase in flourescence on the walls of the cortex. On the contrary, in roots infected by *F*. *proliferatum,* fluorescence decreased in the central cylinder and in parts of the cortex. The decrease was obvious on the inner endodermal layer (Fig. 4.5c, arrows). Gradual addition of ammonia to sections can be used to detect ferulic acid and a change from blue to greenish fluorescence is an indication of this acid. This change was manifested by some cell walls of the cells within the central cylinder and the endodermal wall in sorghum roots infected by Foxy 2, but not in roots infected by *F*. *proliferatum* (not shown).

4.5.2. Colonization of wounded sorghum roots by Foxy 2 and F. proliferatum

As Foxy 2 hyphae were not able to penetrate the endodermal layer and enter the central cylinder, the root tips were cut and the wounded tissues of the root were inoculated with hyphae of Foxy 2 and *F. proliferatum*. With the naked eye, mycelia of both species could be seen to have grown towards and around the wounded sorghum root two days after inoculation. Three days after inoculation, Foxy 2 hyphae did not grow into the central cylinder but after 5 days, hyphae were observed to have penetrated the central cylinder at the cut, but did not digest cells of the central cylinder (Fig. 4.6a). At a distance of 3000 µm from the cut, Foxy 2 hyphae were completely absent from the central cylinder but had invaded the cells of the cortex (Fig. 4.6b). On the contrary, three and five days after inoculation, hyphae of *F. proliferatum* had digested the cells of the central cylinder and the cortex both close to the cut and also at a distance of 3000 µm from the cut (Fig. 4.6c, d). All non-infected wounded control roots were free of hyphae (Fig. 4.6e).

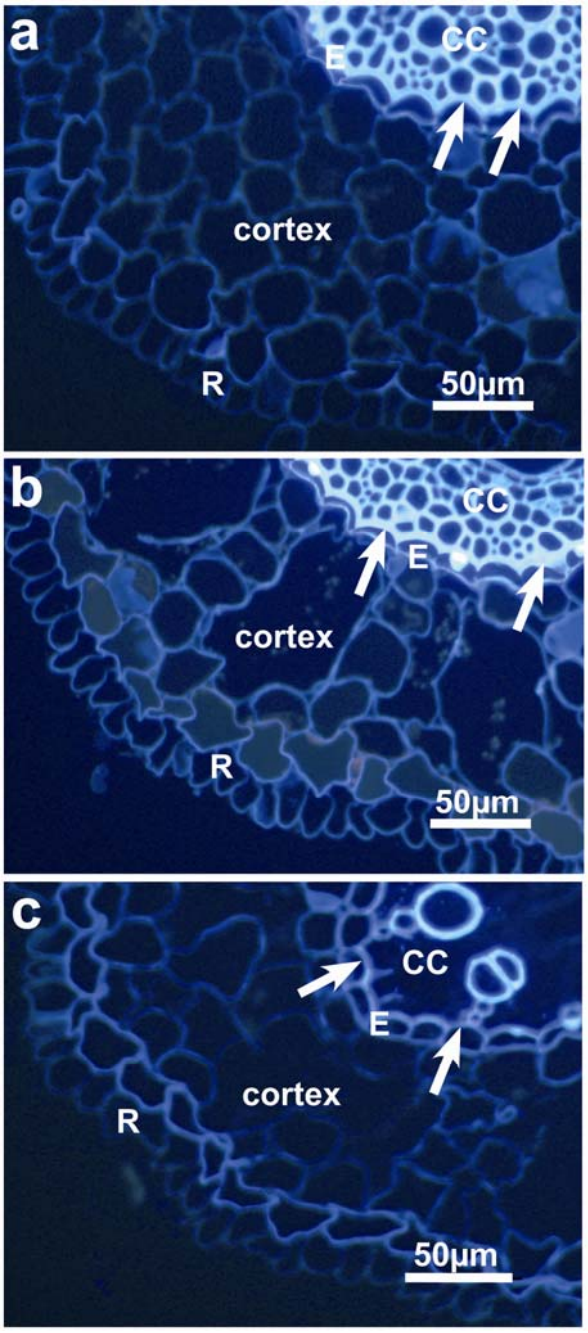

Fig. 4.5. Light micrographs of cross sections of sorghum roots, two weeks after sowing showing blue autofluorescence.

a. Parts of a non-infected root with intense fluorescence of the cell walls of cells within the central cylinder (CC) and the endodermis (arrows). There is also little fluorescence of the cell walls of the cortex. R, rhizodermis.

b. Part of a Foxy 2 infected root. There is a slight increase in fluorescence of the cells within the central cylinder (CC) especially the thick inner endodermal (E) wall (arrows). Cortical cells exhibit less fluorescence than the central cylinder but more than the cortical cells in the control roots. R, rhizodermis.

c. Part of a *F. proliferatum* infected root. There is a decrease in the fluorescence of the cell walls of the inner endodermal (E) layer (arrows) and the central cylinder. Some thick walled vessel remnants are in the CC. Some remaining cell walls within the cortex also show reduced fluorescence. R, rhizodermis.

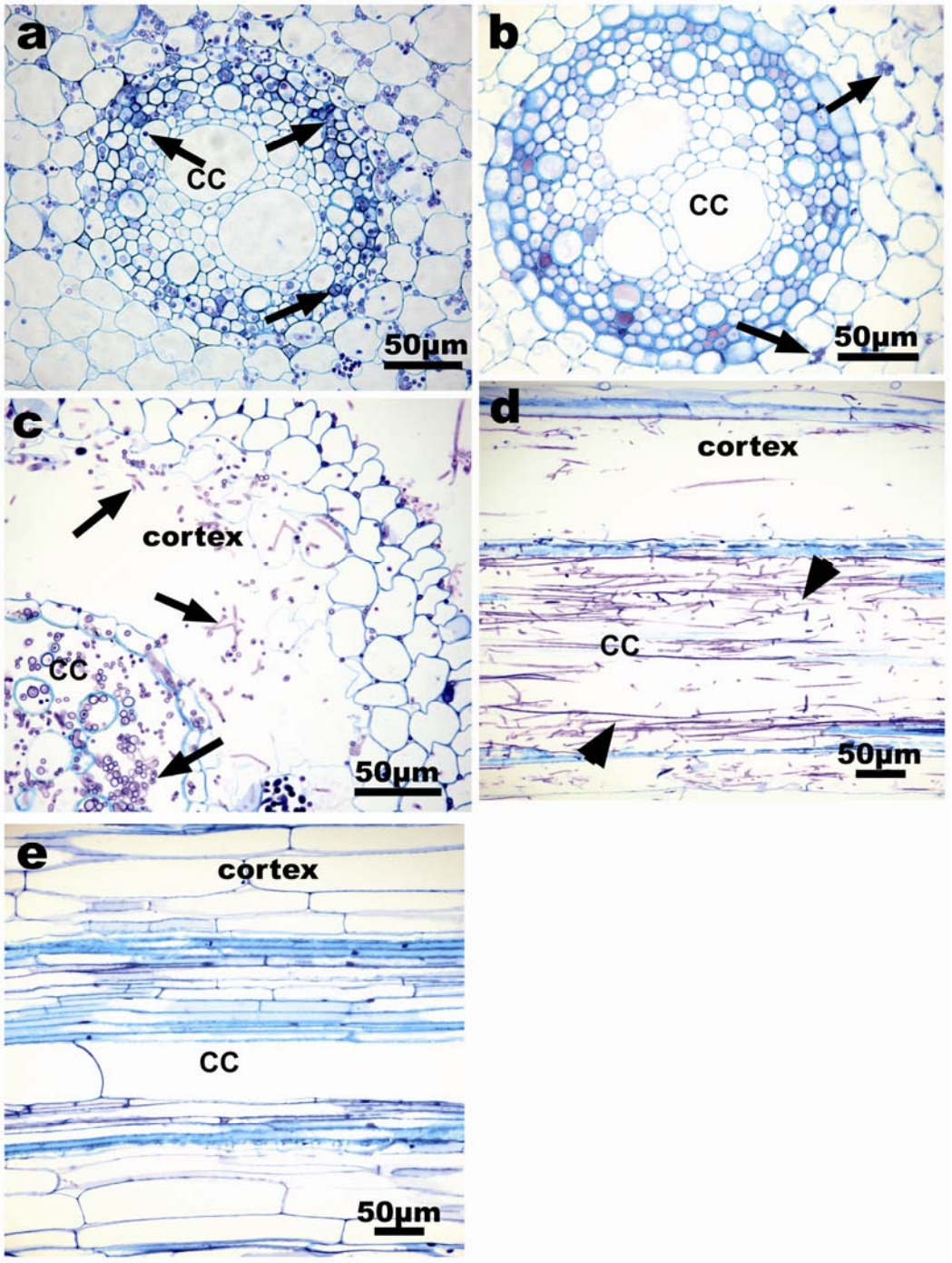

Fig. 4.6. Light micrographs of cross and longitudinal sections of wounded sorghum roots showing colonization by hyphae.

a. Five days after inoculation and close to the cut, Foxy 2 hyphae are growing in vessels and xylem parenchyma of the central cylinder (CC, arrows).

b. Five days after inoculation, Foxy 2 hyphae can be observed only in the cortical parenchyma in a distance of 3000 µm (arrows) but not in the CC.

c. Three days after inoculation, in a distance of 3000 µm, *F. proliferatum* hyphae digested both cells of the CC (arrows) and the cortex.

d. Longitudinal section, five days after wounding, showing long strands (arrow heads) of *F. proliferatum* hyphae destroying the cells within the CC at a distance of 3000 µm from the cut. Digestion has created a gap within the cortex.

e. Longitudinal section of non-infected root, three days after wounding, showing complete absence of hyphae within central cylinder (CC) and cortical cells.

4.6. Discussion

Pathogenicity towards non-target plants could be a major constraint to the use of any biocontrol agent. It is thus essential that host specificity and risk assessment be made before the release of a biocontrol organism into the environment.

4.6.1. The colonization of Foxy 2 and F. proliferatum in sorghum roots

Foxy 2 proved to be non-pathogenic to sorghum. This was attributed to several differences in the colonization of sorghum roots compared to the behavior and abilities of the pathogenic *F. proliferatum.* Firstly, it was noted that the ingress of hyphae of Foxy 2 into the cortical root cells was much slower than with *F. proliferatum*. This might have given the host more time to build up a defense against the invasion of Foxy 2. Secondly, Foxy 2 was not able to penetrate the endodermis and therefore not able to colonize the root central cylinder, confirming previous observations (Chapter 2). In contrast, ultrathin sections revealed that parts of the suberized layer of the endodermal wall were digested by secretions from the hyphae of *F. proliferatum* allowing it to enter the central cylinder.

The inability of Foxy 2 to penetrate the endodermis of sorghum could have been due to host reactions. Changes in autofluorescence of plant cell walls might be indicative of alterations in cell wall phenolic composition caused by pathogens (Krishnamurthy, 1999). We observed an increase in blue fluorescence in the endodermis of Foxy 2 infected roots probably reflecting an increase in lignin or other phenolics in the cell wall. Lignification renders the wall more rigid and also makes it less liable to enzyme digestion. Additionally, an increased presence of wall bound ferulic acid and other phenolics, as indicated by its greenish colour upon addition of ammonia, could have further protected the underlying tissues by creating an enforced mechanical barrier which appeared to have restricted invasion by hyphae of Foxy 2. The behaviour of Foxy 2 and associated host reaction was in line with observations with the pathogenic nematode, (*Radopholus similis*), which was reported to be restricted from invading the vascular bundle of banana roots due to high levels of lignification and suberization of the endodermis (Valette et al., 1998). In contrast, such reactions were not observed with *F. proliferatum* because the endodermal cells had been digested away.

It was also eminent that the fluorescence of the cell walls of the endodermis and central cylinder decreased in the roots infected by *F. proliferatum* due to the digestion

of their cells and cell walls. The different actions on the endodermal wall of the two investigated fungal species might be related to their enzyme production profile. It has been shown that *Fusarium* spp. secrete CWDEs (Cell Wall Degrading Enzymes) during root penetration and colonization including polygalacturonases, pectate lyases, xylanases and proteases (Beckman, 1987). The ability of Foxy 2 to digest exodermal and cortical cells but not the endodermis could be attributed to the absence of enzymes required for digestion of the special cell walls of the endodermal cells or that the enzymes were not expressed at that point in the infection cycle. Evidence for this might be provided by the observation that *xyl3* (gene encoding xylanases) is expressed throughout the disease cycle, whereas *xyl2* is only expressed during the final stages in tomatoes infected by *F. oxysporum* f.sp. *lycopersici* (Ruiz-Roldán et al., 1999) indicating that genes are differentially expressed during a disease cycle.

Earlier studies have been carried out to compare the action of pathogenic and non-pathogenic fungi. Benhamou and Garand (2001) compared the cytology of pea root infection by a pathogenic strain (*F. oxysporum* f.sp. *pisi*) with a non-pathogenic strain *F. oxysporum* Fo47. Fo47 caused the production of wall appositions and accumulation of electron opaque material around the sites of potential hyphae penetration and the formation of papillae. In the present study, Foxy 2 hyphae were not restricted to the outer cortex as observed for Fo47 but were able to invade all cortical layers. Our observations also were not in line with those of Olivain and Alabouvette (1997) who studied the colonization of tomato (*Lycopersicon esculentum* Miller) roots by *F. oxysporum* Schlecht. (*emended* by Snyder and Hansen) and showed the presence of defense reactions of the plant such as cell wall thickenings, wall appositions, intercellular plugging and intracellular osmiophilic deposits. No papillae or such reactions in both Foxy 2 and *F. proliferatum* were observed except an increase in lignification for Foxy 2 infected roots. This was unlike in the case of *F. oxysporum* f. sp. *callistephi* race 3 on staghorn sumac (*Rhus typhina*) where cell wall thickenings and tyloses in vessels were observed as cell reactions to invasion (Ouellette et al., 2006).

4.6.2. A further barrier against Foxy 2 invasion in sorghum roots?

Apart from not being able to penetrate the endodermis of sorghum, Foxy 2 did not colonize the central cylinder to a greater extent even upon exposure to the tissues of the central cylinder as it was not observed at a distance of 3000 µm from the

wounding site. This was contrary to our initial hypothesis. It indicates that not only was the endodermis a anatomical barrier, but also there could be other mechanisms that acted as a barrier within the central cylinder (still unknown physiological defense mechanisms) and prevented further growth of Foxy 2 within the central cylinder of the root, therefore not allowing further spread of the fungus within the plant. Spread of a microorganism along the central cylinder can be facilitated by two pathways: a) digestion of the vessel walls as observed with *F. proliferatum*, or b) movement of the organism within the free space of the transport vessels. Foxy 2 was obviously restricted in its movement within the central cylinder in both pathways. Firstly, movement of Foxy 2 was restricted by the observed increase in vessel wall lignification as seen in fluorescence microscopy and its apparent deficiency in expression of relevant CWDE genes. Secondly, Foxy 2 was also not able to proceed further within the vessels and therefore could not cause wilting of the plant.

Our observations thus indicated that exposure of the vascular system (wounds) is not a prerequisite for Foxy 2 to acquire pathogenic behaviour and to cause destruction of the non-host vessels and disease symptoms, even if it enters the vascular system of sorghum roots by chance. This is an important feature when using Foxy 2 as a mycoherbicide; since in our recent investigations (Chapter 3) we showed that hyphae of Foxy 2 penetrated within *Striga* haustoria into the central cylinder of sorghum roots. This situation can also occur during attack of roots by either nematodes or bacteria causing an exposure of the vascular system. The observations in this study are not in line with the reports of Doling (1963) and Sequeira et al. (1958). The former showed that there must be mechanical damage to pea roots before *F. oxysporum* f.sp. *pisi* can invade the vascular system of the plant, while Sequeira et al. (1958) showed that cutting the vascular tissue to expose the vessels and puncturing the roots of bananas resulted in rapid penetration of *F. oxysporum* f.sp. *cubense* into the vessels. The results from the current study show a very tissue specific action towards Foxy 2. Nevertheless, taking into consideration its function as a mycoherbicide, this mode of action could be seen as a very advantageous characteristic in that the digestion of cortical cells was necessary for the survival of the fungus in readiness for the attack of any *Striga* radicals (germinated seed) which might attach to the sorghum roots. Meanwhile, the fungus caused no harm to the sorghum plant in general as it did not proliferate within the central cylinder to invade the stem to cause wilting and can thus be said to be non-pathogenic to the sorghum plant as a whole. It should be noted that

our previous investigations showed that Foxy 2 can survive in sorghum roots for up to four weeks which coincides with the peak number of *Striga* that get attached to host roots and is appropriate for enabling the fungus to infect nearly all attached *Striga* seedlings (Chapter 2). Further experiments showed that *Striga* seedlings were damaged within 26 days after sowing sorghum seeds (Chapter 3). Foxy 2 can thus be considered appropriate and well suited for biological control. This is supported by the fact that vascular colonization or otherwise invasion of the vascular system has been described as an essential feature of wilt pathogens (MacHardy and Beckman, 1981). Also, Agrios (2005) defines pathogenicity as the ability of a pathogen to cause disease i.e. the ability of a parasite to interfere with one or more of the essential functions of the plant thus causing disease.

4.7. Acknowledgements

The authors would like to thank Frau Rassow Bärbel for all her technical assistance in the laboratory. The first author is grateful to KAAD (Katholischer Akademischer Ausländer-Dienst) for financial support.

CHAPTER 5

The phytotoxic mycotoxin beauvericin produced by biocontrol agent *Fusarium oxysporum* f.sp. *strigae* (Foxy 2) accumulates in *Striga hermonthica* but not in sorghum grains

5. The phytotoxic mycotoxin beauvericin produced by biocontrol agent *Fusarium oxysporum* f.sp. *strigae* (Foxy 2) accumulates in *Striga hermonthica* but not in sorghum grains [d]

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5.1. Abstract

The potential biocontrol agent *Fusarium oxysporum* f.sp. *strigae* 'Foxy 2' has been shown to cause death of *Striga hermonthica* plants but its specific mode of action is yet to be fully understood. Many *Fusarium* strains have been reported to produce toxins. Therefore, HPLC-MS-MS analyses were performed to investigate the toxin production ability of Foxy 2 *in vitro* and in infected *Striga* shoots. Additionally, sorghum grains derived from *Striga* parasitized plants infected from Foxy 2 on coated sorghum seeds were analyzed for the presence of toxins to ensure the safety of this mycoherbicide. Of the toxins tested (beauvericin, fumonisins B1, B2, B3, C and P series, enniatins A, A1, B and B1, and moniliformin), Foxy 2 was able to produce only beauvericin in rice substrate and in *Striga* shoots. Severely diseased *Striga* shoots (six weeks after emergence) contained 720 μg beauvericin kg^{-1} *Striga* shoot sample

[d] **This chapter has been submitted as:**
Ndambi, B., Döll, K., Karlovsky, P., Cadisch, G., Spring, O. & Heller, A. (2011). The phytotoxic mycotoxin beauvericin produced by biocontrol agent *Fusarium oxysporum* f.sp. *strigae* (Foxy 2) accumulates in *Striga hermonthica* but not in sorghum grains. Submitted to BioControl. *Table 5.1, Figures 5.1 and 5.2 are not included in the submitted manuscript.*

whereas three weeks old less infected shoots had only 60 μg kg^{-1}. Effects of beauvericin were examined by applying the pure toxin on shoots. Transmission electron microscopy revealed that beauvericin caused cell death in all types of tissues at concentrations of 10 μM and 50 μM and, interestingly, some membranes of the cells appeared dissolved. None of the mycotoxins tested was found in sorghum grains harvested from plants growing from Foxy 2 coated sorghum seeds, thus confirming the safety of Foxy 2 as biocontrol agent.

5.2. Keywords: fumonisin, HPLC-MS-MS, transmission electron microscopy, biocontrol, beauvericin, sorghum, *Striga hermonthica*

5.3. Introduction

The giant witchweed, *Striga hermonthica* causes serious yield losses in tropical cereals and legumes especially to subsistence farmers (Lendzemo and Kuyper, 2001). As there are no single effective and economically feasible methods for controlling *S. hermonthica* (Lagoke et al., 1991; Debrah, 1994), an integrated approach involving biocontrol is gaining more grounds. The biocontrol agent, *Fusarium oxysporum* f.sp. *strigae* Elzein et Thines (Foxy 2) effectively infects and kills *S. hermonthica* (Elzein and Kroschel, 2004a) but the specific mode of action is yet to be fully understood and its safety with respect to mycotoxin accumulation in the crop has not been proven. Recently, we showed that Foxy 2 killed underground *S. hermonthica* seedlings with a massive degradation of cells (Chapter 3). The few seedlings that were not immediately killed by Foxy 2 managed to emerge to the surface showing impaired development and signs of wilting as their vessels were blocked by hyphae. With ongoing infection, Foxy 2 hyphae also attacked vessels, digested the vessel walls and caused degradation of the surrounding xylem parenchymal cells (Chapter 3). Since most species of *Fusarium* have been shown to produce mycotoxins (i.e. metabolites toxic to humans and animals, as well as phytotoxic compounds), the involvement of toxins in the infection process of Foxy 2 in *S. hermonthica* is likely. The ability of Foxy 2 to synthesize mycotoxins in infected plants has not been studied. It is only known that Foxy 2 produces fusaric acid and 9,10- dehydrofusaric acids in axenic cultures (Amalfitano et al., 2002). Considering that plant pathogenic fungi often produce toxins facilitating the infection of host plants (Strange, 2007), the objective of this study was to investigate the possibility of the involvement of toxins in the pathogenicity of Foxy 2 to *S. hermonthica* shoots. In this

light, pure fungal cultures, rice substrate and *Striga* shoots at various stages of Foxy 2 infection were investigated for the ability of Foxy 2 to produce toxins.

Mycotoxins in food and feed pose a health risk to humans and animals (Bhat et al., 2010). Numerous mycotoxins have been demonstrated to occur in grains for which biological control of *Striga* by *Fusarium* is being developed. *Fusarium* mycotoxins in African grain with fumonisins in the first place are prime suspects in the chronic condition of rural populations dubbed "the maize disease" (Dutton, 2009). Co-occurrence of fumonisins with zearalenone (Doko et al., 1996), enniatins (Adejumo et al., 2007a), and aflatoxins (Kimanya et al., 2008) has been reported; trichothecenes (Adejumo et al., 2007b) and ochratoxin A (Adebajo et al., 1994) have been found in African grain, too. The use of *Fusarium* species as biological control agents may increase the risk of mycotoxin exposure to consumers and farm animals. We therefore investigated sorghum grains from plants grown from Foxy 2 coated seeds for contamination with mycotoxins so as to verify the safety of the use of this mycoherbicide. In addition, considering that certain *F. oxysporum* strains were able to produce fumonisins (Seo et al., 1996; Abbas et al., 1995) which are among the most dangerous mycotoxins of *Fusarium* spp. (Marasas, 1995), and the mycotoxin beauvericin (BEA) (Logrieco et al., 1998), we tested Foxy 2 for the ability to produce fumonisins and BEA and analyzed its genome for the presence of the FUM1 gene which is an essential gene involved in fumonisin synthesis.

5.4. Methodology

5.4.1. Plant and fungal material

Striga hermonthica (Del.) Benth seeds were collected from Sudan in 2006. Two *Sorghum bicolor* L. Moench cultivars were used in the experiments: the *Striga*-susceptible cultivar “Cowbaula” from Burkina Faso (provided by the International Institute of Tropical Agriculture (IITA) Benin Station), and the *Striga*-tolerant cultivar “Wad Admed” from Sudan (provided by the agricultural Research Corporation (ARC), Sudan). All seeds were surface sterilized by complete immersion in 1% sodium hypochlorite (NaOCl) for five minutes, and then rinsed with tap water before use. The fungus used in the study was *Fusarium oxysporum* f.sp. *strigae* Elzein et Thines (Foxy 2) isolated from diseased *S. hermonthica* plants in Northern Ghana by Abbasher et al. (1995). Confirmation of its identification was by the Federal Biological Research Center for Agriculture and Forestry, Berlin, Germany, and the strain has been

deposited under accession number BBA- 67547-Ghana. *F. verticillioides* VP2 (Visentin et al., 2009) was used as positive control for the presence of the gene FUM1, and *F. oxysporum* 121 obtained from Dr. E. Möller (University of Hohenheim, Stuttgart, Germany) was used as positive control for the specific detection of the species *F. oxysporum* in plants.

5.4.2. Cultivation of Striga *parasitizing sorghum and inoculation with Foxy 2*

5.4.2.1. Inoculation of sorghum seeds and cultivation

Sorghum seeds were film-coated with a homogenized suspension of gum arabic and dried Foxy 2 chlamydospores (Elzein et al., 2006) by SUET (Saat u. Ernte-Technik GmbH, Eschwege, Germany) company in order to provide inoculation for the infection of *S. hermonthica*. The viabilities i.e. colony forming units (CFU) of Foxy 2 per coated sorghum seed were 2.1 x 10^2 CFU for the tolerant cultivar and 2.3 x 10^2 CFU for the susceptible cultivar. *S. hermonthica* seeds (50 mg) and three Foxy 2 inoculated (coated) sorghum grains were sown together in soil in pot experiments and cultivated up to four months in the glasshouse as described in Chapter 3. Emerged *S. hermonthica* shoots were collected from Foxy 2 infected plants, three weeks after emergence when still no symptoms of Foxy 2 infection were visible on the shoots, and again six weeks after emergence in the advanced state of Foxy 2 infection when shoots showed severe symptoms of browning and wilting.

In the same experiment, mature sorghum grains were harvested four months after sowing from *S. hermonthica* parasitized sorghum plants which were growing from Foxy 2 coated seeds. As controls, *S. hermonthica* shoots and mature grains were harvested at the same time from plants growing from non-coated sorghum seeds.

5.4.2.2. Direct Foxy 2 inoculation of S. hermonthica shoots

Because *Striga* plants which emerged from sorghum seeds film-coated with Foxy 2 (naturally infected) did not show immediate symptoms of wilting, we did an additional inoculation of these infected shoots with Foxy 2 directly. About 500 µL of Foxy 2 microconidia suspension (2.45 x 10^9 conidia mL^{-1} grown in the liquid media potato dextrose broth) was injected with a syringe into the xylem part of previously Foxy 2 infected *S. hermonthica* shoots. This was done two weeks after emergence when *Striga* plants were between 30 and 45 cm in height. Samples were collected one week after injection with early symptoms (some leaves started getting brown tips and the

plant showed early signs of wilting) and four weeks after injection (severe symptoms occured, plants showed browning and advanced state of wilting). All *Striga* samples (Foxy 2 infected and injected) consisted of the whole shoot (heights of 35 – 52 cm above ground). Shoots from four different *S. hermonthica* plants were combined and replicates from three planting seasons were pooled together for each treatment.

5.4.3. Mycotoxin analysis

5.4.3.1. Sample preparation and extraction

Three sorts of samples were used for mycotoxin analysis: *S. hermonthica* shoots, sorghum grains, and rice substrate. *S. hermonthica* shoots were cut into pieces, frozen in liquid nitrogen, brought to a fine powder in a mortar and freeze dried. Sorghum grains were dried overnight at 45°C and milled to a fine powder. Rice substrates were prepared by adding 70 ml deionised water to 50 g polished rice, autoclaving twice, inoculating with small agar blocks overgrown with Foxy 2 mycelium and incubating for four weeks at 25°C (Fig. 5.1). Samples for mycotoxin analysis (4 g) were extracted with 40 mL of methanol/bidistilled water (3:1 v/v) by shaking overnight at 170 rpm at room temperature, and cleared by centrifugation at 4,500xg for 10 min. The extract was used for HPLC-MS-MS detection of the toxins.

Fig. 5.1. Incubation of rice cultures inoculated with Foxy 2 and non-inoculated (controls).

5.4.3.2. Cleanup for fumonisin analysis

For fumonisin analysis, extracts of *S. hermonthica* shoots and sorghum grains were cleaned up (Fig. 5.2) on a strong anion exchange (SAX) solid phase cartridge (Varian, Darmstadt, Germany) according to Shephard et al. (1990) with modifications. The columns were preconditioned with 8 mL of methanol (HPLC grade) followed by methanol:water (3:1 v/v). 10 mL of sample extract was applied to the cartridge which was subsequently washed with 8 mL methanol/water (3:1 v/v) and with 4 mL methanol. Elution was done using 10 mL of 5% acetic acid in methanol. The flow rate was kept below 1 mL min^{-1} during the whole procedure. The elute was evaporated in a speed vacuum concentrator at 40°C. The residue was dissolved in 500 µL of methanol/water (1:1 v/v).

Fig. 5.2. SAX cartridges used for cleanup which leads to 20-fold enrichment, in order to improve the sensitivity for fumonisin detection.

5.4.3.3. HPLC-MS-MS analysis

The production of the toxins enniatins A, A1, B and B1, BEA and fumonisins B1, B2, B3, C, and P by Foxy 2 was evaluated in *S. hermonthica* shoots, rice cultures of Foxy 2, and in sorghum grains. Chromatographic separation of the mycotoxins was performed by high-performance liquid *chromatography* (HPLC) on a reverse phase *Kinetex* C18 column, *50.0 x 2.1 mm, particle 2.6 µm,* equipped with C_{18} security guard

cartridge, 4 mm × 2 mm i.d., both from Phenomenex (Aschaffenburg, Germany). The mobile phase consisted of (A) water with 5% acetonitrile and (B) methanol, both containing 7 mM acetic acid. Only moniliformin separation was performed on a HILIC system (Khorassani et al., 2011). The flow rates were set to 0.2 ml min^{-1}. HPLC was coupled with an electrospray ion source and ion-trap tandem mass spectrometer 500-MS or triple quadrupole mass spectrometer 1200L (both from Varian, Darmstadt, Germany)

BEA was identified and quantified in a positive ionization mode by using sodium adduct with m/z of 806.4 as precursor ion and daughter ions of m/z 645.5, 545.5 and 384.4. No matrix effect was observed, therefore pure standards in methanol/water (1/1;v/v) were used for quantification. For the detection of fumonisins belonging to the C and P series, HPLC-MS in full-scan mode was performed on the ion trap scanning for the specific masses described by Musser et al. (1996) and Seo et al. (1999). Enniatins A, A1, B and B1 and fumonisins B1, B2 and B3 were analyzed using HPLC coupled with an electrospray ion source and a triple quadrupole mass spectrometer essentially as described by Adejumo et al. (2007a). For fumonisins, extracts were analyzed after cleanup on SAX cartridges, which leads to 20-fold enrichment.

The limits of quantification and detection were 10 g kg^{-1} and 3 g kg^{-1} for BEA, enniatin A1, B and B1, 20 μg kg^{-1} and 7 μg kg^{-1} for enniatin A1, 5 g kg^{-1} and 2 g kg^{-1} for fumonisin B and 300 g kg^{-1} and 100 μg kg^{-1} for moniliformin, respectively.

5.4.4. Real-time PCR analysis

Fungal strain Foxy 2 was grown in 100 ml of potato dextrose broth for one week at 25°C without shaking. After harvesting the mycelium by filtration, 400 µl of ethanol (99%) was added to the mycelium in a 2 ml reaction tube, the mixture was vortexed and the mycelium dried in a speed vacuum concentrator. *S. hermonthica* shoots from pot experiments were freeze-dried and ground. DNA from fungal mycelium and *S. hermonthica* tissue was extracted using the CTAB method as described by Brandfass and Karlovsky (2008).

Real-time PCR was used to confirm the presence of *F. oxysporum* within *S. hermonthica* shoots and to detect the FUM1 gene in Foxy 2 pure cultures. To detect the presence of *F. oxysporum* in *S. hermonthica*, the primer pair Clox1

(CAGCAAAGCATCAGACCACTATAACTC) and Clox2 (CTTGTCAGTAACTGGACGTTGGTACT), specific for *F. oxysporum* (Mulè et al., 2004) was used. For the detection of the FUM1 gene in the DNA of Foxy 2, the primer pairs rp32 (ACAAGTGTCCTTGGGGTCCAGG) and rp33 (GATGCTCTTGGAAGTGGCCTACG) (Proctor et al., 2004), as well as FUM1F7328 (ATGGAACTTGGAACCTGCAC) and FUM1R7664 (AGCTGGTACTCGGGATGATG) (Döll et al., unpublished) were used. PCR was carried out using PCR premix QPCR SYBR Green Mix (Abgene/Thermo Fisher, Schwerte, Germany) with 1.5 mM $MgCl_2$ and thermocycle program described for species-specific PCR for *F. verticillioides* (Nutz et al., 2011).

5.4.5. Tests for the effect of beauvericin on S. hermonthica *shoots*

5.4.5.1. Toxin preparation and application

BEA was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.), dissolved in methanol to 1 mg mL^{-1} and diluted to concentrations of 10 μM and 50 μM using bidistilled water. These concentrations were chosen as it has been shown to be the concentration range produced by most *Fusarium* species in various plants (Marasas et al., 1984; Logrieco et al., 2002).

S. hermonthica shoots from plants attached to roots of non-coated sorghum seeds were cut into pieces of about 2 cm each and placed on wet filter paper in petri dishes. A drop of 5 μL of toxin was placed in the middle of each shoot piece. For the control, a drop of methanol-water of the same concentration as for the toxin application was also placed on other shoots. Eight replicates were used for each treatment. The experiment was carried out at room temperature and repeated twice.

5.4.5.2. Preparation for transmission electron microscopy

21 and 24 hours after application of BEA or methanol-water, *Striga* shoot samples (1 - 3 mm in length) were cut with a razor blade from the area of the drop and fixed immediately in 2.5 % (v/v) buffered glutaraldehyde (0.1 M phosphate buffer, pH 7.2) for one hour and postfixed in 1% (w/v) buffered osmium tetroxide (0.1 M phosphate buffer, pH 7.2) for an other hour at RT. The samples were then rinsed in double distilled water, three times, 10 minutes each. Then they were dehydrated in a graded series of ethanol [30%, 50%, 70%, 100%, 100% (v/v)], at RT for one hour each, infiltrated and embedded in LR White (Science Services, Munich) and polymerized in

gelatine capsules at 60ºC. An Ultracut UCT (Leica) microtome with a diamond knife was used to get ultrathin sections of about 80 nm. The sections were transferred onto Pioloform (Science Service, Munich) and carbon coated copper grids, stained with uranyl acetate and lead citrate (Reynolds, 1963), and examined with a transmission electron microscope (EM 10, Zeiss) at 60 kV. The negatives were scanned (Epson Perfection 2450 Photo) and the pictures were adjusted for contrast and brightness in Photoshop CS2, version 10.

5.5. Results

5.5.1. Toxins in Striga hermonthica shoots

S. hermonthica plants attached to sorghum plants grown from Foxy 2 coated seeds showed severe symptoms such as browning and wilting within six weeks after emergence of shoots. To obtain a higher infection rate and to accelerate the infection process, Foxy 2 microconidia were injected directly into some *Striga* shoots of already infected plants. In these shoots, symptoms were already visible one week after injection with early symptoms of wilting like browning of the tips of some leaves.

Results of the HPLC-MS/MS analysis revealed that among the analyzed toxins (BEA, fumonisins B1, B2, B3, C and P series, enniatins A, A1, B and B1, and moniliformin), only BEA (Fig. 5.3, Table 5.1) was detected in Foxy 2 infected *S. hermonthica* shoots. At the same stage of growth (6 weeks after emergence), *Striga* shoots which were additionally directly injected with Foxy 2 inoculum contained a slightly higher amount of BEA (930 μg BEA kg^{-1} *Striga* shoot weight) as *Striga* shoots infected by seed coated Foxy 2 (760 μg BEA kg^{-1}, Table 5.2).

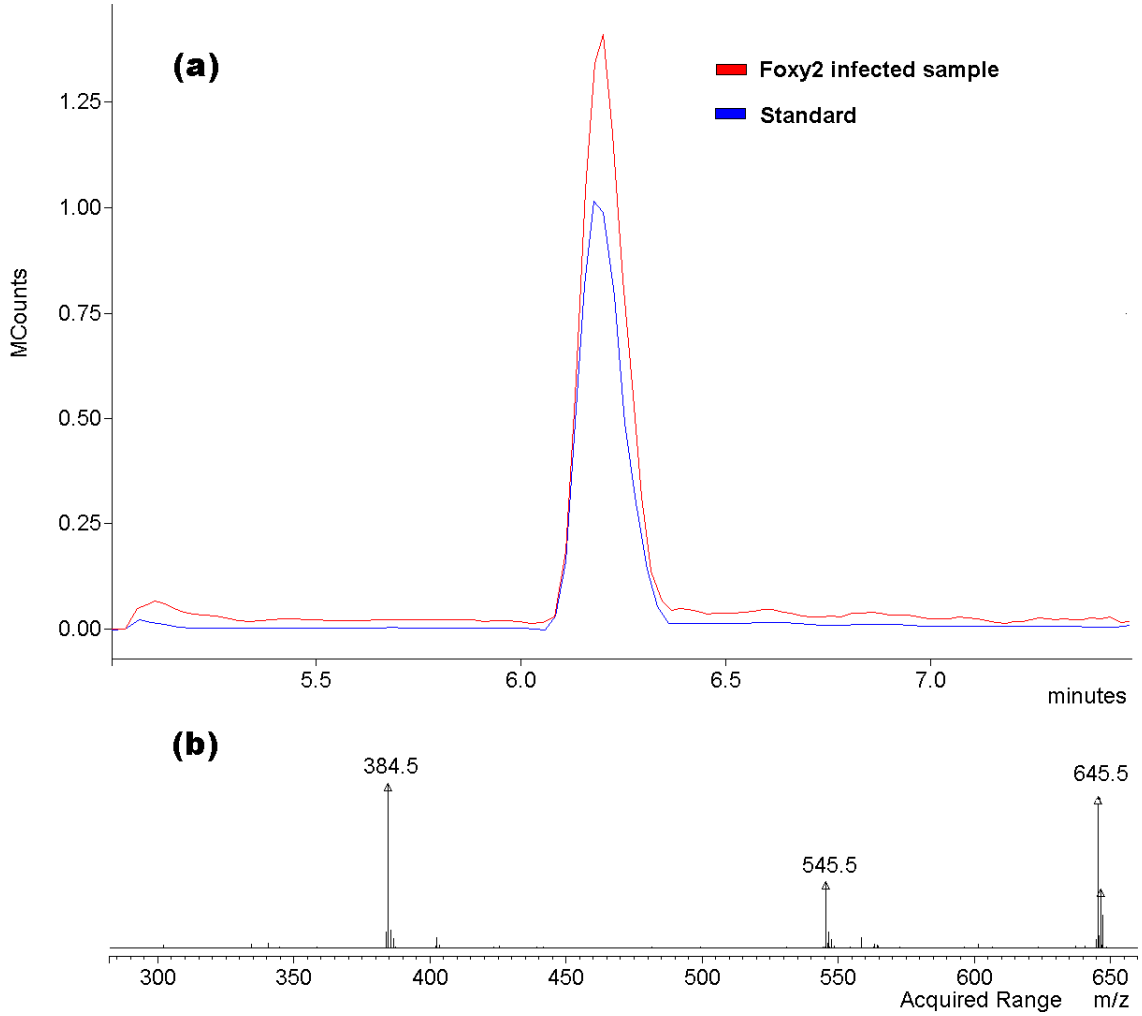

Fig. 5.3. Chromatogram (a) and fragmentation pattern (b) of beauvericin analyzed with HPLC-MS/MS in Foxy 2 infected *S. hermonthica* shoots six weeks after emergence in comparison to pure beauvericin standard. Ions labeled in the mass spectrum are sodium adducts of daughter ions of beauvericin.

Real-time PCR using species specific primer pairs was used to confirm the presence of *F. oxysporum* within the *S. hermonthica* shoots. *F. oxysporum* was detected by PCR in infected shoots while non-infected *S. hermonthica* shoots gave negative results (Table 5.3).

Table 5.1: Toxin production by Fusarium oxysporum f.sp. strigae in Striga hermonthica shoots as revealed by HPLC-MS/MS analysis.

Treat-ment	Weeks after emer-gence	Monili-formin LOD=100 μg kg^{-1}	Beauve-ricin LOD=3 μg kg^{-1}	Fumonisins LOD=2 μg kg^{-1}			Enniatins LOD=3 μg kg^{-1}			
				B1	B2	B3	A	A1*	B	B1
Control	6	ND	ND	ND	ND	ND	ND	ND	ND	ND
Injected control	6	ND	ND	ND	ND	ND	ND	ND	ND	ND
Foxy 2** infected	3	ND	D	ND	ND	ND	ND	ND	ND	ND
Foxy 2 infected	6	ND	D	ND	ND	ND	ND	ND	ND	ND
Foxy 2*** injected	3	ND	D	ND	ND	ND	ND	ND	ND	ND
Foxy 2 injected	6	ND	D	ND	ND	ND	ND	ND	ND	ND

ND: Not detected
D: Detected
LOD: Limit of detection
[a] *Controls not injected*
[b] *Controls injected with water*
**LOD of Enniatin A1 was 7*μg kg^{-1}
*****Striga plants naturally infected from Foxy 2 colonized sorghum roots*
*******Striga plants which were injected with Foxy 2, two weeks after emergence.*

5.5.2. Toxins in Foxy 2 rice substrate and pure cultures

In the rice cultures of Foxy 2, HPLC-MS/MS analysis revealed that amongst the toxins tested (BEA, fumonisins B1, B2, B3, C and P series, enniatins A, A1, B and B1, and moniliformin), only BEA was detected (detection limit 3 μg kg^{-1}). None of the toxins were detected in the controls.

The genomic DNA of pure mycelium of Foxy 2 was analyzed by PCR for the presence of the FUM1 gene involved in the synthesis of fumonisins using two primer pairs (see Materials and Methods). The results were negative. The positive control *F. verticillioides* (VP2) and the negative control *F. oxysporum* 121 generated expected results with both primer pairs (Table 5.3).

Table 5.2: Beauvericin production by Fusarium oxysporum f.sp. strigae, Foxy 2 in emerged S. hermonthica shoots.

Shoots collected from:	Treatment	Weeks after emergence	Weeks after injection	Disease symptoms	Beauvericin (μg kg^{-1})
Controls	Control [a]	6		None	ND
	Injected Control[b]	6	4	None	ND
Foxy 2 treated	Foxy 2 infected*	3		None	60
		6		Severe	760
	Foxy 2 injected**	3	1	Early	550
		6	4	Severe	930

[a] *Controls not injected*
[b] *Controls injected with water*
*****Striga plants naturally infected from Foxy 2 colonized sorghum roots*
******Striga plants which were injected with Foxy 2, two weeks after emergence*
ND = Not detected with limit of detection (LOD) of 3 μg kg^{-1}

5.5.3. Toxins in sorghum grains

BEA as well as the other toxins tested were not detected in grains of sorghum plants which were hosts for *S. hermonthica,* grown from both Foxy 2 coated and non-coated (control) sorghum seeds (limit of detection for BEA was 3 μg kg^{-1}) (Table 5.4). Also fumonisins could not be detected in sorghum grains (limit of detection for fumonisin B1 was 3 g kg^{-1}).

5.5.4. Transmission electron microscopy of Striga shoots treated with beauvericin

Application of BEA drops at concentrations of 10 and 50 μM on *Striga* shoots caused necrosis around the drops within 21 hrs after application. The effect of 50 μM was only slightly more severe than that of 10 μM. The tissue near the application point such as the epidermal layer and the cortical parenchyma showed more severe effects compared to the tissues of the central cylinder and pith parenchyma. All cells of the cortical parenchyma were collapsed. The cytoplasm was disintegrated; the plasma membrane, the tonoplast, and also the envelope membranes of chloroplasts and nuclei appeared dissolved (Fig. 5.4a, b). Vacuoles and other organelles such as endoplasmic reticulum and dictyosomes, were no more discernable in the irregular electron-dense cytoplasm. Only remnants of nuclei and chloroplasts with extremely

Table 5.3: Detection of F. oxysporum DNA with specific primers clox1 and clox2 and FUM1 gene by real-time PCR with specific primers pairs FUM1F7328/ FUM1R7664 and rp32/rp33.

Treatment (weeks after emergence)	***F. oxysporum* Clox1/Clox2[1]**	**FUM1 FUM1F7328/ FUM1R7664[2]**	**rp32/rp33[3]**
Control [a] (6)	Negative		
Injected control [b] (6)	Negative		
Foxy 2 infected* (3)	Positive		
Foxy 2 injected (3)**	Positive		
Foxy 2 infected* (6)	Positive		
Foxy 2 injected (6)**	Positive		
F. oxysporum* 121	Positive	negative	negative
***F. oxysporum f.sp. strigae,* Foxy 2 ***	Positive	negative	negative
F. verticillioides* VP2	Negative	positive	positive

[1] *Mulè et al. (2004)*
[2] *Döll et al. unpublished*
[3] *Proctor et al. (2004)*
[a] *Controls of S. hermonthica shoots not injected*
[b] *Controls of S. hermonthica shoots injected with water*
***** *S. hermonthica plants naturally infected from Foxy 2 colonized sorghum roots*
****** *S. hermonthica plants which were injected with Foxy 2, two weeks after emergence*
******* *Pure cultures*

distorted grana stacks (Fig. 5.4b) and some mitochondria were visible (not shown). Also the xylem parenchyma cells (XP) were collapsed completely and showed the same severe destructions of the cytoplasm as the cortical parenchyma cells (Fig. 5.4c). At that stage no signs of degradation of vessels were visible (Fig. 5.4d). At the more advanced state (24 hours after BEA application), the degradation process also extended to the cell walls. Parts of the cell walls were degraded (Fig. 5.5), but the secondary cell walls of the vessels remained intact (not shown). Degradation of the cytoplasm progressed with accumulation of vesicles and lipid bodies (Fig. 5.5) within remains of chloroplasts.

Table 5.4: Testing for presence of beauvericin in sorghum grains grown from Fusarium oxysporum f.sp. strigae, Foxy 2 coated and non-coated (control) sorghum seeds.

Treatment	Number of sorghum plants harvested	Beauvericin (μg kg^{-1})
Control SS	9	ND[a]
Control ST	10	ND
Foxy SS	6	ND
Foxy ST	8	ND

[a]*ND = Not detected with limit of detection (LOD) of 3 μg kg^{-1}*
SS = Striga susceptible sorghum cultivars, 'Cowbaula'
ST = Striga tolerant sorghum cultivars, 'Wad Admed'

In the controls, all tissues of the shoots were undamaged. In the different cell types, the cytoplasm showed no signs of degradation and all cell organelles appeared intact (Figs. 5.6a and b). The loss of turgor pressure and the partly detached plasma membrane might be due to the methanol/water drops applied on the control shoots, but is often also a typical artefact of chemical fixation in the preparation process for transmission electron microscopy (Figs. 5.6a and b).

5.6. Discussion

5.6.1. Ability of Foxy 2 to produce toxins in vitro

The *S. hermonthica* biocontrol agent, Foxy 2 was investigated for its ability to produce mycotoxins using HPLC-MS-MS and real-time PCR. Foxy 2 produced high amounts of BEA but other tested mycotoxins were not detected in the rice substrate. Other *F. oxysporum* strains, both pathogenic and non-pathogenic, have also been reported to produce BEA *in vitro* (Logrieco et al., 1998). BEA is a cyclic hexadepsipeptide which was first isolated from the culture of the insect pathogenic fungus *Beauverina bassiana* (Hamill et al., 1969). It possesses insecticidal (Grove and Pople, 1980) and antibiotic activity (Castlebury et al., 1999) and is toxic to mammalian cells (Krska et al., 1997). Some *Fusarium oxysporum* strains are known to also produce the mycotoxins enniatins, fusaric acid, moniliformin, naphthazarins, sambutoxin (Desjardins, 2006), and fumonisins of the C series (Seo et al., 1996). Previously, the occurrence of fusaric acid and 9,10 dehydrofusaric acids in culture filtrates (Amalfitano et al., 2002) has been reported for Foxy 2. Another potential biocontrol agent against

S. hermonthica, *F. oxysporum* M12-4A, produced small amounts of fusaric acid and dehydrofusaric acids when cultured in artificial media (Saward et al., 1997).

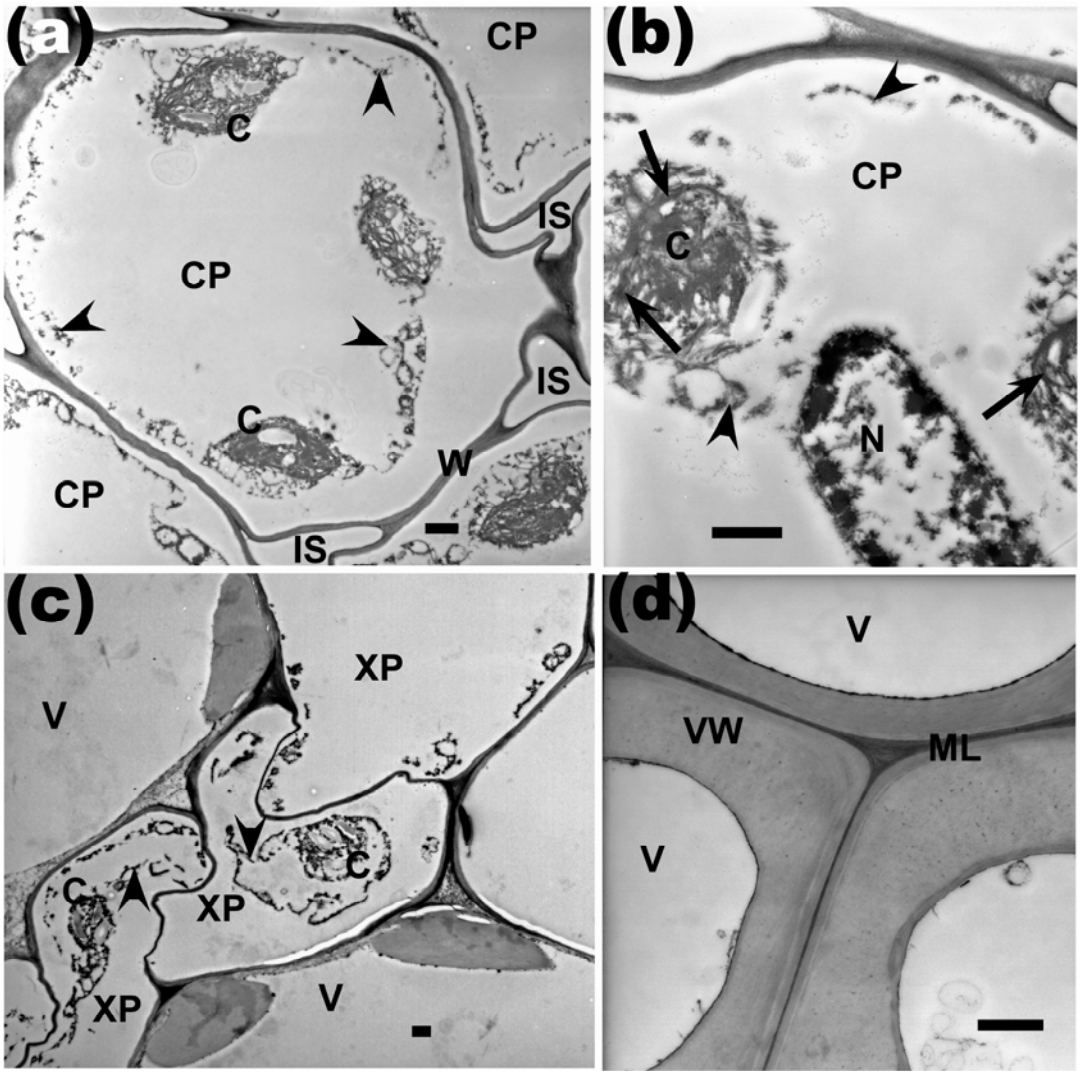

Fig. 5.4. Transmission electron micrographs of cross sections of *Striga* shoot cortical and xylem parenchymal cells showing degenerated cells, 21 hours after application of beauvericin (50 µM).

- **a.** Collapsed cortical parenchyma cell (CP): Plasma membrane and tonoplast appear dissolved. The cytoplasm is degraded (arrowheads) with remains of chloroplasts (C). Only the cell walls (W) are intact; intercellular space (IS).
- **b.** Detail of a collapsed cortical parenchyma cell (CP): Degraded electron dense cytoplasm (arrowheads) with part of a nucleus (N) and chloroplasts (C) with extremely distorted thylakoids (arrows).
- **c.** Collapsed xylem parenchyma cells (XP): Severely degraded electron dense cytoplasm (arrowheads), remnants of chloroplasts (C); vessel (V).
- **d.** Detail of vessels: the vessel walls (VW) including middle lamella (ML) are intact.

Bars = 1 µm.

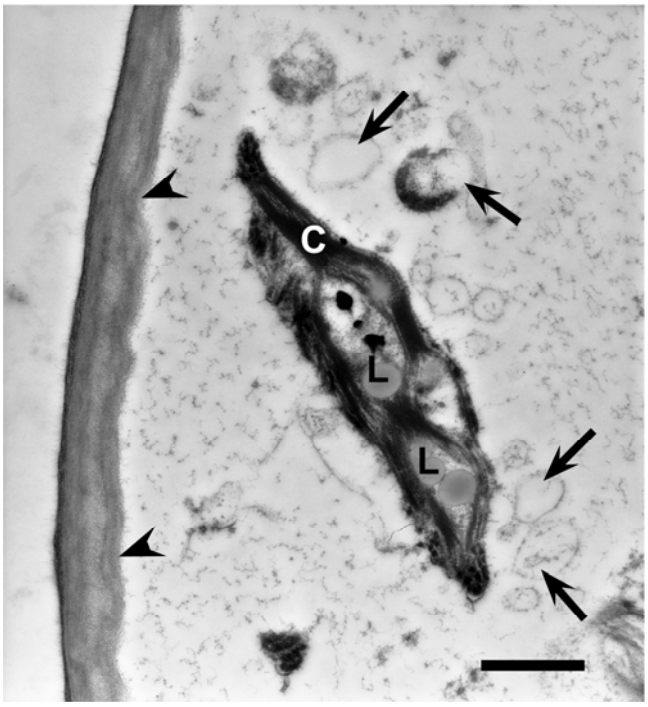

Fig. 5.5. Transmission electron micrograph of a cross section of *Striga* shoot showing an advanced state of degradation of a cortical cell, 24 hours after application of beauvericin. Vesiculation of cytoplasm (arrows) can be seen and a severely degraded chloroplast (C). Lipid bodies (L) have accumulated and degradation of the cell wall (arrowheads) has begun.
Bar = 1µm

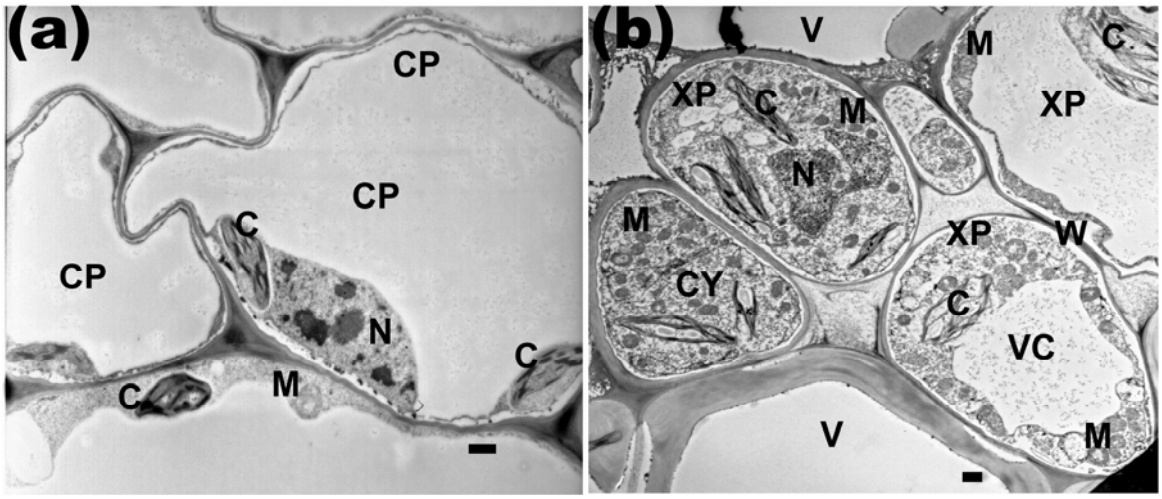

Fig. 5.6. Transmission electron micrographs of cross sections of control *Striga* shoots collected 21 hours after application of methanol and water.

- **a.** Intact cortical parenchyma cells (CP): Non-degraded cytoplasm with intact organelles like nucleus (N), chloroplasts (C), and mitochondrion (M). Parts of the plasma membrane has parted from the cell wall due to chemical fixation.
- **b.** Intact xylem parenchymal cells (XP): Non-degraded, dense cytoplasm (CY); chloroplasts (C), mitochondria (M), nucleus (N), vacuole (VC), cell wall (W), vessel (V). Some parts of the plasma membrane are separated from the cell walls due to chemical fixation.

Bars = 1 µm.

In the current study, fumonisins B1, B2, B3, C and P series, enniatins A, A1, B and B1, and moniliformin were not detected to be produced by Foxy 2. Furthermore, fumonisin was not detected and PCR assays with two FUM1-specific primer pairs suggest that the gene is missing or extensively diversified from FUM1 sequence of *F. verticillioides* for which both primer pairs were designed. This result could be seen as advantageous for the use of Foxy 2 as biocontrol agent since fumonisins in food and feed pose a risk to human and animal health (Marasas, 1995; Dutton, 2009).

5.6.2. Beauvericin in S. hermonthica *shoots*

Our previous cytological investigations showed that the biocontrol agent Foxy 2 controls *S. hermonthica* by the complete degradation of seedlings by hyphae at the early infection stages or in later stages by hyphal clogging of vessels causing wilting of the emerged shoots (Chapter 3). The current results support further evidence of the involvement of mycotoxins in this process at least during the later infection stages, probably when Foxy 2 has reached its saprophytic stage of development. Amongst the toxins tested, only BEA was detected in significant amounts in Foxy 2 infected *S. hermonthica* shoots. BEA was already detectable at low concentrations at early stages of infection of *S. hermonthica* shoots, but at that stage no symptoms appeared. This is in line with the results of investigations of the early infection stages when Foxy 2 hyphae are growing in the vessels and the neighbouring xylem parenchyma cells do not show any negative effects (Chapter 3).

In the current study, higher BEA content in shoots was associated with severe symptoms of necrosis and wilting as could also be seen in the slightly higher amounts of BEA detected in 'injected' *S. hermonthica* plants, where the concentration of Foxy 2 was artificially raised. Obviously, hyphae of Foxy 2 secreted BEA from the very beginning, but toxic concentrations were reached probably only at the later stages of infection when hyphae had spread, thus leading to the death of the *S. hermonthica* plant. The deleterious effect of BEA was demonstrated in this study, when it was directly applied on *S. hermonthica* shoots. BEA killed all cell types of the shoots; moreover, membranes of the cell organelles appeared dissolved. BEA is an ionophoric molecule that can form stable lipophilic complexes with cations. It interacts with the cell membrane and forms cation selective channels in membranes, which can affect the ionic homeostasis (Hilgenfeld and Saenger, 1982, Kouri et al., 2003). These effects of BEA contribute to its toxicity. In contrast to the present study, there was no

membrane dissolution effect visible in *S. hermonthica* shoots infected by Foxy 2 hyphae in the previous study (Chapter 3). Probably the toxic effect of BEA was overlaid by the severe lytic activities of hyphae of Foxy 2. An additional effect besides membrane dissolution and degradation of the cytoplasm was that the cell wall started to degrade, 24 hours after BEA application. Certainly BEA does not exhibit enzymatic activities, but when the integrity of the cytoplasm is destroyed, lytic enzymes of the plant cell will be set free and may start an autolytic breakdown of the cell walls.

For necrotrophic plant pathogenic fungi, mycotoxins are part of their infection strategy (van Kan, 2006). It is already known that some mycotoxins play a role in the pathogenesis of *Fusarium,* for example certain trichothecenes (Proctor et al., 1995), fusaric acid (Gapillout et al., 1996) and enniatins (Herrmann et al., 1996). *Striga,* being a parasite, attaches to the roots of its host plant underground. When *Striga* seedlings attach to sorghum roots, the hyphae of Foxy 2 invade the *Striga* plant to colonize its vessels to the top of the plant and the hyphae are restricted to the vessels (Chapter 3). It is thus likely that BEA was secreted within the vessels and diffused into the surrounding xylem parenchyma and then to the adjacent cells. Although BEA is also produced by the fungus *Fusarium proliferatum*, causing tracheomycosis in sorghum (Waskiewicz et al., 2010), in our study the toxin showed no specific activity on the xylem tissue of *S. hermonthica*. There are tissue specific acting toxins, e.g. phomozin secreted by *Phomopsis helianthi*, a host specific pathogen growing in bundles of leaves of sunflower, where it is only destroying the tissue of the phloem and the surrounding bundle sheath (Heller and Gierth, 2001). In sunflower leafs only the tissues of the bundle and bundle sheath were sensitive to the toxin (Heller, pers. communication).

There is not much information about BEA produced *in planta*. BEA induces cell death and alterations to the ascorbate metabolism in tomato protoplasts (Paciolla et al., 2004). Paciolla et al. (2008) investigated the role of BEA and T-2 mycotoxin in the antioxidant defence response of tomato seedlings. Cut tomato shoots incubated with 50 µM BEA showed no signs of wilting after 24 hrs and the BEA treated plants had more lignified xylem vessels than the controls. In another study, there was loss of plasma membrane integrity and cell death four to five days after treatment of wheat seedlings with 40 µM BEA (Šrobárová et al., 2009). It is clear that *Striga* shoot tissues in our study were much more sensitive to BEA compared to the investigations

mentioned above as 10 µM BEA was able to kill *Striga* tissues within 21 hrs. Therefore, the factors responsible for the toxic effects of Foxy 2 to *Striga* shoots during late stages of infection could involve BEA as well as fusaric acid and 9,10 dehydrofusaric acid which were already detected by Amalfitano et al. (2002). It has also been reported that fusaric acid, 9,10 dehydrofusaric acid, and their methyl esters produced by *F. nygamai* (also a suggested biocontrol agent) cause chlororis, necrosis and inhibition of root elongation when assayed on leaves and seedlings of tomatoes (Capasso et al., 1996).

5.6.3. Safety of the use of the mycoherbicide Foxy 2

A thorough understanding of the mechanisms of action of biocontrol agents is needed to maximize consistency and efficacy of biocontrol (Fravel et al., 2003) and the quality of the crop produced. *Fusarium* spp. that produce trichothecenes and fumonisins are infamous for causing, both acute and chronic mycotoxicoses, in farm animals and in humans (Bhat et al., 2010). None of the tested mycotoxins were found in the grains of sorghum and also BEA, which was found in *Striga* shoots, was absent in the grains. Foxy 2 has been shown to be pathogenic to *Striga*, colonizing seedlings and shoots (Chapter 3), but non-pathogenic to sorghum where it is only colonizing the cortex of the root (Chapter 2). When sorghum roots are parasitized by *Striga,* Foxy 2 hyphae are able to gain access to the central cylinder of sorghum through the *Striga* haustorial cells which are connected to the sorghum xylem. In this case it could be possible that mycotoxins secreted by Foxy 2 are translocated into the developing grains of the sorghum plant. However, BEA did not accumulate in the grain of sorghum plants in detectable amounts. Even if hyphae of Foxy 2 get access to the xylem of sorghum roots through the haustorium of *Striga*, the hyphae would not grow within the tissues of the central cylinder of the roots as has previously been shown (Chapter 4). Therefore, the risk that Foxy 2 hyphae may secrete considerable amounts of mycotoxins at the base of sorghum plants is low. Furthermore the FUM1 gene was not detected in the Foxy 2 genome. Therefore Foxy 2 might not be of major concern for human and animal health when used as a biocontrol agent in the field, thus remaining a promising mycoherbicide component.

5.7. Conclusions

Of the toxins tested (BEA, fumonisins B1, B2, B3, C and P series, enniatins A, A1, B and B1, and moniliformin), Foxy 2 was able to produce only BEA in detectable

amounts in *S. hermonthica* shoots and rice substrates. The production of BEA by Foxy 2 within *Striga* shoots increased with increased Foxy 2 infection. When applied on *S. hermonthica*, BEA caused severe degradation of shoot cells of both the cortical and xylem parenchyma at a concentration of 50 µM. None of the toxins tested was identified in sorghum grains harvested from plants growing from Foxy 2 coated sorghum seeds. The FUM1 gene was not identified in pure cultures of Foxy 2, therefore this strain might not produce fumonisins. Foxy 2 could thus be considered as safe for use as a mycoherbicide against *S. hermonthica*.

5.8. Acknowledgements

Many thanks go to SUET for coating the sorghum seeds used in this study. We are also grateful to Dr. Abuelgasim Elzein for kindly providing the *Striga* and sorghum seeds. The first author is grateful to KAAD (Katholischer Akademischer Ausländer-Dienst) and IITA (Benin; through BMGF project) for financial assistance during the study period.

CHAPTER 6

General Discussion

6. General Discussion

Fungal biocontrol agents have been suggested for the control of weeds as an alternative to the use of chemicals (Boss et al., 2007). Amongst such weeds is the parasitc witchweed *S. hermonthica* which is of major concern as there is growing evidence that the *Striga* problem is worsening (Ejeta, 2007a) particularly in Africa. Successfully tackling *S. hermonthica* damage to improve crop performance would greatly support food security in Sub-Saharan Africa. The intimate life cycle of *S. hermonthica* and its hosts makes biocontrol a better option as opposed to the common practice of hand weeding since most of the damage is done before emergence (Ejeta and Butler, 1993). The use of fungal biocontrol agents against *S. hermonthica* is advantageous as these agents are very host specific and attack the underground stages especially seeds which can help to reduce the seed bank (Kroschel et al., 1996; Thomas et al., 1999; Fravel et al., 2003). Some *Fusarium* isolates have been studied and proposed as biocontrol agents against *S. hermonthica* (Abbasher et al., 1995; Ciotola et al., 1995; Kroschel et al., 1996). Among them, *F. oxysporum* f.sp. *strigae* (Foxy 2) has been shown to be effective and specific to *S. hermonthica* and *S. asiatica* (Elzein, 2003; Elzein and Kroschel, 2004a). However, to ensure the safe use of biocontrol methods, strategies to assess the associated risks need to be developed (Boss et al., 2007). Moreover, a thorough understanding of the mechanisms of action is needed to maximize consistency and efficacy of biocontrol (Fravel et al., 2003). Thus, in-depth studies were carried out to understand the mechanisms underlying the action of Foxy 2 in both its target *S. hermonthica* and non-target sorghum. In order to evaluate the safety of the use of Foxy 2 as biocontrol agent, its toxin production ability was also investigated. It is important to note that in this host-parasite-mycoherbicide complex interaction, sorghum represents the host of *Striga*, while *S. hermonthica* is its parasite and Foxy 2 is the biocontrol agent against *S. hermonthica*.

6.1. The potential of sorghum seed coated Foxy 2 as mycoherbicide to control *Striga hermonthica*

The efficacy of Foxy 2 causing disease in up to 95% of attached *S. hermonthica* seedlings portrays it as a good potential mycoherbicide (Chapter 2). There was a trend showing that a combination of Foxy 2 and the *Striga* tolerant sorghum cultivar was more effective than with the *Striga* susceptible sorghum cultivar (Fig. 6.1). Hence, integration of Foxy 2 with *Striga* tolerant sorghum cultivars could decrease the seed

bank and chance for adaptation making this option a promising approach to fight *Striga* and or its effects on crops especially when such crop cultivars are desired by farmers. Microscopic investigations disclosed that Foxy 2 destroyed all cell types of *S. hermonthica* seedlings including haustorial intrusive cells, hyaline tissue, vessels, central xylem elements and *Striga* cortical parenchyma within 26 days after sowing Foxy 2 coated sorghum seeds (Chapter 3). These results also ascertain the efficacy of the seed coating technology used for the application of this biocontrol agent. From the sorghum coated seeds, Foxy 2 hyphae could grow along the root as it penetrated into the soil (Chapter 2) and it is likely that *S. hermonthica* seeds within the vicinity of the roots could also have been destroyed by Foxy 2. This has been shown in the case of *Orobanche cumana* that were attacked by *F. oxysporum* f.sp. *orthoceras,* destroying the seeds completely (Thomas et al., 1999). However, it is not known how far Foxy 2 could grow into the surrounding soil therefore it was not possible to estimate its ability to destroy seeds in this study. The colonization of sorghum roots by Foxy 2 started with the germination of sorghum seeds, and hyphae were still in the root cortex after four weeks, when roots were already in the state of senescence. They were active and ready to attack *S. hermonthica* seedlings over this long period of time, in the sorghum root cortex and root surface which is the site where *S. hermonthica* seedlings attach (Chapter 2). The death of young *S. hermonthica* seedlings as demonstrated in this study implies that the impact of *S. hermonthica* on crop loss will be minimized, the seed bank in the soil is depleted and additionally further *Striga* seed production is reduced. This is advantageous as strategies to deplete the *Striga* seed bank or reduce it to tolerable levels have been suggested to be the focus of management options and therefore imperative for food security in Africa (Van Mourik et al., 2008). However, the colonization of sorghum roots by Foxy 2 was slow. This is disadvantageous as *Striga* seedlings which attach to sorghum roots at points not yet colonized by Foxy 2 could cause damage to the sorghum plant before Foxy 2 reaches these areas of the root. Evidence for this is provided by the fact that in our experiments, some *Striga* plants succeeded to emerge but were killed by Foxy 2 some weeks thereafter when some damage to the sorghum plants already had occurred.

The seed treatment technology (Elzein et al., 2006) which is applicable to all varieties of cereal crops attacked by *S. hermonthica*, and compatible with other *Striga*-control methods, could accelerate the adoption and implementation of this biocontrol option

by farmers. Besides, Foxy 2 also shows potential to control both *S. hermonthica* and *S. asiatica* (Elzein and Kroschel, 2004a).

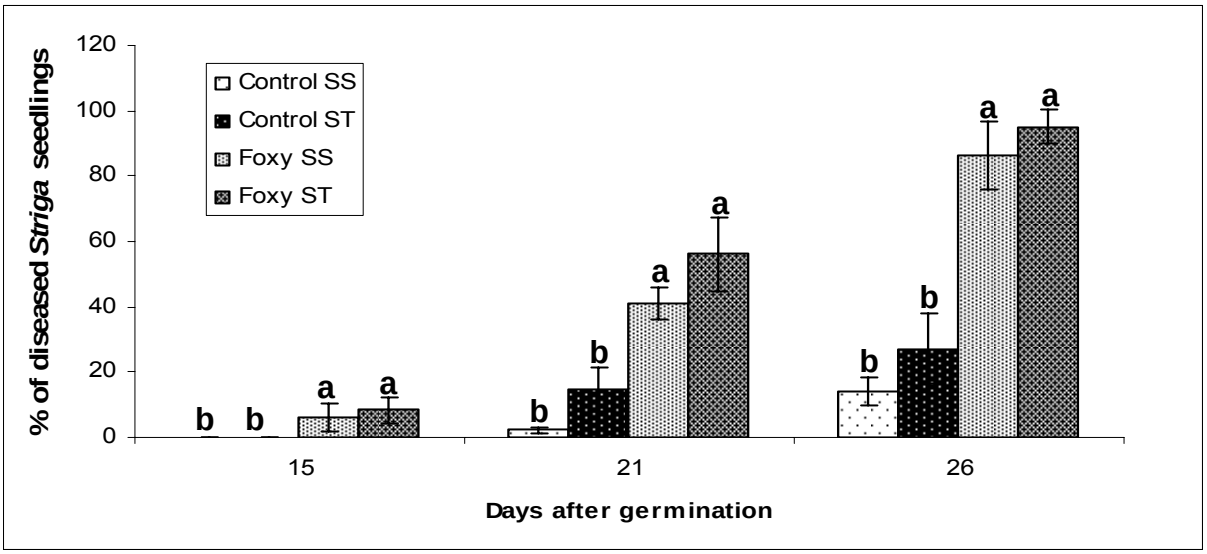

Fig. 6.1. Temporal pattern of percentage of diseased and dead *S. hermonthica* seedlings as affected by application of Foxy 2 as film-coat on susceptible (SS) or tolerant (ST) sorghum varieties. Vertical bars indicate the standard error of the means. Means with the same letter within each time interval are not significantly different at P≤0.05 after LSD-Test.

Another important issue relates to the tolerance and stability of inoculated chlamydospores on seeds that can withstand environmental extremes even over a period of 12 years (Elzein et al., 2009). In fact the option is promising as the seeds of farmers' favorite varieties (high yielding and locally known tolerant or resistant varieties) could be film-coated with Foxy 2 using inexpensive locally available materials like arabic gum making the seeds easy to handle. However, it is not certain if after death of the sorghum root, Foxy 2 can produce enough chlamydospores to be able to survive in the soil in the absence of *S. hermonthica* and if it can still be effective in subsequent years against *S. hermonthica* or the farmer has to purchase coated seeds every planting season. Field studies need to be carried out to investigate this ability. If the effectiveness is only for one season then it would be expensive for subsistence farmers to purchase coated seeds every season. Other factors to be taken into consideration include accessibility and purchase of coated seeds by farmers especially those in remote areas. This might limit the use of Foxy 2. Currently, field experiments are being developed by Real IPM (Integrated Pest Management: Company for designing IPM programmes of real value so as to deliver cost effective, crop protection solutions, which significantly reduce chemical inputs

and promote biological control) Kenya which can multiply Foxy 2 and should be able to produce locally coated seeds with Foxy 2.

6.2. Mechanisms of action of Foxy 2 in *Striga hermonthica*: what is the cause of wilting and death?

Pathogenic *Fusarium* strains infect roots through a wound or lateral roots and penetrate through the cortex to the xylem and upwards to the stem, causing tracheomycosis thus affecting the host water supply (Smith et al., 1993; Fravel et al., 2003; Agrios, 2005). Hyphal clogging of the vessels which affects host water supply or production of toxins could lead to death of the host plant. It was observed that Foxy 2 used similar mechanisms to kill the *S. hermonthica* seedlings and plants (Chapters 3 and 5).

The mechanisms involved in the pathogenicity of Foxy 2 to *S. hermonthica* were the complete digestion of attached seedlings belowground, the obstruction of water flow in vessels of emerged *Striga* shoots (Chapter 3) and a phytotoxic effect (Chapter 5). TEM showed a massive digestion of all tissues including vessel walls in young *S. hermonthica* seedlings by Foxy 2 hyphae which suggested the involvement of cell wall degrading enzymes. The specific action of Foxy 2 against *S. hermonthica* and not sorghum could be observed in *Striga* seedlings at their point of attachment to sorghum roots belowground (Chapter 3). The distinctive behaviour of Foxy 2 which selectively degraded only cells of the *Striga* haustoria protruding into sorghum vessels but not destroying the nearby sorghum vessels (as seen using light microscopy in Chapter 3) was particularly noted showing the complex tripartite interaction between mycoherbicide, parasite and its host. Most *S. hermonthica* seedlings were destroyed by the fungus within 26 days after sowing. An important point to note is that in the *Striga*-sorghum-Foxy 2 interaction, there is pathogenicity of the specific natural enemy (Foxy 2) to its target weed *S. hermonthica* (Chapter 3) unlike in the case of the *Striga*-sorghum-arbuscular mycorhiza interaction where the antagonistic effect of arbuscular mycorhiza protects sorghum roots from attack by *S. hermonthica* (Lendzemo et al., 2004).

In emerged *S. hermonthica* shoots, hyphae were mainly located within the vessels and were observed to have clogged the vessels over long distances, and were identified even in the top of the shoots. However, few vessels contained hyphae which

mostly appeared pressed on each other and spaces or gaps were rare or could hardly be observed (Chapter 3). Though not digesting the vessel walls (Chapter 3), the high level of intertwining and cross-linking of these hyphae within vessels were the factors contributing to the intensive blockage of the vessels (Appendix A.1). In fact, considering the disposition of the hyphae, it can be affirmed that water conduction within such vessels would greatly be impeded if not interrupted. Mechanical plugging of vessels has previously been reported by Beckman (1987) in oak wilt disease. Using radioactive rubidium movement in the transpiration stream, the water flow in oak vessels dropped five days before wilting by 85-90%. Meanwhile, the interference to the upward flow of water in *S. hermonthica* shoot vessels could not be strictly attributed to hyphae blockage only because unknown substances were also identified within these vessels (Appendix A.2). These substances (even though not examined in detail) could be of Foxy 2 origin or otherwise produced by *S. hermonthica* in reaction to the presence of Foxy 2. It has previously been reported that for pathogenic *Fusarium* strains, infected vessels in vascular wilts could be filled with pathogens, or substances secreted by the pathogen or by the plant itself in response to attack by the pathogen which could lead to clogging of the vessels (Agrios, 2005). Similarly, Bishop and Cooper (1983) showed that large fragments of wall materials could probably form plugs which are involved in water flow resistance. Therefore, not surprisingly, the *S. hermonthica* vessels having Foxy 2 hyphae and substances within, could not function accurately such that water flow was affected contributing to the death of the plant.

In dead *S. hermonthica* shoots, Foxy 2 was observed to have spread widely and degraded vessel walls and xylem parenchymal cells (Chapter 3). These degradations could be due to cell wall degrading enzymes or toxins produced by Foxy 2 to kill the plant. Amongst the toxins tested (beauvericin, fumonisins B1, B2, B3, C and P series, enniatins A, A1, B and B1, and moniliformin), beauvericin (BEA) was detected in emerged *S. hermonthica* shoots infected by Foxy 2 (Chapter 5). The results showed that beauvericin was already produced at early stages of infection of *S. hermonthica* shoots (60 µg BEA/kg) which had not yet symptoms of disease and at later stages, 760 µg/kg were produced indicating that the toxin increased with increased infection (Chapter 5). However, in sorghum grains harvested from plants growing from Foxy 2 coated sorghum seeds, none of the tested toxins were detected (Chapter 5), thus pointing to the specificity of Foxy 2 to *S. hermonthica*. After the application of 10 µM and 50 µM of beauvericin, all *S. hermonthica* cell types were observed to be necrotic

such that only remains of few organelles like chloroplasts could be observed (Chapter 5). This is in line with the report of Paciolla et al. (2004) who showed that beauvericin induced 97% of premature tomato protoplast death at concentrations of 50 µM. The effects of other *Fusarium* toxins on plants have previously been studied. For example, application of fumonisin B_1 on jimsonweed leaves (*Datura stramonium* L.) caused soft rot and killed some of the plants (Abbas et al., 1991; Hershenhorn et al., 1992). Also, fusaric acid, 9,10 dehydrofusaric acid and their methyl esters produced by *F. nygamai* can inhibit *Striga* seed germination (Capasso et al., 1996). Other toxins like T-2, Deoxinevalenol and enniatin are as well able to inhibit *Striga* seed germination (Zonno and Vurro, 1999). Meanwhile, toxins have been reported to play a role in *Fusarium* diseases. For example, *Gibberella zeae* (*F. graminearum*) mutants unable to produce trichothecene deoxynivalenol showed reduced virulence compared with the virulence of the trichothecene-producing parents thus demonstrating the contribution of the toxin trichothecene to the disease caused by this fungus on cereals (Proctor et al., 1995).

To summarize, the mechanisms employed by Foxy 2 to kill *S. hermonthica* were the complete digestion of underground *S. hermonthica* seedlings, hyphal clogging of vessels in emerged *S. hermonthica* plants and production of the toxin beauvericin. The specificity of Foxy 2 to *S. hermonthica* and not to sorghum was due to a tissue specific action which enabled it to damage all cell types of *S. hermonthica* seedlings and expressively colonize *S. hermonthica* shoot vessels to the top, and in spite of this, not being able to digest vessels of sorghum roots and not being able to expressively colonize the vascular system in sorghum; producing beauvericin and maybe cell wall degrading enzymes thus killing *S. hermonthica*. Given that specific cell wall degrading enzymes and their actions were not investigated in this study, further research could be directed at investigating gene expression and enzyme activity of Foxy 2 in *S. hermonthica* shoots and sorghum roots so as to provide more information about the specific destruction by Foxy 2 of *S. hermonthica* and not sorghum.

6.3. Reactions of the non-target plant sorghum against invasion by Foxy 2

Roots growing from Foxy 2 coated sorghum seeds, i.e. of sorghum plants which acted as hosts to the parasite *S. hermonthica,* were extensively colonized by Foxy 2. These colonized roots showed reactions to prevent destruction by Foxy 2. Using fluorescence microscopy, it was observed that there was an increase in blue fluorescence in the endodermis of Foxy 2 infected roots probably reflecting an

increase in lignin or other phenolics in the cell wall (Chapter 4). Lignification renders the wall more rigid and also makes it less liable to enzyme digestion. The inability of Foxy 2 to penetrate the endodermis of sorghum could have been due to this reaction. Movement of Foxy 2 into the central cylinder was thus restricted (Chapter 4) and therefore further damage to the root was hindered. This was not the case neither for the pathogenic *F. proliferatum* in sorghum roots (Chapter 4) and also for Foxy 2 within *S. hermonthica* shoot vessels (Chapter 3) since hyphae, being already present within the vessels, could be propelled to the top of the plant within the free space of the vessels. Even when already present within the central cylinder of wounded sorghum roots, Foxy 2 did not further proliferate within these vessels as one would have expected (Chapter 4). The parasitic phase of *F. oxysporum* has been studied and shown to be divided into three phases which determine the success or failure of the pathogen (Beckman, 1987); the 'Determinative Phase I' in which roots are infected by direct penetration or wounds, through the cortex to the vascular tissue; the 'Determinative Phase II', in which the fungus colonizes the xylem vessels and xylem sap stream and finally, fungal growth expands to adopt a necrotic-like 'Expressive Phase III'. Foxy 2 failed in penetrating the sorghum vascular system and also failed in colonizing the vascular system – both determinative phases I and II were not effected in sorghum roots thus Foxy 2 could be said to be non-pathogenic to sorghum which was not the case for *F. proliferatum* which was pathogenic in sorghum (Chapter 4). A plausible explanation for the failure of Foxy 2 in phase I could be the probable increase in phenolics as indicated by an increase in fluorescence (Chapter 4). Both plant and pathogen factors contribute to the success of the pathogen (Beckman, 1987). As Foxy 2 hyphae were observed not to be able to grow within wounded inoculated sorghum roots, there might be the possibility of the existence of a physiological mechanism within the sorghum root vessels against Foxy 2 which prevented the further spread of hyphae (Chapter 4). This observation was drawn from the fact that five days after wounding and inoculating sorghum roots, Foxy 2 hyphae invaded the central cylinder very close to the cut but were completely absent from the central cylinder at a distance of 3000 µm from the cut, meanwhile *F. proliferatum* hyphae had digested the cells of the central cylinder at this distance. The barrier, which needs to be further investigated, prevented the further spread of Foxy 2 hyphae in sorghum roots and was obviously absent in *S. hermonthica* as hyphae were identified at the top of shoots up to a height of 17 cm above the ground (Chapter 3).

Another aspect of reactions in sorghum roots is in the area of the production of pathogenesis related (PR) proteins. Foxy 2 probably did not cause the production of PR proteins in sorghum roots (Appendix A.3). PR proteins are induced by different stress stimuli and play an important role in plant defence against pathogenic constraints (mostly an antifungal effect) and in general, adaptation to stressful environments (Edreva, 2005). Even though not a sensitive enough technique, MALDI results (Matrix-Assisted Laser Desorption/Ionization) showed that protein bands of roots growing from Foxy 2 coated and non-coated sorghum seeds were the same. Digestion and analysis of these bands suggested the same proteins in both infected and uninfected roots (Appendix A.3) but attempts to use DIGE (Differential in Gel Electrophoresis) for further tests which allows for simultaneous separation of up to three samples were not fruitful due to the difficulty in extracting proteins from sorghum roots. DIGE is a technique used to see changes in protein abundance between samples which allows for simultaneous separation of samples on one gel, bringing a new level of statistical confidence and reliability therefore overcoming the limitations in 2D electrophoresis that are due to inter-gel variation. Even though the MALDI method is seen as not very efficient, it gives an idea or overview of the proteins produced within the infected and uninfected roots suggesting that Foxy 2 might not cause a stressful reaction of sorghum roots.

6.4. Reactions of the target plant *Striga hermonthica* against Foxy 2 invasion

Reactions of *S. hermonthica* to Foxy 2 invasion on the other hand were observed only in aboveground stages (Chapter 3). In fact, underground young *S. hermonthica* seedlings were completely destroyed and showed no counter reactions to Foxy 2 invasion. The attack by Foxy 2 could have been too fast such that the *S. hermonthica* seedling could not coordinate a response. Whereas, as Foxy 2 was growing slowly on sorghum roots, there were areas towards the root tips which were not yet colonized by hyphae. *S. hermonthica* seedlings that attached to these areas could have established and developed without getting in contact immediately with Foxy 2. In such cases the seedlings might have been able to outgrow the fungus and therefore emerged to the surface as shoots. These shoots thus had enough time to co-ordinate a defence reaction. Unlike in young *S. hermonthica* seedlings, vessels in emerged shoots had an electron dense wall layer which could be a cell wall modification produced by *S. hermonthica* as a defense mechanism against Foxy 2. This layer might have acted as a barrier, thus preventing vessel wall digestion. However, further attack and damage

by Foxy 2 was not prevented in a major way, as hyphae spread extensively. Additionally, attempted penetration of xylem parenchymal pit membranes was foiled as the underlying xylem parenchyma cells produced a wall apposition just beneath the xylem parenchyma pit membrane (Chapter 3). Successful colonization of a particular host entails that pathogens have to develop the ability to circumvent the defense barriers of the plant to prevent infection (Jackson and Taylor, 1996). After breaching these barriers, the plant faces pressure to develop countermeasures that block invasion by the pathogen. When the plant has generated this new resistance response, the pathogen again has to respond with an alternative mechanism to restore virulence. Disease symptoms appear when the pathogen is able to overcome the complexities of the plant defense responses (Jackson and Taylor, 1996). This scenario could be related to *S. hermonthica* shoots in the advanced state of destruction by Foxy 2 (Chapter 3). From the TEM investigations, digestion of *S. hermonthica* vessel walls and xylem parenchyma could be noticed in dead shoots. Effectively, there were no longer defense barriers or Foxy 2 had overcome the reaction which allowed the digestion of vessels by hyphae which was not the situation when *S. hermonthica* was alive. It is more likely that there were no longer defense barriers as the plant was dead and eventually, Foxy 2 was able to get access to (degrade) substrates of these *S. hermonthica* cells (Chapter 3).

One of the noticeable effects of the attack by Foxy 2 was that it affected the general physiology of the *S. hermonthica* plant as seen from DIGE (Differential In Gel Electrophoresis) analysis (Appendix A.4). Many proteins for stress response were up regulated while some tubulin proteins for GTP binding were down regulated thus interfering with GTP (guanosine triphosphate) binding (Appendix A.4). Since hyphae interrupted the flow of water in vessels, the plants were stressed as reflected in the up regulation of these proteins. The observed differences in the expressed proteins indicated an increase in biological processes in the infected *S. hermonthica* plants such as glycolysis or ATP binding as opposed to the controls. This showed a stress/defense reaction of *S. hermonthica* to the presence of Foxy 2.

6.5. Is Foxy 2 a safe mycoherbicide?

Before the release of a biocontrol organism into the environment, it is very important to make a risk assessment. So far there has been considerable uncertainty about the kind of risk assessment studies that would be necessary for the registration of a

biocontrol agent and there are as yet no well-defined regulations. But several researchers have discussed the need to examine product toxicity as well as the fate of toxic metabolites produced after application of fungal biocontrol agents (Strasser et al., 2000; Strasser, 2003; Skrobek and Butt, 2005). The action of biocontrol agents in *Striga*-hosts as well as reactions of those hosts towards the presence of the biocontrol agents need to be known as part of the risk assessment to ensure their safe use. Other approaches include quantifying the relative susceptibility of target and non-target plant species as well as microscopic and histological examination of infection events (Watson, 1986; Bruckart et al., 1996). Also, the specificity of the biocontrol agent to its target plant is a factor of concern. Actually, wilt-inducing strains of *F. oxysporum* show a high level of host specificity attacking only one or few plant species and sometimes only certain cultivars of that plant (Fravel et al., 2003). A host range of 25 plants have previously been screened in the greenhouse for possible attack by Foxy 2 (Table 1.1) and had shown no negative effects, suggesting the safety of the use of Foxy 2. In the case of *Striga*, greenhouse experiments clearly indicated that Foxy 2 almost completely prevented emergence of *S. hermonthica* and *S. asiatica* but had no effect on *S. gesneriodes* thus expressing pathogenicity only to these species.

There were some observations of the action of Foxy 2 in sorghum roots from this study which point to the safety of this biocontrol agent. First of all, Foxy 2 was observed to be a slow but constant colonizer of sorghum roots. While only few hyphae were detectable on sorghum roots after one week, there were thick mycelia on the upper parts getting thinner towards the root tips after three weeks. Concurrently, Foxy 2 was also colonizing and digesting sorghum root cortical parenchymal cells (Chapters 2 and 4). Secondly, it was observed that although Foxy 2 was digesting the cortex of roots, there were no negative effects to the growth of the sorghum plants. Moreover, application of Foxy 2 as film-coat on sorghum seeds caused an increase in root length as compared to the control (Chapter 2, Table 2.1). Thirdly, in the presence of *S. hermonthica*, Foxy 2 entered into sorghum root vessels but strictly within the *Striga* haustorial cells (Chapter 3). Given the opportunity of finding itself within the sorghum root vessels, Foxy 2 could have digested both *S. hermonthica* and sorghum vessels but this was not the case as it did not digest sorghum vessels (Chapter 3). Fourthly, coating with Foxy 2 did not cause any direct or indirect negative effects on germination and growth of the non-target sorghum plant (Chapter 2). These interactions between Foxy 2 and sorghum roots points to the category of fungi that are

able to colonize some plant roots but do not cause disease (Shoresh et al., 2010). Such fungi have been described as endophytic plant symbionts (Harman et al., 2004). To establish such a symbiotic association (interaction between two different organisms in which both benefit from the association), the fungi first colonize the roots of the plant (Harman, 2011). The next step is physical penetration and colonization of the root outer epidermal layer, cortex, and establishment of chemical communication with the plant (Yedidia et al., 1999; Harman et al., 2004; Bae et al., 2011). Experiments with *Trichoderma virens* have demonstrated that plant-derived sucrose is critical for and associated with the control of plant root colonization by this strain (Vargas et al., 2009; Vargas et al., 2010). This is backed up by the presence in the fungal genome of a plant-like sucrose transporter: the specific sucrose／H2 symporter which is induced during the early stages of root colonization. Their results also suggested the presence of a sucrose-independent network in the fungal cells that regulates the symbiotic association (Vargas et al., 2010). We suspect a similar process for Foxy 2 in sorghum roots as in the case of *Trichoderma virens*. Digestion of sorghum root cortical cells by Foxy 2 hyphae as observed in Chapters 2 and 3 is thereby understandable, since nutrients were essential for Foxy 2. Effectively, one could say that digestion of root cortical cells promoted growth of Foxy 2 hyphae such that even after 4 weeks hyphae could still be observed. In essence, mycelia continued to increase in the root cortical cells from 2 to 4 weeks (Chapter 2), implying that with the digestion of these cells, the survival of Foxy 2 was guaranteed. The outcome of the interaction between an endophytic plant symbiont and a plant is that there are beneficial effects for the plant which can last for at least the growing season for an annual plant because the fungi grow and continue to colonize the roots as they, in turn, also grow and increase (Harman, 2000). Systemic effects in plants are induced, so that while only roots are typically colonized by the fungi, the effects also occur in leaves and in stems of the plant (Harman, 2011). Thus, the addition of even a small amount of such fungi as seed treatment confers significant advantages on a wide variety of crops such as increase in plant growth especially roots, increase in photosynthesic rate in leaves (Harman, 2000; Vargas et al., 2009; Shoresh et al., 2010; Harman, 2011), enhanced nutrient uptake, improved nitrogen use efficiency by plants (Harman and Mastouri, 2010; Shoresh et al., 2010), and resistance to plant stresses like drought, salt and temperature (Mastouri et al., 2010; Shoresh et al., 2010; Harman, 2011). From the report of Harman (2011), we could deduce the explanations for the significant increases in shoot dry weight (110%), panicle yield

(100 %) and height (70%) in sorghum plants inoculated with Foxy 2 observed by Elzein and Kroschel (2004a). The reason for these increases could be as a result of the systemic effect induced by Foxy 2 colonization of sorghum roots. Indeed, colonization of sorghum roots by Foxy 2 can be seen as a beneficial relationship to sorghum as the overall plant growth is not negatively affected by Foxy 2. Thus accounting for the absence of defense reactions such as cell wall thickenings, wall appositions, intercellular plugging, intracellular osmiophilic deposits and papillae as observed in cortical cells of Foxy 2 infected sorghum roots (Chapter 4), except for an increase in lignification of the central cylinder. Explicitly, one could say that in the presence of Foxy 2, the sorghum plant produces more roots which could enhance nutrient uptake. However, this can only be true in cases of reduced parasitic losses due to *S. hermonthica*. It could also be possible that new roots which develop later on might not be colonized by Foxy 2 such that late attack by *Striga* might cause nutrient losses for the sorghum plant.

Pathogenicity has been defined as the ability of the pathogen to interfere with one or more of the essential functions of the plant, thereby causing disease (Agrios, 2005). Foxy 2 proved to be non-pathogenic to sorghum. This was attributed to several differences in the colonization of sorghum roots compared to the behavior and abilities of the known pathogenic *F. proliferatum* (Chapter 4). Foxy 2 did not penetrate the thickened walls of the endodermal cells and was never found in the central cylinder of the root even after four weeks. Instead, its hyphae were blocked at the outer endodermis and were somehow inhibited or could not penetrate this wall. TEM revealed that the central cylinder of the Foxy 2 infected roots had intact vessels and xylem parenchyma with functional cytoplasm. Meanwhile, ultrathin sections showed that parts of the suberized layer of the endodermal wall were digested by secretions from the hyphae of the pathogenic *F. proliferatum* allowing it to enter the central cylinder and digest all cell types within it (Chapter 4). The endodermis contains additional substances different from cortical cell walls, implying that it is not digested by cell wall degrading enzymes (CWDE) like cellulase and pectinase (Schreiber et al., 1999). Lignin and manganese peroxidases, laccases, glyoxal oxidase and flavin adenine dinucleotide enzymes are responsible for degradation of lignin in white rot basidiomycetes (Cullen and Kersten, 2004). The ability of Foxy 2 to digest rhizodermal and cortical cells but not the endodermal cells of sorghum roots might be attributed to the absence of enzymes required for digestion of these special cells or probably, it

could be that the Cell Wall Degrading Enzymes genes for Foxy 2 might not have been expressed at the appropriate stage during its invasion process. The role of gene expression in pathogenicity which gives an insight into the requirements for root colonization (Di Pietro et al., 2001) and invasion by *F. oxysporum* has been studied in great detail. For example, *FOW2*-targeted mutants lose pathogenicity in melon plants just as fga1 disruptants do in cucumber plants (Jain et al., 2002; Jain et al., 2005; Imazaki et al., 2007). Also, impaired colonisation of *F. oxysporum* f.sp *lycopersici* on tomato roots is correlated with reduced CWDE gene expression (Jonkers et al., 2009). Thus, the inability of Foxy 2 to digest cells of the endodermal barrier in non-wounded roots might be due to non-expression of some genes or a defect in the activation of enzyme-encoding genes as in the FOW2-targeted mutants. There is little doubt therefore that the inability of Foxy 2 to digest cells within the central cylinder in wounded roots (Chapter 4) could be because it lacks the genes or simply because the genes were not expressed. Therefore Foxy 2 could not cause tracheomycosis in sorghum while *F. proliferatum* could do so as it invaded the vessels and could be conveyed to the shoots. This is one of the factors indicating the non-pathogenicity of Foxy 2 to sorghum. Definitely, Foxy 2 could as well not grow further within the central cylinder after wounding and inoculation (Chapter 4). The overall effect of this deficiency or inability is that it prevents it from negatively affecting the plant as a whole, therefore making Foxy 2 a safe mycoherbicide.

Beyond the roots, there was a complete absence of Foxy 2 within all cells of sorghum shoots at all stages (Chapter 2) as hyphae were not detected in shoots even after 11 weeks of sowing. This was expected, as hyphae did not enter the vascular tissue of the root to grow through the vessel into the shoot of sorghum as pathogenic *Fusarium* species would do, and thus further proving evidence of the non-pathogenic behaviour of Foxy 2 to sorghum even at a later stage of growth. It is an established fact that after colonizing the cortex and vascular system, further dispersal through the plant is easily achieved by mycelia or conidia (Isaac, 1992) through the vessels. Also, hyphae did not grow from the coated seed towards the shoots to colonize it.

Alternatively, when examining the safety of Foxy 2, its ability to produce secondary metabolites, particularly toxins that have been reported to be potential risks to human and animal health (Marasas 1995, Dutton 2009), should be taken into consideration. None of the toxins tested (beauvericin, fumonisins B1, B2, B3, C and P series,

enniatins A, A1, B and B1, and moniliformin), were detected in grains of sorghum (Chapter 5) from plants whose roots where colonized by Foxy 2. In addition, the FUM1 gene (which is the key gene for fumonisin synthesis) was not detected after real-time PCR amplification using two specific primer pairs (rp32, rp33 and FUM1F7328, FUM1R7664) (Proctor et al. 2004 and Döll et al. unpublished) in the Foxy 2 pure culture indicating the inability of Foxy 2 to produce fumonisin which is among the toxins with potential risk to human and animal health (Bhat et al. 2010). Given that human and animal health problems could be caused by the accumulation of such toxins in agricultural commodities with a subsequent absorption through nourishment (Desjardins and Hohn, 1997), it is obvious that without proper knowledge on the type of toxins produced by Foxy 2, adoption and implementation by farmers might be limited as farmers would be interested to be sure that consuming crops from seeds coated with Foxy 2 would be of no negative health consequences.

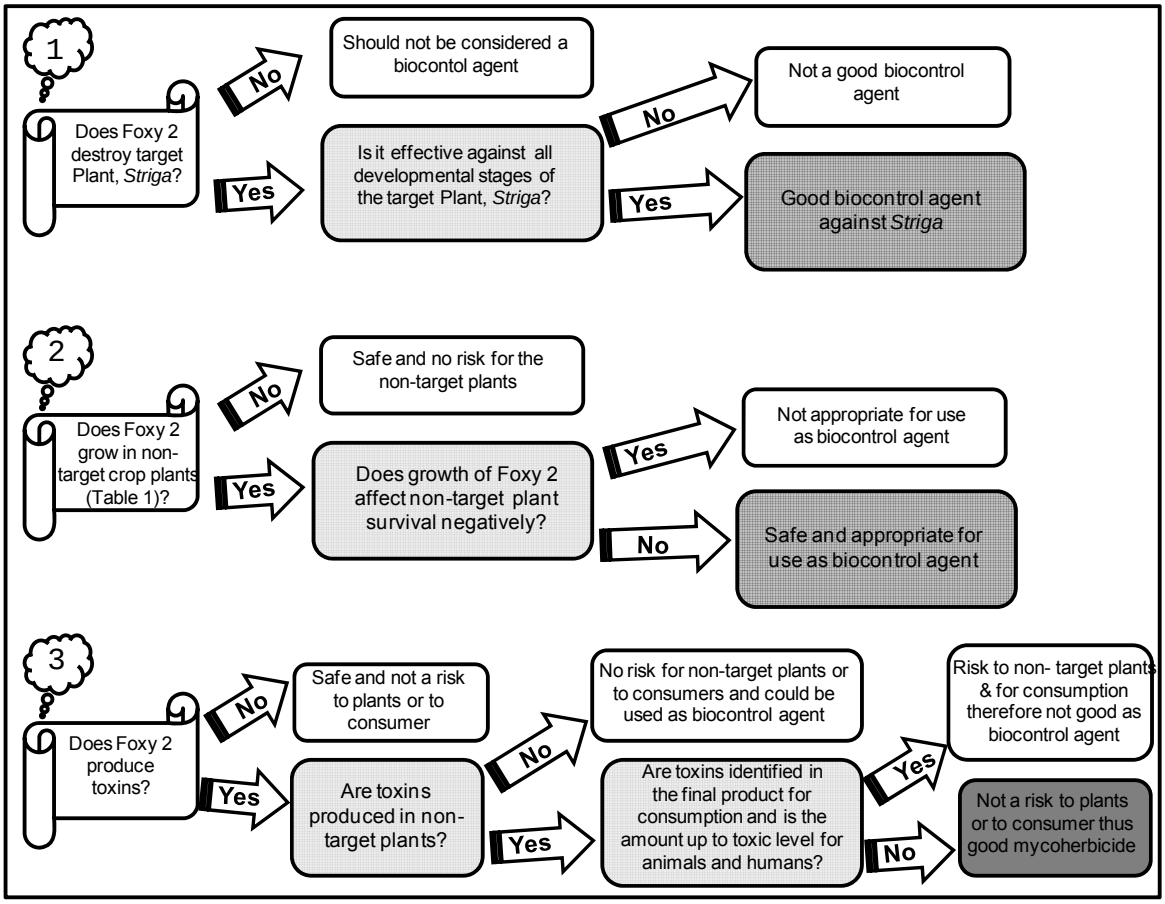

Fig. 6.2. Decision scheme to evaluate if Foxy 2 is a safe and good biocontrol agent with regards to its specificity to *Striga* and risks towards non-target plants (crops). Adopted from Boss et al. (2007).

A guide to the decision as to whether or not Foxy 2 is a safe mycoherbicide has been summarized in Fig. 6.2. The derived message from these findings is that Foxy 2 is a safe and promising mycoherbicide to be used against *Striga*. It should however be noted when talking about safety of Foxy 2 that, concerns have been expressed in that *F. oxysporum* strains might acquire genetic traits through mutation, parasexuality, or other ways of horizontal gene transfer to become pathogenic to crops (Amsellem et al., 2001). Unfortunately, there is no further information about the degree of this probability. Therefore frequent tests for possible pathogenicity should be carried out on a regular basis to ensure that the strain has not become pathogenic e.g. quality control during inoculum preparation.

6.6. Implementation of the biocontrol agent Foxy 2 in Africa: its challenges and the way forward

Having confirmed the efficacy and specific action of Foxy 2 against *S. hermonthica* but not sorghum using cytological techniques and taking into consideration its specificity on a broader scale as reported by Elzein and Kroschel (2006b), it is time for scaling up. The factors influencing the adoption potential of a control method for *S. hermonthica* in Africa have been summarised in Fig. 6.3. Generally, the seed coating option with Foxy 2 proves promising for the African farmer as no specialized training or extra resources are required and additionally, there is an increase in yield when it is used. Foxy 2 shows compatibility when co-delivered with the fungicide Apron XL® (Elzein et al., 2009) such that *S. hermonthica* and fungal cereal diseases could be controlled simultaneously. Furthermore the fungus is easy to culture, to manipulate and can grow on minimal medium (Sands and Pilgeram, 2009). Seed treatment allows Foxy 2 to grow along the root which is the infection zone of *Striga* thus Foxy 2 can readily attack *S. hermonthica* young stages therefore reducing effect on host crops. However, these results are based on laboratory and greenhouse observations but extensive testing under different field conditions is required as it has been reported that the efficacy of mycoherbicides is often lower and not reliable under natural conditions compared to pot experiments (Sauerborn et al., 2007). For example, Elzein et al. (2007) demonstrated in field experiments in Benin that when coated on resistant maize seeds, Foxy 2 reduced the number of emerged *S. hermonthica* plants by 83%. This efficacy however dropped to 21% when integrated with the susceptible maize cultivars. Another challenge is the genetic variability of *Striga* spp. which is high (Beed et al., 2007) and therefore may impact the efficacy of Foxy 2 mycoherbicide when

used in the field. Thus, the efficacy of Foxy 2 needs to be evaluated against multiple *Striga* populations from different hosts across varied environments.

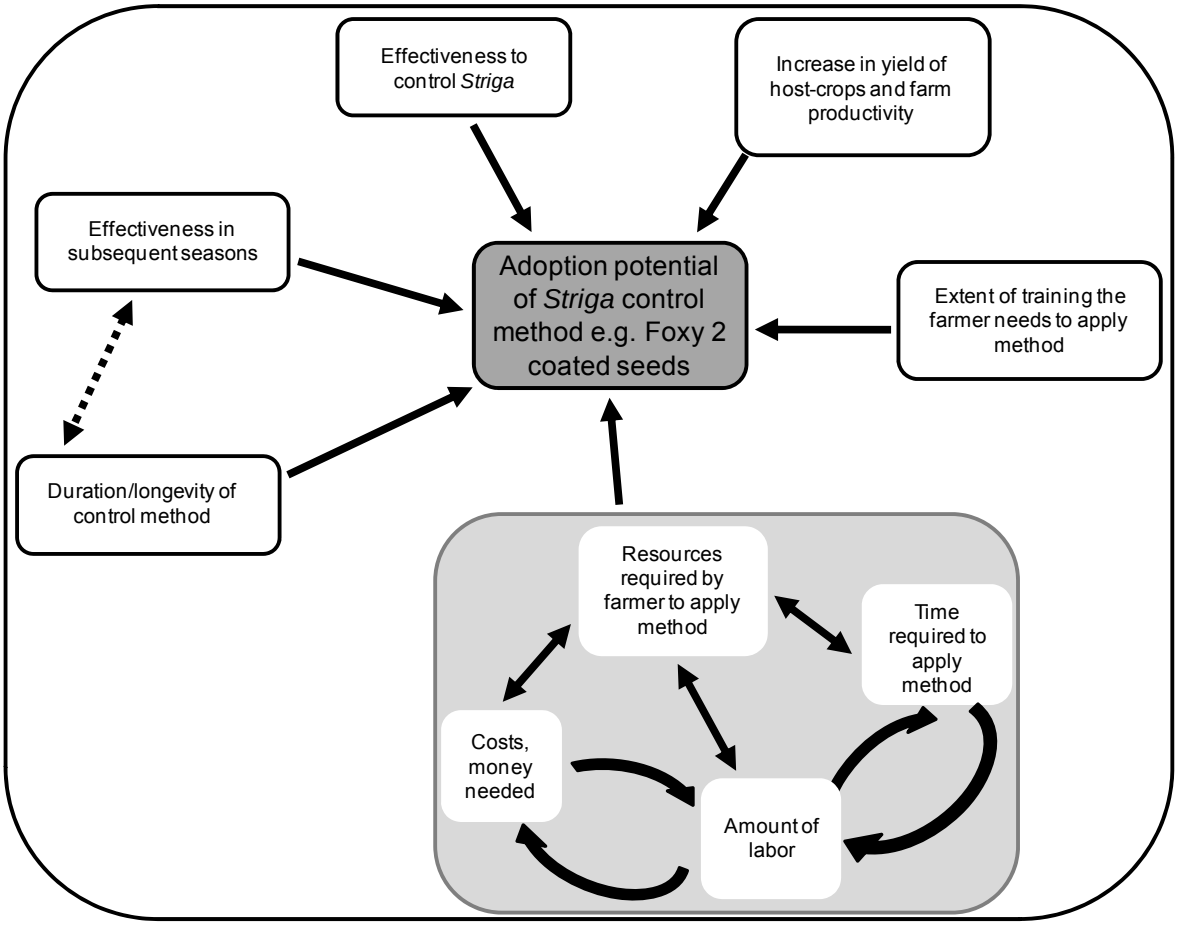

Fig. 6.3. Factors influencing the adoption potential of Foxy 2 coated sorghum seeds for the control of *Striga*. Drawn using data adopted and modified from Oswald (2005).

Successful implementation of this biocontrol agent would require local seed coating companies and its acceptance into countries which are not its origin and which sometimes is a challenging procedure. Areas of concern for the release of any biocontrol agent are: displacement of non-target microorganisms, allergenicity to humans and other animals, toxigenicity to non-target organisms, and pathogenicity to non-target organisms (Cook et al., 1996). In the case of the *F. oxysporum* species including Foxy 2, there are concerns as to whether the biocontrol agent is truly non-pathogenic or whether it may be pathogenic on a species of plant on which it has not yet been tested (Fravel et al., 2003). Notwithstanding, Cook et al. (1996) also pointed out that any adverse effects from biological control are likely to be short-term and can

be eliminated by terminating use of the biocontrol agent. Another constraint with the mycoherbicide approach remains with the quarantine regulations of affected countries. As part of the registration procedure of Foxy 2 in Kenya, field experiments have been designed based on the requirements of the Kenyan authorities. The Real IPM Company (Integrated Pest Management) under the managing director of Dr. Henry Wainwright and Louise Labuschagne now has an ongoing project for this process. Efficacy of Foxy 2 and its effects on height, dry weight and other measures in *S. hermonthica* and sorghum shoots are being evaluated under field conditions. These experiments are very valuable as efficacy needs to be evaluated in varied environments. Effect of seeds coated by SUET (Saat-u. Ernte-Technik GmbH, Eschwege, Germany) will be compared to seeds coated locally by the Real IPM (Integrated Pest Management) Company so as to evaluate and correct any differences and also future commercialization is envisaged. Meanwhile, another project which has recently been funded by the Bill and Melinda Gates Foundation focuses on field validation which involves evaluation of the efficacy of Foxy 2 at different agro-ecological sites i.e. Kenya and Nigeria under the supervision of IITA Nigeria. Thereafter the Gates Foundation plans to move to the phase of dissemination of the mycoherbicide and other effective measures to improve food security in these countries.

New studies could be directed towards the identification of genes involved in biological control which should assist in making such control more effective. Additionally, developing molecular diagnostic (primers) and tools to monitor Foxy 2 in the field would be of great interest for bio-safety assurance, quality control and persistence evaluation. Experiments could also be carried out in the field to evaluate the effectiveness of the biocontrol agent in subsequent planting seasons and to investigate its interaction with naturally occurring microorganisms in the soil to evaluate if it potentially induces undesired negative side-effects on the natural sorghum associated soil microbial population. Alternatively, field experiments need to be done to test the hypothesis that soil microorganisms have a negative impact on the population of Foxy 2 in the rhizosphere. Even though it is known that chlamydospores which are used to coat the seeds in the seed treatment technology, can withstand adverse environmental conditions, more field research needs to be done to evaluate the impact of abiotic conditions on the performance of Foxy 2 in nature so as to understand its interactions with the environment and optimize efficacy.

Conclusively, Foxy 2 is effective and shows a specific action to *S. hermonthica* but not sorghum (Chapters 2, 4 and 5). The biocontrol agent did not cause disease symptoms in the crop sorghum (Chapter 3) but it was highly pathogenic to its specific target weed *S. hermonthica*. Based on the results of this study, it can be stated that the mycoherbicide, Foxy 2 completely digested most *S. hermonthica* seedlings belowground and had a dual action in *S. hermonthica* shoots: a blocking effect which created a resistance to water flow (Chapter 4) in the plant and a phytotoxic effect (Chapter 5) which lead to destruction and final death of the whole plant. On the other hand no toxins were detected in sorghum grains from plants whose roots where colonized by Foxy 2. Foxy 2 applied as chlamydosopores on sorghum seeds is a method which reduces inoculum amount and the resulting coated seeds are easy to handle. Seed coated Foxy 2 thus is a promising biocontrol agent and option to control *S. hermonthica* and reduce its effect on crops thereby enhancing food security in Sub-Saharan Africa (Chapters 2 and 4). However, for a successful integrated approach, Foxy 2 should not be used alone but in combination with the local tolerant cultivars to ensure greater efficiency.

CHAPTER 7

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Summary

Amongst the factors that are a threat to food security in Africa, is the parasitic weed *Striga hermonthica* which affects mostly cereals that constitute the staple food for subsistence farmers, thus affecting the livelihood of millions of people. Popularly known as witchweed, attack due to *S. hermonthica* can completely destroy the yield of cereal crops. Efforts to combat *Striga* have had very limited success since farmers rarely adopt control methods due to the mismatch between technologies and farmers' socio-economic conditions. Being such a severe problem, an appropriated method for *Striga* management adapted for African farmers is very much needed. The use of soil-borne fungi for biocontrol is now being developed as an alternative to the use of chemicals considering the specificity of such fungi and the fact that most of the damage by *Striga* is done before its emergence. The fungus *Fusarium oxysporum* f.sp. *strigae* has been identified and shown to be effective and specific to *S. hermonthica* and *S. asiatica* but its mode of action is not yet well known. It is required that the mechanisms underlying the mycoparasitic process of this natural antagonistic agent be well understood before its use. Thus, studies on the effectiveness, specificity and timely colonization of Foxy 2 on *S. hermonthica* are necessary as well as studies on the effect of Foxy 2 in *Striga*-host plants which should demonstrate its non-pathogenicity to food crops. The objective of this study was therefore to investigate the mode of action of Foxy 2 in its target *S. hermonthica* and non-target *Sorghum bicolor* and also to examine the safety of the use of this mycoherbicide by evaluating its ability to produce toxins.

In the first part of the thesis, the ability of Foxy 2 to colonize sorghum roots and possibly shoots was investigated using light and transmission electron microscopy. The efficacy of Foxy 2 to cause death of *S. hermonthica* seedlings attached to Foxy 2 colonized sorghum roots was also evaluated. Microscopic investigations revealed that the intensity of root colonization by Foxy 2 increased with time and Foxy 2 could survive and colonize the sorghum rhizodermis, root hairs and cortical parenchyma up to four weeks after sowing. This behaviour is well adapted for *Striga* control as it corresponds to the peak of *Striga* seedling attachment. Hyphae were completely absent from the sorghum root central cylinder even after four weeks and also absent from the sorghum shoots up to 11 weeks after sowing indicating the non-pathogenity of Foxy 2 to sorghum. Furthermore, Foxy 2 was effective in controlling *S. hermonthica* by causing disease in 95% and 86% of *S. hermonthica* seedlings when coated on

seeds of tolerant and susceptible sorghum cultivars respectively. Therefore, Foxy 2 could be combined with the tolerant sorghum variety in an integrated approach against *S. hermonthica and S. asiatica*.

The effect of Foxy 2 on various growth stages of *S. hermonthica* was investigated subsequently so as to understand the mechanisms of action of Foxy 2 within *S. hermonthica* in the real living complex between the mycoherbicide Foxy 2, the parasite *S. hermonthica* and its host sorghum. Light, scanning and transmission electron microscopy were used to evaluate the pattern of colonization and control of *S. hermonthica* seedlings and shoots by Foxy 2. Results showed that 26 days after sowing Foxy 2 coated sorghum seeds, all tissues of the young *S. hermonthica* seedlings attached to sorghum roots were completely degraded and destroyed by Foxy 2 including the haustorial intrusive cells, hyaline tissue, vessels, central xylem elements and *Striga* cortical parenchyma. Some *S. hermonthica* plants which attached to areas of the sorghum root which were not yet colonized by Foxy 2 (towards the root tips), were able to outgrow the fungus and emerged. In the emerged *S. hermonthica* shoots, hyphae had subsequently penetrated and colonized vessels clogging them over long distances and were identified up to the top of the plants. In some vessels there was an intensive blockage of the vessels by hyphae such that spaces or gaps were rare. Ultrathin sections showed that the diseased *S. hermonthica* shoots reacted to Foxy 2 invasion by forming an electron dense wall coating along the secondary vessel walls probably to prevent fungal digestion of the walls. The study thus identified two mechanisms by which Foxy 2 contributed to wilting and death of *S. hermonthica* which included complete digestion of underground *S. hermonthica* seedlings and hyphal clogging of vessels in emerged *S. hermonthica* plants which interfered with water conduction.

In order to understand the reactions of sorghum towards the presence of Foxy 2 as part of the risk assessment to ensure the safe use of this biocontrol agent, the action of Foxy 2 and a known pathogenic *Fusarium* species, *F. proliferation,* were compared in the fourth chapter. Sorghum roots were also wounded to expose the vascular system so as to investigate whether removal of the endodermal barrier could give access to Foxy 2 into the vessels which could lead to digestion resulting in wilting of the sorghum plants. The colonization processes of the two Fusaria species were quite different at all stages of growth. While *F. proliferatum* degraded the endodermis, invaded the central cylinder and digested the xylem parenchyma two weeks after

sowing, Foxy 2 was restricted to the cortex even up to four weeks after sowing. Hyphae of Foxy 2 filled the intercellular spaces at the outer endodermal wall but could not penetrate the endodermis. Sorghum roots were observed to react to Foxy 2 invasion by reinforcing the central cylinder as seen by an increase in blue auto fluorescence especially of the endodermis. Five days after wounding and inoculating sorghum roots, Foxy 2 hyphae invaded the central cylinder very close to the cut but were completely absent from the central cylinder at a distance of 3000 µm from the cut, meanwhile *F. proliferatum* hyphae had digested the cells of the central cylinder at this distance. This indicated that not only the endodermis was a barrier but there could also be a physiological barrier within the central cylinder of the sorghum root which did not allow further spread of Foxy 2. Hence, exposure of the vascular system did not serve as a route for the invasion of Foxy 2 which therefore implied that it could not cause wilting of the plant.

In the last part of the thesis, *S. hermonthica* shoots were analyzed by HPLC-MS/MS to investigate the possible production of toxins by Foxy 2 to kill the plant. Amongst the toxins tested (beauvericin, fumonisins B1, B2, B3, C and P series, enniatins A, A1, B and B1, and moniliformin), only beauvericin (BEA) was detected to be produced by Foxy 2 in *S. hermonthica* shoots. The concentration of this toxin increased with increased infection e.g. 60 µg BEA/kg *Striga* shoot tissue (dry weight) were detected three weeks after emergence rising to 720 µg BEA/kg *Striga* shoot tissue after six weeks in the severely diseased *S. hermonthica* shoots. When beauvericin was applied on *S. hermonthica* shoots at concentrations of 50 µM, transmission electron microscopy showed that all cell types became necrotic. However, beauvericin as well as all the other toxins were not detected in sorghum grains harvested from sorghum plants which were hosts to the *S. hermonthica* plants and growing from Foxy 2 coated sorghum seeds. Given that some *F. oxysporum* strains were previously shown to be able to produce fumonisins which are among the toxins which have been reported to be of potential risks to human and animal health, a pure culture of Foxy 2 was evaluated for its fumonisin production ability. Results from real-time PCR using two specific primer pairs for the FUM1 gene (which is the key gene for fumonisin synthesis), were negative confirming that Foxy 2 was not able to produce fumonisins and might not be of major concern for human and animal health when used as a biocontrol agent in the field, therefore safe for use as a biocontrol agent.

To conclude, Foxy 2 showed potential to control *S. hermonthica* by completely destroying young underground stages and clogging vessels in aboveground stages, as well as producing the toxin beauvericin, both actions contributing to wilting of the plants. Its non-pathogenicity to sorghum and its inability to produce fumonisins could be seen as factors which make it well suited as a biocontrol agent. Further research needs to be done to evaluate its efficacy under field conditions and the impact of naturally occurring soil microorganisms and abiotic conditions on performance of Foxy 2 so as to understand its interactions with the environment and to optimize its efficacy.

Zusammenfassung

Zu den Faktoren, die die Nahrungssicherung in Afrika bedrohen, gehört das parasitische Unkraut *Striga hermonthica*, das speziell das Getreide der Landwirte schädigt, die in Subsistenzwirtschaft leben. Somit wird der Lebensuntethalt von Millionen Menschen negativ beeinflusst. Bei einem starken Befall kann das Unkraut *S. hermonthica,* auch „witchweed“ genannt, zum vollständigen Getreideertragsverlust führen. Bemühungen *Striga* zu bekämpfen, hatten bisher sehr begrenzten Erfolg, da die Landwirte selten die geeigneten Bekämpfungsmethoden anwenden können, da solche Technologien mit den Sozioökonomischen Umständen der Landwirte oft nicht vereinbar sind. Daher ist eine für einen afrikanischen Bauern geeignete Methode für *Striga*-Management notwendig. Angesichts des Lebenszyklus von *S. hermonthica* bietet sich die Anwendung pathogener Pilze als Alternative zur Anwendung von Chemikalien an. Dafür sprechen die Wirtsspezifität solcher Pilzarten sowie die Tatsache, dass ein Großteil der Schädigung durch *Striga* bereits vor dem Auflaufen erfolgt. Zwar wurde der Pilz *F. oxysporum f.sp. strigae* identifiziert und als wirksam und spezifisch für *S*. *hermonthica* charakterisiert, aber die Mechanismen seiner Vorgehensweise und Wirkung sind noch weitgehend unbekannt. Es ist für die Anwendung notwendig, die dem mykoparasitischen Prozess zugrundeliegenden Mechanismen eines natürlich entgegenwirkenden Erregers zu verstehen. Daher sind Studien zur Wirksamkeit, Spezifizität und spontanen Kolonisation von Foxy 2 auf *S. hermonthica,* sowie Untersuchungen zur pathogenen Wirkung von Foxy 2 auf *Striga*-Wirtspflanzen notwendig. Ziel dieser Studie war daher das Verhalten von Foxy 2 auf *S. hermonthica* und *Sorghum bicolor* zu untersuchen, aber auch die Sicherheit bei der Anwendung dieses Mykoherbizides zu erforschen, in dem die Fähigkeit zur Toxinproduktion bewertet wird.

Im ersten Teil (Kapitel II) wurde die Fähigkeit von Foxy 2 Sorghumwurzeln und möglicherweise Sprosse zu kolonisieren mit Hilfe von Licht- und Transmissionselektronenmikroskopie untersucht. Ebenso wurde die Wirksamkeit von Foxy 2 bewertet, mit der es zu einem Absterben von *S. hermonthica* Jungpflanzen kommt, welche Sorghumwurzeln befallen die mit Foxy 2 kolonisiert wurden. Mikroskopische Untersuchungen zeigten, dass sich die Intensität der Wurzelkolonisation durch Foxy 2 mit der Zeit erhöhte und Foxy 2 dabei noch bis vier Wochen nach dem Säen überleben konnte und Sorghum-Rhizodermis, Wurzelhaare und kortische Parenchyme kolonisieren konnte. Dies ist von Vorteil bei der

Bekämpfung von *Striga*, da dieser Zeitpunkt dem Höhepunkt im Befall durch *Striga* Jungpflanzen entspricht. Hyphen waren auch noch vier Wochen nach der Saat im Zentralzylinder der Sorghumwurzel und bis zu 11 Wochen nach der Saat im Sorghum Schössling nicht vorhanden. Dies weist darauf hin dass Foxy 2 gegenüber Sorghum kein Pathogen darstellt. Darüber hinaus war Foxy 2 effektiv bei der Bekämpfung von *S. hermonthica,* da es bei 95% bzw. 86% der Jungpflanzen von *S. hermonthica* Erkrankungen verursachte, wenn das Saatgut von toleranten bzw. anfälligen Sorghum Sorten mit Foxy 2 umhüllt wurde. Foxy 2 kann daher mit der toleranten Sorte von Sorghum in einem integrierten Ansatz gegen *Striga* kombiniert werden.

Die Wirkung von Foxy 2 auf unterschiedliche Wachstumsphasen von *S. hermonthica* wurde im zweiten Teil untersucht, um das Verständnis der Verhaltensmechanismen von Foxy 2 innerhalb des lebenden Systems zwischen dem Mykoherbizid Foxy 2, dem Parasit *S. hermonthica* und seinem Wirt Sorghum zu verbessern. Licht-, Scan- und Transmissionselektronenmikroskopie wurden verwendet, um die Art des Befalls und den Einfluss auf Keimlinge und Schösslinge von *S. hermonthica* durch Foxy 2 zu bewerten. Die Ergebnisse zeigten, dass alle Gewebe der Jungpflanzen von *S. hermonthica* (an Sorghumwurzeln) 26 Tagen nach der Aussaat von mit Foxy 2 umhülltem Saatgut vollständig abgeschwächt und zerstört wurden, einschliesslich der intrusiven Zellen, den Haustorien der Hyalingewebe, der Gefäße der Zentralxylemelemente und des Rindenparenchyms. Einige Pflanzen von *S. hermonthica*, die sich in der Umgebung der Wurzelspitzen von noch nicht mit Foxy 2 kolonisierten Sorghumwurzeln angeheftet hatten, konnten dem Pilz entwachsen und sich entwickeln. In einigen Gefäßen solcher *Striga*pflanzen war eine intensive massenhafte Hyphenbesiedelung zu finden, die über weite Strecken das Leitsystem der Sprösslinge bis in die Sprossspitzen fast vollständig verstopften und zur Welke der Schösslinge beitrugen. Ultradünnschnitte zeigten, dass die erkrankten Schösslinge von *S. hermonthica* auf den Eingriff von Foxy 2 reagiert haben indem sie eine elektronendichte Wand (wahrscheinlich zur Vermeidung einer Zersetzung der Wände durch den Pilz) entlang der sekundären Gefäße bildeten. Die Studie identifizierte daher zwei Mechanismen wodurch Foxy 2 zum Welken und Absterben von *S. hermonthica* beigetragen hat. Zum einen die komplette Zersetzung der noch unterirdischen Keimlinge von *S. hermonthica* und zum anderen die durch Hyphenbefall der Gefäße gestörte Wasserversorgung von *S. hermonthica*.

Zur Risikobewertung dieser Pflanzenschutzanwendung wurde im vierten Kapitel die Reaktion von Sorghum auf die Anwensenheit von Foxy 2 untersucht, in dem das Verhalten von Foxy 2 mit dem eines bekannten *Fusarium* Pathogens, *F. proliferatum* verglichen wurde. Zudem wurde an Sorghumwurzeln, bei denen das Gefäßsystem freigelegt wurde untersucht, ob die Entfernung der Endodermisbarierre das Eindringen von Foxy 2 in die Gefäße erleichtert, so dass eine Zersetzung stattfindet die zum Welken der Sorghumpflanze führt. Die Infektionsprozesse beider Pilzarten unterschieden sich in allen Wachstumsphasen grundlegend. Während *F. proliferatum* das Endoderm zersetzte, in den Zentralzylinder eindrang und das Xylem-Parenchym zwei Wochen nach dem Säen zersetze verblieb Foxy 2 bis zu vier Wochen nach der Aussaat in der Rinde. Hier verblieben die Hyphen von Foxy 2 in den Interzellularräumen außerhalb der Endodermis, während die Sorghumwurzeln mit einer Verstärkung des Zentralzylinders auf das Eindringen von Foxy 2 reagierten, wie durch den Anstieg der Autofloureszenz der Endodermis im blauen Bereich gezeigt werden konnte. Fünf Tage nach einer artifiziellen Infektion und der Inokulation der Sorghumwurzel haben Foxy 2 Hyphen den Zentralzylinder nahe dem Auschnitt befallen, waren jedoch in einer Entfernung von 3000 µm im Zentralzylinder nicht nachzuweisen, wohingegen *F. proliferatum*-Hyphen die Zellen des Zentralzylinders bis zu dieser Entfernung bereits zersetzt hatten. Dies zeigt, dass nicht nur die Endodermis eine Barriere darstellte, sondern auch eine physiologische Barriere innerhalb des Zentralzylinders der Wurzel existieren könnte, die eine weitere Ausbreitung von Foxy 2 verhinderte. Deshalb konnte das freigelegte Gefäßsystem von Foxy 2 nicht als Infektionsweg genutzt werden, was zur Folge hatte dass die infizierte SorghumPflanze kein Welken zeigte.

Im letzten Teil der Arbeit wurde mithilfe der HPLC-MS/MS Schösslinge von *S. hermonthica* auf eine mögliche Produktion von Toxinen durch Foxy 2 untersucht, die zum Absterben der Wirtspflanzen führen könnten. Bei den untersuchten Toxinen (Beauvericin, Fumonisine der B1, B2, B3, C und P Serie, Enniatin A, A1, B und B1, und Moniliformin) wurde festgestellt, dass Beauvericin in den Schösslingen von *S. hermonthica* von Foxy 2 produziert wurde. Die Konzentration dieses Toxins stieg mit steigender Infektionsintensität. So wurden drei Wochen nach dem Auflaufen der *Striga* Sprosse 60 µg BEA/kg Trochengewicht festgestellt, nach sechs Wochen in den schwer geschädigten Schösslingen dagegen 720 µg BEA/kg Trochengewicht. Als Gaben von Beauvericin in Konzentrationen von 50 µM auf die Schösslinge von *S. hermonthica* untersucht wurden, zeigte sich im

transmissionselektronenmikroskopischen Befund, dass alle Zellarten nekrotisch waren. Weder Beauvericin noch die anderen Toxine wurden in den geernteten Getreidekörnen, welches von Sorghum Pflanzen stammt, die von *S*. *hermonthica* befallen waren und welche aus mit Foxy 2 umhüllten Saatgut angezogen wurden, festgestellt. Da Fumonisin zu den für Menschen und Tiere gesundheitschädlichsten Toxinen gezählt wird, wurden Reinkulturen von Foxy 2 auf die Fähigkeit zur Fumonisinproduktion getestet, indem die PCR-Methode untersucht wurde, ob das wichtigste Gen der Fumonisinsynthese (FUM1) anwesend war. Die Anwendung von zwei spezifischen Primerpaaren zeigte negative Ergebnisse so, dass gefolgert wurde, dass Foxy 2 Fumonisin nicht produzieren kann, und daher als ungefährlich im Einsatz als Pflanzenschutzmittel angesehen wird.

Zusammenfassend kann festgestellt werden, dass Foxy 2 das Potenzial hat den Befall durch *S*. *hermonthica* zu kontrollieren, indem es die jungen noch unterirdischen Pflanzen völlig zerstört und in den oberirdischen Pflanzen die Gefäße verstopft, wo auch das Toxin Beauvericin produziert wird. Beides führt zum Welken der Pflanze. Aufgrund der fehlenden Pathogenität gegenüber Sorghum, sowie der fehlenden Möglichkeit Fumonisin zu produzieren, kann Foxy 2 als geeignetes Pflanzenschutzmittel angesehen werden. Weitergehende Untersuchungen müssen die Eignung von Foxy 2 unter dem Einfluss natürlicher Bodenmikroorganismen und abiotischen Faktoren im Freiland zeigen, um die Interaktion mit der Umwelt zu verstehen und die Wirkung zu optimieren.

Résumé

Le *striga hermonthica,* une mauvaise herbe parasitique, est un des facteurs qui menacent la sécurité alimentaire en Afrique. Le *S. hermonthica* affecte principalement des céréales qui constituent l'aliment de base des agriculteurs de subsistance, et par conséquence, il met en péril les conditions de vie de millions de personnes. Communément appelé *l'herbe des sorcières*, une infestation par cette herbe peut causer la perte complète de rendement de céréale. Les efforts fournis pour combattre le *Striga* sont demeurés sans aucun succès notable, étant donné que les agriculteurs adoptent rarement les méthodes de contrôle dû à la disparité entre les moyens technologiques et la condition socio-économiques des agriculteurs. Vu la gravité du problème, la lutte contre le *Striga* requière des méthodes adaptées aux agriculteurs Africains. De nos jours, l'emploi de fongicides transmis par le sol pour la lutte biologique se développe comme alternative à l'emploi des produits chimiques, et cela en ayant égards à la spécificité de ces fongicides et au fait que la majorité des ravages causés par le *Striga* s'effectuent avant qu'il ne sorte du sol. Le fungus, *Fusarium oxysporum f.sp strigae,* a été identifié et s'est avéré effectif et approprié dans la lutte contre le *S. hermonthica* et le *S. asiatica*. Toutefois, le mode d'action de ce fungus reste encore inconnu. Il est impératif avant l'emploi de cet agent naturel antagoniste de bien comprendre les mécanismes sur lesquels le processus myco-parasitique est fondé. Ainsi, des études sur l'effectivité, la spécificité et la colonisation ponctuelle du Foxy 2 dans le *S. hermonthica* sont aussi bien nécessaires que des études sur l'effet du Foxy 2 dans les végétaux hôtes du *S. hermonthica* afin de démontrer sa non-pathogénicité. L'objectif de cette étude était donc de tant découvrir le mode d'action du Foxy 2 dans l'organisme cible, notamment le *S. hermonthica,* et dans l'organisme non cible, à savoir le *Sorghom bicolor,* qu'aussi d'examiner à quel point l'emploi de ce myco-herbicide est sans risques en évaluant sa capacité de produire des toxines et sa propagation parmi les plantes.

La première partie de la thèse se focalise sur l'observation de la capacité du Foxy 2 de coloniser les racines du sorgho et éventuellement ses pousses au moyen de La microscopie à lumière polarisée et électronique à transmission. L'efficacité du Foxy 2 de provoquer la mort des plantules du *S. hermonthica* attachées aux racines du sorgho colonisées par Foxy 2 a été également évaluée. Les recherches effectuées au microscope ont révélé qu'avec le temps, la colonisation de la racine par Foxy 2 a augmenté en intensité et que Foxy 2 a pu survivre et coloniser le rhizoderme du

sorgho, les poils radiculaires et le parenchyme cortical jusqu'à quatre semaines après l'ensemencement. Cette observation du comportement de Foxy 2 est bien utile pour le contrôle du *Striga* vu que ca correspond au point culminant de l'attachement des semis du *Striga*. Quatre semaines après l'ensemencement, les hyphes étaient complètement absentent tant dans la racine centrale du sorgho que dans les pousses jusqu'à onze semaines après l'ensemencement. Cela indique la non-pathogénicité de Foxy 2 par rapport au sorgho. En outre, Foxy 2 était effectif dans le contrôle du *S. hermonthica* en entraînant l'infection de 95% et de 86% des plantules du *S. hermonthica* enduites dans des graines tolérantes et chacune susceptibles aux cultivars de sorgho. Foxy 2 peux donc être combiné avec une variété du sorgho tolérante dans une approche intégrée contre le *Striga*.

La seconde partie des recherches se focalise sur L'effet du Foxy 2 lors de divers étapes de la croissance du *S. hermonthica*. Il s'agit de comprendre les mécanismes d'action du Foxy 2 à l'intérieur du *S. hermonthica,* et cela dans une situation complexe proche de la vie réelle entre le myco-herbicide Foxy 2, le parasite *S. hermonthica* et son hôte le sorgho. La microscopie à lumière polarisée, électronique à balayage et à transmission a été employée pour évaluer le modéle/type de colonisation et le contrôle des plantules et pousses du *S. hermonthica* par Foxy 2. Les résultats ont démontré que 26 jours après avoir planté des graines de sorgho recouvertes de Foxy 2, tous les tissus des jeunes plantules du *S. hermonthica* attachées aux racines du sorgho étaient complètement dégradés et détruits par Foxy 2, y compris les cellules intrusives du complexe haustorien, les tissus hyalines, les vaisseaux, les éléments centraux du xylème et le parenchyme cortical du *Striga*. Certaines plantes du *S. hermonthica* attachées aux endroits des racines du sorgho non-colonisées par Foxy 2 (notamment vers les pointes des racines) étaient capables de se développer indépendamment du fongicide et d'émerger. Dans les pousses émergeantes du *S. hermonthica*, les hyphes avaient pénétré et colonisé les vaisseaux, obstruant ceux-ci sur de longues distances. Elles furent également identifiées au sommet des plantes. Certains vaisseaux étaient si intensément bouchés par les hyphes qu'il n y avait que peu d'espace ou d'intervalle. Des sections ultraminces ont démontré que les pousses colonisées du *S. hermonthica* ont réagi à l'invasion du Foxy 2 en formant un mur dense en électrons couvrant les murs du vaisseau secondaire, probablement pour prévenir la digestion fongique des murs. L'étude a donc identifié deux mécanismes par lesquels Foxy 2 a contribué au flétrissement et à la mort du *S. hermonthica,* à savoir la digestion des plantules souterraines de *S. hermonthica* et le colmatage des

vaisseaux par les hyphes dans les plantes du *S. hermonthica* qui avaient émergé, interférant avec la conduction d'eau.

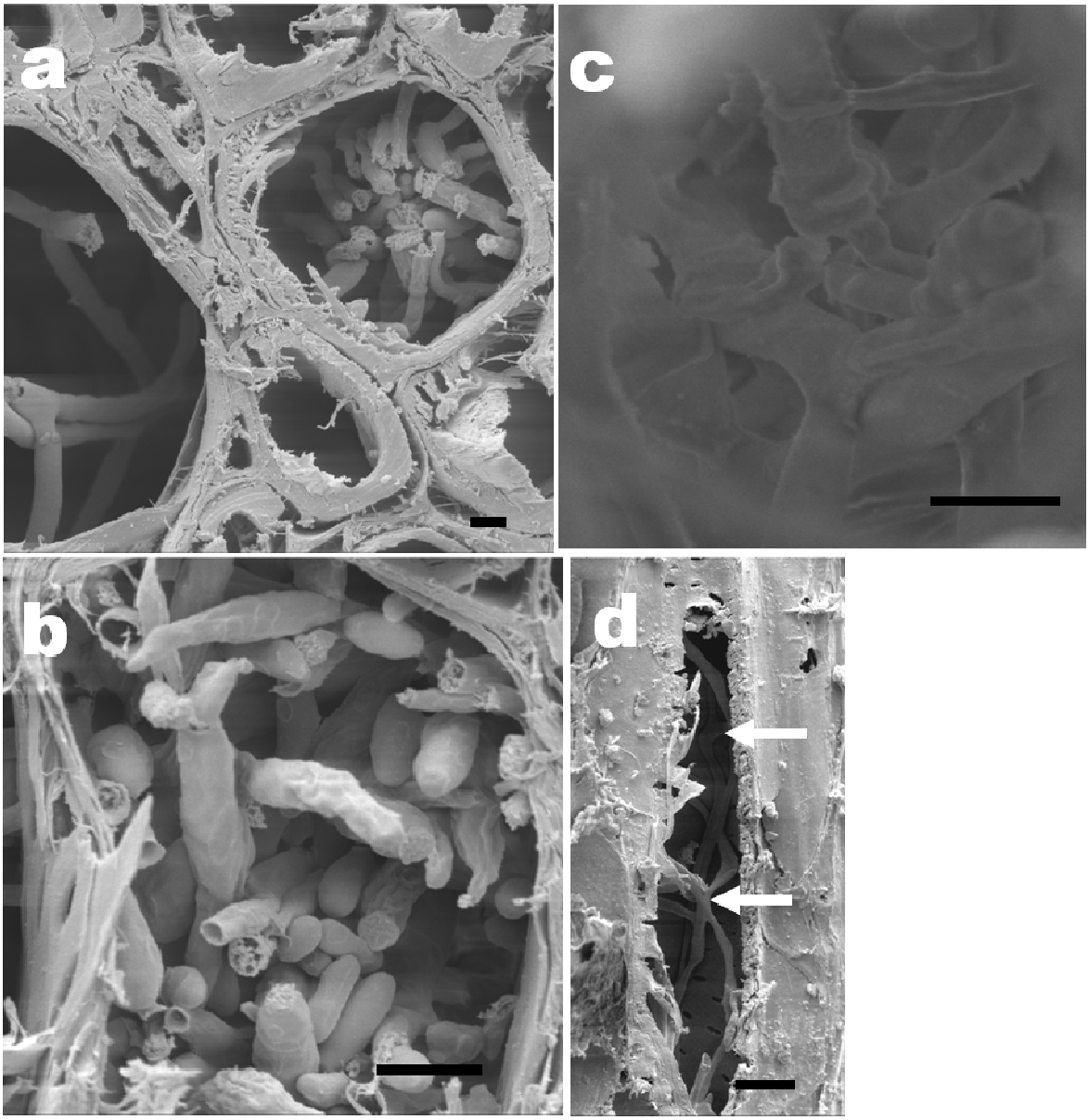
Pour comprendre les réactions du sorgho à l'égard de Foxy 2, dans l'optique d'une évaluation de risque afin d'assurer un usage contrôlé de cet agent de lutte biologique, le quatrième chapitre compare l'action du Foxy 2 et celle d'une espèce connue de *Fusarium* pathogénique, *F prolifération.* Les racines du sorgho furent également blessées pour exposer le système vasculaire afin d'observer si le fait d'enlever la barrière endodermique donnerait à Foxy 2 un accès vers les vaisseaux, ce qui pourrait entraîner une digestion de l'agent et causer par conséquence le flétrissement des plantes du sorgho. Le processus de colonisation des deux espèces était tout à fait différent au cours des diverses étapes de croissance. Tandis que deux semaines après l'ensemencement, le *F. proliferatum* provoquait une dégradation de l'endoderme, envahissait le tronc central et digérait le parenchyme xylémique, Foxy 2 lui, se voyait restreint au cortex même jusqu'à quatre semaines après l'ensemencement. Bien que les hyphes du Foxy 2 aient rempli les espaces intercellulaires du mûr extérieur de l'endoderme, ils ne purent pénétrer l'endoderme même. Les racines du sorgho réagissaient à l'invasion du Foxy 2 en renforçant le tronc central, - observation faite grâce à une augmentation d'auto fluorescence bleue particulièrement de l'endoderme. Cinq jours après avoir blessé et inoculé les racines du sorgho, les hyphes du sorgho avaient envahi le tronc central à une distance de 3000 µm à partir de la coupure. Entre temps les hyphes du *F. proliferatum* avaient ravagé dans la même distance les cellules du tronc central. Ceci indique que non seulement l'endoderme fut une barrière mais qu'il pouvait aussi y avoir une barrière physiologique dans le tronc central de la racine du sorgho empêchant au Foxy 2 de continuer à se répandre. Ainsi, l'exposition du système vasculaire n'a pas été un moyen d'invasion pour Foxy 2, ce qui signifiait donc que la plante n'a pas pu subir de flétrissement.

Dans la dernière partie de la thèse, les pousses du *S. hermonthica* ont été analysées par HPLC-MS/MS pour observer une potentielle production de toxines par Foxy 2 qui provoqueraient la mort de la plante. Parmi les toxines testées (les enniatines, la beauvericine, les fumonisines, la moniliformine), Foxy 2 n'a produit que de la beauvericine (BEA) dans les pousses du *S. hermonthica*. La concentration de cette toxine s'augmentait au fur et à mesure que l'infection se répandait. Un échantillon de 60 µg BEA/kg des pousses du *Striga* a été détecté trois semaines après l'émergence

tandis qu'un échantillon de 720 µg BEA/kg des pousses de *Striga* a été détecté après six semaines dans des pousses du *S. hermonthica* fortement infectées. Lorsque la beauvericine a été appliquée sur des pousses du *S. hermonthica* d'une concentration de 50 µM, la microscopie électronique à transmission montrait que toutes les types de cellules étaient nécrotiques. Cependant, la beauvericine ainsi que d'autres toxines n'étaient pas détectées dans les grains de sorgho récoltés des plantes de sorgho qui avaient servi comme hôtes aux plantes du *S. hermonthica* et qui poussaient des semences du sorgho couvertes du Foxy 2. En plus, étant donné qu'avait déjà été détecté qu'une souche de *F. oxysporum* était capable de produire de la fumonisine C, une des toxines signalées être un risque potentiel à la santé humaine et animale, la capacité de Foxy 2 de produire de la fumonisine a été évaluée sans risques pour la culture. Les résultats des tests lors desquels on a utilisé deux paires d'amorces spécifiques pour le gène FUM1 (gène clé de la synthèse de la fumonisine) se sont avérés négatifs, suggérant que Foxy 2 ne serait pas capable de produire de la fumonisine et par conséquence sans risques comme agent de lutte biologique.

En conclusion, Foxy 2 s'est avéré à mesure de contrôler le *S. hermonthica* en détruisant complètement l'herbe mauvaise dans ses étapes précoces dans le sous-sol, et en colmatant les vaisseaux dans des étapes plus avancées à la surface du sol, ainsi qu'en produisant de la toxine beauvericine contribuant au flétrissement de la plante. Sa non-pathogénicité au sorgho et son incapacité de produire de la fumonisine pourraient être vu comme des facteurs qui le qualifieraient comme agent approprié de la lutte biologique. D'autres recherches sont nécessaires pour évaluer son efficacité sur terrain, de même que l'impacte des microorganismes propres au sol et des conditions abiotiques sur la performance de Foxy 2, afin de comprendre ses interactions avec l'environnement et d'optimiser l'efficacité.

Appendix A.1.

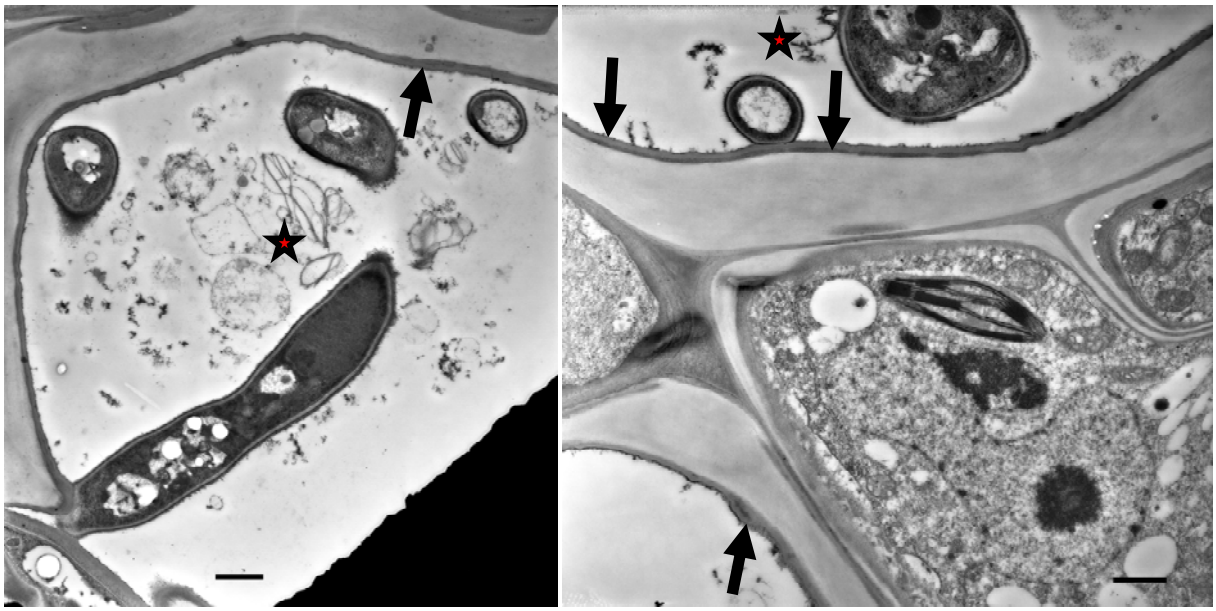


Scanning electron micrographs of *S. hermonthica* shoots infected by Foxy 2 showing hyphae within vessels.

- **a.** Cross section of a shoot three weeks after emergence. Few vessels are blocked.
- **b.** Higher magnification showing hyphae being pressed on each other within a vessel. Gaps or spaces for waterflow can hardly be seen.
- **c.** View from within a vessel showing intertwinning of hyphae within vessels. Shoot was collected four weeks after *Striga* emergence.
- **d.** Longitudinal section showing intertwining of hyphae as they grow within vessels five weeks after *Striga* emergence. Arrows indicate such positions.

Bars = 10 µm

Appendix A.2



Transmission electron micrographs of Foxy 2 infected *S. hermonthica* shoots one week after emergence. A wall layer (arrows) is formed around infected vessels and unknown substances are observed within vessels (asterisks). *Bars* = 1 µm

Appendix A.3. Summary of Maldi results of Sorghum roots

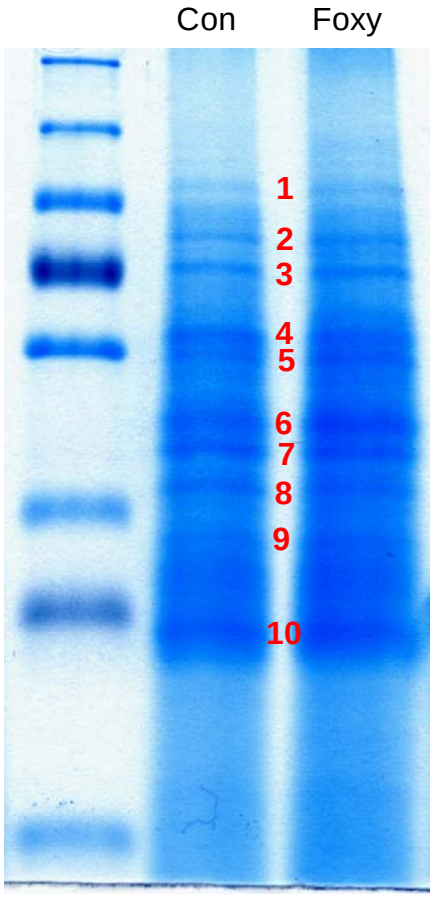


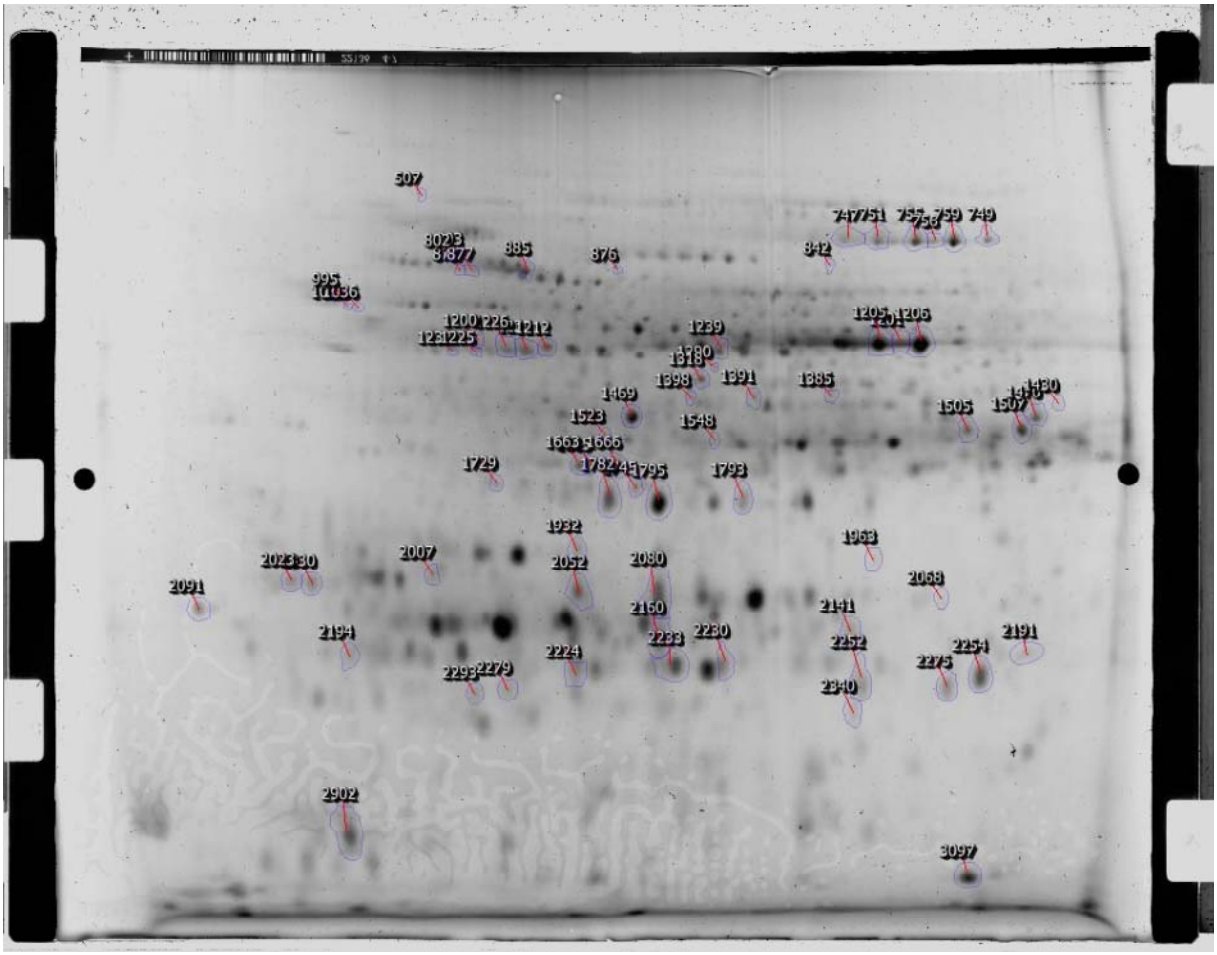
Table A.2. Summary of the identified proteins in Foxy 2 infected and non-infected sorghum roots after digestion of bands as indicated on the gel above.

Position on gel	Treatment	Estimated size on gel	Protein name		Protein size
1	Control	104 KDa	Best match	hypothetical protein SORBIDRAFT_02g009150 [Sorghum bicolor]	116754
			2nd best match	sucrose synthase [Sorghum bicolor]	92053
	Foxy	104 KDa	Best match	sucrose synthase [Sorghum bicolor]	92053
			2nd best match	hypothetical protein SORBIDRAFT_01g020910 [Sorghum bicolor]	90698
2	Control	90 KDa	Best match	methionine synthase protein [Sorghum bicolor]	84135
			2nd best match	5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase [Zea mays]	84759

	Foxy	90 KDa	Best match	methionine synthase protein [Sorghum bicolor]	84135
			2nd best match	5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase [Zea mays]	84759
3	Control	75 KDa	Best match	hypothetical protein SORBIDRAFT_04g034980 [Sorghum bicolor]	81671
			2nd best match	exoglucanase precursor [Zea mays]	67356
	Foxy	75 KDa	Best match	hypothetical protein SORBIDRAFT_04g026510 [Sorghum bicolor]	76011
			2nd best match	phenylalanine ammonia-lyase [Zea mays]	75919
4	Control	65 KDa	Best match	hypothetical protein SORBIDRAFT_01g020010 [Sorghum bicolor]	61322
			2nd best match	glucose phosphate isomerase [Zea mays]	62488
	Foxy	65 KDa	Best match	hypothetical protein SORBIDRAFT_01g020010 [Sorghum bicolor]	61322
			2nd best match	Os08g0478800 [Oryza sativa (japonica cultivar-group)]	67742
5	Control	55 KDa	Best match	enolase1 [Zea mays]	48262
			2nd best match	RecName: Full=Catalase isozyme 3	57159
	Foxy	55 KDa	Best match	enolase1 [Zea mays]	48262
			2nd best match	unknown [Zea mays]	53884
6	Control	51 KDa	Best match	allene oxide synthase [Zea mays]	53257
			2nd best match	UDP-glucose pyrophosphorylase [Saccharum officinarum]	52431
	Foxy	51 KDa	Best match	RecName: Full=UTP--glucose-1-phosphate uridylyltransferase;	51632
			Second best match	UDP-glucose pyrophosphorylase [Saccharum officinarum]	52431
7	Control	46 KDa	Best match	enolase1 [Zea mays]	48262
			2nd best match	transaldolase 2 [Zea mays]	46274

	Foxy	46 KDa	Best match	NADPH producing dehydrogenase of the oxidative pentose phosphate pathway [Zea mays]	53307
			2nd best match	enolase1 [Zea mays]	48262
8	Control	42 KDa	Best match	unknown [Zea mays]	43016
			2nd best match	Os01g0654500 [Oryza sativa (japonica cultivar-group)]	46356
	Foxy	42 KDa	Best match	unknown [Zea mays]	43016
			2nd best match	Os01g0654500 [Oryza sativa (japonica cultivar-group)]	46356
9	Control	35 KDa	Best match	RecName: Full=Glyceraldehyde-3-phosphate dehydrogenase, cytosolic	36605
			2nd best match	cytosolic glyceroldehyde-3-phosphate dehydrogenase GAPC2 [Zea mays]	36633
	Foxy	35 KDa	Best match	RecName: Full=Glyceraldehyde-3-phosphate dehydrogenase, cytosolic	36605
			2nd best match	cytosolic glyceroldehyde-3-phosphate dehydrogenase GAPC2 [Zea mays]	36633
10	Control	29 KDa	Best match	LOC100285226 [Zea mays]	28960
			2nd best match	14-3-3-like protein [Saccharum hybrid cultivar CP65-357]	28978
	Foxy	29 KDa	Best match	14-3-3-like protein [Saccharum hybrid cultivar CP65-357]	28978
			2nd best match	LOC100285226 [Zea mays]	28960

Appendix A.4. Summary of *Striga hermonthica* shoot analysis - DIGE



Reference image for spots on 2D gel from the analysis of *Striga hermonthica* Foxy 2 infected and non-infected shoots – experiment carried out in 3 replicates.

Table A.4. DIGE: Identification of spots on the gel from the analysis of S. hermonthica shoots.

SN	Protein name in plant	Function (activity affected)	Biological process	Effect (fold)	Anova (p)	Average Normalised Volumes	
						Striga Foxy	*Striga* Control
1	**ATP synthase subunit beta, mitochondrial precursor**	Mitochondrial membrane ATP synthase	ATP synthesis coupled proton transport	Up regulated in Foxy 2 infected *Striga* (2.0)	0.002	2.058	1.016
2	**Heat shock cognate 70 kDa protein 2**	ATP-binding Nucleotide-binding	response to stress	Up regulated in Foxy 2 infected *Striga* (2.7)	0.003	2.272	0.846
3	**Os05g0565200 [Oryza sativa (japonica cultivar-**	ATP binding	nickel ion binding	Up regulated in Foxy 2 infected *Striga* (2.0)	0.003	2.268	1.112

	group)						
4	**Fructose-bisphosphate aldolase**	- fructose-bisphosphate aldolase activity - zinc ion binding	glycolysis	Up regulated in Foxy 2 infected *Striga* (2.3)	0.007	2.097	0.916
5	**Luminal-binding protein 5**	endoplasmic reticulum lumen	ATP binding	Up regulated in Foxy 2 infected *Striga* (2.2)	9.490e-004	3.143	0.541
6	**Probable phospholipid hydroperoxide glutathione peroxidase (PHGPx)**	- Stress response -Oxidoreductase Peroxidase -glutathione peroxidase activity -phospholipid-hydroperoxide glutathione peroxidase activity	- oxidation reduction - response to oxidative stress	Up regulated in Foxy 2 infected *Striga* (3.5)	0.008	2.863	0.824
7	**Hsc70 [Solanum lycopersicum]**	ATP-binding RuleBase RU003322V2 Nucleotide-binding	ATP-binding RuleBase RU003322V2 Nucleotide-binding	Up regulated in Foxy 2 infected *Striga* (2.1)	0.010	2.069	0.966
8	**Caffeic acid 3-O-methyltransferase**	Catalyzes conversion of caffeic acid to ferulic acid and of 5-hydroxyferulic acid to sinapic acid - products may be incorporated into lignins.	lignin biosynthetic process	down regulated in Foxy 2 infected *Striga* (2.4)	0.016	0.710	1.694
9	**Tubulin beta-5 chain**	GTP binding structural molecule activity	- microtubule-based movement -protein polymerization	Down regulated in Foxy 2 infected *Striga* (3.9)	0.003	0.468	1.813
10	**Beta-tubulin [Zinnia elegans]**	- Microtubule RuleBase RU000352V1 -microtubule-based movement	microtubule-based movement	down regulated in Foxy 2 infected *Striga* (4.5)	0.027	0.379	1.706

Presentations in Seminars and Conferences within the scope of the Ph.D. study

Date	Conference Theme	Organizers	Title of presentation
7th – 9th Oct 2008	Tropentag (Competition for resources in a changing World – New drive for Rural Development)	The Universities of Berlin, Göttingen, Hohenheim, Bonn and Kassel-Witzenhausen held in Hohenheim	Action of the mycoherbicide *Fusarium oxysporum* f. sp. strigae 'Foxy 2' on *Striga hermonthica*: an anatomical study *Available at:* http://www.tropentag.de/2008/proceedings/node232.html
26th – 27th March 2009	Tagung der DPG - Arbeitskreise Mykologie und Wirt-Parasit-Beziehungen'	Deutsche Phytomedizin held in the Technischen Universität Kaiserslautern.	*Fusarium oxysporum* f.sp *strigae*: Pathogenic to the parasite *Striga* and Non-pathogenic to its host sorghum? *Available at:* http://www.phytomedizin.org/show_abstracts.html
8th – 12th June 2009	10th World Congress on Parasitic plants	International Parasitic Plant society (IPPS) held in Kusadasi, Turkey	Colonisation of *Striga hermonthica* and its host sorghum by the mycoherbicide *Fusarium oxysporum* f.sp. *strigae* and its implication for *Striga* control *Available at:* http://parasiticplants.org/docs/IPPS_10th_Congress_Abstracts_Kusadasi_Turkey.pdf
6th – 10th October 2009	Tropentag - Conference on Biophysical and Socio-economic Frame Conditions for the Sustainable Management of Natural Resources	The Universities of Berlin, Göttingen, Hohenheim, Bonn and Kassel-Witzenhausen held in Hohenheim	Interactions between the Mycoherbicide *Fusarium oxysporum* F. sp. *strigae* and Sorghum Roots *Available at:* http://www.tropentag.de/2009/abstracts/links/Beninweck__Endah_b4vBvEaF.php
25th – 26th March 2010	Tagung der DPG - Arbeitskreise Mykologie und Wirt-Parasit-Beziehungen'	Deutsche Phytomedizin held in Universität Konstanz, Germany	Infection of the parasitic weed *Striga hermonthica* by *Fusarium oxysporum* f.sp. *strigae*: an anatomical study *Available at:* http://dpg.phytomedizin.org/aktuell_detail.html