CROP PROTECTION PROGRAMME

Epidemiology and variability of *Gibberella xylarioides*, the coffee wilt pathogen

R8188 (ZA0505)

FINAL TECHNICAL REPORT

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1. Executive Summary

Coffee wilt disease (CWD), caused by *Gibberella xylarioides(Fusarium xylarioides)* causes major damage to *Coffea canephora* grown by smallholder farmers in the Democratic Republic of Congo and Uganda and threatens the crop in Rwanda and Tanzania; it is also present on *Coffea arabica* in Ethiopia and causes substantial losses in some areas. This project provided an important research element that is directly linked with a larger regional coffee rehabilitation and disease management programme (the Regional Coffee Wilt programme, RCWP), led by the Common Fund for Commodities (CFC). Associated funding for complementary scientific studies was also made available under the European Union (EU) INCO-DEV programme. In planning and undertaking this research, project scientists have collaborated closely with partners involved in other components of the RCWP, and specifically INCO-DEV project ICA4-CT-2001-10006 ('Development of a long-term strategy based on genetic resistance and agro-ecological approaches against coffee wilt disease'), coordinated by CIRAD. By taking this approach a more comprehensive, regional perspective of CWD and prospects for future management of the disease was gained, and this report is presented in this context.

The ultimate aim of the project was to provide new knowledge on the variability and epidemiology of the CWD pathogen, about which very little was previously known. Through acquisition of a comprehensive collection of *F. xylarioides* isolates from CWD affected areas in Ethiopia, Tanzania, Uganda and other parts of Africa, application of a broad range of approaches to study genetic and pathogenic variability and initiation of a range of relatively simple on-farm and on-station field studies, this aim has been achieved. The project research has revealed, for example, that very little genetic variability exists within the CWD pathogen and suggests that two populations, possibly clonal, are currently responsible for the disease in these countries. Host specificity would also appear to be operating within the pathogen, in that isolates obtained since re-emergence of the disease on *C. canephora* are pathogenic either to *C. arabica* or to *C. canephora*, but not to both coffee species, and clearly relates to the two genetic groups. Given the occurrence of the two coffee species within Eastern Africa, the two variants are also therefore geographically separated. Field studies, while still in their infancy, have helped to clarify the mechanisms of survival and transmission of the CWD pathogen in confirming that, for example, the disease can be spread from plant to plant through use of a machete. However, no evidence was acquired to indicate that the pathogen survives on plants other than coffee, or that insects are involved in disease transmission. The various findings have major implications with regard to future management of CWD through cultural approaches, by providing information of relevance to the current search for durable resistance in particular.

Unfortunately, problems experienced with regard to finalisation of formal contractual agreements between CABI Bioscience and project collaborators in Ethiopia (EARO), Tanzania (TaCRI) and Uganda (NARO) led to considerable delays in initiation of in-country activities. Such delays were not unique to project R8188 but were experienced within the RCWP as a whole where, for example, delays were incurred in delivery of vehicles to NARS (and would also have an indirect effect on this project). Despite the provision by CPP of a five-month, no cost extension to the project, some key areas of research (including in-country pathogenicity screening and field trials) could not be initiated or completed as originally planned. Nevertheless, a considerable amount of research has been undertaken and the findings of the research will be taken up, through pathways including RCWP, NARS, agriculture ministries and regional networks such as ASARECA, for further validation and to help develop and strengthen efforts to contain spread of the disease and to minimise further damage. The new knowledge generated by the project will enhance the capacity of those with a vested interest in coffee production to counteract the serious impact of CWD experienced to date and therefore protect coffee production as their main source of revenue. It will ultimately help to improve the livelihood of millions of resource-poor farmers, traders, processors, exporters and other stakeholders across Africa whose very existence depends on coffee as a commodity crop.

The research components from this project, the related INCO-DEV project and indeed the RCWP as a whole are essential to provide knowledge for formulating strategies to help control the disease that may be validated and promoted in the target countries by the broader research programme memorandum. Outputs of R8188 have already contributed to the efforts of the RCWP and the various stakeholder groups during the course of the project and will continue to be taken up for on-farm validation, with impact at farm level expected to become apparent within the next five years.

2. Background

Coffee is currently Uganda's premier export, accounting for up to 55 % of the countries foreign exchange. The country is the second largest producer of coffee in Africa after Ivory Coast and the industry is worth about US$350 million. However, currently 70% of the coffee growing areas are affected by CWD and production has declined from 4.4 million bags in 96-97 to 3.6 million bags in 97-98 (latest data available). The coffee industry in Uganda employs more than 2.5 million people. The Ugandan Coffee Developmental Authority (UCDA) has estimated that the disease is causing a financial loss per coffee growing household of approx. 63US$ compared to a per capita income in Uganda of 190 US$ (World Bank, 1996). Hence a significant proportion of income is being lost to the country due to the disease. In Ethiopia, losses due to CWD are patchy and range from 44% in Gera and 46% in Jimma to 61% in Bebeka, although some small holders have suffered 100% loss (Girma, 1997).

CWD, also known as tracheomycosis or vascular wilt of coffee, has been known for over 70 years, having first been observed in the Central African Republic (CAR) in 1927 on *Coffea excelsa* (Figueres, 1940). Since then, CWD has spread to many parts of West, Central and East Africa to become the most serious disease of *C. excelsa*, *C. arabica* and *C. canephora*, with *F. xylarioides* (teleomorph *Gibberella xylariodes*) being confirmed as the causal fungus. Field resistance or tolerance of coffee to CWD has been observed in several instances, with resistance being deployed as a component of successful CWD eradication programmes in some areas, including Ivory Coast (Saccas,1956; Meiffren,1961). However, reports of variation in field resistance of particular coffee species or clones, involving species showing resistance in some areas but being completely susceptible in others, has led to speculation that pathogenic variability may exist within the pathogen and may be responsible (Meiffren, 1961). Although CWD declined from the late 1950s and became considered as a minor disease on *C. canephora*, the disease began to manifest itself again in parts of Central Africa in the 1970s, and was widespread on *C. canephora* in DRC by 1992 (Flood, 1996) and in Uganda by 1996 Lukwago & Birikunzira, 1997). Currently, CWD does not appear to affect *C. arabica* in Uganda nor in DRC, but continues to be a problem on *C. arabica* where *C. canephora* is unaffected. In Ethiopia the risk to *C. arabica* posed by the latest epidemic needs to be assessed. In Tanzania symptoms similar to those of CWD have been reported on *C. canephora* coffee trees in the Kagera Region (G.Oduor, pers comm). Similarly, CWD-like symptoms have been reported in Rwanda. If the disease has indeed spread to these areas from Uganda or elsewhere, the etiology, extent and severity of the outbreaks needs to determined and efforts to limit further spread put in place.

Lack of knowledge of CWD generally, and the biology of the fungal pathogen in particular, is a serious limitation to the development of rational and sustainable options for disease control. As a consequence, national coffee institutions in the target countries are not in a position to offer adequate, reliable advice to farmers and other coffee stakeholders on possible control options. Increased knowledge of the underlying causes of the recent upsurge in CWD and how it spreads will enable current measures to be redefined and new measures formulated to contain further development and spread of the problem across Uganda and into countries, such as Rwanda, currently unaffected by the disease. Additional knowledge of variability within the pathogen, in particular the relationship between strains attacking *C. arabica* and *C. canephora*, needs to be determined to again help explain why the disease has re-emerged but

also to assess the threat to coffee producers across the region and to assist the development and selection, by growers, of durable resistance to the disease. This, coupled with knowledge of, for example, how the pathogen is transmitted, how and for long it survives and how it infects its host will also enable specific control options such as the application of sanitary measures and implementation of appropriate quarantine restrictions.

Following the recent re-emergence of CWD firstly in DRC and subsequently in Uganda, official delegations to the ICO requested that a programme to alleviate the problem be formulated and submitted for international funding. In November 1996, an extraordinary assembly of the Inter African Coffee Organization (IACO) adopted a resolution expressing concern about the spread of and devastation caused by CWD in the DRC and they recommended that a workshop be held in the region to draw up strategies to contain the disease. A regional workshop was held in Uganda in July 1997 under the co-ordination of COREC (now the Coffee Research Institute, CORI). The workshop was funded by the EU and was attended by more than 60 participants from fifteen African countries plus International Organizations such as ICO, IACO, CABI, ASIC and CIRAD. Participants agreed a draft work programme and CABI was charged with formulating a project proposal for submission to donors and was also mandated to act as Project Executing Agency and CIRAD as Assisting Agency. The project proposal formed the basis of the larger regional programme submitted for funding by CFC and the EU which has been endorsed by country delegations to ICO and approved for multilateral funding by CFC. Subsequently, the coffee research network (CORNET) of ASARECA gave the topic the highest level of priority at a coffee sector stakeholders meeting in Nairobi (May 2000). CABI Bioscience was requested to investigate relevant aspects of the biology of the pathogen, and to clarify the mechanisms behind the recent resurgence of the disease and provide information on the variability and field behaviour of the pathogen necessary to devise rational control strategies.

3. Project Purpose

The project research will enable strategies to be developed to help reduce the impact of CWD and to stabilise productivity of coffee grown by smallholder farmers in the target countries and important to the livelihood security of poor people in Forest Agriculture systems. New knowledge generated by the project will form an integral element of the broader RCWP funded by CFC and EU aimed at validating and promoting effective and sustainable management of CWD. This project will underpin the larger, field-orientated, regional programme. Specifically, acquiring knowledge of pathogen variability and of the methods of spread and infection of CWD will enable the identification of cultural control suitable for validation by farmers, contribute to the search for durable resistance and assist in the overall efforts to contain the disease.

4. Research Activities

4.1 Collect samples from CWD affected coffee trees and establish a collection of isolates of *F. xylarioides* and other *Fusarium* species

During the project, several visits were made by counterparts from the Tanzania Coffee Research Institute (TaCRI), Moshi, Tanzania and Jimma Agricultural Research Center (JARC), Addis Ababa, Ethiopia, led by Deusdedit Kilambo and Million Abebe respectively, to the primary coffee growing areas of Tanzania and Ethiopia. Where CWD was encountered, plant material was collected from affected trees (Appendix 2, Plates 1-5) and returned to the laboratory for isolation and purification (see below) of fusaria (Appendix 2, Plate 6). Wood pieces, exhibiting blue/black discoloration immediately below the bark which is typical of infection by *F. xylarioides* were usually obtained for this purpose (Appendix 2, Plate 7). Where possible, isolations were made in-country, an original, purified culture being retained

for in-country activities[1] and a subculture being sent to CABI UKC for confirmation of identity to species level and for use in research activities undertaken at CABI. In some instances, the plant sample was forwarded directly to the UK for isolation of fusaria. Where available, isolates were also obtained from collections held elsewhere and from the CABI Genetic Resources Collection (GRC). These, together with isolates of *F. xylarioides* and other fusaria obtained from CWD affected regions in Africa through EU INCO-DEV project ICA4-CT-2001-10006, were maintained at CABI UKC and their variability investigated using a range of morphological and genetic methodologies. A number of fusarium isolates were also collected from a farm in Uganda selected as a CWD mapping site (see Section 4.4).

Unless otherwise stated, fungal isolations were made from coffee plant material by removal and rehydration (if the samples are exceptionally dry, as in the case of wood) of root, shoot or wood pieces in sterile distilled water for 3-5 mins. These were then surface sterilised by immersion in sodium hypochlorite solution (1.4% a.i.) for 1-2 mins, rinsed several times in sterile distilled water and 1-4 small pieces of material placed on tap water agar (TWA) medium (Booth, 1971). Agar plates were incubated at 25^{o}C (under daylight fluorescent light tubes) for several days and emerging fungal colonies aseptically transferred to synthetic low nutrient agar (SNA, Nirenberg, 1976) medium and cultured at 25^{o}C. Cultures were single-spored by removing fungal material from an SNA culture in a loopful of sterile distilled water and streaking the loop across a TWA plate. Plates were incubated at 25^{o}C for 24-36 hours and, with the aid of a binocular microscope, a single spore with emergent germ tube removed on an agar block (using a scalpel) and transferred to fresh SNA medium. The block was examined under a high power microscope at x 40 magnification to ensure that a single spore had been removed and the SNA plates incubated at 25^{o}C. For the purposes of identification of fusaria to species level, purified isolates were grown at 25^{o}C on SNA and potato sucrose agar (PSA, Booth, 1971) medium; fusaria usually producing pigmentation characteristic of particular species on the latter. For isolation of fusaria from soil, a few grams of air-dried soil were sprinkled onto TWA or SNA agar plates which were then incubated at 25^{o}C. Fungal colonies emerging from soil particles were subcultured onto fresh SNA medium for purification and/or identification. Where bacterial contamination was considered problematic, TWA medium was amended with either 150 mg/L streptomycin sulphate (when isolating from stem pieces, roots and twigs) or 300 mg/L (when isolating from soil) to inhibit bacterial growth.

All isolates retained at CABI UKC were purified either by overseas counterparts prior to donating to the Centre or by CABI UKC scientists on receipt. At CABI, the identity[2] of the majority of isolates (and of all isolates characterised by the various approaches) was determined or confirmed to species level in the first instance by Dr Mike Rutherford. Where difficulties arose, the isolate was subsequently examined by Dr Paul Cannon (mycologist).

Sixty-one isolates representative of geographic origin, host species and other attributes were selected from the collection established at CABI UKC for in-depth characterisation (Table 1). These comprised isolates of the various *Fusarium* species (the majority being *F. xylarioides*) obtained from *C. canephora*, *C. arabica* and *C. excelsa* trees cultivated in differing countries and localities, isolates obtained during the current CWD outbreak in Eastern and Central Africa (i.e. since the late 1970s) and a small number of *F. xylarioides* isolates obtained from coffee during the earlier wilt outbreak in Western Africa in the 1950s/1960s. The latter will hereafter be referred to as 'historical isolates'.

Isolates were maintained at 5^{o}C on SNA over the short to medium term and prior to deposition in the CABI UKC Genetic Resources Collection (GRC, see below). Subcultures

[1] The reintroduction of plant and fungal material to the country of origin following removal from Ethiopia, Tanzania, Uganda or DRC is prohibited.

[2] Identifications based on morphological characteristics as described by Booth, C. (1971)

prepared from SNA slopes were maintained at 25^{o}C on SNA plates while undergoing morphological and genetic investigation. The majority of the representative isolates were

deposited in the CABI GRC under liquid nitrogen and also in a freeze-dried state for secure, long-term storage. The remaining isolates will be deposited in early 2005. As standard practice, the recovery of individual isolates will be periodically assessed by GRC staff as a means of determining fungal viability over time.

Table 1. *Fusarium* isolates obtained from coffee and selected for morphological and genetic characterisation at CABI UKC (i'e' 'representative isolates)

Species[1]	Known isolate accession no.s				Host species or substrate	Country of origin	Locality	Date isolated or received
F. xylarioides	IMI204746		CAB009		*Coffea* sp.	Ethiopia		1976
F. xylarioides	IMI375907	Gx26			*Coffea arabica*	Ethiopia	Teppi	1997
F. xylarioides	IMI375908	Gx31	CAB007		*Coffea arabica*	Ethiopia		1997
F. xylarioides	IMI375909	Gx43			*Coffea arabica*	Ethiopia	Agaro Gera Res. Subcentre	1997
F. xylarioides	IMI375916	RIGx43	CAB008		*Coffea arabica*	Ethiopia		1997
F. xylarioides	IMI369711	W5106a	CAB013		*Coffea canephora*	DRC	Rutchuru area, North Kivu	1995
F. xylarioides	IMI392246	W5263c	CAB014	B	*Coffea canephora*	DRC	Umbru plantation, Mandimbo	1996
F. xylarioides	IMI392247	W5267a	CAB015	C	*Coffea canephora*	DRC	Apoyo village	1996
F. xylarioides	IMI392248	W5272b	CAB016	E	*Coffea canephora*	DRC	Nzuki plantation, Capaco	1996
F. xylarioides	IMI392249	W5280b	CAB017	A	*Coffea canephora*	DRC	Seeurs plantation, Orantes	1996
F. xylarioides	IMI392250	W5432a			*Coffea canephora*	Uganda	Milambi village	1997
F. xylarioides	IMI392251	W5433a	CAB001		*Coffea canephora*	Uganda	?	1997
F. xylarioides	IMI392252	W5440a			*Coffea canephora*	Uganda	Kamirundi village	1997
F. xylarioides	IMI392253	W5448a			*Coffea canephora*	Uganda	Wabilongo village, Mukon District	1997
F. xylarioides	IMI392254	W5543a			*Coffea canephora*	Uganda	Mpigi District	1997
F. xylarioides	IMI392255	W5543b			*Coffea canephora*	Uganda	Mpigi District	1997
F. xylarioides	IMI392256	W5543c			*Coffea canephora*	Uganda	Mpigi District	1997

F. xylarioides	IMI392257	W5543e			*Coffea canephora*	Uganda	Mpigi District	1997
F. xylarioides	IMI392258	W5554a			*Coffea canephora*	Uganda	Mubende District	1997
Suspect *F. xylarioides*	IMI392259	H1	S11		*Coffea canephora*	Uganda	Kakuk-uulu	
F. xylarioides	IMI392260	R	47		*Coffea canephora*	Uganda	Katoma	
F. xylarioides	IMI392261	W	24		*Coffea canephora*	Uganda	Kasawo	
F. xylarioides	IMI389563	W7276c			*Coffea arabica*	Ethiopia	Karo Mariam	
F. xylarioides	IMI389567	W7279a			*Coffea arabica*	Ethiopia	Tsanu	
F. xylarioides	IMI389571	W7283a			*Coffea arabica*	Ethiopia	Wesheka	
F. stilboides	IMI389578	W7277a	CAB011		*Coffea arabica*	Ethiopia	Gumero Abo	
F. lateritium	IMI389579	W7284a	CAB012		*Coffea arabica*	Ethiopia	(Kebele) Kosa	
F. stilboides	IMI389580	W7291a			*Coffea arabica*	Ethiopia	(Kebele) Jato	
F. oxysporum	IMI389581	W7291b			*Coffea arabica*	Ethiopia	(Kebele) Jato	
F. stilboides	IMI389582	W7292a			*Coffea arabica*	Ethiopia	Kabo	
F. xylarioides	IMI392263	W 5440a - (16)	CAB 003		*Coffea canephora*	Uganda	Karimundi	11 Oct 2000
F. xylarioides	IMI392264		OUG 008		*Coffea canephora*	Uganda	Mukono	Aug 1997
F. xylarioides	IMI392265	C12	OUG 151		*Coffea excelsa*	Uganda	Clonal trial, CORI, Kituza	Oct-02
F. xylarioides	IMI392681	C12	OUG 152		*Coffea excelsa*	Uganda	Clonal trial, CORI, Kituza	Oct-02
F. xylarioides	IMI392682	C13	OUG 154		*Coffea excelsa*	Uganda	Clonal trial, CORI, Kituza	Oct-02
F. xylarioides	IMI392266	C13	OUG 155		*Coffea excelsa*	Uganda	Clonal trial, CORI, Kituza	Oct-02
F. xylarioides	IMI392267	C14	OUG 157		*Coffea excelsa*	Uganda	Clonal trial, CORI, Kituza	Oct-02
F. xylarioides	IMI392268	MUCL 14186	RDC 002		*Coffea canephora*	DRC	Isiro 1992	

Suspect *F. xylarioides*	IMI392269		RDC 004		*Coffea canephora*	DRC	Oicha -Ngadi	Sep-02
Suspect *F. xylarioides*	IMI392270		RDC 051		*Coffea canephora*	DRC	Mutwanga - Bulongo	Sep-02
Suspect *F. xylarioides*	IMI392271	IMU 0101	RDC 068		*Coffea canephora*	DRC	Isiro (Mungbere)	Dec-02
F. xylarioides	IMI392272	MUCL 44508	FYMY access no. SR 20/10 SCO1					
F. xylarioides	IMI392273	MUCL 44543	FYMY access no. SR 20/(SS)19		*Coffea canephora*	DRC	Butembo region, Itendi sector	12/09/2002
F. xylarioides	IMI392274	W7477b			*Coffea canephora*	Tanzania	Bukoba (Bugabo)	Feb 2003
F. xylarioides	IMI392275	W7489a			*Coffea canephora*	Tanzania	Muleba (Kamachumu)	Feb 2003
F. xylarioides	IMI392276	W7494a			*Coffea canephora*	Tanzania	Karagwe (Kaisho/Murongo, Kaisho – Karakwanzu)	Feb 2003
F. xylarioides	IMI392277	W7498a			*Coffea canephora*	Tanzania	Karagwe (Kaisho/Murongo, Kaisho - Ruwensiga)	Feb 2003
F. xylarioides	IMI392278	W7500b			*Coffea canephora*	Tanzania	Karagwe (Kituntu-Mabira)	Feb 2003
F. xylarioides	IMI392279		TZ002			Tanzania	Karagwe (Kaisho/Murongo, Kaisho - Ruwensiga)	Feb 2003
F. solani	IMI392280	NRLL 25804			*Coffea canephora*	Guinea	Unknown	Mar-03
F. stilboides	IMI392281	NRLL 13277	FRC L101		*Coffea canephora*	Guinea	Unknown	Mar-03
F. lateritium	IMI392282	NRLL 13275	FRC L84		Coffee berry	Guinea	Unknown	Mar-03
Suspect *F. xylarioides*	IMI127629 Also IMI 127629	DSMZ 62457	FUS001	ATCC 36326.	*Coffea excelsa*	Central Africa Republic	Unknown	1955
Suspect *F. xylarioides*	IMI392674	CBS25852	FUS002		*Coffea sp.*	Ivory Coast	Unknown	1951
Suspect *F. xylarioides*	IMI392675	CBS749.79	FUS003		*Coffea canephora*	Guinea	Unknown	1963

Suspect *F. xylarioides*	IMI392676	ATCC15664	FUS004			Unknown	Unknown	1960s
Suspect *F.solani*	IMI392677	ATCC36325	FUS005		*Coffea excelsa*	French E Africa		1960s
F. xylarioides	IMI392678		TZ008			Tanzania	Karagwe (Kaisho/Murongo, Kaisho – Karakwanzu)	2003
F. xylarioides	IMI392679		TZ009			Tanzania	Karagwe (Kaisho/Murongo, Kaisho – Karakwanzu)	2003
F. xylarioides	IMI392680	G3P22						

1 - as confirmed by project scientists or CABI mycologist. Where the identity has not been confirmed, a 'suspect' species based on the designation provided by the donor

4.2 Evaluate methodologies for assessing genetic variability and apply selected methodologies to determine variability of the CWD pathogen in Tanzania and Ethiopia.
Genetic diversity within the representative fusarium isolates (*F. xylarioides, F. solani, F. stilboides, F. lateritium* and *F. oxysporum*) was evaluated using molecular techniques. This was a comprehensive study employing a range of primarily PCR-based approaches to investigate genetic variation at and below species level in order to (i) identify any population structure existing within the CWD pathogen (ii) demarcate individual *F. xylarioides* isolates and (iii) discriminate between isolates of *F. xylarioides* and other fusaria found on coffee.

A block of agar approx. 1 cm^3 and bearing fungal mycelium was removed from an SNA plate on which a fusarium isolate was growing. The block was chopped into small pieces, placed in a conical flask containing 60 ml liquid glucose yeast medium (GYM, Mugnai *et al.*, 1989) and incubated for five days at 27°C to stimulate fungal growth. Fungal mycelium was harvested by vacuum filtration and freeze-dried. The dried mycelium was then disrupted using a pestle and mortar and total DNA extracted as described by Cubero *et al*. (1999) for analysis and stored in a deep freeze at –20°C until required. PCR-based non-specific and targeted techniques were evaluated for their ability to amplify the DNA at loci scattered across the fungal genome and to provide an insight into intraspecific genetic variation. These techniques included anchored inter-simple-sequence-repeat (ISSR) analysis (Grünig *et al*., 2001), single-enzyme, agarose gel-based amplified fragment length polymorphism (AFLP) analysis (Mueller *et al*., 1996; Vos *et al*., 1995), sequencer-based AFLP analysis, amplification and enzymatic digestion of the IGS region (Appel & Gordon, 1995) and analysis of A+T-rich DNA (i.e. presumptive mtDNA RFLPs (Freeman *et al.*, 1993; Marriott *et al*, 1984; Typas *et al*., 1992; Varga *et al*., 1994). These approaches enabled a study not only of the entire fungal genome, but also of specific regions of the genome (e.g. ribosomal intergenic spacer [IGS] region) and of DNA located in different parts of the fungal cells (e.g mitochondria).

Although not a molecular approach, the project also investigated the possible existence of vegetative compatibility groups (VCG) within the CWD pathogen as a measure of genetic variability. VCG have been used to identify and discriminate genetic populations within a broad range of *Fusarium* species, including *F. oxysporum* where more than 20 VCG have now been identified. In some instances (e.g. *F. oxysporum* f. sp. *cubense*) strong correlation has been found between VCG and other genetic markers, including mitochondrial and RFLP profiles. This is indicative of the existence of clonally reproducing populations that may, for example, have become geographically isolated or have a strong relationship with a specific host. VCG determination involves the generation and pairing of nitrate non-utilising mutants. Where potentially compatible mutants complement one another for nitrate utilisation, they are considered to share identical alleles at loci governing incompatibility and therefore to be vegetatively compatible (Leslie, 1993). The existence of VCG within 16 selected isolates was evaluated as described by Correll *et al*. (1987).

4.3 Evaluate pathogenic variability of *F. xylarioides* isolates against a range of coffee germplasm.
As part of the project research, Dr Girma Adugna Senbata[3] (Jimma Agricultural Research Center, Addis Ababa, Ethiopia) undertook a six-month research attachment at CIRAD, Montpellier, France, under the supervision of Dr Daniel Biyesse. The objective of Dr Adugna's work was to investigate variation in pathogenicity of *F. xylarioides* isolates obtained from various *Coffea* species and lines cultivated in different regions of East and

[3] Dr Adugna was also pursuing a PhD thesis at the Institute for Plant Diseases, University of Bonn, Bonn, Germany, under the supervision of Professor H. Hindorf. The thesis was successfully defended in July 2004 and when Dr Adugna returned to Ethiopia to continue research on coffee as a member of staff at JARC.

Central Africa. *F. xylarioides* isolates selected for this work were obtained from C. *arabica*, *C. canephora* and *C. excelsa* trees in Ethiopia, Uganda, Tanzania, Central African Republic (CAR) and DRC and were representative of most regions where CWD is a serious constraint to coffee production. *C. arabica*, *C. canephora* and *Coffea liberica* germplasm was obtained from Ethiopia, DRC, Ivory Coast, Kenya and Costa Rica for inoculation with the pathogen (Table 2).

Three separate trials were established in which coffee seedlings, grown in a 1:2 humin substrate (Neuhaus): vermiculite mix under glasshouse conditions, were inoculated by stem injection with a spore suspension (2×10^6 spores per ml) of *F. xylarioides*. Between 25 and 30 seedlings of each coffee cultivar/line were inoculated with each *F. xylarioides* isolate at fully expanded the cotyledonary leaf stage, and between three and five seedlings injected with sterile distilled water as a control. Treated seedlings were maintained at 25^{o}C and 70-80% RH in a growth chamber under a 12 hr light/dark cycle. Seedlings were assessed at two week intervals for the development of external symptoms of CWD for up to six months, the number of affected plants being recorded and expressed as a percentage of the total number inoculated. For further details of this study refer to Appendix 1).

Table 2. *Coffea* species and lines and *F. xylarioides* isolates used in pathogenicity testing at CIRAD

Trial no.	*Coffea* species			*F. xylarioides* isolates		
	Coffea spp.	Country of origin	CIRAD ref. code	Isolate ref. no.	Host species	Country of origin
I	*C. arabica*	Kenya	1686 (K7)	G3P22	*C. arabica*	Ethiopia
			1688 (SL)	CAB003	*C. canephora*	Uganda
	C. canephora	DRC	1650	TZ009	*C. canephora*	Tanzania
			1653	DSMZ	C. *excelsa* (old type)	CAR
		Ivory Coast	1655			
			1673			
II	*C. arabica*	Ethiopia	1689	G3P22	*C. arabica*	Ethiopia
			1690	CAB003	*C. canephora*	Uganda
				TZ008	*C. canephora*	Tanzania
	C. canephora	Ivory Coast	1669	RDC002	*C. canephora*	DR Congo
			1670	OUG152	*C.excelsa*	Uganda
III	*C. arabica*	Costa Rica	1716	G3P22	*C. arabica*	Ethiopia
			1743	CAB003	*C. canephora*	Uganda
	C. canephora	DRC	1620	DSMZ 62457	C. *excelsa* (old type)	CAR
			1626			
	C. liberica	Costa Rica	1722			
			1723			

4.4 Collect isolates from specific field sites in Uganda and use molecular markers to characterise pathogen populations and identify individuals in the field.

As described more fully in Section 4.5.4, and as part of part of linked project ICA4-CT-2001-10006, the development of CWD has been monitored at selected field sites in Uganda with a view to generate maps of disease spread spatially and over time.

During two visits to one of these sites, a farm located in Kavule village, Mayuge, owned and managed by Mr Samuel Kirunda (Appendix 2, Plate 8), in December 2003 and May 2004 wood samples were collected from CWD-affected trees. *F. xylarioides* was isolated from the wood and purified as described in Section 4.1. DNA was extracted from each isolate and molecular analysis undertaken based on the approaches described in Section 4.2. The aim of this work was to ascertain if any genetic variability existed within the pathogen at the site and, if so, to try to identify molecular markers that would be suitable for tracking the movement of *F. xylarioides* populations or individual *F. xylarioides* isolates. To facilitate this, and where possible, wood pieces were obtained during the second visit from affected coffee trees that had not exhibited symptoms on the first visit but were adjacent to trees that were affected, and from which wood was obtained, during the first visit. This information would support the findings of the disease mapping study undertaken at this particular site and also the small-scale trials and associated activities described under Section 4.5.

4.5 Undertake small-scale field experimentation in Uganda to investigate survival and transmission of CWD.

A number of activities were undertaken in partnership with the Coffee Research Institute (CORI) at Kituza, Uganda to provide further information on the survival and transmission of *F. xylarioides* under field conditions, and to investigate possible sources of infection. These were undertaken at the Mayuge on-farm disease-mapping site and at CORI, and involved laboratory, screen house (Appendix 2, Plate 9) and field studies on-station and on-farm. They included small-scale trials to the study possible transmission of *F. xylarioides* by a machete, survival in (and infection from) soil and coffee wood pieces, an investigation of the occurrence of *F. xylarioides* on a number of bean and weed species and the occurrence of *F. xylarioides* on, and possible transmission by, insects commonly associated with coffee.

Note: With respect to activities undertaken in Uganda, the severity of CWD on coffee trees and on coffee seedlings (Appendix 2, Plate 10) was recorded according to the symptoms described in Tables 3 and 4 respectively.

4.5.1 On-farm and on-station trials

In 2004, a number of on-farm and on-station field trials were established with counterparts at CORI, to investigate survival of *F. xylarioides* and possible mechanisms of CWD spread in order to provide new information on which disease management recommendations may be based. All trials were designed to provide at least preliminary data within a few months but may continue well beyond the life of the current project. The trials were also designed to utilize or replicate, as far as possible, on-farm conditions and usual farm management practices, and to determine if changes in management practice may be of benefit in reducing *F. xylarioides* transmission and CWD spread. Four trials were established as described below.

Trial 1. Transmission of F. xylarioides and spread of CWD through use of a machete.

The machete and hoe are the two implements most commonly used by a farmer. In many cases they are the only implements that a farmer may own, yet are used for a wide range of activities including pruning, weeding and digging. They may therefore play an important role in pathogen transmission and disease spread. A study was undertaken at CORI, both in a coffee field and in a screen house, to investigate transmission of *F. xylarioides* inoculum on a machete and subsequent development of CWD symptoms on coffee plants:

Coffee field

A coffee field (Block 38) at CORI was selected that had both mature CWD affected coffee trees and trees considered to be susceptible to the disease but on which no external wilt symptoms had developed. Twelve trees showing no external symptoms of CWD were selected for wounding at three points, the stem base, mid-way along a primary stem and mid-way a length of fresh, green growth at the top of a stem. For each tree, only one wound was made and only at one of the three

points. Six of the trees were wounded, two per wound position, by making several horizontal wounds through the bark to a depth of approx. 5 mm with a machete blade. The blade was cleansed prior to wounding each tree by swabbing with 70% (vol:vol) alcohol and allowing to air dry.

A piece of stem, approx. 1m long and exhibiting clear discoloration of the wood due to CWD throughout it's length, was cut from a tree affected by CWD (grade 5) for use as a potential source of inoculum. Six trees were again wounded as described above but, for each tree, by first making several wounds through the bark of the infected stem piece. As before, the machete blade was cleansed with alcohol and allowed to dry prior to cuts being made in the infected stem piece for the first time and after wounding each tree. In line with standard management practice, treated trees were ring weeded and desuckered. Trees were checked weekly for the development of external symptoms of CWD, visible as described in Table 3, and the date on which these were first observed recorded.

Table 3. Grading and associated external symptoms applied for assessing severity of CWD on coffee trees.

Severity grade	Description
0	Tree missing at start of mapping (due to previous death by CWD or other reasons)
1	No CWD symptoms apparent
2	Leaves curling inwards
3	Dieback and defoliation of 1-24% of tree
4	Dieback and defoliation of 25-50% of tree
5	Dieback and defoliation of 51-75% of tree
6	Dieback and defoliation of 76-100% of tree, or tree dead
D	Tree dead and cut back to stump by farmer

Screen house

Soil was collected from a field site at CORI where coffee has not been grown, steam sterilised and used to fill cylindrical pots measuring 8 cm (diameter) by 16 cm (height). A single, 6-8 month old *C. canephora* coffee seedling[4] was planted in each pot (Appendix 2, Plate 11). In a similar manner to the field trial, five coffee seedlings were wounded by carefully making a single wound on the stem, 5cm from the base, with a machete blade cleansed beforehand with 70% alcohol and allowed to dry. A further five seedlings were wounded in the same way but, for each seedling, by first making cuts through an infected stem piece. The machete was cleansed after wounding each seedling as described above.

Several cuts were then through the infected stem piece with the machete blade, the surface edge of which was then washed with sterile cotton wool and sterile distilled water into a sterile glass beaker (Appendix 2, Plate 12). The blade was cleansed with 70% alcohol, allowed to dry and a second washing made in a similar manner into a second beaker. The volume of washing in each beaker was adjusted to 100 ml with sterile distilled water. Each washing was used to treat another 10 seedlings, five by dipping a sterile scalpel blade into the washing and then making a single cut in the seedling stem, the other five by pouring 20 ml of the remaining wash onto the soil surface around the stem base. A further two 100 ml washings were prepared in a similar manner, but one after making cuts in the infected wood piece and the other after cleansing the machete blade. Five seedlings per washing were uprooted, the roots dipped in the washing for a few seconds and the seedlings replanted. Each remaining washing was applied as a soil drench to a further five seedlings. Several cuts were once again made through the infected stem piece during which

[4] For investigations of CWD on coffee seedlings, 6-8 month old plants bearing 3-4 pairs of fully opened leaves were selected

wood chippings displaced from the stem were gathered and placed in approx. equal quantities on the surface of five seedlings. Five seedlings were planted without any of the above treatments. For a summary of all treatments, see Table 5.

Seedlings were maintained in the screen house at CORI where daily temperatures rise to a maximum of between 22 and 27^0C during the day and fall to between 15 and 18^0C during the night. Relative humidity ranges from 65 to 70%. Plants were watered twice weekly and checked at 2-3 day intervals for the development of external symptoms of CWD. Where symptoms developed (see Table 4), the date on which these were first observed was recorded and fungal isolations made onto TWA medium, as described in Section 4.1, to confirm the presence of *F. xylarioides.* After thorough mixing, a small amount of each washing was also removed aseptically, plated onto TWA medium, incubated at 25^oC and checked for up to seven days for growth of *F. xylarioides*. Isolations were also attempted from sterilised soil to check for the presence of the pathogen.

Table 4. Grading and associated external symptoms applied for assessing severity of CWD on coffee seedlings

Severity grade	Description
0	No CWD symptoms apparent
1	Brown lesions observed on, and/or defoliation of, 1-24% of leaves
2	Brown lesions observed on, and/or defoliation of, 25-50% of leaves
3	Brown lesions observed on, and/or defoliation of, 51-75% of leaves
4	Brown lesions observed on, and/or defoliation of, 76-100% of leaves or plant dead

Table 5. Treatments applied to investigate transmission of CWD through use of a machete.

Method of inoculation	Inoculum source
None ('Control')	None
Stem wound with machete	'Contaminated' machete
	Cleansed machete
Stem wound with scalpel	Scalpel dipped in 'contaminated' machete washing
	Scalpel dipped in cleansed machete washing
Washing used as soil drench	Washing from 'contaminated' machete
	Washing from cleansed machete
Machete washing used as root dip	Washing from 'contaminated' machete
	Washing from cleansed machete
Soil placement	Wood chips from infected stem piece

Trial 2. Survival of F. xylarioides in, and inoculum potential of, coffee wood pieces over time.

Farmers consider coffee wood to be an excellent source of firewood. As such, old or damaged trees, including those that have been killed by CWD, are usually cut down and physically dragged across the farm to an area adjacent to the home. Here they are cut into short pieces of timber and stored for gradual use as firewood. This practice may have obvious implications for the transfer of CWD inoculum to disease free areas of the farm, partly through fallen wood pieces remaining on the ground, and for the establishment of a new inoculum source in the form of a wood stack near the home.

A trial was established in the screen house at CORI, Kituza, to investigate the survival of *F. xylarioides* in coffee wood pieces and the role of wood pieces as a source of CWD. Soil

(sufficient to fill 125 cylindrical pots, each 8 cm in diameter and 16 cm in height) was collected from a field site at CORI where coffee has not been grown and was steam sterilised. Trees exhibiting severe (grade 5, see Table 3) symptoms of CWD were identified from an affected site at CORI as a potential source of inoculum and stem pieces, 2-3 cm in diameter and exhibiting clear discoloration of the wood due to CWD throughout their length, were removed. Stem pieces were cut into 5 cm lengths until 144 wood pieces were available.

On establishment of the trial, all 125 pots were filled with sterilised soil. Ten pots were each planted with a single, 6-8 month old coffee seedling of *C. canephora* variety Is/6, known to be highly susceptible to CWD. A single wood piece was placed horizontally on the surface of the soil of five of the pots planted with seedlings and at a distance of approximately 2 cm from the seedling to ensure that no contact was made with the seedling stem. The remaining five pots were intended as an untreated 'control' throughout the course of the trial, on which CWD should not develop. Wood pieces were also placed in a similar manner in the remaining 115 pots without seedlings. At approximate one month intervals a further five pots with a wood piece were each planted with a seedling. The remaining wood pieces were stored under cover in a CWD-free area and fungal isolations made as described (see Section 4.1) from a single piece of stored wood at each stage at which seedlings were newly planted to check for the presence of *F. xylarioides.*

Seedlings were maintained and checked for development of external symptoms of CWD as described for trial 1 (screen house component. On seedling death, fungal isolations were made from leaves, stem and roots of a few randomly selected plants as described in Section 4.1 to check for the presence of *F. xylarioides* in plant tissues. Should no external symptoms of CWD developed on any seedlings after six months, one seedling per treatment would be removed and fungal isolations made to determine whether *F. xylarioides* is present in the plant tissues.

Trial 3. Survival/ inoculum potential of F. xylarioides in soil over time.

Circumstantial evidence (e.g. the use of susceptible seedlings as bait plants) strongly suggests that the CWD pathogen can survive in soil and act as a source infection to coffee plants for many months. This would appear to be true both for naturally infested field soil or soil artificially inoculated with the pathogen. In an example of the latter, soil obtained from a disease free site, artificially inoculated with *F. xylarioides* and stored in sealed containers under laboratory conditions retained the capacity to cause CWD on planted, susceptible seedlings for many months (G. Hakiza, pers. comm.). However, the fungus retained the ability to infect seedlings for a longer period where the soil had been sterilised prior to inoculation in comparison to soil that was not sterilised.

Information on the ability of *F. xylarioides* to remain viable in soil and retain the ability to infect coffee again has implications with respect to management of CWD. Where coffee plants affected by CWD are removed and destroyed by farmers, for example, it would help to clarify how long land must remain as a fallow or under an alternative crop before susceptible coffee may be replanted. A trial was therefore established in the screen-house at CORI to evaluate the survival/inoculum potential of *F. xylarioides* in field soil over time as described below.

Soil was obtained from three field sites, Block 17 (CORI), Block 36 (CORI) and a forest site (where wild coffee types were growing) for use in the trial. CWD had been prevalent on coffee trees at all three sites for several years at high incidence and severity. A single coffee tree exhibiting severe symptoms of CWD was selected at each site and uprooted and removed. On uprooting, and at approximate monthly intervals following uprooting, soil from the hole was used to fill 20 cylindrical pots (8 cm diameter x 16 cm high). Each pot was planted with four 6-8 month old *C. canephora* coffee seedlings. Seedlings were maintained and assessed as described for trial 2 above, but both the date on which symptoms were first

observed and the date on which plant death occurred were recorded (the period between the two to be used as a measure of infectivity). In addition, and on each occasion that seedlings were planted, a few grams of soil from each site was sprinkled onto TWA medium and checked for growth of *F. xylarioides* as (Section 4.1).

Trial 4. Role of aerial vectors in transmission of F. xylarioides and disease spread.
Given the manner in which CWD symptoms develop and how other vascular wilt fusaria behave, it is suspected that *F. xylarioides* most likely infects coffee trees via the roots or the lower stem, either directly or through wound sites. However, it is also possible that infection may also occur via aerial parts of the plant, including lateral branches, flowers and berries, where a number of possible vectors, including pollinators, may be involved. The following trial was established at the on-farm mapping site at Mayuge to determine whether CWD can develop as a result of infection of the aerial plant parts and, coupled with information obtained from activities described in Section 4.5.2 below, to help clarify the role that insect vectors may have in pathogen transmission.

Soil was obtained from mid-way between coffee trees at the Mayuge site that were moderate-severely affected by CWD, half of which was steam-sterilised at CORI. Twenty 6-8 month old *C. canephora* seedlings were planted in pots (8 cm diameter x 16 cm high), 10 in unsterilised soil (to provide a natural source of CWD inoculum) and 10 in sterilised soil. A ring of *Vaseline* was smeared around the outer rim of each pot to prevent insects and other organisms crawling up the side of the pots. All pots were placed on a raised, wooden platform. Five of the pots containing unsterilised soil and five containing sterilised soil were enclosed in fine netting to prevent insects and other small animals, including birds, gaining access to the seedlings. The remaining 10 plants were left uncovered.

On establishment of the trial, soil was plated onto TWA medium (Section 4.1) to check for the presence of *F. xylarioides.* Seedlings were also assessed for development of CWD symptoms and the date on which they appeared recorded, as described in Section 4.5.1).

4.5.2 Occurrence of *F. xylarioides* on insects associated with coffee.
To help determine what vectors may be involved in transmission of *F. xylarioides*, a range of insect pests, bees and other pollinators and termites were collected from coffee fields at CORI and at Kawanda Agricultural Research Institute (KARI), Uganda, where CWD is prevalent on *C. canephora*. Insect pests and coffee fauna were also collected at field sites in Mukono, Luwero, Mpigi, Bushenyi, Kanungu and Kamwenge districts of Uganda. Fungi present on the surface of the insects were investigated by washing insects in sterile distilled water, the insect removed and the washing from each insect transferred onto a TWA plate amended with 150mg/L streptomycin sulphate. Washed insects were dried carefully by placing on sterile, absorbent paper, aseptically cut into several pieces and also placed onto TWA, one per plate, amended with 150mg/L streptomycin sulphate. Plates were incubated for three days at 25^{o}C and monitored for growth of fungal organisms. Emerging fungal colonies were subcultured onto SNA and PDA agar and identified to genus or species level.

4.5.3 Occurrence of *F. xylarioides* on other plants
It was previously reported that *F. xylarioides* was recovered from surface sterilised roots of the banana cultivar 'Kayinja' (syn. 'Pisang Awak') obtained from two farms, in Mpigi and Kibale Districts of Uganda, where banana was intercropped with coffee (Serani, 2000). Kayinja and other crops are frequently grown alongside coffee, particularly in Central Uganda, and the finding suggests that these may provide an alternate host for the CWD pathogen and therefore have some influence on survival of *F. xylarioides* and disease spread. They may also be of relevance with respect to CWD management where, for example, their cultivation is being considered as an intercrop or rotation crop or to help reduce pathogen inoculum levels in soil following removal of coffee affected by CWD.

During a visit to the Mayuge on-farm mapping site, plants of a number of commonly cultivated bean types (white haricot beans, a local 'black bean', and beans used for drying ('K132') and weed species (Table 6) growing within 1m of coffee trees exhibiting moderate to severe external symptoms of CWD (i.e. severity grades 5-6) were uprooted (Appendix 2, Plates 13 & 14). The plants were taken to the laboratories at CORI, where adhering soil was shaken off and the roots and lower stem washed under running water. Fungal isolations were attempted from the lower stem and roots by cutting root and stem pieces, 0.5-1.0 cm in length, which were placed onto TWA medium (2-3 pieces per plate) both before and after surface sterilisation. Root and stem pieces were surface sterilised by immersing in sodium hypochlorite solution (2% a.i.) for 1-2 minutes, rinsed 2-4 times in sterile distilled water and gently dried by placing on sterile tissue paper. Roots and stems were placed on separate agar plates and the TWA medium amended with 150 mg/L streptomycin sulphate to inhibit bacterial growth. Plates were incubated at 25^{o}C for up to seven days and, where emerging fungal colonies emerged, these were picked off on agar blocks, subcultured onto fresh SNA medium and identified to species level where possible.

Table 6. Weed species and bean types collected from Samuel Kirunda's farm for isolation of *Fusarium* spp from lower stem and roots

Weed species	Common local name	Common English name
Synedrella nodiflora	Makai (Lugandan)	
Solanum nigram	Katukuma (Lugandan)	
Ageratum conzyoides L.		Goat weed
Oxygonum sinuatum	Kafumita bagenda (Lugandan)	
Euphophia heterophylla	Kisandasanda (Lugandan)	
Senecio discifolius		
Bidens pilosa	Sere (Lugandan)	Black jack
Rottboellia cochinchinensis (Graminae)	Lajanawara (Luo)	

4.5.4 Spatial and temporal mapping of CWD spread and relationship with climatic factors

As part of linked project ICA4-CT-2001-10006, the development of CWD at a number of selected field sites at CORI and coffee farms in Mayuge and Iganga districts (Eastern Uganda) and Masaka and Rakai districts (Central Uganda) has been closely monitored by CORI staff since 2002. Visits were made to each site at one or two month intervals and the incidence and severity of CWD on selected coffee trees within a demarcated area recorded. The objective of this work is to use the data so obtained to generate disease distribution maps to help determine the rate and manner in which CWD has spread at these sites, both spatially and temporally, and to speculate as to the underlying mechanisms of spread and how it may be influenced by farm management practices and climatic factors such as rainfall. The sites have been selected based on, for example, their physical characterisitics.

Given the related research activities being undertaken at the Mayuge site in particular as part of project R8188, an opportunity was taken to collate data collected to date from two of these sites, the Mayuge farm and a field site at CORI (Block 36), and to undertake a preliminary analysis of the data in consultation with staff[5] from the University of Reading Statistical Services Centre (URSSC). At Block 36 the development of CWD on four *C. canephora* clones known to be susceptible to CWD has been monitored since 2002. Block 36 was originally planted with 396 seedlings of clones Is/2, Is/3, Is/6 and 258, arranged in 12 rows of 33 plants, each clone planted

[5] Dr Fiona Underwood

as a single row in each of three blocks. Clones were randomly allocated to rows within each block as shown in Figure 1. The number of plants exhibiting external symptoms of CWD was recorded monthly from January 2002, when several plants had already died due to CWD and been removed, to September 2004. The severity of symptoms on each plant was also recorded using the grading provided in Table 3. The data was analysed and used to generate graphical summaries, or disease 'maps', to help determine the rate and manner in which the disease has spread spatially and temporally. The data was also compared with monthly data for rainfall and temperature recorded at CORI over the assessment to period to determine if fluctations in rainfall and temperature have any apparent influece on CWD development.

In a similar manner, the severity of CWD on 130 mature coffee trees growing within a selected area within the Mayuge farm was assessed. At this site the trees were originally planted in such a way that the plants assessed more or less formed 26 rows of five trees, as presented in Figures 8 & 9. Trees were assessed for CWD during the period December 2003 to May 2004 and the data subjected to preliminary analysis, again by primarily producing graphical summaries. At both the Mayuge farm and Block 36, a number of plants were already exhibiting CWD symptoms when assessment commenced or had already been removed due to being killed by the disease.

Figure 1. Layout of CWD mapping site Block 36, showing allocation of *C. canephora* clones into blocks and rows

	Coffee clone	Plant no.																															
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33
Block I	**Is/2**															X																		
	Is/6										X									X														
	Is/3				X									X			X																	X
	258										X			X																				
Block II	**Is/2**																															X		
	Is/3						X	X															X										X	
	258								X	X											X							X						
	Is/6																				X		X									X		
Block III	**Is/2**								X						X																			
	258								X																									
	Is/6					X			X																									
	Is/3		X																															

X =denotes coffee plant absent
Shaded cell denotes coffee plant exhibiting external symptoms of CWD

4.6 Database development

Development of an electronic database was initiated at CABI UKC in 2002 as part of linked EU project ICA4-CT-2001-10006 to maintain background information relating to isolates obtained through both that project and through project R8188. The database was developed using *Excel* software, due to its general availability to partners based in both Europe and Africa and it's relative ease of use, and was gradually expanded during the course of the project as fusaria were isolated from newly acquired coffee material or obtained from other sources. Data input to the database related to: mycological identity; known accession numbers, including those relating to the internationally recognised collections at CABI, the American Type Culture Collection (ATCC), the Centraalbureau voor Schimmelcultures (CBS) and Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microorganisms and Cell Cultures, DSMZ) numbers; geographic origin (e.g. country, locality); host species (e.g. coffee species) or other substrate and tissue from which isolated; date isolated and date received at CABI UKC; and details of donor. Background notes referring to, for example, unusual mycological attributes of an isolate were also input where considered appropriate.

The database was periodically forwarded to project partners during the project to ensure that they were kept aware of fusaria available for the various research activities and of any newly acquired information, and to stimulate and facilitate interaction between research groups.

5. Outputs

Note: To avoid presenting the outputs of this project in isolation and provide the most complete overview of the current situation regarding the CWD pathogen in Africa, some of the following sections report findings and conclusions based on the work undertaken through both this project and related EU INCO-DEV project ICA4-CT-2001-10006.

5.1 Collect samples from CWD affected coffee trees and establish a collection of *F. xylarioides* isolates

A large number of fusarium isolates were obtained through this project and through project ICA4-CT-2001-10006. Of all isolates obtained, 284 are currently held at CABI UKC. The majority (249) of these were isolated, primarily from stem wood, by project scientists from coffee species found to be affected by CWD on-farm and on-station (Table 7). Several isolates were recovered from stocks already held in the CABI UKC GRC while a number were donated from collections held elsewhere. Many were successfully identified to species level by CABI UKC scientists or by project partners, or are suspected to be a particular species based on information received on receipt (e.g. accession data provided by other collections. A total of 206 *F. xylarioides* isolates were obtained from *C. canephora* trees cultivated in Tanzania, Uganda[6], DRC[6] and Guinea, 21 from *C. arabica* trees in Ethiopia and 10 from *C. excelsa* trees in Uganda[6] and the Central African Republic. In addition, isolates were obtained from wood and root samples collected from coffees trees with and without external CWD plants at a farm in Mayuge, Uganda (for further details see Section 5.4 below). This farm is one of six on-farm sites where the development of CWD has been monitored spatially and temporally since 2002. Although both *C. arabica* and *C. canephora* are cultivated in Tanzania CWD was only observed on *C. canephora*, in Muleba, Bukoba and Karagwe (Table 1). Conversely, although *C. arabica* is cultivated much more widely than *C. canephora* in Ethiopia, *C. arabica* is affected by CWD while *C. canephora* remains free of the disease.

[6] Isolates from Uganda and DRC obtained primarily through EU INCO-DEV project ICA4-CT-2001-10006

Table 7. *Fusarium xylariodes* isolates obtained from coffee trees exhibiting coffee wilt disease symptoms

Coffee host	Country of origin	Number of isolates	Total
C. arabica	Ethiopia	21	**21**
C. canephora	Uganda	124	**206**
	DRC	62	
	Tanzania	18	
	Guinea	2	
C. excelsa	Uganda	9	**10**
	CAR	1	
Coffea sp.	Various (Ethiopia, Uganda, DRC, Cote d'Ivoire)	12	**12**
Total			**249**

Sixteen isolates belonging to other fusaria were also obtained from coffee trees in CWD affected areas (Table 8). Of these, six (*F. stilboides*, *F. lateritium*, *F. oxysporum* and *F. decemcellulare*) were obtained in Ethiopia and two (*F. solani*) in Tanzania. A number of these isolates were recovered from coffee trees exhibiting external (and in some cases internal) symptoms similar to those of CWD and, in some cases, from wood pieces from which *F. xylarioides* was also recovered. This suggests that they may co-exist on the crop as components of a disease 'complex'. However, the precise role of each of these species, all of which may individually cause specific diseases of coffee, remains unclear. They may collectively contribute to the development or expression of CWD-like symptoms, which are often not definitive with respect to disease diagnosis. Perhaps some species reduce host vigour or by providing entry points for *F. xylarioides*. Alternatively, they may invade host tissues as opportunistic pathogens or saprophytes subsequent to infection by *F. xylarioides*. To the author's knowledge, the pathogenicity of these isolates to *C. canephora*, *C. arabica* and *C. excelsa* has not yet been tested, either alone or in combination with pathogenic forms of *F. xylarioides.* Nevertheless, based on these isolations and feedback from in-country counterparts, it is strongly suspected that sometimes symptoms caused by other fusaria are mistakenly attributed to CWD. This highlights the need, firstly, to increase awareness and understanding of the various fusaria that may affect coffee and enhance capacity among in-country counterparts to recognise and differentiate the various symptoms that may be encountered[7]. Secondly, in-country disease surveys should be supported by isolation of fungal organisms from diseased plants and any fungi identified to species level as a means of ascertaining what may be responsible for the symptoms observed.

[7] A training manual '*Isolation and identification of Fusaria from Coffee*' was produced by CABI UKC specifically for this purpose in 2003 using CFC funding. Eight scientists investigating CWD as part of the RCWP, from Ethiopia, Tanzania, Uganda, DRC and Rwanda also completed a training workshop on and isolation and identification of fusaria occurring on coffee from coffee' in 2003, led by CABI Bioscience and hosted by CORI, Uganda. Funding for the workshop was also provided by CFC.

Table 8. Fusaria other than *F. xylarioides* obtained from coffee trees exhibiting coffee wilt disease symptoms

Coffee host	Country of origin	*F. stilboides*	*F. lateritium*	F. *solani*	*F. oxysporum*	*F. decemcellulare*
C. arabica	Ethiopia	3	1		1	1
C. canephora	Uganda		1	4	1	
	DRC					
	Tanzania			2		
	Guinea			1		
C. excelsa	French East Africa			1		

The various fusaria, including *F. xylarioides*, were routinely isolated with relative ease from coffee wood pieces excised from symptomatic trees. In contrast, *F. xylarioides* could not be isolated from soil particles sprinkled onto agar media, including a medium selective for *Fusarium* species (Komada, 1975). Plates usually became overgrown by other fusaria without *F. xylarioides* becoming apparent, a recurring problem where pot and field based experimentation were concerned (see Section 6). These fusaria may actively compete with or inhibit the growth of *F. xylarioides* and therefore restrict or inhibit its growth or simply obscuring its presence. This is supported by reports that *F. xylarioides* was readily isolated from soil that had been collected from a field site affected by CWD, steam sterilised and subsequently inoculated with the pathogen. However, the fungus could not be re-isolated from soil that had not been sterilised prior to inoculation (Hakiza, pers. comm.). The problem could not, as had been hoped, resolved by serial dilution plating, despite several attempts being made. In many such cases it was suspected or known that the pathogen was in fact present in soil, as shown by the development of CWD symptoms on susceptible coffee seedlings transplanted into the soil. While the use of susceptible plants as a means of 'baiting' *F. xylarioides* from soil is usually effective, a more rapid and straightforward means of confirming the presence of, and isolating, the fungus from soil is necessary to support fundamental and critical studies on, for example, the ability of *F. xylarioides* to remain viable in soil and persist as a source of inoculum (see Section 6).

In several cases an absolute species confirmation could not be assigned due to the morphological condition (e.g. poor sporulation) of a culture. Furthermore, in a number of instances, isolates were not found to exhibit the morphological characters typical of the species to which they had been assigned prior to receipt. Several *F. stilboides* and *F. lateritium* isolates, for example, were originally received as *F. xylarioides*, which highlights difficulties associated with morphological similarity of some members of these species. Molecular characterisation of a number of these isolates, however, tended to support their morphological attributes. A number of cultures were also found to comprise more than one *Fusarium* species (i.e. mixed cultures).

5.2 Evaluate methodologies for assessing genetic variability and apply selected methodologies to determine variability of the CWD pathogen in Tanzania and Ethiopia.
Single-enzyme, agarose gel-based AFLP analysis and amplification and enzymatic digestion of the IGS region (Figure 2), while revealing very little genetic variation among the *F. xylarioides* isolates, suggested that two distinguishable pathogen populations exist. Of these, one comprises *F. xylarioides* isolates obtained from *C. arabica* in Ethiopia, the other *F. xylarioides* isolates from *C. canephora* in Uganda, DRC and Tanzania.

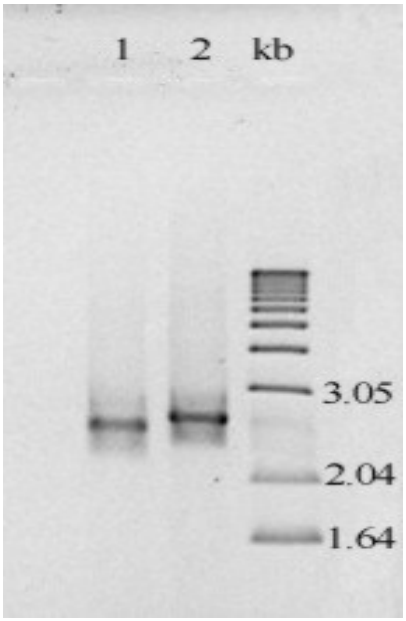


Figure 2. DNA banding patterns produced by PCR amplification of the ribosmal DNA (rDNA) intergenic spacer (IGS) region of *F. xylarioides* isolates from coffee. Isolates originating from *C. arabica* in Ethiopia (lane 1) show a smaller IGS fragment than those obtained from *C. canephora* in Uganda, DRC and Tanzania (lane 2). To the right is a DNA ladder (kb) with band sizes inserted. When digested with restriction enzymes such as *Hae*III and *Eco*R1, the two bands also produce different DNA profiles, suggesting fundamental differences in base sequence.

A number of additional isolates, obtained from *C. canephora* trees at an on-farm CWD mapping site at Mayuge in Uganda (see also Section 5.4), were also analysed and were found to be genetically similar to those of the larger *C. canephora* group. Isolates of the other *Fusarium* species had molecular profiles markedly distinct to one another and to those of *F. xylarioides*.

These findings were confirmed by minor differences revealed by PCR analysis of ISSR DNA sequences (Figure 3) and by analysis of mtDNA RFLPs (Figure 3a). They also apply to all *F. xylarioides* isolates obtained from coffee between 1976 and 2004 and correlate with the results of microsatellite-based PCR research undertaken at CIRAD as part of project ICA4-CT-2001-10006 (Bieysse, 2004). As shown in (Figure 3a), the few historical isolates available for molecular analysis were found to differ genetically from those of either the *C. arabica* group or the *C. canephora* group. These isolates, accession no.s DSMZ 62457, CBS 25852, CBS749.79 and ATCC 15664, were obtained in the 1950s and 1960s from *C. canephora* and *C. excelsa* trees apparently affected by CWD (Table 1). CBS 25852 and CBS749.79, from Ivory Coast and Guinea, respectively, appear to be genetically identical and, according to the mitochondrial DNA analysis, are more closely related to ATCC 15664 (geographic origin unknown) than to DSMZ 62457, from CAR. The former three isolates also have more genetic similarity with the *C. canephora* group than DSMZ 62457, which shows almost the same level of dissimilarity to the *C. arabica* group as to the *C. canephora* group. Furthermore, DSMZ 62457 has also been shown to be pathogenic to a broader range of *Coffea* species but, most significantly and unlike any other isolates investigated, is capable of causing CWD on both *C. arabica* and *C. canephora* seedlings (see Section 5.3). These findings are intriguing, as they may indicate that the *C. arabica* group, the *C. canephora* group and the historical isolates may have evolved independently, perhaps due to selection pressures related to geographic isolation or the coffee type cultivated. Alternatively they may have evolved sequentially, with one or more genetic forms emerging from another. In this sense it is therefore feasible that the re-emergence of CWD on *C. canephora* is due to a genetic form of *F. xylarioides* that has evolved (and perhaps become more specialised) from the *C. arabica* group or, more likely, from one or more of those genetic forms responsible for the earlier CWD outbreak in West Africa.

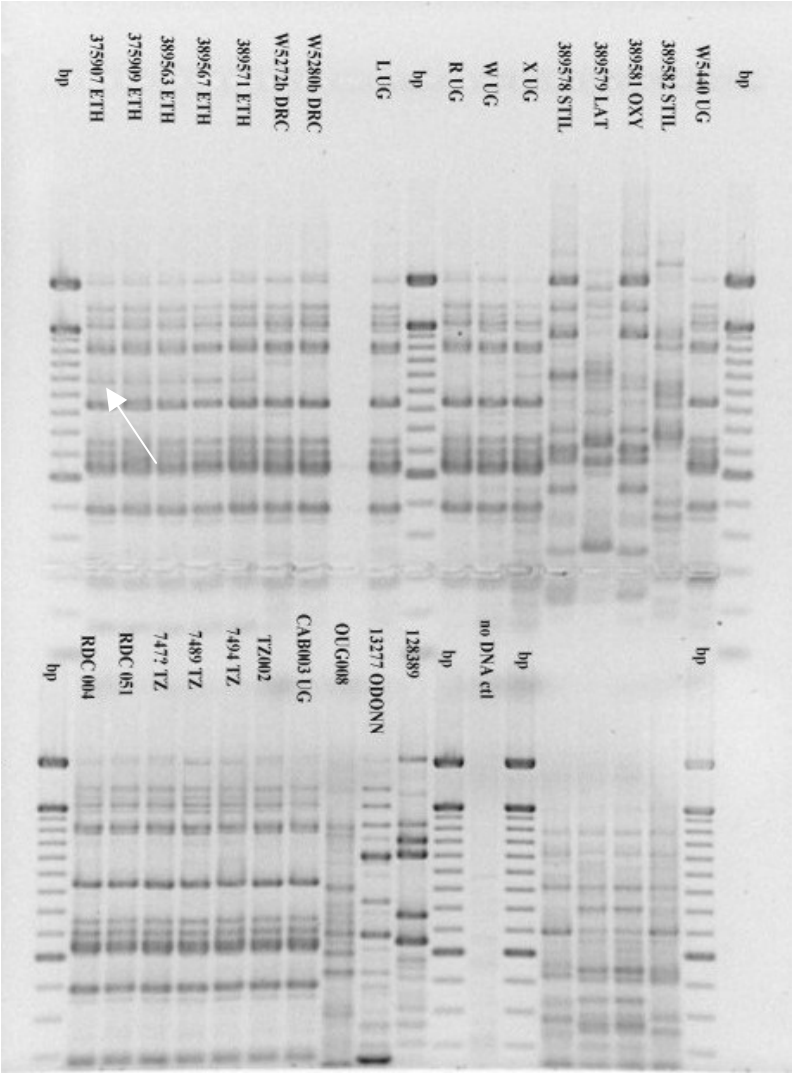
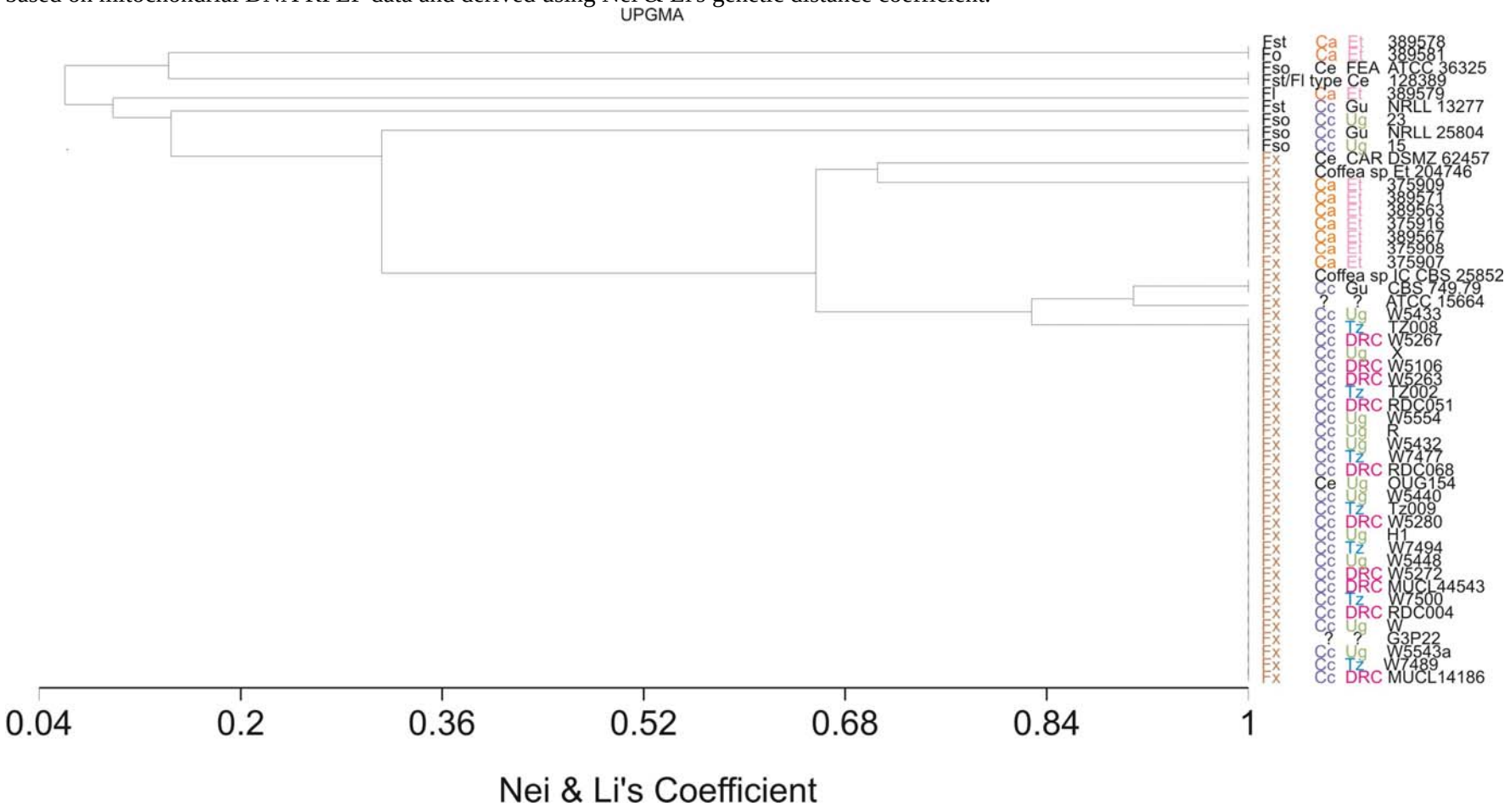


Figure 3. DNA profiles obtained for fusaria isolated from coffee following amplification of total DNA with inter simple sequence repeat (ISSR) primers. While *F. xylarioides* isolates are generally very similar, those from *C. arabica* in Ethiopia (denoted by 'ETH') show a slightly different DNA profile (e.g. at point denoted by arrowed) to that for isolates from *C. canephora* in Uganda, DRC and Tanzania (denoted by 'UG', 'DRC or RDC' and 'TZ' respectively. *F. stilboides* ('STIL'), *F. lateritium* ('LAT') and *F. oxysporum* ('OXY') isolates have very different profiles, as does isolate NRLL 13277 ('13277 ODONN').

bp = DNA size marker (ladder)

Very recent work involving the use of sequencer-based AFLP analysis, whilst showing greater resolution of AFLP fragments compared to agarose AFLPs, has not revealed any greater degree of discrimination at subspecies level. Dendrograms depicted in Figure 4 and based on sequencer AFLP data clearly show the extent of similarity within and between the two *F. xylarioides* populations and between these and other *Fusarium* species.

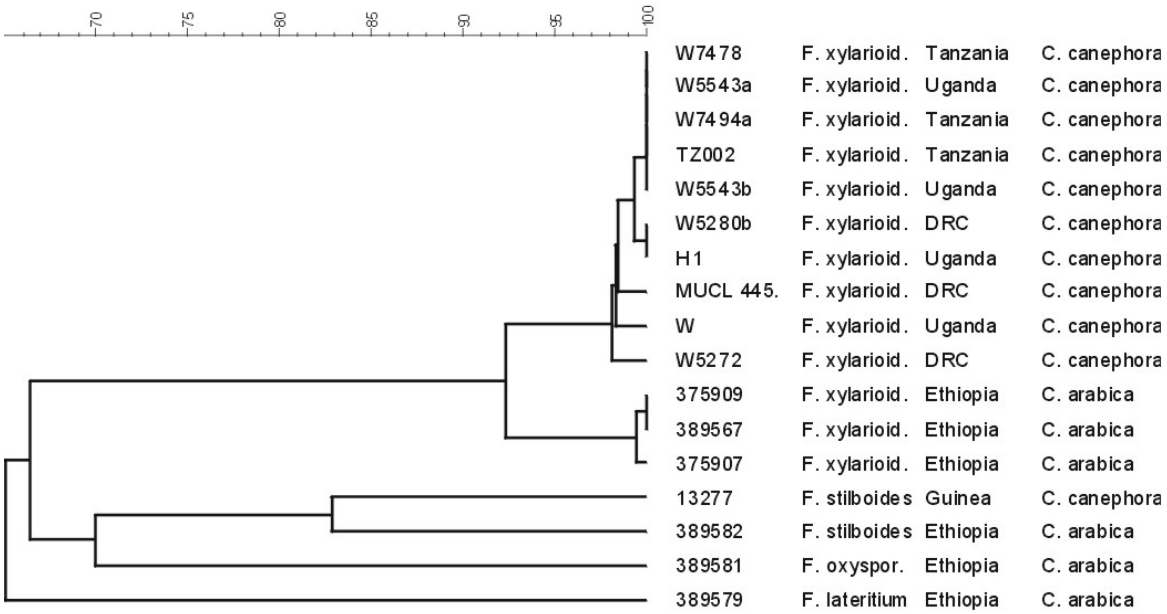
Figure 3a. UPGMA dendrogram showing % genetic similarity between fusarium isolates obtained from coffee affected by CWD. The dendrogram was based on mitochondrial DNA RFLP data and derived using Nei & Li's genetic distance coefficient.

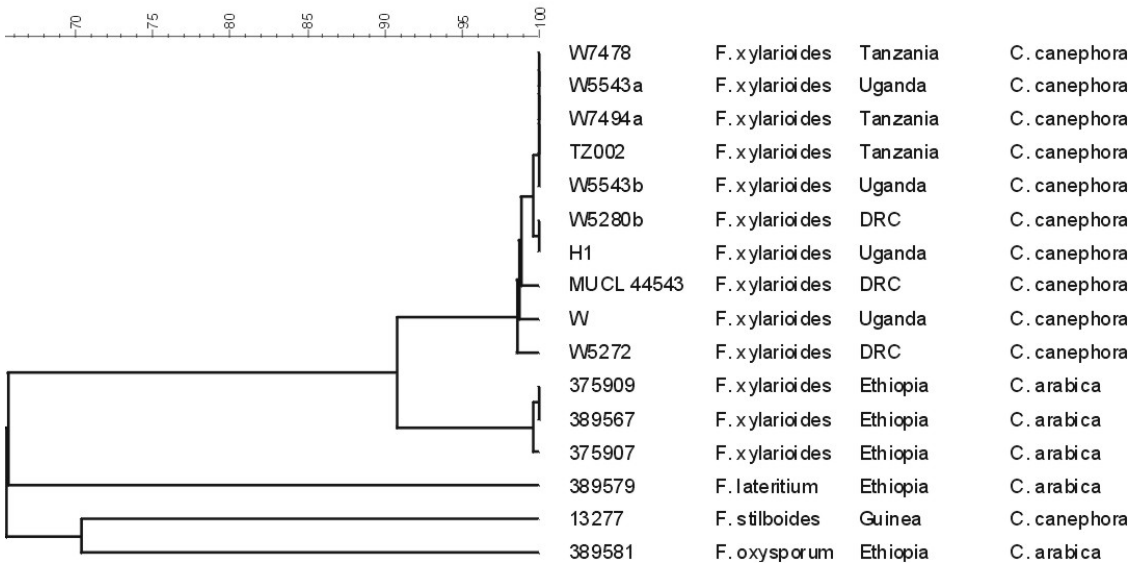


Abbreviations: Fx, *F. xylarioides;* Fo, *F.oysporum*; Fs, *F. solani*; Fst, *F. stilboides*; Fl, *F. lateritium*; Ca, *C. arabica*; Cc, *C. canephora;* Ce, *C. excelsa* Et, Ethiopia; Ug, Uganda; DRC, Democratic Republic of Congo; Tz, Tanzania, Gu, Guinea; CAR, Central African Republic; IC, Ivory Coast; FEA, French East Africa

Figure 4. Dendrograms showing genetic similarity between selected isolates of *F. xylarioides* obtained from *C. canephora* and *C. arabica* in Ethiopia, Uganda, DRC, Tanzania and Guinea and other fusaria based on dual enzyme amplified fragment length polymorphisms (AFLP) analysis. The upper and lower dendrograms were generated using combined AFLP data for three (AFLP ATACC + AFLP ATCTC + AFLP CGCTC) and four (AFLP ATACC + AFLP ATCTC + AFLP CGACC + AFLP CGCTC) primer sets respectively. Data was analysed using GelComparII software (Applied Maths, Sint-Martins-Latem, Belgium) and similarity matrices calculated using Dice's co-efficient. The dendrograms constructed using the unweighted pair generated method of arithmetic averages (UPGMA).

Percentage similarity is shown along the top of each dendrogram.





It is therefore possible that one or more relatively minor genetic changes is responsible for the species specificity observed in the pathogen (see also Section 5.3) and perhaps for the re-emergence of CWD on *C. canephora*. The differences observed in IGS DNA profiles, for example, may be due to DNA insertions or deletions. Attempts are currently underway to sequence the entire IGS region of a small number of isolates from the *C. arabica* and *C. canephora* subgroups, as well as that of an historical isolate, to determine the precise nature of the variability observed and to investigate prospects of developing a rapid, DNA-based, diagnostic technique (see also Section 6).

The molecular techniques applied were chosen due to their fundamentally distinct approaches and to provide an opportunity to reveal the occurrence of any artefactual results. Differences in IGS sequence have previously been used to delimit special forms of *F. oxysporum* (Appel & Gordon, 1996), and have shown a level of resolution in *F. xylarioides* similar to that found here in a previous study (Flood & Brayford, 1998). ISSR-PCR has been employed to successfully determine genetic variability among fungi in a similar manner to RAPDs. However, compared to RAPD-PCR this method is deemed to provide more accurate discrimination among individual isolates as it produces more variable banding patterns and better reproducibility due to longer primers and more stringent amplification conditions (Grünig *et al*., 2001). It has shown to be discriminatory at the inter- and even intra-specific levels for fungi (i.e. a similar level of differentiation to that obtainable from single-enzyme, agarose gel-based AFLP analysis). Differences in A+T rich DNA banding patterns have been used to discriminate among different fungi at a similar level but with the added feature, particularly for sexually reproducing fungi, that mitochondrial inheritance is normally uniparental. This technique makes use of an attribute seen in most fungi in that the mitochondrial DNA is very rich in A+T. Hence, when total fungal DNA is restricted with a 4-base cutting enzyme such as *Hae*III (or *Msp*I) that recognise sites containing G+C (GGCC for *Hae*III; CCGG for *Msp*I), the chromosomal DNA (in which these sites are extremely common) is digested into a smear of small fragments. However, as such sites are scarce in mitochondrial DNA, larger mitochondrial fragments are obtained that may be visualised and clearly discriminated after electrophoresis.

There has been an increasing realisation amongst fusarium molecular taxonomists that, despite being viewed as a near 'universal' species delimiter for fungi, ITS regions are not suitable for use with fusarium due to the presence of non-orthologous copies of the ITS (O'Donnell & Cigelnik, 1997). This has led to the adoption of translation elongation factor 1α (TEF) by researchers (e.g. O'Donnell *et al*., 1998; O'Donnell *et al*., 2000) and its incorporation into a fusarium identification sequence database (FUSARIUM-SEQFUSARIUM-ID v.1.0; Geiser *et al*. 2004). As a logical step in our studies, part of the TEF region of a small number of isolates was analysed as described by O'Donnell *et al*. (1998) in an attempt to discern the taxonomic significance (i.e. inter or intra-specific variation) of the polymorphisms observed between the two *F. xylarioides* populations. Comparison of sequences for several *F. xylarioides* isolates belonging to the two genetic populations described above with those held on GenBank (using its version of the BLAST algorithm) revealed a high level of similarity (>99% homology, 1 mismatch over almost 300 bases) to sequences deposited as 'pseudogenes' of *Gibberella xylarioides* deposited in January, 2003, by Université Catholique de Louvain (UCL), Belgium. However, there was no evidence to suggest differentiation of the two populations at species level.

In contrast, when BLAST-searched using the Fusarium Research Center's FUSARIUM-ID v.1.0 database, very little homology was shown between these isolates and the representative sequence of *F. xylarioides* (NRRL 13277[8]). This isolate, received from the Biologische Bundesanstalt für Land- und Forstwirtschaft (BBA) in Berlin as isolate 62455 and deposited in the Fusarium Research Center (FRC) at the Pennsylvania State University as L-101, is

[8] Isolated from *Coffea canephora* in Guinea

depicted in Nelson *et al*. (1983) as having clear *F. xylarioides* morphology. Morphological examination of a culture of NRRL 13277, obtained from the Agricultural Research Service Culture Collection [NRRL], Illinois), also revealed characters atypical of *F. xylarioides* (and more in line with *F. stilboides*, the causal agent of bark disease [syn. Storey's bark disease] of coffee) while ISSR, IGS and single enzyme AFLP profiles for the isolate also differed markedly to those of the other *F. xylarioides* isolates tested. When compared against the FUSARIUM-SEQFUSARIUM-ID v.1.0 database using the BLAST algorithm, a partial TEF sequence obtained for this isolate gave a >99% match against the NRRL 13277 sequence held, but was ranked as low as 145th taxon against the *G. xylarioides* sequences held on the GenBank database. The NRRL 13277 sequence has since been removed from the FUSARIUM-SEQFUSARIUM-ID database, replaced with isolates confirmed to be *F. xylarioides* and consistent in molecular and morphological characters with those described here (D. Geiser, pers. comm.).

The various molecular approaches employed in this work were consistent in clearly discriminating the *Fusarium* species recovered form coffee exhibiting CWD-like symptoms. The findings also reinforce earlier work by Flood & Brayford (1998) in suggesting that two, genetically distinct populations of *F. xylarioides* are associated with CWD in East Africa, but reveal very limited genetic diversity within either of these populations. The close relationship between the two populations and coffee species strongly suggests some level of host specialisation that, in terms of current efforts to develop host resistance to the disease, is a positive development. Host specificity is also largely supported by pathogenicity studies undertaken to date by African counterparts and involving assessment of a number of the isolates reported here (G. Hakiza and G. Adugna, unpublished). For further details of pathogenicity studies undertaken through this project, see Section 5.3 and Appendix 1).

As mentioned above, several isolates received at CABI as *F. xylarioides* were shown, through subsequent morphological examination, to belong to other fusaria (namely *F. solani*, *F. stilboides* and *F. lateritium*) or to be mixed cultures. In most of these cases the morphological findings were supported by molecular DNA profiles, the latter occasionally alerting the need for revised morphological examination, and emphasized the ability of the genetic approaches selected. However, this again clearly highlights the need for accurate morphological identifications to support field diagnoses and to provide confidence to conclusions based on other investigatory approaches, including studies of genetic and pathogenic variability.

Of a subset of 16 of the representative isolates investigated for vegetative compatibility (i.e. VCG), mutant types required for isolate pairing were successfully generated for 14 isolates, providing a total of 91 separate mutant lines. The majority of these were identified as nit M types and not the nit 1 and nit 3 types also required for pairing. Only one isolate, IMI 375916 (ex. *C. arabica*, Ethiopia) produced suitable nit1 and nit3 mutants, while some lines were wild type revertants. When the nit 1 and nit 3 mutants IMI 375916 were paired against nit M mutants of each other isolate, vegetative compatibility was only observed (as would be expected) in the self-pairing test between the IMI 375916 nit 1 and nit 3. Repeat attempts to generate more mutants of the correct type through adaption of the mutant inducing conditions proved unsuccessful and, given the time commitment required to undertake such work, this approach was not pursued further.

5. 3 Evaluate pathogenic variability of *F. xylarioides* isolates against a range of coffee germplasm.

For the most part, and in line with previous reports and the results of molecular analyses reported above, *F. xylarioides* obtained from *C. arabica* coffee cultivated in Ethiopia induced CWD symptoms on *C. arabica* seedlings (obtained from Ethiopia, Kenyan and Costa Rica) but not on *C. canephora*, while isolates from *C. canephora* cultivated in Uganda, Tanzania and DRC induced symptoms on *C. canephora* seedlings (from DRC and Ivory Coast) but not

on *C. arabica* (Appendix 1) While an isolate (OUG152) obtained in 2002 from *C. excelsa* in Uganda was also found to be pathogenic to *C. canephora* but not to *C. arabica*, an isolate (DSMZ 62457) obtained in 1955 from *C. excelsa* in CAR induced symptoms not only on a large number of the Kenyan and a proportion of the Costa Rican *C. arabica* seedlings inoculated, but also on a (albeit smaller) percentage of *C. canephora* seedlings from DRC and Ivory Coast. It also induced symptoms on almost all seedlings of two *C.liberica* lines obtained in Costa Rica. These two lines were also found to show susceptibility, but contrasting responses, to an Ethiopian *C. arabica* isolate (G3P22 [syn. IMI392680]) and a Ugandan *C. canephora* isolate (CAB03 [syn. IMI392263]). Differential responses were also observed among other lines of the same coffee species, including those from the same country, when challenged with a particular isolate. *C. canephora* line 1673 from Ivory Coast, for example, had a relatively low percentage of seedlings affected by *F. xylarioides* isolates (from Uganda and DRC) over the course of the trial than other *C. canephora* lines including 1655, also from Ivory Coast.

As highlighted in the Executive Summary, it was not possible to undertake in-country evaluation of coffee germplasm against specific *F. xylarioides* isolates during the course of the project. However, through visits made to the major coffee producing areas during the project, *F. xylarioides* isolates were obtained from areas affected by CWD and are therefore representative of differing coffee hosts, geographic locations and agroecological conditions. These were purified and are being maintained at TaCRI (Tanzania) and JARC (Ethiopia) in a stable condition for use in future pathogenicity studies. Coffee germplasm was also collected for this purpose. As part of the project, two scientists (Mr Deusdedit Kilambo and Mr Nyabisi Ng'homa) and a technical assistant (Mrs Grace Monyo) from TaCRI also received in-depth training in appropriate pathogenicity methods from highly experienced pathologists while on a four day training attachment at CORI, Uganda, in the latter stages of the project. This training covered various techniques related to pathogenicitiy testing, including media preparation, isolation of fusaria from plant roots, stem and leaves, subculturing fungi, preparation and standardisation of pathogen inoculum, differing methods of host plant and microscopy. The visit also provided a useful opportunity for in-depth discussion and exchange of ideas in relation to the current status of CWD in the two countries.

The various preparative activities should ensure that evaluation of germplasm against a range of indigenous isolates may proceed in Tanzania without further delay, and is currently anticipated to commence during 2005.

5.4 Collect isolates from specific field sites in Uganda and use molecular markers to characterise pathogen populations and identify individuals in the field.

Application of a broad range of molecular approaches to help identify genetic variability within *F. xylarioides* isolates obtained from the CWD mapping site at Mayuge, Uganda, showed that these were genetically identical to one another and also genetically identical to *F. xylarioides* isolates recovered from *C. canephora* in other parts of Uganda, from DRC and from Tanzania. The approaches applied included AFLP analysis, widely considered to be one of the most powerful tools for detecting low level genetic variability (i.e. to individual isolate) in fungi and other organisms. These findings support those obtained for the larger and more diverse representative group of *F. xylarioides* isolates, in that variability within the pathogen generally, and within the *C. canephora* and *C. arabica* groups in particular, is very limited. However they also rendered monitoring of small populations or individual isolates at this mapping site, as part of the studies on possible mechanisms of CWD spread and management reported in Section 5.5 below, an impractical proposition at this stage.

5.5 Undertake small-scale field experimentation in Uganda to investigate survival and transmission of CWD.

5.5.1 On-farm and on-station trials

Note: Repeated attempts to isolate *F. xylarioides* from soil in the various trials in the manner described in Section 4.1. Although several other fungi were recovered, including other fusaria, these attempts proved unsuccessful. As such, observation of CWD symptoms on plants and isolation of *F. xylarioides* from plant parts was the only means by which the presence of *F. xylarioides* was confirmed. Further information and suggestions on why isolation from soil is so problematic are provided in Section 5.1.

Trial 1. Transmission of F. xylarioides and spread of CWD through use of a machete.

Coffee field

Of the 12 trees wounded with a 'contaminated' or a cleansed machete in Block 38 at CORI, one of the trees wounded with a 'contaminated ' machete within the fresh, green growth at the top of a stem developed symptoms of CWD above the wound point. The symptoms were first observed 49 days after wounding, and were classed as severe (grade 5). *F. xylarioides* was successfully isolated from a piece of the stem showing disease symptoms. A second main stem on the same plant, which had not been wounded, also developed CWD symptoms four weeks later and by termination of the project, the entire plant had died. *F. xylarioides* was also successfully isolated from this stem. A second plant, wounded mid-way along a primary stem with a contaminated machete, also developed CWD symptoms 223 days later. However, these symptoms developed on both of the two remaining untreated stems on this plant (one three weeks after the first) and have not as yet developed on the stem that was wounded. Symptoms of CWD were not observed on any of the other trees wounded with a cleanse machete. Although it may be considered unusual for symptoms to develop so rapidly[9] on one of the two trees, these observations nevertheless suggest that wounding healthy trees with a machete previously used to cut or prune infected trees can lead to transmission of *F. xylarioides* and subsequent development of CWD. This would likely be true also should a machete become contaminated with pathogen inoculum in other ways.

Screen house

At 203 days after treatment, none of the seedlings treated by wounding with a machete or a scalpel, immersing roots in an aqueous suspension, applying a soil drench or adding wood chippings had developed symptoms of CWD. This may be considered to be unusual as the *C. canephora* seedlings used in this trial are considered to be very susceptible to CWD. When inoculated by immersing roots in a spore suspension of *F. xylarioides* ($1x10^6$ spores/ml), seedlings of a similar level of susceptibility to CWD have previously been found to develop external symptoms of the disease after 36 to 40 days when maintained under screenhouse conditions (Olal, pers. comm.) Similarly, when planted in pots filled with naturally infested soil, symptoms have developed after 50 to 55 days. However, attempts to isolate the pathogen from 1 ml aliquots of washing from the contaminated machete blade proved unsuccessful, suggesting that either the fungus was absent or so dilute in the washing that propagules were not transferred to the agar medium. Alternatively, prevailing climatic conditions in Uganda, namely the onset of a abnormally dry period, may be restricted or prevented fungal growth and therefore CWD development on seedlings not only in this but also several of the other reported trials (for further discussion refer to Section 6). *F. xylarioides* did not emerge from soil obtained from several of the pots and plated onto TWA medium.

[9] On wounding a mature tree with a cleansed machete and subsequently applying a conidiospore suspension ($1x10^6$ spores/ml) of *F. xylarioides* to the wound site, CWD symptoms are usually observed within 14-21 days and the tree killed after approximately 220 days (S. Olal, pers. comm.).

Trial 2. Survival of F. xylarioides in, and inoculum potential of, coffee wood pieces over time.

None of the coffee seedlings planted in the absence of a wood piece on initiation of the trial had developed symptoms of CWD. However, symptoms were observed on five seedlings in pots containing wood pieces. These seedlings were planted on the day that wood pieces were placed in pots (one seedling) as well as 33 days (two seedlings) and 64 days (two seedlings) after wood pieces were placed. Symptoms were first observed on these plants 95, 68, 76, 67 and 69 days respectively after the wood pieces were placed in pots. Formation of perithecia was also observed on several of these wood pieces. *F. xylarioides* was readily isolated from stored wood pieces at monthly intervals for up to six months after collection of the wood from the field[10]. These findings confirm that wood pieces obtained from CWD affected coffee trees can act as a source of infection for healthy coffee plants, and that seedlings planted in soil bearing wood pieces are at risk of infection for at least 64 days after first contact between wood and soil, at least where seedlings and wood are in close proximity. However, from this trial it is unclear whether seedlings have become infected directly from the wood piece or indirectly via the underlying soil.

Trial 3. Survival/ inoculum potential of F. xylarioides in soil over time.

Nine of the coffee seedlings planted in soil obtained from the three CWD affected sites had developed clear external symptoms of CWD (Table 9). All seedlings were subsequently killed by the disease. These seedlings were among those planted in soil obtained immediately after tree uprooting and also at 47 and 95 days after uprooting.

Table 9. Development of CWD on seedlings planted in soil obtained from CWD affected sites, Block 17, Block 36 and a forest area.

Period between tree removal and collection of soil/planting of seedlings (days)	Source of soil	Number of seedlings with CWD symptoms	Period between planting to observation of symptoms (days)	Period between first symptoms to seedling death (days)
0	Block 36	2	55[1]	15[2]
	Block 17	1	55	39
	Forest	3	49[2]	10[2]
47	Forest	1	105	22
95	Block 36	1	150	15
	Forest	1	150	13
		Total = 9	**Mean = 94**	**Mean = 19**

1 - mean for 2 plants
2 - mean for3 plants

Although this represents a relatively small proportion of seedlings planted, development of disease on seedlings planted in August showed that the soil had remained infective for 95 days, or approximately three months. Furthermore, this was the case for soil obtained from all three sites. Although based on a small number of plants, a gradual increase was also observed

[10] CORI staff report that *F. xylarioides* has previously been isolated directly from standing coffee trees for a similar period following death of the trees due to CWD

in the time taken for disease symptoms to first appear on seedlings through the consecutive plantings, rising from 49-55 days to 150 days (CWD symptoms would be expected to develop on susceptible seedlings after approximately 50 days where they are planted in sterile soil artificially inoculated with *F. xylarioides* [S. Olal, pers. comm.). This indicates that the level of infectivity of the field soil to newly plated coffee does decline over time following removal of diseased trees. Little difference was observed in the time taken from initial symptom development to seedling death (on average 19 days), with the exception of those seedlings planted in soil from Block 17 (39 days). Attempts to isolate *F. xylarioides* from soil by sprinkling onto TWA agar and by dilution plating proved unsuccessful.

Trial 4. Role of aerial vectors in transmission of F. xylarioides and disease spread.
At 140 days after planting, none of the seedlings planted at the Mayuge on-farm mapping site had developed symptoms of CWD. While this may be expected for seedlings planted in sterilised soil, it is somewhat unusual for seedlings planted in unsterilised farm soil given that susceptible coffee seedlings planted on field soil naturally infested with CWD have previously been shown to exhibit external symptoms of the disease after 50-55 days (Olal, pers. comm.). Again, it is possible that adverse climatic conditions prevailing at the time of initiation of the trial may have had a detrimental effect on pathogen survival or disease development.

5.5.2 Occurrence of *F. xylarioides* on insects associated with coffee.

F. xylarioides was not isolated from any of the insects obtained from CWD affected sites. Other fusaria were recovered, including *F. oxysporum* from Coffee Berry Borer and scales, *F. solani* from termites, bees, leaf skeletonisers and attendant ants, *F. stilboides* from beetles and *F. semitectum* from fruit flies and beetles. A *Curvularia* species was isolated from coffee berry moths.

5.5.3 Occurrence of *F. xylarioides* on other plants

Although a number of *Fusarium* species were recovered from both sterilised and non-sterilised roots of the various weed species and beans collected from the Mayuge farm site, these did not include *F. xylarioides*. No fungi were recovered from the plant stem pieces. This would indicate that, at least at the time of investigation, none of these plants were harboring the CWD pathogen. The finding is strongly supported by a comprehensive survey undertaken across six coffee growing districts in Uganda having a high CWD incidence (Kangire *et al*., 2003). Of 270 plant samples obtained, covering 105 species of cultivated crops and a number of weed specuies, *F. xylarioides* was not recovered from any plant parts including roots. Several other fusaria were again recovered, including *F. oxysporum* from Kayinja, a species responsible for Panama disease of banana in Uganda and elsewhere. Of note, *F. xylarioides* isolated from cotton seed has previously been reported to pathogenic to cotton seedlings under laboratory and (less so) glasshouse conditions (Pizzinatto and Menten, 1991). It has also been isolated, among other known fungal pathogens, from rot of tomatoes obtained from fruit markets in Nigeria. Artificial inoculation of tomato fruits confirmed that it could cause a slow-spreading soft rot (Onesirosan and Fatunla, 1976).

Screen house studies, involving artificial plant inoculation with *F. xylarioides*, have been initiated by CORI (not part of R8188) to assess pathogenicity of the fungus to seven solanaceous crops, a *Phyllanthus* species and Kayinja, and to assess their ability to act as hosts for the pathogen. These studies are ongoing.

5.5.4 Spatial and temporal mapping of CWD spread and relationship with climatic factors

On 2 January 2002, only nine of the 368 *C. canephora* present in Block 36 exhibited external symptoms of CWD (Figure 5)[11]. These were located at five focal points of the trial site, four of which were adjacent to points where a plant had already been removed due to CWD symptoms being observed. Twenty-eight months later, on 2 May 2004, the number of

symptomatic plants had risen to 166 (Figure 6)[11], with expanded areas of disease being found adjacent to the original focal points. This represents an increase from 2.4% to 45.1% of those plants present or, on average, six plants per month. Clones 1s/2, 1s/3, 1s/6 and 258/24 showed some variation in development of CWD, where the percentage of plants exhibiting symptoms of CWD increased from 3, 0, 3 and 1 respectively to 76, 63, 72 and 54 respectively during the assessment period.

[11] Figures 5 and 6 shown the occurrence and distribution of CWD at the beginning and end of the assessment period. Data for intermediate assessments is available but not presented.

Figure 5. *C. canephora* plants exhibiting external symptoms of CWD in Block 36 on 2 January 2002

	Coffee clone	Plant no.																																
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33
Block I	**Is/2**															X																		
	Is/6										X									X														
	Is/3				X									X			X																	X
	258										X			X																				
Block II	**Is/2**																															X		
	Is/3						X	X															X										X	
	258								X	X											X						X							
	Is/6																				X		X									X		
Block III	**Is/2**								X						X																			
	258								X																									
	Is/6					X			X																									
	Is/3		X																															

X =denotes coffee plant absent
Shaded cell denotes coffee plant exhibiting external symptoms of CWD

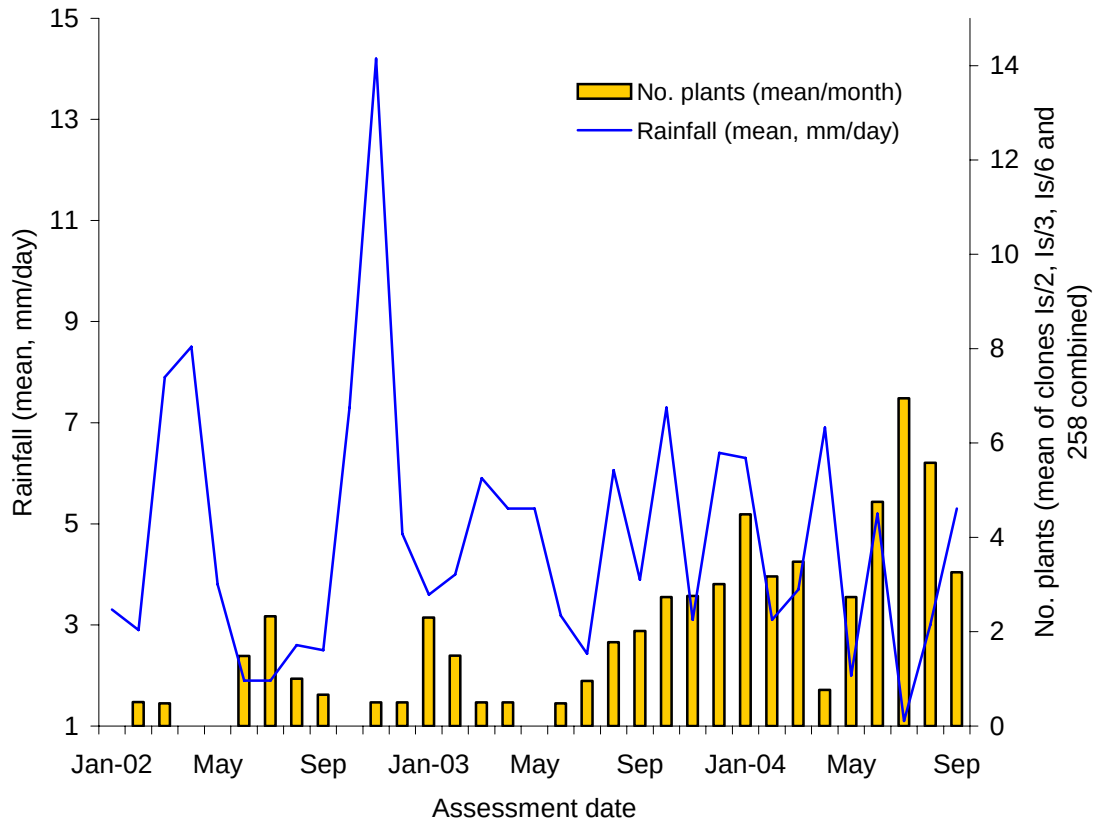
Figure 6. *C. canephora* plants exhibiting external symptoms of CWD in Block 36 on 2 May 2004

	Coffee clone	Plant no.																																
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33
Block I	**Is/2**															X																		
	Is/6										X									X														
	Is/3				X									X			X																	X
	258										X			X																				
Block II	**Is/2**																															X		
	Is/3						X	X															X										X	
	258								X	X											X						X							
	Is/6																				X		X									X		
Block III	**Is/2**								X						X																			
	258								X																									
	Is/6					X			X																		X							
	Is/3		X																															

X = coffee plant absent
Shaded cell denotes coffee plant exhibiting external symptoms of CWD

As shown in Figure 7, mean monthly rainfall at CORI fluctuated markedly during the assessment period, reaching a maximum of 14.2 mm in November 2002. Uganda has two rainy seasons, between about March and May and between about September and November. Rainfall is usually heavier and more prolonged during the former season. Some correlation was apparent between the number of coffee plants found to exhibit CWD symptoms for the first time (expressed as a mean for all four coffee clones) and rainfall, at least during the first 18 months of assessment, when the number of plants increased and reached a peak during periods of low rainfall that followed periods of high rainfall. Two possible explanations for this are, firstly, that symptoms such as leaf yellowing and wilting become more recognizable during dry as opposed to wet periods, due to plants already being under greater water stress. Secondly, increased rainfall (and hence relative humidity, for which data is not available) has caused an increase in fungal activity in terms of infection and colonisation of the host plant, for example, and led to an increase in damage to the plant and expression of symptoms. However, due to a lag period being required for symptoms to develop and become apparent, these are not recognizable until some weeks or months later.

Figure 7. Development of CWD on *C. canephora* plants in Block 36 at CORI, Uganda, in terms of plants newly exhibiting symptoms, in relation to monthly rainfall.



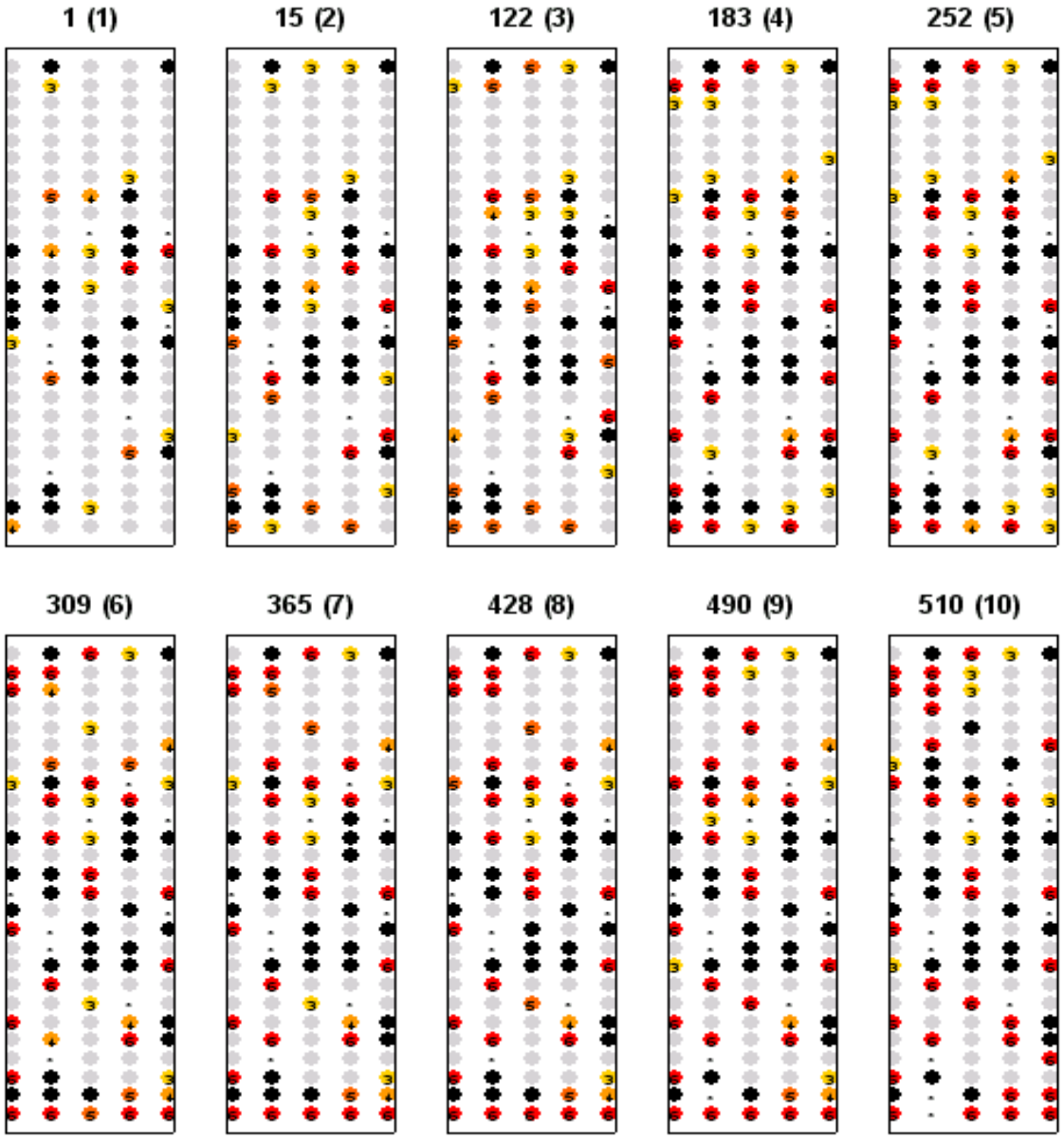
There was no apparent effect of temperature on CWD development (not shown). However, temperatures do not fluctuate markedly in Uganda, with average, minimum and maximum monthly means of mean 22.1, 19.9 and 27.3°C during the assessment period. Indeed, the monthly mean was between 19.9 and 24.2 °C for 27 of the 28 months.

Analysis of the data obtained over an 18 month period at the Mayuge on-farm site suggests that, for the most part and once the farm was affected by CWD, the disease again spread outward from affected trees or clumps of affected trees (i.e. foci of infection) to adjacent trees

that previously appeared disease free. CWD did not appear to develop across the trial site in any particular direction as might be expected where the movement of water or soil is facilitating dispersal of inoculum. Although CWD symptoms did develop on some trees that were not adjacent to affected trees, these tended to be at the edge of the trial site and where perhaps affected by trees adjacent to the monitored area. While it is difficult to draw firm conclusions the small number of such cases, coupled with the apparent tree to tree spread, suggests that (although confirmed as a means of pathogen transmission in R8188) use of a machete is perhaps not the primary means of CWD spread and that root to root transmission or localised movement of the pathogen in soil are more likely. Figures 8 and 9 provide examples of graphical representations of CWD development at this site.

These analyses have provided initial information on the rate and pattern of CWD development at the two sites and have already proved useful in helping to determine how *F. xylarioides* is being transmitted, what factors may influence disease development and, consequently, what measures may be taken to restrict or prevent initial disease development and subsequent spread. More in-depth analysis of the data is required, including for symptom severity and for individual coffee clones in Block 36, and will be undertaken in the near future as part of project ICA4-CT-2001-10006. Collection of data from these and the other on-farm sites in Uganda will also continue under that project, at least until the end of 2005, and will be subjected to full analysis based on the approaches described here.

Figure 8. Graphical summary showing the spatial layout of trees and CWD development at an on-farm mapping site in Mayuge, Uganda.



In the summary above, the trial plot is depicted as a rectangle, each rectangle representing one of 10 time points, or assessment visits. The headings give the day of each visit, the first (day 1) being on 20 December 2002 and the last (day 510) on 12 May 2004, and in brackets the number of the visit (1-10). Each coffee tree is shown as a coloured spot and shaded as white (missing), grey (no symptoms) or from yellow (denoting early symptoms severity with severity score 2 or 3), through orange (intermediate symptoms, severity scores 4 or 5) to red (severe symptoms, severity score 6). Trees that have died from CWD and been cut back are shaded black.

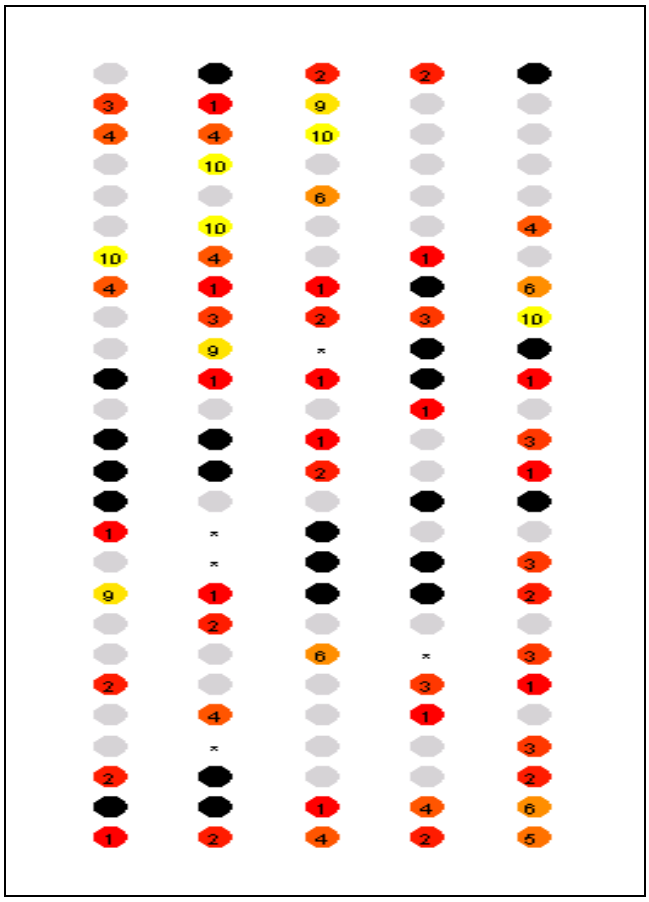


Figure 9. Graphical summary showing the spatial layout of trees and CWD development at an on farm mapping site in Mayuge, Uganda.

In this summary, the data for all 10 time points is combined into one map and highlights when CWD symptoms were first observed. Missing trees are again shaded white, dead trees black and trees that remain uninfected throughout the set of visits in grey. However, trees shaded red through to yellow started to exhibit symptoms (severity score 2 to 6) during the course of the study, those first developing symptoms in the earlier, intermediate and latter visits shown in red, orange and yellow respectively. The numbers in each spot states the visit at which symptoms were first noted.

5.6 Database development

Where available, information relating to the many fusarium isolates obtained through the project has been added to the *Excel* database. This database now holds comprehensive data on almost 300 fusarium isolates obtained from coffee plants in producing areas of East, Central and West Africa and provides a fundamental tool to support future decision-making with respect to CWD research and management in the region. The database is fully searchable and allows information relating to a particular attribute(s) (e.g. geographic origin, coffee host species, date of collection) to be extracted through selection of identifiable parameters.

A copy of the full database is available on request from the project leader.

6. Overview of findings and implications for CWD management

A number of very significant findings emerged from this research that may have important implications with regard to future approaches to managing CWD. In-depth genetic analysis of the pathogen undertaken at CABI UKC has revealed that, while overall variability is limited, two distinct forms of *F. xylarioides* exist in East and Central Africa. The first (referred to here as Type 'A') is found only in Ethiopia and only on *C. arabica*, while the second (Type 'C') occurs in Tanzania, Uganda and the Democratic Republic of Congo (DRC) and only on *C. canephora*. Variability within each of these two groups is very limited indeed, suggesting that they may represent two clonal lines. Several isolates obtained from *C. canephora* and *C. excelsa* in the 1950s and 1960s, towards the end of the previous CWD outbreak in West Africa, were found to be genetically distinct from either Type 'A' or Type 'C' isolates, suggesting that the *C. arabica* group, the *C. canephora* group and the historical isolates may have evolved independently. It is also possible that the re-emergence of CWD on *C. canephora* is due to a

genetic form of *F. xylarioides* that has evolved from the *C. arabica* group or, more likely, a genetic form(s) previously responsible for the earlier CWD outbreak in West Africa. Furthermore, limited studies of pathogenic variability undertaken during the project have also revealed that type 'A' is only pathogenic to *C. arabica* coffee variants while type 'C' is only pathogenic to *C. canephora*. i.e. cross-pathogenicity is not occurring and the two forms are exhibiting species specificity. It is also possible that the minor genetic difference found between these two forms, which preliminary studies suggest may be due to straightforward DNA insertions or deletions, may be linked to pathogenicity traits (see below also). One *F. xylarioides* isolate ('historical' isolate DSMZ 62457) assessed in this work exhibited pathogenicity to two coffee species other than that from which it was obtained. This isolate was recovered from *C. excelsa* affected by CWD in the Central Africa Republic in 1955 and was capable, through articificial inoculation, to elicit CWD symptoms on *C. arabica* (from Ethiopia and Kenya), *C. canephora* (from DRC, Ivory Coast and Congo) and *C.liberica* (from Costa Rica). It was also much more pathogenic to the *C.liberica* and Kenyan *C. arabica* than to the other coffee. This was not the case with a second isolate obtained from *C. excelsa* in Uganda in 2002, which was found to be pathogenic to *C. canephora* but not to *C. arabica*. While isolate DSMZ does exhibit host specialisation in terms of its aggressiveness to the different coffee species and lines, it was found to be pathogenic to a wider range of coffee than any of the other isolates assessed, all of which were since the re-emergence of CWD on *C. canephora*. It is therefore possible that, for whatever reason, the pathogen has become more specialised since the original outbreak on *C. canephora*. Isolates from *C. canephora* may also have become more aggressive to this particular species, as shown by the number of seedlings developing CWD following inoculation with isolates from *C. canephora* being much higher than following inoculation with DSMZ.

These findings have considerable implications with regard to the search for host resistance to CWD since the genetic base within the pathogen for which resistance is currently being sought would appear to be narrow. Theoretically, therefore, coffee showing resistance or tolerance to a type 'A' isolate from Ethiopia should show this trait against any type 'A' isolate, at least within Ethiopia. Similarly, resistance or tolerance to a type 'C' isolate from Tanzania, Uganda or DRC should exhibit this characteristic when challenged by isolates obtained from throughout the region. Host specificity operating within the pathogen may also explain why CWD has not been observed on *C. arabica* in Tanzania nor (although cultivation is limited) on *C. canephora* in Ethiopia. Should the genetic variability observed between the two forms of *F. xylarioides* be due to differences at specific DNA sites (e.g. intron or deletion), prospects of developing a rapid, DNA-based diagnostic technique appear good. Preliminary studies on this possibility have already been undertaken but further research is required. Unfortunately in-country studies of pathogenic variability within the two *F. xylarioides* forms were not possible during the project, and acquisition of further information on host specificity and aggressiveness in particular would be beneficial. However, based on the molecular approaches applied in this work and the limited variability observed, it is highly unlikely that any pathogenic variability existing within either the Type A or the Type C populations would be accurately or reliably reflected in genetic traits.

The project research has helped to clarify the mechanisms of CWD transmission and disease spread. Early indications from a number of small-scale on-farm and on-station trials established during the project suggest that wounds made with a machete (a multipurpose implement used daily by farmers) previously used to cut infected coffee wood would be sufficient to transmit the pathogen to, and induce CWD development on, healthy coffee, at least in the case of mature trees. Coffee wood pieces, an important source of fuel for farmers, can also act as a source of infection to young coffee seedlings where the wood is already affected by CWD, while infested field soil can remain infective to seedlings for at least three months after removal of affected coffee trees from fields. However, the infectivity of such soil, as shown by time taken for CWD symptoms to appear on newly planted seedlings, does appear to decline in the absence of an affected tree. Although the rate of development of the disease on seedlings was slow, with few plants developing symptoms, these plants (and perhaps other sources of inoculum) may be

sufficient to act as subsequent foci of infection from which CWD may rapidly spread. Despite speculation that insects may play a role in transmission of *F. xylarioides*, the fungus was not found on the body parts of a range of insects commonly found on coffee farms and associated with coffee itself, including pests, bees and termites. Nor was the fungus been found on a wide range of potential alternative hosts, including crops commonly cultivated alongside coffee and weed species occurring on coffee farms. These findings, while still somewhat preliminary, do generally confer with currently held perceptions on CWD and, more importantly, provide evidence to support current recommendations being promoted in some areas with respect to on-farm CWD management. These relate to regular cleansing of farm implements, at least following use on trees exhibiting CWD symptoms and prior to use on trees that appear to be healthy. This may be achieved by wiping down with a household disinfectant (e.g. 'Jic'), although this is often not possible or practical for many farmers. Heating tools in a fire is an alternative. Efforts should also be made to minimise inadvertent wounding of trees, including through the use of implements such as machetes, spades and hoes during planting and weeding, improper pruning and the tethering of domestic animals to tree stems or sufficiently close to coffee plants to cause damage. Diseased trees should be cut down as soon as CWD symptoms are first observed and preferably burnt *in situ*. Tree stumps should also be removed and burnt, and not left to rot (as is current practice) as they constitute a source of inoculum. Coffee wood is a valuable source of firewood for farmers, where unhealthy trees including those affected by CWD are often cut down and wood pieces stored as firewood on-farm, usually adjacent to the home, for many months. Where coffee wood is required for firewood on farms affected by CWD, this should be obtained in a manner that minimises inoculum spread – cutting into required lengths *in situ* is again recommended, as is transfer of cut wood to, and its storage at, the homestead in an enclosed container such as a bag or sack. If feasible, the wood should also be moved through an already affected part of the farm as opposed to disease-free areas. Depending on availability, both seeds and vegetative cuttings may be used as planting material. Although farmers and nursery operators prefer seeds, which are easier to handle, semi-soft wood cuttings from upright growing shoots are also taken from mother bushes maintained to provide such a supply. Planting materials, in the form of either vegetative cuttings or seedlings, are also distributed from private nurseries to farmers by local authorities. It is important to consider that while coffee material, including trees, stem pieces and seedlings, may not exhibit symptoms of CWD, it may still be contaminated or infected with *F. xylarioides* and therefore act as a source of infection. Even where symptoms are present these may not be recognizable to the inexperienced eye or may be overlooked. As such, local and national distribution systems may constitute an important and very efficient means of spread for CWD (and indeed other diseases and pests), and may be at least partly explain the rapid and widespread development of CWD in countries such as Uganda. It is recommended that nurseries and distribution systems be investigated further, specifically with respect to how farms from which seed[12] or cuttings may be obtained are selected and whether, and to what extent, contamination of plant material by *F. xylarioides* is assessed. New coffee plantings established with distributed plants should also be monitored to determine what risk they may present in terms of the disease.

In some areas affected by CWD, recommendations are already being made for farmers to leave land fallow or to replant with an alternative crop following removal of diseased trees. This would appear to be sound advice in terms of reducing the inoculum potential of soil (Appendix 2, Plate 15), certainly given the failure to recover *F. xylarioides* from the broad range of crops and weeds investigated to date. Indeed, a previous report of the fungus on Kayinja bananas has not since

[12] Coffee berries do not appear to constitute an important source of infection. CORI previously assessed 43 coffee berry samples obtained from 43 trees slightly, moderately and severely affected by CWD using standardised protocols whereby 400 berries per sample were plated onto PDA. *F. xylarioides* did not emerge from any of the berries. Plantlets were also raised from 50 berries from each sample. Of the plants raised from all of the berries, and after one year of growth, only one plant developed symptoms of CWD.

been confirmed. The work reported here suggests that previously affected fields should be kept as fallow or planted with an alternative crop(s) for a minimum of three months, but only continued monitoring of the established trials and experimental and controlled replanting of field sites will allow a more precise period to be defined. This could feasibly be several years. Where areas are replanted with coffee after implementing such measures, the *F. xylarioides* type originally present should be determined and coffee known to have resistance to that type planted if possible. Replanted areas should also be closely monitored for re-emergence of the disease and recommendations revised accordingly. Care should also be taken to prevent re-introduction of CWD and spread of the disease between farms generally, by restricting or preferably preventing exchange of planting material and sharing of uncleansed farm implements (particularly between disease-afflicted and disease-free areas). Insects associated with coffee, including coffee pollinators, have been considered as a possible means by which *F. xylarioides* may spread and, more significantly, a plausible explanation for the rapid geographic spread of the disease in countries such as Uganda. However, the investigations reported here suggest that this is not the case. As such, management recommendations relating specifically to insect transmission would appear unwarranted at this stage and indeed may be an unnecessary burden for farmers in particular. Further investigation of the role of insects, including continuation and expansion of the work already initiated, is recommended.

It must be appreciated, that some of the practices recommended above may be impracticable given farm resources, not least farmer access to labour. To ensure that measures are implemented in the most effective manner, CWD levels in differing coffee growing areas should be determined, the risk of further disease development (particularly to previously unaffected areas) ascertained, areas ranked with respect to the urgency to contain the disease and available resources mobilised. Where necessary and feasible, farmers should be provided with external resources, including personnel, to ensure that management procedures may be implemented as required.

In a number of the on-station and on-farm trials initiated through the project and described above, only a small proportion of coffee trees and seedlings planted in *F. xylarioides* infested soil, artificially inoculated with the pathogen or treated with contaminated implements or materials developed symptoms of CWD prior to termination of the project. In some instances this may simply be due to the relatively low inoculum levels involved (e.g. through wounding with a machete), or to the time required for symptoms to develop and become clearly visible. Discussions with CORI staff, however, have also suggested that poor failure of CWD to develop when it may otherwise have been expected may have been related to the onset of an unusually dry period coinciding with when the majority of the trials were initiated, in May 2004). Average daily rainfall during May 2004 was only 2 mm, as opposed to 3.8 mm and 5.3 mm in May 2002 and May 2003 respectively (see Section 5.5.4 and Figure 7). It may ultimately become necessary to repeat at least some of these trials, and this has already been discussed with CORI and is likely for 2005. With some modification, similar trials to those described above could also be established to assess the effects of using other farm implements, including a hoe and pruning saw. However, given the development of CWD observed on albeit a small number of plants in the current trials, and assuming that the prevailing conditions have not had an adverse and irrecoverable effect on the pathogen itself, further development of CWD symptoms may be observed over the coming months. It will therefore be equally important to continue to monitor these trials during 2005 (and preferably beyond), as was the intention when they were originally designed, to ensure that output is maximized and sound conclusions are drawn where possible. Funding for continued monitoring of the trials beyond the current project has already been requested in a research proposal submitted to DFID CPP in September 2004.

Isolation of *F. xylarioides* from soil, as an indicator of the existence of viable propagules of the pathogen, proved problematic throughout the project. Development of CWD symptoms on seedlings raised in soil was, albeit based on somewhat circumstantial evidence, found to be the

only means of confirmation. Successful development of a DNA-based diagnostic technique, while it would not differentiate between living and non-living fungal material, would constitute a major step forward in this respect. Further research is required to investigate why recovery of *F. xylarioides* from soil is problematic and to develop an effective approach to isolation.

Through the project, a large and comprehensive collection of fusaria from coffee has been established at CABI, while smaller collections have also been developed in Ethiopia, Tanzania and other countries affected by the disease. These collections provide the backbone for research on CWD, and therefore constitute an extremely useful resource for future research initiatives. It is important that they are adequately maintained and, where feasible, built upon.

The project has greatly enhanced the capacity of UK scientists and overseas counterparts to undertake research on CWD. This has been achieved directly, for example, through specific training components provided to Ethiopian and Tanzanian scientists on approaches to pathogenicity testing. However, it has also been achieved indirectly through the extensive interaction between members of the project team and between the project team and farmers, extension, scientists, traders, processors and other specialists with whom relationships were developed on-farm, in the laboratory and at conferences, workshops and meetings.

7. Contribution of Outputs to developmental impact

Overall, and considering delays incurred in the initiation of a number of in-country activities, the outputs of the project been achieved. Considerable new knowledge of the CWD pathogen has been gained, with respect to pathogen variability in particular, and may now be taken up in the wider context to tackle what is a highly destructive and rapidly spreading disease of a crop whose profitability has already been severely damaged by the recent decline in world coffee prices. The project outputs will be taken up by national coffee improvement programmes for validation, promotion and implementation. This will be achieved in part under the auspices of the CFC/EU RCWP, in which a component is devoted to the validation and promotion of improved CWD management methods for farmers. This component specifically addresses the development of extension programmes and training for small coffee producers, and is directly dependent on knowledge generated by activities undertaken through initiatives such as project R8188 and linked INCO-DEV project ICA4-CT-2001-10006. Regional workshops are held each year to summarise outputs from various activities under the RCWP, to identify those suitable for extension and dissemination, identify relevant promotional pathways and allocate resources to appropriate promotional activities. The process of promotion and dissemination of the findings of R8188 has already been initiated, directly and via the RCWP, through contributions to the regional and national workshops, international conferences, advisory and training materials and courses for farmers, extensionists and indeed scientists, preparation of posters, leaflets etc. and project reports.

As partners in the RCWP, the national programmes will be directly funded by the CFC and EU, at least until 2005, to develop, validate, promote and disseminate management measures for CWD. The RCWP will also be seeking to support, financially, the formulation, promotion and implementation of regional management strategies, including those relating to quarantine, and to promote new knowledge to the global coffee producing community. An application for funding has also been submitted to DFID CPP to facilitate synthesis of current knowledge on pests of coffee in East and Central Africa and development of up-to-date learning and advisory materials for uptake and application by coffee stakeholders. This application also includes a component to support continued management and monitoring of the field trials established under this project.

Uptake of the new knowledge generated by this project will allow more appropriate, effective, applicable and sustainanble management strategies to be developed, evaluated and ultimately applied to reverse the very destructive effects of CWD experienced to date. By enhancing the

capacity of those struggling top counteract the disease, it will help to safeguard future coffee production in Africa, maintain a vital source of revenue for resource-poor farming communities and others dependant on coffee production and stabilize national economies so dependant on the crop as an income generating commodity.

8. Publications

8.1 Internal Reports

RUTHERFORD, M.A. (2004). Epidemiology and variablility of *Gibberella xylarioides*, the coffee wilt pathogen. DFID CPP project R8188 Final Technical Report (in prep.). Egham, CABI Bioscience.

RUTHERFORD, M.A. (2004). Epidemiology and variablility of *Gibberella xylarioides*, the coffee wilt pathogen. DFID CPP project R8188 Project Completion Summary Sheet, December 2004. Egham, CABI Bioscience.

RUTHERFORD, M.A. (2004). Epidemiology and variablility of *Gibberella xylarioides*, the coffee wilt pathogen. DFID CPP project R8188 Project Progress Report 1, September 2004. Egham, CABI Bioscience.

RUTHERFORD, M.A. (2004). Epidemiology and variability of *Gibberella xylarioides*, the coffee wilt pathogen Progress report submitted to coffee wilt programme coordinator, June 2004. Egham, CABI Bioscience.

SENBATA, G. A. (2004). Cross-inoculation studies of the coffee wilt pathogen, *Gibberella xylarioides* (*Fusarium xylarioides*) with different *Coffea* spp. Progress report for CPP project R8118: Epidemiology and variability of *Gibberella xylarioides*, the coffee wilt pathogen.

ODUOR, G. AND AKIRI, M. (2004) Improvement of coffee production in Africa by the control of coffee wilt disease (tracheomycosis). Coffee wilt disease programme CFC/ICO/13: Progress report, January-June 2004. Nairobi, CAB International.

CAN DE KLASHORST, G. AND MREMA, H. (2004). Improvement of Coffee Production in Africa by Control of Coffee Wilt Disease. Regional Coffee Wilt Programme CFC/ICO/13: Mid-term evaluation, April 2004 (draft)

RUTHERFORD, M.A. (2004) R8118: Epidemiology and variablility of *Gibberella xylarioides*, the coffee wilt pathogen. DFID Crop Protection Programme, Annual Report. March 2004. CABI International, Egham, UK.

RUTHERFORD, M.A. (2004) R8118: Epidemiology and variablility of *Gibberella xylarioides*, the coffee wilt pathogen. DFID Crop Protection Programme, Project Progress Report 2. January 2004. CABI International, Egham, UK.

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RUTHERFORD, M A (2003) DFID CPP Coffee Wilt Project (R8188): Progress Report submitted to Regional Coffee Wilt Programme co-ordinator, July 2003. CABI International, Egham, UK.

FLOOD, J. (2003) R8118: Epidemiology and variablility of *Gibberella xylarioides*, the coffee wilt pathogen. DFID Crop Protection Programme, Annual Report. March 2003. CABI International, Egham, UK.

FLOOD, J. (2003) R8118: Epidemiology and variablility of *Gibberella xylarioides*, the coffee wilt pathogen. DFID Crop Protection Programme, Project Progress Report 2. January 2003. CABI International, Egham, UK.

FLOOD, J. (2002). Epidemiology and variablility of *Gibberella xylarioides*, the coffee wilt pathogen. DFID CPP project R8188 Project Progress Report 1, September 2002. Egham, CABI Bioscience.

8.2 Journal publications

RUTHERFORD, M.A., BUDDIE, A., CROZIER, J. AND FLOOD, J. (2005) Genetic variability in *F. xylarioides*, the causal agent of coffee wilt disease (*in prep*.).

*RUTHERFORD, M.A., BUDDIE, A., CROZIER, J. AND FLOOD, J. (2005) Current knowledge of coffee wilt disease, a major constraint to coffee production in Africa (in prep).

8.3 Oral presentations

RUTHERFORD, M.A., BUDDIE, A., INESON, J., CROZIER, J. & FLOOD, J. (2004). Regional Coffee Wilt Programme: CABI UK Centre Research Activities. Regional Coffee Wilt Programme Stakeholders' and Planning Workshop, Nairobi, Kenya, Nairobi, 9-13 November 2004 (*presented by M.A.Rutherford*).

RUTHERFORD, INESON, J., BUDDIE, A. & FLOOD, J. (2004). Regional Coffee Wilt Programme: CABI UK Centre Research Activities. Regional Coffee Wilt Programme Stakeholders' and Planning Workshop, Nairobi, Kenya, 3-5 December 2003 (*presented by M.A.Rutherford*).

FLOOD, J., RUTHERFORD, M., BUDDIE, A., BRAYFORD, D., INESON, J., CANNON, P., HAKIZA, G., BYESSE, D. AND KALONJI-MBUYI, A. (2003). Fusarium wilt of coffee in Africa - re-emergence of an old problem. International Fusarium Symposium, Sydney, Australia, February 2003. Egham, CAB International. (*presented by J. Flood*).

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8.4 Workshop proceedings

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8.5 Websites

CABI commodities website: http://www.cabi-commodities.org/Coffee/Cfp/Cfp.htm

8.6 Listing and reference to key datasets generated:

* RUTHERFORD, M. A. (2004) Electronic Excel data files of baseline information relating to *Fusarium xylarioides* and other fusaria isolated from coffee affected by coffee wilt disease in Africa. CABI Bioscience, Egham, Surrey, UK.

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Appendix 1

Cross-Inoculation Studies of the Coffee Wilt Pathogen, *Gibberella xylarioides* (*Fusarium xylarioides*) with Different *Coffea* spp.

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Introduction

Coffee wilt disease (CWD) also tracheomycosis is a typical vascular disease incited by the fungus *Gibberella xylarioides* (*Fusarium xylarioides*) is known to attack almost all species of coffee. Since the late 1980s, the disease re-emerged as one of the major threats to coffee production in Africa causing serious losses of Arabica and Robusta coffee in various countries of East and Central Africa, mainly in Uganda, Democratic Republic of Congo (DRC), Tanzania and Ethiopia. Surveys conducted in these countries implicated the presence of regional variation in CWD incidence and severity across the affected region. In Uganda and the Democratic Republic of Congo only Robusta coffee was seriously attacked (Flood 1996, 1997) while Arabica coffee trees was found infected in Ethiopia (Girma 1997). The results of very recently conducted greenhouse experiments in Ethiopia, consisting of 10 *G. xylarioides* strains from Arabica and 1 isolate from Robusta indicated host specificity in the CWD pathogen populations (Girma et al. 2002). Thus, in order to manage the coffee wilt problem in sustainable ways, it is imperative to carry out detailed cross inoculation experiments and investigate regional diversity and host specificity of the pathogen populations in Africa. The main objective of this investigation was to determine regional diversity of the coffee wilt pathogen *Gibberella xylarioides* (*Fusarium xylarioides*) strains collected from *Coffea* spp. in different regions of East and Central Africa.

Materials and Methods

The cross inoculation experiment was undertaken in 3 sets comprised of different combinations of *G. xylarioides* (*F. xylarioides*) strains and *Coffea* spp. (Table 1) representing almost the regions where tracheomycosis is a serious problem. Coffee seedlings were raised from cultivars/lines of the respective *Coffea* spp. in Humin substrate (Neuhaus) and vermiculite mixed in a 1:2 ratio in the glasshouse at CIRAD, Montpellier, France. Almost all seedlings were inoculated at fully expanded cotyledonary leaf stage by stem inoculation method with spore suspensions of each strain, adjusted to 2×10^6 per ml (Girma et al. 2000).

About 25 – 30 seedlings/cultivar or line per isolate were used and 3 – 5 seedlings treated with sterile water were included as check in the same box. The inoculated seedlings were then kept in a growth chamber with 12 hr light/dark cycle at 25 ^{o}C temperature. The number of seedlings with characteristic wilting symptoms were recorded every two weeks interval for about 5 to 6 months following disease development.

Results

In this study, *G. xylarioides* strain collected from Arabica coffee in Ethiopia, isolate G3P22, infected only the Arabica coffee cultivars, *cvs*. coded as 1686 and 1688, without any wilting symptoms on the Robusta lines. On the other hand, stains isolated from Robusta coffee in Uganda (CAB003) and in Tanzania (TZ009) showed characteristic infection symptoms of tracheomycosis only on the Robusta lines obtained from Democratic Republic of Congo (1650 and 1655) and Ivory Coast (1669 and 1670). Some seedlings of Arabica and that of Robusta coffee inoculated with the old strain of the fungus collected from Excelsa coffee (DSMZ) showed wilting symptoms (Table 2).

Similar trends were observed in set II where infections of Arabica cultivars, *cvs*. 1689 and 1690 with the strain isolated from Arabica coffee (G3P22), both from Ethiopia. Seedlings of Robusta lines (1669 and 1670) obtained from Ivory Coast infected with *G. xylarioides* strains of Robusta coffee in Uganda (CAB003), Tanzania (TZ009) and DR Congo (RDC002) (Table 3). The recently isolated strain from Excelsa coffee in Uganda (isolate OUG152) demonstrated clear wilting symptoms and high seedling deaths the Robusta lines but not on seedlings of the Arabica cultivars (Table 4). These host-pathogen interaction and specificity pattern were also obsereved in set III experiment. In this set of experiment, the old strain DSMZ collected from Excelsa coffee in Central African Republic caused high infection on *C. liberica*. However this isolate did not infect seedlings of Arabica and Robusta coffees.

Table 1. *Coffea* spp/ lines and *G. xylarioides* strains used in cross inoculation experiment at CIRAD, Montpellier, France

Coffea spp.				*G. xylarioides* strains		
SET	*Coffea* spp.	Country	CIRAD code	Strain	Host species	Country
I	*C. arabica*	Kenya	1686 1688	G3P22 CAB003	*C. arabica* *C. canephora*	Ethiopia Uganda
	C. canephora	DR Congo	1650 1653	TZ009 DSMZ	*C. canephora* C. excelsa (old)	Tanzania CAR
		Ivory Coast	1655 1673			
II	*C. arabica*	Ethiopia	1689 1690	G3P22 CAB003 TZ008	*C. arabica* *C. canephora* *C. canephora*	Ethiopia Uganda Tanzania
	C. canephora	Ivory Coast	1669 1670	RDC002 OUG152	*C. canephora* *C.excelsa*	DR Congo Uganda
III	*C. arabica*	Costa Rica	1716 1743	G3P22 CAB003	*C. arabica* *C. Canephora*	Ethiopia Uganda
	C. canephora	DR Congo	1620 1626	DSMZ	C. excelsa (old)	CAR
	C. liberica	Costa Rica	1722 1723			

Table 2. Percent infection of seedlings of *C. arabica* and *C. canephora* inoculated with *Gibberella xylarioides* strains from different hosts in a growth room at CIRAD, set – I experiment

Coffea spp.	CIRAD Code	Country of Origin	*G. xylarioides* strains			
			C. arabica G3P22	*C. canephora*		*C. excelsa* DSMZ (Old)
				CAB003	TZ009	
C. arabica	1686	Kenya	90.0	0.0	0.0	58.3
	1688		93.3	0.0	0.0	66.7
C. canephora	1650	DR Congo	0.0	61.3	34.5	13.3
	1653		0.0	41.9	74.2	6.9
	1655	Ivory Coast	2.7	70.0	61.9	2.8
	1673		0.0	19.4	11.4	0.0

Table 3. Percent infection on seedlings of *C. arabica*, *C. canephora* and *C. liberica* inoculated with *G. xylarioides* strains in agrowth room at CIRAD Set – III experiment

Coffea spp.	CIRAD Code	Country of origin	*G. xylarioides* strains				
			C. arabica G3P22	*C. canephora*			*C. excelsa* OUG152
				CAB003	TZ008	RDC002	
C. arabica	1689	Ethiopia	53.3	0.0	0.0	0.0	0.0
	1690		30.0	0.0	0.0	0.0	0.0
C. canephora	1669	Ivory Coast	4.0	63.0	48.1	59.3	65.4
	1670		8.3	21.7	38.5	46.2	40.9

Table 4. Percent infection of seedlings of *C. arabica*, *C. canephora* and *C. liberica* inoculated with *G. xylarioides* strains in agrowth room at CIRAD Set – III experiment

Coffea spp.	CIRAD code	Country of origin	*G. xylarioides* strains		
			C. arabica G3P22	*C. canephora* CAB003	*C. excelsa* DSMZ
C. arabica	1716	Costa Rica	95.5	0.0	4.2
	1743		85.2	0.0	33.3
C. canephora	1620	DR Congo	0.0	51.9	15.4
	1626		0.0	42.3	0.0
C. liberica	1722	Costa Rica	5.0	100	80.0
	1723		42.1	8.3	94.4

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