INCO : International Scientific Cooperation Projects

Contract number : ICA4-CT-2001-10006

Development of a long term strategy based on genetic resistance and agroecological approaches against Coffee Wilt Disease in Africa.

Fourth **annual report:** covering period 1/11/2004 to 31/10/2005

Acronym: COWIDI

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Key words: *Fusarium xylarioides*, coffee wilt disease, *Coffea canephora*, genetic resistance.

Report n° 7 - 2006
CIRAD/AMIS
UMR : BGPI

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COWIDI INCO Project YEAR 4

SUMMARY

Coffee wilt disease is the major constraint to robusta culture in the different partner countries. The disease spread quickly in Tanzania. In Ethiopia, arabica culture area, the disease remained at a very low threshold for many years, but the damage increased significantly since the end of 1990s. Consideration of the status of *C. arabica* is essential, because to develop resistance it is necessary to accumulate résistance genes for the two forms of the pathogen. Currently, every form of *Fusarium xylarioides* is species specific. But there are some indicators leading to the thinking that the evolutionary potential of the fungus can drive to emergence of new virulence with no specific species reaction.

Variety resistance is considered the best option for an effective control of the coffee wilt disease and therefore the main objective of this project remains, to develop a global strategy to fighting the disease, based on durable tolerance/resistance, that is adapted to small holder agro-system and to the economic conditions prevailing in Africa.

In year 4, the work carried out built on results obtained in year 3. i.e:

1- Study the genetic diversity of the pathogen with different technics.
2- Assess the aggressiveness of isolates and characterize interactions
3- Identify coffee trees bearing resistance factors
4- Analyse genetic diversity of *Coffea canephora* from Uganda
5- Analyse disease distribution in the field
6- Assess correlations between field resistance and artificial inoculation tests

Genetic variability was assessed among 15 single-spore derived isolates obtained from wild *C. canephora* trees suspected affected by CWD in Ngogo-Kibuguta and Itwara primaries forests of western Uganda. Phylogenetic analysis, based on ITS1-P3 sequences revealed 6 isolates with 100% of homology with reference strain CAB003.

The discovery of wild *C. canephora* plants exhibiting symptoms of CWD in forest sites in western Uganda and confirmation of the presence of *F. xylarioides* in at least some of these plants raises questions as to the origin of the disease in the country and perhaps elsewhere. For many decades and perhaps centuries, forests have been viewed as a source of virgin planting material from which seeds and other parts of species including *Coffea* species were obtained and subsequently cultivated, some on a major scale. Indeed, coffee berries were collected for the purposes of trade. Such material would, presumably, have appeared health, including with regard to pests and diseases such as CWD. Depending on the length of time over which *F. xylarioides* has been associated with wild coffee, it is feasible that the pathogen has co-evolved with its host and, at some stage in time, carried with planting material removed from forest areas to be cultivated in areas adjacent to forest areas and subsequently further afield. In the case of *C. canephora,* transfer may have occurred during the 1920s and 1930s, when when plantations of the species first became established. If so, co-evolution may offer prospects in terms of a pool of resistance genes perhaps being available amongst wild coffee. A simpler explanation may be, of course, that the forest coffee has become affected by CWD subsequent to emergence of the disease on cultivated coffee, perhaps through the intervention of man and perhaps relatively recently.

The various molecular approaches, through RAPD, microsatellites markers analysis and sequencing of MAT-1 and MAR-2 gene, TEF 1-α, calmodulin and histone 3 genes, ITS, applied by the project partners continue to unravel the underlying genetic variability existing within the CWD pathogen. They have further clarified the taxonomic structure within the *F. udum* and *F. lateritium* complex, and helped to more accurately determine the nature (species or otherwise) of isolates that have proved difficult to identify on the basis of morphology, including some that appear to have been misidentified previously. Of note, the recent results of the mating tests and MAT-2/TEF sequencing has shown that *G. xylarioides* encompasses at least three apparent groups: *G. xylarioides* sensu strictu Ia (defined by historical West African isolates CBS 25852 and CBS 74979); *G. xylarioides* sensu strictu Ib (defined by historical Central African isolates DSMZ 62457 and ATCC 15664; and *G. xylarioides* sensu lato II (DRC, Ugandan, and Tanzanian *C. canephora and C. excelsa* isolates). These three groups was define like the *Giberella xylarioides* complex and permit their placement within the *G. fujikoiri* complex.

Assessment of 20 clones in the field trial at Kituza still found clone J/1/1 resistant to coffee wilt disease and clone Q/3/4 had only 4.2% mortality). These clones are being propagated for further evaluation in other locations. In contrast, assessment of 35 specific cross hybrid progenies in the field hybrid trial did not identify any progeny completely resistant to CWD.
Representative genotypes of wild coffee from primary forests were collected from 5 populations in Kibale forest and 6 populations of Itwara forest. Sample genotypes were also collected from 4 populations in Kalangala forest.

The study on genetic diversity of the *C. canephora* of the Ugandan wild genotypes were, using 25 microsatallites markers revealed that Ugandan *canephora* were different from other known groups (Congolese and Guinean) and the nganda and erecta types from Uganda being different from each other and also different from the forests genotypes. Results also found distinct variations between coffee from different forests but not significant difference between populations within Itwara or Kalangala forests. However there were distinct differences between populations from Kibale forest.

The pathogenicity tests indicate confirm a host specificity of contemporary isolates. Isolates collected on *C.arabica* are pathogenic on *C. Arabica* and isolates from *C. canephora* are pathogenic on *C. canephora*. Furthermore, one "historical" isolate collected on *C.excelsa* in CAR (1950) was found to be cross pathogenic on *C. excelsa*, *C.arabica* and *C. canephora*.

In UGANDA and DRC, systematic screening was carried out on cuttings and open pollinated seedlings progenies are in progress. Individuals still alive after inoculation are planted in mother garden for vegetative propagation. Resistant genotypes identified in previous screening were planted out in mother gardens and are being propagated vegetatively for further evaluation in field trials.

In Uganda, hybrid progenies of specific crosses between CWD resistant (J/1/1 and Q/3/4) and susceptible (E/3/2 and 257s/53) are being raised in coffee nursery for inoculation and analyzing inheritance of CWD resistance.

Both in Uganda and DRC, observations on farms indicate an increase in wilt incidence. The human activities are clearly correlated to the spread of the disease. The survival of the fungus in infected wood in at least 6 months. The survival in the soil is still unknown.

Year 4: November 2004 – October 2005

Introduction

Coffee wilt disease is the major constraint to the robustaculture in the different partners 's countries. The disease spread quickly in Tanzania. In Ethiopia, arabicaculture area, the disease stayed to a very low threshold during a lot of years, but the damage increase significantly since end of 1990s. The consideration of the status of *C. arabica* is essential, because to develop resistance strategies it's necessary to cumulate résistance genes to the two forms of the pathogen. At present time, every form of *Fusarium xylarioides* is species specific. But some indicators draw to think that the evolutionary potential of the fungus can drive to emergence of new virulence with no specific species reaction.

The collect of wild C. canephora in primaries forests in Uganda or hot spots in DRC and Uganda, to enrich germplasm is pursued.

Variety resistance is considered the best option for an effective control.

The main objective of this project remains, to develop a global strategy to fighting the disease, based on durable tolerance/resistance, that is adapted to small holder agro-system and to the economic conditions prevailing in Africa.

En année 4, les travaux engages s'inscrivent dans la suite des résultats obtenus en année 3:

Study the genetic diversity of the pathogen with different technics.
Assess the aggressiveness of isolates and characterize interactions
Identify coffee trees bearing resistance factors
Analyse genetic diversity of *Coffea canephora* from Uganda
Analyse disease distribution in the field
Assess correlations between field resistance and artificial inoculation tests

WP1: Pathogen diversity

Task 1 (CORI-UNIKIN with participation of CIRAD-CABI): Survey and collection of anamorphic and teleomorphic forms of the fungus on various parts of trees, possibly on alternative hosts, in infested regions. Maintenance and dispatch to European labs.

During this period, partners continued to enrich the collection of *Fusarium xylarioides*

Task 2: (CABI) Identification, storage, and exchange of isolates. Use a designed facility to ensure the successful maintenance of the pathogen strains. All data used to characterize the strains to be maintained on a database.

An extensive collection of more than 300 isolates of various *Fusarium* species has been established at the CABI UK Centre, maintained over the short to medium term on synthetic nutrient agar (SNA) slopes at 5°C. These isolates are considered representative of the range of *Fusarium* species, geographic origins, host plant species/clones and time of recovery from CWD affected trees in relation to the larger collection available. Six *F. xylarioides* isolates obtained from *C. arabica* in Ethiopia and included in analyses of genetic variability have also been deposited. Isolates obtained from trees forming an on-farm site in Uganda where CWD spread has been monitored since 2002 will also be deposited over the coming months.

The CABI collection will continue to be maintained for the duration of the project and should also provide an invaluable source of reference material for researchers throughout the world for many years after project completion.

Task 3 (CORI-UNIKIN CIRAD-UCL): Evaluation of the variability in isolate aggressiveness using standard inoculation tests.

Differences were observed in the symptom precocity and the leaves drying induced by different isolates but no difference was observed with the percentage of dead plants due in fact to the strain variabilty(UNIKIN).

Task 4: (CIRAD, CORI, UNIKIN UCL) Description of the fungal life cycle, asexual and sexual phases.

At UCL an investigation of mycelial growth, has shown varying effects of temperature on colony growth *in vitro*. While similar optimal and maximum growth temperatures (25°C and 32.5°C respectivey) were observed for contemporary isolates. In particular, the maximum temperature for growth of some historical isolates was higher than for others. These findings, which may relate to adaption to specific environments, also have implications with regard to pathogen characterisation, including the use of pathogenicity screening.

At UCL earlier attempts to cross individual isolates of *F. xylarioides in vitro*, supported by an investigation of the mating type (*MAT*) gene within the fungus, suggested that the coffee wilt pathogen is heterothallic corresponding to Booth female strains. Further genetic studies have now shown that genetical material of both parents used in these crosses can be present among ascopore progeny, confirming that the progeny can indeed arise through sexual recombination.

At UoK (DRC) a study of the role of *F. xylarioides* and other fusaria in development of CWD by observation of morphological attributes, growth rate *in vitro* (on agar medium), pathogenicicity testing (under glasshouse conditions) and genetic sequencing of the ITS1, ITS2 and TEF 1-α regions,.examination of the production and form of conidia, along with colony growth, separated isolates into two groups and facilitated identification to species level. The first group comprised slower growing isolates of *F. xylarioides* and *F. stilboides*, the second other fusaria including *F. equiseti*, and *F. falciforme*. Molecular analyses generally supported the grouping and species designations, and also confirmed the findings of project partners in that the *F. xylarioides* isolates formed a genetically homegeneous group which differed genetically to the fusaria. When inoculated onto susceptible coffee plantlets, the *Fusarium sp.* differed in terms of the time take for symptom appearance, the severity of symptoms and mortality. *F. xylarioides* alone induced symptoms characteristic of CWD and caused death of treated plants.

Task 5 (CIRAD-CABI-UCL): Evaluation of genetic diversity using a range of techniques including PCR and microsatellites.

Evaluation of genetic deversity at UCL was undertaken through RAPD analysis and sequencing of MAT-1 and MAR-2 gene, TEF 1-α, calmodulin and histone 3 genes. These approaches enabled separation of *F. xylarioides* isolates and other fusaria, including *F. udum* and *F. phyllophilium*, into putative subdivisions or clades.

The results, were somewhat inconsistent RAPD, calmodulin and histone analysis, for example, could discriminate between recent *C. canephora* isolates and recent *C. arabica* isolates. To varying extents these approaches could also differentiate between these recent isolates and/or the historical isolates as well as other fusaria. While TEF analysis failed to differentiate between recent *C. canephora* and recent *C. arabica* isolates, but separated the historical isolates and other fusaria. It also showed that the *G. xylarioides* complex (GxC) is nested within a larger group comprising *F. udum, F. phyllophilium* and other fusaria, with two clades making up the GxC: one comprising the recent DRC, Ethiopian, Tanzanian and Ugandan isolates from *C. arabica*, *C. canephora* and *C. excelsa*, along with historical isolates DRC (ex. *C. canephora*) and Ethiopia (ex. *C. arabica*). The second comprises two sub-groups for the historical isolates from western Africa. Calmodulin and H3 analysis also revealed a number of isolate groupings/clades. Of note, the placement of the GxC as a sister clade to the *F. udum/Fusarium* species complex was consistent.

In conclusion, the results allow at the identification of at least three distinguishable groups of strains within *G. xylarioides* populations and their placement within the *G. fujikoiri* species complex.

At CIRAD, genetic variability was assessed among the 15 single-spore derived isolates obtained from wild *C. canephora* trees suspected affected by CWD in Ngogo-Kibuguta and Itwara primaries forests of western Uganda.

Phylogenetic analysis, based on ITS1-P3 sequences, revealed five subgroups within the two main groups as follows:

- *Subgroup 1: A F. xylarioides* group, comprising the three historical strains (DSMZ 62457, ATCC 15664, CBS 74979), contemporary strain CAB003 and six isolates from wild *C. canephora* (OUG163, OUG164, OUG165, OUG166, OUG182 and OUG184
- *Subgroup 2:* Isolates OUG180-1, OUG180-2, OUG175, OUG170, OUG178-1 and OUG178-2, which appear to be fusaria other than *F. xylarioides.*
- *Subgroup 3: F. decencellulare* reference strain CAB010
- *Subgroup4:* Two *F. equisetii* reference strains
- *Subgroup 5:F.lateritium* reference strain and isolate OUG 186, from Itwara forest, which shows BLAST homology with *F. lateritium*

The discovery of wild *C. canephora* plants exhibiting symptoms of CWD in forest sites in western Uganda and confirmation of the presence of *F. xylarioides* in at least some of these plants raises questions as to the origin of the disease in the country and perhaps elsewhere. For many decades and perhaps centuries, forests have been viewed as a source of virgin planting material from which seeds and other parts of species including *Coffea* species were obtained and subsequently cultivated, some on a major scale. Indeed, coffee berries were collected for the purposes of trade. Such material would, presumably, have appeared health, including with regard to pests and diseases such as CWD. Depending on the length of time over which *F. xylarioides* has been associated with wild coffee, it is feasible that the pathogen has co-evolved with its host and, at some stage in time, carried with planting material removed from forest areas to be cultivated in areas adjacent to forest areas and subsequently further afield. In the case of *C. canephora,* transfer may have occurred during the 1920s and 1930s, when plantations of the species first became established. If so, co-evolution may offer prospects in terms of a pool of resistance genes perhaps being available amongst wild coffee. A simpler explanation may be, of course, that the forest coffee has become affected by CWD subsequent to emergence of the disease on cultivated coffee, perhaps through the intervention of man and perhaps relatively recently.

Task 6 (CABI): Synthesis of the results of all above tasks in order to propose an explanation for the evolution of the fungus

The various molecular approaches applied by the project partners continue to unravel the underlying genetic variability existing within the CWD pathogen. They have further clarified the taxonomic structure within the *F. udum* and *F. lateritium* complex, and helped to more accurately determine the nature (species or otherwise) of isolates that have proved difficult to identify on the basis of morphology, including some that appear to have been misidentified previously. Of note, the recent results of the mating tests and MAT-2/TEF sequencing has

shown that *G. xylarioides* encompasses at least three apparent groups: *G. xylarioides* sensu strictu Ia (defined by historical West African isolates CBS 25852 and CBS 74979); *G. xylarioides* sensu strictu Ib (defined by historical Central African isolates DSMZ 62457 and ATCC 15664; and *G. xylarioides* sensu lato II (DRC, Ugandan, and Tanzanian *C. canephora and C. excelsa* isolates). How the observed genetic variability relates to the various genetic and non-genetic definitions applied to fungi, including species, subspecies and *formae speciales*, is still not clear.

WP1 : Pathogen diversity : Milestones

Achievements

Task 1.
Collection of samples from the different growing zones is virtually finished.
Samples from new infested zones in DRC are currently undergoing isolation.
Collection of samples from Ugandan primaries forests is ended.
Long term keeping is operational.

Task 2
Development of an Excel electronic data base available for each partner

Task 3
Confirmation of the variability in isolate aggressiveness.

Task 4
Confirmation: *G. xylarioides* is a heterothallic fungus
In vitro crosses of *F.xylarioides* isolates, confirm that the progenies can indeed arise through sexual recombination.
F. xylarioides alone induce symptoms characteristics of CWD

Task 5
Genetics markers MAT1, MAT2, TEF, CL, H3 permit to separate F.xylarioides isolates and others Fusarium sp.
These results allow to distinguished 3 groups of strains within Fusarium xylarioides and the proposition of a *Giberella xylariodes* complex, and their placement within the *Giberella fujikoiri* species complex.
Identifcation of *F. xylarioides* in Uganda primaries forests.

Task 6
Confirmation of a fungus specialisation to the host species
Confirmation of sexual type that can origin recombination mechanism
Definition of the *Giberella xylarioides* complex
Clarification of the taxonomic stucture within the *Giberella fujikoiri* complex.

Still to be done

Task 1
To complete the collection of strains collected on identified clones.

Task 3
Check the aggressivness of isolates from Ugandan primaries forests.

Task 4
Check the variability and aggressiveness of ascospores produced when strains of opposite mating types are compared.

5
Ennance knowledge of crosses between "canephora" x "arabica" isolates.
Analyse the progeny of fertile crosses.

WP2 - Host/Pathogen interaction

I (CIRAD-UCL-CORI-UNIKIN) Identification of *Fusarium* isolates representing a wide range of host susceptibility/resistance by screening tests on seedlings both in Africa and in Europe using available germplasm.

The results observed confirm the host specificity of isolate CAB003, a contemporary strain collected from *C. canephora*, inducing symptoms on *C. canephora* only (trial 57).

Isolate CAB007 induced symptoms on the Arabica progenies tested, except with totally resistant genotype ET19. That genotype displays cumulative resistance to CAB007 and DSMZ. Isolate DSMZ overcomes the resistance of the other 4 *C. arabica* genotypes and expresses greater aggressiveness than isolate CAB007.

The ability of isolate DSMZ to induce symptoms on the three species (*C. canephora, C. deweivri, C. arabica*) is confirmed.

Task 2 (CORI-UNIKIN) Conduct similar field inoculations in Africa to validate the inoculation method

An assessment of the resistance of *canephora* coffee trees to isolate CAB003 revealed substantial variation in susceptibility within this species, ranging from total resistance to considerable susceptibility. An assessment of the resistance of the cross-fertilizing species *C. canephora* is not easy, because the seeds are derived from uncontrolled pollination. Consequently, only the maternal effect is assessed. Nevertheless, assessing resistance remains a strong indicator for detecting genotypes displaying resistance traits, particularly when infection rates are around 0 to 10%. Selection thresholds will be defined for identifying candidate genotypes displaying a good probability of having traits of resistance to vascular wilt disease.

A few genotypes, LK56/1, S19/29, and TR-CI 22/21, display good levels of resistance, notably cumulative resistance to isolates CAB003 and DSMZ.

Task 4 Combine all these observations to propose a hypothesis on the nature of the resistance

This result indicates that the "historical" *Fusarium* population, particularly DSMZ, displays quite a broad host spectrum, compared to the contemporary population, which seems restricted to specific reactions. We put forward the hypothesis that the contemporary population could have arisen from a strong foundation effect that counter-selected an isolate specific to the species *C. canephora*. This result also clearly shows that the contemporary strains of *Fusarium xylarioides* display a potential to evolve towards the acquisition of complementary virulence that could prove pathogenic on other *Coffea* species. The question is raised as to the existence of races within the species *Fusarium xylarioides*. For the moment, we do not have available the plant material needed to detect races, as it requires genotypes propagated by vegetative multiplication, or obtained by controlled pollination.

WP2 : Host/Pathogen interaction

Achievements

Task 1
- Confirmation d'une spécificité d'hôte chez les souches contemporaines collectées sur une espèce.
- Absence de spécificité d'hôte chez l'isolat historique collecté sur C. excelsa.

Task 2
- Planting of healthy plants after double inoculation in an infected area is in progress (Kituza experimental station) (CORI).
-

Task 4
Idem to WP1, task 6
Hypothesis of a fungus specialisation to the host species

Still to be done

Task 1
- Pursuit search for any specific reactions on a range of hosts.
- Analyze resistance of C. arabica

Task 2
- Increase the number of genotypes inoculated in the greenhouse to confirm field resistance (CORI-UNIKIN)
- Poursuivre l'installation au champ des boutures résistantes sélectionnées par inoculations artificielles

Task 4
- Pursue analyse on hypothesis on the nature of the resistance.

WP3 - Breeding for resistance

WP3 – Breeding for resistance

Variety resistance is still considered the most appropriate and long method of controlling coffee wilt disease. The search involved acquisition and assessing locally available germplasm in Uganda and the DR. Congo and exotic materials outside the two countries.

Task 1: Identify sources of resistance through field assessment

UGANDA
Assessment was carried out in field trials of *C. canephora* clones and specific cross hybrid progenies at Kituza. Assessment of 20 clones in the field trial at Kituza still found clone J/1/1 resistant to coffee wilt disease and clone Q/3/4 had only 4.2% mortality (1 out of 24 trees died). These clones are being propagated for further evaluation in other locations. The study on this trial also shows that different clones have varying levels of resistance and that CWD resistance is controlled by many genes. Assessment of 35 specific cross hybrid progenies in the field hybrid trial did not identify any progeny completely resistant to CWD but found individuals among progenies that are still resisting. The trials also revealed varying levels of mortality between the progenies. Monitoring the trees that are still surviving will continue.

Task 2: Collect seeds and cuttings from representative genotypes of available germplasm

UGANDA
Seeds and cuttings were collected from genotypes in wilt devastated gardens in Iganga, Wakiso and Kibale districts and they are being raised in the nursery for later inoculations. The study on genetic diversity of the *C. canephora* in co-working with CIRAD continued. Representative genotypes of wild coffee from primary forests were collected from 5 populations in Kibale forest and 6 populations of Itwara forest. Sample genotypes were also collected from 4 populations in Kalangala forest. The wild genotypes were, together with genotypes of the nganda and erecta *canephora* types from the collections at CORI, analyzed for genetic diversity using 35 microsetallite markers although only 25 markers gave good results. Representative genotypes of the known Guinean and Congolese *canephora* groups were included this study as controls. The analysis found Ugandan *canephora* different from other known groups (Congolese and Guinean) and the nganda and erecta types from Uganda being different from each other and also different from the forests genotypes. Results also found distinct variations between coffee from different forests but not significant difference between populations within Itwara or Kalangala forests. However there were distinct differences between populations from Kibale forest.

Cuttings and seeds collected from the representative genotypes of each of the forests are being raised in the coffee nursery at CORI and they will be inoculated for screening against coffee wilt disease. Any resistant genotypes identified will be included in the genetic improvement program for incorporating resistance into the current commercial clones.

DRC
Seeds from Kiyaka was receipted to UNIKIN Garden for planting and inoculate.

Task 3: Conduct screening tests in both Africa and Europe using isolates with a wide range of aggressiveness.

UGANDA
Systematic screening was carried out on young rooted cuttings of 85 genotypes and 31 open pollinated seedlings progenies raised from the germplasm plots at CORI. Rooted cuttings of 117 genotypes and 45 seedling progenies collected from trees surviving in wilt devastated gardens were also screened. A number of individuals were still alive after the second inoculation by the end of this reporting period. Data collection will however continue and wilt resistant individuals or clones will be identified and planted in mother garden for vegetative propagation. Resistant genotypes identified in previous screening were planted out in mother gardens and are being propagated vegetatively for further evaluation in field trials.

Task 4: Establish multi-location field trials of wilt resistant/tolerant varieties.

UGANDA
Clones Q/3/4 and J/1/1/ that have shown field resistant at Kituza have been cloned and included in multi-location field trials planted in Kyenjojo, Bushenyi, Hoima and Kanungu districts. Assessment on the trials is progress.

DRC
To cofirm the resistance of genotypes inoculated by artificial inoculation, seeds are in dispatching in different hot spots infected by CWD (Beni, Isiro).

Task 5: Study inheritance of CWD resistance

UGANDA
Hybrid progenies of specific crosses between CWD resistant (J/1/1 and Q/3/4) and susceptible (E/3/2 and 257s/53) are being raised in coffee nursery for inoculation and analyzing inheritance of CWD resistance.

WP3: Breeding for resistance: milestones

Achievements

Task 1
- Identification of various levels of field resistance (clonal trial CORI)
- Identification of resistant clone J1/1 and Q3/4 (clonal trial CORI)
- Vegetative multiplication of "survivors" plants resistant after double artificial inoculation in a mother garden (CORI)
- Dispatching in various hot spots of J1/1 and Q3/4, to confirm resistance

Task 2
- Prospection of wild coffee plants in primary forests, Kibale, Itwara, Kalangala
- Genetic analysis using microsatellites markers of wild Ugandan coffee germplasm
- Ugandan C. canephora different from other knows groups

Task 3
- Identification of resistant progenies (CORI, CIRAD, UNIKIN)
- Screening of seeds and cuttings of wild coffee by artificial inoculation
- Vegetative multiplication of "survivors" for further evaluation in field trials

Task 4
- Assessment of field resistance of some clones in various geographical areas, in progress.

Task 5
- Progenies from crosses between resistant and susceptible clones were inoculated to analyze inheritance of CWD resistance.

Task 6
- Confirmation on the existence of a Ugandan group of forest coffee trees.
- Hypothesis of different genetic factors controlling the CWD resistance.

Still to be done

Task 1
- Continue identification of new plants resistants after artificial inoculation. (CORI - UNIKIN)
- Vegetative multiplication of "survivors" in a mother garden(CORI)
- Confirmation of CWD resistance of J1/1 and Q3/4 in hot spot.

Task 2
- Continue analyze of genetic diversity of Ugandan forest germplasm (CORI –CIRAD)

Task 3
- Continue identification of resistant progenies (CORI, CIRAD, UNIKIN)
- Screening of C. Arabica germplasm (CIRAD)

- Task 4
- Continue the planting of cuttings identified after double artificial inoculation.

Task 5
- Scoring of artificial inoculation of the cross pollinations between resistant and susceptible clones (CORI).

Task 6
- Continue the study of the genetic diversity of the Uganda canephora coffee with microsatellites markers .(CORI – CIRAD)

WP4 - The disease: epidemiology

Task 2 (CORI, UNIKIN): Description of the spatio-temporal spread of the disease.

- Observations on farm indicate, increase in wilt incidence (CORI).
- Human activities and movement of dry infected stems accelerate the spread of CWD
- The spread of the disease indicate the evolution in 4 years of the quasi total destruction of 4 commercial varieties in experimental block 36 (CORI).

Task 3 (CORI,UNIKIN): Evaluation of the duration of survival form of the pathogen.

- Wood pieces remain infective for at least 6 months from the death of trees.
- Inoculum is present in the soil
- Contaminated tools (machete) transmit the disease

Achievements

Task 2
- In Uganda, confirmation of the spread of the disease in the farms studied.
- Confirmation of the human activities in the spread of CWD.
- Confirmation of the high susceptibility of 4 commercial varieties.

Task 3 & 4

- Confirmation of the survival of the fungus during 6 months in the infected wood.
- Confirmation of the survival of the fungus in the soil, duration unknown.

Task 5
- The role of rainfall in the appearance of visible symptoms is currently being studied
- A statistical analysis and graphic representation method has been adopted
- The first results raise the hypothesis of disease spread from tree to tree

Still to be done

Task 2
- Data analysis of CWD development at on-farm mapping and trial block 36.
- Field mapping to assess disease spread tree-by-tree since the beginning of the experiment.

Task 3
Continue the observations
Statistical analyse of data collected since March 2003.

Task 5
- Statistics analyze of the datas scored

Outline Plan Year 4

Tasks		Partners	
1-1	Survey and isolate collection	CORI	Collection of isolates from identified clones- Ended
		UNIKIN	Collection of geographical isolates - Ended
1-2	Identification, storage and exchanges of isolates	CABI	Ended
1-3	Evaluation of aggressiveness	UNIKIN-CIRAD	In progress
1-4	Description of the fungus cycle	UCL-UNIKIN-CORI	In progress
1-5	Evaluation of genetic diversity by RAPD and microsatellites	CABI	Ended
		CIRAD	Ended
1-6	Synthesis of the results	CABI	Partial conclusions
2-1	Identification of isolates representative of genetic diversity and aggressiveness	CIRAD	In progress
		CORI	In progress
		UNIKIN	In progress
2-2	Field inoculations in Africa	CORI	Planting "survivors" coffee plants in infected soils. In progress
		CORI	Dispatching of resistant clones in different areas. In progress
		UNIKIN	Dispatching of seeds from resistant clones in different areas. In progress
2-3	Cyto-histological study, types of reaction	CIRAD	Continue the task
2-4	Hypothesis on the nature of resistance	CIRAD-CORI	Partial conclusions
3-1	Identification of sources of resistance in the field and wild forest	CORI	In progress
		UNIKIN	In progress
3-2	Collect and dispatch seeds and cuttings	CORI	In progress
		UNIKIN	In progress
3-3	Screening tests on seedlings and cuttings in Africa and Europe	CORI-UNIKIN-CIRAD	In progress
3-4	Multi-locational trials with tolerant varieties	CORI-UNIKIN	In progress
4-1	Identification of sites representative of the epidemic	UNIKIN-CORI	Ended
4-2	Description of the spatio-temporal distribution of the disease in plantations	UNIKIN	Continue observation and statistical analyze
		CORI	Continue the task
4-3	Definition of the conditions conducive to the sexual phase	CORI-UCL	In progress
4-4	Evaluation of how and how long the pathogen survives	CORI-UCL	In progress

MANAGEMENT ANNUAL REPORT

Organisation of the collaboration

During this fourth year, regularly pooling and discussion of the results obtained has been pursuit by the different teams.
A joint UCL-CABI-CORI-CIRAD meeting was held on 29 June 2005 at UCL. In addition, important interrelations were established between the teams through an exchange of researchers undergoing training for periods of several months between between CORI and CIRAD. It was possible to conduct joint experiments and the results are currently being exploited.

CABI as a result of a successful application to the EU for an extension to the project CABI has been able to continue its research. However, given that the extension was not funded this research has relied on funds remaining from the original phase of the project and, as such, has been somewhat limited. Nevertheless, CABI is pleased to be able to continue to support it's partners in pursuing activities, including secure storage of fusaria and database maintenance, considered fundamental to the ongoing success of the project.

Meetings

UCL-CABI-CORI-CIRAD
29/06/2004. Pr Maraite, Mrs Pascale Lepoint, Mike Rutherford, Pascal Musoli, Daniel Bieysse held a meeting in UCL to discuss the results on genetic diversity, sexual cycle and host pathogen interaction and draw up work program.

Exchanges - Trainings

UCL

Thesis formation, "Etude de la variation de *G. xylarioides* au cours de son cycle sexuel et/ou parasexuel" second year, Mrs Pascale Lepoint.

CIRAD – CORI

Mission Prospection wild Canephora and identification CWD in primary forests
Visit to field trials
Pascal Musoli – Daniel Bieysse
Kampala 23-11 to 4-12 2004

Mission Prospection wild Canephora and identification CWD in primary forests
Pascal Musoli – Daniel Bieysse
Visit to field experimentations
Georgina Hakiza – Daniel Bieysse
Kampala 23-11 to 4-12 2004

Thesis formation, second year, Montpellier University, M. Pascal Musoli.
Thesis: Use of genetic resources for resistance to control wilt disease, *Fusarium xylarioides* of *Coffea canephora*.
Training in CIRAD avril 2005 to September 2005.

Problems

Inexcusable delays in the submission of financial report and scientific reports, which consequently prevented the release of funding.

OUTPUT

CABI

Internal Reports

Rutherford, M A (2004). Development of a long-term strategy based on genetic resistance and agro-ecological approaches against Coffee Wilt Disease (CWD). Annual Report 2005. INCO-DEV contract ICA4-CT-2001-10006. April 2006. Egham, CAB International.

RUTHERFORD, M. A. (2005). Development of a long-term strategy based on genetic resistance and agro-ecological approaches against Coffee Wilt Disease (CWD). Progress Report submitted to Regional Coffee Wilt Programme Co-ordinator. August 2005. Egham, CAB International.

UNDERWOOD, F. (2005). Analysis of spread of coffee wilt. Consultancy report provided for INCO-DEV contract ICA4-CT-2001-10006 'Development of a long-term strategy based on genetic resistance and agro-ecological approaches against Coffee Wilt Disease'. Reading, University of Reading Statistical Services Centre.

Oral presentations

RUTHERFORD, M.A., BUDDIE, A., INESON, J., CROZIER, J. AND FLOOD, J. (2005). Newly acquired knowledge of coffee wilt disease and its implications for disease management. Regional Coffee Wilt Programme Stakeholders' and Planning Workshop, Nairobi, Kenya, Nairobi, 8 – 9 December (presented by M.A.Rutherford).

RUTHERFORD, M. (2005) Current knowledge of coffee wilt disease, a major constraint to coffee production in Africa. Annual Meeting of the American Phytopathological Society, Austin, Texas. 30 July–3 August.

Poster presentations

RUTHERFORD, M., J. CROZIER, A. BUDDIE, J. INESON, S. LEA and J. FLOOD. (2005) Poster: Coffee Wilt Disease. Presented at the Regional Coffee Wilt Stakeholders Workshop, Nairobi, Kenya. 8–9 December 2005.

Related dissemination

SIMONS, S, AKIRI, M., PHIRI, N., KIMANI, M., RUTHERFORD, M., NZANZU, T. S., ADUGNA, G., MUGUNGA, M., KILAMBO, D. AND HAKIZA, G. (2005) Improvement of coffee production in Africa by the control of coffee wilt disease (tracheomycosis). Coffee wilt disease programme CFC/ICO/13: Project Progress Report, January-June 2005. July 2005. Nairobi, CAB International.

(i) RUTHERFORD, M. (2005) Epidemiology and variability of the coffee wilt pathogen. In: Perspectives on Pests II. Chatham, NR International.

RUTHERFORD, M., FLOOD, J., LEA, S. AND CROZIER, J. (2005). Coffee Wilt Disease. Advisory leaflet. 10,000 copies. 8 pp. Egham, CAB International.

CIRAD

Internal Reports

BIEYSSE, D. (2005). Development of a long term strategy based on genetic resistance and agroecological approaches against Coffee Wilt Disease in Africa. Third Annual Report, 1 November 2004 - 31 October 2005. INCO-DEV contract ICA4-CT-2001-10006. April 2005. Montpellier, CIRAD-AMIS

Oral presentations

Fabrice Pinard. Coffee Wilt Disease. Giberella xylarioides: genetic diversity. Presented at the Regional Coffee Wilt Stakeholders Workshop, Nairobi, Kenya. 8–9 December 2005.

Student training

CUBRY Philippe Analyse de la diversité et evaluation du déséquilibre de liaison chez quelques populations naturelles et cultivées de caféiers *Coffea canephora.* DEA Université de ontpellier II ; 01/07/2005.

Others

Collection of *Fusarium xylarioides* from wild *C. canephora* collected in Ugandan forests.

CORI

Internal Reports

HAKIZA, G. (2005). Development of a long term strategy based on genetic resistance and agroecological approaches against Coffee Wilt Disease in Africa. Annual report for Work Package 1-4, November 2004-31 October 2005. INCO-DEV contract ICA4-CT-2001-10006. April 2005. Kituza, Uganda, Coffee Research Institute.

MUSOLI, P. (2005). Development of a long term strategy based on genetic resistance and agroecological approaches against Coffee Wilt Disease in Africa. Annual report for Work Package 3, November 2004-31 October 2005. INCO-DEV contract ICA4-CT-2001-10006. April 2005. Kituza, Uganda, Coffee Research Institute.

Others

Carry out of a mother garden with new resistant plant material in Kituza.
Introduction in germplasm plots of 168 accessions from wild forests.

UCL

Internal Reports

LEPOINT, P. (2005). Development of a long term strategy based on genetic resistance and agroecological approaches against Coffee Wilt Disease in Africa. Annual report for Work Package 1-, November 2004-31 October 2005. INCO-DEV contract ICA4-CT-2001-10006. April 2005. Kituza, Uganda, Coffee Research Institute.

Publications

P. Lepoint, F. Munaut, H. Maraite. 2005. *Gibberella* xylarioides Sensu Lato from Coffea canephora : a new mating population in the Gibberella fujikuroi species complex. Applied and Environmental Microbiology. 71:8466-8471.

Others

Introduction of referenced and "historitical" strains

UNIKIN

Internal Reports

KALONJI A., TSHILENGE P. (2005). Development of a long term strategy based on genetic resistance and agroecological approaches against Coffee Wilt Disease in Africa. Annual report for

Work Package 1-2-4, November 2004-31 October 2005. INCO-DEV contract ICA4-CT-2001-10006. April 2005. Kituza, Uganda, Coffee Research Institute.

Publications

P. Tshilenge, F. Munaut, A. Kalonji, H. Maraite. Caractérisation des Fusarium sp. Associées au dépérissement du caféier Robusta en RDC. Parasitica. 2004, 60(3-4) : 67-82.

Annexe 2

Contract number : ICA4-CT-2001-10006

Year 4

Data sheet

for annual report

(to be compiled by **the co-ordinator** at 12-monthly intervals from start of contract. Figures to be up-dated **cumulatively** throughout project lifetime)

1. Dissemination activities
(cumulative)

	Totals
Number of communications in conferences (published)	3
Number of communications in other media (internet, video,)	
Number of publications in refereed journals (published)	2
Number of articles/books (published)	
Number of other publications	

2. Training

Number of PhDs	2
Number of MScs	1
Number of visiting scientists	5
Number of exchanges of scientists (stays longer than 3 months)	1

3. Achieved results

Number of patent applications	
Number of patents granted	
Number of companies created	
Number of new prototypes/products developed	
Number of new tests/methods developed	
Number of new norms/standards developed	
Number of new softwares/codes developed	
Number of production processes	

4. Industrial aspects

Industrial contacts	no
Financial contribution by industry	no
Industrial partners : - Large	no
- SME [1]	no

S. Comments

Other achievements (use separate page if necessary)

[1] Less than 500 employees.

CIRAD

INTERNATIONAL SCIENTIFIC COOPERATION PROJECT (INCO)

Development of a long-term strategy based on genetic resistance and agro-ecological approaches against Coffee Wilt Disease

Fourth Annual Report (November 2004 to October 2005)

CIRAD-AMIS
TA41/K
Campus International Baillarguet
34 398 Montpellier Cedex 5
France

WP1: PATHOGEN DIVERSITY

Task 5: Evaluation of genetic diversity

During two surveys to collect wild *Coffea canephora* materials in the primary forests of West Uganda, in the forests of Kibale and Itwara, a search was made for plants affected by vascular wilt. The search was made along coffee tree sampling transects.

During the survey in the forests of Kibale district, 5 forests were visited. The distance between the two forests furthest apart was 20 km.

In the course of that survey, trees displaying vascular wilt symptoms were found solely in the forest of Ngogo-Kibuguta, site A. Identifying symptoms on plants in forests is quite tricky because the architecture of the coffee trees is very different from cultivated coffee trees and the symptoms are more diffuse. The typical symptoms of black streaks at the base of trunks after cutting into the wood were clearly visible, but no perithecium was found.

The second survey was carried out in the forest of Itwara, located 40 km north of the Kibale Forest. This forest, measuring around 10-12 km in diameter, is a natural reserve, located in an environment of tea plantations to the South and West, and savannah zones to the East and North. Five survey blocks were defined. Coffee trees displaying similar symptoms to vascular wilt were found in 4 blocks.

Wood and bark samples were coded and single spore isolations were carried out. The term sampling was used for the samples taken from a single tree. Some gave several single spore samples. After culturing on agar medium, some samplings were very far from the genus *Fusarium* and were discarded from the study.

Figure 1. Map-sites of the primary forests visited,

Forest	Site	Samplings	Single spore code
Kibale Forest	Ngogo-Kibuguta Site A	1	OUG163
		1	OUG164
		1	OUG165
		1	OUG166
Itwara Forest	Rutoona	2	OUG168
	Kanaaba	3	OUG170
		4	OUG172
		4	OUG173
		5	OUG175
		5	OUG176
	Kyamukoro	6	OUG178
		7	OUG180
	Itwara	8	OUG182
		9	OUG184
		10	OUG186

Table 1. Origin of isolates.

Identification of isolates

The isolates were observed under the microscope to check for the existence of typical *Fusarium* conidia, and the culture profiles were also observed. 15 isolates were selected.

In order to have a rapid diagnosis, ITS1and P3 sequencing was carried out for comparison with the reference sequences (Scientific report Year 3).

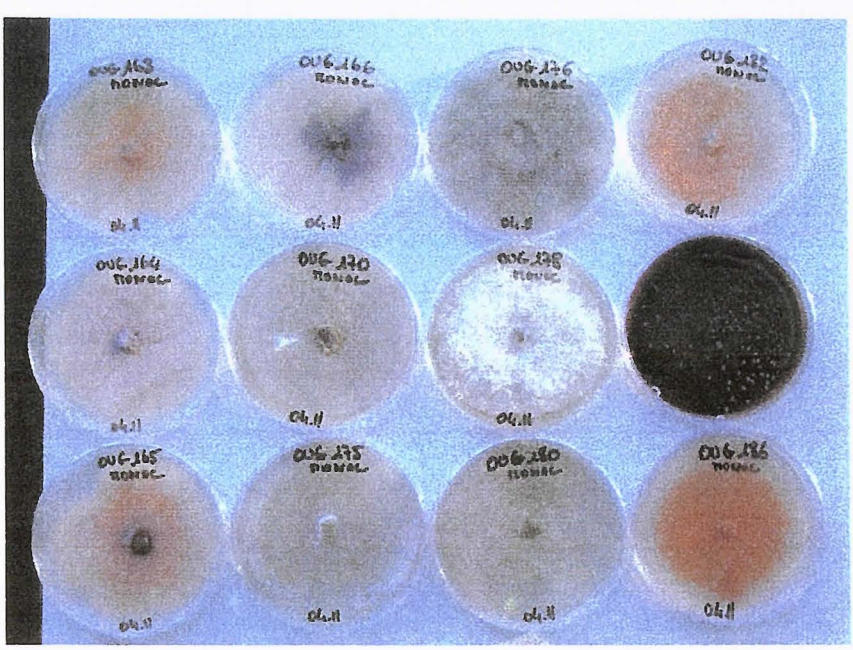


Photo 1 : Culture of isolates collected on affected *Coffea canephora* in Uganda primary forests.

Materials and methods

All the strains were single spore cultures grown on Potato Dextrose Agar (DIFCO) used to inoculate 100ml of potato dextrose broth (PDB). The liquid cultures were stirred for 7 days (100 rpm) at 25°C, then vacuum filtered and freeze-dried. The freeze-dried mycelium was stored at -20°C.

DNA extraction

Fungal DNA was extracted and purified from the freeze-dried mycelium using the protocol described by Rogers and Bendisch (1988), modified for *F. xylarioides*. A volume of 1.5 ml of 1X CTAB extraction buffer (1% cetyltrimethyl-ammonium bromide (CTAB), 50 mM Tris-HCl pH 8.0, 10mM EDTA pH 8.0, 0.7M NaCl pH 8.0, 1% β-Mercaptoethanol) was added to a 2-ml Eppendorf tube containing 80-100 mg of ground, freeze-dried mycelium. The mixture was incubated for 45 min at 65°C, then an equal volume of chloroform/isoamylic alcohol (24:1) was added. After emulsion, centrifugation was carried out for 10 min at 10 000 rpm. A second chloroform/isoamylic alcohol (24:1) purification was carried out. A 500-µl aliquot of supernatant was transferred to another 2-ml Eppendorf and 600µl of isopropanol were added to precipitate DNA. After centrifugation for 15 min at 13 000 rpm at 4°C, the supernatant was eliminated and the deposit was rinsed with 1 ml of 70% ethanol, vacuum dried and resuspended and incubated for 10 min at 65°C in 200 µl of TE+Rnase A buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0, RNase A at 100µg/ml). The DNA concentration was estimated visually on agar gel by comparing with a standard range of known concentrations of calf thymus DNA. The DNA solution was then adjusted to a concentration of 50ng/µl.

PCR amplification and sequencing

Primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') (White *et al.*, 1990) and P3 (5'-CCTTGGTCCGTGTTTCAAGACGGG-3') (Guadet *et al.*, 1989) were used to amplify a rDNA fragment including the ITS1 region, the 5.8S rDNA, the ITS2 region, and 630 nucleotides of the 5'end of the 28S region of rDNA. The primers used were synthesized by the Eurogentec company (France). Each amplification was performed in a final volume of 100 µl of reagent mixture comprising 2 µl of DNA (50 ng/ml), 0.5 µM of each primer (ITS1/P3), 1.5 mM of $MgCl_2$, 1X PCR buffer (67 mM Tris-HCl pH 8.8, 16 mM $(NH_4)_2SO_4$, 0.1% Tween 20), 100 µM of triphosphate deoxyribonucleotides (dATP, dCTP, dGTP, dTTP), and 5 units of *Taq* DNA polymerase. Amplifications were performed in a PT 100 thermocycler (MJ Research, Inc) following an established programme consisting of an initial denaturing phase of 5 min at 94°C followed by 30 cycles of 45 sec at 94°C, 45 sec at 55°C and 1 min at 72°C, with a final elongation phase of 5 min at 72°C. Each PCR amplification for sequencing was carried out by the GENOME Express company (Meylan, France) with ITS1 forward and P3 reverse universal primers.

The CLUSTAL W multiple alignment program (Thompson, Higgins & Gibson 1994) was used to align the set of sequences obtained. The alignment generated under CLUSTAL W was used to estimate the pairwise distances between the nucleic sequences of the different *Fusarium* strains studied using the Kimura method (1980) with the TREECON computer program (Van de Peer, Y., De Wachter, R.1993). A dendrogram using the distance matrix was constructed using the "unweighted neighbour-joining" method with the DARWIN 4.0 program. The reliability of the branches was tested by a bootstrap analysis with 100 replications.

ification and phylogenetic analysis based on nucleotide sequence alignment.

Due to sequencing constraints, the dendrogram obtained from the rDNA nucleotide sequence alignment only takes into account ITS1 alignment for the 13 isolates derived from wild *Coffea canephora*. For the phylogenetic analysis, referenced isolates were included.
The distances were calculated by comparing nucleotide sequences two by two using Kimura's method, and the representation used followed the unweighted neighbour-joining method.

Two groups of isolates were differentiated. The group of 11 strains belonging to the species *Fusarium xylarioides* consisting of the reference strain CAB003 and 3 "historical strains", ATCC 15664, DSMZ 62457, CBS 74979, and 6 strains isolated from wild coffee trees from the Kibale and Itwara forests. The second group differed from the first by a minimum 6% of dissimilarity (OUG186) to 16% (OUG180-2). The latter strain might belong to the species *F. lateritium* from which it differs by 1.7%

(FLAT4). BLAST analysis of isolate OUG175 indicated 98% homology with the species *F. solani* (NJM0271 1). After BLAST searching, isolates OUG180-1, OUG180-2, OUG175, OUG170, OUG178-1, OUG178-2 displayed homology rates of around 98% with *Fusarium,* but without any indication of the name of the species.

The bootstrap value of the branch separating the *F. xylarioides* group from the other isolates was 100 indicating the clear separation between groups.

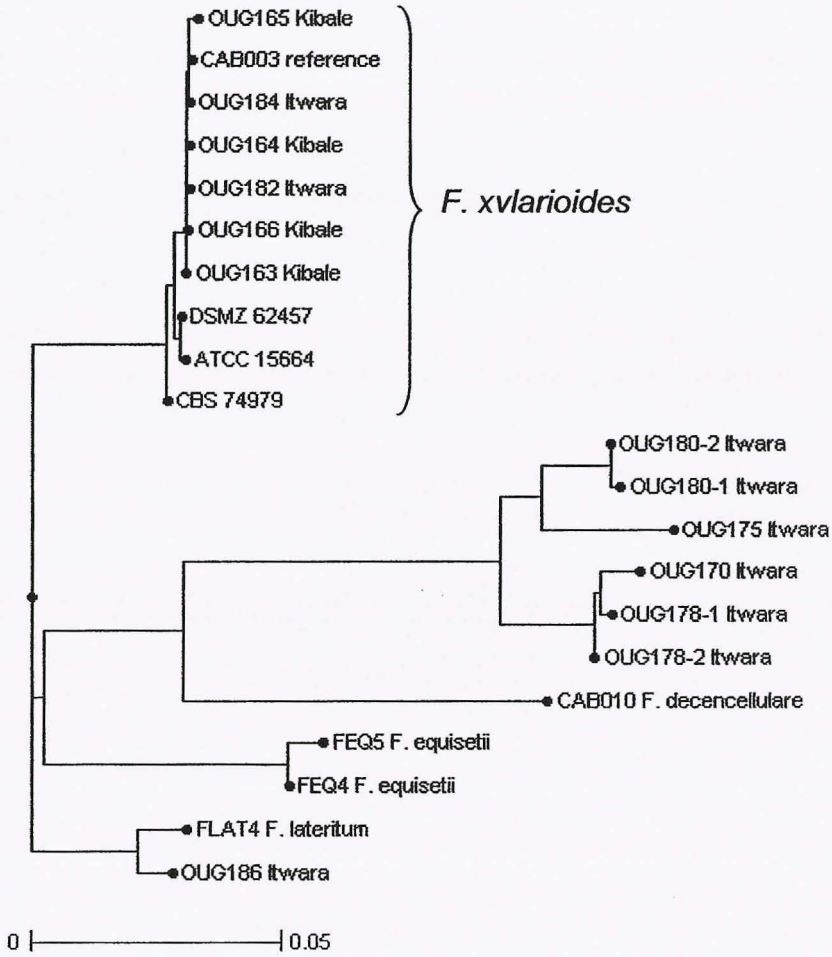


Fig. 2 - Dendrogramme produced from the alignment sequences of 21 nucleic sequences of rDNA (ITS-P3).

Conclusion

A phylogenetic analysis based on an analysis of the ITS1-P3 sequences revealed 5 groups:

The first *F. xylarioides* group, made up of the historical strains (DSMZ 62457, ATCC 15664, CBS 74979) that stood out from the contemporary strain CAB003. The strains (OUG163, OUG164, OUG165, OUG166, OUG182 and OUG184) newly collected from wild coffee trees belonged to the species *F. xylarioides*. The disease was present in the forests surveyed: Kibale and Itwara. Isolates OUG163 to OUG166 were isolated from the only plant displaying symptoms during the Kibale survey and isolates OUG182 and OUG184 were collected from the same block in the Itwara forest on trees a few dozen metres apart.

The second group (OUG180-1, OUG180-2, OUG175, OUG170, OUG178-1 and OUG178-2, made up of *Fusarium sp.* after a BLAST search.

The third group, corresponding to the *F. decencellulare* referenced strain and the fourth group corresponding to the *Fusarium equisetii* referenced strains.

The fifth group was made up of the *F.lateritium* referenced strain and isolate OUG 186 which, during a BLAST comparison, displayed the maximum of homology with a strain of the species *F. lateritium.*

The host spectrum of the newly collected strains belonging to the species *Fusarium xylarioides* is to be tested to check for the specificity of those isolates.

The existence of *Fusarium xylarioides* in the primary forests of Uganda raises the question of the origin of that species.

To date, the most commonly accepted hypothesis has been the absence of the disease in primary forests, since exploitation of forest resources in highly diverse forms by the local populations goes back to time immemorial. In particular, from the period, during which *canephora* coffee plantations were developed in the 1920s-1930s. The forest constituted a reservoir for seeds for numerous coffee smallholdings. Likewise, coffee berries was collected for trade. Thus numerous comings and goings involving the coffee tree can be found in an environment close to cultivated coffee trees. There was a high probability of contaminating cultivated plots. Yet, the disease was identified in those zones around 1993-1994. It is accepted that the disease came from RDC, with foci gradually spreading alongside roads. Did the disease occur in primary forests before or after it appeared in plantations?

If the existence of the disease in the forest goes back to ancient times, it could mean co–evolution of the pathogen and *Coffea canephora* and provides hope for a reservoir of genes of resistance to vascular wilt.

WP2: Host/Pathogen interaction

1 Identification of isolates of *Fusarium xylarioides* representative of genetic diversity and aggressiveness

This year, assessment of the plant material from RDC has continued with the reference isolate CAB003. Emphasis was then placed on analysing the pathogenicity of historical isolate DSMZ collected from *Coffea excelsa*. This isolate had displayed non-specific reaction, being pathogenic on *C. liberica*, *C. deweivri*, *C. canephora* and *C. arabica*. We wished to confirm those results.

An analysis of interactions was completed with the work undertaken at CIRAD in association with Pascal Musoli on genotypes from Uganda. The results can be found in the CORI section.

Material and methods

The evaluation of the susceptibility/resistance of plant material from DRC is carried out on open pollinated seeds harvested from individual identified trees. In the text they are referred to as "progenies". No agronomic information is available on these genotypes kept in germplasm collections at different stations in the country.

This year, 3 trials (55, 56, 57) were undertaken on 8-month-old plants to evaluate wilt resistance. The protocol was the standard technique for inoculating a calibrated suspension of conidia of isolates CAB003 and DSMZ by syringe into the stem.

For the other trials (63,64) we inoculated young 7-week-old seedlings by wounding under the cotyledon leaves.

The plants were kept in a climatic chamber at 25°, with a 12 h/12 h photoperiod. Symptoms were observed 100 days after inoculation. Statistical analyses were carried out using the General Linear Model.

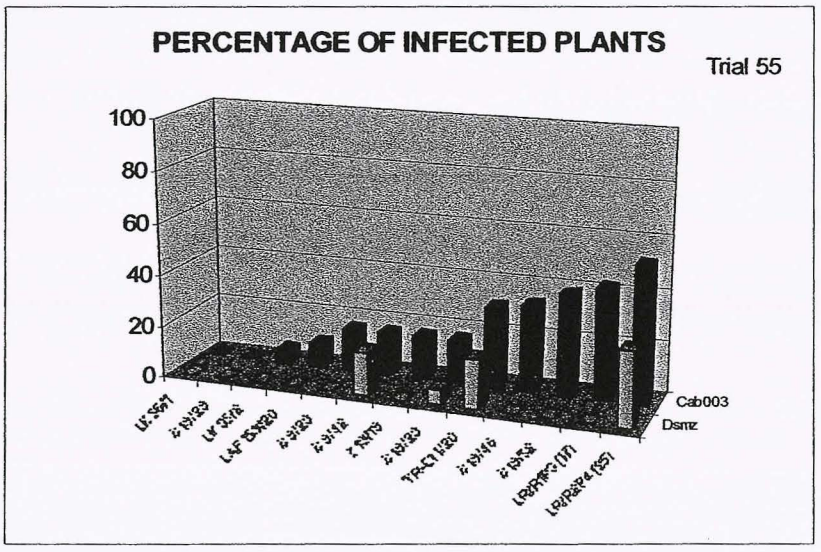
Results

Trial 55

Table 1. Evaluation of the resistance of progenies from DRC with *F. xylarioides* CAB003 and DSMZ

Trial	Isolates	CIRAD Code	DRC Code	Number of plants	% infected plants	Groups
55	CAB003	1639	LK 56/1	18	0	
		1644	S 19/29	18	0	
		1637	LK 35/2	18	6	a
		1632	LAF 159/20	21	10	a
		1640	S 9/23	18	17	ab
		1641	S 9/42	18	17	ab
		1642	S19/19	18	17	ab
		1643	S 19/23	18	17	ab
		1657	TR-CI 1/20	16	31	ab
		1645	S 19/46	18	33	ab
		1646	S 19/52	18	39	ab
		1653	LR/R1P3517°	21	43	ab
		1655	LR/R2P4 525°	21	52	b
	DSMZ	1639	LK 56/1	18	0	
		1644	S 19/29	18	0	
		1637	LK 35/2	15	0	
		1640	S 9/23	18	0	
		1632	LAF 159/20	21	0	
		1642	S19/19	18	0	
		1645	S 19/46	18	0	
		1646	S 19/52	18	0	
		1653	LR/R1P35 (17)°	20	0	
		1643	S 19/23	18	6	a
		1641	S 9/42	18	17	a
		1657	TR-CI 1/20	16	19	a
		1655	LR/R2P45 (25)°	21	29	a

Fig.3 - Percentage of infected plants 100 days after inoculation with CAB003 and DSMZ



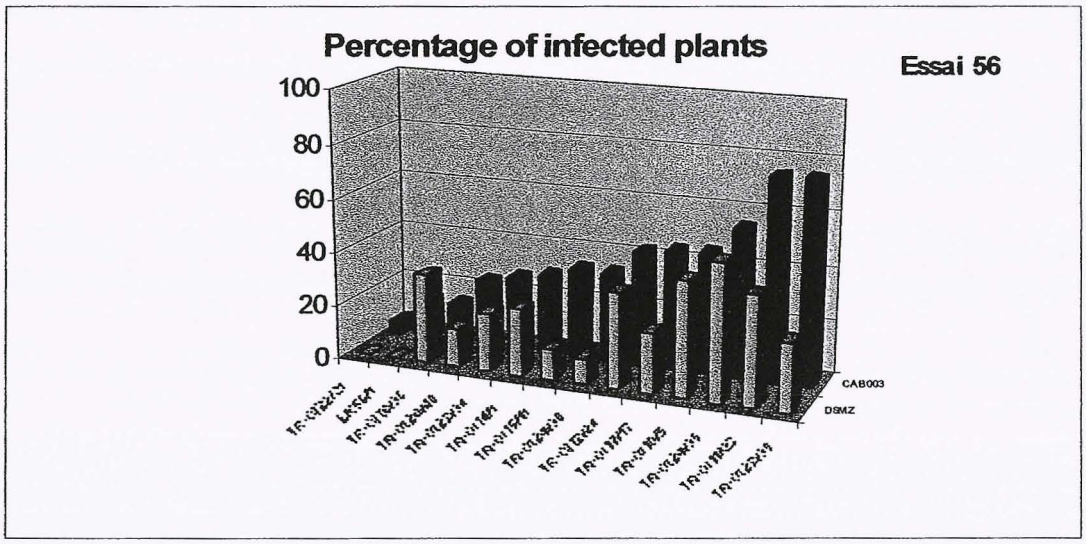
The percentage of infected plants with CAB003 varied depending on the progenies. Progenies LK 56/1 and S 19/29 were resistant, LR/R1P35 (17) and LR/R2P45 (25) very susceptible. Between these extremes, we observed a continuum in the percentage of infected plants. With DSMZ, 3 progenies were susceptible, the others presented 0% of infected plants. These progenies were also susceptible with CAB003

Trial 56

Table 2. Evaluation of the resistance of progenies from DRC with *F. xylarioides* CAB003 and DSMZ

Trial	Isolates	Code	DRC Code	Number of plants	% infected plants	Groups
56	CAB003	1677	TR-CI 22/21	19	5	a
		1639	LK 56/1	21	14	a
		1674	TR-CI 18/36	21	14	a
		1676	TR-CI 20/40	20	25	ab
		1678	TR-CI 23/34	18	28	ab
		1666	TR-CI 14/1	20	30	ab
		1667	TR-CI 15/11	18	33	abc
		1680	TR-CI 24/30	21	33	abc
		1665	TR-CI 12/24	21	43	abc
		1671	TR-CI 17/17	18	44	abc
		1663	TR-CI 10/5	20	45	abc
		1681	TR-CI 24/35	20	55	bc
		1664	TR-CI 11/23	20	75	c
		1679	TR-CI 23/39	20	75	c
	DSMZ	1677	TR-CI 22/21	18	0	a
		1639	LK 56/1	21	0	
		1680	TR-CI 24/30	21	10	a
		1667	TR-CI 15/11	18	11	a
		1676	TR-CI 20/40	21	14	a
		1678	TR-CI 23/34	14	21	a
		1671	TR-CI 17/17	18	22	a
		1666	TR-CI 14/1	20	25	a
		1679	TR-CI 23/39	12	25	a
		1674	TR-CI 18/36	21	33	a
		1665	TR-CI 12/24	20	35	a
		1664	TR-CI 11/23	20	40	a
		1663	TR-CI 10/5	19	42	a
		1681	TR-CI 24/35	12	50	a

Fig. 4 - Percentage of infected plants inoculated with CAB003 and DSMZ



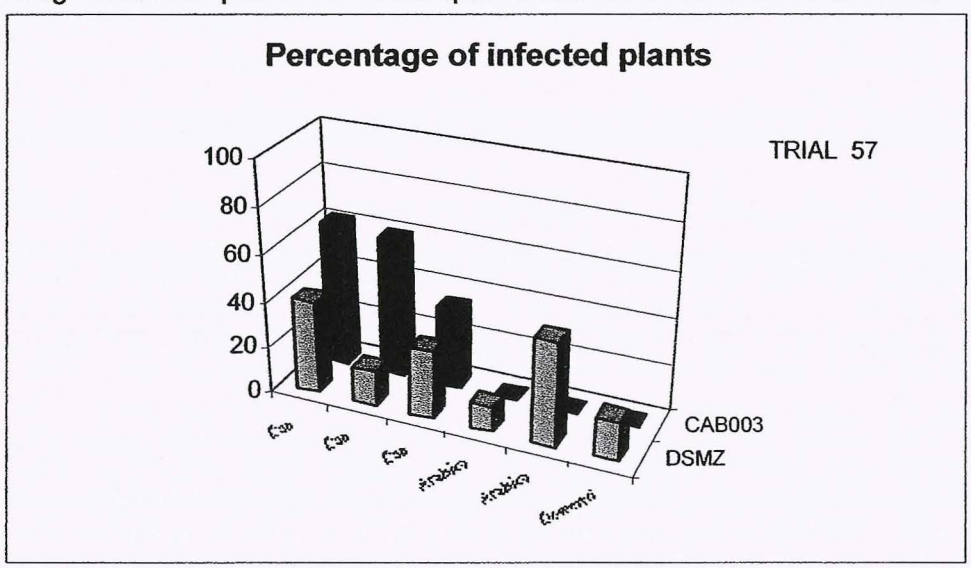
All the plants inoculated with CAB003 presented symptoms. With DSMZ, all the progenies were susceptible, except LK56/1 which was resistant. In general, the percentage of infected plants with DSMZ was lower than the percentage of infected plants with CAB003.

Trial 57

Table 3. Evaluation of the resistance of a set of different *Coffea* species with CAB003 and DSMZ

Trial	Isolates	CIRAD Code	DRC Code	Number of plants	% infected plants	Groups
57	CAB003	1826	Deweivri	21	0	
		1764	Caturra	21	0	
		1763	Java	21	0	
		1793	02576	27	35	
		1768	121	24	60	
		1497	B/6/2	26	63	
	DSMZ	1763	Java	20	11	
		1768	121	23	15	
		1826	Deweivri	21	17	
		1793	02576	22	29	
		1497	B/6/2	20	40	
		1764	Caturra	26	44	

Fig.5 - Percentage of infected plants of 3 *Coffea* species inoculated with CAB003 and DSMZ

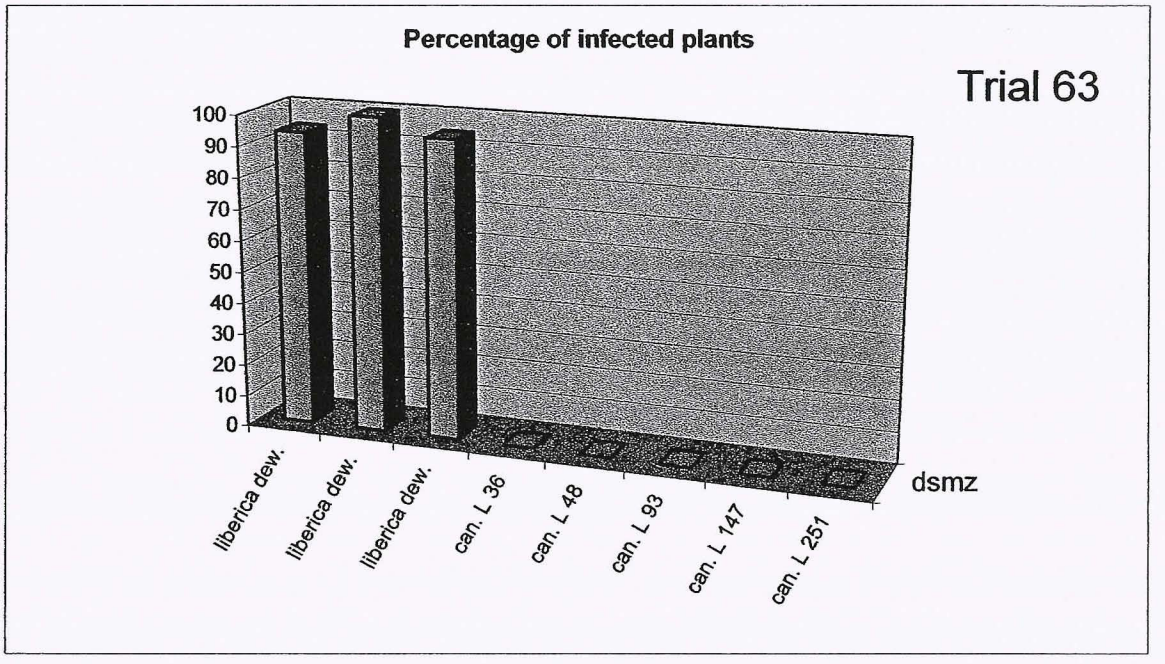


With CAB003, only the progenies belonging to *C. canephora* were susceptible. The progenies belonging to *C. Arabica* and *C. deweivri* were resistant. On the other hand, DSMZ attacked the three *Coffea* species.

Trial 63

Isolate DSMZ induced a percentage of infected plants exceeding 90% on coffee trees of the species *Coffea liberica deweivri*. On the other hand, no symptoms appeared on *C. canephora*. These *Coffea* species came from DRC, but we do not have any information available regarding their belonging to any particular genetic group.

Fig.6 - Percentage of infected plants of 3 *Coffea* species inoculated with DSMZ

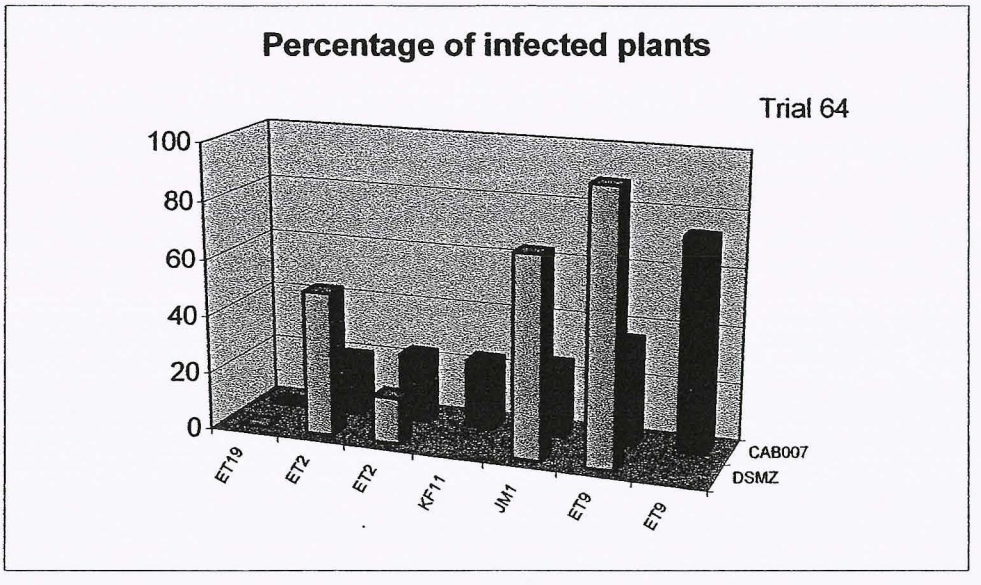


Is it a specific reaction of isolate DSMZ to these *canephora* materials?

Trial 64

Isolate DSMZ induced symptoms on 4 out of 5 of the coffee tree genotypes tested. Genotype ET19 displayed 0% of infected plants. For the genotypes evaluated (ET2, JM1, ET9) with the two isolates, the percentage of symptoms was highest with isolate DSMZ, whereas isolate CAB007 was the specific isolate collected from *Coffea arabica*. Genotype ET19 was resistant to both isolates.

Fig.7 - Percentage of infected plants of *Coffea canephora* inoculated with DSMZ



Conclusion

An assessment of the resistance of *canephora* coffee trees to isolate CAB003 revealed substantial variation in susceptibility within this species, ranging from total resistance to high susceptibility. An assessment of the resistance of the cross-fertilizing species *C. canephora* is not easy, because the seeds are derived from uncontrolled pollination. Consequently, only the maternal effect is assessed. Nevertheless, assessing resistance remains a strong indicator for detecting genotypes displaying resistance traits, particularly when infection rates are around 0 to 10%. Modelling of the choice of candidate coffee trees to be selected for their resistance based on the results obtained is going to be developed. Selection thresholds will be defined for identifying candidate genotypes displaying a good probability of having traits of resistance to vascular wilt disease.

A few genotypes, LK56/1, S19/29, and TR-CI 22/21, display good levels of resistance, notably cumulative resistance to isolates CAB003 and DSMZ.

The results observed confirm the host specificity of isolate CAB003, a contemporary strain collected from *C. canephora*, inducing symptoms on *C. canephora* only (trial 57).

In trial 64, isolate CAB007 induced symptoms on the Arabica progenies tested, except with totally resistant genotype ET19. That genotype displays cumulative resistance to CAB007 and DSMZ. Isolate DSMZ overcomes the resistance of the other 4 *C. arabica* genotypes and expresses greater aggressiveness than isolate CAB007.

The ability of isolate DSMZ to induce symptoms on the three species (*C. canephora, C. deweivri, C. arabica*) is confirmed.

This result indicates that the "historical" *Fusarium* population, particularly DSMZ, displays quite a broad host spectrum, compared to the contemporary population, which seems restricted to specific reactions. We put forward the hypothesis that the contemporary population could have arisen from a strong foundation effect that counter-selected an isolate specific to the species *C. canephora*. This result also clearly shows that the contemporary strains of *Fusarium xylarioides* display a potential to evolve towards the acquisition of complementary virulence that could prove pathogenic on other *Coffea* species. The question is raised as to the existence of races within the species *Fusarium xylarioides*. For the moment, we do not have available the plant material needed to detect races, as it requires genotypes propagated by vegetative multiplication, or obtained by controlled pollination.

WP3 : Breeding for resistance

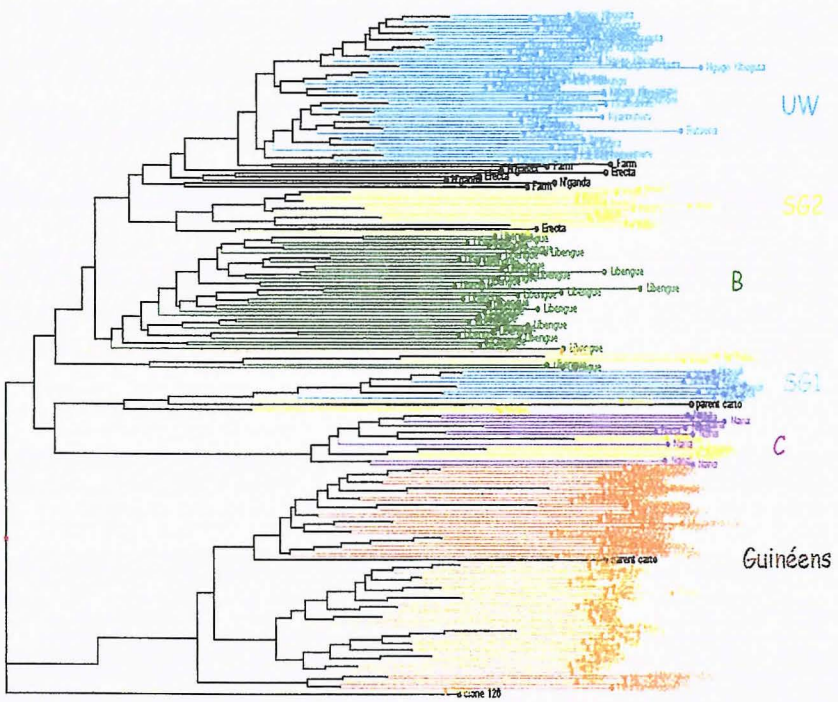
Evaluating genetic diversity among potential sources of CWD resistance in UGANDA

This study was divided in two parts:

- a co-working with Pascal Musoli – Thierry Leroy during his training in CIRAD. Results are presented in CORI report
- a study carry out during the training of a DEA student Philippe Cubry.

GENETIC DIVERSITY ANALYSES AND LINKAGE DISEQUILIBRIUM EVALUATION IN SOME NATURAL AND CULTIVATED POPULATIONS OF *Coffea canephora*.

Robusta coffee, produced by *Coffea canephora*, represents about 35% of the world coffee production. As part of studies aiming at the improvement of coffee cup quality, the knowledge of the genetic diversity of the wild and cultivated populations is an important task.
First, a diversity study was undertaken with 34 SSR markers distributed on the whole genome. This study concerned several populations belonging to the main known coffee diversity groups, as well as wild and cultivated East African populations that were never analyzed for diversity. A dissimilarity-based multivariate analysis and a Neighbour-Joining phylogenetic reconstruction were laid in order to understand the structure of the species diversity. The results confirmed the group structure previously described and stressed out the original features of the East African populations which seems to have some genetic divergence from the previously described Congolese groups and appears to be related to SG2 in compliance with the supposed geographic origin and historical data. Fst calculations confirmed the observations made on the diversity analysis and shown a higher homogeneity within the Ugandan populations (Fst = 0.20 for this group, Fst = 0.42 for the species). Moreover a confirmation of this supposed structure was made with the "Structure" software (Pritchard *et al*, 2001). Ugandan populations formed clusters well differentiated from the others material origins. This new group should be tested in selection and could allow the breeders to broaden the genetic basis they use for coffee improvement as hybrids are known to be of good value. Second, an estimation of linkage disequilibrium for markers for which the genetic distance is known was made for 25 SSR markers on the same populations as in the diversity study. The results of this work will be useful to breeding programs and will be the basis for future association studies.



NJ-tree for 34 SSRs markers on several individuals representing diversity of *C. canephora* from a collection of Ivory Coast, and including wild populations from Uganda.

UCL

INTERNATIONAL SCIENTIFIC COOPERATION PROJECT (INCO)

Development of a long-term strategy based on genetic resistance and agro-ecological approaches against Coffee Wilt Disease

Fourth Annual Report (November 2004 to October 2005)

Université catholique de Louvain
Unité de Phytopathologie
Croix du Sud 2bte 3
B-1348 Louvain la Neuve - Belgium

WP I: PATHOGEN DIVERSITY

Task II: Identification, storage, and exchange of isolates

Table 1 lists all isolates received this past year from CABI, CIRAD as well as from international culture collections.

Twenty isolates collected in the Equator Province (DRC) by UNIKIN (September-October 2004) from *C. canephora* trees showing typical coffee wilt symptoms were purified by single spore isolation. Molecular analysis and characterization of mating type is being carried out to complete the distribution within the DRC and for confirmation that the pathogen present in these regions is identical to the causal agent isolated in the other Congolese, Ugandan and Tanzanian regions.

Thirteen monospore isolates and 3 hyphal tip isolates from *C. arabica* were added to our existing ***Gibberella xylarioides* "Type A"** (from Ethiopia) collection. Three *Fusarium udum* strains, a close relative of *G. xylarioides* causing wilt on *Cajanus* spp. in India and Malawi, were received from CABI in addition to a "*F. oxysporum*" isolate from arabica.

Two historical isolates were obtained from BBA through **Dr. H. Nirenberg**. Two of these isolates are "historical", dating back respectively from 1964 (**BBA 62455**, Guinea, *C. canephora*) and from 1971 (**BBA 62458**, Ethiopia, *C. arabica*), and previously studied by Girma. A historical isolate (**709**) as well as 5 herbarium samples were sent by the Museum d'histoire Naturelle de Paris via **Dr. Cony Decock**. In total, our historical *G. xylarioides* collection now accounts seven isolates of which one originates from arabica.

Strains **NRRL 26064** (*Fusarium* sp.) and **NRRL 22540** (*F. udum*), identified as *G. xylarioides* closest relatives based on translation elongation factor 1-∝ (*tef* 1-∝) sequence analysis, were ordered via **Dr. K. O'Donnell** for mating type (*MAT*) identification, partial gene sequencing, and cross fertility studies with *G. xylarioides*.

In order to complete our cross fertility study, thus identifying *G. xylarioides* as a new mating population (MP) within the *G. fujikuroi* species complex (GFC), 18 standard mating population tester strains (MP A-I) were ordered at the Fungal Genetics Stock Center (**FGSC**).

UCL will forward historical isolates BBA 62455 and 62458 to CIRAD for assessment of pathogenicity once the transfer agreement has been received from Dr. H. Nirenberg.

Task IV : Description of the fungal life cycle, asexual and sexual phases

Growth rate and Morphological study

Nine representative *G. xylarioides* isolates (**Table 2**) were chosen to determine the optimal growth temperature of the coffee wilt pathogen. PDA plates were inoculated with three equi-distant 4mm plugs of SNA culture, and two colony diameters were noted for each inoculation point from days 4 to 12. Plates were incubated in the dark at 16, 21, 25, 28, 32.5, and 37.5°C (±0.5°C) and mean growth rates were calculated. The growth rate-temperature curve is shown in **Figure 1**.

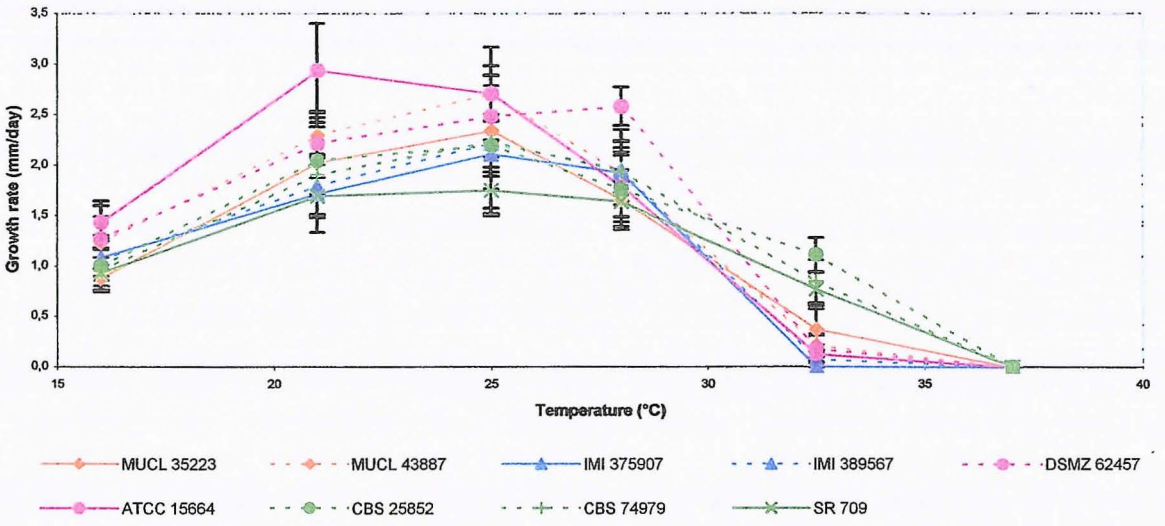
Accession N°		Origin/Host	Date	*MAT-PCR*	Profile
MUCL 35223	**FGSC 9928**	DRC/*C. canephora*	1992	1	**A**
MUCL 43887	**FGSC 9929**	Uganda/*C. canephora*	2002	2	**A**
IMI 375907		Ethiopia/*C. arabica*	1997	1	**A**
IMI 389567		Ethiopia/*C. arabica*	?	2	**A**
ATCC 15664		Guinea?/*Coffea* sp.	1964	1	**B**
BBA 62457	**IMI 127629 = ATCC 36326**	CAR/*C. excelsa*	1971	2	**C**
CBS 25852		Côte d'Ivoire/*Coffea* sp.	1951	2	**D**
CBS 74979	**BBA 62721**	Guinea/*C. canephora*	1963	2	**D**
SR 709		CAR?/*Coffea* sp.	1950?	1	**D**

Table 2: List of *Coffea* spp. strains used in the growth-temperature study

Physiologically, recently isolated *C. canephora* (**MUCL 35223 & MUCL 43887**) and *C. arabica* (**IMI 375907 & IMI 389567**) strains present a similar growth rate-temperature response (profile A) with optimum and maximum growth temperatures at 25 and ~32.5°C. Historical isolates showed three distinct profiles. The first and second profiles incorporate respectively strains **ATCC 15664** (profile B) and **DSMZ 62547** (profile C) with optimum/maximum growth temperatures respectively of 21°C and ~32,5°C for the first, and 28°C and ~32,5°C for the latter. A third and distinctive profile (profile D) represented by strains **CBS 25852, CBS 74979** and **SR 709**, intermediate in optimum growth temperature (25°C), reveals a higher inhibiting temperature situated in the region of 33 to 37°C. A growth assay at 33°C on PDA allows discrimination of this historical group from other coffee wilt isolates.

Strains **ATCC 15664, CBS 74979** and **BBA 62457** produce on PDA a pinkish-brown coloration of the colony and diffuse a similar pigment in the agar. This pigment production, untypical of *G. xylarioides* isolates, was most intense for ATCC 15664 at 25°C (**Figure 2a**).

Figure 1: Mean diametral growth rate response to temperature for *G. xylarioides Coffea* spp. isolates



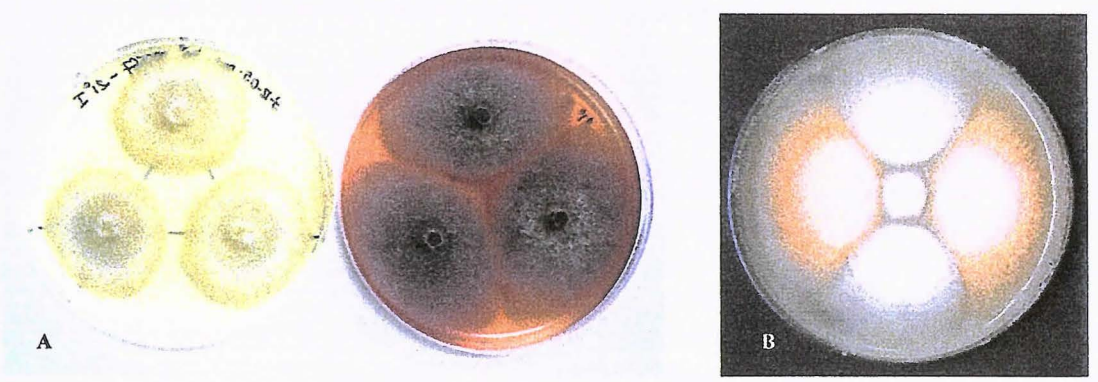


Figure 2: A. IMI 389567 and ATCC 15664 cultures grown at 21°C on PDA
B. Arabica (white) x robusta (orange) isolates confronted on carrot agar

Morphological characterization of the anamorph is underway. Nevertheless, macroscopic observation of PDA cultures exposed to a light source distinguishes *C. arabica* strains from other *G. xylarioides* strains (**Figure 2b**). Colonies of the latter produce an orange pigment, lacking in arabica strains. The teleomorph will equally be characterized on the basis of recent stem samples from the DRC and if possible from Ethiopia, in addition to historical herbarium samples received from the Museum d'Histoire Naturel de Paris.

Crossing assays

In the third annual report (1/11/2003 - 1/11/2004 period) the coffee wilt pathogen was described as a **heterothallic** fungus based on *in vitro* crosses and *MAT*-PCR analysis. In order to be sure of the heterothallic nature of the resulting perithecia formed in confrontations, the progeny of three crosses were analysed by *MAT*-PCR. Both mating types were present in each cross, indicating that the progeny originated from the sexual recombination of both parents and were not of homothallic origin (from one of the parents).

As described previously, fertile perithecia can be readily produced respectively in crosses among canephora strains and in crosses among arabica strains. Fertile fructifications (perithecia containing full sized ascospores and presenting a cirrhus at maturity) have never been observed in canephora x arabica, historical x historical, canephora x historical, or arabica x historical crosses. However, in some of these crosses, sterile perithecia, or perithecia containing micro-ascospores can be observed but never exude ascospores.

All 20 isolates obtained from the Equator Province are *MAT-1* and ~50% have produced the teleomorph in crosses with female fertile canephora testers. This is most unusual in two aspects and could eventually explain the decrease of the number of cases of wilt observed in the region. Firstly, isolates analysed in previous Congolese prospections from numerous provinces have revealed the presence of both mating types whilst only one mating type has been identified in the Equator province (*MAT*-1). Secondly, the sterility of some of the crosses between canephora testers and Equator field isolates can be due either to the fact that the latter are sterile (phenomena frequently observed), or that they are incompatible with the canephora tester strains. This can only be confirmed by sequencing of specific genes, and crossing assays.

Results from crosses between recently acquired historical isolates BBA 62455 and 62458 with canephora, arabica and previous historical isolates are expected shortly.

<u>Task V.</u> Evaluation of genetic diversity within *F. xylarioides*

The sequencing process is in its finale stage for non-MAT genes (translation elongation factor, calmodulin and histone 3), with recently obtained BBA and Equator province strains still awaiting characterization. **Table 3** summarizes sequencing results.

In depth characterization and sequencing of the entire *MAT*-1 idiomorph is in process and should provide additional information on the status of the different *G. xylarioides* isolates. If time and funds permit, UCL will characterization and sequencing the *MAT*-2 idiomorph.

RAPD

Random amplified polymorphic DNA (**RAPD**) polymerase chain reaction (PCR) was carried out using four decamer primers (A3, A14, A15, and A17) from kit A (Operon Technologies Inc.) that indicated certain levels of interspecies variation in the genus *Fusarium* (unpublished, Munaut). 25µl-PCR reaction mixtures contained 20 ng of template DNA, 1x PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.75µg/ml of each primer and 2.5 U of Taq DNA polymerase. PTC-200 thermocycleur conditions were 7 min at 94°C, followed by 45 cycles of 1.5 min at 94°C, 2 min at 35°C and 3 min at 72°C, followed by a final elongation at 72°C for 7 min (Sreenivasaprasad *et al.*, 1992).

Out of the four decamers tested, primers A3 and A14 discriminated three distinct profiles, recent canephora/arabica strains, group Ia (DSMZ 62457 & ATCC 15664) as well as group Ib (CBS 25852 & CBS 74979) within the historical strains. Primer A17 was the only primer that generated a distinct polymorphism for recent arabica isolates as well as differentiating the recent canephora and two historical groups. Strain MUCL 14186's (Yangambi, 1976) profile is identical to recent canephora isolates.

Translation elongation factor 1-∝ (tef 1-∝)

Translation elongation factor (*tef 1-∝*) sequences for *G. xylarioides* and closely related *Fusarium* spp. strains reveal that the ***G. xylarioides* complex (GxC)** is nested (98% bootstrap support) within a larger complex comprised of *F. udum*, *F. phyllophilium* and *Fusarium* spp. strains. Two main clades make up the GxC; the first contains all recent Congolese, Ugandan, Tanzanian and Ethiopian isolates from *C. canephora*, *C. excelsa*, and *C. arabica* as well as "historical" isolates MUCL 14186 (DRC, *C. canephora*, 1968) and BBA 62458 (Ethiopia, *C. arabica*, 1972). The second is formed by two sub-clades consisting respectively of historical isolates BBA 62457 & ATCC 15664 and CBS 25852 & CBS 74979 & SR 709.

F. udum strains IMI 193652, IMI 271070, IMI 275452 are identical in sequence but differ from *F. udum* "reference" strain NRRL 22540 and *Fusarium* sp. NRRL 26064. Isolate IMI 389581 isolated from *C. arabica* in Ethiopia and received from CABI as being '*F. oxysporum*' shows the closest similarity (95%) to *Fusarium* sp. BVS2005a of the GFC.

Calmodulin (CL)

Sequencing of the **calmoduline (CL)** gene distinguishes two groups supported by a 100% bootstrap value. The first group encompasses the GxC formed by two clades, recent as well as "historical" BBA 62458 (1971) and IMI 204746 (1976) arabica strains which are basal to the clade formed by all the other recent and historical *C. canephora* and *C. excelsa G. xylarioides* strains.

The second group contains equally two clades, the first of which is formed by all the *F. udum* strains isolated from *Cajanus* spp. and *Crotalaria* sp. in addition to NRRL 22540 and NRRL 26064.

The latter being basal to *F. phyllophilum*. IMI 389581 shows a 98% similarity to *F. nygamai*, a member of the African clade in the GFC.

Histone 3 (H3)

A mega clade (96% bootstrap) divided into two sister groups corresponding to the GxC and to the *F. udum* / *Fusarium* spp. complex was identified on the basis of the **H3** sequences. The GxC is formed by three clades. The first contains all recent *C. canephora* isolates, the second historical isolates CBS 25852, CBS 74979 and SR 709, whilst the third group contains recent and historical (BBA 62458, IMI 204746) arabica isolates in addition to historical isolates BBA 62457 and ATCC 15664.

The second group encloses two clades (90%), the first contains *F. udum* IMI strains to which NRRL 22540 is basal to (86% bootstrap value) and *Fusarium* sp. strain NRRL 26064 is basal to the later. BAST results for IMI 389581 show a 97% similarity to *F. nygamai*. Unfortunately no sequence is available for *F. phyllophilum* and *F. udum* reference strain NRRL 22949 in international databases.

Mating type 1 (MAT-1)

Partial ***MAT-1*** gene sequencing (~300bp) has revealed very little polymorphism within the GxC with the exception of Oubangui strain SR 709 which differs by 2 bp (~0.8%) from the rest of the strains. Identical sequences were obtained for recent and historical *C. canephora*, *C. excelsa*, and *C. arabica* isolates, which in turn were identical to the *F. udum* IMI 271070 sequence. NRRL 22540 and NRRL 26064 strains differ from each other and from this main clade.

IMI 389581 revealed a 98% similarity to *G. fujikuroi*. Unfortunately no sequence was available for *F. phyllophilum*.

Mating type 2 (MAT-2)

The amplified *MAT-2* fragment using previously described primer sets (Yoshida *et al.*, 1998; Kerényi *et al.*, 1999; Steenkamp *et al.*, 2000) discriminates three clades within the *G. xylarioides/F. udum* complex. The first clade is formed by ***MAT-2*** sequences from recent *C. canephora* and *C. excelsa* isolates and historical *C. canephora* strain MUCL 14186 (1976) that are 100% identical to each other regardless of their geographical, historical, or host origin and differ by 2bp (~0.3%) from historical excelsa isolate BBA 62457. *F. udum* strains IMI 193652 and IMI 275452 differ by 3 bp from each other and form a sister clad (64% bootstrap) to the first. The third clade (82% bootstrap support) is formed by recent *C. arabica* strains identical in sequence with the exception of IMI 389563 differing by 2 bp. Historical strains CBS 25852 and CBS 74979 are identical in sequence and differ from the canephora group by four nucleotides (99% homology). Unfortunately no sequence was available for *F. phyllophilum*.

CONCLUSION

The sequence analysis of the **four nuclear genes** studied (tef, CL, H3, *MAT*) is **incoherent**, the putative clades/subdivisions within the actual and historical *G. xylarioides* pathogen population are inconsistent. Nevertheless, the placement of the GxC as a sister clade to the *F. udum/Fusarium* spp. complex **(Table 3)** is consistent.

It should be noted that only a small part of the ***MAT*-1** idiomorph has been sequenced, which could explain the poor phylogenetic differentiation obtained. Indeed, the *G. fujikuroi MAT-1* idiomorph, and supposedly that of *G. xylarioides*, spans more than 4600 bp containing three open reading frames (ORF) of which only ~300 bp (~1/10 of the coding region) in the *MAT-1-1* ORF have been sequenced. Currently, UCL is working on the amplification of the entire *MAT*-1 idiomorph in order to compare the phylogenetic utility of the different ORF and non-coding regions compared to non-*MAT* genes.

It should equally be noted that the ***MAT-2*** gene, spanning more than 3800 bp, contains a single ORF of which approximately a third has been sequenced. These partial sequences are very informative, discriminating 5 different types of sequences within the GxC whilst tef, CL and H3 discriminated respectively 3, 2, and 3 groups. Solely the *MAT-2* gene distinguished *F. udum* isolates IMI 193652 and IMI 275452, originating from different hosts, as well as identifying a "mutant" arabica isolate (IMI 389563). The sequencing of the entire *MAT* idiomorph in *G. xylarioides / F. udum* strains should enable the differentiation of eventual "clades" within the actual and historical *G. xylarioides* population, as well as help in the understanding of the functions controlled by the *MAT* ORFs.

Historical arabica isolates **BBA 62458** (1971) and **IMI 204746** (1976) are identical in sequence to recent *C. arabica* isolates for all 4 genes studied. This report contradicts Girma's (2004) observation of a different RAPD profile for isolate BBA 62458. Historical Congolese canephora isolate **MUCL 14186** (1968) is identical to recent canephora and excelsa isolates from the DRC, Uganda and Tanzania. Two groups of historical isolates have been identified using *tef*, H3, *MAT-1* and *MAT-2* sequences. Group Ia contains **CBS 25852, CBS 74979** and **SR 709** and group Ib is composed of isolates **BBA 62457** and **ATCC 15664**. Only the calmodulin (CL) gene was unable to distinguish these two groups.

We would like to remark that historical strain **BBA 62455** (Guinea, 1964), currently being sequenced in our facilities, has been identified morphologically by UCL as being *G. xylarioides*, confirming Dr. A. Girma's (2004) results. However, this same strain (renamed FRC L-101 = NRRL 13277), has been identified by Geiser *et al.* (2005) as belonging to the *Lateritum* Clade I, and by CABI

(= IMI 39228) as *F. stilboides* (with an erroneous date of isolation noted). It would seem that the strain has been contaminated somewhere along the way.

The growth rate assay allowed amongst other things, to confirm that the **optimum growth** temperature for the GxC is **~25°C** and that the **lethality** temperature is **~33-37°C**, depending on the isolate. This could explain why inoculations in UCL facilities yielded poor results. Indeed, during the summer, regulation of the temperatures in the greenhouse is difficult, with temperatures of ~40° being recorded on some occasions. A complementary assay is being carried out to identify the incubation period necessary for spores to be non-viable at 37,5°C.

MAT ratio results obtained for Equator province isolates indicates a clonal introduction of the pathogen in this region, and possibly reflects the epidemiological history of the pathogen. The existence of only *MAT-1* isolates in this region needs to be validated by UNIKIN staff by the fact that the teleomorph was not observed in fields surveyed.

The study has allowed the opening of new doors, such as the clarification of the taxonomy within the *F. udum* and *F. lateritium* complex, and the existence of previously undescribed Fusarium (*Fusarium* sp. IMI 389581 previously identified as *F. oxysporum* by CABI). However, the exact status of the different clades as *formae speciales*, subspecies or species is still under discussion.

A first manuscript "*Gibberella xylarioides sensu lato* from *Coffea canephora*: a new mating population in the *G. fujikuroi* species complex" has been published in AEM [Dec 2005, Vol 71 (12): 8466-8471]. Two other manuscripts treating respectively of the diversity within the *G. xylarioides/F. udum* population and the characterization of the *MAT*-1 idiomorph in the *G. xylarioides* complex are in preparation.

Gene /Method	Recent Canephora	Recent Arabica	BBA 62455	BBA 62457	BBA 62458	ATCC 15664	CBS 25852	CBS 74979	SR 709	IMI 193652	IMI 271070	IMI 275452	NRRL 22540	NRRL 26064	IMI 389581
RAPD	1	2	-	3	-	3	4	4	-	-	-	-	-	-	-
Tef	1	1	NT	2	1	2	3	3	3	4	4	4	5	6	7
CL	1	2	NT	1	2	1	1	1	1	3	3	3	3	3	4
H3	1	2	NT	2	2	2	3	3	3	4	4	4	5	6	7
Haplotype	**A**	**B**	**NT**	**C**	**B**	**C**	**D**	**D**	**D**	**E**	**E**	**E**	**F**	**G**	**H**
***MAT*-1**	1	1	NT	-	1	1	-	-	2	-	1	-	3	4	5
***MAT*-2**	1	2 3)	NT	4	-	-	5	5	-	6	-	7	-	-	-
Crosses	**A**	**B & C**	**NT**	-	-	-	-	-	-	**D**	**E**	**E**	-	-	-

Table 3 : Clades and putative haplotypes for tef, CL, H3 and *MAT* genes analyzed within the *G. xylarioides* complex and closely related *Fusarium* spp.

NT: not yet tested

-: data unavailable

TABLE 1: Single-spore *Fusarium* spp. Isolates selected for crossing assays and genetic characterization at UCL

Accession N°	Other Code	Site Code	Locality	Collector	Date Isol./Rec	Host	Tissue	MAT PCR	Identification	Remarks
NRRL 22540	MUCL 46372	Brasil	-	-	-	*Crotalaria* sp.	-	1	*F. udum*	ex holotype *F. oxysporum* sp. *crotalariae*
NRRL 26064	MUCL 46371	Tanzania	-	-	-	*Sorghum bi.*	seed	1	*Fusarium* sp.	African clade closely related to *F. xylarioides* & *F. udum*
Gx3P22	IMI 39 26 8	Ethiopia	-	-	-	*C. arabica*	-	1	*F. xylarioides*	
IMI 37 59 07	Gx26	Ethiopia	Teppi	-	1997	*C. arabica*	-	1	*F. xylarioides*	
IMI 37 59 09	Gx43	Ethiopia	Agaro Gera Res	-	1997	*C. arabica*	-	1	*F. xylarioides*	
IMI 38 95 63	-	Ethiopia	-	-	-	*C. arabica*	-	2	*F. xylarioides*	
IMI 38 95 67	-	Ethiopia	-	-	-	*C. arabica*	-	2	*F. xylarioides*	
IMI 38 95 71	-	Ethiopia	-	-	-	*C. arabica*	-	2	*F. xylarioides*	
IMI 38 95 81	-	Ethiopia	-	-	-	*C. arabica*	-	1	*F. xylarioides*	
IMI 19 36 52	-	India	Hyderabad	Y. L. Nene	<1975	*Cajanus indicu*	61	2	*F. udum*	
IMI 27 10 70	-	Malawi	-	D. W. Makina	<1982	*Cajanus cajan*	var. ICP/8869	1	*F. udum*	
IMI 27 54 52	-	Malawi	-	E. B. Khonga	<1983	*Cajanus cajan*	A1 - stem	2	*F. udum*	
MUCL 47 039	Notre-Dame	DRC	Loeka	Kalonji & Dibue	5-09 / 22-11-200	*C. canephora*	stem	1	*F. xylarioides*	Equator Province
MUCL 47 040	Vil basalaka	DRC	Itimbiri	Kalonji & Dibue	1-09 / 22-11-200	*C. canephora*	stem	1	*F. xylarioides*	Equator Province
MUCL 47 041	Mongene	DRC	Itimbiri	Kalonji & Dibue	0-10 / 22-11-200	*C. canephora*	stem	1	*F. xylarioides*	Equator Province
MUCL 47 042	Ngwa	DRC	Itimbiri	Kalonji & Dibue	0-09 / 22-11-200	*C. canephora*	stem	1	*F. xylarioides*	Equator Province
MUCL 47 043	Yeboka	DRC	Itimbiri	Kalonji & Dibue	9-09 / 20-11-200	*C. canephora*	stem	1	*F. xylarioides*	Equator Province
MUCL 47 044	Yangoyi	DRC	Itimbiri	Kalonji & Dibue	1-09 / 20-11-200	*C. canephora*	stem	1	*F. xylarioides*	Equator Province
MUCL 47 045	Maleka	DRC	-	Kalonji & Dibue	5-09 / 22-11-200	*C. canephora*	stem	1	*F. xylarioides*	Equator Province
MUCL 47 046	Bandazwa	DRC	Itimbiri	Kalonji & Dibue	9-09 / 20-11-200	*C. canephora*	stem	1	*F. xylarioides*	Equator Province
MUCL 47 047	Mindembo 17	DRC	Lisala	Kalonji & Dibue	7-09 / 20-11-200	*C. canephora*	stem	1	*F. xylarioides*	Equator Province
MUCL 47 048	Yabia	DRC	Loeka	Kalonji & Dibue	5-09 / 22-11-200	*C. canephora*	stem	1	*F. xylarioides*	Equator Province
MUCL 47 049	Zobolia	DRC	Loeka	Kalonji & Dibue	5-09 / 20-11-200	*C. canephora*	stem	1	*F. xylarioides*	Equator Province
MUCL 47 050	Isangi	DRC	Lisala	Kalonji & Dibue	7-09 / 20-11-200	*C. canephora*	stem	1	*F. xylarioides*	Equator Province
MUCL 47 051	Bunduki	DRC	Proche Oriental	Kalonji & Dibue	9-09 / 20-11-200	*C. canephora*	stem	1	*F. xylarioides*	Equator Province
MUCL 47 052	Payipayi	DRC	-	Kalonji & Dibue	5-09 / 22-11-200	*C. canephora*	stem	1	*F. xylarioides*	Equator Province
MUCL 47 053	Mindembo 5	DRC	Lisala	Kalonji & Dibue	7-09 / 20-11-200	*C. canephora*	stem	1	*F. xylarioides*	Equator Province
MUCL 47 054	Mabanda	DRC	Itimbiri	Kalonji & Dibue	9-09 / 20-11-200	*C. canephora*	stem	1	*F. xylarioides*	Equator Province
MUCL 47 055	Moboko	DRC	Axe Itimbiri	Kalonji & Dibue	9-09 / 22-11-200	*C. canephora*	stem	1	*F. xylarioides*	Equator Province
MUCL 47 056	Mangbakapal	DRC	-	Kalonji & Dibue	6-09 / 22-11-200	*C. canephora*	stem	1	*F. xylarioides*	Equator Province
MUCL 47 057	Adongo	DRC	Loeka	Kalonji & Dibue	4-09 / 22-11-200	*C. canephora*	stem	1	*F. xylarioides*	Equator Province
MUCL 47 058	Yanangi	DRC	Itimbiri	Kalonji & Dibue	9-09 / 20-11-200	*C. canephora*	stem	1	*F. xylarioides*	Equator Province
709	-	CAF	-	Laguière	Dec. 1950	*Coffea* sp.	-	1	*F. xylarioides*	
BBA 62455	-	Guinea	-	on Fassi (isolation	1964	*C. canephora*	-	2	*F. xylarioides*	
BBA 62458	-	Ethiopia	-	ummland (isolatio	1971	*C. arabica*	-	1	*F. xylarioides*	
BBA 71975	-	Ethiopia	-	Girma Senbeta	-	*C. arabica*	-	1	*F. xylarioides*	
BBA 71976	-	Ethiopia	-	Girma Senbeta	-	*C. arabica*	-	1	*F. xylarioides*	
BBA 71977	-	Ethiopia	-	Girma Senbeta	-	*C. arabica*	-	1	*F. xylarioides*	
BBA 71978	-	Ethiopia	-	Girma Senbeta	-	*C. arabica*	-	1	*F. xylarioides*	
BBA 71979	-	Ethiopia	-	Girma Senbeta	-	*C. arabica*	-	1	*F. xylarioides*	
BBA 71980	-	Ethiopia	-	Girma Senbeta	-	*C. arabica*	-	2	*F. xylarioides*	
BBA 72205	-	Ethiopia	-	H. Nirenberg	2002	*C. arabica*	-	-	*F. xylarioides*	Hyphal tip isolates
BBA 72206	-	Ethiopia	-	H. Nirenberg	2002	*C. arabica*	-	-	*F. xylarioides*	Hyphal tip isolates
BBA 72207	-	Ethiopia	-	H. Nirenberg	2002	*C. arabica*	-	-	*F. xylarioides*	Hyphal tip isolates
Herbier n°1	-	CAF	Boukoko	A. M. Saccas?	15/10/1950	*C. excelsa*	perithecia from bark	-	*F. xylarioides*	
Herbier n°2	-	CAF	Boukoko	A. M. Saccas?	30/10/1950	*C. excelsa*	perithecia from bark	-	*F. xylarioides*	
Herbier n°3	-	CAF	Boukoko	A. M. Saccas?	Avril 1950	*C. excelsa*	perithecia from bark	-	*F. xylarioides*	
Herbier n°4	-	Côte d'Ivoir	Divo	J-Felix / ID More	31/07/1950	*C. liberica*	perithecia from bark	-	*F. xylarioides*	n°6.140
Herbier n°5	-	Côte d'Ivoir	Divo	J-Felix / ID More	31/07/1950	*C. canephora*	perithecia from bark	-	*F. xylarioides*	n°6.143
Herbier n°6	-	DRC	Yangambi	?	1971	*C. canephora*	perithecia from bark	-	*F. xylarioides*	

ANNEXE

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 2005, p. 8466–8471 Vol. 71, No. 12
0099-2240/05/$08.00+0 doi:10.1128/AEM.71.12.8466–8471.2005

Gibberella xylarioides Sensu Lato from *Coffea canephora*: a New Mating Population in the *Gibberella fujikuroi* Species Complex

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Received 27 January 2005/Accepted 7 September 2005

Gibberella xylarioides* Heim & Saccas (presumed anamorph, *Fusarium xylarioides* Steyaert) is the causal agent of coffee wilt disease, an economically important tracheomycosis in Africa. In vitro crosses carried out with Congolese, Ugandan, and Tanzanian single-ascospore/conidial isolates originating from diseased *Coffea canephora/excelsa* demonstrated a heterothallic mating system, controlled by a single locus with two alleles, *MAT-1* and *MAT-2*. Compatible isolates produced fertile perithecia within 2 to 8 weeks after mating. Mating type (*MAT*) was characterized by PCR with primer pairs previously developed for the *Gibberella fujikuroi* species complex (GFC) and for *Fusarium oxysporum*. All strains analyzed were morphologically identical and corresponded to Booth's description of the "female" *F. xylarioides* strain. Based on crossing results and *MAT-2*/translation elongation 1-α (*tef*) sequence data, *G. xylarioides*, as currently understood, is demonstrated to encompass at least three "groups": *G. xylarioides* sensu strictu Ia, defined hitherto by two "historical" West African strains originating from the severe 1930s to 1950s epidemic (CBS 25852 and CBS 74979); *G. xylarioides* sensu strictu Ib, defined by two "historical" Central African lowland strains (DSMZ 62457 and ATCC 15664); and *G. xylarioides* sensu lato II, containing Congolese, Ugandan, and Tanzanian *C. canephora/excelsa* isolates. Infertility of crosses between the coffee wilt pathogen and known GFC mating populations demonstrates that *G. xylarioides* sensu lato constitutes a new biological species within the *G. fujikuroi* complex. MUCL 44532/MUCL 43887 and MUCL 35223/MUCL 44549 are proposed as *G. xylarioides* sensu lato II *MAT-1/MAT-2* reference mating type tester strains.

Coffee wilt disease (CWD) was first noticed around 1927 in Oubangui-Chari (Central African Republic [CAR]) on *Coffea excelsa* (27). Between 1937 and 1939 the disease spread to *C. canephora* and *C. liberica* in Cameroon, Guinea, Côte d'Ivoire, and the Democratic Republic of Congo (DRC), where up to 40% of plantations were infected (8, 11, 12, 19, 26, 28). Since then, CWD has reemerged on *C. canephora/excelsa* in portions of the DRC (mid-1980s), affecting up to 90% of plantations (6), and more recently (1993) in Uganda (7), and the Lake Victoria region of Tanzania.

Gibberella xylarioides sensu strictu Heim & Saccas (1950) (supposed anamorph, *Fusarium xylarioides* Steyaert [1948]) was determined as the causal agent of the severe coffee tracheomycosis or carbuncullariosis, more commonly known as CWD, reported in the 1930s-50s epidemic. On the basis of a probable initial misidentification as *F. oxysporum* (8) and *F. oxysporum* f. *xylarioides* (5), the pathogen was thought to be a saprophyte endemic in intertropical African soils invading coffee bushes through wounds. *Gibberella xylarioides* was considered by Booth (3) as heterothallic, with sex-linked morphological characteristics. "Female" strains produced highly curved, 0-3-septate conidia, and masses of small bluish-black stromata, some of which represented perithecial initials. "Male" strains had a slimy appearance due to the presence of pionnote sporodochia containing long, thin, 5-7-septate conidia. Perithecia, occurring frequently in nature (3, 8, 11, 12, 19, 26, 28), were produced in vitro if the correct mating types were brought together under suitable conditions (3). However, representative mating type strains were never deposited in a culture collection and crossing conditions were not specified. As a consequence, von Blittersdorff and Kranz (31) were unable to repeat Booth's in vitro production of the teleomorph and the "male" strain was in fact reidentified as *F. stilboides* (20, 31) and more recently as belonging to the "*Lateritium* clade" (9).

Sexual reproduction in heterothallic filamentous ascomycetes is controlled by a single mating type (*MAT*) gene with two functional alleles/idiomorphs. The *MAT-1* idiomorph contains three open reading frames (ORFs), one of which (*MAT-1-1*) encodes a protein with a motif called the alpha-box, while the *MAT-2* idiomorph contains a single ORF (*MAT-2-1*) encoding a regulatory protein with a DNA-binding domain of the high-mobility-group (HMG) type (4). The conservation of certain amino acids in these regions could enable the PCR amplification of the as-yet-undescribed *G. xylarioides MAT-1/MAT-2* alleles using previously developed *G. fujikuroi* species and *F. oxysporum* primer pairs (30).

In addition to mating type, mating success in heterothallic fungi is also influenced by an isolate's ability to produce the required sexual structures. One of the parents must be "female fertile," i.e., capable of producing perithecia, and the other parent must be "male fertile," i.e., capable of fertilizing the female structure. Self-sterile hermaphroditic individuals can function as either the male or female parent in a cross.

The objectives of the present study were (i) to establish the heterothallic nature of the pathogen, (ii) to test Booth's hy-

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TABLE 1. Recent and historical isolates analyzed in this study

Accession no.[a]	Origin/host	Date	Fertility type[b]	*MAT* PCR	Current identification	Accession no.	
						MAT	*tef*[c]
MUCL 14186	DRC/*C. canephora*	1968	m	2	*G. xylarioides* sensu lato (group II)	AJ876536	AM072523
MUCL 35223 = FGSC 9928	DRC/*C. canephora*	1992	h	1	*G. xylarioides* sensu lato (group II)	AJ876532	AM072524
MUCL 46057	DRC/*C. canephora*	2002	m	2	*G. xylarioides* sensu lato (group II)	AJ876541	AM072525
MUCL 46056	DRC/*C. canephora*	2002	h	1	*G. xylarioides* sensu lato (group II)	AJ876534	AM072526
MUCL 45584	DRC/*C. canephora*	2002	NT	2	*G. xylarioides* sensu lato (group II)		
MUCL 45596	DRC/*C. canephora*	2002	NT	2	*G. xylarioides* sensu lato (group II)		
MUCL 44532 = FGSC 9930	DRC/*C. canephora*	2002	h	1	*G. xylarioides* sensu lato (group II)	AJ876533	AM072527
MUCL 44536*	DRC/*C. canephora*	2002	h	2	*G. xylarioides* sensu lato (group II)	AJ876539	AM072528
MUCL 44549* = FGSC 9929	DRC/*C. canephora*	2002	h	2	*G. xylarioides* sensu lato (group II)	AJ876540	AM072529
14/SS08*	DRC/*C. canephora*	2002	NT	1	*G. xylarioides* sensu lato (group II)		
15/SS02*	DRC/*C. canephora*	2002	NT	1	*G. xylarioides* sensu lato (group II)		
20/SS19*	DRC/*C. canephora*	2002	NT	2	*G. xylarioides* sensu lato (group II)		
20/SS24*	DRC/*C. canephora*	2002	NT	2	*G. xylarioides* sensu lato (group II)		
09B/SS03*	DRC/*C. canephora*	2002	NT	1	*G. xylarioides* sensu lato (group II)		
09B/SS06*	DRC/*C. canephora*	2002	NT	2	*G. xylarioides* sensu lato (group II)		
CAB 003	Uganda/*C. canephora*	1997	h	1	*G. xylarioides* sensu lato (group II)	AJ876531	AM072530
OUG 008	Uganda/*C. canephora*	1997	f	2	*G. xylarioides* sensu lato (group II)	AJ876535	AM072531
MUCL 43887 = FGSC 9931	Uganda/*C. canephora*	2002	h	2	*G. xylarioides* sensu lato (group II)	AJ876537	AM072532
MUCL 43889	Uganda/*C. canephora*	2002	m	2	*G. xylarioides* sensu lato (group II)	AJ876538	AJ539581
OUG 036	Uganda/*C. canephora*	2001	h	1	*G. xylarioides* sensu lato (group II)	AM072512	AM072533
OUG 151	Uganda/*C. excelsa*	2002	h	1	*G. xylarioides* sensu lato (group II)		
OUG 152	Uganda/*C. excelsa*	2002	h	1	*G. xylarioides* sensu lato (group II)	AM072513	AM072534
OUG 158	Uganda/*Coffea* sp.	2002	m	1	*G. xylarioides* sensu lato (group II)	AM072514	AM072535
OUG 159	Uganda/*C. canephora*	2002	h	2	*G. xylarioides* sensu lato (group II)	AM072515	AM072536
W7477b	Tanzania/*C. canephora*	2003	h	2	*G. xylarioides* sensu lato (group II)	AM072516	AM072537
W7489a	Tanzania/*C. canephora*	2003	h	2	*G. xylarioides* sensu lato (group II)		
W7494a	Tanzania/*C. canephora*	2003	h	1	*G. xylarioides* sensu lato (group II)	AM072517	AM072538
W7498a	Tanzania/*C. canephora*	2003	h	1	*G. xylarioides* sensu lato (group II)	-	AM072539
TZ002	Tanzania/*Coffea* sp.	?	h	2	*G. xylarioides* sensu lato (group II)	AM072518	AM072540
CBS 25852	Côte d'Ivoire/*Coffea* sp.	1951	ND	2	*G. xylarioides* sensu strictu (group Ia)	AM072519	AY707136
CBS 74979 = BBA 62721	Guinea/*C. canephora*	1963	ND	2	*G. xylarioides* sensu strictu (group Ia)	AM072520	AY707120
ATCC 15664 DSMZ 62457 = ATCC 36326	Guinea?/*Coffea* sp.	1964	ND	1	*G. xylarioides* sensu strictu (group Ib)	AM072521	AM072541
ATCC 36325	CAR/*C. excelsa*	1955	ND	2	*G. xylarioides* sensu strictu (group Ib)	AM072522	AM072542
	CAR/*C. excelsa*	1960s?	ND	ND	*Fusarium* sp. strain ATCC 36325		AM072543

[a] Culture collection abbreviations: MUCL, Mycothèque de l'Université catholique de Louvain, Louvain-la-Neuve, Belgium; FGSC, Fungal Genetics Stock Center, University of Kansas Medical Center, Kansas City, KS; CBS, Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands; BBA, Biologische Bundesanstalt für Land- und Forstwirtschaft, Berlin-Dahlem, Germany; ATCC, American Type Culture Collection, Manassas, VA; DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany. Ugandan and Tanzanian isolates not carrying the MUCL single-prefix were donated by CABI (CABI Bioscience UK Centre, Egham, Surrey, United Kingdom) and CIRAD (CIRAD/UMR BGPI, TA41/K, Campus International de Baillarguet, Montpellier, France) within the framework of the European Community-funded INCO-COWIDI project. *, *G. xylarioides* ascospore strains isolated from freshly collected samples.

[b] f, female fertile; m, male fertile; h, hermaphrodite; ND, not determined; NT, not tested.

[c] CBS *tef* sequences are identical to those previously submitted by Geiser et al. (9).

pothesis of sex-linked morphological dimorphism, and (iii) to determine whether cryptic speciation has occurred within the recent *C. canephora/excelsa* pathogen population and/or between existent and historical pathogen populations. Our working hypothesis was that *G. xylarioides* strains isolated from *C. canephora/excelsa* trees of diverse geographic origins produce the teleomorph when opposite mating types are paired under in vitro conditions. We tested here the utility of the biological and phylogenetic species concept in the *G. xylarioides* complex, allowing us to improve our knowledge of the reproduction mode and diversity of this important fungus.

MATERIALS AND METHODS

Fungal isolates and culturing conditions. Accession numbers, geographical origins, and host substrates are listed for the isolates studied here (Table 1). Single conidia and ascospore-derived *F. xylarioides/G. xylarioides* strains were obtained from stem samples of diseased *C. canephora* trees recently collected in the DRC and Uganda. Ugandan and Tanzanian isolates not carrying a MUCL number were donated by CABI and CIRAD. Five "historical" strains associated with coffee wilt symptoms were obtained from international culture collections. An in-depth study of fertility and mating type was carried out on strains and *MAT* and translation elongation factor 1-α (*tef*) genes were sequenced. The *MAT/tef* accession numbers are indicated in Table 1. Cultures were routinely grown on synthetic low-nutrient agar (SNA) (10) and incubated at 25 ± 2°C with a 12-h photoperiod under light banks of cool white fluorescent lights (General Electric 35099 F36W/33) and black lights (Philips TLD 36W/80) in a 3:2 ratio spaced 15 cm apart and 40 cm from the petri dishes. Correspondence with the morphological characteristics described for *F. xylarioides* (3, 5, 19, 26, 28), as well as with *Fusarium* sp./*Lateritium* clade (strain ATCC 36325), was established for strains after 7 days with conidia produced on SNA plates. All strains with a MUCL prefix are stored for long-term conservation on SNA slants, in lyophilized form and by cryopreservation (−130°C), at the Mycothèque de l'Université Catholique de Louvain (BCCM/MUCL) culture collection.

Standard *G. fujikuroi* mating populations mating-type tester strains from the Fungal Genetics Stock Center (FGSC; University of Kansas Medical Center, Kansas City, Kans.) (18), were used for the cross-fertility study: FGSC 7600 (*G. moniliformis*, *MAT-1*), FGSC 7603 (*G. moniliformis*, *MAT-2*), FGSC 7611 (*G. sacchari*, *MAT-1*), FGSC 7610 (*G. sacchari*, *MAT-2*), FGSC 8931 (*G. fujikuroi*, *MAT-1*), FGSC 8932 (*G. fujikuroi*, *MAT-2*), FGSC 7615 (*G. intermedia*, *MAT-1*), FGSC 7614 (*G. intermedia*, *MAT-2*), FGSC 7616 (*G. subglutinans*, *MAT-1*), FGSC 7617 (*G. subglutinans*, *MAT-2*), FGSC 7057 (*G. thapsina*, *MAT-1*), FGSC 7056 (*G. thapsina*, *MAT-2*), FGSC 8934 (*G. nygamai*, *MAT-1*), FGSC 8933 (*G. nygamai*, *MAT-2*), FGSC 9022 (*G. circinata*, *MAT-1*), FGSC 9023 (*G. circinata*, *MAT-2*), FGSC 8910 (*G. konza*, *MAT-1*), and FGSC 8911 (*G. konza*, *MAT-2*).

Sexual compatibility tests and mating type. Crosses were made on carrot agar (CA), modified carrot agar (MCA), and coffee twig agar (CTA). CA (14) was prepared with 200 g of carrots per liter of media. MCA was prepared by using only 50 g of carrots and then filtering the juice through a fine cheesecloth. For the preparation of CTA plates, *C. canephora* twigs (≤2 cm in diameter) were cut into 2- to 3-cm-long segments. Segments were split lengthwise, and half of their bark was removed. These segments were autoclaved at 121°C for 30 min on each of two consecutive days before being embedded into 2% water agar with the cambium side exposed above the agar surface.

Crosses were set up in triplicates on at least two separate occasions. Diallel CA/MCA crosses were carried out according to the protocol established for *F. moniliforme* (14) except that the male parent was inoculated onto a SNA plate and a Tween 20 (Merck, Munich, Germany) water solution (25 μl of Tween 20 per 100 ml of distilled water) was used. Each female parent was self-inoculated ("selfed") to test for potential homothallism and/or contamination. Cultures were incubated at 25 ± 2°C with a 12:12 h light-dark cycle. Crosses on CTA plates were tested by placing 4-mm mycelial plugs of the isolates on either side of the twig and incubating the plates at 25°C for 2 weeks in total darkness until the colonies had intermingled. Plates were placed in the light and examined weekly for perithecial development for up to 12 weeks. Crosses were scored as positive when ascospores were observed oozing out of perithecia in at least two different confrontations. Tests for interfertility between the 18 MP (A-I) tester strains of the GFC and *F. xylarioides*/*G. xylarioides* isolates were carried out in triplicates on CA.

DNA extraction, amplification and nucleotide sequencing. Isolates were grown in the dark at 25°C for 3 days in a 2% malt extract broth medium (Duchefa, Haarlem, The Netherlands) on a rotary shaker (100 rpm). Mycelium was harvested by centrifugation (2,250 × *g*, 4°C, 15 min), and the pellets were lyophilized. Fungal DNA was extracted from 30-mg mycelium samples by using a procedure based on the method of Lee et al. (15), and crude nucleic acids were precipitated with a double volume of absolute ethanol and kept 1 h at −80°C before dissolving the pellet in 100 μl of a sterile water solution. A second purification was carried out on DNA samples using the GeneClean III kit (Q-Biogene, Carlsbad, CA) according to the manufacturer's recommendations before being quantified with an Eppendorf Bio Photometer (Eppendorf, Hamburg, Germany). Purified DNA and dilutions were kept at −20°C. PCR amplification of the *tef* 1-α gene and sequencing of the amplicon was performed with primer pair ef1/ef2 (22). Previously described degenerate *F. oxysporum* primers Fα1/Fα2 (2) and *F. oxysporum*-specific PCR primer pairs GfHMG1/GfHMG2 (13) and Gfmat1a/1b and Gfmat2c/2d (27) were used to amplify and sequence parts of the *G. xylarioides MAT* gene. Primers were suspended in ultra pure water (W4502; Sigma, Steinheim, Germany) and stock solutions (100 μM) stored at −20°C. *MAT* and *tef* PCRs were performed with *Taq* DNA polymerase recombinant (Invitrogen Life Technologies, Carlsbad, CA) in an Eppendorf Mastercycle thermocycler (Eppendorf). PCR mixtures and amplification conditions for the chosen primer pairs were identical to those described previously (2, 13, 22, 27). PCR products were purified with a QIAQuick PCR purification kit 250 (QIAGEN, Inc., Hilden, Germany) according to the manufacturer's recommendations. Sequencing reactions were performed by using a CEQ DTCS Quick Start kit (Beckman Coulter, Inc., Fullerton, CA), and nucleotide sequence chromatograms were obtained with a CEQ 2000 XL capillary automated sequencer (Beckman Coulter). Sequences were assembled and corrected manually using the Sequencher 4.1 program (Gene Code Corp., Ann Arbor, MI). *MAT* and *tef* sequences were deposited in the EMBL database (Table 1).

Phylogenetic analysis. Similarity searches were done against the GenBank/EMBL databases by using the BLASTN 2.2.9 program (1). Based on the results, sequences were aligned by using CLUSTAL W 1.82 (24) with files containing available DNA sequences representing known mating populations within the *G. fujikuroi* species complex (27) (for *MAT-2-1*), part of the African clade (21) (for *tef*), and the *Lateritium* clade sensu Geiser (9) (for *tef* analysis of historical strain ATCC 36325). The phylogenetic relationship between the coffee wilt pathogen and other closely related *Fusarium* spp. was inferred from the maximum-parsimony analysis of aligned sequences by using the PAUP* version 4.0b10 phylogenetics package (29). Heuristic searches were performed by using random sequence addition with a tree-bisection-reconnection branch-swapping algorithm. Confidence in the branching points was established by performing 1,000 bootstrap replicates using maximum-parsimony as the criterion and random sequence addition. For the *F. xylarioides* analysis, trees were generated for *MAT-2-1* and *tef* using, respectively *F. oxysporum* AB011378 and AF160312 sequences as outgroups. *F. xylarioides* AJ539581 was used as the outgroup in the ATCC 36325 *tef* analysis.

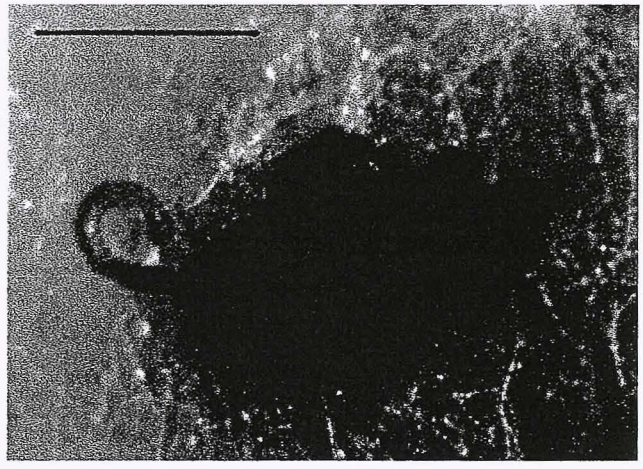

FIG. 1. Purple-black *G. xylarioides* sensu lato perithecia producing an orange cirrhus of ascospores on a carrot agar plate. Scale bar, 500 μm.

RESULTS

Sexual reproduction by *G. xylarioides*. The perfect state was not produced when strains were selfed on any of the crossing media used. Based on CTA crosses, strains could be divided into two mating groups, *MAT-1* and *MAT-2*, confirmed with PCR assays. The *MAT-1*/*MAT-2* ratio was 6:9 in isolates originating from the DRC, 5:4 in isolates from Uganda, and 2:3 in the Tanzanian collection. Strains could be divided into either female fertile, male fertile, or hermaphrodite classes (Table 1). Ascospore-derived strains examined were hermaphrodites.

Perithecia oozing ascospores (Fig. 1) appeared on CA/MCA 2 to 6 weeks after spermatization, whereas on CTA mature perithecia were observed 4 to 8 weeks after inoculation. To confirm outcrossing potential in *G. xylarioides*, *MAT*-PCR was used to analyze nine ascospore progeny from each of the three following crosses, CAB003 × OUG008, CAB003 × MUCL 44549, and MUCL 46056 × MUCL 14186. *MAT-1*/*MAT-2* ratios were, respectively, of 4:5, 7:2, and 5:4. Based on the abundance of perithecia (>30) formed in CTA pairings, hermaphroditic strains MUCL 44532/MUCL 43887 and MUCL 35223/MUCL 44549 were chosen as *MAT-1*/*MAT-2 C. canephora* reference mating type tester pairs and have been deposited at the FGSC (Table 1).

Crosses between *C. canephora* tester strains and the historical isolates did not produce fertile perithecia when opposite mating types were paired. Warty bodies presenting external perithecial characteristics contained either unidentified or asci-like structures enclosing "pseudo-microascospores" in DSMZ 62457 × MUCL 44532, ATCC 15664 × MUCL 44536, and

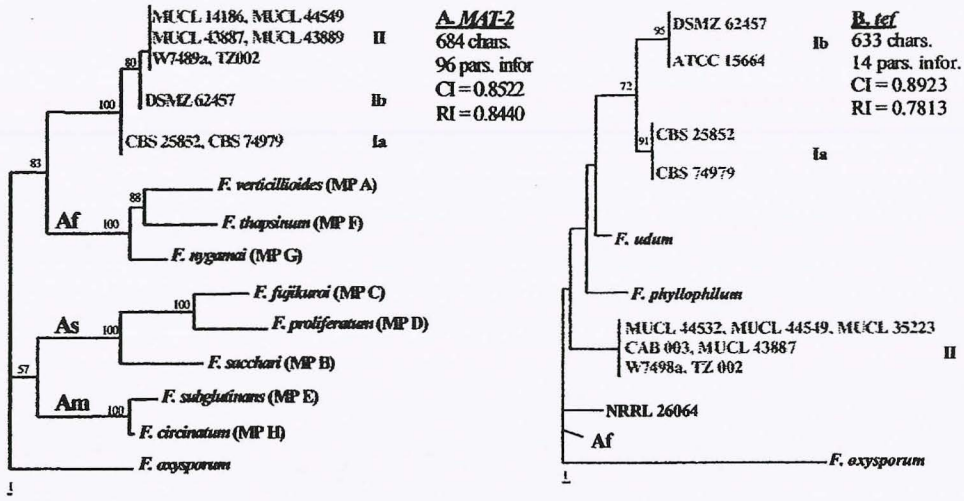

FIG. 2. Maximum-parsimony phylograms based on partial *MAT-2* (27) (A) and *tef* (21) (B) gene sequences of species belonging to the *G. fujikuroi* species complex including representative coffee wilt isolates (in gray) from *Coffea canephora/excelsa* and historical strains. Trees were generated with PAUP* v.4.0 b10 (29) with *F. oxysporum* as outgroups. Bootstrap values based on 1,000 replications are indicated in percentages above the internodes when replication frequencies exceed 50%. African (Af), Asian (As), and American (Am) clades sensu O'Donnell (21) are shown. MP, mating population.

CBS 74979 × MUCL 44532 crosses. Perithecial initials were observed in the DSMZ 62457 × ATCC 15664 crosses. The MP A, B, D, E, and F control crosses all produced mature perithecia under *G. xylarioides* crossing conditions (25 ± 2°C with a 12-h photoperiod), while MP C, G, H, and I produced only protoperithecia under these conditions. No intercrossing was observed between *G. xylarioides* and any of the tested mating populations in the GFC.

Lack of diversity in *MAT-1*. A degenerate *F. oxysporum* primer pair (2) and a specific *Fusarium* primer pair (27) could be used to amplify a 400- and a 300-bp band, respectively, for recently collected *MAT-1* strains and for historical strain ATCC 15664. The resulting 329-bp sequences (AJ876531 to AJ876534, AM072512 to AM072514, AM072517, and AM072521) were 100% identical and 96% similar to the *F. nygamai* (MP G) *MAT-1-1* gene (AF236763) and 92% similar to the *F. oxysporum* f. sp. *niveum MAT-1-1* gene (AY040736.1).

Diversity at *MAT-2* level. Recent *F. xylarioides/G. xylarioides* strains and historical isolates CBS 74979, CBS 25852, and DSMZ 62457 produced a single amplification product of ~200 and 850 bp using specific primer pairs (13, 27). The resulting 627-bp nucleotide sequences from recent *C. canephora* strains (AJ876535 to AJ876541, AM072515, AM072516, and AM072518) were 100% identical to each other and 94% similar to both *F. oxysporum* f. *radici-lycopersici* (AB011378.1) and to *F. nygamai* (MP G, AF236771). CBS strains 25852 (AM072519) and 74979 (AM072520) have identical sequences but differ from the historical *G. xylarioides* isolate DSMZ 62457 (AM072522) and the recent *C. canephora* isolates by 4 and 6 bp, respectively.

Diversity at *tef* level. All strains had an ~800-bp fragment amplified when the ef1/ef2 primer pair was used; historical strain ATCC 36325 produced a slightly larger fragment. Four sequence patterns were identified. The first group contained the previously described (9) historical isolates CBS 25852 and 74979 that differed at 14 of 633 sites (2.2%) from recent *F. xylarioides* strains, confirming available sequences for these strains (AY707136 and AY707120). The second group, consisting in historical isolates DSMZ 62457 (AM072542) and ATCC 15664 (AM072541), differed at 20 of 633 sites (3.2%) from recent *F. xylarioides* isolates and at 6 of 633 sites (0.9%) from the historical CBS isolates forming the first group. The third and quantitatively most important group consisted in all recent *MAT-1/MAT-2 C. canephora* isolates originating from the DRC, Uganda and Tanzania (AM072523 to AM072540) and was 100% similar to previously sequenced *F. xylarioides* strains deposited by Munaut (AJ539579 to AJ539582) and Geiser et al. (9) (AY70719 and AY707121 to AY707135). BLAST searches of the 667-bp ATCC 36325 historical isolate *tef* sequence presented the highest similarity (93%) to *Lateritium* clade I sensu Geiser strain L-376 (from coffee seed in Brazil).

Phylogenetic comparison with related *Fusarium* spp. Partial *MAT-1* and *MAT-2* sequences obtained from recent and historical *F. xylarioides/G. xylarioides* isolates were compared to available *G. fujikuroi* MP sequences (A to H, AF236757 to AF236772). The resulting phylogenetic trees are very similar to those of Steenkamp et al. (27) with the exception of the placement of *F. xylarioides*. *MAT-1-1* sequences from the recent and historical DSMZ isolates could not be associated to a particular portion of the phylogenetic tree with any statistical confidence (data not shown). *MAT-2-1* sequences of recent and historical isolates (Fig. 2A) form a strongly supported clade (100%) and group as a sister clade (83% bootstrap value) with those from *F. verticilloides* (MP A), *F. nygamai* (MP G), and *F. thapsinum* (MP F) of the previously described "African" clade (9, 21). The *F. xylarioides/G. xylarioides* clade is composed of three subclades (or "alleles"): all recent *C. canephora* isolates from DRC, Uganda, and Tanzania form a well-supported clade (group II), while historical isolate DSMZ 62457 and CBS isolates 25852 and 74979 form two distinct clades, respectively, called "historical" groups Ib and Ia.

The same three clades can be differentiated with the *tef*

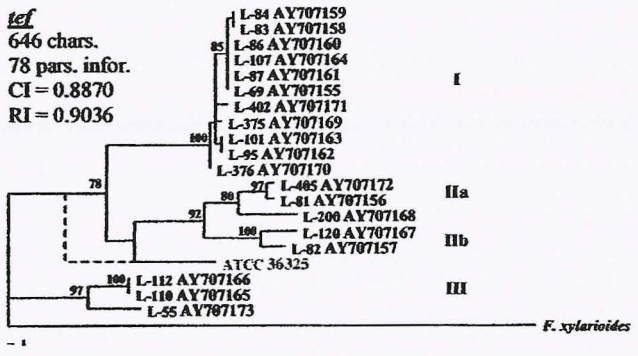

FIG. 3. Maximum-parsimony phylogram based on *tef* gene sequences of *Lateritium* clade fusaria (9) including historical coffee wilt isolate ATCC 36325 from *Coffea excelsa* (in gray). The figure was generated with PAUP* v.4.0 b10 (29) with *F. xylarioides* strain MUCL 43889 (AJ539581) as an outgroup. Bootstrap values, based on 1,000 replications, are indicated in percentages above the internodes when replication frequencies exceed 70%.

sequences (Fig. 2B), with "historical" isolate ATCC 15664 joining DSMZ 62457 in its placement in group Ib within the African clade of the GFC. However, the *F. xylarioides/G. xylarioides* isolates do not form a monophyletic clade as in the *MAT-2* phylogeny. Historical isolates nest together and form two well-supported sister "clades" (72%), while recent isolates form a well-supported clade but of uncertain relationship. *F. udum*, *F. phyllophilum*, and *Fusarium* sp. strain NRRL 26064 could not be placed unambiguously in the tree. Historical isolate ATCC 36325 can be placed either basally to the *Lateritium* clades I/II or to *Lateritium* clade II (Fig. 3).

DISCUSSION

***G. xylarioides*, a heterothallic fungus corresponding to Booth's "female" strain.** Results show that recent isolates of the *G. xylarioides* complex represent a heterothallic dimictic fungus. Single ascospore/conidia-derived hermaphroditic strains MUCL 44532/MUCL 43887 and MUCL 35223/MUCL 44549 were selected as *MAT-1/MAT-2* reference mating type tester pairs. Both mating types are present in an approximately 1:1 ratio in the different regions sampled, which was expected since the perfect stage was frequently observed in situ. Analysis of progeny from three fertile crosses confirmed that the coffee wilt pathogen can outcross.

Strains of opposite mating type are morphologically identical and similar to those previously described as *F. xylarioides* "female" strains (3), which contradicts Booth's claim of sex-linked morphological characteristics and the existence of a morphologically distinct "male" strain in this species. Through carrot agar diallel crosses we identified hermaphroditic, male-fertile only, and female-fertile only (isolate OUG 008) strains within our CW isolates.

Among other *G. fujikuroi* MPs, Leslie and Klein (17) reported that, on a worldwide basis, the percentage of hermaphroditic strains ranges from 10 to 50%. Leslie (16) suggested that the loss of female functions is relatively common in these organisms because perithecium formation can be interrupted by mutation at any one of the many genes involved in the process. The observation of a female-fertile only strain (OUG 008) within isolates from *C. canephora* was unexpected since it is generally expected that all strains are capable of acting as the male parent (fertilizing agent) but only a subset are capable of acting as the female parent. Moreover, this female-fertile only strain, like all of the strains in the present study, produces abundant conidia, suggesting that a more detailed study of this strain is warranted to identify the genetic basis for its low/nonexistent male fertility observed.

Diversity within the historical and actual *G. xylarioides* population. A recent publication (9) hypothesized that the usual concept of *F. xylarioides/G. xylarioides* could encompass cryptic species. This suggestion resulted from the discovery that two groups had different *tef* alleles. The larger group was composed of isolates from recently wilted *C. canephora* and *C. arabica* trees in Uganda and Ethiopia, while the second group consisted of two "historical" strains (1950s to 1960s) originating from Guinea (CBS 74979) and Côte d'Ivoire (CBS 25852). On the basis of this observation, a possible cryptic speciation event was suggested to separate Eastern and Western African strains.

In the present study, recent ascospore-derived isolates from the DRC corresponded consistently morphologically, sexually, and molecularly (*MAT* and *tef* sequence data) with recent *F. xylarioides* isolates from the DRC, Uganda, and Tanzania. This confirms that the perfect stage observed in recent outbreaks corresponds to the anamorph isolated in the DRC, Uganda, and Tanzania since 1968. However, these isolates differ from "historical" isolates in their sexual compatibility and in *tef* and *MAT-2-1* sequences, differences that are consistent with cryptic speciation. We have been unable to access the *F. xylarioides* (28) type material, so the connection between historical *G. xylarioides* and historical *F. xylarioides* cannot be proven. For the moment it remains unclear whether recent epidemics observed on *C. canephora* and those reported in the 1950s and 1960s from Central Africa are due to the same pathogen or whether they constitute different cryptic species. The apparent sterility of crosses between these two groups is being evaluated in more detail and may enable us to understand some of the mechanisms underlying mating and fertility and to better characterize pathogen diversity. Historical strains may have limited sexual cross fertility with any strain due to long-term storage under less-than-ideal conditions, making that it essential to increase the number of "historical" strains studied. However, perithecial initials were observed in historical isolate DSMZ 62457 cultures and selfings, implying potential female fertility. We recommend that the term *G. xylarioides* sensu strictu refer to all that are strains molecularly identical to (and sexually compatible with) historical CBS strains 25852 and 74979 (group Ia) and DSMZ 62457 and ATCC 15664 (group Ib), whereas the term *G. xylarioides* sensu lato (group II) would also include strains that are molecularly identical to (and sexually compatible with) recently isolated *C. canephora/C. excelsa* strains.

Within the "*F. xylarioides*" strains received from international culture collections, strain ATCC 36325 had clearly different conidial characteristics, resembling *F. lateritium* sensu lato, and had a strong phylogenetic connection to the "*Lateritium* clade" sensu Geiser (9). This observation further confirms that historically strains belonging to this clade were incorrectly

identified as *F. xylarioides* "male" strains, by authors such as Booth, most likely due to their frequent coisolation from wilted samples.

***G. xylarioides* sensu lato is a biological species of the GFC.** The *MAT* sequences obtained with previously described primer pairs were 94 to 96% similar to those already available for species in the GFC as well as for *F. oxysporum*. *MAT-2* sequence similarities combined with *tef* sequencing results are consistent with the recent placement of the *G. xylarioides* groups in the "African" clade of the *G. fujikuroi* species complex rather than in *Fusarium* section *Lateritium* (9), suggesting the eventual use of the *MAT* loci for phylogenetics in heterothallic fungi (23, 27). The inability of *G. xylarioides* strains to cross with known GFC MP mating-type tester strains leads us to suggest that *G. xylarioides* sensu lato is the 11th biological species in the GFC, i.e., MP K, after the recently described *G. konza* (32) and *G. gaditjirrii* sp. nov. (25). The status of the historical strains as a biological species within the GFC is unclear since the perfect state has not been obtained in in vitro conditions.

In conclusion, the identification of at least three distinguishable groups of strains within *G. xylarioides* populations, their placement within the *G. fujikuroi* species complex, and the ability to reliably cross strains of interest should enable genetic analysis of critical traits such as pathogenicity, and mycotoxin production that were not previously possible. The frequent recovery of perithecia under field conditions suggests that new genotypes for multigenic traits can be formed much more readily in this species than in other species of the *G. fujikuroi* species complex.

ACKNOWLEDGMENTS

This study was supported by a grant from the Université catholique de Louvain (Louvain, Belgium) within the framework of the INCO-COWIDI ICA4-CT-2001-10006 European Community-funded project. We acknowledge the financial support received from the Belgian Federal Science Policy Office (contracts BCCM C2/10/007 and C3/10/003).

We thank A. Kalonji and P. Tshilenge for providing diseased coffee tree samples, M. Rutherford (CABI) and D. Bieysse (CIRAD) for supplying Ugandan and Tanzanian isolates (all except MUCL 43887 and MUCL 43889), and C. Decock for helpful insights in the interpretation of results and preparing the final manuscript.

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NATIONAL AGRICULTURAL RESEARCH ORGANISATION (NARO)

INTERNATIONAL SCIENTIFIC COOPERATION PROJECT (INCO)

Development of a long-term strategy based on genetic resistance and agro-ecological approaches against Coffee Wilt Disease

Progress report (November 1, 2004 to October 30th 2005)

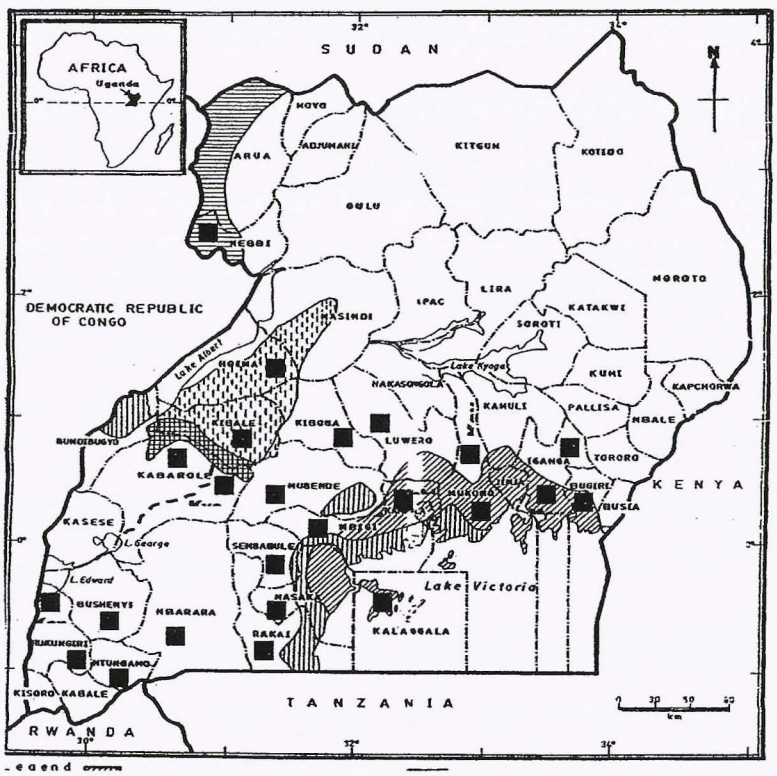
Coffee Research Institute (CORI), Kituza
P.O. BOX 185
Mukono
UGANDA

Task 1

Collection of *Fusarium xylarioides* isolates from different coffee cultivars/lines.

All the isolates of *F. xylarioides* collected during the project period were from different locations as indicated in the map below. In addition, isolates from specific clones at the research institute were also collected. The robusta clones were: 1s/2, C/1/7, B/1/1, 257/53/ 1S/3, 223/32, L/2/7, 1S/6/ Q/3/4, B/6/2, H8 CS 16, 236/26 CS 1, H4 CS 3, H4 CS 9, H16 CS 7, 218/32 CS 21, H1/CS 20, 236/26 and 258/24 (0).

Fig. 1: Locations from which *F. xylarioides* isolates were collected in Uganda (2002/4).



Impact of activities.

Isolates of *Fusarium xylarioides* in our collection came from different locations in Uganda and can be considered representative of the pathogen population in the country. It is therefore not necessary, at least for the moment to collect more isolates.

Through this extensive collection of samples, it was established, to date that, *Fusarium xylarioides* has not been identified from *Coffea arabica* in Uganda. Even in the districts of Rukungiri, Bushenyi, Mbarara, Arua and Nebbi, where both *C. arabica* and *C. canephora* are grown, arabica coffee has remained free from *F. xylarioides*. However, there is need for surveillance to be maintained on arabica coffee and quick response to reports on wilting arabica coming from the districts is essential.

WP 3 : BREEDING FOR RESISTANCE AGAINST CWD

Pascal Musoli

I INTRODUCTION

Coffee wilt disease (CWD) continued to be a major threat to coffee production and productivity in Uganda during the reporting period and variety resistance is still considered the most appropriate and a cost effective option for controlling the disease and therefore all breeding activities in the Work Package 3 of this project were given due consideration.

II OBJECTIVES

The objectives of WK3 remained unchanged i.e.

1) Identify sources of resistance against CWD through screen house tests on young seedlings and cuttings and field assessments
2) Assess inheritance of resistance to CWD among robusta coffee in Uganda
3) Evaluate genetic diversity among and between different sources of resistance to CWD in Uganda
4) Initiate breeding towards developing varieties with durable resistance to CWD.

III ACTIVITIES AND PROGRESS

The research activities carried out during this reporting period are described below and they are grouped according the work package objectives although activities of aiming at achieving the different objectives are however interlinked

A. IDENTIFIFYING SOURCES OF RESISTANCE TO CWD

The anticipated sources of resistance to CWD remained to be local germplasm available in Uganda and germplasm from exotic sources, mainly other African countries with history of having controlled the disease using variety resistance. The local germplasm include:

i) On-station robusta collections and their intraspecific hybrids
ii) Arabica collections and their intraspecific hybrids
iii) Arabusta (interspecific hybrids between robusta and arabica)
iv) On-farm robusta coffee trees surviving in wilt 'hot spots
v) Wild forest robusta coffee from its natural forest habitant.
vi) Other coffee species available in the germplasm collections/fields at CORI and KARI
vii) Exotic/imported germplasm (imported or anticipated to be imported from Ivory Coast and Cameroon).

The resistance is to be identified through:

a) Carrying out tests on young coffee seedlings and rooted cuttings under screen house (uncontrolled) conditions
b) Carrying out tests on young coffee seedlings and rooted cuttings under controlled room conditions
c) Field evaluation of young and mature coffee.

Basing on the results of previous tests carried out at CORI that found arabica coffee resistant to CWD (using isolates in Uganda) screening arabica for resistance against CWD is not being carried out. Screening tests are only carried out on robusta coffee seedlings and rooted cuttings and field evaluations are on both robusta clones and intraspecific hybrids and arabusta clones.

i) Screen house tests on young rooted cuttings and seedlings of robusta coffee germplasm at CORI/KARI.

During the reporting period

i) Survivor plants (rooted cuttings and seedlings) that have under gone two rounds of inoculation in the screen house were planted out in mother gardens for multiplication

ii) Survivor cuttings and seedlings that had under gone through only one round of inoculation were re-inoculated.

iii) More robusta coffee seedlings and rooted cuttings (raised from plants in the germplasm collection at CORI and from collections of the CWD field hotspots) were inoculated

iv) Data collection continued on inoculated seedlings and cuttings

Table 1 shows mortality of 82 *C. canephora* rooted cuttings from germplasm collections at CORI recently inoculated and or re-inoculated with CWD under screen house conditions at CORI. Figure 1 shows the frequency distribution of the mortalities between the clones. After the first inoculation, many cuttings survived and 16 clones did not have any of their cuttings dying of the CWD, giving an impression that many clones be have high level of resistance to the disease. However after the second inoculation, the mortality increased drastically and only two (2) clones had all its rooted cuttings surviving. From the results, it is clear that level of mortality and therefore resistance varies between the clones. Only 4 clones had a mortality of less than 20%. This shows that the resistance against CWD in *C. canephora* is controlled by more than one gene/resistance factors and that the resistance is not equally distributed among the clones. High level of resistance exists in a smaller proportion of the population. The phylogeny seems to contribute positively to the resistance of the descendants.

Figure 1: Frequency distribution for % mortality of *C. canephora* rooted cuttings for clones from the germplasm plots at CORI artificially infected with CWD under screen house conditions atKituza

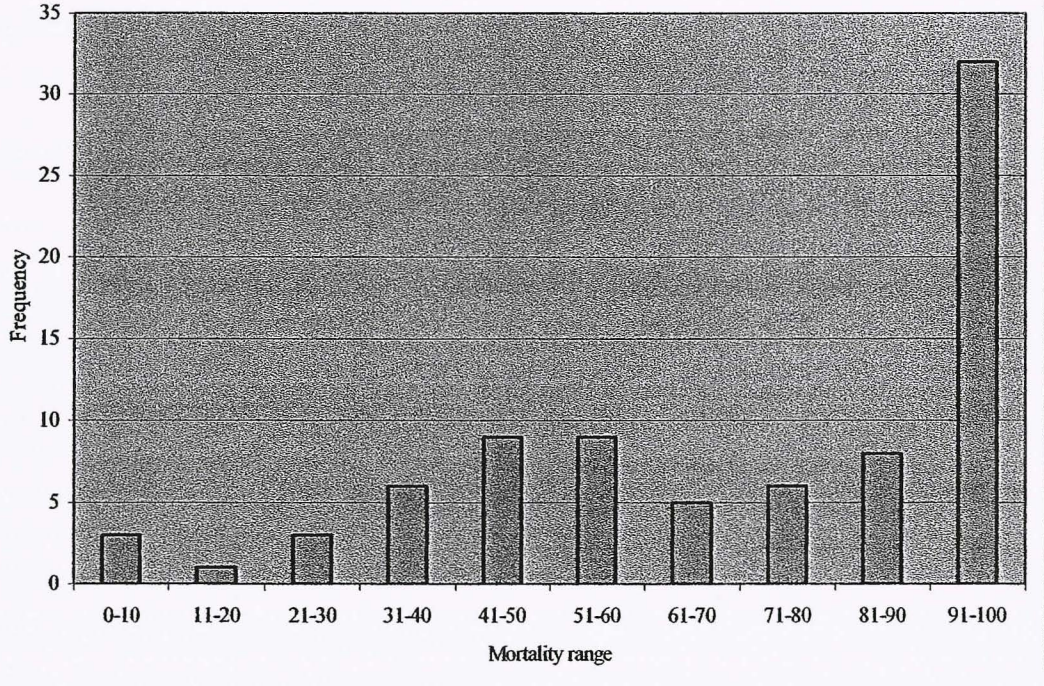


Table 1: Mortality of rooted cuttings of 82 C. canephora clones from the germplasm collection at CORI after artificial infection with CWD under screen house conditions

		29/3/2004	30/3/2005.	Score by 22/09/2005			By 22/9/05	
	Progeny	Plants Inoc	Plants re-inoc	H	S	D	% mortality	Phylogeny mean
1	1/11	7	6	1	1	1	71.4	80.9
2	1/12	12	11	0	0	11	100.0	
3	1/15	76	67	1	0	66	98.7	
4	1/3	3	3	0	0	3	100.0	
5	1/48	18	16	4	5	7	50.0	
6	1/7	7	7	2	1	4	57.1	
7	1/70	18	14	2	0	12	88.9	
8	13/15	2	0	0	0	0	100.0	66.5
9	13/71	91	82	22	39	21	33.0	
10	14/50	11	11	0	0	11	100.0	100
11	14/60	9	9	0	0	9	100.0	
12	14/70	8	8	0	0	8	100.0	
13	$1^{S}/2$	103	95	40	12	43	49.5	61.8
14	$1^{S}/3$	136	124	2	0	122	98.5	
15	$1^{S}/6$	51	45	28	4	13	37.3	
16	2/13	18	11	3	0	8	83.3	76.6
17	2/57	2	2	1	0	1	50.0	
18	2/86	31	21	0	1	20	96.8	
19	202/30	181	144	75	27	45	43.6	55.9
20	202/63	47	20	6	9	5	68.1	
21	203/14	13	12	2	0	10	84.6	76.4
22	203/32	9	5	2	2	1	55.6	
23	203/74	9	9	0	1	8	88.9	
24	$207^{S}/15$	4	4	0	0	4	100.0	100
25	209/29	20	18	6	5	7	45	45.7
26	209/29	9	7	1	3	3	55.6	
27	209/29	11	11	5	2	4	36.4	
28	218/32	2	2	2	0	0	0.0	0
29	22/2	10	9	3	3	3	40.0	40
30	222/65	35	27	3	3	21	82.9	82.9
31	223/32	31	28	10	1	20	64.5	64.5
32	227/53	38	36	0	0	36	100.0	56.3
33	227/54	5	4	0	0	4	100.0	
34	227/56	12	11	6	2	3	33.3	
35	227/58	4	3	3	0	0	25.0	
36	227/59	26	23	3	17	3	23.1	
37	228/13	11	10	1	0	9	90.9	82.3
38	228/57	25	23	3	2	18	80.0	
39	228/63	12	9	4	1	4	58.3	
40	228/65	4	4	0	0	4	100.0	
41	234/37	19	13	0	3	10	84.2	84.2
42	238/29	121	113	27	6	80	72.7	72.7
43	245/25	89	85	81	0	4	9.0	9.0

		29/3/2004	30/3/2005.	Score by 22/09/2005			By 22/9/05	
	Progeny	Plants Inoc	Plants re-inoc	H	S	D	% mortality	Phylogeny mean
44	254/04	1	1	0	0	1	100.0	74.5
45	254/28	63	55	25	1	29	58.7	
46	254/62	14	12	0	0	12	100.0	
47	254/80	189	181	101	14	66	39.2	
48	256/20/6	2	2	0	0	2	100.0	100
49	258/28	70	61	36	5	20	41.4	69.4
50	258/58/3	76	71	2	0	69	97.4	
51	261^{s}/2	70	57	1	0	56	98.6	97.2
52	261^{s}/21	23	19	1	0	18	95.7	
53	266^{s}/11	65	55	15	19	21	47.7	47.7
54	267/5	3	2	0	0	2	100.0	75.6
55	267^{s}/6	41	37	16	4	17	51.2	
56	286	20	18	6	2	10	60.0	60
57	288	110	96	38	10	48	56.4	56.4
58	3/20	48	41	6	8	27	70.8	56.9
59	3/59	2	1	0	0	1	100.0	
60	3/62	2	2	1	1	0	0.0	
61	J1/14/19	4	2	0	0	2	100.0	88.6
62	J1/14/5	4	3	1	0	2	75.0	
63	J1/14/51	11	7	1	0	6	90.9	
64	J105203/11	13	12	0	1	11	92.3	92.3
65	J124.9/1	16	13	2	3	8	68.8	68.8
66	J15109.4/5	23	22	4	4	14	65.2	65.2
67	J24/13/5	52	43	18	10	15	46.2	70
68	J24/13/52	7	7	1	0	6	85.7	
69	J56/20/5	7	5	0	0	5	100.0	95.8
70	J56/20/50	3	3	0	0	3	100.0	
71	J56/20/57	8	6	1	0	5	87.5	
72	J74/2/13	9	8	2	3	3	44.4	44.4
73	J94/2/13	3	1	0	0	1	100.0	100
74	JB5109.4/1	12	7	1	0	6	91.7	88.8
75	JB5109.4/2	4	2	0	0	2	100.0	
76	JB5109.4/3	9	8	0	0	8	100.0	
77	JB5109.4/5	33	27	10	2	21	63.6	
78	NGREDOG	42	36	19	17	0	14.3	25.9
79	NGREDOG/3	8	5	5	0	0	37.5	
80	Unknown 1	47	47	0	1	46	97.9	
81	Unknown 2	41	16	7	2	7	78.0	
82	Unknown 3	5	3	1	1	1	60.0	

Note: % mortality =((Plants inoculated - (Healthy plants + Sick plants)/Plants inoculated)*100

Table 2 shows mortality of 31 *C. canephora* open pollinated seedling progenies from the germplasm collections at after artificial infection with CWD under screen house conditions at CORI. The high level of mortality may reflect very low level of resistance among the progenies but conditions of the screen house may have influenced the effect. Only progeny A/4/13 had 0.0% mortality and can be considered very resistant.

Table 2: Mortality of 31 C. canephora open pollinated seedling progenies from the germplasm collection at CORI after artificial infection with CWD

		29/09/2004	21/05/2005	05/10/2005			5/10/2005
	Progeny	Inoc	Re-inoc	H	S	D	% mortality
1	1/11	16	0	0	0	0	100.0
2	1/13	141	4	1	1	2	98.6
3	13/2/1	170	0	0	0	0	100.0
4	14/70	1	0	0	0	0	100.0
5	203/14	74	9	5	3	1	89.2
6	228/15	27	3	0	1	2	96.3
7	228/18	1	0	0	0	0	100.0
8	228/57	9	0	0	0	0	100.0
9	234/37	62	0	0	0	0	100.0
10	238/29	93	9	2	5	2	92.5
11	258S/58	87	0	0	0	0	100.0
12	261^{S}/21	250	22	2	10	10	95.2
13	288	125	5	0	1	4	99.2
14	3/14	22	2	0	0	2	100.0
15	3/54	125	9	6	2	1	93.6
16	3/59	2	0	0	0	0	100.0
17	3/62	7	0	0	0	0	100.0
18	A/4/13	16	16	16	0	0	0.0
19	B/1/1	136	47	11	17	19	79.4
20	B/2/1	145	44	12	8	24	86.2
21	B/6/2	60	7	2	4	1	90.0
22	C/6/1	197	42	12	14	16	86.8
23	J/1/1	324	156	46	15	95	81.2
24	J/24.9/1	94	0	0	0	0	100.0
25	J105203/11	37	0	0	0	0	100.0
26	J56/20/5	37	2	2	0	0	94.6
27	J56/20/61	3	0	0	0	0	100.0
28	JB51094/3	53	3	2	1	0	94.3
29	NGREDOG	27	0	0	0	0	100.0
30	Q/6/1	188	64	27	20	17	75.0
31	R/1/4	66	8	2	3	3	92.4

Note: % mortality =((Plants inoculated - (Healthy plants + Sick plants)/Plants inoculated)*100

ii) Screen house tests on young robusta coffee seedlings and rooted cuttings collected from wilt hotspots

During the reporting period, plants (*C. canephora* seedlings and rooted cuttings) collected during the previous reporting period from wilt hotspots in districts of Luwero, Kanungu, Wakiso, Kiboga, Rukungiri, Mubende, Bundibugyo, Mayuge, Bushenyi, Iganga, Mukono and Kyenjojo were raised in the nursery at CORI. More plants were collected from Jinja and Masaka and are also being raised in the coffee nursery shade at CORI for inoculation. Data collection continued on materials inoculated /re-inoculated in previous reporting periods and more inoculations were conducted under the screen house at CORI.

Table 3 shows results of 45 open pollinated *C. canephora* seedling progenies raised from coffee trees surviving in CWD hotspots after artificial inoculation and re-inoculation (28/2/2004 and survivors of first inoculation were inoculated on 15/9/2004) with CWD under screen house conditions at CORI. Figure 2 shows frequency distribution of the mortality between and among the seedling progenies. These results show that although the open pollinated progenies were obtained from parents that had been presumed resistant/partially resistant after surviving natural infection in the field, their resistance is varied. Only one (1) progeny had percentage mortality of less than 20% (>80% resistance) and four progenies had mortality of between 21 and 40%. Majority of the progenies had mortality of between 41 and 80%. The results again show that resistance to CWD is controlled by more than one (1) gene and the genes are not equally distributed among the parents and their progenies. Source farm seems to influence the response of the progenies.

Figure 2: Frequency distribution of % mortality among *C. canephora* open pollinated seedling progenies, from trees surviving in wilt infected field, after artificial inoculation with CWD under screen house conditions at Kituza

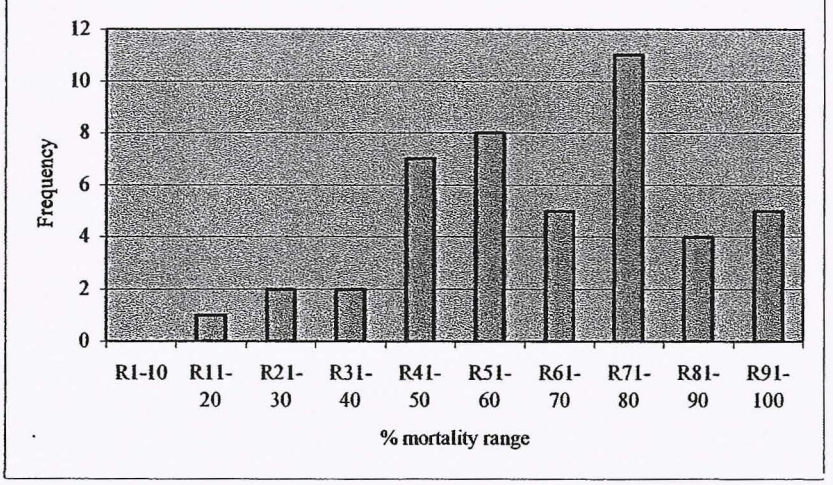


Table 3: Mortality of 45 *C. canephora* open pollinated seedling progenies, raised from trees surviving in wilt infected gardens, after artificial infection with CWD under screen house at CORI

		Plants inoculated	*Plants re-inoculated*	*Healthy status at 25/11/2005*			*% dead (overall)*	*Source (farm)*
	Progeny	*28/02/2004*	*15/09/2004*	*Healthy*	*Sick*	*Dead*		*Mean mortality*
1	Haji Kawoya/1	161	130	19	5	106	85.1	75.3
2	Haji kawoya/3	115	75	24	5	46	74.8	
3	Haji kawoya/4	30	23	6	1	16	76.7	
4	Haji Kawoya/5	105	97	32	13	58	57.1	
5	Hajikawoya/2	93	73	13	3	58	82.8	
6	Kanu/africabenard/2	34	28	7	2	19	73.5	73.5
7	Kanu/bwengyebukye/3	104	68	5	0	63	95.2	95.2
8	Kanu/kagambagye j /1	68	58	17	0	41	75.0	75
9	Kanu/kagumira/1	119	98	59	2	37	48.7	60.9
10	Kanu/kagumira/10	76	53	28	2	23	60.5	
11	Kanu/kagumira/11	138	127	44	18	65	55.0	
12	Kanu/kagumira/12	142	120	30	13	77	69.7	
13	Kanu/kagumira/13	31	21	13	0	8	58.1	
14	Kanu/kagumira/15	21	21	12	1	8	38.1	
15	Kanu/kagumira/2	-	70	21	7	42	40.0	
16	Kanu/kagumira/2	83	70	20	8	48	66.3	
17	Kanu/kagumira/3	49	37	6	2	29	83.7	
18	Kanu/kagumira/4	42	37	9	2	26	73.8	
19	Kanu/kagumira/5	60	58	14	6	38	66.7	
20	Kanu/kagumira/6	48	39	8	2	29	79.2	
21	Kanu/kagumira/7	95	82	33	6	43	58.9	
22	Kanu/kagumira/8	44	33	23	2	8	43.2	
23	Kanu/kagumira/9	42	33	12	0	21	71.4	
24	Kanu/kahgye p	34	30	2	0	28	94.1	94.1
25	Kanu/kanahe/2	63	51	14	5	32	69.8	74.2
26	Kanu/kanahe/5	89	76	9	10	57	78.6	
27	Kanu/lubinga/1	174	149	71	5	73	56.3	59.7
28	Kanu/lubinga/2	113	91	16	9	66	77.9	
29	Kanu/lubinga/3	111	85	60	1	24	45.0	
30	Kanu/mwebehire/1	160	135	50	15	70	59.4	79.7
31	Kanu/mwebehire/2	2	2	0	0	2	100.0	
32	Kanu/ndyabagira/2	24	23	17	1	5	25.0	25.0
33	Kanu/nkumbi w/4	23	16	4	1	11	78.3	55
34	Kanu/nkumbi w/5	16	8	8	0	0	50.0	
35	Kanu/nkumbi w/7	4	3	2	0	1	50.0	
36	Kanu/pkabi/1	24	19	12	2	5	41.7	
37	Kanu/sausa a	37	26	4	1	21	86.5	86.5
38	Kanu/tindiwegiwilison/1	19	16	8	0	8	57.9	55.4
39	Kanu/tindiwegiwilison/4	22	13	4	1	8	77.3	
40	Kanu/turinamasiko/1	7	5	0	0	5	100.0	100
41	Nansubuga M/2	88	79	46	1	32	46.6	73.3
42	Nansubuga M/3	8	5	0	0	5	100.0	
43	No label 1	58	52	45	1	6	20.7	20.7
44	No label 2	28	23	12	1	10	53.6	53.6
45	Walakira md/tr2	5	4	2	0	2	60.0	60.0

Note: % mortality =((Plants inoculated - (Healthy plants + Sick plants)/Plants inoculated)*100

Table 4 shows mortality of rooted cuttings of 117 C. canephora genotypes collected from CWD hotspots after two (2) rounds of artificial infection with CWD under screen house conditions at CORI. Figure 2 show frequency distribution of the mortality among the genotypes. The results show that after the two rounds of infection about 15% of the genotypes did not have cuttings dying of coffee wilt, i.e. they are resistant to the disease. Over 20% were completely susceptible (100% mortality). Majority however had mortality of between 20-90%. These results also reflect that resistance to CWD in *C. canephora* is controlled by many genes/resistance factors that are not equally distributed among genotype. However the level of resistance is relatively higher among progenies from on-farm CWD hotspots as compared to progenies from on-station collections. This is likely to be due to the fact that most parents of the progenies from on-farm have gone through screen after the natural field infection and therefore have some level of resistance that can enable them survive the disease, and which resistance has been passed on to their progenies.

Figures 4 & 5 show diagrammatic comparison between the rooted cuttings of some of *C. canephora* genotypes from wilt hotspots and their seedling progenies studied in table 3. These results show that the progenies are more venerable to CWD when infected with CWD than their parents. And resistant genotypes tend to give rise relatively resistant progenies but not always as exhibited by progenies of genotypes Adalphus Sausa-Sel, Haji Kawoya Tr2, Kagumira Tr6, Kagambirwe Tr1 and Nansubuga Tr3. Such situations complicate breeding for resistant varieties and it may therefore necessitates that clonal selections of resistant individuals are used.

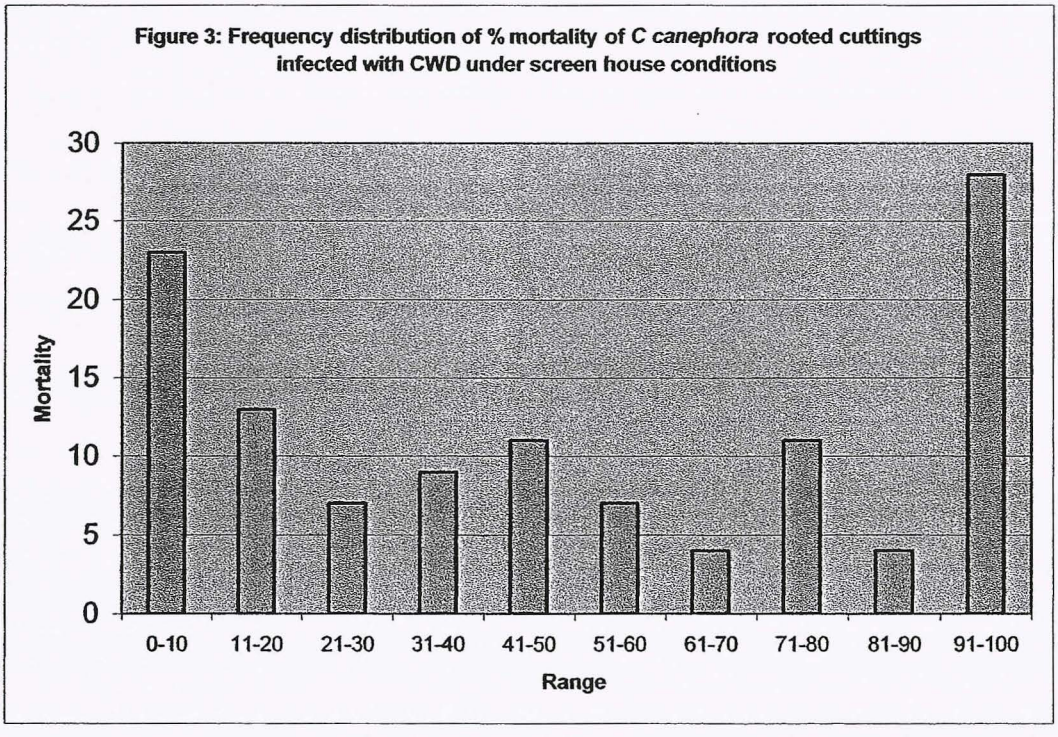


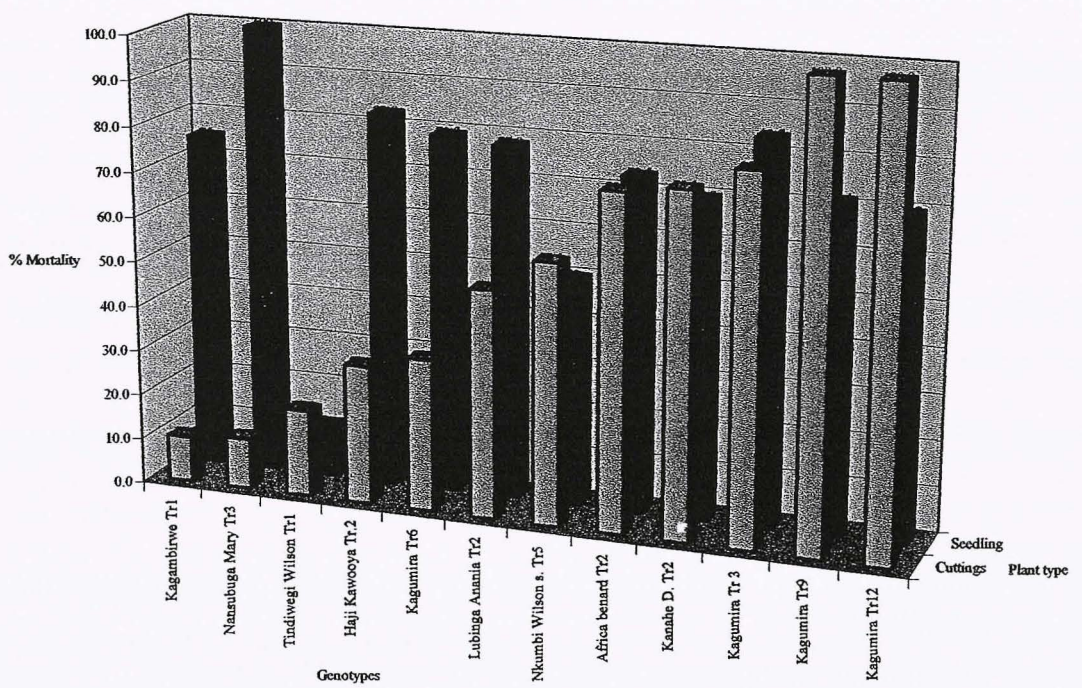
Table 4: Mortality of rooted cuttings of *C. canephora* genotypes, collected from wilt hot spots, after 20 months from time of inoculations with CWD under screen house conditions

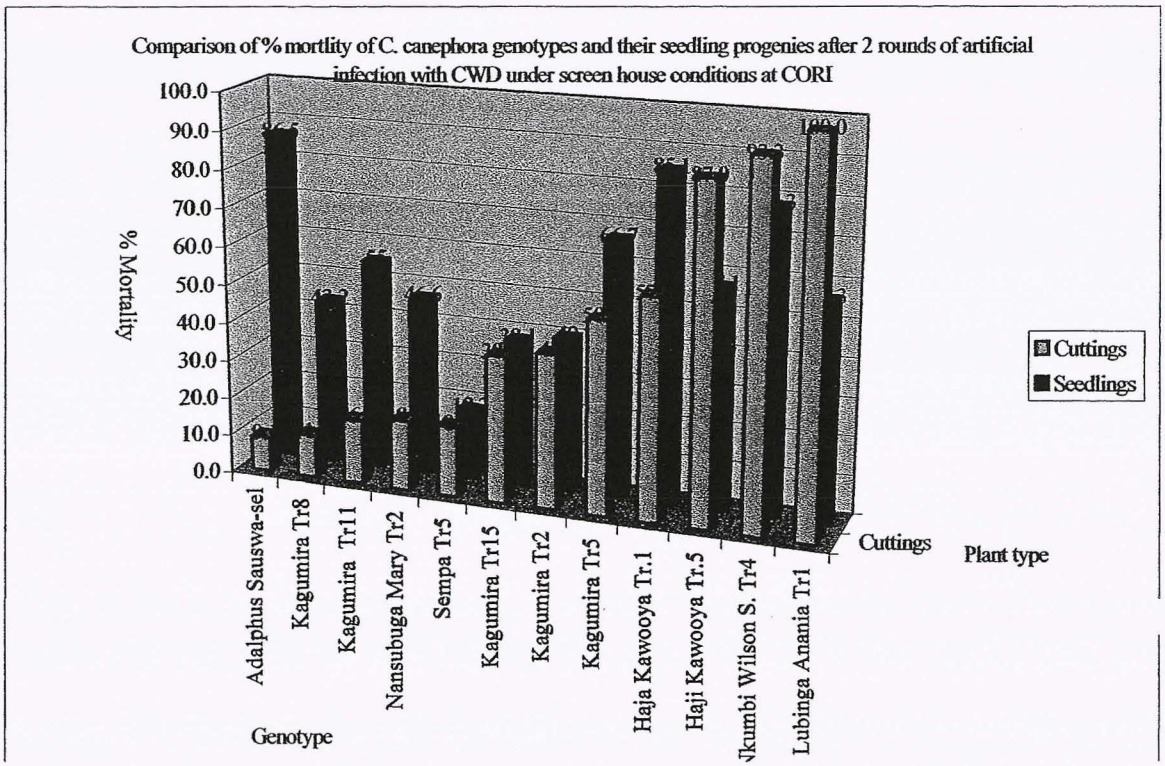
	Cuttings	Plants inoculated 28/3/2004	Plants re-inoculated *15/09/2004*	30/11/2005			Overall % Mortality after 20 months
				H	S	D	
1	Adalphus Sauswa-sel	11	11	8	2	1	9.1
2	Africa Benard/1	7	7	7	0	0	0.0
3	africa Bernard/2	9	1	0	0	1	100.0
4	Africa Bernard/3	3	3	3	0	0	0.0
5	Buteraba Tr.1	14	9	6	0	3	57.1
6	Buteraba Tr.3	3	3	3	0	0	0.0
7	Buteraba Tr.4	7	7	1	1	5	71.4
8	Buteraba Tr.5	1	1	1	0	0	0.0
9	Buteraba Tr.6	4	1	1	0	0	75.0
10	Buteraba Tr.8	4	2	1	0	1	75.0
11	Buteraba Tr.9	5	4	2	0	2	60.0
12	Bwengye Buk.Rut.Tr.3	11	11	4	2	5	45.5
13	Bwengye Bukye Rut.Tr2	11	11	0	0	11	100.0
14	Byekwaso Tr.1	5	2	0	0	2	100.0
15	Byekwaso Tr.2	9	4	0	2	2	77.8
16	Byekwaso Tr.3	8	5	3	1	1	50.0
17	Byekwaso Tr.4	9	2	2	0	0	77.8
18	Byekwaso Tr.6	5	4	3	0	1	40.0
19	Byekwaso Tr.8	14	7	0	0	7	100.0
20	Edith Tr.1	23	9	6	0	3	73.9
21	Edith Tr.2	1	1	0	1	0	0.0
22	Edith Tr.3	4	1	1	0	1	75.0
23	EdethTr.4	10	1	0	1	0	90.0
24	Enocka Mwebehire/1	1	1	0	1	0	0.0
25	Enoka Mwebehire/2	1	1	0	0	1	100.0
26	Enoka Mwebehire/3	6	5	4	1	0	16.7
27	Haji Kawooya Tr.1	7	4	3	0	1	57.1
28	Haji Kawooya Tr.2	23	18	14	2	2	30.4
29	Haji Kawooya Tr.4	9	8	6	1	1	22.2
30	Haji Kawooya Tr.5	23	13	2	1	10	87.0
31	Kagambirwe 2	3	2	2	0	0	33.3
32	Kagambirwe/1	10	10	7	2	1	10.0
33	Kagambirwe/3	10	10	9	0	1	10.0
34	Kagumira /17	4	4	3	0	1	25.0
35	Kagumira 11	6	5	4	1	0	16.7
36	Kagumira 10	1	1	1	0	0	0.0
37	Kagumira 12	2	1	0	0	1	100.0
38	Kagumira 14	5	5	5	0	0	0.0
39	Kagumira 16	3	2	2	0	0	33.3
40	Kagumira 7	2	1	1	0	0	50.0
41	Kagumira 8	9	9	7	1	1	11.1
42	Kagumira 9	1	1	0	0	1	100.0
43	Kagumira Tr 3	5	4	1	0	3	80.0
44	Kagumira Tr.1	1	1	0	0	1	100.0

45	Kagumira Tr.5	2	2	1	0	1	50.0
46	Kagumira Tr.6	9	7	5	1	1	33.3
47	Kagumira Tr.No. 4	9	7	6	1	0	22.2
48	Kanahe D. Tr.2	4	1	1	0	0	75.0
49	Kanahe D. Tr.3	6	5	5	0	0	16.7
50	Kanahe D. Tr.4	17	17	15	2	0	0.0
51	Kanahe/5	2	2	2	0	0	0.0
52	Kanahe/6 (selection)	3	3	2	1	0	0.0
53	Kanahe/7	6	6	4	0	2	33.3
54	Kanahe/8	10	10	7	0	3	30.0
55	Kigoye Tr.1	3	3	0	0	3	100.0
56	Kigoye Tr.10	10	1	0	0	1	100.0
57	Kigoye Tr.7	7	1	0	0	1	100.0
58	Kigoye Tr.8	3	2	0	0	2	100.0
59	Kigoye Tr.9	5	2	0	0	2	100.0
60	Kiryowa Tr.4	15	14	12	1	1	13.3
61	Kiryowa Tr.6	7	4	3	0	1	57.1
62	Lubinga Anania 1	4	1	0	0	1	100.0
63	Lubinga Anania Tr.2	6	3	3	0	0	50.0
64	Lubinga Anania Tr.3	3	2	1	0	1	66.7
65	Mayinja Tr.1	4	3	0	1	2	75.0
66	Mayinja Tr.3	3	2	0	0	2	100.0
67	Mayinja Tr.4	3	3	3	0	0	0.0
68	Mayirane Tr.2	8	6	6	0	0	25.0
69	Mayirane Tr.1	8	8	7	1	0	0.0
70	Mayirane Tr.3	5	4	3	1	0	20.0
71	Mayirane Tr.4	6	2	1	0	1	83.3
72	Mrs.Walakira Tr.1	22	2	0	0	2	100.0
73	Mukasa Tr.10	5	5	0	0	5	100.0
74	Mukasa Tr.3	3	3	0	0	3	100.0
75	Mukasa Tr.5	3	3	1	0	2	66.7
76	Mukasa Tr.8	4	3	2	0	1	50.0
77	Mukasa Tr.9	1	1	1	0	0	0.0
78	Muza Tr.1	5	4	4	0	0	20.0
79	Muza Tr.2	4	2	2	0	0	50.0
80	Muza Tr.3	9	6	2	1	3	66.7
81	Nakazibwe Tr.4	2	1	1	0	0	50.0
82	Nakazibwe Tr.2	2	2	2	0	0	0.0
83	Nakazibwe Tr.3	1	2	1	0	1	0.0
84	Nakazibwe Tr.5	7	3	1	0	2	85.7
85	Namutebi Tr.2	8	4	2	0	2	75.0
86	Namutebi Tr.3	6	1	0	0	1	100.0
87	Namutebi Tr.4	4	2	0	0	2	100.0
88	Namutebi Tr.5	4	3	2	1	0	25.0
89	Namutebi Tr.6	13	7	7	0	0	46.2
90	Nansubuga Mary Tr.3	18	16	15	1	0	11.1
91	Nansubuga MaryTr.2	11	10	8	1	1	18.2
92	Ndyabagira 11	3	3	3	0	0	0.0
93	Ndyabagira 2	1	1	1	0	0	0.0

99	Nkumbi Wilson S.Tr.1	21	17	14	0	3	33.3
100	Nkumbi Wilson S.Tr.2	16	8	5	2	1	56.3
101	Nkumbi Wilson S.Tr.3	24	20	16	2	2	25.0
102	Nkumbi Wilson S.Tr.4	15	4	1	0	3	93.3
103	Nkumbi Wilson s.Tr.5	21	15	8	1	6	57.1
104	Nkumbi Wilson S.Tr.7	24	23	0	0	23	100.0
105	Patricia Rutonde	1	1	1	0	0	0.0
106	Peter Kabi/2	5	3	0	0	3	100.0
107	Sempa Tr.1	6	6	5	0	1	16.7
108	Sempa Tr.10	4	2	2	0	0	50.0
109	Sempa Tr.4	3	1	1	0	0	66.7
110	Sempa Tr.5	11	10	9	0	1	18.2
111	Ssemakula Sam Tr.2	6	3	0	0	3	100.0
112	Tindiwegi Wilson Tr.1	21	18	17	0	1	19.0
113	Tindiwegi Wilson Tr.3	9	8	8	0	0	11.1
114	Tindiwegi Wilson Tr.4	5	3	2	0	1	60.0
115	Turinamasiko/1	2	1	1	0	0	50.0
116	Unknown	14	14	12	1	1	7.1
117	Unknown	1	1	0	0	1	100.0

Comparison of % mortality of C. canephora genotypes and their open pollinated seedling progenies after 2 rounds of artificial infection with CWD under screen house conditions at CORI





iii) Screen house tests of young seedlings and cuttings of wild *C. canephora* collected from forests

During the reporting period plant materials (seeds and cuttings) of wild robusta coffee were collected from Kibale National Park forest, Itwara and Kalangala forests. The seeds and cuttings were planted in the coffee nursery at CORI to raise seedlings and rooted cuttings for inoculation with CWD. Seedlings and rooted cuttings raised from collections made in the previous reporting period were maintained in the nursery awaiting inoculation. The plants are likely to be inoculated during the next reporting period and results will then be available.

iv) Screen house tests on young seedlings and cuttings of imported *C. canephora*

During the previous reporting period seeds were obtained from Ivory Coast and planted in the coffee nursery at CORI to raise seedlings. During the reporting period the seedlings were being raised for inoculation.

The inoculation will be carried out during the next reporting period. 69 seedlings of 14 clones received from Ivory Coast through CIRAD in the previous reporting period were planted out in an isolated field at CORI for field evaluation.

v) Tests under controlled room conditions on young *C. canephora* seedlings

Studies under controlled room conditions were carried out in collaboration with scientists at CIRAD. Seeds were processed at CORI and submitted to CIRAD for raising seedlings. The seedlings were inoculated at CIRAD. CIRAD is in France, which is a non-coffee growing zone (neutral) thus a suitable venue for testing *C. canephora* from Uganda with CWD isolates from other countries or other coffee species. Such tests are not acceptable in Uganda.

During the reporting period two inoculations were carried out on more or less similar *C. canephora* open pollinated seedling progenies were inoculated with 3 CWD isolates; CAB003, DSMZ and CAB007. CAB003 was isolated at CABI from infected *C. canephora* specimen originating from Uganda. CAB007 was isolated by CABI from *C. arabica* specimen collected from Ethiopia and DSMZ is an old isolate kept at CIRAD, originally isolated from *C. excelsa*. The plants were inoculated by wounding using a scalpel and introducing a drop of the inoculum into the wound by the same scalpel. Data was collected from the first inoculation for 22 weeks and from the second inoculation for 14 weeks. Figures 6 & 7 shows response of the inoculated plants to the 3 CWD isolates in experiment 2. Figure 6 presents mean % incidence (sick plus dead plants) by progeny while figure 7 presents mean % mortality (only dead plants). Unfortunately only open pollinated seedlings were used in these studies. The results reveal that isolate CAB007 is able to induce initial symptoms of CWD on *C. canephora* (figure 6) but it is not able to advance to a point where it causes death (figure 7). Isolate DSMZ induces symptoms up to death as exemplified by its effect on progeny H/4/1 but it is far less aggressive than CAB003 (figure 7). It is not known whether this situation is true in the field. If so, then it is of a serious concern, especially about DSMZ, which shows less host specificity.

Figure 8 and figure 9 show progression of disease incidence (dead plus sick plants) among and between the progenies in experiments 1 & 2 respectively over a period of 14 weeks. These results show that in both experiments progenies had individuals showing the disease symptoms by 4th week from time inoculation, save for progeny Q/3/4, that had none, but the disease progressed at varied rate between the progenies and experiments. The progression was faster in experiment 2 than in experiment and by the 10th week some progenies (P/3/6 and Q/1/1) in experiment 2 had all their plants showing the disease symptoms. Even by 4th week the progenies in experiment 2 were showing high level of disease as compared to same progenies in experiment 1. The results also show inconsistence between and among progeny responses to the disease in experiments 1&2. Progenies B/1/1, C/6/1 and Q/3/4 that seemed to have good level of resistance among their seedlings in experiment behaved negatively in experiment 2. Such cases may exist for diseases whose resistance is expected to be polygenic and for open pollinated progenies as is the case with *C. canephora*.

Figure 10 and figure 11 show percent mortality realized among the progenies in inoculation experiments 1 & 2 respectively after 14 weeks from time of the inoculation. In both experiments, death of plants was not realized until in the sixth week except for 1 plant of progeny Q/1/1 in experiment 1. The mortality also progressed at varied rates for the different progenies. Progeny Q/3/4 had the least death and therefore most resistant in experiment 1 and progenies H/4/1, P/5/1 and P/3/6 realized the highest deaths and therefore most susceptible. In experiment 2 progeny J/1/1 (not included in experiment 1) was the most resistant and progenies G/3/7, Q/1/1, P/3/6 and B/6/2 were the most susceptible. Therefore the mortality also revealed inconsistence between experiments 1 & 2, probable for the same reasons (dealing with progenies of polygamous crop and polygenic resistance. Figure 12 shows pictorial comparison of the mortalities in experiments 1&2.

Figure 6: % disease incidence on open pollinated *C. canephora* open pollinated progenies after 14 weeks from time of artificial infection with 3 CWD isolates under controlled room conditions at CIRAD

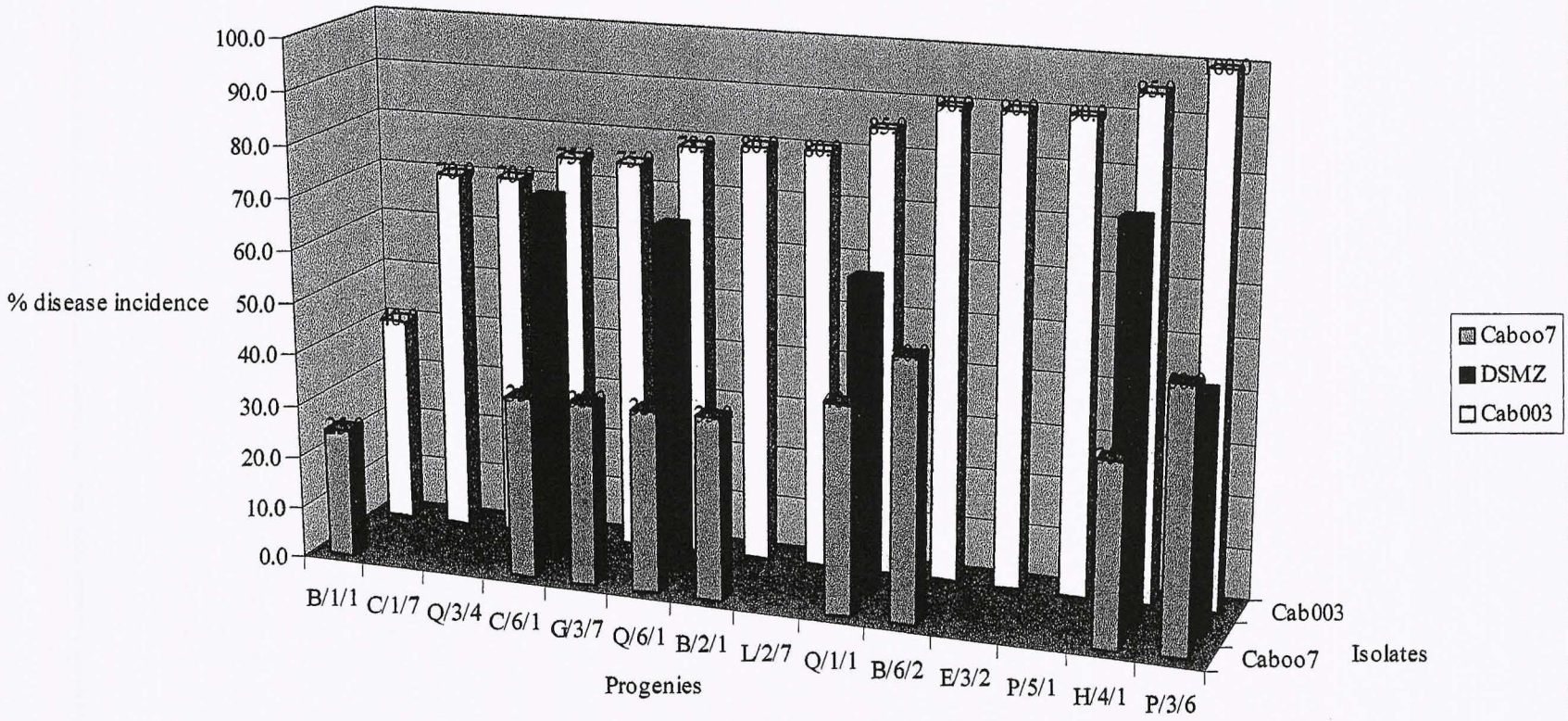


Figure 7: % Mortality of *C. canephora* open pollinated seedlings after 22 weeks from time of artificial infection with 3 CWD isolates under controlled room conditions at CIRAD

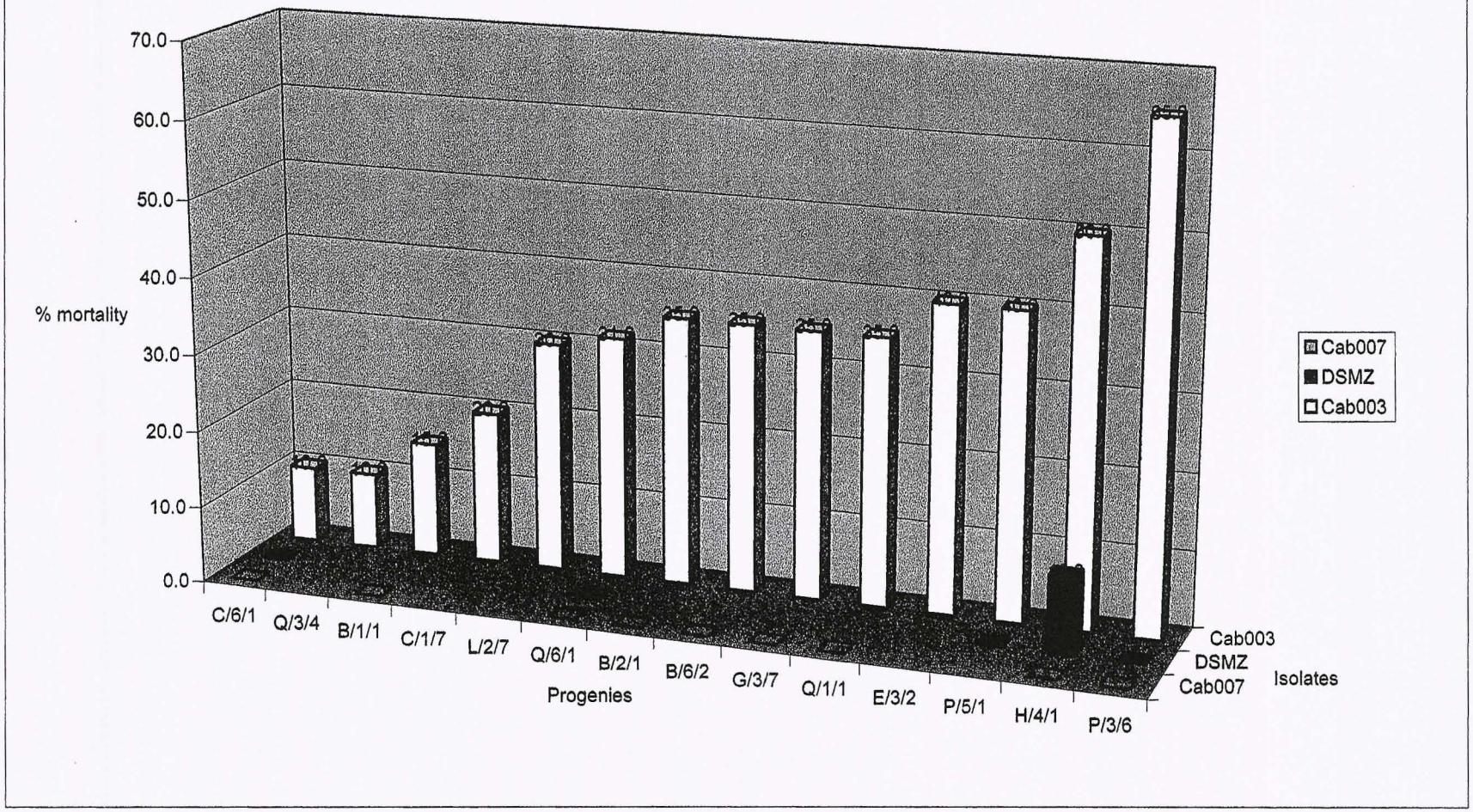


Figure 8: Progression of CWD disease among *C. canephora* open pollinated progenies in expt 1 artificlly infected with CWD under controlled room conditions at CIRAD

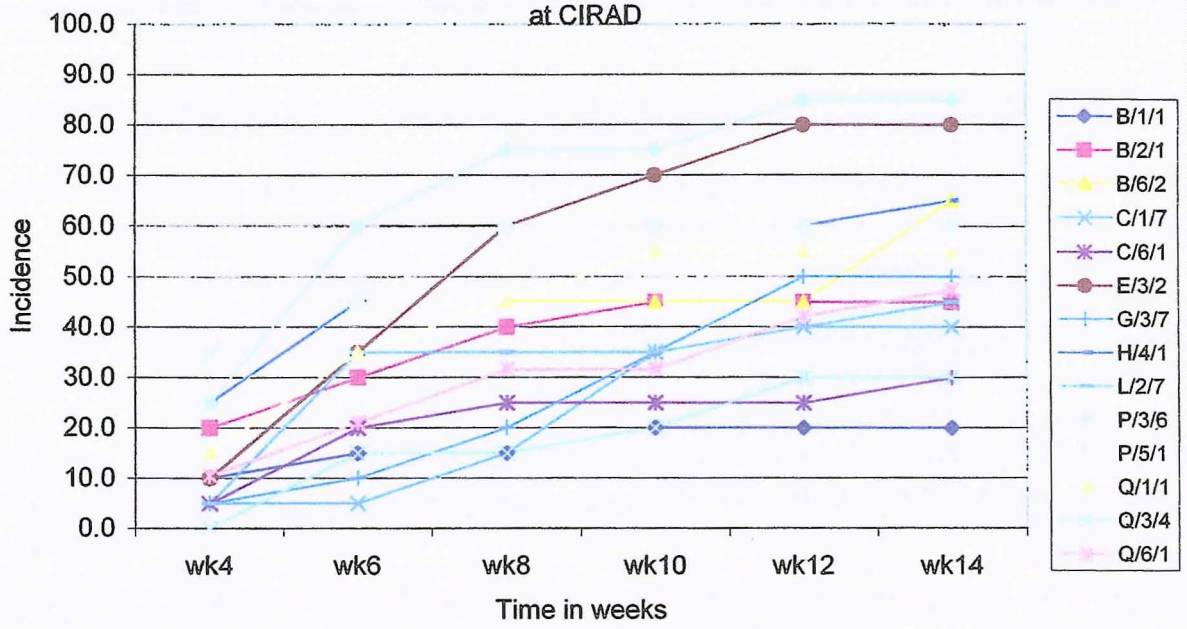


Figure 9: Progression of CWD disease among C. canephora open pollinated progenies artificlly infected with CWD in expt 2 under controlled room conditions at CIRAD

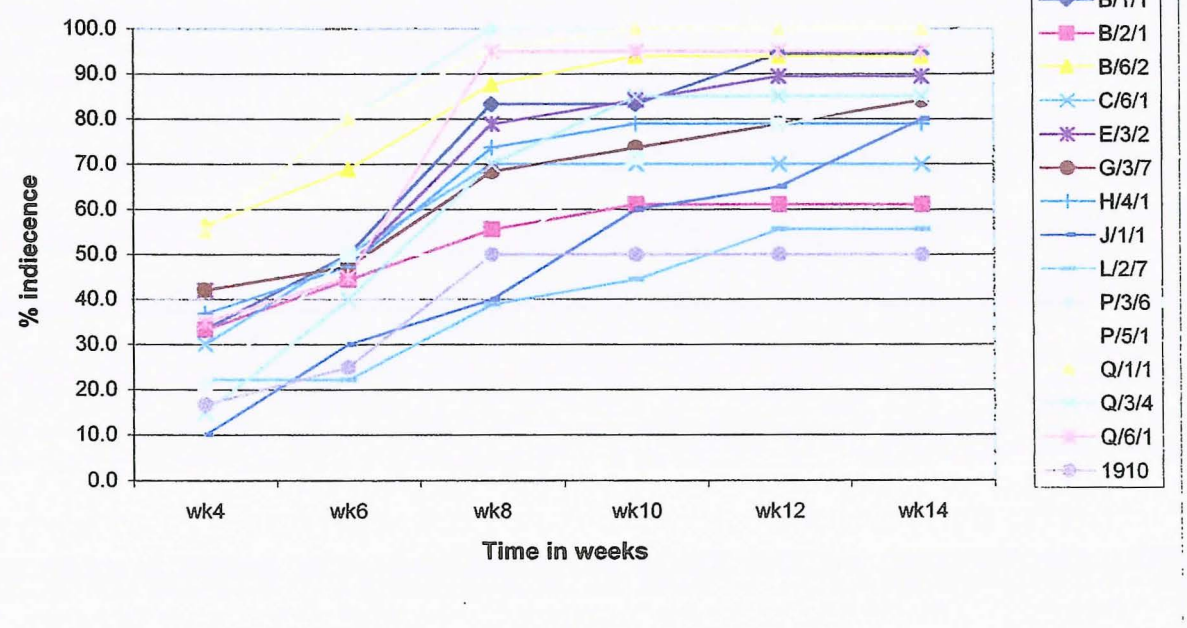


Figure 10: Progression of mortality among C. canephora open pollinated seedling progenies artificially infected with CWD in expt. 1 under controlled room conditions

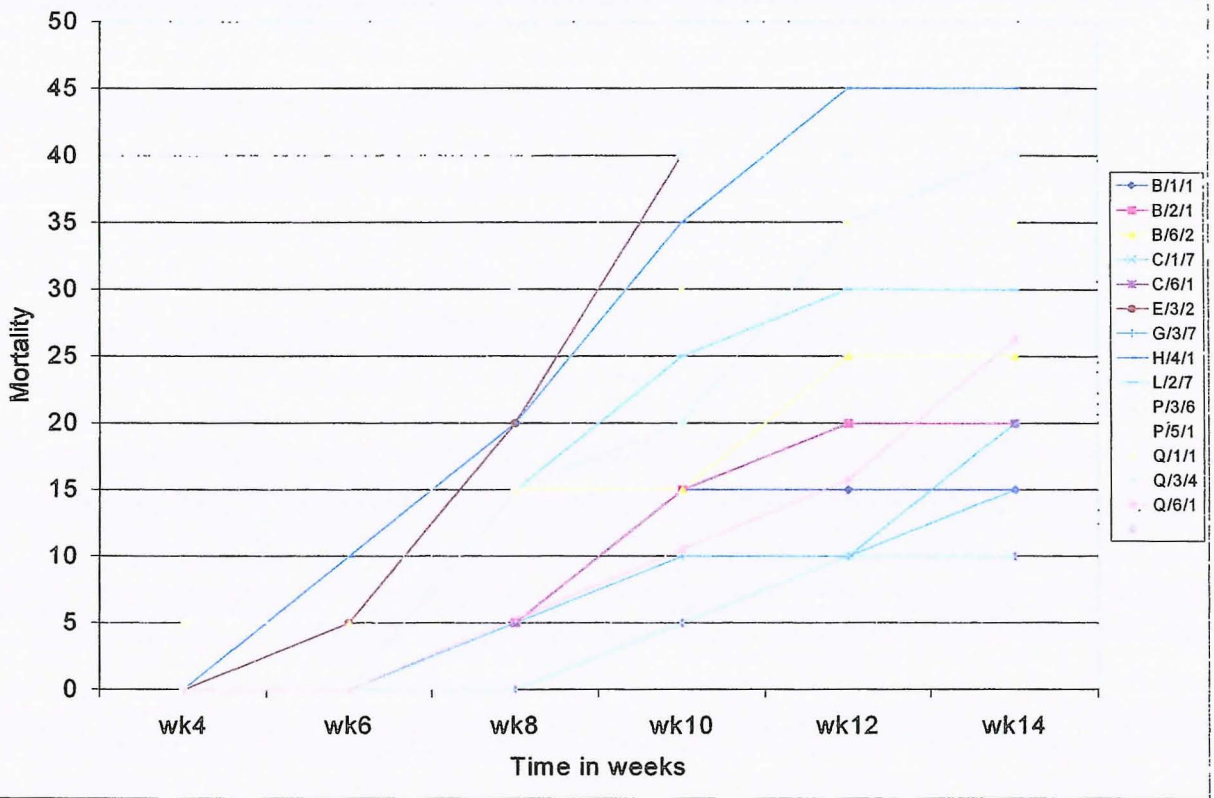


Figure 11: Progression of mortality among C. canephora open pollinated seedling progenies artificially infected with CWD expt 2 under controlled room conditions at CIRAD

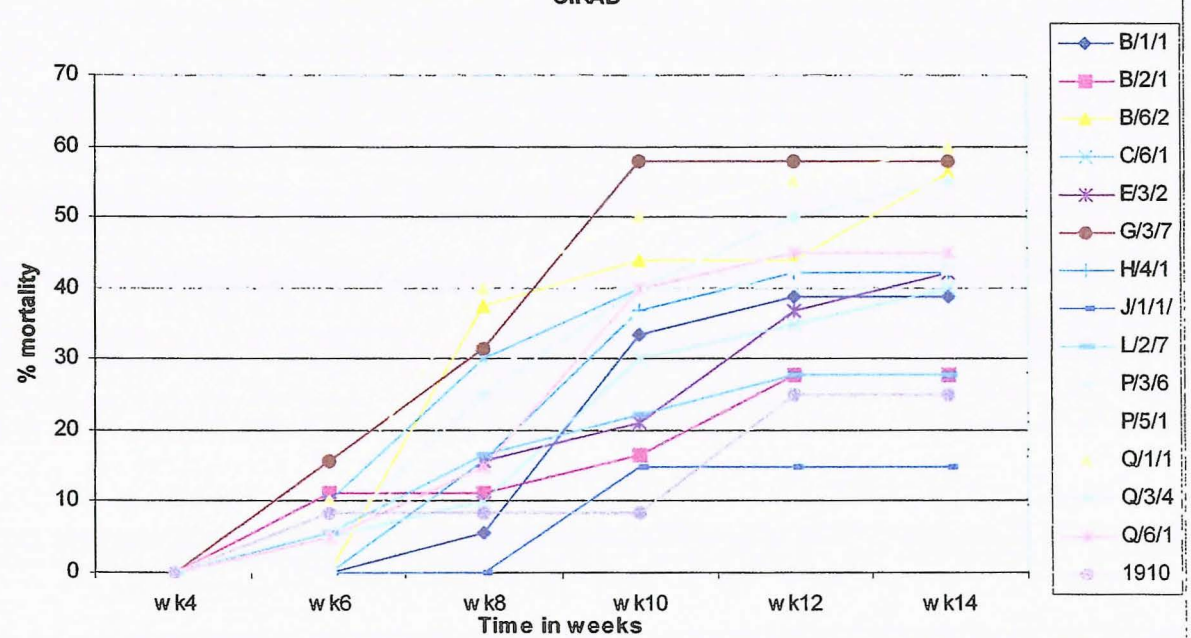
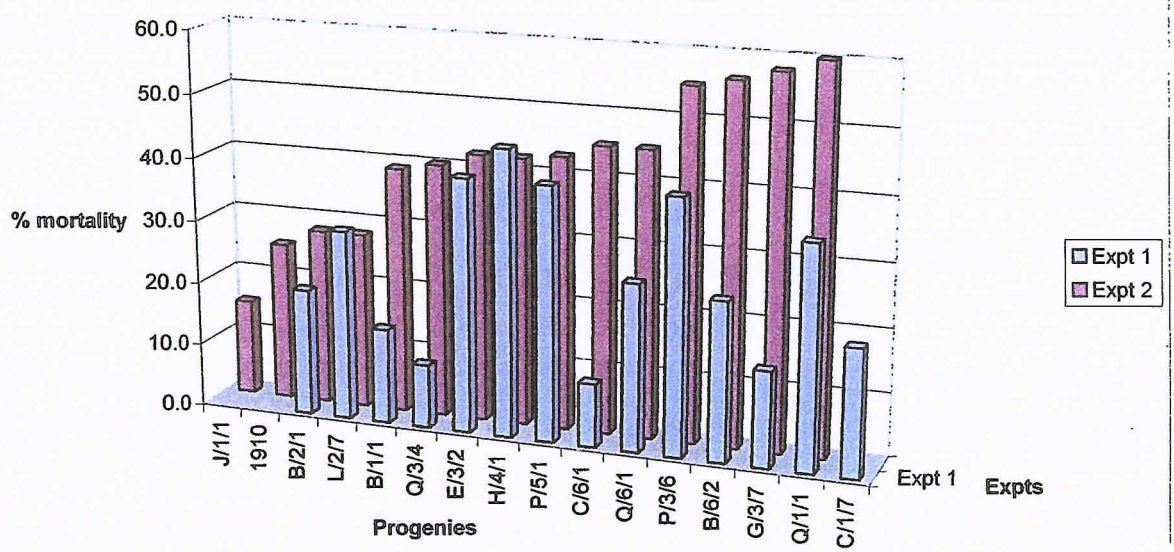


Figure 12: Comparison of mortality of among *C. canephora* open pollinated seedling progenies in expts. 1 & 2 artificial infection with CWD under controlled room conditions at CIRAD



2) Evaluation for CWD resistance in field trials

Field evaluation was conducted on a complete randomized block design trial consisting 20 *C. canephora* clones in 4 replications and another randomized block design trial consisting of 35 *C. canephora* specific cross progenies with their 10 clonal parents in two replications. The two trials are located at Kituza, the headquarters of the Coffee Research Institute (CORI). The clones in the clonal trial are progenitors of the open pollinated seedling progenies inoculated in the controlled room experiments carried out at CIRAD. During the reporting period yield and CWD data collection continued on the two trials. The trial fields were maintained following recommended procedures for maintaining robusta coffee farms. Results of the yield data are not presented in this report but results of CWD incidence and severity since 2001 up to September 2005 for the clonal trial and since 2002 to for the hybrid trial are presented in this report.

The results reveal a general increase of incidence of the diseases across varieties during the reporting period but varieties J/1/1 remained completely resistant while Q/3/4 maintained only the 4% mortality realized in 2003. By September 2005, mortality attained varied between clones. This also shows that different clones have different level of resistance. This is also a reflection that the resistance is controlled by many genes/resistance factors and the resistance factors are not equally distributed among the clones.

Table 5: Percentage mortality of 20 *C. canephora* clones naturally infected with CWD under field conditions at CORI, Kituza

	Clone	April 2001	April 2002	April 2003	April 2004	April 2005	September 2005
1	J/1/1	0.00	0.00	0.00	0.00	0.00	0.00
2	Q/3/4	0.00	0.00	4.17	4.17	4.17	4.17
3	1S/3	12.50	12.50	12.50	12.50	33.33	33.33
4	C/6/1	12.50	16.67	16.67	20.83	29.17	33.33
5	R/1/4	11.11	27.78	33.33	33.33	33.33	33.33
6	Q/6/1	50.00	50.00	50.00	50.00	50.00	50.00
7	B/2/1	29.17	41.67	50.00	50.00	54.17	54.17
8	223/32	12.50	20.83	41.67	41.67	58.33	58.33
9	L/2/7	12.50	33.33	45.83	54.17	58.33	58.33
10	Q/1/1	41.67	50.00	50.00	50.00	66.67	66.67
11	B/1/1	29.17	37.50	50.00	66.67	70.83	75.00
12	257/53	29.17	62.50	70.83	75.00	70.83	83.33
13	G/3/7	25.00	37.50	58.33	70.83	79.17	83.33
14	1S/2	4.17	4.17	20.83	41.67	83.33	87.50
15	E/3/2	20.83	70.83	87.50	87.50	87.50	87.50
16	P/5/1	54.17	70.83	70.83	75.00	87.50	87.50
17	B/6/2	37.50	66.67	83.33	83.33	87.50	91.67
18	P/3/6	54.17	87.50	91.67	91.67	91.67	91.67
19	H/4/1	27.78	77.78	83.33	94.44	94.44	94.44
20	C/1/7	45.83	58.33	83.33	87.50	91.67	95.83

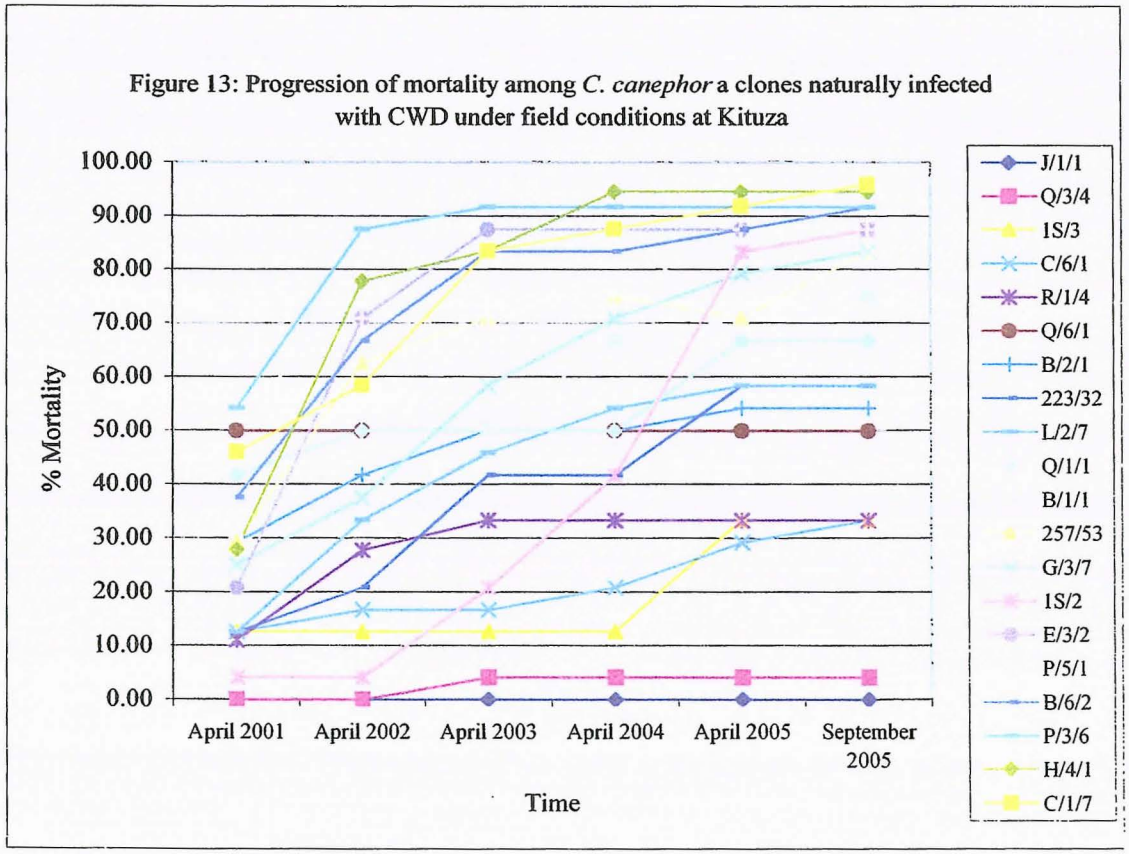


Figure 14: % Mortality among *C. canephora* clones under field conditions after 6 years from time of noticing first symptoms

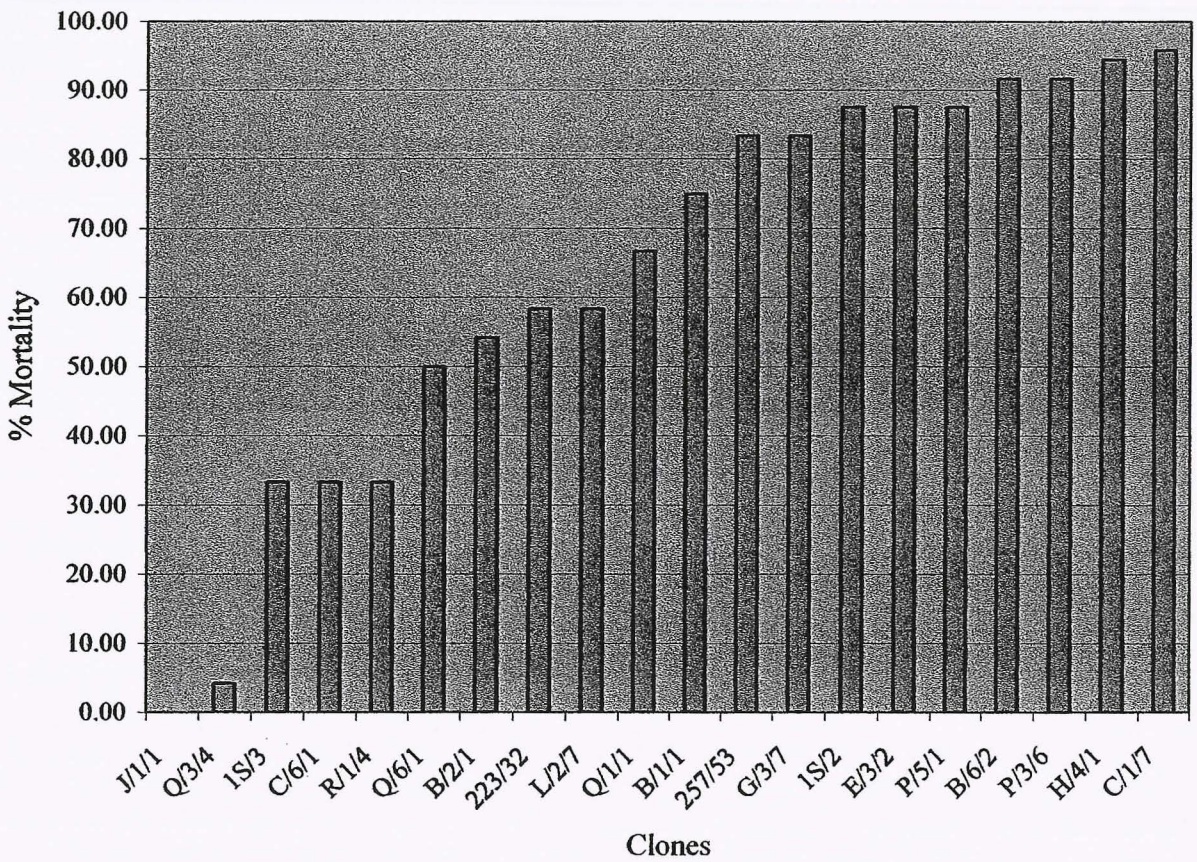
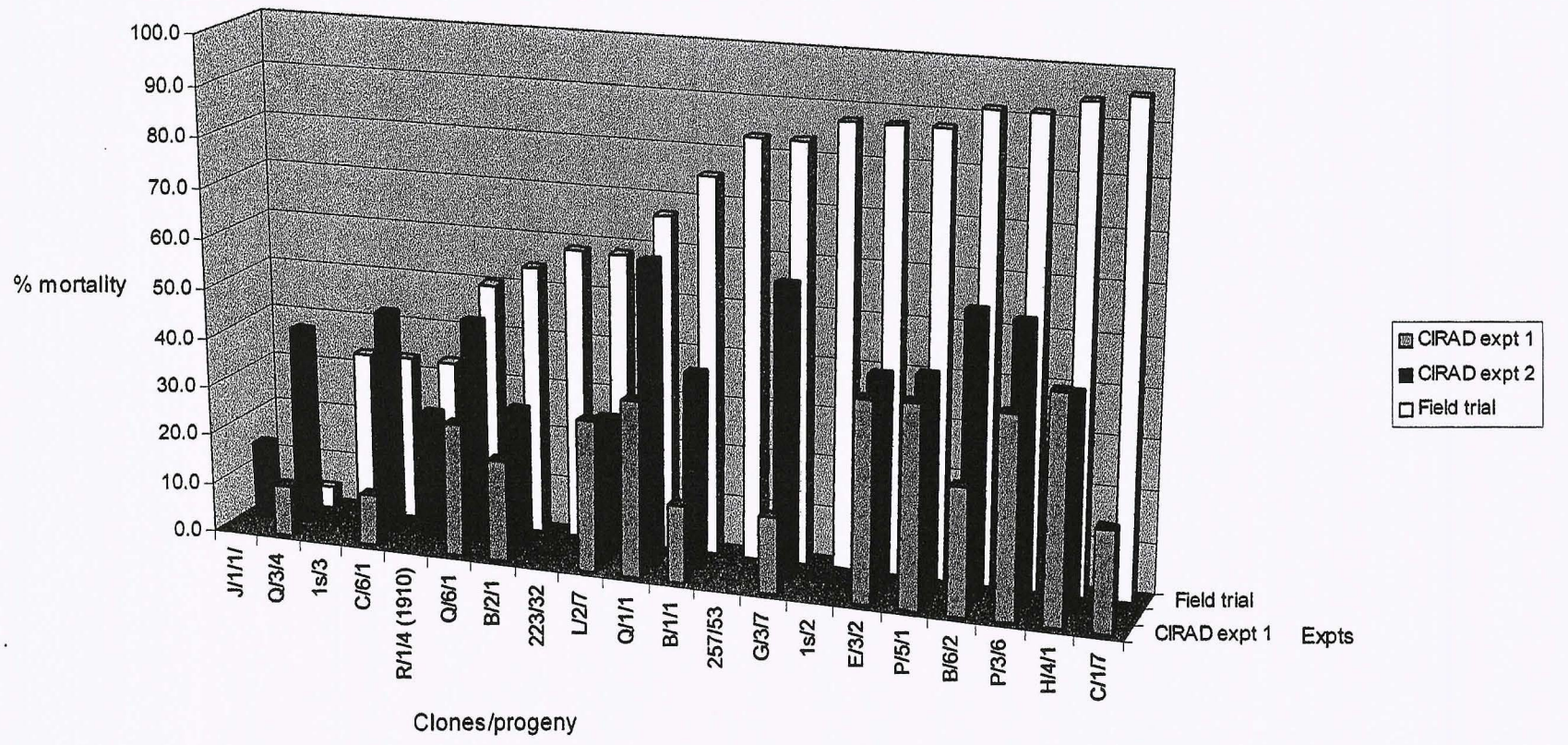


Table 6 and figure 15 show a comparison between field mortality of the clones and the mortality of their open pollinated seedling progenies in two experiments conducted under controlled climatic conditions. As noted earlier, there is inconsistence between results of the two experiments on the seedling progenies. It is also observed that clone Q/3/4 that has high field resistance gave rise to progenies that very susceptible to the disease (experiment 2). This most likely due to an give earlier (the seedling progenies are open pollinated and from a cross pollinated parents and therefore they are bound to behave quite variedly. Secondly the resistance to CWD is polygenic and therefore is not unexpected that a resistance parent gives progenies with varied levels of susceptibility. The high disease level in the field is only a consequence of time. . The clones in the field have been under infection for about 7 years while the laboratory experiments were of 14 weeks from time of inoculation. A statistical analysis has not been carried out but it will be done to establish whether there is significant correlation between the clones in the field and their seedling progenies in controlled climatic conditions and also between the two laboratory experiments.

Table 6: Comparison between mortality of *C. canephora* clones naturally infected with CWD under field conditions and their open pollinated seedling progenies artificially infected under controlled room conditions

	CIRAD expt 1	CIRAD expt 2	Field trial
J/1/1/		15.0	0.0
Q/3/4	10.0	40.0	4.2
1s/3			33.3
C/6/1	10.0	45.0	33.3
R/1/4 (1910)		25.0	33.3
Q/6/1	26.3	45.0	50.0
B/2/1	20.0	27.8	54.2
223/32			58.3
L/2/7	30.0	27.8	58.3
Q/1/1	35.0	60.0	66.7
B/1/1	15.0	38.9	75.0
257/53			83.3
G/3/7	15.0	57.9	83.3
1s/2			87.5
E/3/2	40.0	42.1	87.5
P/5/1	40.0	42.9	87.5
B/6/2	25.0	56.3	91.7
P/3/6	40.0	55.0	91.7
H/4/1	45.0	42.1	94.4
C/1/7	20.0		95.8

Figure 15: Comparison of mortality between *C. canephora* clones infected naturally with CWD under field conditions and their open pollinated seedling progenies artificially infected under controlled room conditions



B: STUDIES ON INHERITANCE OF CWD RESISTANCE

Activities carried out during the reporting period towards under standing transmission of CWD resistance from parents to progenies include conducting artificial crosses/pollination between CWD resistant and susceptible parents, sowing of available hybrid seed of the crosses between CWD resistant and susceptible parents and maintenance of available hybrid seedlings. The seedlings will be inoculated in the next reporting period.

C: EVALUATION OF GENETIC DIVERSITY OF *C. CANEPHORA* IN UGANDA.

During the reporting period seeds, cuttings and leaves were collected from wild robusta coffee in Kibale and Itwara forests. The 232 leaf samples were submitted to CIRAD in France were their DNA was extracted and analyzed for diversity using microsetallite markers but the cuttings and seeds were planted in the coffee nursery at CORI for raising to appropriate age before inoculating them with CWD pathogen. Seedlings and rooted cuttings of the previous collection were also maintained in the nursery. Table 6 shows populations/leaf samples analyzed for genetic diversity.

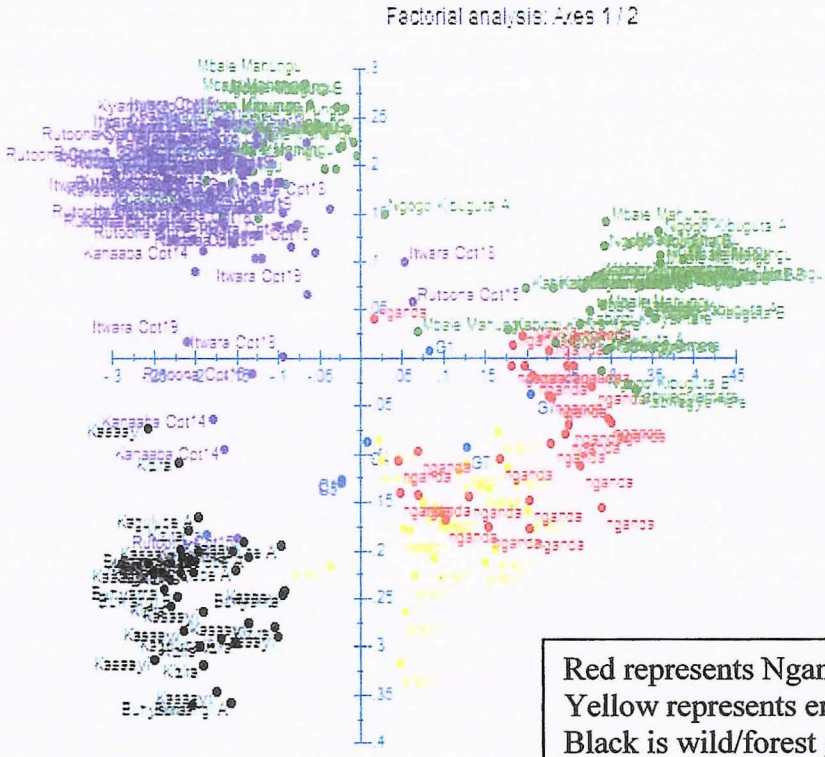
Figures 10 & 11 shows graphical representation of the DNA dissimilarity among and between populations. The results show that Ugandan *C. canephora* populations are genetically different from other known coffee groups such as the Congolese and the Guinean. It is also evident that populations from Kalangala forest are genetically different from populations in Itwara and Kibale forests but there are two genetically distinct groups among Kibale populations. One of the Kibale sub-group is closer to collections in Itwara forests probably because Kibale and Itwara were at one time one continues forest that became separated due to human activity in time. The second kibale sub-group is distinct to Kibale. It is also seen that Itwara populations are a distinctive group of robusta coffee.

The erecta type of robusta from the germplasm collections at Kituza (CORI) is also a distinct group of coffee. The nganda seem to be a hybrid between erecta and one of the Kibale populations. With such genetic variation between the different populations it anticipated that there will also be variations in their response to CWD infection. The test for response of the various coffee groups to CWD will be carried out during the next reporting period.

Table 6: Plant populations of C. canephora genotypes analyzed for genetic diversity using microsetallite markers

Source	Population	Type of material	Samples analyzed
CORI	Nganda-type	Spontaneous	33
	Erecta type	Spontaneous	24
	Sub-total		**57**
Kibale National Park Forest	Ngogo-Kibuguta	Wild spontaneous	12
	Ngogo-Kisita	Wild spontaneous	1
	Mbale- Mahungu	Wild spontaneous	30
	Kabirizi-Kinyantale	Wild spontaneous	7
	Kabwegyemere	Wild spontaneous	4
	Sub-total		**54**
Itwara Forest	Rutooma compart. 15	Wild spontaneous	23
	Kanaaba compart. 14	Wild spontaneous	13
	Kyamuhuro compart. 11	Wild spontaneous	9
	Compartment 19	Wild spontaneous	10
	Compartment 18	Wild spontaneous	12
	Compartment 27	Wild spontaneous	4
	Sub-total		**71**
Kalangala Forests	Bunyama Island	Wild spontaneous	6
	Kasayi	Wild spontaneous	13
	Bugulube	Wild spontaneous	9
	Kazira	Wild spontaneous	12
	Bumangi	Wild spontaneous	3
	Sub-total		**43**
CIRAD (Controls)	*Congolese*	DNA of wild Congolese populations group	4
CIRAD (controls)	Guinean	DNA of wild Guinean population group	3
	Sub-total		**7**
Total			**232**

Figure 10:Graphical presentation of factorial analysis.



Red represents Nganda genotypes
Yellow represents eracta genotypes
Black is wild/forest genotypes from Kalangala
Purple is wild/forest genotypes from Itwara forests
Green is wild/forest genotypes from kibale forest
Blue C is genotypes of the Congolese group
Blue G is genotypes of the Guinean group

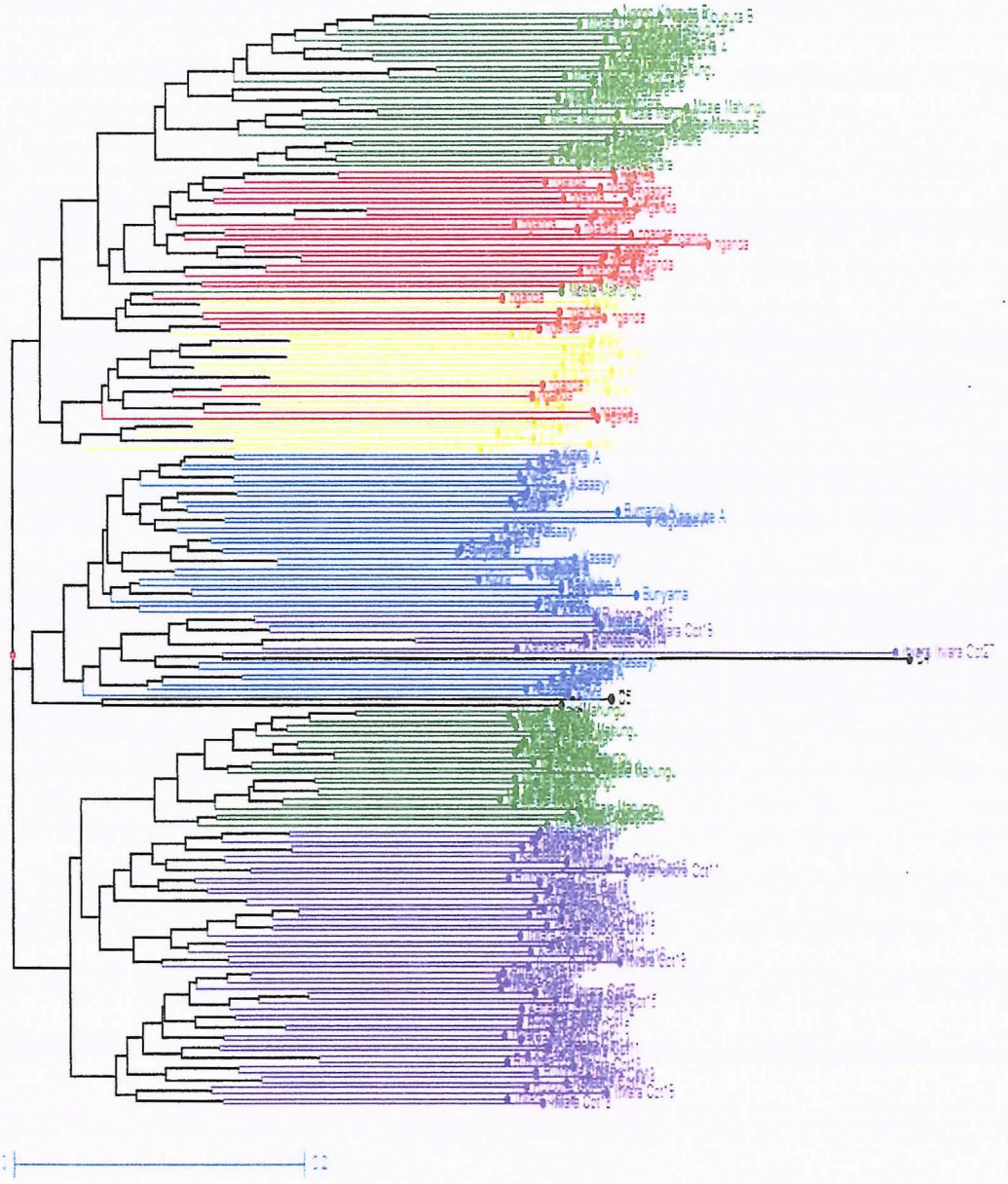


Figure 11: Dendogram showing distribution of the various robusta coffee population analyzed using microsetallite markers at CIRAD.

Table 12: Plant materials collected from wild coffee in Kibale and Itwara forests

D: BREEDING FOR RESISTANCE AGAINST CWD DISEASE

Following successful identification of CWD resistant clones through screen house inoculations and assessments at CORI, a mother garden of the clones was established and vegetative cuttings raised and planted in a field evaluation trials. Some of the plants in the mother gardens were however allowed to mature and function as breeding parents as well as initial clone performance indicators through visual assessments. It is some of these plants that were therefore used for generating various hybrids. Some of the hybrids were planted out in the field for field evaluation but a few were inoculated to assess their response to CWD. Table 6 shows the response of 14 controlled cross hybrid progenies to artificial CWD infection under screen house conditions at CORI. The results reveal that crosses between two resistant parents do not necessarily give rise to progeny seedlings that are all resistant to the disease. However their progenies tend to be more resistant as compared to progenies of other crosses such as RXMR, RXS AND SXS. As indicated earlier, such progenies are better evaluated on individual basis and selected as clone.

Table 1: Mortality of 14 hybrid progenies of *C. canephora* after 14 months from time of infection with CWD under screen house conditions

	Progeny	Type of cross	No. inoc	Dead plants	% Mortality	Category mortality mean
1	254/80/2xJB5109.4/5/1	RXR	28	12	43	
2	254/80/2x202/63/1	RXR	16	12	75	
3	254/80/2xF.Ruk/1	RXR	49	15	31	33.4
4	JB5109.4/5/1xF.Ruk/1	RXR	16	7	44	
5	F.Ruk/1x254/80/2	RXR	12	1	8	
6	F.Ruk/1x202/63/1	RXR	5	0	0	
7	254/80/2x258s/24(0)	RXMR	16	5	31	
8	254/80/2x1s/3	RXMR	13	9	69	37.6
9	254/80/2x1s/6	RXMR	10	5	50	
10	F.Ruk/1x1s/3	RXMR	4	0	0	
11	254/80/2x257/53	RXS	21	3	14	
12	254/80/2x1s/2	RXS	9	8	89	58.2
13	286/1x1s/2	RXS	7	5	71	
14	1s/2x223/32	SXS	26	20	77	77

Key

R=Resistant parent
MR=Moderately resistant parent
S=Susceptible parent

CONCLUSION

Since initiation of WKP3, many activities have been carried out that have positively contributed towards achieving the WKP objectives. During the reporting period (year 4), activities initiated in the previous reporting periods such as conducting CWD screen house tests, field evaluations for CWD resistance, controlled room studies on CWD evolution and analyzing genetic diversity of different *C. canephora* population using microsetallites were consolidated.

Screen house tests continued to be used at CORI as a mean of identifying genotypes resistant to CWD among robusta coffee progenies. Results of this study revealed that CWD resistance varies among and between *C. canephora* genotypes and their corresponding progenies and that the resistance is controlled by many genes/resistance factors that are not equally distributed in the individuals. Only a small proportion, usually less than 10%, of the population has a high level of resistance that can minimize mortality caused by CWD to less than 10%. The studies revealed that resistant genotypes tend to give rise to relatively resistant progenies (both open pollinated progenies and progenies of controlled crosses) but cases were resistant parents give rise to highly susceptible progeny or highly susceptible parents giving rise to relatively resistant progenies exist. Results of field evaluations also show that resistance level varies between the genotypes/clones. Preliminary work on controlled cross hybrids of resistant and susceptible parents shows that the inheritance of the resistance may not follow a normal mendelian behaviour. Such situations make clonal selections, basing on individual performances the most suitable short breeding approach to identifying CWD resistant cultivars for farmers.

Studies under controlled room conditions revealed that *F. xylarioides* isolates causing CWD of *C. arabica* induce mild symptoms of CWD on *C. canephora* but it does not induce death. However the isolate that causes CWD of *C. excelsa* may induce death of *C. canephora* although it is less aggressive. The behaviour of this isolate therefore should be of concern if it behaves similarly under field conditions. Under such situation it would imply that it has potential to give rise to a more aggressive strain that can over come resistance of robusta coffee varieties bred basing only on the C. *canephora* isolates.

Results of analyses of the genetic diversity revealed genetic distinction of Ugandan *C. canephora* from other known *C. canephora* groups such as the Congolese and the Guinean. The studies also revealed distinctive genetic subgroups among the Ugandan *canephora*. I.e. wild populations from Itwara, Kibale and Kalangala forests and the nganda and erecta types of the on-station germplasm collections all appeared as different genetically variable subgroups.

With the current results not giving clear information on the inheritance of CWD among *C. canephora*, there is need for carrying out more systematic studies using specific hybrid progenies of resistant and susceptible parents to be able to systematically estimate the inheritance. Representative samples of the different genetic subgroups of Ugandan *C. canephora* should also be systematically studied for resistance so as to ascertain whether any such group has unique resistance genes that make it peculiar and useful for breeding against CWD. Better understanding of the inheritance of the CWD resistance and knowledge on the response of the different sub-groups will be useful in developing a long term and more sustainable breeding programme for generating CWD resistant varieties adaptable for cultivation in different agro-ecological areas.

Dr Georgina Hakiza

Task 2: Description of the spatio-temporal diffusion of the disease in the plantations

Spread of coffee wilt disease has been monitored on farmers' coffee on 6 farms in 4 districts. In addition, similar observations and recordings have been in progress on robusta clonal trial at Kituza, CORI.

Observations both on-farms and on station indicated increase in wilt incidence over time. On-station differences in susceptibility to CWD among varieties/clones were observed. Some clones rapidly succumb to the disease. Some individual plants may develop symptoms but disease progress was very slow and the plant lingered on for more than 6 months without full development of symptoms or death of plants. Cracks normally occur on the affected stems of coffee trees. This could probably be some form of reaction to the disease in cultivars that might of some tolerance to CWD. Yet in most cultivars death occurs within a few weeks (3 – 4 weeks) from first symptoms.

Rate of spread was observed to be greatly enhanced by human activities, particularly with respect to removal and transport of infected stems. In the table below an analysis of different farm situations was attempted.

Wilt incidence increased with time at all the farms. However, rate of spread varied from farm to farm. This could probably be partly due to environmental conditions and partly to some unidentified factors. In Table 2 an attempt was made to analysis situations at each farm and to link them with the spread of the disease on the farms. These were age of coffee, site of farm whether on flat, level, or steep slope, pruning practices, cropping system, presence or absence of shade and how the diseased trees were managed.

Generally all the coffee trees were over 40 years old. It was observed at several of these farms that even young coffee seedlings growing from fallen berries were attacked by CWD,

soil being the most likely source of infection. Young seedlings planted to replace dead trees were infected and died within 3 months from planting. It is apparent that the disease attacks coffee from seedling to mature plants.

Farm sites and farmer practices

All farms regardless of the sites were affected by wilt, though to varying degrees. At Mr. Samanya's farm (Mayuge district) the disease started on coffee under Ficus shade trees and spread out. While at Mr. Mukasa's (Rakai district) CWD progressed down the valley. The rest of the fields/farms had random distribution of disease. The highest wilt incidence was recorded on farms in Iganga, which were situated on more or less flat or level ground.

All farmers never practised any form of pruning apart from removal of dry or broken branches. The influence of this practice on CWD incidence could not be elucidated.
Weed control was taken care of mostly through intercropping; otherwise weeding by hoeing was done occasionally. No soil fertility amendments were practised by any of the farmers.

Movement of infected dry coffee stems

Farms sites varied from level land to steep slopes. In Mayuge district, both farms were more or less on level land. One of the farmers in Mayuge, Mr. Kirunda previously stored dry coffee stems from off farm sources outside his Kitchen house next to his coffee field. CWD started from near the heap of firewood and spread out further afield. In the same general area, the farmer used to tether 3 heads of cattle and some goats for over night. Tethering animals on coffee stems cause wounding of the tree stems, it is not clear if this could have contributed to increase in wilt incidence by providing entry points for the pathogen.

In Rakai, CWD started on one slope. The farmer cut down the dry stem, dragged it down the slope into the valley and up the slope to his home. This was repeated, as more stems dried up. Consequently, more and more CWD appeared along the track he was following.

It was apparent that movement of dry infected stems accelerated the spread of CWD on-farms, and to neighbouring farms. These observations support the recommendation of burning infected plants *in situ*. Movement of infected plants within and out of the infected

field appeared to cause rapid spread of the disease. All farmers carried off dry infected stems for use as firewood and in so doing accelerated the spread of disease.

On station field observations on spread of CWD Block 36.

Unlike farmers' coffee, station coffee is in pure stand (monocrop), without shade or intercrops. In addition, management practices are also different. Weeding is done regularly using herbicide (Glyphosate), hoeing or slashing. Pruning and desuckering are done as required and stumping is done every 5 – 6 years for renewal of growth cycle. Soil fertility is maintained by application of inorganic fertiliser (NPK: 17:17:17) or organic manure.

Disease spread under field conditions was studied on 4 robusta clones, 1s/2, 1s/3, 1s/6 and 258/24 since 2002. Rate of increase varied among clones. At the beginning wilt progress was highest in clone 1s/6, followed by 1s/2 and 1s/3. Clone 258/24 had the lowest rate of spread. In the end the highest mortality was 258/24 and lowest in 1s/6.

Block 36

CWD continued to spread within the field destroying most of the trees. The tables below indicate wilt incidence at the start of the observation in January 2002 and the latest recording done in March 2006.

Table 1: CWD incidence on 4 robusta clones in block 36 at the start of the observation in January 2002 to December 2005.

Clone	Total number of trees January 2002	Number of trees that were healthy January 2002	Number of trees infected and with CWD symptoms	CWD incidence (%)
1s/2	95	92	3	3.2
1s/3	90	90	0	0
1s/6	91	87	4	4.4
258/24	92	90	2	2.2

Table 2: CWD incidence on 4 robusta clones in block 36 in April 2006.

Clones	Total number of trees at start (2002)	Number of trees currently healthy (April 2006)	Total number of trees infected/died of CWD	CWD incidence to date (April 2006)
1s/2	95	7	88	92.6%

1s/3	90	5	85	94.4%
1s/6	91	8	83	91.2%
258/24	92	2	90	97.8

Task 4: Evaluation of the duration of survival form of the pathogen

Trial 1

Survival of *F. xylarioides* in and inoculum potential of CWD infected coffee wood pieces:

Coffee trees with severe wilt symptoms were identified and selected from an infected coffee plot at CORI on July 28 2004 for use as potential source of inoculum. Stem pieces of 2 –3 cm diameter showing clear discolouration of the wood due to CWD throughout their length were cut off. These pieces were further cut into smaller pieces of 5 cm lengths until 144 wood pieces were obtained.

To begin the experiment, sterile soil was used to fill 125 black polypots size 8 cm diameter x 16 cm height/length. Ten pots were each planted with a single robusta coffee seedling 6- 8 months old. A single wood piece was placed horizontally on the soil surface of 5 pots planting with seedlings. The wood pieces were placed about 2 cm away from the stems of seedlings to avoid contact with the stems. The remaining 5 pots were left without wood pieces as control, where CWD was not expected to develop. The infected wood pieces were placed in the remaining 115 pots from which 5 pots were planted with seedlings at 4 weekly intervals. The rest of the wood pieces were kept in CWD free area in the screen house from which isolations of the wilt pathogen was done every 4 weeks. Seedlings were maintained in the screen house and observed for CWD symptoms. Data was collected on days from planting to appearance of symptoms and days from symptoms to death of plants.

Isolations were made from leaves (leaf veins), stems and roots of randomly selected dead seedlings to confirm the cause of death. In cases where no external symptoms were observed, after 6 months, random samples of these apparently healthy seedlings were checked to establish presence/absence of *F. xylarioides* in the plant tissues. The experiment was first initiated in July 2004 and will be continued.

Trial 2

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

On May 24 2005 soil was collected from three sites at CORI where coffee wilt disease has been severe. These sites were Forest near the institute, Block 36 and Block 38. A single tree with severe wilt symptoms was selected at each site, uprooted and soil collected from the hole for the experiment. A portion of the sampled soil from each site was taken and used to fill 10 polypots and each pot planted with one coffee seedling immediately after up rooting. The rest of the soil was stored and used for planting at 4 weekly intervals in the same way. Data collected were on days from planting to symptom appearance and days from symptoms to death of seedlings. Isolations were made from leaves (leaf veins), stems and roots of randomly selected dead seedlings to confirm the cause of death. In cases where no external symptoms were observed, after 6 months, random samples of these apparently healthy seedlings were checked to establish presence/absence of *F. xylarioides* in the plant tissues. The experiment was first initiated in July 2004 and will be continued.

Trial 3

Role of farm tools/machete or panga in transmission of CWD

Coffee field

The farm tool investigated to start with was the panga or machete, which is commonly used by farmers for cutting. The machete was first used for cutting infected coffee trees with symptoms and later used to wound healthy trees in the field. A total of 12 trees were wounded in 3 positions, stem base, middle of the stem and top below the shoot tip. Trees were observed for symptoms to develop.

Screen house

Robusta coffee seedlings of 6-8 months old were transplanted, one seedling in each of 20 pots filled with sterile soil. The pots of seedlings were treated as follows:

Number of plants	Treatment
5 seedlings	Seedlings wounded 5cm from stem bases with clean/sterile machete/panga

5 seedlings	Seedlings wounded as above with contaminated machete/panga
5 seedlings	Seedlings inoculated by root dipping in washing from wilt contaminated machete.
5 seedlings	Seedlings inoculated by root dipping in sterile water.

Seedlings were maintained in the CORI screen house at 22 – 27 C day time and 15 – 18 C nighttime with relative humidity ranging from 65 – 70%. Watering was done twice a week and plants checked once in 2 days for symptoms of CWD.

Results/Progress

Trial 1

Survival of *F. xylarioides* in and inoculum potential of CWD infected coffee wood pieces

The highest number of infected seedlings (32%) occurred during the first three months with incubation periods varying 60 – 158 days. Wilt infection declined over time (4 – 5 month 12% infected seedlings), with prolonged incubation periods, varying from 113 – 298 days. No plants developed symptoms in pots planted 6 months from placement of wood pieces. During 2005, seedlings planted with stored infected wood pieces did not develop symptoms. Control plants did not develop wilt symptoms. The wilt pathogen was not recovered from the apparently healthy plants six months from planting in pots. Isolations from dead seedlings confirmed presence of *F. xylarioides*.

Isolations from stored wood pieces continued to reveal presence of *F. xylarioides* for 6 months. From the 7 month there was no growth on either Synthetic Nutrient Agar (SNA) and Tap water agar (TWA) or Potato dextrose agar (PDA). It appears that wood pieces remain infective for at least six months from the death of trees.

From these observations wood pieces whether buried in soil or in form of diseased dead plants standing in the field can provide inoculum source for coffee wilt disease. Uprooting and burning infected trees in *situ* still goes a long way to reducing infection.

Trial 2

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

Seedlings (3 out of 10 seedlings) planted in freshly sampled wilt infected soil from the Forest site developed wilt symptoms 76 - 83 days and died within 6 - 8 days from first symptoms.

No infection occurred in soil collected from Blocks 36 and 38. Subsequent monthly plantings from the 3 sites on stored soil continued to reveal more wilting from Forest soil up to the 5th month of storage. Soil from Block 36 was infective only in the first and second month of storage while Block 38 had no infection by end of 2005. Although the number of infected seedlings was small, it serves to demonstrate that inoculum is actually found in the soil. Nursery operators normally collect fertile soil from forests. If this soil is not properly sterilized, coffee wilt disease can be initiated or spread to various destinations.

Trial 3

Role of farm tools/machete or panga in transmission of CWD

Coffee field

4 of the 6 trees in the field (Block 38) inoculated with contaminated panga developed symptoms above inoculation points. Two of these particular plants were cut at the top in the fresh green growth. CWD symptoms were observed about 177 days (mean for both) from the date of inoculation. *F. xylarioides* was recovered from a piece of stem with symptoms. Weeks from first symptoms the whole plant had died. The third plant to show symptoms was cut/ wounded mid-way along the main stem with a contaminated panga, and developed CWD symptoms 173 days later and the fourth wounded/ cut at about 15 cm from the ground level developed symptoms after about 597 days. No symptoms developed on plants wounded with a sterile panga. It appears possible that heavily contaminated panga could transmit the disease from diseased to healthy plants.

Screen House

All seedlings inoculated in various ways did not develop wilt symptoms in the first and subsequent trials. Probably the inoculum adhering to tools was too low to initiate infection.

UNIKIN

INTERNATIONAL SCIENTIFIC COOPERATION PROJECT (INCO)

Development of a long-term strategy based on genetic resistance and agro-ecological approaches against Coffee Wilt Disease

Fourth Annual Report (November 2004 to October 2005)

University de Kinshasa
B.P. 866, Kinshasa XI RDC

WP 1: PATHOGEN DIVERSITY

Task 3: Evaluation of the variability in isolates aggressiveness from Equateur

Objectif

Nous avons procédé à l'évaluation de l'agressivité des isolats obtenus des échantillons récoltés dans la Province de l'Equateur. L'objectif poursuivi est la recherche de la diversité génétique au sein des souches du *Gibberella (Fusarium) xylarioides* en provenance de différentes origines géographiques. Nous voudrions comprendre si le pathogène aurait subi des changements dans le temps ou dans l'espace.

Matériel et méthodes

Souches étudiées

Au cours de cet essai, nous avons utilisé 6 souches choisies en fonction de différents axes de récolte des échantillons. Des souches de référence MUCL 35223, et MUCL 45580 de la Mycothèque de l'UCL et B10101 (2) J de l'UNIKIN, conservée à la MUCL sous n°46044 ont également été utilisé. (Tableau 1)

Tableau 1 : Liste de souches en provenance de la province de l'Equateur en compagnie des souches historiques de référence MUCL et UNIKIN.

Souche	Province d'origine en RDC	Localité d'origine en RDC	Date de récolte par [1]	Date d'isolement par [2]	Identifié par [3]
Bunduki	Equateur	Bunduki	09/09/2004 K.D.	20/11/2004 T.	T.
Mangbakapale	Equateur	Mangbakapale	15/09/2004 K.D.	20/11/2004 T.	T.
Mindembo Bloc 5	Equateur	Lisala	17/09/2004 K.D.	20/11/2004 T.	T.
Moboko	Equateur	Itimbiri	09/09/2004 K.D.	22/11/2004 T.	T.
Notre-Dame	Equateur	Loeka	15/09/2004 K.D.	22/11/2004 T.	T.
Zobolia	Equateur	Loeka	15/09/2004 K.D.	20/11/2004 T.	T.
B10101(2)J	Nord-Kivu	Mutwanga	02/12/02 K.T. D.	02/01/03 T.	T.
MUCL35223	Orientale	Isiro	Nov. 1992 P.	Nov.1992 Decock	Decock
MUCL 45580	Orientale	Yangambi (L147)	15/12/02 K.T. D.	21/05/03 L.	L.

[1] K.D. = Kalonji et Dibue; P. = Pochet.
[2] K.D.T. =: Kalonji, Dibue et Tshilenge; L.= Lepoint.
[3] T : Tshilenge; L. = Lepoint.

Matériel végétal et inoculation

Les plantules utilisées dans cet essai sont issues des graines du clone L251 récoltées à la Station de l'Institut National d'Etudes et Recherche Agronomiques (INERA) Bongabo (Province de Equateur). Ce clone est choisi pour sa susceptibilité déjà observée dans un essai antérieur (Kalonji, 1975). L'essai comporte 3 répétitions et pour chaque traitement, nous avons utilisé 12 plantules. L'inoculum pour chaque

souche est constitué d'une suspension de conidies ajustée à 10^6 conidies/ml de solution stérile de Tween® 20 (une goutte de Tween® 20 dans 100 ml d'eau)., Il a été produit à partir des subcultures sur milieu SNA. Au moment de l'inoculation, les plantules ont l'âge de 3 mois avec 3 à 4 paires des feuilles. L'inoculation est réalisée par injection à la seringue de marque Terumo Myjector U-40 insulin, Terumo Europe N.V., 3001 Leuven, Belgium. Au moment de l'injection, la tige est désinfectée superficiellement avec de l'éthanol 70 % que l'on a laissé s'évaporer pendant 10 minutes. L'injection est faite en un point situé au niveau du premier entrenœud sous la première feuille. Un volume minimum de 0,025 ml est injecté avant le refoulement (fig.1).Un traitement témoin, inoculé uniquement avec la solution Tween 20, a été inclus.

Figure 1 : Brunissement des feuilles

Figure 2 : Déssechèment des feuilles

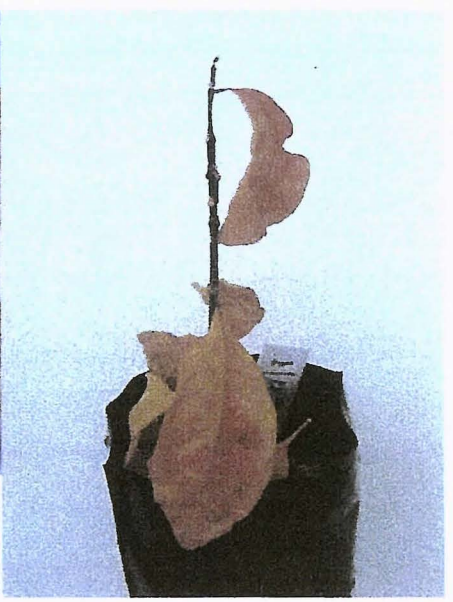


Figure 3 : Mortalité de la plantule

Manifestations pathologiques observées (symptômes)

Les observations ont porté sur les manifestations pathologiques et la production des périthèces. Les symptômes observés sont le flétrissement, le brunissement, le dessèchement des feuilles ainsi que la mortalité de la plantule correspondant au dessèchement total de la tige (figures 1 à 3). On a enregistré la vitesse d'apparition de différents symptômes à partir de la date d'inoculation. La sévérité d'attaque a été évaluée sur base des valeurs de AUDPC (Area Under the Disease Progress Curve" ou surface sous la courbe de progression de la maladie) établies suivant l'évolution dans le temps des symptômes enregistrés. Ce paramètre est calculé suivant la formule : AUDPC = $\sum[(x_1+x_2).0,5][t_2-t_1]$ où x_1 et x_2 représentent la sévérité au temps 1 et au temps 2; t_2-t_1 : intervalle de temps entre 2 observations. A la fin de l'essai des réisolements ont été réalisés.

Les résultats enregistrés ont été analysés à l'aide de Microsoft Excell Office 2003 suivant le modèle ANOVA 2 facteurs sans répétition et les moyennes ont été

comparées par le test LSD au niveau de probabilité 5%. La variation individuelle autour de la moyenne est indiquée dans les tableaux de résultats en terme d'écart-type et dans les graphiques par la barre d'erreur.

Résultats

Le moment d'apparition des différentes anomalies foliaires (flétrissement, brunissement et dessèchement) ainsi que le niveau de mortalité des plantules sont repris dans le tableau 2.

Tableau 2 : Vitesse d'apparition des symptômes enregistrés après inoculation avec différentes souches de la province de l'Equateur.

Souche	Délai (jour) d'apparition des différents symptômes observés			
	Flétrissement des feuilles (moyenne ± SD*)	Brunissement des feuilles (moyenne ± SD*)	Dessèchement des feuilles (moyenne ± SD*)	Mortalité (moyenne ± SD*)
Bunduki	17,33 ± 1,52	16,73 ± 1,10	23,00 ± 2,29	23,13 ± 2,12
Mangbapale	17,40 ± 0,53	18,33 ± 1,62	22,83 ± 0,29	22,83 ± 0,29
Mindembo 5	17,10 ± 0,79	17,30 ± 2,10	19,30 ± 0,98	18,57 ± 0,51
Moboko	18,07 ± 0,60	17,57 ± 1,40	21,07 ± 1,10	22,40 ± 2,51
N-Dame	21,83 ± 2,84	19,87 ± 1,40	23,33 ± 0,58	23,07 ± 0,12
Zobolia	16,00 ± 0	18,50 ± 1,14	21,67 ± 0,58	20,00 ± 2,00
B10101(2)J	21,00 ± 5,19	18,77 ± 0,80	22,00 ± 0,87	23,00 ± 0,87
MUCL35223	17,00 ± 0,86	19,33 ± 1,26	21,00 ± 0,00	22,00 ± 1,73
MUCL45580	18,00 ± 0	16,43 ± 2,06	21,93 ± 0,90	24,43 ± 2,25
Témoin	N.O.**	N.O.**	N.O.**	N.O.**
Délai moyen	18,19	18,09	21,79	22,16
LSD (0,05)	NS	NS	0,58	0,79
CV (%)	12,13	8,51	5,03	6,7

SD* = Ecart-type.
N.O.**=Non Observé

L'examen du tableau 2, montre en moyenne pour l'ensemble des souches que le flétrissement et le brunissement sont les premiers symptômes qui apparaissent chez les sujets infectés en moyenne 18 jours après inoculation. Le dessèchement des feuilles et la mortalité surviennent après 22 jours d'inoculation.

La comparaison des souches de la province de l'Equateur avec les souches historiques ne montre pas de différence significative en ce qui concerne le flétrissement et le brunissement des feuilles. Par contre, des différences apparaissent en ce qui concerne le dessèchement des feuilles ($LSD_{0,05}$ = 0,58) et la mortalité des plantules (LSD 0,05 = 0,79). Dans l'un et l'autre cas, on remarque que ces différences ne sont liées ni à la répartition géographique ni à l'âge des souches. Dans le cas du dessèchement des feuilles, on peut citer le cas de deux souches provenant de deux zones et des époques différentes : Moboko (provenant de l'Equateur et isolée en 2004) et MUCL35223 (provenant de la province orientale et isolée en 1992) ; les deux présentent le même délai moyen (21 jours) d'apparition de ce symptôme. Pour ce qui concerne la mortalité, les souches B10101(2)J (provenant

du Nord Kivu et isolée en 2003), et Bunduki et Notre Dame (provenant de la province de l'Equateur et isolées en 2004) présentent un délai moyen (23 jours) d'apparition de ce symptôme. Ces résultats montrent qu'il n'existe pas de diversité au sein des souches du *G. xylarioides*.

Les figures 4 à 7 permettent d'exprimer la sévérité de différents symptômes par les valeurs AUDPC.

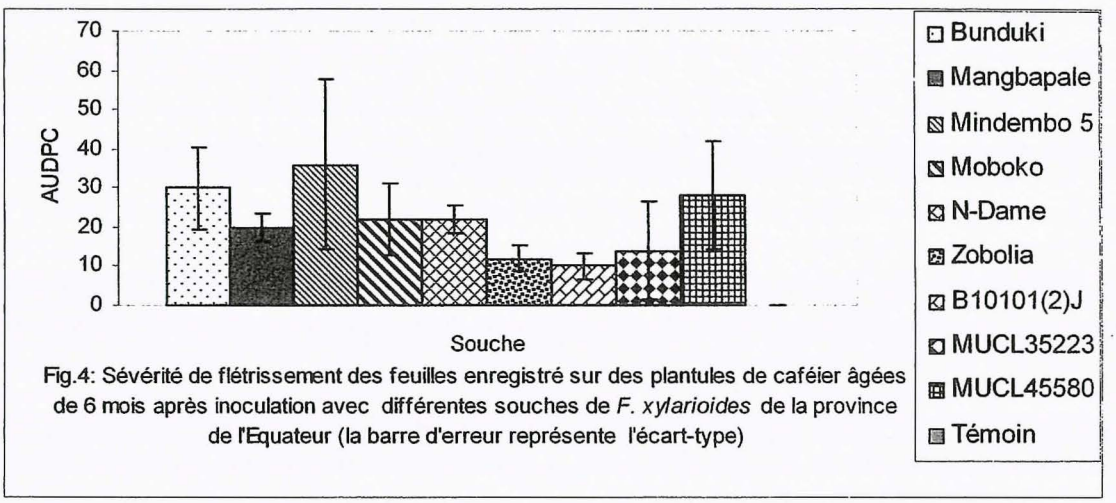


Fig.4: Sévérité de flétrissement des feuilles enregistré sur des plantules de caféier âgées de 6 mois après inoculation avec différentes souches de *F. xylarioides* de la province de l'Equateur (la barre d'erreur représente l'écart-type)

Les surfaces représentant l'augmentation des flétrissements (fig.4) montrent des différences significatives entre les souches ($LSD_{0.05}$ =6,17).La valeur AUPDC la plus élevée est enregistrée avec la souche Mindembo Bloc 5 et les valeurs les moins élevées avec B 10101 (2) J et Zobolia dont les valeurs sont respectivement de 10 et 12.

En ce qui concerne le brunissement (fig.5), les valeurs AUDPC présentent également des différences significatives (LSD 0.05 = 3,16). Ce symptôme est plus sevère dans le cas des souches, Bunduki, Mindembo Bloc 5, Notre Dame et Moboko avec des valeurs AUDPC respectivement de 50, 52, 52 et 50

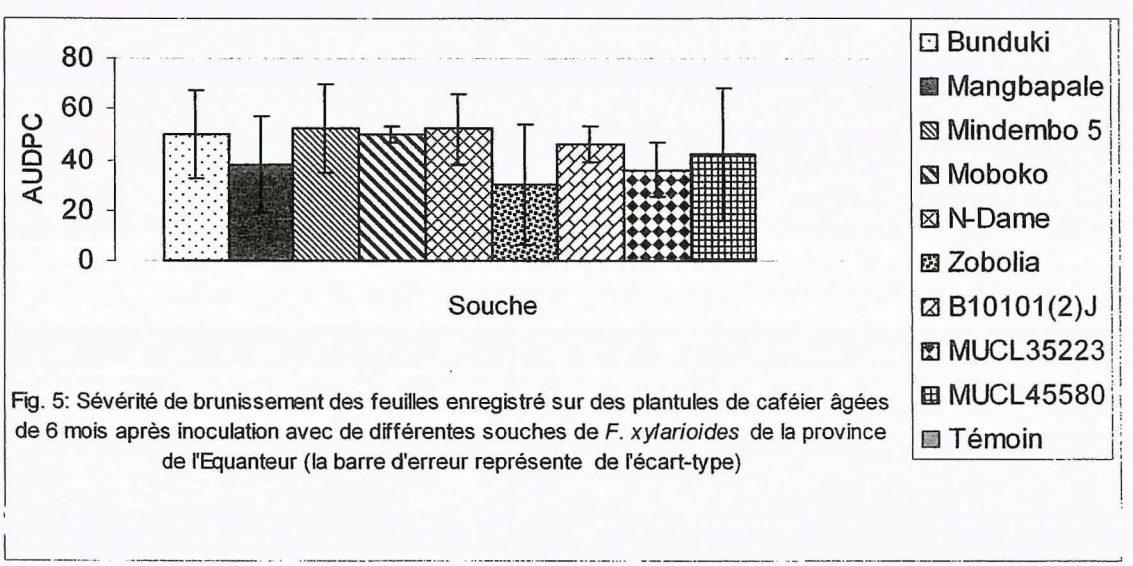


Fig. 5: Sévérité de brunissement des feuilles enregistré sur des plantules de caféier âgées de 6 mois après inoculation avec de différentes souches de *F. xylarioides* de la province de l'Equanteur (la barre d'erreur représente de l'écart-type)

L'aire sous les courbes du progrès du symptôme dessèchement (AUDPC) représentée par la figure 6 revèle des différences significatives (LSD 0.05 = 5,79). La surface la plus importante est obtenue avec la souche Mindembo bloc 5 tandis que la souche B 10101 (2) J parait la moins sévère.

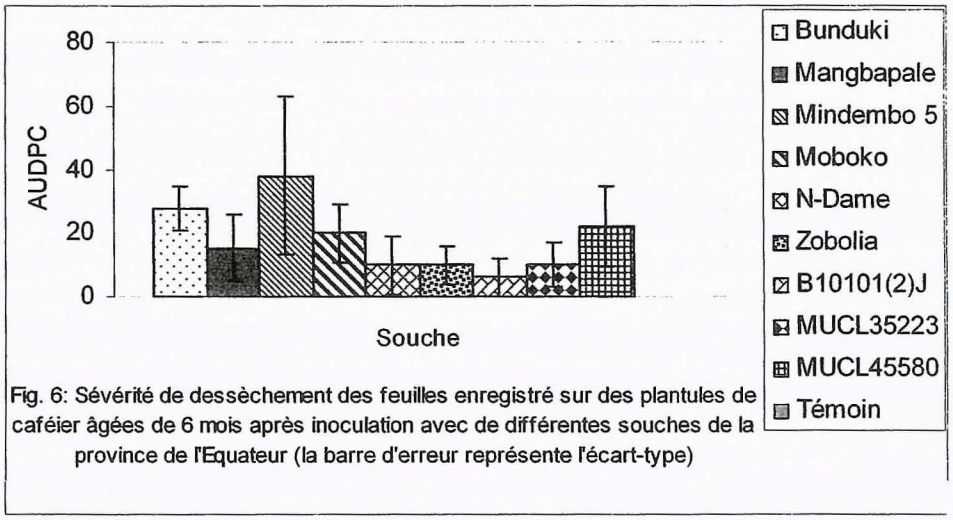


Fig. 6: Sévérité de dessèchement des feuilles enregistré sur des plantules de caféier âgées de 6 mois après inoculation avec de différentes souches de la province de l'Equateur (la barre d'erreur représente l'écart-type)

Les valeurs AUDPC (fig. 7) correspondantes aux différentes souches ne présentent pas de différences significatives en ce qui concerne la mortalité des plantules. Ceci s'expliquerait par la grande variabilité observée au sein des souches (écart-types) et leur chevauchement.

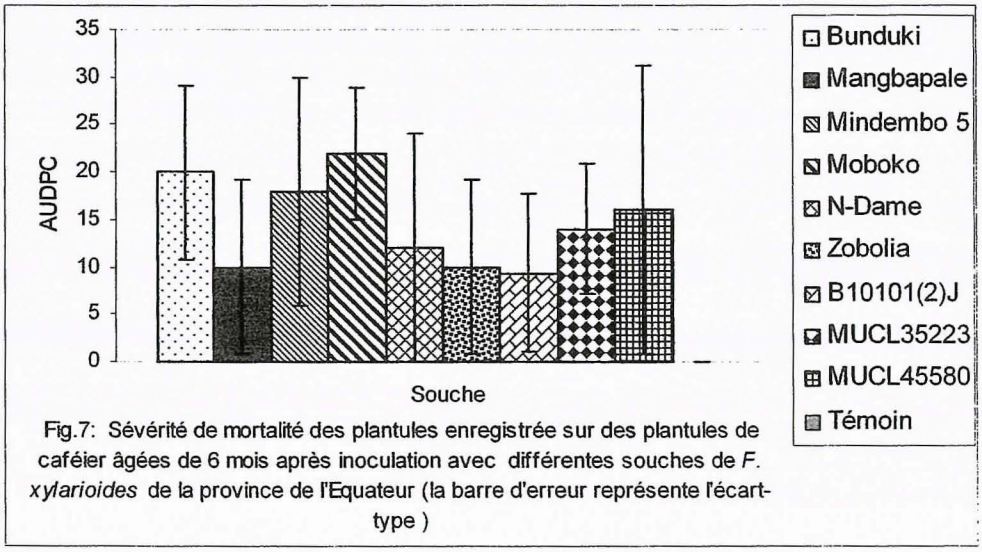


Fig.7: Sévérité de mortalité des plantules enregistrée sur des plantules de caféier âgées de 6 mois après inoculation avec différentes souches de *F. xylarioides* de la province de l'Equateur (la barre d'erreur représente l'écart-type)

Conclusion

Il ressort de l'analyse des données sur la vitesse d'apparition (tableau 2) des symptômes montre qu'il existe des différences entre les symptômes induits par les différentes souches pour ce qui concerne le dessèchement et la mortalité. Par contre, il n'y a pas eu de différence significative observée avec cette dernière en

ce qui concerne sa sévérité exprimée par AUDPC. Ainsi en s'appuyant sur la mortalité, on peut signaler comme Bieysse, (2003) et Tshilenge *et al.,(* 2004) qu'il n'existe pas de diversité dans le temps et dans l'espace au sein de la population de souches du *Gibberella xylarioides* étudiées dans le présent essai.

Task 4: Description of the fungal life cycle, asexual and sexual phases

Essai 1 : Monitoring of perithecia production of *Gibberella xylarioides*

Objectif : Cet essai a pour but de quantifier la production des ascospores à partir des plants inoculés dans les différents essais effectué à l'UNIKIN.

Matériel et méthode

Les observations des périthèces sont effectuées mensuellement durant une période de 6 mois (du O3 mai au 03 octobre 2005) sur 9 plants identifiés attaqués par la trachéomycose. Ils sont choisis au hasard parmi les sujets inoculés au cours des essais précédents. Nous présentons dans le tableau 3, les caractéristiques des plantes mis sous observations.

N° Plant	Caractéristiques
1	Sujet Inoculé en 1999
2	Plant inoculé par blessure et insertion de morceau de culture gélosé du pathogène au cours de l'essai portant sur les méthodes d'inoculation (2003)
3	Plant inoculé par blessure au cours de l'essai test d'agressivité des isolats en provenance du Nord Kivu (2004)
4	Plant inoculé par blessure au cours de l'essai test d'agressivité des isolats en provenance du Nord Kivu (2004)
5	Plant inoculé par blessure au cours de l'essai test d'agressivité des isolats en provenance du Nord Kivu (2004)
6	Plant inoculé par blessure au cours de l'essai test d'agressivité des isolats en provenance du Nord Kivu (2004)
7	Plant inoculé par badigeonnage d'une suspension des conidies du pathogène au cours de l'essai portant sur les méthodes d'inoculation (2003)
8	Plant inoculé par badigeonnage d'une suspension des conidies du pathogène au cours de l'essai portant sur les méthodes d'inoculation (2003)
9	Plant inoculé par blessure et insertion de morceau de culture gélosé du pathogène au cours de l'essai portant sur les méthodes d'inoculation (2003)

La méthode d'observation proposée par Maraite (First intermediate report, pp. 42-45 Report n° 28/2002 CIRAD/AMIS) a été appliquée avec légères modifications portant principalement sur les dimensions du gabarit en fonction des grosseurs des tiges.

Des sections transversales sont marquées à l'aide d'un stylo à encre indélébile sur la tige pour faire l'objet d'examens sous loupe binoculaire de marque Wild Heerbrugg. Sur la tige sèche, les périthèces se retrouvent suivant les individus soit au dessus du point d'inoculation, soit en dessous ou dans les deux directions. On marque les portions distantes de 1 cm sur une longueur de 6 cm de façon à permettre une observation sur toute la section circonscrite sous loupe binoculaire.

L'abondance et l'état des périthèces ont été évalués sous une loupe binoculaire de marque Wild Heerbrugg sur chacune des portions tracées sur la tige selon l'échelle suivante:

Abondance des périthèces:

0= Pas de périthèce

1= Jusqu'à 3 périthèces simples ou groupés par portion observée de 1 cm de hauteur et sur toute la circonférence de la tige.

2= Plus de 3 jusqu'à 10.

3= Plus de 10 périthèces.

L'état des périthèces:

S= Stroma sombre sans périthèces

G= Stroma à périthèce avec ostiole visible.

E= Périthèce vide et dégradé.

Les périthèces individuels sont prélevés avec les scalpels à lame n°11 et montés dans une goutte du lactophénol bleu pour vérification de l'identité d'après la description de *G. xylarioides* et l'examen de maturation des asques et des ascospores au microscope.

Les données météorologiques de la période concernées fournies par la Station climatologique du Centre Régional d'Etudes Nucléaires de Kinshasa (CREN-K) indique que la période d'observation présentait une amplitude thermique moyenne de 25°C et un air humide.

Résultats

Les résultats représentant l'abondance (A) et l'état de maturation (E) des périthèces sont repris respectivement dans les tableaux 3 et 4.

Tableau 3 : Abondance des périthèces (fréquences observées)

Niveau d'observation (cm)	Fréquence observée			
	Côte 0 (pas de périthèces)	Cote 1 (jusqu'3 périthèces)	Cote 2 (entre 3 et 10 périthèces)	Cote 3 (plus de 10 périthèces
1	6	25	14	1
2	8	18	12	8
3	12	11	15	8
4	5	7	20	14
5	13	8	19	6
6	15	12	4	14
Total	59	81	84	54

Tableau 4 : Etat de maturité des périthèces

Niveau d'observation (cm)	Fréquence observée			
	Côte 0 (pas de périthèces)	Cote S (stroma sombre sans périthèces)	Cote G (stroma avec périthèces à ostiole ouvert)	Cote E (stroma sombre et périthèces dégradés)
1	9	5	7	25
2	7	7	13	19
3	12	11	9	14
4	6	23	5	12
5	12	17	7	10
6	11	14	10	10
Total	57	77	51	90

Le classement des fréquences observées montre une importante variabilité et l'importance des différences entre les niveaux d'observation varie selon que l'on considère l'abondance ou l'état de maturité des périthèces.

Sur base de la production totale des périthèces, l'évolution mensuelle reste semblable pour les cotes 1 et 2 avec cependant une supériorité de la production pour la cote 2 représentant la présence sur les sujets 3 à 10 périthèces.

En ce qui concerne, l'état des périthèces,on retrouve de nombreux cas des stromas sombres sans périthèces (77 cas) et un peu plus des stromas avec périthèces mais dégradés. On conviendra que le rythme mensuel d'observation doit être raccourci ; il ne permet pas de visualiser suffisamment les états intermédiaires (cote G).

Essai 2 : Etude de fertilité de Mating types *in vivo* en pépinière (IN)

Cet essai concerne l'étude de fertilité de mating types in vivo en pépinière. Elle est réalisé à partir de deux souches MUCL 44532 et MUCL 44536 fournies à l'UNIKIN par l'UCL (Pascale Lepoint). Il s'agit de confirmer la fertilité de ces deux souches en conditions naturelles de la trachéomycose à Kinshasa et à Beni. Il est prévu que l'analyse de la fertilité des périthèces sera étudiée à l'UCL où les échantillons seront envoyés à la fin de l'essai.

Conduite de l'essai

Les 2 souches sont inoculées au même moment, mais en des endroits différents sur la tige de jeunes caféiers. Le matériel végétal utilisé a été représenté par des plantules caféiers issues de la germination des graines récoltées sur un sujet sensible attaqué par la trachéomycose et bien identifié (un caféier Robusta : I1010203/OG) récolté à Beni. A l'inoculation elles ont été âgées de 2 ans et 3 mois. La technique d'inoculation a été celle de blessure pratiquée par entaille dans la tige et l'insertion d'un fragment de culture du pathogène sur milieu SNA. Les plantules ont été inoculées au mois de juillet.

Les objets ont été répartis de la manière suivante :

Objet 1 : Mating type 1 ou 2 inoculé seul à 1 cm en dessous des feuilles cotylédonaires sur le plan de la première paire de vraies feuilles;
Objet 2 : Les souches inoculées à 1 cm en dessous des feuilles cotylédonaires sur le plan de la première paire de vraies feuilles en position face à face.
Objet 3 : les deux souches inoculées en position décalées Mat1 au dessus de Mat2 à partir du niveau de 1 cm en dessous des feuilles cotylédonaires sur le plan de la première paire de vraies feuilles.
Objet 4 : l'inverse de l'objet 3, c'est-à-dire Mat2 au-dessus de Mat1.

Résultats
Les observations sont en cours.

WP3 Breeding for resistance

Task 1 : Screening tests

Varietal Evaluation

Previous trials carried out on screening different genotypes in greenhouse at CIRAD-Montpellier and at Université de Kinshasa revealed some interesting genotypes with less level of susceptibility to CWD according the established threshold (0 to 15 % of mortality). To confirm the durability of this resistance, these genotypes must be evaluated in the areas where CWD is naturally present i.e. Beni, Isiro, Yangambi, etc. The current step is then to obtain seeds.

Ripe fruits were carefully harvested and processed by the wet technique to guaranty the quality. This technique consists in removing manually the pulp while cherries are still fresh. Seeds were immersed in water order to eliminate twigs, light fruits (pierced, immature, etc) which are floating on the surface. Then they were drained off by letting the water overflow. The heavy fruits were then pulped handily to remove the skin. After staying in water during 24 h, seeds were cleaned to remove the remaining pulp. Afterwards, they were dried under the shade and packed in polybags.

The following genotypes seeds (table) are in dispatching in different hot areas infected by CWD (Beni, Isiro) to be sown, in order to get plantlets for varietal evaluation trials.

Since some genotypes were highly productive, it appeared interesting to test their resistance level. Then the seed were harvested too for this goal.

Table5 : Seed list harvested in Bloc-Coffee at INERA Kiyaka (DRC) and planting in Unikin garden

N°	Genotype	Weight (g)	Date de récolte	Date de semis	Germoir
1	KR 1/1	246	13/06/05	18/07/05	**A/2**
2	KR 1/2	96	13/06/05	18/07/05	**C/1**
3	KR 1/3	1206	14/06/05	18/07/05	**A/1 B/3**
4	KR 2/5	500	13/06/05	18/07/05	**A/7**
5	KR 3/5	820	14/06/05	18/07/05	**B/1**
6	KR 8/8	406	13/06/05	18/07/05	**A/8**
7	KR 9/8	318	13/06/05	18/07/05	**B/2 C/2**
8	KR 10/7	590	13/06/05	18/07/05	**A/9**
9	KR 11/12	65	13/06/05	18/07/05	**C/3**
10	KR 12/4	70	13/06/05	18/07/05	**A/4**
11	KR 12/5	20	13/06/05	18/07/05	A/5
12	KR 12/6	206	14/06/05	18/07/05	**A/6 C/4**
13	KR 16/5	280	15/06/05	18/07/05	**A/10**
14	KR16/13	140	13/06/05	18/07/05	A/11
15	KR 19/1	260	14/06/05	18/07/05	A/3
16	KR 19/12	200	13/06/05	18/07/05	A/12
17	KR 20/10	440	13/06/05	18/07/05	**A/13**
18	KR20/51	127	13/06/05	18/07/05	**C/7**
19	KR A/6	540	15/06/05	18/07/05	**C/5**
20	KR C/3	1600	15/06/05	18/07/05	C/6

Tableau 8 : Seed list harvested in Bloc-Coffee at INERA Luki (DRC) and planting in Unikin garden

N°	Abre-mère	Quantité récoltée (g)	Date de récolte	Matériel	Date de semis	Bloc récolté
1	R1P1L3(1)SO	600	17/08/05	Robusta		Essai d'adaptation locale
2	R1P1L4(1)SO	300	17/08/05	Robusta		Essai d'adaptation locale
3	R1P3L1(5)SO*	200	17/08/05	Robusta		Essai d'adaptation locale
4	R1P4L2(3)SO	400	17/08/05	Robusta		Essai d'adaptation locale
	R2P3L2(5)SO	400	17/08/05	Robusta		Essai d'adaptation locale
	R2P3L3(7)SO	400	17/08/05	Robusta		Essai d'adaptation locale
	LAF159/L2(9)	150	18/08/05	Kouillou		Bloc Kouillou
	LAF159/L5(2)	50	18/08/05	Kouillou		Bloc Kouillou
	LAF159/L6(5)	50	18/08/05	Kouillou		Bloc Kouillou
	LK/L22(6)	200	18/08/05	Kouillou		Bloc Kouillou
	S23/L1(3)	50	18/08/05	Kouillou		Bloc Kouillou
	S23/L6(7)	150	18/08/05	Kouillou		Bloc Kouillou
	S23/L7(3)	100	18/08/05	Kouillou		Bloc Kouillou

ANNEXE

Parasitica, 2004, 60(3-4):19-34

Caractérisation des *Fusarium* spp. associées au dépérissement du caféier Robusta en République Démocratique du Congo[1]

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Résumé

Les auteurs ont isolé et caractérisé différentes espèces de *Fusarium* à partir des caféiers Robusta atteints de la trachéomycose en République Démocratique du Congo en vue de déterminer leur causalité dans cette maladie. Les caractères culturaux et microscopiques ainsi que les profils moléculaires par Random Amplified Polymorphic DNA (RAPD) ont été étudiés. Pour les caractères observés en culture, des différences ont été observées entre les souches du *F. xylarioides* et celles des *F. equiseti, F. falciforme, F. solani* et *F. stilboides*. Les inoculations artificielles ont révélé que seules les souches du *F. xylarioides* ont causé une sévère défoliation et la mortalité des plants. La responsabilité suspectée d'autres *Fusarium* spp. dans le dépérissement du type trachéomycose chez le caféier n'est pas établie.

Mots-clés: trachéomycose, caféier Robusta, *Fusarium equiseti*, *Fusarium falciforme*, *Fusarium solani*, *Fusarium stilboides*, *Fusarium xylarioides*, PCR-RAPD.

Introduction

En République Démocratique du Congo (RDC) les plantations caféicoles, situées dans les provinces Orientale et du Nord-Kivu, sont fortement attaquées par la trachéomycose qui entraîne le dépérissement des caféiers. Cette maladie, causée par le *Gibberella xylarioides* Heim et Saccas, anamorphe *Fusarium xylarioides* Steyaert, est déjà décrite en détails par d'autres auteurs (STEYAERT, 1948; FRASELLE, 1950; SACCAS, 1951).

Après plusieurs décennies de lutte depuis son apparition à Yangambi en 1949, la maladie a été combattue et maîtrisée jusqu'en 1982, l'année où elle a été de nouveau signalée dans le district du Haut-Uelé (KATENGA, 1987).

[1] Reçu le 24 mars 2005, accepté le 2 septembre 2005

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Parmi les hypothèses émises sur cette résurgence, on a pensé au rôle possible, dans la pathogenèse, d'autres espèces de *Fusarium* (MARAITE, 2003). En rapport avec cette hypothèse, plusieurs *Fusarium* spp. avaient été observées et suspectées comme responsables de la trachéomycose chez le caféier Robusta. STEYAERT (1948) a identifié l'agent pathogène responsable comme le *Fusarium xylarioides*. FRASELLE (1950) a pensé au *F. oxysporum* associé avec le flétrissement du feuillage et la nécrose des racines. BOOTH (1971) distinguait au sein de la population de *F. xylarioides,* des souches femelles et des souches mâles. Plus tard, VON BLITTERSDORF & KRANZ (1976) ont rattaché la souche "mâle" de Booth au *F. stilboides*. MESSIAEN & CASSINI (1981) ont considéré que le champignon responsable appartenait à la grande espèce de *F. lateritium* sensu Snyder et Hansen et devrait, dans ces conditions, s'appeler *F. lateritium* f.sp. *xylarioides*.
Le présent travail vise à déterminer le rôle de différentes espèces fusariennes dans la pathogenèse de la trachéomycose à travers une étude comparative des caractères morphologiques, moléculaires, parallèlement aux caractères pathogéniques des *Fusarium* spp. isolées des caféiers Robusta en RDC.

Matériel et méthodes

SOUCHES DES *FUSARIUM* SPP. ÉTUDIÉES

Les isolements des *Fusarium* spp. ont été faits à partir des morceaux de bois collectés sur des caféiers Robusta atteints de la trachéomycose au cours des prospections réalisées en 2002 dans diverses régions caféicoles de la RDC. Des échantillons en forme des rondelles des dimensions variables (3-5 cm de longueur et 2-4,5 cm de diamètre), en fonction de la grosseur des tiges, ont été désinfectés en surface avec de l'éthanol 70 % puis flambés rapidement afin d'évaporer l'excès d'alcool. Ils ont été ensuite fendus en deux, dans des conditions aseptiques à l'aide d'un biseau de menuisier et d'un maillet en caoutchouc en essayant de passer par des zones présentant des "rubans noirs" sous-corticaux dénudés, symptôme de la trachéomycose chez le caféier. A l'intérieur du bois, sur des endroits de coloration sombre, de minuscules fragments d'ordre de quelques millimètres ont été prélevés à l'aide de pointe de scalpel n° 11 et déposés sur milieu Eau Agar Streptomycine (Agar Oxoid®, LTD, Basingstoke, Hampshire, UK: 15 g; H_20 déminéralisée: 1000 ml; Streptomycine: 100 mg). Après émission du mycélium, les extrémités d'hyphes ont été repiquées sur milieu Synthetic Nutrient Agar: SNA (KH_2PO_4: 1 g; KNO_3: 1 g; $MgSO_4.7H_2O$: 0,5 g; KCl: 0,5 g; Glucose: 0,2 g; Saccharose: 0,2; Agar Oxoid®, LTD, Basingstoke, Hampshire, UK: 20 g; H_20 déminéralisée: 1000 ml). Une collection de souches monoconidiennes a été constituée et conservée à la Mycothèque de l'Université catholique de

Louvain (MUCL). Des souches de référence, isolées en 1960 et en 1992, ont été reçues de la MUCL et font aussi partie de la collection en étude (Tableau 1). Le nombre de souches a été élargi pour l'analyse des profils RAPD (figure 1).

TABLEAU 1
Liste de souches étudiées des *Fusarium* spp. de la collection
List of Fusarium *spp. studied from the collection*

Fusarium sp.	Souche	n° MUCL	Origine en RDC	Date de récolte
F. equiseti	PTFsp4	46054	Isiro	17 déc. 2002
F. falciforme	MUCL43880	43880	Bas-Congo	12 mars 2002
F. solani	PTFsp1	-	Isiro	17 déc. 2002
	PTFsp2	46055	Yangambi	15 déc. 2002
	PTFsp5	-	Isiro	17 déc. 2002
	SR21/03	45431	Butembo-Njiya	12 sept. 2002
F. stilboides	SR17/09	45428	Butembo-Njiya	12 sept. 2002
F. xylarioides	B10101(2)J	46044	Beni-Mutwanga	02 déc. 2002
	B10101(5)	46045	Beni	02 déc. 2002
	MUCL14186	14186	Yangambi	1960
	MUCL35223	35223	Isiro	nov. 1992
	SR01A/05a	44516	Beni	02 déc. 2002
	SR01B/10a	45580	Yangambi	15 déc. 20/02
	SR12B/01a	45592	Isiro	17 déc. 20/02
	SR22/01a	44512	Butembo	12 sept. 2002

CARACTÉRISATION MORPHOLOGIQUE

Caractères culturaux

Les subcultures destinées à l'étude des caractères culturaux ont été obtenues à partir d'un fragment de 5 mm de diamètre, découpé à l'aide d'un perce-liège en périphérie du mycélium sur SNA et placé au centre d'une boîte de Petri sur 15 ml de PDA (Oxoid®, LTD, Basingstoke, Hampshire, UK). Chaque souche a été repiquée dans 5 boîtes de Petri et l'opération a été répétée 4 fois. Les observations ont été enregistrées journellement à la lumière du jour. Elles ont porté sur la croissance radiale, le contour et la pigmentation des colonies. Le diamètre des colonies a été mesuré jusqu'à l'occupation totale de la boîte de Pétri. Ces mesures ont permis de déterminer la vitesse journalière de croissance radiale (cm/j), calculée sur la phase linéaire de l'évolution de la courbe de croissance. L'analyse de la variance a été effectuée à l'aide de Microsoft Excel Office 2003 suivant le modèle ANOVA 2 facteurs et la comparaison des moyennes par le test LSD. Les cultures ont été incubées à 27±3°C dans l'obscurité pendant 10 jours. La description de la couleur du mycélium aérien et de l'envers de la colonie a été faite au 10ème jour de culture en utili-

sant l'échelle proposée par NELSON *et al.* (1983). Le contour des colonies a été décrit par rapport au diagramme présenté par AINSWORTH (1971).

Caractères microscopiques

L'analyse des caractères microscopiques a été effectuée à partir des subcultures sur SNA incubées en chambre de culture à 25±2°C pendant 7 jours à 50 cm sous un banc de lumière blanche (Color white, F 36 W/133, SYLVANIA Standard, Germany) alternée à 1/1 avec la lumière proche UV (Philips, TLD 36 W/08, Holland), à une photopériode de 12 heures. Les caractères biométriques ont été observés sur des coupes microscopiques faites à partir des fragments prélevés dans les subcultures à des endroits où une forte densité des conidies a été observée préalablement au microscope inversé (Olympus, CK2). Les coupes ont été montées dans une goutte de lactophénol bleu et observées au microscope à contraste des phases (Zeiss, Axiolab, E re 450907 01, Germany) à l'oculaire 10x équipé d'une échelle micrométrique et l'objectif 100x sous huile à immersion. Les mesures, prises sur un échantillon 40 conidies, ont porté, pour les macroconidies, sur la fréquence de différentes formes, la longueur et la largeur au niveau de la section la plus large ainsi que le nombre de cloisons. La forme, la longueur et la largeur des microconidies ont été également décrites ainsi que leur fréquence. La description de la morphologie s'est référée à celle proposée par AINSWORTH (1971).

CARACTÉRISATION MOLÉCULAIRE

L'analyse moléculaire des souches de *Fusarium* spp. a visé un double objectif. Le premier est la confirmation de l'identité des *Fusarium* spp. faite initialement sur base morphologique après confrontation des valeurs de différents caractères observés dans la présente étude avec celles établies par BOOTH (1971). La recherche de diversité au sein des souches de *F. xylarioides* a constitué le deuxième objectif de cette partie de l'étude.

Identification des **Fusarium** *spp.*

L'unité de transcription ribosomique (rDNA), plus précisément les zones hautement conservées que sont les espaces internes transcrits (Internal Transcribed Spacers: ITS) et le facteur d'élongation alpha 1 (EFα1) ont été analysés. L'amplification par réaction de polymérisation en chaîne (Polymerase Chain Reaction: PCR) des régions ITS1 et ITS2 a été réalisée en utilisant les amorces universelles de ces régions (WHITE *et al.*, 1990, cités par MUNAUT *et al.*, 1998). Après amplification, les séquences ont été comparées avec celles disponibles dans la banque des données European Molecular

Biology Laboratory (EMBL) utilisant l'algorithme Basic Local Alignment Search Tool (BLAST) développé par ALTSCHUL *et al.* (1990, cités par BOTTU & VAN RANST, 2003).

***Diversité au sein des souches de* F. xylarioides**

A partir de la biomasse mycélienne des souches de *F. xylarioides,* l'ADN a été extrait et purifié avant la réalisation du Random Amplified Polymorphic DNA (RAPD) après amplification par PCR. L'extraction de l'ADN a été faite par la méthode phénol/chloroforme et la purification, par la méthode Geneclean® III (F. MUNAUT, 2004, comm. pers.). La PCR-RAPD a été réalisée avec trois amorces choisies dans le kit A 10 mers (Operon Technologies Inc., Alameda, California, USA): A-14 (5'-TCTGTGCTGG-3'), A-15 (5'-TTCCGAACCC-3') et A-17 (5'-GACCGCTTGT-3') dans un volume de 25 µl. Celui-ci est constitué de 2 µl d'ADN (10 ng/µl), 2,5 µl d'amorce (15 µl/ml), 0,5 µl de chaque nucléotide, 0,5 µl de *Taq* polymérase, 1,5 µl de $MgCl_2$, 2,5 µl de tampon et de 15,5 µl d'eau déionisée (eau milliQ). Le thermocycleur Eppendorf Mastercycler AG (Hamburg, Germany) a été utilisé et programmé suivant les étapes ci-après: une étape initiale de dénaturation à 94°C pendant 7 minutes pour la dénaturation complète des brins d'ADN, suivie de 45 cycles de 1 minutes 30 secondes à 94°C, 2 minutes à 35°C et de 3 minutes à 72°C et une étape finale d'élongation de 7 minutes à 72°C. Les produits d'amplification ont été séparés par électrophorèse dans un gel d'agarose à 1,4 % et visualisés sous UV après ajout de bromure d'éthidium. L'échelle GeneRuler™ DNA Ladder Mix #SMO331/2/3 de la Firme Fermentas (Alameda, California, USA) a été utilisée comme référence.

CARACTÉRISATION DU POUVOIR PATHOGÈNE

La pathogénicité des *Fusarium* spp. a été évaluée à travers des inoculations artificielles sur des plantules de caféier dans un essai conduit durant la période du 1er janvier au 24 mai 2004 dans la logette Patho II des serres tropicales de l'Unité de phytopathologie de l'UCL. Pendant la période de l'étude, la température journalière a été en moyenne de 28,6±3,5°C avec les maxima moyens de 35,5±5,4°C et les minima moyens de 21,8±2,0°C. La variation de l'humidité relative pendant la même période a été en moyenne de 54,1±7,4 % avec les maxima moyens de 75,3±11,3 % et les minima moyens de 33±8,1 %.

Matériel végétal

Les plantules inoculées sont issues de la germination des graines en provenance de Kiyaka en RDC. Ces graines ont été récoltées en juin 2003 sur des arbres-mères identifiés comme KR 12 (Kiyaka Robusta de la 12ème ligne) dans le champ d'essai d'introduction variétale à Kiyaka. Elles ont été semées en serres dans de la vermiculite en terrines de germination à la température de 35°C dans une atmosphère saturée d'humidité relative. Après la germination et la sortie de premières vraies feuilles, les plantules ont été repiquées dans des sachets en polyéthylène noir (100 µm d'épaisseur, 12 cm de largeur et 25 cm de hauteur) perforés à la partie inférieure. Les sachets ont été remplis du substrat constitué de terreau pour plantes de bruyère DCM® (De Ceuster Meststoffen N.V./S.A., Grobbendonk, Belgique) avec la composition en matière sèche (35 %), matière organique (25 %), pH (3,5-5,0), NPK (7-7-10). A l'inoculation les plantules étaient âgées de 7 mois et portaient 3 à 5 paires de feuilles sur une tige non encore aoûtée. Le dispositif expérimental a comporté 3 répétitions de 4 plantules soit au total 12 plantules pour chaque souche. Les arrosages au goulot ont eu lieu tous les 2 jours.

Souches utilisées

En fonction de leur appartenance à de différentes espèces fusariennes et de leurs diverses origines en RDC, 7 souches figurant au Tableau 1 ont été utilisées dans cet essai: MUCL 43880 (*F. falciforme*), MUCL 45428 (*F. stilboides*), MUCL 45431 (*F. solani*), MUCL 45580 (*F. xylarioides*), MUCL 46044 (*F. xylarioides*), MUCL 46054 (*F. equiseti*) et PTFsp1 (*F. solani*).

Technique d'inoculation

L'inoculation par injection à la seringue (Terumo Myjector U-40 insulin, Terumo Europe N.V., 3001 Leuven, Belgium) a été utilisée. L'inoculum est constitué d'une suspension de conidies ajustée à 10^6 conidies/ml de solution stérile de Tween 20 (une goutte de Tween 20 dans 100 ml d'eau). Pour chaque souche, l'inoculum a été produit à partir des subcultures sur milieu SNA maintenues dans les conditions précédemment décrites pour l'étude des caractères microscopiques. Avant l'injection, la tige est désinfectée superficiellement avec de l'éthanol 70 % que l'on a laissé s'évaporer pendant 10 minutes. L'injection est faite en un point situé au niveau du premier entrenœud sous la première feuille. Un volume minimum de 0,025 ml est injecté avant le refoulement. Un traitement témoin, inoculé uniquement avec la solution Tween 20, a été inclus.

Observations

Au rythme hebdomadaire, les observations enregistrées ont porté sur la détermination de l'intervalle de temps entre le moment de l'inoculation et l'apparition de différents symptômes d'une part et la sévérité de la maladie d'autre part. Lorsqu'ils apparaissent, les symptômes foliaires ci-après ont été enregistrés: le flétrissement, le brunissement, le dessèchement et la défoliation. La sévérité a été évaluée par le rapport entre le nombre de feuilles tombées et le nombre de feuilles formées. Son évolution dans le temps a donné lieu à la détermination de l'aire en dessous de la courbe retraçant l'évolution de la maladie et l'axe du temps ou "Area Under Disease Progress Curve: AUDPC". La mortalité est exprimée par le pourcentage de plantules complètement desséchées par rapport au nombre total de plantules inoculées. Les données enregistrées ont été analysées à l'aide de Microsoft Excel Office 2003 suivant le modèle ANOVA 2 facteurs. La comparaison des moyennes a été effectuée par le test LSD. A la fin de l'essai, des réisolements ont été effectués dans le but de vérification des postulats de Koch sur un échantillon de 28 plantules inoculées à raison de 4 plantules par souches.

Résultats

CARACTÉRISATION DES SOUCHES DES *FUSARIUM* SPP. EN CULTURE

Caractères culturaux

Les observations faites sur les cultures de différentes souches ont permis de constater une variabilité dans les caractères culturaux observés (Tableau 2) en fonction des espèces.

L'examen des caractères présentés par les souches de *Fusarium* spp. révèle que du point de vue de la pigmentation, la couleur du mycélium aérien met en évidence le *F. stilboides* par sa coloration violacée, à l'opposé d'autres espèces qui présentent un mycélium blanc. Les colonies de *F. equiseti* se sont caractérisées par un contour irrégulier et profondément lacinié tandis que les autres ont présenté un contour sinueux.

L'analyse de variance de la vitesse de croissance révèle une nette différence ($LSD_{0,01}$ = 0,09) entre le groupe de *F. equiseti, F. falciforme* et *F. solani* d'une part et celui de *F. stilboides* et de *F. xylarioides* d'autre part. Le premier se caractérise par une croissance plus rapide (0,99-1,10 cm/j) que le second (0,43-0,49 cm/j). Au sein du premier groupe, le *F. falciforme* est le moins rapide.

TABLEAU 2
Caractères culturaux après 10 jours sur PDA des *Fusarium* spp. associées au dépérissement du caféier Robusta en RDC
Fusarium *spp. associated with coffee wilt disease in DRC, characters on PDA after 10 days*

Espèce	Souche	Couleur		Contour des colonies	Vitesse de croissance (cm/jour)
		du mycélium aérien	de l'envers de la culture		
F. equiseti	PTFsp4	blanc	crème	lacinié	1,10 ± 0,03
F. falciforme	MUCL43880	blanc	orange	sinueux	0,99 ± 0,12
F. solani	PTFsp1	crème	rouge carmin	sinueux	1,02 ± 0,06
	SR21/03	crème	rouge carmin	sinueux	1,03 ± 0,06
F. stilboides	SR17/09	violacée	violacée	sinueux	0,48 ± 0,01
F. xylarioides	B10101(2)J	blanc	beige bleuté au centre	sinueux	0,47 ± 0,05
	MUCL35223	blanc	beige bleuté au centre	sinueux	0,49 ± 0,01
	SR01A/05a	blanc	beige bleuté au centre	sinueux	0,43 ± 0,00
	SR01B/10a	blanc	beige bleuté au centre	sinueux	0,49 ± 0,01
	SR12B/01a	blanc	beige bleuté au centre	sinueux	0,48 ± 0,01
	SR22/01a	blanc	beige bleuté au centre	sinueux	0,48 ± 0,01
LSD (0,01)					0,09

Morphologie et fréquence des conidies

Les caractères microscopiques des conidies sur SNA révèlent des différences dans la proportion de différentes morphologies des conidies (Tableau 3) et dans leur dimension (Tableau 4).

Les données du Tableau 3 montrent que les formes incurvées et fusoïdes ont été absentes chez le *F. equiseti*, lequel présente par contre les macroconidies falciformes dans 100 % des cas. Le *F. stilboides* a produit des macroconidies falciformes à 91 % des cas contre 9 % fusoïdes. Des macroconidies n'ont pas été observées dans les cultures de *F. solani* et de *F. falciforme*. Chez le *F. xylarioides* il a été constaté une prédominance des macroconidies incurvées (56-94 %) suivies des falciformes (6-42 %) et des fusoïdes (2-9 %).

Les dimensions des conidies présentées dans le Tableau 4 montrent des différences suivant les espèces. Le *F. equiseti* révèle des macroconidies de 46-47 µm x 3-4 µm avec 7 à 8 cloisons. Des microconidies n'ont pas été observées chez cette espèce. Par contre elles ont été observées chez *F. falciforme* et *F. solani*. Leurs dimensions ont été de 11-15 µm x 4-5 µm pour le *F. falci-*

forme et de 7-15 µm x 3-5 µm pour le *F. solani*. Le *F. stilboides* n'a pas produit des microconidies et ses macroconidies ont mesuré 30-45 µm x 3-4 µm.

TABLEAU 3
Fréquence de différentes morphologies des macroconidies des *Fusarium* spp. associées au dépérissement du caféier Robusta en RDC
Macroconidia shape frequency of different Fusarium *spp. associated with coffee wilt disease in DRC*

Espèce	Souche	Morphologie		
		Incurvée (%)	Falciforme (%)	Fusoïde (%)
F. equiseti	PTFsp4	NO*	100	NO*
F. falciforme	MUCL43880	NO*	NO*	NO*
F. solani	PTFsp1	NO*	NO*	NO*
	SR21/03	NO*	NO*	NO*
F. stilboides	SR17/09	NO*	91	9
F. xylarioides	B10101(2)J	94	6	NO*
	MUCL35223	56	42	2
	SR01A/05a	84	16	NO*
	SR01B/10a	94	6	NO*
	SR12B/01a	92	8	NO*
	SR22/01a	76	24	NO*

NO*= Non Observé *(Not observed)*

TABLEAU 4
Caractéristiques biométriques des conidies produites sur SNA par différentes souches des *Fusarium* spp. associées au dépérissement du caféier Robusta en RDC
Biometrical characters of conidia produced on SNA medium by different Fusarium *spp. strains associated with coffee wilt disease in DRC*

Espèce	Souche	Dimension				
		macroconidies			microconidies	
		longueur (µm)	largeur (µm)	nombre de cloisons des macroconidies	longueur (µm)	largeur (µm)
F. equiseti	PTFsp4	46-47	3-4	7-8	NO*	NO*
F. falciforme	MUCL43880	NO*	NO*	NO*	11-15	4-5
F. solani	PTFsp1	NO*	NO*	NO*	12-14	4
	SR21/03	NO*	NO*	NO*	11-15	4-5
F. stilboides	SR17/09	30-45	4-3	4-5	NO*	NO*
F. xylarioides	B10101(2)J	11-31	1-3	1-3	5-7	1-2
	MUCL35223	29-30	3-4	1-2	5-7	1-3
	SR01A/05a	28-30	2-3	1-2	7-8	1-2
	SR01B/10a	27-30	3	1-2	7-8	1-2
	SR12B/01a	25-35	3	1-2	7-8	1-2
	SR22/01a	22-31	3	1-3	6-7	1-2

NO*= Non Observé *(Not Observed)*

Au sein des souches de *F. xylarioides* les dimensions des macroconidies ont été de 27-31 µm x 2-3 µm. Les microconidies de cette espèce ont révélé des formes cylindrique, allantoïde et incurvée avec de longueur entre 6-8 µm et de largeur de 1- 2 µm.

CARACTÉRISATION MOLÉCULAIRE

Identification des* Fusarium *spp.

L'analyse combinée des ITS1 et ITS2 ainsi que le facteur d'élongation α1 ont confirmé à 100% l'identité des espèces PTFsp1, PTFsp2 et PTFsp5 comme *F. solani*. L'espèce PTFsp4 a été rattachée au *Fusarium equiseti* à 99% d'homologie avec les régions ITS (Tableau 5).

***Diversité au sein des souches de* F. xylarioides**

Outre son intérêt primordial dans la recherche de diversité au sein d'une espèce, la RAPD a permis de confirmer la différence entre les souches de *F. xylarioides* et celles d'autres *Fusarium* spp. Les premières présentent une grande homogénéité dans leurs profils (Figure 1). Cette homogénéité a été reproduite lors de deux autres amplifications avec l'amorce A-14 et A-15 (résultats non présentés).

TABLEAU 5

Analyse des séquences d'ADN ribosomal et de facteur d'élongation (EF α1) des *Fusarium* spp. associées avec le dépérissement du caféier en RDC

DNA and EF α1 sequencing analysis of Fusarium *spp. associated with coffee wilt disease in DRC*

Souche identifiée	Identification morphologique	Identification par homologie des séquences			Conclusion
		ITS 1	ITS 2	EF α1	
PTFsp1	*F. solani*	*Nectria haemato-cocca** 100%	*Nectria haemato-cocca* 100%	*Nectria haemato-cocca* 100%	*F.solani*
PTFsp2	*F. solani*	*Nectria haemato-cocca* 100%	*Nectria haemato-cocca* 100%	*Nectria haemato-cocca* 100%	*F. solani*
PTFsp4	*F. equiseti*	*F. equiseti* 99%	*F. equiseti* 99%	*Gibberella zeae* 85% **	*F. equiseti*
PTFsp5	*F. solani*	*Nectria haemato-cocca* 100%	*Nectria haemato-cocca* 100%	*Nectria haemato-cocca* 100%	*F. solani*

* *Nectria haematococca* est la forme sexuée de *F. solani*. (Nectria haematococca *is the sexual form of* F. solani).

** Il n'existait pas de séquences de EF α1 pour *F. equiseti* jusqu'au moment de l'analyse (26 février 2004). (*There were no EF α1 sequences of the* F. equiseti *available at the date of analysis, 26th February 2004).*

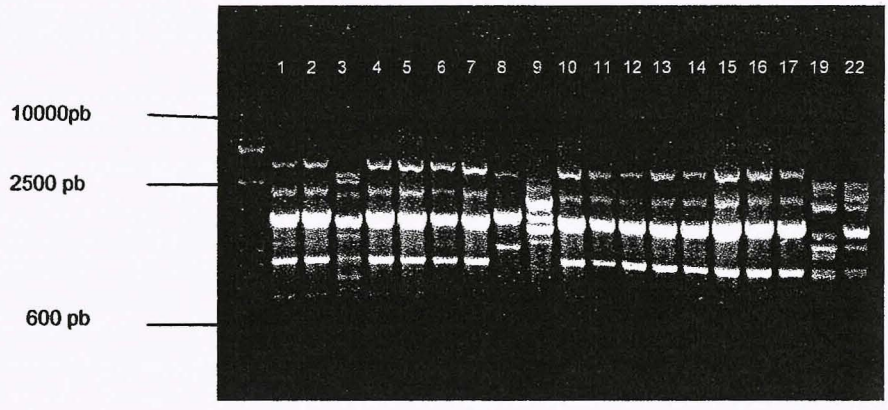

Figure 1. Profils RAPD des espèces fusariennes isolées des caféiers atteints de dépérissement avec l'amorce A-15.
RAPD patterns of Fusarium *spp. associated with coffee wilt disease in DRC obtained with A-15 primers.*
1 = MUCL 14186 (*F. xylarioides*); **2** = MUCL 35223 (*F. xylarioides*); **3** = MUCL 43880 (*F. falciforme*); **4** = SR 01B/10a (*F. xylarioides*); **5** = SR 01A/05a (*F. xylarioides*); **6** = SR 22/01a (*F. xylarioides*); **7** = SR 12B/01a (*F. xylarioides*); **8** = SR 17/09 (*F. stilboides*); **9** = SR 21/03 (*F. solani*); **10** = B10101(2)J (*F. xylarioides*); **11** = B103 (*F. xylarioides*); **12** = IPMO 2(2)G (*F. xylarioides*); **13** = Abdoul (*F. xylarioides*); **14** = Kambale (*F. xylarioides*); **15** = Lebruniana (*F. xylarioides*); **16** = Mayimoya (*F. xylarioides*); **17** = Twaze (*F. xylarioides*); **19** = PTFsp 2 (*F. solani*); **22** = PTFsp.5 (*F. solani*).

CARACTÉRISATION DU POUVOIR PATHOGÈNE

Evolution de différents symptômes

Après une période d'incubation variable en fonction des souches (Tableau 6), les premiers symptômes induits par celles-ci ont varié et ont consisté en un flétrissement ou en un brunissement des feuilles. Les deux évoluent vers le dessèchement suivi de la défoliation.
Les résultats du Tableau 6 dénotent une précocité dans l'apparition des symptômes sur les plantules inoculées avec les souches de *F. xylarioides*. L'absence des cas de flétrissement et de mortalité sur les plantules inoculées avec les autres *Fusarium* spp. a été notée. Les souches de *F. xylarioides* (B10101(2)J et SR 01B/10a) ont été les seules à entraîner la mortalité avec les taux de 17 et 25 % respectivement après 62 et 67 jours d'inoculation.

TABLEAU 6

Délai moyen d'apparition et sévérité de différents symptômes enregistrés sur matériel Kiyaka inoculé avec différentes *Fusarium* spp. associées avec le dépérissement du caféier Robusta en RDC

Average time of occurrence and severity of different symptoms recorded on coffee plantlets inoculated with different Fusarium *spp. associated with coffee wilt disease in DRC*

Fusarium spp.	Souche	Moment (jour) d'apparition de différents symptômes				Mortalité (%)	AUDPC
		Flétris-sement des feuilles	Brunis-sement des feuilles	Dessèchement des feuilles	Défoliation		
F. equiseti	PTFsp4	NO*	43±9	46±4	62±11	0	1,52 ± 1,54
F. falciforme	MUCL43880	NO*	47±6	56±3	61±4	0	2,20 ± 0,51
F. solani	PTFsp1	40±12	47±4	53±4	56±5	0	1,48 ± 0,28
	SR 21/03	NO*	42±3	49±3	56± 3	0	4,10 ± 3,63
F. stilboides	SR 17/09	NO*	34±15	45±14	57±7	0	3,71 ± 3,20
F. xylarioides	B10101(2)J	35±11	19±9	47±19	48±9	17	9,20 ± 3,89
	SR01B/10a	39±8	40±11	44±10	46±9	25	10,89 ± 3,72
Témoin		NO*	47±4	53±4	57±9	0	3,56 ± 3,18
LSD $_{0,05}$		N.S.	8,6	N.S	8,2		4,80

NO*= Non Observé *(Not Observed)*.

Réisolement du pathogène

L'examen de l'intérieur du bois des plantules mortes à l'issue des inoculations avec les souches de *F. xylarioides* a permis le réisolement de cette espèce à des distances plus ou moins éloignées (2-4 cm) des points d'inoculation tandis que les *F. solani* et les *F. stilboides* ont été retrouvés autour de la zone d'inoculation à environ 1 cm de part et d'autre du point d'inoculation. Aucun cas de réisolement n'a été enregistré sur des échantillons des plantules inoculées avec le *F. equiseti* et le *F. falciforme* ainsi que sur des plantules témoins.

Discussion et conclusion

L'étude des caractères des *Fusarium* spp. associées au dépérissement du caféier Robusta en RDC, menée en culture, au niveau moléculaire et au niveau du pouvoir pathogène, a montré des différences dans les caractères étudiés. En culture, du point de vue de la croissance radiale, de nettes différences ont permis un regroupement des espèces en 2 catégories distinctes (Tableau 2). Le *F. equiseti*, *F. falciforme* et *F. solani* ont constitué un groupe à croissance plus rapide que le groupe des *F. stilboides* et *F. xylarioides*. La morphologie et les dimensions des conidies ont révélé, pour certaines souches, des valeurs qui se situent dans l'intervalle de celles déjà établies par d'autres auteurs (BOOTH, 1971; HOLLIDAY, 1980; NELSON *et al.*, 1983). Après confrontation avec la description morphologique de ces auteurs, une

première identification a été établie (Tableau 5). En ce qui concerne l'espèce PTFsp4 la confrontation de certains critères d'identification a permis de la rapprocher à l'espèce *F. equiseti.* Ces critères sont: l'absence des microconidies, la morphologie falciforme accompagnée d'une sorte de "bec" qui accentue sa courbure ainsi que le nombre de cloisons allant de 4 à 7 avec des dimensions comprises entre 22-60 µm x 3,5-5,9 µm (BOOTH, 1971). Cependant à eux seuls ces caractères culturaux ne suffisent pas pour une identification complète. Il devient important de recourir à d'autres critères, comme la caractérisation moléculaire.

L'analyse des régions ITS1 et ITS2 de l'ADN ribosomique et du facteur d'élongation α 1 a contribué à cette identification (Tableau 5). On remarque cependant que, même cette approche moléculaire ne donne pas toujours une certitude absolue dans l'identification. C'est les cas de l'espèce PTFsp4 dont le rapprochement a été établi avec l'espèce *F. equiseti* à 99% par l'analyse de l'ADN ribosomique et que le facteur d'élongation α1 l'assimile à 85 % au *Gibberella zeae* (Tableau 5). SUMMERELL *et al.* (2003) ont fait remarquer l'intérêt d'une identification basée sur la complémentarité des critères morphologiques, biologiques et moléculaires. Le deuxième aspect de la caractérisation moléculaire, visant la recherche de diversité à l'aide des marqueurs RAPD au sein des souches de *F. xylarioides,* a montré qu'il existe une homogénéité dans la collection analysée (Figure 1) et confirme les résultats obtenus à l'aide des marqueurs microsatellites (BIEYSSE, 2004). Par contre des différences sont trouvées entre les profils de *F. xylarioides* et ceux d'autres *Fusarium* spp. (Figure 1). Des résultats similaires ont été obtenus par GIRMA (2004), qui a aussi observé un polymorphisme entre les profils des *Gibberella xylarioides,* anamorphe *Fusarium xylarioides,* et ceux des *F. solani, F. stilboides, F. eumartii* et *F. lateritium* isolés des caféiers morts de trachéomycose.

Au niveau de pathogénicité, des différences ont également existé entre les *Fusarium* spp. étudiées en ce qui concerne le délai d'apparition des symptômes, la sévérité de la maladie et la mortalité. Les souches *F. xylarioides* se distinguent des autres par leur capacité à induire précocement les symptômes et par leur sévérité plus élevée (Tableau 6). Il en est de même de la mortalité qui a été enregistrée uniquement avec les souches *F. xylarioides*. Les réisolements ont révélé leur présence dans les tissus internes de bois à des distances de 2 à 4 cm dans le sens longitudinal de part et d'autre du point d'inoculation. Les souches de *F. solani* et *F. stilboides* quant à elles, ont été réisolées localement au point d'inoculation. Il s'avère en outre que les souches de *F. xylarioides* sont les seules à induire tous les signes du syndrome de la trachéomycose (Tableau 6). L'évolution de ces signes débute par le brunissement des feuilles (à 19 jours après inoculation pour la souche B10101(2)J) ou par leur flétrissement (à 39 jours après inoculation pour la souche

SR01B/10a) et progresse vers leur dessèchement, suivi de la défoliation et de la mortalité de la plantule. En observant l'absence du flétrissement des feuilles et de la mortalité chez *F. equiseti, F. falciforme, F. solani* et *F. stilboides* et chez le témoin, on pourrait penser que ces deux symptômes constitueraient des signes externes liés au *Fusarium xylarioides* dans la trachéomycose du caféier. En effet, malgré la présence des symptômes enregistrés sur les plantules inoculées avec les *F. equiseti, F. falciforme, F. solani* et *F. stilboides*, il ne paraît pas évident qu'ils soient réellement induits par ces espèces, du fait de leur présence enregistrée aussi sur les plantules témoins (Tableau 6). Il s'agit du brunissement et dessèchement des feuilles, la défoliation et le dessèchement du plant. Les valeurs AUDPC ($LSD_{0,05} = 4,80$) qui regroupent le témoin, les *F. equiseti, F. falciforme, F. solani* et *F. stilboides*, pourraient aussi laisser penser à une cause physiologique dans la défoliation observée avec ces *Fusarium* spp.

De ce fait l'hypothèse d'une responsabilité majeure des espèces *F. equiseti, F. falciforme, F. solani* et *F. stilboides* dans le dépérissement du type trachéomycose chez le caféier Robusta ne semble pas se justifier. Toutefois par le fait que le *F. solani* et le *F. stilboides* se retrouvent souvent associés au *F. xylarioides* dans le dépérissement des caféiers et qu'ils soient réisolés dans le présent travail, il apparaît utile de mener une étude plus approfondie pour déterminer une éventuelle interaction, synergique ou antagoniste, avec le *F. xylarioides* dans l'expression de la trachéomycose du caféier. GIRMA (2004) a également pensé que la détermination du rôle des différentes *Fusarium* spp. dans le dépérissement du caféier représente à ce jour un sujet qui mérite une investigation. En effet, diverses *Fusarium* spp. ont été isolées et identifiées dans le dépérissement du *C. arabica* en Ethiopie dans les proportions évaluées à 62% pour le *F. xylarioides*, 17% pour le *F. stilboides* Wollenw., téléomorphe *Gibberella stilboides* Gordon, 9% pour le *F. solani* (Mart.) téléomorphe *Nectria hematococca* Berk. & Br. et à 6% pour le *F. oxysporum* Schlecht. D'autres moisissures ont été représentées en proportion de 6% (GIRMA & HINDORF, 2001). Dans diverses autres parties du monde la présence de différentes *Fusarium* spp. dans le dépérissement des caféiers a été signalée également par plusieurs auteurs (WELLMAN, 1961; BAKER, 1972; CLOWES & HILL, 1985; FLOOD, 1997; WALLER & HOLDENESS, 1997; cités par GIRMA, 2004).

Remerciements

Nous témoignons toute notre gratitude au Projet Coffee Wilt Disease (COWIDI) qui a financé notre formation en Belgique à l'Université catholique de Louvain auprès du laboratoire de l'Unité de Phytopathologie. Les critiques et les commentaires du Dr. Bieysse, Coordonnateur du Projet, ont été utilement exploités dans la rédaction du présent article et nous lui sommes particulièrement reconnaissant.

Abstract

CHARACTERISATION OF *FUSARIUM* SPP. ASSOCIATED WITH COFFEE WILT DISEASE OF ROBUSTA COFFEE IN THE DEMOCRATIC REPUBLIC OF CONGO

The authors isolated and characterized various *Fusarium* spp. from wilting coffee trees in order to find the causal agent of coffee wilt disease in the Democratic Republic of Congo. Isolates were characterized by morphology and ITS sequences. Artificial inoculation experiments revealed that only *Fusarium xylarioides* caused severe defoliation and mortality of coffee plantlets.

Key words: coffee wilt disease, tracheomycosis, *Fusarium* spp., PCR-RAPD

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INCO-DEV COFFEE WILT PROJECT

(Contract no. ICA4-CT-2001-10006)

Work Package 1

Progress Report, Nov. 04 – Oct. 05

Dr Mike Rutherford
CABI Bioscience (UK)

Contents

1. Project partners

CABI Bioscience (CABI), United Kingdom (leading Work Package 1)
Centre de Cooperation Internationale en Recherche Agronomique pour Le Developpement (CIRAD), France
Coffee Research Institute (CORI), Uganda
Université Catholique de Louvain (UCL), Belgium
University of Kinshasa (UoK), Democratic Republic of Congo

2. Overall objective

To improve knowledge of the coffee wilt pathogen, *Fusarium xylarioides*, with respect to genetic diversity and variation in aggressiveness.

3. Specific activities

Specific activities for which CABI UK Centre (CABI UKC) is responsible for, or involved in, are:

3.1 Establish a collection of the anamorph (*F. xylarioides)* and teleomorph (*Gibberella xylarioides)* forms of the CWD pathogen obtained from wilt-affected coffee trees in Uganda, DRC and other regions of Africa as appropriate. Deposit representative isolates in a designated facility to facilitate secure, long-term storage.

3.2 Investigate the extent of diversity among isolates, across geographical locations and over time, principally using a range of genetic approaches.

3.3 Establish and maintain baseline data relating to those isolates acquired and held by CABI and its partners on an electronic database.

3.4 Synthesise results of research activities undertaken by project partners contributing to WP1.

4. Research progress

Note: *It was expected that, under the original contractual arrangements, CABI's inputs to the project would cease at the end of October 2004. However, as a result of a successful application to the EU for an extension to the project CABI has been able to continue its research since that time. However, given that the extension was not funded this research has relied on funds remaining from the original phase of the project and, as such, has been somewhat limited (as reflected in this report). Nevertheless, CABI is pleased to be able to continue to support it's partners in pursuing activities, including secure storage of fusaria and database maintenance, considered fundamental to the ongoing success of the project.*

4.1 Establish a collection of anamorph and teleomorph forms of the CWD pathogen, deposit representative isolates for secure, long-term storage

An extensive collection of more than 300 isolates of various *Fusarium* species has been established at the CABI UK Centre. The majority of these are from coffee plants that exhibited symptoms of CWD and were confirmed as *F. xylarioides*. Isolates identified as being species other than *F. xylarioides*, including the recognised pathogens of coffee *F. stilboides*, *F. lateritium*, *F. solani* and *F. oxysporum*, are also held along with fusaria from a number of other plant hosts. Several of the latter were requested from other collections as specific characters (e.g. genetic profiles) were already known. These isolates are therefore suitable for inclusion in project activities for comparative purposes. Isolates added to the collection during 2005 include seven recovered from *C. arabica* in Ethiopia. Project partners continued to request and utilise material from the CABI collection for use in their own research activities (e.g. mating tests undertaken at UCL, Belgium). Dr Mike Rutherford, supported by mycologist Dr Paul Cannon and plant pathologist Jim Waller, continued, through examination of morphological characters, to determine or confirm the identity[1] of isolates received or already held at CABI. In the course of this work several isolates, including a number deposited in the CABI GRC prior to initiation of the project, were found to differ from their original species designations. Notes to this effect have been included in the database (see Section 4.3). Where possible, documentation available from the CABI herbarium and GRC, international collections including ATCC, DSMZ and CBS and other sources was obtained to clarify the identity of the various fungi to species level.

All isolates held at CABI UKC are maintained over the short to medium term on synthetic nutrient agar (SNA) slopes at 5°C. The entire set of sixty representative isolates, selected for in depth analysis of variability at CABI using a range of approaches, has now been deposited for long term storage in the GRC at Egham under liquid nitrogen and also in a freeze-dried state. These isolates are considered representative of the range of *Fusarium* species, geographic origins, host plant species/clones and time of recovery from CWD affected trees in relation to the larger collection available. Six *F. xylarioides* isolates obtained from *C. arabica* in Ethiopia and included in analyses of genetic variability (see Section 4.2) have also been deposited. Isolates obtained from trees forming an on-farm site in Uganda where CWD spread has been monitored since 2002 will also be deposited over the coming months.

The CABI collection will continue to be maintained for the duration of the project and should also provide an invaluable source of reference material for researchers throughout the world for many years after project completion.

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[1] Identifications based on morphological characteristics as described by Booth, C. (1971) *The Genus Fusarium*. Kew, Commonwealth Agricultural Bureaux.

4.2 Investigate the extent of diversity among isolates, principally using a range of genetic approaches

Analysis of genetic variability within a representative group of 60 isolates, including *F. xylarioides*, *F. solani*, *F. stilboides*, *F. lateritium* and *F. oxysporum*, has been completed. Variability within these isolates was evaluated through inter simple sequence repeat (ISSR) analysis, presumptive mtDNA restriction fragment length polymorphism (RFLP) analysis and analysis of the intergenic spacer (IGS) region. During the reporting period ISSR and IGS analysis has also been undertaken on additional isolates, including seven (six being *F. xylarioides*) from *C. arabica* in Ethiopia; 15 from *C. canephora* in Tanzania and a number of *F. xylarioides* isolates obtained during two separate visits to an on farm site in Uganda where the spatio-temporal spread of CWD is being monitored. These studies have supported the genetic population structures reported previously, in terms of the limited genetic variability observed within the pathogen as a whole, the genetic dissimilarity observed between isolates obtained from *C. arabica* and *C. canephora* since re-mergence of CWD and the ease with which *F. xylarioides* may be distinguished from other fusaria occurring on coffee. A manuscript detailing the findings of this work is currently in preparation.

4.3 Establish and maintain baseline data relating to those isolates acquired and held by CABI and its partners on an electronic database.

Input of data to the electronic database continued during the reporting period, including for newly acquired isolates. The database now holds comprehensive information relating to more than 300 of the isolates held at CABI, UCL, CIRAD and by project partners in Africa. Information on outputs of the various studies of, for example, genetic variability, pathogenic variability and mating type remains limited. This information should be provided and input by CABI and it's partners as a priority during the remaining time of the project.

4.4 Synthesise results of research activities undertaken by CABI UKC and partners contributing to WP1.

In addition to the collection established at CABI, collections are also held by project partners at CIRAD, UCL, CORI and UoK. At each organisation isolates are held that are unique to that particular collection. However a number of the more important isolates, namely those representative of specific circumstances and characterised by varying approaches, are now held in at least two collections. This replication will help to ensure the security of isolates. Additions were made to the CIRAD, UCL, CORI and UoK collections during the reporting period.

In Uganda, a comprehensive collection of *F. xylarioides* isolates has now been established that can be considered to be representative of the pathogen population. This comprises material obtained from CWD affected coffee plants in many districts located across the south, south western, western, central and eastern parts of the country. A number of isolates were also obtained from specific coffee clones held at CORI. The majority of these were recovered from *C. canephora*. To date CWD has not been observed on *C. arabica*, even in districts where the species *was* cultivated alongside affected *C. canephora*, nor has *F. xylarioides* been recovered from *C. arabica*. Scientists from CIRAD also undertook two systematic surveys of primary forests located in Kibale (5 forests) and Itwara, western Uganda, in order to identify wild *Coffea canephora* plants affected by CWD from which the pathogen, *F. xylarioides*, could be isolated. During the survey in Kibale trees affected by CWD, as confirmed by typical black streaks visible in wood at the base of the trunk, were only observed in Ngogo-Kibuguta forest. Perithecia were not observed. At Itwara, a natural reserve situated 40 km north of Kibale and bordered by tea plantations to the south and west and savannah to the east and north, CWD was again observed. Of the fungi isolated from wood and bark pieces collected from the forests, 15 were confirmed as fusaria. DNA of purified isolates was sequenced in order to determine the genetic similarity of the isolates to other fusaria from coffee for which reference DNA sequences were available (see below).

Twenty *F. xylarioides* isolates were obtained from *C. canephora* trees exhibiting CWD symptoms in Equator Province, DRC, by scientists from the UoK. Isolates were purified by single sporing and are now available for molecular characterization and investigation of mating types at UCL, Belgium to determine whether they conform to forms of the pathogen already recovered from elsewhere in DRC and from Ugandan and Tanzania. Sixteen isolates recovered from *C. arabica* in Ethiopia were also deposited in the *G. xylarioides* collection at UCL. Three *F. udum* strains, originally obtained from *Cajanus* spp. in India and Malawi, along with an isolate from *C. arabica* considered to be *F. oxysporum*, were provided from the CABI collection for research purposes. Two new 'historical' isolates, one isolated from *C. canephora* in Guinea in 1964 and the other from *C. arabica* in Ethiopia in 1971, were donated to UCL by BBA courtesy of Dr. H. Nirenberg. A third historical isolate, along with five herbarium specimens, were donated by the Museum d'histoire Naturelle de Paris, courtesy of Dr Cony Decock. The UCL collection now holds seven historical *G. xylarioides* isolates, one from *C. arabica*. Several strains have been requested from Dr K. O'Donnell to support UCL's on identification of mating types (*MAT*), partial gene sequencing and cross fertility studies. To facilitate the latter, 18 standard mating population tester strains (MP A-I) were also requested from the Fungal Genetics Stock Centre (FGSC).

An investigation of mycelial growth among isolates of the coffee wilt pathogen undertaken at UCL, albeit on a small number of isolates, has shown varying effects of temperature on colony growth *in vitro*. While similar optimal and maximum growth temperatures (25°C and 32.5°C respectivey) were observed for isolates obtained from CWD affected *C. arabica* and *C. canephora* trees since 1992 (i.e. since re-emergence of the disease on *C. canephora*), some variability was found historical isolates. In particular, the maximum temperature for growth of some historical isolates was higher than for others. These findings, which may relate to adaption to specific environments, also have implications with regard to pathogen characterisation, including the use of pathogenicity screening. While the effect of temperature on conidiospore viability are currently being investigated also, a more comprehensive study of the effects of temperature on fungal growth and development and how it relates to disease development would be beneficial.

At UoK (DRC) a study of the role of *F. xylarioides* and other fusaria in development of CWD continued. As part of this study *F. xylarioides* and other fusaria associated with CWD on *C. canephora* were characterised by observation of morphological attributes, growth rate *in vitro* (on agar medium), pathogenicicity testing (under glasshouse conditions) and genetic sequencing of the ITS1, ITS2 and TEF 1-α regions. Examination of the production and form of conidia, along with colony growth, separated isolates into two groups and facilitated identification to species level. The first group comprised slower growing isolates of *F. xylarioides* and *F. stilboides*, the second other fusaria including *F. equiseti*, and *F. falciforme*. No clear difference in growth rate was observed among *F. xylarioides* isolates. Molecular analyses generally supported the grouping and species designations, and also confirmed the findings of project partners in that the *F. xylarioides* isolates formed a genetically homegeneous group which differed genetically to the fusaria. Of significance, and when inoculated onto susceptible coffee plantlets, the species differed in in terms of the time take for symptom appearance, the severity of symptoms and mortality. While several species, including *F. xylarioides*, induced discoloration, drying and abcision of leaves, *F. xylarioides* alone induced symptoms characteristic of CWD and caused death of treated plants. Furthermore, and unlike other fusaria, *F. xylarioides* could be reisolated some distance from the point of inoculation, confirming its unique status as a vascular pathogen. The findings reinforce the need to confirm the nature and cause of wilt-like disorders of coffee through thorough investigation of internal as well as external symptoms, isolation of fungi present in host tissues and confirmation of their pathogenic nature.

In Uganda, monitoring of the spread of CWD on coffee trees (generally 40 or more years old) on six selected farms in four districts, and on seedlings and young plants on trial sites at CORI,

Uganda, continued. As expected, the disease has continued to spread in all cases, although the rate of increase in incidence varied across the farms. Factors (e.g. cropping practices) possibly contributing to this variation are currently being investigated. Furthermore, variability was observed in the rate of CWD symptom development on plants of different *C. canephora* clones cultivated in the on-station trials, suggesting some level of field tolerance. Further statistical analysis of disease spread at the on-farm and on-station has been undertaken by statisticians in the UK in consultation with CABI project staff. A more comprehensive report of the findings of the mapping studies to date, including the results of statistiacal analyses, is included in the report for Work Package 4.

At UCL earlier attempts to cross individual isolates of *F. xylarioides in vitro*, supported by an investigation of the mating type (*MAT*) gene within the fungus, suggested that the coffee wilt pathogen is heterothallic with either *MAT*-1 and *MAT*-2 mating type alleles found in individual isolates. Further genetic studies have now shown that genetical material of both parents used in these crosses can be present among ascopore progeny, confirming that the progeny can indeed arise through sexual recombination. Related to this work, an analysis of the 20 *F. xylarioides* isolates obtained from *C. canephora* in Equator Province, DRC, has shown all to be of *MAT*-1 mating type. This is a somewhat unusual finding given that both mating types have been observed in other provinces. Furthermore, only half of the isolates produced a fertile teleomorph when crossed with female fertile *C. canephora* tester isolates, the remaining isolates possibly being either sterile or incompatible with the testers. These findings suggest a clonal introduction of the CWD pathogen to Equator Province, and would be supported should the teleomorph stage not occur under field conditions.

Mating type within the fungus has been further characterised by PCR analysis with primer pairs previously developed for the *Gibberella fujikuroi* species complex (GFC) and *F. oxysporum*. Partial sequencing of the *MAT*-1 gene revealed little polymorphism within the *Gibberella xylarioides* complex (GxC) , identical sequences being obtained for recent and historical *C. canephora*, *C. excelsa*, and *C. arabica* isolates. *MAT*-2 sequencing, however, was much more informative, distinguishing five discrete sequences within the GxC and revealing three clades within the *G. xylarioides/F. udum* complex, one comprising recent *C. canephora* and *C. excelsa* isolates and a historical *C. canephora* isolate, all of which were genetically identical. Two *F. udum* strains formed a sister clade to the first, while a third clade comprised recent *C. arabica* strains which are again identical in sequence with the exception of one isolate. Two historical strains (CBS 25852 and CBS 74979) were identical in sequence and show 99% homology to the *C. canephora* group. It is important to note that the limited phylogentic differentiation observed from *MAT*-1 analysis may be due to only a small part of the coding region being sequenced to date. In contrast, approximately one third of the *MAT*-2 open reading frame has been read.

Evaluation of genetic deversity at UCL was also undertaken through RAPD analysis and sequencing of a TEF 1-α, calmodulin and histone 3 genes. These approaches enabled separation of *F. xylarioides* isolates and other fusaria, including *F. udum* and *F. phyllophilium*, into putative subdivisions or clades. The results, coupled with those for the *MAT* genes, were somewhat inconsistent RAPD, calmodulin and histone analysis, for example, could discriminate between recent *C. canephora* isolates and recent *C. arabica* isolates. To varying extensts these approcahes could also differentiate between these recent isolates and/or the historical isolates as well as other fusaria. While TEF analysis failed to differentiate between recent *C. canephora* and recent *C. arabica* isolates, but separated the historical isolates and other fusaria. It also showed that the *G. xylarioides* complex (GxC) is nested within a larger group comprising *F. udum, F. phyllophilium* and other fusaria, with two clades making up the GxC: one comprising the recent DRC, Ethiopian, Tanzanian and Ugandan isolates from *C. arabica*, *C. canephora* and *C. excelsa*, along with historical isolates DRC (ex. *C. canephora*) and Ethiopia (ex. *C. arabica*). The second comprises

two sub-groups for the historical isolates from western Africa. Calmodulin and H3 analysis also revealed a number of isolate groupings/clades. Of note, the placement of the GxC as a sister clade to the *F. udum/Fusarium* species complex was consistent. The Equator Province isolates have not been included in these analyses as yet.

At CIRAD, genetic variability was assessed among the 15 single-spore derived isolates obtained from wild *C. canephora* trees affected by CWD in Ngogo-Kibuguta and Itwara forests of western Uganda. DNA was extracted for each isolate, purified and a rDNA fragment encompassing the ITS1, ITS2 and 5.8S regions, along with 630 nucleotides of the 5' end of the 28S region, amplified using primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and P3 (CCTTGGTCCGTGTTTCAAGACGGG-3'). Each fragment was sequenced and the sequence sets aligned using CLUSTAL W multiple alignment program. Based on the alignment generated, pairwise distances between nucleic sequences of the different isolates was estimated and a dendrogram constructed using the distance matrix. Reference strains of *F. xylarioides* (including three 'historical strains'), *F. lateritium*, *F. decemcellulare* and *F. equisetii* were included in the analysis.

From this study it was only possible to obtain satisfactory results for alignment of the ITS1 region for only 13 of the 15 isolates. Nevertheless, two main isolate groups were distinguishable. The first comprised 10 *F. xylarioides* isolates, which included six of those obtained from either the Kibale or Itwara forests, reference strain CAB003 (ex. *C. canephora*, Uganda) and, perhaps surprisingly, the three 'historical strains' ATCC 15664, DSMZ 62457 and CBS 74979 from W and C Africa. The second, showing between 6% and 16% dissimilarity to the first, comprised seven isolates from wild *C. canephora* (from Itwara forest only) and the *F. lateritium*, *F. decemcellulare* and *F. equisetii* isolates. BLAST analysis of the seven Itwara suggested that may all be fusaria other than *F. xylarioides* (e.g. *F. lateritium*, *F. solani*). Phylogenetic analysis, based on ITS1-P3 sequences, revealed five subgroups within the two main groups as follows:

- *Subgroup 1: A F. xylarioides* group, comprising the three historical strains (DSMZ 62457, ATCC 15664, CBS 74979), contemporary strain CAB003 and six isolates from wild *C. canephora* (OUG163, OUG164, OUG165, OUG166, OUG182 and OUG184
- *Subgroup 2:* Isolates OUG180-1, OUG180-2, OUG175, OUG170, OUG178-1 and OUG178-2, which appear to be fusaria other than *F. xylarioides*.
- *Subgroup 3: F. decencellulare* reference strain CAB010
- *Subgroup4:* Two *F. equisetii* reference strains
- *Subgroup 5:F.lateritium* reference strain and isolate OUG 186, from Itwara forest, which shows BLAST homology with *F. lateritium*

The discovery of wild *C. canephora* plants exhibiting symptoms of CWD in forest sites in western Uganda and confirmation of the presence of *F. xylarioides* in at least some of these plants raises questions as to the origin of the disease in the country and perhaps elsewhere. For many decades and perhaps centuries, forests have been viewed as a source of virgin planting material from which seeds and other parts of species including *Coffea* species were obtained and subsequently cultivated, some on a major scale. Indeed, coffee berries were collected for the purposes of trade. Such material would, presumably, have appeared health, including with regard to pests and diseases such as CWD. Depending on the length of time over which *F. xylarioides* has been associated with wild coffee, it is feasible that the pathogen has co-evolved with its host and, at some stage in time, carried with planting material removed from forest areas to be cultivated in areas adjacent to forest areas and subsequently further afield. In the case of *C. canephora*, transfer may have occurred during the 1920s and 1930s, when when plantations of the species first became established. If so, co-evolution may offer prospects in terms of a pool of resistance genes perhaps being available amongst wild coffee. A simpler explanation may be, of course, that the forest

coffee has become affected by CWD subsequent to emergence of the disease on cultivated coffee, perhaps through the intervention of man and perhaps relatively recently.

The various molecular approaches applied by the project partners continue to unravel the underlying genetic variability existing within the CWD pathogen. They have further clarified the taxonomic structure within the *F. udum* and *F. lateritium* complex, and helped to more accurately determine the nature (species or otherwise) of isolates that have proved difficult to identify on the basis of morphology, including some that appear to have been misidentified previously. Of note, the recent results of the mating tests and MAT-2/TEF sequencing has shown that *G. xylarioides* encompasses at least three apparent groups: *G. xylarioides* sensu strictu Ia (defined by historical West African isolates CBS 25852 and CBS 74979); *G. xylarioides* sensu strictu Ib (defined by historical Central African isolates DSMZ 62457 and ATCC 15664; and *G. xylarioides* sensu lato II (DRC, Ugandan, and Tanzanian *C. canephora and C. excelsa* isolates). How the observed genetic variability relates to the various genetic and non-genetic definitions applied to fungi, including species, subspecies and *formae speciales*, is still not clear.

Two articles describing CWD and highlighting the findings of the ongoing research have recently been published or accepted for publication in the journals *Applied and Environmental Microbiology* and *Phytopathology*. A review of current knowledge of CWD, in which reference was made to the findings of the project research, was also presented at the annual meeting of the American Phytopathological Society (Austin, Texas, 2 August 2006). A paper supporting this presentation has been accepted for publication in the journal *Phytopathology*. A number of other manuscripts are currently in preparation (see Section 5).

5. Promotion/dissemination outputs

5.1 Internal Reports

Rutherford, M A (2004). Development of a long-term strategy based on genetic resistance and agro-ecological approaches against Coffee Wilt Disease (CWD). Annual Report 2005. INCO-DEV contract ICA4-CT-2001-10006. April 2006. Egham, CAB International.

RUTHERFORD, M. A. (2005). Development of a long-term strategy based on genetic resistance and agro-ecological approaches against Coffee Wilt Disease (CWD). Progress Report submitted to Regional Coffee Wilt Programme Co-ordinator. August 2005. Egham, CAB International.

RUTHERFORD, M. A. (2005). Development of a long-term strategy based on genetic resistance and agro-ecological approaches against Coffee Wilt Disease (CWD). Progress Report for Work Package 1 (Pathogen Diversity), November 2004-April 2005. INCO-DEV contract ICA4-CT-2001-10006. June 2005. Egham, CAB International.

UNDERWOOD, F. (2005). Analysis of spread of coffee wilt. Consultancy report provided for INCO-DEV contract ICA4-CT-2001-10006 'Development of a long-term strategy based on genetic resistance and agro-ecological approaches against Coffee Wilt Disease'. Reading, University of Reading Statistical Services Centre.

5.2 Journal publications

LEPOINT, P. C. E., MUNAUT, F. T. J. AND MARAITE, H. M. M (2005) *Gibberella xylarioides sensu lato* from *Coffea canephora*: a new mating population in the *G. fujikuroi* species complex. Applied and Environmental Microbiology **71 (12)**: 8466-8471.

RUTHERFORD, M. A. (2005) Current knowledge of coffee wilt disease, a major constraint to coffee production in Africa. *Phytopathology* **96** (*in press*)

TSHILENGE-DJIM, P., MUNAUT, F., KALONJI-MBUYI1, A. AND MARAITE, H. (2006) Characterization of *Fusarium* spp. associated with the deterioration of Robusta coffee in Democratic Republic of Congo. *Phytoparasitica* (*in press*)

5.3 Oral presentations

RUTHERFORD, M.A., BUDDIE, A., INESON, J., CROZIER, J. AND FLOOD, J. (2005). Newly acquired knowledge of coffee wilt disease and its implications for disease management. Regional Coffee Wilt Programme Stakeholders' and Planning Workshop, Nairobi, Kenya, Nairobi, 8 – 9 December (presented by M.A.Rutherford).

RUTHERFORD, M. (2005) Current knowledge of coffee wilt disease, a major constraint to coffee production in Africa. Annual Meeting of the American Phytopathological Society, Austin, Texas. 30 July–3 August.

RUTHERFORD, M. (2005) EU INCO-DEV funded project ICA4-CT-2001-10006 Development of a long term strategy based on genetic resistance and agroecological approaches against coffee wilt disease in Africa: Review of Research Progress on Work Package 1 (Pathogen Diversity). Project review meeting, Louvain, Belgium. 29 June 2005.

5.4 Poster presentaions

RUTHERFORD, M., J. CROZIER, A. BUDDIE, J. INESON, S. LEA and J. FLOOD. (2005) Poster: Coffee Wilt Disease. Presented at the Regional Coffee Wilt Stakeholders Workshop, Nairobi, Kenya. 8–9 December 2005.

5.5 Related dissemination

BIEYSSE, D. (2005). Development of a long term strategy based on genetic resistance and agroecological approaches against Coffee Wilt Disease in Africa. Third Annual Report, 1 November 2003 - 31 October 2004. INCO-DEV contract ICA4-CT-2001-10006. April 2005. Montpellier, CIRAD-AMIS.

BIEYSSE, D. (2005). Development of a long term strategy based on genetic resistance and agroecological approaches against Coffee Wilt Disease in Africa. Annual report for Work Package 1, November 2004-31 October 2005. INCO-DEV contract ICA4-CT-2001-10006. April 2005. Montpellier, CIRAD-AMIS.

HAKIZA, G. (2005). Development of a long term strategy based on genetic resistance and agroecological approaches against Coffee Wilt Disease in Africa. Annual report for Work Package 1, November 2004-31 October 2005. INCO-DEV contract ICA4-CT-2001-10006. April 2005. Kituza, Uganda, Coffee Research Institute.

LEPOINTE, P. (2005). Development of a long term strategy based on genetic resistance and agroecological approaches against Coffee Wilt Disease in Africa. Annual report for Work Package 1, November 2004-31 October 2005. INCO-DEV contract ICA4-CT-2001-10006. April 2005. Louvain, Universite Catholique de Louvain.

SIMONS, S, AKIRI, M., PHIRI, N., KIMANI, M., RUTHERFORD, M., NZANZU, T. S., ADUGNA, G., MUGUNGA, M., KILAMBO, D. AND HAKIZA, G. (2005) Improvement of coffee production in Africa by the control of coffee wilt disease (tracheomycosis). Coffee wilt disease programme CFC/ICO/13: Project Progress Report, January-June 2005. July 2005. Nairobi, CAB International.

RUTHERFORD, M. (2005) Epidemiology and variability of the coffee wilt pathogen. In: Perspectives on Pests II. Chatham, NR International.

RUTHERFORD, M., FLOOD, J., LEA, S. AND CROZIER, J. (2005). Coffee Wilt Disease. Advisory leaflet. 10,000 copies. 8 pp. Egham, CAB International.

Acknowledgements

The author would like to thank the following for their active participation in this work as project collaborators: G. Hakiza (Coffee Research Institute, Mukono, Uganda); M. Abebe (JIMA Agricultural Research Center, Addis Ababa, Ethiopia); D. Kilambo (Tanzania Coffee Research Institute, Moshi, Tanzania); G. Adugna (JIMA Agricultural Research Center, Addis Ababa, Ethiopia/University of Bonn, Bonn, Germany); D. Biyesse (CIRAD/UMR BGPI, Montpellier, France); H. Maraite (Université Catholique de Louvain, Louvain, Belgium). Gratitude is also expressed to all who donated coffee plant material and fusarium isolates for use in this work. P. Cannon and J. Waller (CABI Bioscience, UK) are also acknowledged for assisting in the morphological identification of isolates and provision of advice.

Mike Rutherford
18 May 2006